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In vitro and *in vivo* biofunctional effects of selected coffee compounds, extracts and brews on key elements of adenosine receptor-mediated signaling pathways and on cellular heme oxygenase

Dem Fachbereich Chemie der Technischen Universität Kaiserslautern
zur Erlangung des akademischen Grades
„Doktor der Naturwissenschaften“
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D386

vorgelegt von Gina Alejandra Montoya Parra

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Prof. Dr. Elke Richling

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Este trabajo se lo quiero dedicar especialmente
a la tierra que me vió nacer: mi amado país **Colombia**,
a mi familia por la que corre sangre cafetera
y por supuesto al amor de mi vida Heiko Schulz.

Tengo tu mismo color
Y tu misma procedencia,
Somos aroma y esencia
Y amargo es nuestro sabor...
¡Vamos hermanos, valor,
El café nos pide fe;
Y Changó y Ochún y Agué
Piden un grito que vibre
Por nuestra América Libre,
Libre como su café!

Nicomedes Santa Cruz

Der experimentelle Teil dieser Arbeit entstand zwischen Januar 2008 und Februar 2012 im Fachbereich Chemie, Fachrichtung Lebensmittelchemie und Toxikologie der Technischen Universität Kaiserslautern.

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Table of contents

Abbreviations

5´AMP	Adenosin-5'-monophosphat
5´-NT	5´-Nucleotidase
A_{2A}	Adenosine A_{2A} receptor
A_{2B}	Adenosine A_{2B} receptor
AA	Acrylamide
AAMA	N-acetyl-S-(2-carbamoylethyl)-cysteine
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AFMU	5-Acetylamino-6-formylamino-3-methyluracil
AKAP	A-kinase anchoring proteins
ARE	Antioxidant responsive element
ARs	Adenosine receptors
ATP	Adenosine triphosphate
AUC_{0-24}	Area under the curve from 0 time to 24h
$BMDL_{10}$	Benchmark dose for a 10% response
BP	Blood pressure
BR	Bilirubin
BV	Biliverdin
CA	Caffeic acid
cAMP	Cyclic adenosine monophosphate
CD39	Nucleoside triphosphate phosphohydrolase
CD73	Ecto-50-nucleotidase
CGA	Chlorogenic Acid
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
cN-I	Cytosolic 5´-nucleotidase-I
CQA	Caffeoylquinic acid
CVD	Cardiovascular disease
CYP	Cytochrome P450
CYP1A2	Cytochrome P450 1A2
CYPE1	Cytochrome P450 E1
DAG	Diacylglycerol
diCQA	Dicaffeoylquinic
DM	Diabetes Mellitus
EC_{50}	Half maximal effective concentration

ecto-5´-NT	Ecto-5´-nucleotidase
ENT	Equilibrative nucleoside transporter
FA	Ferulic acid
FQA	Feruloylquinic acid
GA	Glycidamide
GAMA	Glycidamide mercapturic acid
GDP	Guanosine diphosphate
G_i	Inhibitory G protein
GIP	Glucosedependent insulinotropic peptide
GLP1	Glucagon like peptide 1
GP	Glycoprotein
GPCRs	G protein-coupled receptors
G_s	Stimulatory G protein
GSH	Reduced glutathione
GTP	Guanosine triphosphate
Hb	Hemoglobin
Hcy	Homocysteine
HE-NECA	2-Hexynyl-5´-*N*-ethylcarboxamidoadenosine
HO	Heme oxygenase
HO-1	Heme oxygenase-1
I/R injury	Ischemia/reperfusion injury
IBMX	3-Isobutyl-1-methylxanthin
IC_{50}	Half maximal inhibitory concentration
IGF1	Insulin-like growth factor
ILSI	International Life Sciences Institute
IP_3	Inositol
IRPS	Iron regulatory proteins
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KO	Knockout
LDL	Low density lipoprotein
MA	Mercapturic acid
mAKAP	Muscle specific A-kinase anchoring protein
MAPK	Mitogen-activated protein kinase
MK	Megakaryocyte
mRNA	Messenger RNA
NAT2	*N*-acetyltransferase
NECA	5´-*N*-Ethylcarboxamidoadenosine

NF-κB	Nuclear factor-κB
NMP	N-Methylpyridinium
NO	Nitric oxide
NRf2	Nuclear factor (erythroid-derived 2)-like 2
P2Y	Purinoceptor
PAHs	Polycyclic aromatic hydrocarbons
*p*CoA	Coumaric acid
p-CQA	*p*-Coumaroylquinic acids
PD	Parkinson's disease
PDE	Phosphodiesterase
PDTC	Pyrrolidine dithiocarbamate
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PLC	Phospholipase C
PPIX	Protoporphyrin IX
R	Receptor
RACK1	Receptor for activated C-kinase 1
R_i	Inhibitory receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
R_s	Stimulatory receptor
RT	Room temperature
RyR2	Ryanodine receptor 2
SDF	Soluble dietary fiber
sGC	Soluble guanylate cyclase
SMC	Smooth muscle cells
Src	Proto-oncogene tyrosine-protein kinase
SULT	Sulfotransferase
T2D	Type 2 diabetes mellitus
TAG	Triacylglycerol
TP	Thromboxane/prostanoid receptors
TXA_2	Thromboxane A_2
UCR	Upstream conserved region
VLDL	Very low density lipoprotein
vWf	Von Willebrand factor

Summary

Mechanisms underlying the biological effects of coffee and its constituents are incompletely understood. Many effects have been attributed solely to caffeine, neglecting that coffee is a mixture of many chemical substances. Some authors suggest that the main mechanism of action of caffeine is to antagonize adenosine receptors (AR); a second effect is the inhibition of phosphodiesterases with the subsequent accumulation of cAMP and an intensification of the effects of catecholamines. Although the inhibition of phosphodiesterases may contribute to the actions of caffeine, there is growing evidence that most pharmacological effects of this xanthine result from antagonism of AR.

One of the main objectives of this work was to investigate whether substances other than caffeine in coffee may influence the homeostasis of intracellular cyclic nucleotides *in vitro* and *in vivo*. The influence of selected coffee compounds, extracts and brews on key elements involved in the adenosine receptor-mediated signaling pathway have been investigated.

A further aim of this work was also to determine if coffee or some coffee constituents may have a stimulatory effect on the cellular heme oxygenase activity (HO-activity). Two coffee extracts, a slightly (AB1) and an intensively roasted coffee (AB2), were studied along with selected individual compounds. Caffeine and low substituted pyrazines showed no effect on the HO-activity, while NMP, pyrazines with a greater substitution pattern such as Tetramethylpyrazine (TMP) and 2-Ethyl-3,5(6)-dimethylpyrazine (2-E-3,5-DMP) and both coffee extracts significantly induced the HO-activity in liver hepatocellular carcinoma (HepG2), intestinal colo-rectal adenocarcinoma (Caco-2) and in some instances in monocytic leukemia (MM6) cells.

It was found that caffeine, theophylline, coffee extracts from conventional or functional coffees, pyrazines (2,3-DE-6-MP, 2-Isobutyl-3-methoxyP)**,** 5-CQA and caffeic acid all significantly inhibited the basal cytoplasmatic PDE activity in lysates of lung tumour xenograft cells (LXFL529L) and human platelets. To a somewhat lesser extent, PDE inhibition was also found in experiments performed with paraxanthine and other pyrazines (2-E-3,5-DMP, TMP and 2-E-5-MP). Thus the degree of roasting has a considerable impact on constituents of influence for PDE activity. Caffeine, coffee polyphenols and some pyrazines and further, as yet unknown roasting products appear to represent the main modulating constituents.

In two coffee intervention studies, a short-term (8 weeks) and a long-term study (24 weeks), comprising 8 and 84 healthy volunteers respectively, we examined extracellular key elements of the adenosine pathway including plasma adenosine levels and adenosine deaminase activity. Additionally, we studied the intracellular cAMP concentration and the PDE activity in platelets as surrogate biomarkers of adipocytes.

Results of *in vitro* experiments had suggested that the concentrations of caffeine and coffee extracts required to obtain a half maximal inhibition were in the upper range of physiological conditions. Yet, it was demonstrated for the first time *in vivo* that moderate consumption of coffee can modulate the activity of platelet phosphodiesterases in humans in long and short term. In both studies, the first exposure to coffee showed a strong inhibition ($p<0.001$) of the PDE activity in the platelet lysates of the participants while the second coffee phase showed no or a slight effect when compared with the first coffee intervention.

In both studies a significant increase ($p<0.001$) in intraplatelet cAMP concentrations during the wash-out phase (after the first coffee phase) was observed. This response could be due to inhibition of the PDE activity in the previously phase extending in to the wash out phase. However, the behavior of cAMP in the following study phases cannot be easily explained. It may be hypothesized that this effect is attributable to adaptive effects to allow PDE inhibition. One possibility is the modulation of the expression of membrane-bound adenosine receptors in platelet precursors, which still have a nucleus. This may potentially influence adenylate cyclase activity in mature platelets. For the observed effects, in addition to caffeine other ingredients of coffee appear to play a role. The findings suggest that monitoring of cAMP homeostasis in platelets is not a useful surrogate biomarker for effects in other tissues.

Neither the activity of adenosine deaminase nor the adenosine concentrations in plasma were markedly modulated by the coffee consumption in both trials. This may reflect the fact that adenosine is subject to quick and effective enzymatic turnover by phosphorylation (adenosine kinase) or deamination (adenosine deaminase) allowing keep its concentration within a well balanced homeostasis. However, it is also well known, that considerable variability exists in the responses to coffee drinking. In part, such variability is due to caffeine tolerance, but there is also evidence for a genetic background.

Altogether the data reported here provide further evidence for the perception that coffee consumption is associated with beneficial health effects demonstrated for the cAMP enhancement in platelets, known to counteract platelet aggregation. The effects observed for the influence of cellular heme oxygenase (HO) are in line with the well documented antioxidative activity of coffee and its constituents.

1 Introduction

Coffee is one of the most widely consumed beverages in the world and has been linked with both beneficial and harmful biological effects. Epidemiological evidence suggests coffee consumption to be associated with prevention or delay of degenerative diseases including diabetes mellitus type 2 (T2D), parkinson´s, alzheimer´s, cardiovascular disease (CVD) and cancer [Deutscher-Kaffeeverband, 2011; Ranheim and Halvorsen, 2005; Zhang et al., 2009]. These beneficial effects have been partly attributed in part to the antioxidant activity of coffee [Bakuradze et al., 2011a; Bakuradze et al., 2010; Hoelzl et al., 2010]. Additionally, moderate reduction in body weight was reported to correlate with regular coffee consumption [Thom, 2007; Tunnicliffe and Shearer, 2008]. Some harmful substances in coffee, like the carcinogens acrylamide and furan are of major interest concerning consumer health effects and have been studied extensively on the last few years [Berger et al., 2011; Mills et al., 2008; Stadler and Lineback, 2009].

Coffee beans contain thousands of constituents, including lipids, proteins, carbohydrates, vitamins, and minerals. The precise mechanisms underlying the actions of coffee are incompletely understood. Most of the effects have been attributed to caffeine, although coffee is a mixture of many biological active compounds [Ferruzzi, 2010; Ranheim and Halvorsen, 2005]. More recently, the acknowledgement that coffee and caffeine are not physiologically equivalent has increased the exploration of other coffee constituents [Boettler et al., 2011b; Farah et al., 2006; Johnston et al., 2003; Tunnicliffe and Shearer, 2008].

Average caffeine consumption by adult humans varies among different cultures and nations from 80 to 400 mg per person per day. Caffeine elicits a diverse number of pharmacological responses, including increased vigilance, decreased psychomotor reaction time, increased sleep latency and waking time and may also influence intellectual performance. Moreover, caffeine causes relaxation of smooth muscles, enhances the secretion of gastric acid, the release of catecholamines, and increases metabolic activity [Varani et al., 2000]. Some authors suggest that the main mechanism of action of caffeine is to antagonize adenosine receptors; a secondary effect is the inhibition of phosphodiesterases [Ribeiro and Sebastiao, 2010], with the subsequent accumulation of cAMP and an intensification of the effects of catecholamines [Renda et al., 2012]. However, at blood concentrations resulting from normal coffee consumption, caffeine acts mainly as an adenosine receptor antagonist, while the main caffeine metabolite, paraxanthine, has been shown to inhibit the effect of endogenous adenosine on a variety of physiological processes [Fredholm et al., 1999; Varani et al., 2000].

Adenosine receptors are associated with intracellular pathways that influence cAMP production, phospholipase C activity, and the mitogen-activated protein kinase (MAPK)

[Schulte and Fredholm, 2003]. Thus, any tissue that expresses such receptors could respond to caffeine. However, there are several subtypes of adenosine receptors and they are found in most tissues. This results in a vast array of possible primary and secondary responses [Beaudoin and Graham, 2011].

One hypothesis explaining how coffee alters T2D involves weight management and body composition. Individuals who consume coffee on a regular basis would be expected to have a *lower* body mass, a factor that would lower T2D risk [Tunnicliffe and Shearer, 2008]. Caffeine from coffee consumption has been shown to induce thermogenesis, lipolysis, fat oxidation, and insulin secretion in both nonobese and obese individuals [Acheson et al., 2004; Dallas et al., 2008; Greenberg et al., 2006]. This could be a significant contribution to the overall energy balance, if it was maintained over decades of caffeine/coffee ingestion. There is little evidence that coffee consumption promotes significant weight loss or causes alterations in body composition. This appears to be due to habituation to caffeine-induced catecholamine responses and lipolysis with prolonged use [Dekker et al., 2007].

It is also well know that the administration of caffeine to fasted humans also causes an increase in FFA mobilization. Adipocytes express adenosine A_1 receptors and these are tonically active, inhibiting adenylate cyclase and reducing both the level of cAMP and lipolysis [Liang et al., 2002]. The proposed mechanism of action of caffeine for the increased lipolysis is blockage of adenosine receptors [Greenberg et al., 2006]. It has also been shown that it takes much more caffeine to cause fat mobilization *in vitro* than *in vivo* [Winder, 1986], suggesting that may be an active metabolite is needed (together with caffeine) to bring about the increase in lipolysis.

A large prospective study reported that increased caffeine intake correlated with reduced weight gain [Lopez-Garcia et al., 2006a]. A similar effect was seen in decaffeinated coffee consumers. Finally, an intervention study comprising 33 healthy volunteers showed that daily consumption of 3–4 cups of a special Arabica coffee brew exerts beneficial health effects, such as reduced oxidative damage, body fat mass and energy uptake [Bakuradze et al., 2011a].

The mode of action of coffee compounds and their metabolites still is not fully understood. Additional studies are needed to clarify if moderate coffee consumption may influence the homeostasis of intracellular cyclic nucleotides and to elucidate a pattern of compounds responsible for such effects.

2 State of the Art

2.1 Coffee general aspects

“Coffee (coffee beans) includes the seeds of crimson fruits from which the outer pericarp is completely removed and the silverskin (spermoderm) is occasionally removed. The seeds may be raw or roasted, whole or ground, and should be from the botanical genus *Coffea*. The drink prepared from such seeds is also called coffee” cited from Belitz *et al.* [Belitz et al., 2009]. The name coffee is derived from the name of the province Keffa where shepherds from Abyssinia (Ethiopia) discovered the coffee beans in the 6th century. Two hundred years later coffee was sold in Europe, thus introducing the new beverage into Western life. But it was the Dutch who first started the spreading of the coffee plant in Central and South America, where today it reigns supreme as the main continental cash crop. Coffee first arrived in the Dutch colony of Surinam in 1718, to be followed by plantations in French Guyana and the first of many in Brazil in the state of Pará [ICO, 2011; Yanagimoto et al., 2004].

World coffee consumption is estimated at 134 million bags (1 bag ≈ 60 kg) in calendar year 2010 compared to 131.3 million bags in 2009, an increase of 2.1%. The world coffe consumption for exporting countries (Brazil, Colombia, Ethiopia, Indonesia, Mexico, and Guatemala etc.) is 30% whereas importing countries represent around 70%. The European Union, USA and Japan consume 30.5%, 16.3% and 5.4 % respectively. Within Europe, coffee is most popular in Germany (6.9%), France (4.4%) and Italy (4.3%) according to the International Coffee Organization [ICO, 2011; ICO and International-Coffee-Organization, 2011]. The German coffee market has gone through important changes in 2011. Even though the consumption of coffee per capita remained high at 150 liters per year, the trend towards freshly brewed coffee for immediate consumption is getting stronger. This reflects clearly on the higher demand for espresso as well as coffee capsules and pads. At the same time, 75% of German consumers prepare their coffee with roasted grains [Federal Ministry of Economics and Technology, 2011]

The coffee tree or shrub belongs to the family *Rubiaceae* which has some 500 genera and over 6,000 species. The economically two most important species of coffee are *Coffea arabica* (Arabica coffee) - which accounts for over 60 percent of world production - and *Coffea canephora* (Robusta coffee). Two other species, the *Coffea liberica* (Liberica coffee) and *Coffea dewevrei* (Excelsa coffee), are grown on a much smaller scale.

The coffe bean (see Figure 2-1) has a green outer skin (exocarp) that turns deep red when ripe, and encloses the pulp (mesocarp) and the stone-fruit bean which consists of two elliptical hemispheres with flattened adjacent sides. The spermoderm (silverskin) covers each hemisphere. “Covering both hemispheres and separating them from each other is the

strong fibrous endocarp, called the “parchment”. When the fruit is ripe a thin, slimy layer of mucilage surrounds the parchment. Occasionally, 10–15% of the coffee fruits consist of only one spherical bean (“peaberry” or “caracol”), which often brings a premium price” cited from Belitz *et al.* [Belitz et al., 2009].

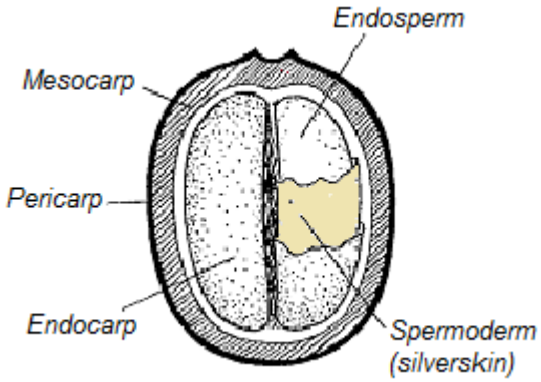


Figure 2-1: Longitudinal section of a coffee fruit. Modified from Belitz *et al.* [Belitz et al., 2009].

Before roasting, coffee beans must be removed from the other fruit parts and dried; this can be done in two ways, known as dry and wet method. When the process is complete the unroasted coffee beans are called as green coffee [ICO, 2011].

In wet processing, ripe coffee cherries are mechanically depulped and the mucilaginous residues of the pulp are degraded by fermentation. The resulting beans are still covered by the parchment, which subsequently must be dried, conditioned and hulled. This method requires the use of specific equipment and substantial quantities of water but ensures that the intrinsic qualities of the coffee beans are better preserved. Coffee produced by this method is generally considered to be from a better quality and commands higher prices. By contrast, during the dry processing entire coffee fruits are dried without removal of the pulp and then are hulled mechanically [Selmar et al., 2008].

The chemical composition of the green coffee beans differs significantly depending on the processing method applied. Quantitative analyses of low molecular sugars in green coffees that were processed in parallel either by the traditional wet or the traditional dry method, revealed a close correlation between the kind of post-harvest treatment and the contents of fructose and glucose. While in *washed* coffee beans (wet processing) only low amounts of both hexoses were present, concentrations in *unwashed* coffees (dry processing) were significantly higher. However, sucrose, the major low molecular sugar in green coffee beans, is not significantly affected by coffee processing [Knopp et al., 2006; Selmar and Bytof, 2008].

2.2 From the raw bean to the roasted coffee

The desired flavour of coffee is produced during roasting; green coffee has no desirable flavour. Chlorogenic acids contribute to body and astringency; sucrose contributes to the color, aroma, bitterness and sourness, and proteins remain perfectly stable, but minor protein components such as free amino acids, are highly reactive. Trigonelline generates pyridine and may consequently be responsible for some objectionable flavours. Caffeine has no function other than a contribution to the bitterness [Flament and Bessiere-Thomas, 2002].

Comparing the content of amino acids (after hydrolysis) in roasted coffee with their content in green coffee serine, cystine and lysine are clearly reduced arginine disappears. Glutamic acid, the most abundant of the amino acids, proportionally increases (see Table 2-3). It has been observed too a partial destruction of the polysaccharides, except cellulose, during roasting. Oligosaccharides and arabinans disappear nearly totally, galactans are strongly degraded, but mannans remain particularly resistant. Monosaccharides, together with with amino acids and amines, are responsible of the formation of melanoidins, pyrazines and other nitrogen-containing heterocycles, resulting mainly from the Strecker and Maillard reactions.

Ho and coworkers [Ho et al., 1993] suggested that deamidation of glutamine and asparagine give more pyrazines than the corresponding acids when heated with reducing sugars. The majority of the volatiles identified in roasted coffee are formed by Maillard reactions, however, sensory-specific investigations have shown that many odorants with a strong flavour impact are also generated by other formation pathways besides the Maillard reaction [Flament and Bessiere-Thomas, 2002; Holscher and Steinhart, 1994].

The volatile fraction of roasted coffee has a very complex composition. Dilution analyses have shown that of the 850 volatile compounds identified only 4.7% contribute to the aroma. Indeed, 28 aroma substances in the concentrations present in a medium roasted Arabica coffee drink (see

Table **2-1**) can largely approximate its aroma. The aroma profile of coffee is composed of the following notes: sweet/caramel-like, earthy, sulfurous/roasty and smoky/phenolic [Belitz et al., 2009].

On roasting, the formation of furfurylthiol is promoted by the water content and the slightly acidic pH value of the beans because under these conditions, the precursor arabinose in the polysaccharides is released by partial hydrolysis.

Table 2-1 Concentrations of potent odorants in medium roast Arabica coffee from Colombia [Belitz et al., 2009]

Group/odorant	Concentration (mg/kg)[a]
Sweet/caramel-like group	
• Methylpropanal	28.2
• 2-Methylbutanal	23.4
• 3-Methylbutanal	17.8
• 2,3-Butandione	49.4
• 2,3-Pentandione	36.2
• 4-Hydroxy-2,5-dimethyl- 3(2H)-furanone	120
• 5-Ethyl-4-hydroxy-2-methyl- 3(2H) furanone (EHM3F)	16.7
• Vanillin	4.1
Earthy group	
• 2-Ethyl-3,5-dimethylpyrazine	0.326
• 2-Ethenyl-3,5-dimethylpyrazine	0.053
• 2,3-Diethyl-5-methylpyrazine	0.090
• 2-Ethenyl-3-ethyl- 5-methylpyrazine	0.017
• 3-Isobutyl-2-methoxy- pyrazine	0.087
Sulfurous/roasty group	
• 2-Furfurylthiol	1.70
• 2-Methyl-3-furanthiol	0.064
• Methional	0.239
• 3-Mercapto-3-methylbutyl- formiate	0.112
• 3-Methyl-2-butene-1-thiol	0.0099
• Methanethiol	4.55
• Dimethyltrisulfide	0.028
Smoky/phenolic group	
• Guaiacol	3.2
• 4-Ethylguaiacol	1.6
• 4-Vinylguaiacol	55
Fruity group	
• Acetaldehyde	130
• Propanal	17.4
• (E)-*β*-Damascenone	0.226
Spicy group	
• 3-Hydroxy-4,5-dimethyl- 3(5H)-furanone	1.58
• 5-Ethyl-3-hydroxy-4- methyl-2(5H)-furanone	0.132

[a] Beverage prepared by percolation of coffee powder (54 g) with water (ca. 90 °C)

Robusta coffees contain significantly higher concentrations of alkylpyrazines than Arabica. Arabica coffees are usually richer in the odorants of the sweet/caramel-like group. The aroma note of raw coffee is produced by 3-alkyl-2-methoxypyrazines, 3-isobutyl-2-methoxypyrazine having the highest aroma value. Being very stable compounds, they easily survive the roasting process. However, this process yields very intensively smelling odorants so that the odor of the methoxypyrazines is largely suppressed. The aroma of coffee is not stable; the fresh note is rapidly lost. The aroma profile changes because the slow-evaporating furanones remain. As a result, the aroma balance can be destroyed by the spicy odor [Belitz et al., 2009].

Volatile aldehydes that play an important role in coffee aroma are formed partially by Strecker degradation of amino acids. Aldehydes are formed by the oxidative degradation of amino acids during their interaction with sugars at high temperatures and during the interaction of amino acids and polyphenols in the presence of polyphenol oxidase at normal temperatures. The formation of various aldehydes and ketones by autoxidation of unsaturated fatty acids via breakdown of hydroperoxide intermediates is well established in the literature. Therefore their presence is not surprising considering that green coffee beans contain lipids and proteins. Hexanal, formed at least partially by oxidation of lipids, was identified by Cantergiani *et al.* [Cantergiani et al., 2001] in a green Mexican arabica (0.24 % of the volatiles) and by Czerny and Grosch [Czerny and Grosch, 2000].

2.3 Coffee constituents, metabolism and physiological effects

Coffee beans contain thousands of constituents, including lipids, proteins, carbohydrates, vitamins, and minerals. In fact, isolating specific compounds responsible for the positive effects of coffee is quite difficult. To date, the majority of research on the biological activity of coffee has mainly been focused on caffeine. More recently, the understanding that coffee and caffeine are not physiologically equivalent has increased the exploration of other coffee constituents [Boettler et al., 2011b; Farah et al., 2006; Johnston et al., 2003; Tunnicliffe and Shearer, 2008].

Green coffee beans smell green-earthy and are further heat treated in a roasting process, generating the typical, characteristic flavour and aroma of coffee. The beans are heated between 180 ºC and 240 ºC for 8 to 15 min, depending on the degree of roast required. The beans increase in volume (50–80%) and change their structure and color. During the roasting process green beans turn brown and a 11–20 % loss in weight occurs. As moisture is lost, the bean "pops" audibly rather like popcorn and a chemical reaction called pyrolysis takes place: starches are converted into sugar, proteins are broken down and the entire cellular structure of the bean is altered. As shown in Table 2-2, the content of proteins, lipids, trigonelline, caffeine and minerals only slightly changed by the roasting process, whereas chlorogenic acids dramatically decreased and substances such melanoidins are formed. Is important to note that the composition of roasted coffee varies greatly, depending on coffee variety and extent of roasting [Belitz et al., 2009].

2.3.1 Proteins and free amino acids

Protein is subjected to extensive changes when heated in the presence of carbohydrates, resulting in a different amino acid composition of coffee protein acid hydrolysates before and after bean roasting (Table 2-3). The total amino acid content of the hydrolysate drops by about 30% because of considerable degradation

Table 2-2 Compositions of green and roasted coffee (medium degree of roasting) [Belitz et al., 2009]

Component	Green coffee		Roasted coffee	
	Arabica (%)*	Robusta (%)*	Arabica (%)*	Robusta (%)*
Carbohydrates	55-65.5	40-55.5	38.0	41.5
Protein	8.5-12		10	
Chlorogenic acids	6.7-9.2	7.1-12.1	2.7	3.1
Lipids	15-18	8-12	17.0	11.0
Trigonelline (niacin)	0.6-1.2	0.3-0.9	1.0	0.7
Caffeine	0.8-1.4	1,7-4	1.3	2.4
Minerals	3-5.4		4.6	
Melanoidins			23.0	

***Values in % of solids**

"Arginine, aspartic acid, cystine, histidine, lysine, serine, threonine and methionine, being especially reactive amino acids, are somewhat decreased in roasted coffee, while the stable amino acids, particularly alanine, glutamic acid and leucine, are relatively increased. Free amino acids occur only in traces in roasted coffee" cited from Belitz *et al.* [Belitz et al., 2009].

Table 2-3 Amino acid composition of the acid hydrolysate of Colombia coffee beans prior to and after roasting [Belitz et al., 2009]

Amino acid	Green coffee (%)	Roasted coffee (%)*
Alanine	4.75	5.52
Arginine	3.61	0
Aspartic acid	10.63	7.13
Cystine	2.89	0.69
Glutamic acid	19.80	23.22
Glycine	6.40	6.78
Histidine	2.79	1.61
Isoleucine	4.64	4.60
Leucine	8.77	10.34
Lysine	6.81	2.76
Methionine	1.44	1.26
Phenylalanine	5.78	6.32
Proline	6.60	7.01
Serine	5.88	0.80
Threonine	3.82	1.38
Tyrosine	3.61	4.35
Valine	8.05	8.05

*** Loss due to roasting amounts to 17.6%.**

2.3.2 Carbohydrates

Carbohydrates are essential for the formation of aroma compounds, mainly by caramelization of the low-molecular-weight sugars and by Maillard reactions with amino acids. When green coffee beans are roasted, 98% of their sucrose undergoes hydrolysis and further degradation reactions occur. Thus, less than 30% of the total carbohydrates in coffee beans are found in brewed coffee. The polysaccharides found in brewed coffee are reported to act as prebiotics and dietary fiber and, as such, may reduce the risk of colon cancer [Arya and Rao, 2007]. Instant or soluble coffee has an amount of soluble dietary fiber (SDF) of aprox. 9 g/L. Mannose and galactose are the main monosaccharides found in these beverages suggesting that arabinogalactan and mannans are most important SDF components [Diaz-Rubio and Saura-Calixto, 2011]. Soluble dietary fiber also plays a role in the antioxidant activity of coffee by binding and enabling passage of phenols to the brewed beverage [Diaz-Rubio and Saura-Calixto, 2007]. Decaffeinated coffee may have slightly lower levels of carbohydrates than regular coffee, depending on the method used in the decaffeination process [Ramalakshmi and Raghavan, 1999].

Brewed coffee contains a significantly higher amount of soluble dietary fiber (0.47-0.75 g/100 mL of coffee) more than other common beverages. As aforementioned, coffee dietary fiber contains a large amount of associated antioxidant phenolics (8.7-10.5 mg/100 mL of brewed coffee) [Diaz-Rubio and Saura-Calixto, 2007]. Díaz-Rubio *et al.* shown that galactose (156-422 mg/100 mL) and mannose (395-246 mg/100 mL) are the major constituents in all infusions, suggesting that galactomannans are the main component of SDF in brewed coffee.

2.3.3 Lipids

The two most important coffee species, Coffea Arabica and Coffea canephora var. Robusta, contain between 7 and 17% fat. The lipid content of green Arabica coffee beans averages some 15%, whilst Robusta coffees contain much less, namely around 10%. Most of the lipids, the coffee oil, are located in the endosperm of green coffee beans [Wilson et al., 1997]; only a small amount, the coffee wax, is located on the outer layer of the bean.

The lipid fraction of coffee is very stable and the roasting process produces only minor changes. The composition of lipids of green coffe is shown in Table 2-4. Linoleic acid is the predominant fatty acid, followed by palmitic acid. The raw coffee waxes, together with hydroxytryptamide esters of various fatty acids originate from the fruit epicarp. These compounds are 0.06–0.1% of normally roasted coffee beans [Belitz et al., 2009].

Table 2-4 Composition of lipids of green coffee [Maier, 1981]

Compounds	(%) dry matter
Triacylglycerols	75.2
Esters of diterpene alcohols and fatty acids	18.5
Diterpene alcohols	0.4
Esters of sterols and fatty acids	3.2
Sterols	2.2
Tocopherols	0.04 - 0.06
Phosphatides	0.1 - 0.5
Tryptamine derivatives	0.6 - 1.0

The diterpenes present are cafestol, 16-O-methylcafestol (found only in Robusta coffee), and kahweol see Figure 2-2. Cafestol and kahweol are partly degraded by the roasting process. They are natural constituents of coffee beans and are released from roast and ground coffee beans by hot water, but are largely trapped by the use of a paper filter in coffee preparation. These components have been identified as hypercholesterolemic, but although the diterpenes are retained in the filter, there is some evidence that consumption of filtered coffee may be associated with a risk of cardiovascular disease [Ranheim and Halvorsen, 2005].

Figure 2-2: Chemical structures of cafestol and kahweol.

Cafestol and kahweol show cholesterol raising potential on one hand and on the other hand chemopreventive potential is reported [Pardo Lozano et al., 2007]. In the first case there is still some confusion, as to whether these components are involved in the deposition of LDL cholesterol but the oxidation of this lipid fraction is not reported to occur, indicating that the potential negative effects of coffee are not as high as hypothesized in the literature [Suzuki et al., 2008].

A number of studies have examined the association between boiled coffee consumption and cardiovascular disease. There is good evidence that nonfiltered, boiled coffee such as Scandinavian-type boiled coffee, cafetière (or French-press) coffee, and Turkish coffee increase serum cholesterol levels. Elevated concentrations of plasma triacylglycerol and LDL cholesterol are important risk factors for the development of cardiovascular disease. In contrast to boiled coffee, consumption of filtered coffee was found to have little or no association with serum concentration of cholesterol [Pietinen et al., 1990; Ranheim and

Halvorsen, 2005]. Unfiltered coffee brews contain 3–6 mg of cafestol per cup. Filtered coffee does not contain cafestol because the diterpene is retained by paper filters [de Roos et al., 2001].

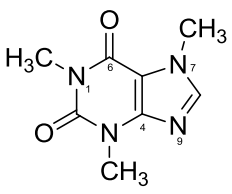
In humans, most triacylglycerol is transported in VLDL (Very Low Density Lipoprotein) particles. VLDL can be further fractionated into two metabolically discrete components: $VLDL_1$ and $VLDL_2$. Compared with $VLDL_1$, $VLDL_2$ particles are smaller, enriched in cholesteryl ester, depleted in triacylglycerol, and have a lower ratio of apolipopotein (apo) E and apo C to apo B. $VLDL_1$ carries almost one-half of the total amount of triacylglycerol in the circulation, whereas $VLDL_2$ carries only about one-fifth. High concentrations of triacylglycerol-rich $VLDL_1$ have been suggested to give rise to a preponderance of small, dense LDL (low density lipoprotein). Small LDL particles appear to be a higher risk factor for the development of cardiovascular disease than the larger. Cafestol raises plasma triacylglycerol predominantly through an increased production rate of $VLDL_1$ apo B after 2 wk of consumption. [de Roos et al., 2001]. In addition, *in vitro* studies suggest that cafestol elevates plasma LDL-cholesterol concentrations at least in part by down-regulation of the LDL receptor. Down-regulation of the LDL receptor appears to be initiated by suppression of bile acid synthesis [van Dam and Feskens, 2002].

Huber *et al.* further suggested that coffee consumers are at lower risk of colon cancer and attributed reduction to high content of the diterpenes, kahweol and cafestol, and their ability to inhibit the CYP450 and SULT (sulfotransferase) enzymes involved in the metabolic activation of carcinogens [Huber et al., 2008].

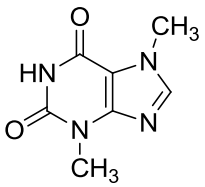
Kahweol and cafestol are effective too in ameliorating H_2O_2-induced oxidative stress and DNA damage, probably via scavenging ROS in mouse embryo fibroblast (NIH3T3) cell line [Lee and Jeong, 2007].

2.3.4 Alkaloids

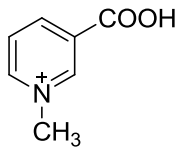
Coffee contains two different kinds of alkaloid delivered from nucleotides (see Figure 2-3). One type are purine alkaloids, such as caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine); while trigonelline (1-methylnicotinic acid) represents pyridine alkaloid, [Ashihara, 2006].



Caffeine



Theobromine



Trigonelline

Figure 2-3: Chemical structures of caffeine, theobromine and trigonelline

2.3.4.1 Purine alkaloids (caffeine)

Caffeine is the most common purine alkaloid in coffee. A large amount of caffeine is found in coffee seeds (mainly in endosperms), but also is found in the pericarp of fruits and leaves of coffee plants [Zheng and Ashihara, 2004]. It has been proposed that caffeine is sequestered in the vacuoles of coffee leaves as a chlorogenic acid complex, in a coffee drink, 10% of the caffeine and about 6% of the chlorogenic acid present occur in this form [Aerts and Baumann, 1994].

Caffeine crystallizes with one molecule of water into silky, white needles, which melt at 236.5 °C and sublime without decomposition at 178 °C. This alkaloid is mildly bitter in taste and has a threshold value in water of 0.8–1.2 mmol/L. The caffeine content of raw green Arabica coffee is 0.9–1.4 %, and 1.5–2.6 % in Robusta. Other purine alkaloids found in coffee are theobromine (Arabica: 36–40 mg⁄kg, Robusta: 26–82 mg⁄kg) and theophylline (Arabica: 7–23 µg⁄kg, Robusta: 86–344 µg⁄kg) which represent demethylated caffeine derivates [Belitz et al., 2009].

The caffeine content of coffee beans is only slightly decreased during roasting. The loss is limited by the increase of the sublimation point of caffeine during roasting by increase of the pressure within the bean and by a poor rate of diffusion through the outer layers. The solubility of caffeine increases more than 10 times from room temperature to 80 °C and can thereby easily be extracted from the coffee beans and grounded coffee. Caffeine is odorless and has a bitter taste. It contributes only to about 10% of the perceived bitterness of brewed coffee beverages. This sensory characteristic is difficult to distinguish, even by a trained panel of assessors, from the astringency caused simultaneously by phenolic compounds [Lee et al., 1992].

For the production of decaffeinated products, decaffeination is performed prior to the roasting process. The most common and least costly caffeine extraction methods use organic solvents such as dichloromethane or ethyl acetate, associated with the use of water/vapor prior to and after extraction. Beans are then dried until they reach a moisture level similar to that prior to processing. Water alone has alternatively been used to replace organic solvents in the process. At the end of the process, caffeine content is usually reduced to 0.02-0.3% [Farah et al., 2006]. Another possibility for the decaffeination is the carbon dioxide method (supercritical fluid extraction), where the caffeine is dissolved from the beans by carbon dioxide in the supercritical fluid state at high pressure. Under certain conditions it allows for a selective caffeine extraction and leaves most of the other coffee bean constituents unaltered. This process has the advantage that it avoids the use of potentially harmful substances [ICO, 2011].

2.3.4.1.1 Caffeine metabolism

Following ingestion, caffeine is rapidly and completely absorbed from the gastrointestinal tract and maximum caffeine concentrations in blood are reached within 1–1.5 h. Absorbed caffeine is readily distributed throughout the entire body. It is lipophilic and penetrates all biologic membranes, passes across the blood–brain barrier, through the placenta into amniotic fluid and the foetus, and into breast milk [Eteng et al., 1997; Heckman et al., 2010; Nawrot et al., 2003].

Caffeine is almost completely metabolized in the liver by CYP1A2 (cytochrome P450 1A2) exclusively to its principal metabolite paraxanthine (1,7-methylxanthine). The metabolic profile of the biotransformation by CYP1A2 averaged 81.5% for paraxanthine, 10.8% for theobromine and 5.4% for theophylline. Formation of the primary metabolites, theophylline and theobromine, is mediated by CYP2E1 and CYP1A2. The main metabolic routes of caffeine are illustrated in Figure 2-4. In the complex metabolism of caffeine, CYP1A2 is solely responsible for the caffeine 3-demethylation to paraxanthine and mainly for the 7-demethylation of 1,7-dimethylxanthine. Thus, paraxanthine is both a product and a substrate of CYP1A2. Paraxanthine further undergoes two reactions, one of which is 8-hydroxylation by CYP2A6 and also CYP1A2. The other reaction, 7-demethylation via CYP1A2, leads to an unstable intermediate (UI). This ringopened compound gives two competing products: 1-methylxanthine and the acetylated product 5-acetylamino-6-formylamino-3-methyluracil (AFMU). 1-methylxanthine undergoes 8-hydroxylation to 1-methyluric acid, mediated by xanthine oxidase with a little contribution of CYP1A2. The polymorphic *N*-acetyltransferase NAT2 mediates the step toward AFMU. CYP1A2 and CYP2E1 catalyze the *N*-demethylation of caffeine at N1 and N7 to produce theobromine and theophylline, respectively. Various intermediates of the pathway have been isolated from the urine of humans and experimental animals fed caffeine rich diets [Eteng et al., 1997; Heckman et al., 2010; Sinues et al., 1999]. Caffeine pharmacokinetics show dose-dependency with greater maximal concentrations, longer half-lives, and lower clearances at higher doses. Longer half-lifes are seen in patients with chronic liver disease whereas shorter half-lifes are observed in smokers [Chou, 1992; Gu et al., 1992; Kaplan et al., 1997].

Caffeine clearance and the paraxanthine/caffeine $AUC_{0–24}$ (area under the curve from 0 time to 24 h) metabolic ratio in plasma are regarded as the ‘gold standard’ measurements for CYP1A2 activity, but the paraxanthine-to caffeine concentration ratio at one time point (usually 4 h) has also shown a high correlation with caffeine clearance as estimated from plasma, saliva and serum samples [Gu et al., 1992; Perera et al., 2010].

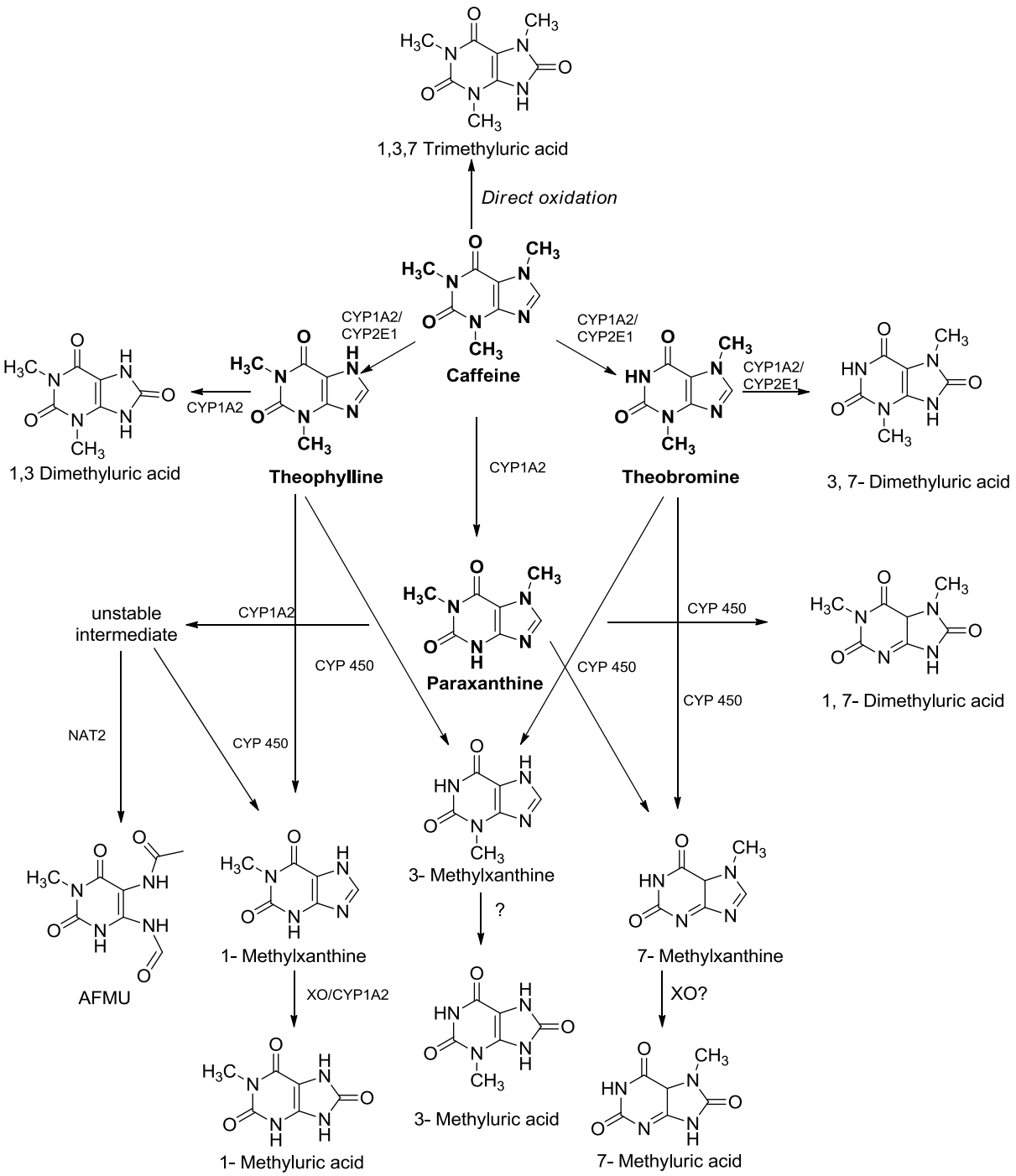


Figure 2-4: Pathways of caffeine metabolism [Eteng et al., 1997; Heckman et al., 2010; Sinues et al., 1999]. CYP 450= cytochrome P450; CYP1A2=cytochrome P450 1A2A; AFMU= 5-acetylamino-6-formylamino-3-methyluracil**;** NAT2= N-acetyltransferase**;** XO=Xanthine oxidase

2.3.4.1.2 Biofunctional effects of caffeine

Caffeine has been widely studied in a variety of areas regarding human health effects. Many studies substantiate caffeine's ability to elicit a diverse number of pharmacological responses, including increased vigilance, decreased psychomotor reaction time, increased sleep latency and waking time and may also influence intellectual performance. Moreover, caffeine causes relaxation of smooth muscles, enhances the secretion of gastric acid and the

release of catecholamines, and increases metabolic activity. The precise mechanism(s) underlying the actions of caffeine remain poorly understood. The biological activity of caffeine could at least in part be based on its structural similarity to biologically important compounds such as adenine, guanine, adenosine, xanthine, and uric acid. A first hypothesis was that caffeine and other methylxanthines primarily acted as inhibitors of phosphodiesterases (PDEs). Although the inhibition of PDEs may contribute to the actions of caffeine, there is growing evidence that most pharmacological effects result from antagonism of adenosine receptors. At blood concentrations resulting from normal coffee consumption, caffeine acts mainly as an adenosine receptor antagonist, while the main caffeine metabolite, paraxanthine, has been shown to inhibit the effect of endogenous adenosine on a variety of physiological processes [Fredholm et al., 1999; Heckman et al., 2010; Varani et al., 2000]. The topic about adenosine and adenosine receptors will be discussed in detail in chapter No 2.5.2.1 and 2.5.2.2.

Epidemiologic studies have shown some evidence that caffeine/coffee could be an effective therapeutic against Alzheimer's (AD) and Parkinson's disease (PD). Cross-sectional human studies have reported that caffeine consumption in both young and aged normal adults is associated with better cognitive performance [Arendash and Cao, 2010; Hameleers et al., 2000; Jarvis, 1993]. Maia and de Mendonca [Maia and de Mendonca, 2002] reported that AD patients consumed markedly less caffeine during the 20 years preceding diagnosis of AD, compared with age-matched individuals without AD.

Caffeine may play a positive role is in the prevention of sunlight-induced skin cancer. The main mutagenic effect of UV radiation is DNA damage in which research suggests caffeine has a protective role in both mice and humans [Kerzendorfer and O'Driscoll, 2009].

T2D is a global health concern estimated to have affected 346 million people worldwide in 2004, approximately 3.4 million people died in the same year from consequences of high blood sugar. WHO projects that related diabetes deaths will double between 2005 and 2030 [WHO, 2011]. There are several studies that have investigated the association between caffeine, particularly caffeine from coffee consumption, and the relative risk for developing type 2 DM. A long-term study [Salazar-Martinez et al., 2004] (in the framework of the The Nurses' Health Study and Health Professionals' Follow-up Study) on consumption of regular coffee, decaffeinated coffee, and other caffeinated beverages, showed an inverse relationship between intakes of caffeine and regular coffee and incidence of DM in both men and women. This relationship was stronger in women than in men. The researchers did not find a relationship between decaffeinated coffee intake and T2D. Rosengren and coworkers [Rosengren et al., 2004] reported that more than two cups of coffee per day had potentially beneficial effects and protected from the development of diabetes in Swedish women. The data indicated that some components in coffee may have a protective effect with respect to

the development of diabetes in women, and this supports the findings of the large-scale, prospective cohorts of van Dam [van Dam et al., 2004], Tuomilehto [Tuomilehto et al., 2004], and Salzar-Martinez [Salazar-Martinez et al., 2004]. In contrast, short-term metabolic studies have shown that caffeine ingestion can acutely decrease insulin sensitivity and glucose disposal [Greer et al., 2001; Keijzers et al., 2002]. Van Dam *et al.* examined the effects of coffee and caffeine on fasting blood glucose and insulin over 2–4 weeks in healthy subjects. They reported that high coffee consumption for 4 weeks increased fasting insulin concentrations compared to no coffee intake, while no such effect was seen with “weaker coffee”. The authors suggested that the increased fasting insulin concentrations reflected decreased insulin sensitivity. No effect on fasting glucose concentrations was observed [Ranheim and Halvorsen, 2005; van Dam et al., 2004]. While the role of caffeine in lowering the risk of type 2 DM is not totally clear, it is evident that the consumption of caffeinated beverages is not associated with increasing the risk for type 2 DM [Pereira et al., 2006].

One hypothesis explaining how coffee alters type 2 DM risk involves weight management and body composition. There is evidence of the association between caffeine intake and weight reduction. Caffeine supplementation has also been recently considered as an effective means of weight management. This alkaloid has been shown to induce thermogenesis, lipolysis, fat oxidation, and insulin secretion in both nonobese and obese individuals [Dulloo et al., 1999; Greenberg et al., 2006; Kovacs et al., 2004]. Individuals who consume coffee on a regular basis would be expected to have a lower body mass, a factor that would lower type 2 DM risk. There is little evidence that coffee consumption promotes significant weight loss or causes alterations in body composition. This appears to be due to habituation to caffeine induced catecholamine responses and lipolysis with prolonged use [Dekker et al., 2007].

A large prospective study reported that increased caffeine intake correlated with lower weight gain [Lopez-Garcia et al., 2006a]. However, the amount was only ~0.5 kg over a 12 year period. A similar effect was seen in decaffeinated coffee consumers.

In addition, caffeine has been shown to exhibit several biological effects, such as increased fat oxidation and mobilization of glycogen in muscle, increased lipolysis, and decreased body fat [Astrup et al., 1990; Ranheim and Halvorsen, 2005; Spriet et al., 1992] and also paraxanthine may play a role in increased lipolysis after caffeine administration in humans [Hetzler et al., 1990].

2.3.4.1.3 Caffeine/coffee safety and adverse effects

Caffeine induced pharmacological and toxicological effects in adults can present a spectrum of clinical symptoms, ranging from nervousness, irritability and insomnia to sensory disturbances, diuresis, arrhythmia, tachycardia, elevated respiration and gastrointestinal

disturbances. Caffeine toxicity in children is manifested by severe emesis, tachycardia, central nervous system agitation and diuresis. Chronic exposure to caffeine has been implicated in a range of dysfunctions involving the gastrointestinal system, liver, renal system and musculature. The acute lethal dose in adult humans has been estimated to be 10 g/person. Death has been reported after ingestion of 6.5 g caffeine, but survival of a patient who allegedly ingested 24 g caffeine was also reported [James, 1991].

Dependency on caffeine is characterized by the development of tolerance, as reflected by increases in dose size with time, persistent desire, and withdrawal symptoms. Withdrawal symptoms include headaches, irritability, anxiety, depression, drowsiness, and fatigue [Greenberg et al., 2006].

Many diseases have been associated with caffeine and coffee consumption. Caffeine and coffee intake have often been associated with hypertension. Blood pressure, as discussed earlier, changes initially but shows little abnormality in the long term. The acute effect of caffeine on blood pressure (BP) is well documented, with increases in the range of 5 to 10 mmHg. Despite the popular belief that tolerance occurs with habitual consumption, it has been demonstrated that tolerance to caffeine related blood pressure effects is incomplete following daily structured intake. However it has been shown that caffeine resulted in an increase in BP in healthy, normotensive, young and older men and women, but the effects of caffeine still not well understood in specific subgroups [Farag et al., 2010].

In summary, the data currently available indicate that moderate caffeine intake (four or fewer cups of coffee per day, or ≤400mg caffeine/day) does not adversely affect cardiovascular health. There are insufficient epidemiological data to draw any conclusions about the risk for coronary heart disease or mortality associated with consumption of 10 or more cups of coffee per day (≥1000 mg caffeine/day) [Nawrot et al., 2003].

Caffeine is globally approved by numerous regulatory authorities as a safe dietary ingredient for use mainly in carbonatedbeverages and dietary supplements. The evaluation and approval process that caffeine underwent has been based on scientific evidence that supported its safety. In 1959, the U.S. Food and Drug Administration (FDA) classified the caffeine in cola drinks to be generally recognized as safe (GRAS, code #21CFR). Since then, the FDA continuosly reevaluates caffeine’s safety as a food ingredient. The latest FDA evaluation stated that there was no evidence to show that the use of caffeine would cause any negative health effects; therefore, caffeine continues to have GRAS status [FDA, 2011].

After reviewing over 200 scientific studies focused on the reproductive effects of caffeine Christian and coworkers [Christian and Brent, 2001] concluded that woman who consumed moderate amounts of caffeine, less than 5 to 6 mg/kg bw/d, do not have an increase in reproductive risks. In 2002, the International Life Sciences Institute (ILSI) assembled another expert panel, to focus on recent caffeine-related research in the area of behavior and

development in children and adults, withdrawal and related issues, bone, and calcium physiology, and reproductive risks in women [Mandel, 2002]. The weight of evidence supported the conclusion that a moderate intake of caffeine does not elevate the risks for adverse effects.

Additionally, caffeine is not present in the category of substances classified as causing "substance dependence" by the American Psychiatric Association, nor is considered a prohibited substance by the World Anti-Doping Agency (WADA) [WADA, 2011].

Recent evidence suggests that there is no overall association between moderate coffee intake and coronary heart disease (CHD). Cardiovascular disease (CVD) is a multi-factorial condition with a wide variety of risk factors, such as high blood pressure, DM, smoking and high serum cholesterol. Heredity plays a role in coronary heart disease and stroke, but both are largely influenced by lifestyle factors such as diet, physical activity and smoking. A large meta-analysis of 21 studies on coffee consumption and coronary heart disease risk found a large variation between the individual results of the studies reviewed. Overall, it did not find a statistically significant association between coffee drinking and long-term risk of coronary heart disease. In fact, habitual moderate coffee consumption was shown to be associated with a lower risk of heart disease in women [Wu et al., 2009]. A prospective cohort study found no association between caffeine and CHD [Lopez-Garcia et al., 2006b] .

Elevated plasma homocysteine (Hcy) concentrations are associated with an increased risk of vascular disease and foetal birth defects. Coffee consumption positively correlates with plasma Hcy concentration [Stolzenberg-Solomon et al., 1999]. Filtered [Urgert et al., 2000] and unfiltered [Grubben et al., 2000] coffee increase plasma Hcy concentration by approximately 10%, while abstention from filtered coffee reduces plasma Hcy [Christensen et al., 2001]. The effect of Hcy can not solely be attributed to caffeine [Verhoef et al., 2002], other components of coffee like chlorogenic acid is shown to inhibit the methylation of Hcy by betaine, catalysed by betaine-homocysteine methyltransferase (see Chapter 2.3.4.2 and 2.3.5).

2.3.4.2 Pyridine alkaloids (trigonelline)

Trigonelline (see Figure 2-3) was first isolated from fenugreek (*Trigonella foenum-graecum*) and is found in various plants and in some animal species including sea urchins and jellyfish. It also appears in mammalian urine after administration of nicotinic acid. Trigonelline in raw coffee materials ('green beans') is thermally converted to nicotinic acid and to certain flavour compounds during roasting [Mazzafera, 1991].

Dry coffee contains 0.6–1.3% trigonelline. When the green beans are roasted, the nicotinic acid increased to a level of 3.6 – 53.4 mg/100 g because of a progressive demethylation of trigonelline and decarboxylation of quinolic acid [Casal et al., 2000; Minamisawa et al., 2004].

A single cup of coffee contains 24–86.2 mg of trigonelline, depending on the origin of the beans and degree of roast [Minamisawa et al., 2004].

In fact, trigonelline decomposes via two major routes under typical temperatures as used during coffee roasting, that is, (1) decarboxylation and methyl rearrangement to form pyridines and (2) N-demethylation to give nicotinic acid. Depending on the nature of the trigonelline salt in the coffee bean, the degradation route may indeed favor the formation of 1-methylpyridinium (see 2.3.4.3), which will have a direct and indirect impact on other physicochemical properties such as flavour and aroma [Stadler et al., 2002a]. Trigonelline has little influence on the quality of coffee brew. Its bitter taste is approximately a quarter of that of caffeine, but its thermal degradation products have more sensory and nutritional importance e.g. consumption of 3.5 standard cups of coffee per day contributes up to one-third of the minimum dietary requirement for an adult of nicotinic acid [Flament and Bessiere-Thomas, 2002].

Trigonelline is believed to have anti-migraine, anti-carcinogenic, antiseptic, antioxidative [Hirakawa et al., 2005], hypoglycemic [Farah et al., 2006; van Dam, 2006] and hypocholesterolemic activities [Zhou et al., 2011]. It may also act as a stimulant and sedative. There are no regulations in place regarding the production or use of trigonelline. There is no evidence that human exposure to trigonelline causes any level of toxicity. Also, no severe side effects of trigonelline have been noted so far.

In 1997 Wu *et al*. detected mutagenic activity with metabolic activation of heated trigonelline samples; however, higher mutagenic activity was found for trigonelline and its combinations without metabolic activation, which suggests that other types of mutagens (direct-acting) were predominant [Wu et al., 1997].

As previously mentioned, coffee consumption correlates with plasma Hcy concentration, some components of coffee could inhibit the methylation of Hcy by betaine, catalysed by betaine-homocysteine methyltransferase (BHMT). Although trigonelline is an analogue of glycine betaine that may competitively inhibit BHMT, is not responsible for the increase in Hcy after coffee consumption, this effect is mainly attributable to caffeine and chlorogenic acid (see 2.3.4.1.3 and 2.3.5) [Slow et al., 2004].

2.3.4.3 N-methylpyridinium (NMP)

The quaternary base N-methylpyridinium (see Figure 2-5) is a major reaction product of trigonelline subjected to pyrolytic conditions. This quaternary base is formed by decarboxylation and represents an important stable intermediate in the thermal reaction pathways of trigonelline. As mentioned earlier, two major parameters have a pronounced impact on the degradation route and formation of N-methylpyridinium: the temperature and the nature of the trigonelline salt [Stadler et al., 2002a].

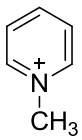


Figure 2-5: Chemical structure of N-methylpyridinium (NMP).

N-methylpyridinium *per se* may further react with nucleophiles present in the reaction mixture, e.g. substituted pyridines, and forms alkylpyridiniums such as 1,2-, 1,3-, and/or 1,4-dimethylpyridinium. Thermally induced methyl rearrangements of quaternary salts such as 1-methylpyridine can procure a range of volatile products in the presence of a nucleophile such as pyridine, in particular good yields of 2- and 4-methylpyridine (30.1 and 34.6%, respectively), and minor formation of 3-methylpyridine (5.8%) and ethylpyridines (1.4%) were observed. Alkylpyridines thus act as nucleophiles and participate in methyl displacement reactions with 1-methylpyridinium to afford pyridine and the new disubstituted dialkypyridinium products (see Figure 2-6) [Stadler et al., 2002a].

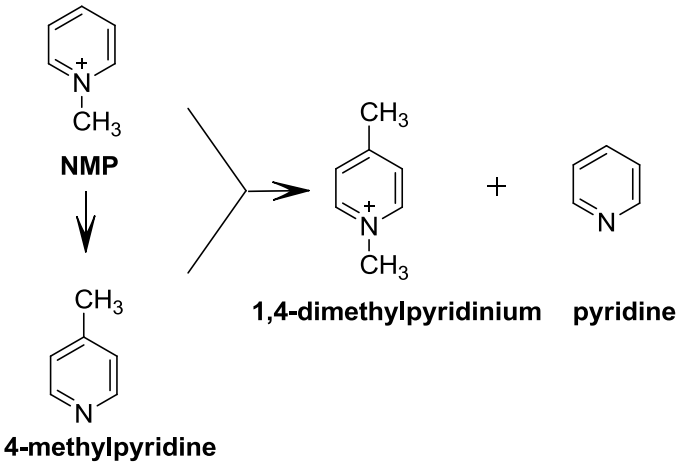


Figure 2-6: Reaction pathways leading to the formation of alkylpiridiniums [Stadler et al., 2002a].

The levels of NMP in roasted coffee are positively correlated to the degree of roasting. As already mentioned, NMP is not present in raw coffee beans but it is formed during the roasting process from its chemical precursor, trigonelline. The degradation is about 50% of the trigonelline content and the concentration of N-methylpyridinium in roasted coffee is up to 0.25% on a dry weight basis [Stadler et al., 2002b].

Additionally, NMP has been identified as a suitable dietary biomarker for coffee consumption [Lang et al., 2011]. The reported method allows the precise quantitative analysis of NMP besides trigonelline and creatinine and can be considered a non-invasive, high-throughput technology enabling the rapid and accurate analysis of NMP as a coffee-specific candidate biomarker.

NMP has been shown to have a chemopreventive activity *in vitro* and *in vivo*. Evidence is supported by the shown induction of chemopreventive phase II enzymes by dietary coffee beverages containing *N*-methylpyridinium [Somoza et al., 2003]. The molecular mechanism for this induction is hypothesized to be based on kinase-mediated reactions rather than binding to the arylhydrocarbon receptor (AhR) which is the main receptor involved in the phase I/II enzyme induction [Iba et al., 2002].

2.3.5 Chlorogenic acids

In coffee, chlorogenic acids (CGA) are the major antioxidants. They are a family of esters formed between transcinnamic acids, mainly caffeic or ferulic acid, and quinic acid. CGA can be ester-linked of several positions, and there can be one or two phenolic acids per quinic acid moiety, resulting in a complex pattern. The main nine isomers in green coffee beans are: 5-CQA (5-caffeoylquinic acid), 4-CQA, 3-CQA, 3,5-diCQA, 4,5-diCQA, 3,4-diCQA, 5-FQA (5-feruloyl quinic acid), 4-FQA and 3-FQA (see Figure 2-7) which depends on variety, location, roasting and processing [Clifford, 2000; Williamson et al., 2011].

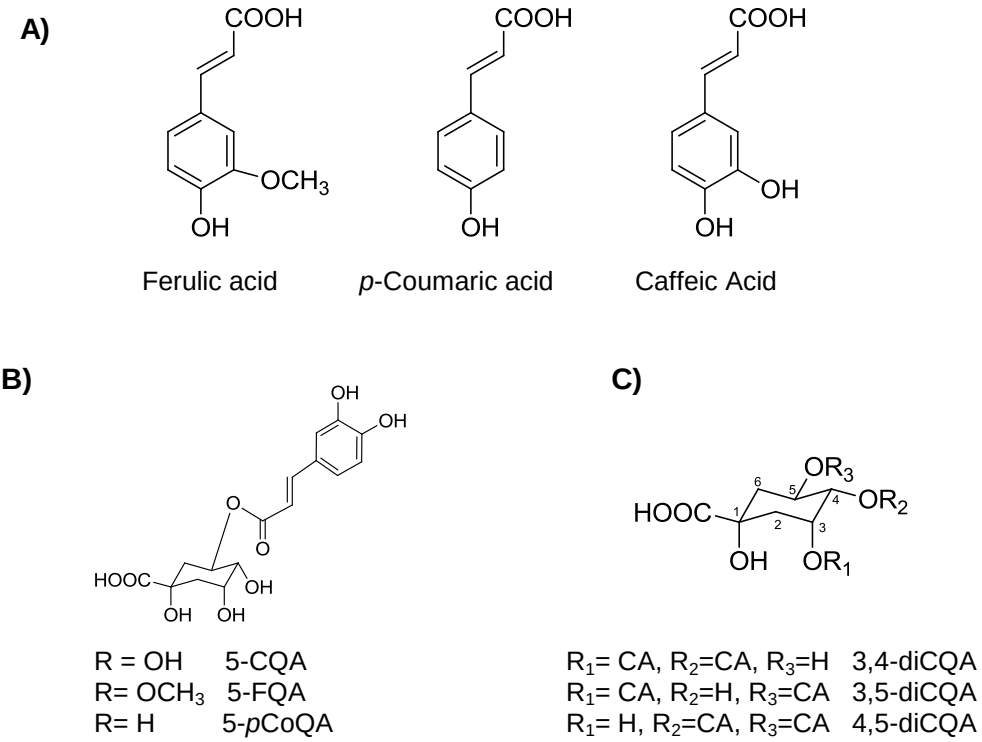


Figure 2-7: Chemical structures of monomeric hydroxycinnamates (A). Main chlorogenic acid monoesters (B) and diesters (C) of quinic acid (QA). Esterification also occurs in carbons 3 and 4 of the quinic acid. CA=caffeic acid, FA= ferulic acid, *p*CoA= coumaric acid, CQA= caffeoylquinic acid, FQA= feruloylquinic acid, diCQA= dicaffeoylquinic, *p*-couma-roylquinic acids (*p*-CQA).

Coffee roasting causes a progressive destruction and transformation of CGA with some 8±10% being lost for every 1% loss of dry matter. During the early stages, CGA isomerisation occurs accompanied by partial hydrolysis yielding quinic acid and the various

cinnamic acids. The released cinnamates may be decarboxylated, degraded to a range of simple phenols and further transformed. CGA in which position 5 is not substituted may lactonise forming caffeoyl and feruloyl-1,5-γ-quinides referred to collectively as chlorogenic lactones (CGL) [Clifford, 2000].

The CGA content in brewed coffee is influenced by the used coffee beans. Arabica beans contain less CGA than Robusta varieties. The roasting method also plays an important role in the CGA content of the final coffee product. For example, the light medium roasting condition was found to result in the highest amount of transformation from CGA to the corresponding lactones, suggesting that this process reduced the amount of CGA in coffee [Farah et al., 2005].

During the decaffeination process, losses of key flavour components of coffee generally occur, especially when solvents that lack specificity, such as water are used. Among the compounds lost may be the chlorogenic acids (CGA) and their related compounds [Farah et al., 2006].

When consumed by healthy volunteers, phenolic acids from coffee are absorbed and metabolized by complex pathways involving reduction, hydrolysis, methylation, sulfation and glucuronidation. Phenolic acids are partially absorbed in the small intestine, but mostly after microbial catabolism in the colon. More studies on chlorogenic acid bioavailability are needed on healthy humans after consumption of coffee, including dose–response studies [Del Rio et al., 2010; Williamson et al., 2011].

Ferulic acid and *p*-coumaric acid are often ester-linked to plant cell wall polymers and cannot be absorbed in this complex form. Enzymes able to break the ester bonds and release free phenolic acids are therefore required as the first step in the uptake and metabolism of dietary hydroxycinnamates. The presence of such esterase activity has been demonstrated in the large intestine (microflora enzymes) of rats and humans. The esterase(s) are able to hydrolyze and release the hydroxycinnamates ferulate and *p*-coumarate, which then were available for absorption into the circulatory system [Andreasen et al., 2001]. An esterase activity able to hydrolyze chlorogenic acid has been demonstrated in human colonic microflora [Plumb et al., 1999]. Chlorogenic and caffeic acid can also be absorbed from the small intestine into the circulatory system of humans [Olthof et al., 2001a].

A series of health benefits have been associated with the consumption of CGA and their lactones, such as reduction of the relative risk of cardiovascular disease, T2D, and Alzheimer's disease [Ranheim and Halvorsen, 2005]. Even antibacterial and antiinflammatory activities were reported [Almeida et al., 2006; dos Santos et al., 2006].

Different studies have shown the antioxidant activity of CGA and caffeic acid, mainly by using *in vitro* studies but also as a result of *in vivo* and *ex vivo* investigations [Bakuradze et al., 2010; Gomez-Ruiz et al., 2007; Nenadis et al., 2004].

Since only one-third of ingested CGA is absorbed in the small intestine, large amounts reach the colon, where it is hydrolyzed by the microflora to caffeic acid and quinic acid [Olthof et al., 2001a]. These compounds are extensively metabolized to a range of products including *m*-coumaric, dihydroferulic, 3-hydroxyphenylpropionic, and hippuric acids [Olthof et al., 2003]. Some authors attribute the antioxidant activity especially to these phenolic compounds generated from CGA by microbial metabolism [Gomez-Ruiz et al., 2007; Nenadis et al., 2004], other to the presence of original constituents particularly 5-CQAs and its isomers 3- and 4-CQA [Bakuradze et al., 2010]. Some authors point out that CGA represents a potent activator of Nrf2 translocation in different cell lines [Boettler et al., 2011a; Feng et al., 2005].

Johnston *et al.*[Johnston et al., 2003] reported that the ingestion of either caffeinated or decaffeinated coffee containing equal amounts of chlorogenic acid and glucose caused acute changes in gastrointestinal hormone concentrations. They concluded that chlorogenic acid attenuated the rate of glucose uptake in the proximal small intestine and moved it to more distal regions of the small intestine. Their findings suggest that chlorogenic acid or some other noncaffeine coffee constituents antagonizes caffeine's stimulation of glucose uptake in the small intestine. Additionally, Nieuwenhoven *et al* [Van Nieuwenhoven et al., 2000] using a sugar absorption test of intestinal permeability shown that the addition of caffeine to a sports drink expedited glucose uptake in the small bowel in 10 athletes. The implication is that chlorogenic acid slows the absorption of glucose from the gut, whereas caffeine accelerates it. However, 3 wk of intravenous infusion of chlorogenic acid significantly lowered the postprandial peak response to a glucose challenge in insulin-resistant Zucker rats [Rodriguez de Sotillo and Hadley, 2002]. Several mechanisms have been suggested for the effects of chlorogenic acid on glucose metabolism including decrease of glucose output in the liver, helping preserve *β*-cell function by promoting the synthesis of the homeodomain transcription factor IDX-1 (which helps beta cells respond to increases in plasma glucose) [Greenberg et al., 2006; McCarty, 2005], inhibition of glucosidase and glucose-6-phosphatase (suggesting that it may delay intestinal glucose uptake and also reduce hepatic glucose output) and a stimulatory effect on the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) [Olthof et al., 2011]. Manzano and Williamson [Manzano and Williamson, 2010] showed that polyphenols, phenolic acids and tannins-rich extracts from strawberry and apple were able to influence glucose uptake into the cells and transport to the basolateral side by inhibiting activities of the glucose transporters (GLUT2 >SGLT1).

Chlorogenic acid is marketed under the tradename Svetol® in Norway and the United Kingdom as a food active ingredient used in coffee, chewing gum, and mints to promote weight reduction. There is evidence that Svetol® significantly inhibited glucose-6-phosphate

hydrolysis in intact human liver microsomes in a competitive manner [Henry-Vitrac et al., 2010].

A correlation between chlorogenic acids and stomach discomfort after ingestion of coffee brew has been described [Weiss et al., 2010]. Moreover there is some evidence that CGA raise total Hcy concentrations in plasma and could be partly responsible for the higher homocysteine concentrations observed in coffee drinkers [Olthof et al., 2001b].

2.3.6 Pyrazines

Pyrazines are monocyclic heteroaromatic substances containing nitrogen atoms in the 1- and 4-position of the aromatic ring (see Figure 2-8). Many substituted pyrazines occur naturally. These pyrazines carry substituents at one or more of the four ring carbon atoms. Alkylpyrazines contain only carbon and hydrogen substituents. From these alkylpyrazines alone, 70 different compounds have been identified in nature. Other substituents include oxygenated functional groups like alkoxy groups and acyl groups or sulphur containing thiol or sulphide groups [Adams et al., 2002; Wagner et al., 1999].

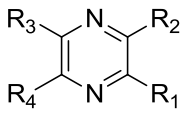
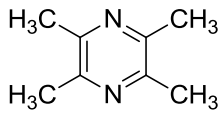


Figure 2-8: General structure of pyrazines.

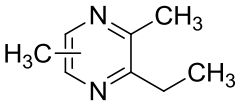
Many of these compounds have intensive smells and very low odour threshold values. Therefore, these compounds contribute to many aromas and flavours. In coffee, a number of methyl-, dimethyl-, ethyl-, diethyl- and mixed ethyl–methyl pyrazines have been detected and are considered to be important for flavour and aroma [Adams et al., 2002; Czerny et al., 1999; Muller and Rappert, 2010; Richling et al., 2005]. In 2000, Czerny and Grosch [Czerny and Grosch, 2000] demonstrated that the aroma of raw coffee is primarily caused by 3-isobutyl-2-methoxypyrazine (3-Isobutyl-2-methoxyP). Parallel, 2-methoxy-3,5 dimethylpyrazine (2-Methoxy-3,5-DMP) was identified as an additional aroma-active compound of raw as well as roasted coffee. During roasting, the concentrations of other aroma compounds (like methional and phenols) increase drastically and mask, together with other odorants that are exclusively formed by the roasting process, the peasy odor of 3-isobutyl-2-methoxypyrazine.

From aroma-extract dilution analysis, 2,3-diethyl-5-methylpyrazine (2,3-DE-5-MP) was found to be the dominant component of the aroma of a medium roasted Arabica coffee sample [Grosch, 2001]. On the other hand, our research group identified 2-methylpyrazine (2-MP), 2,5-dimethylpyrazine (2,5-DMP) and 2,6-dimethylpirazine (2,6-DMP) as the most abundant

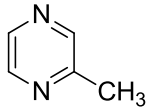
pyrazines in coffee brews [Pickard et al., 2011]. Some of the pyrazines found in coffee are illustrated in Figure 2-9.



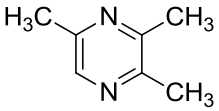
2,3,5,6-Tetramethylpyrazine (TMP)



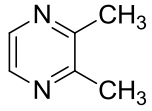
2-Ethyl-3,5(6)-Dimethylpyrazine (2-E-3,5-DMP)



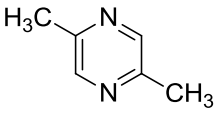
2-Methylpyrazine (2-MP)



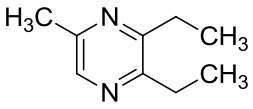
2,3,5-Trimethylpyrazine (2,3,5-TriMP)



2,3-Dimethylpyrazine (2,3-DMP)

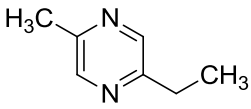


2,5-Dimethylpyrazine (2,5-DMP)

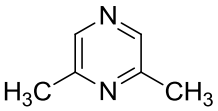


2,3-Diethyl-5-methylpyrazine (2,3-DE-5-MP)

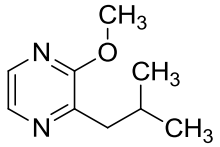
Pyridin



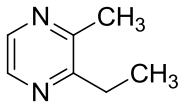
2-Ethyl-5-Methylpyrazine (3-E-5-MP)



2,6-Dimethylpyrazine (2,6- DMP)



2-Isobutyl-3-Methoxypyrazine (2-Isobutyl-3-methoxyP)



2-Ethyl-3-Methylpyrazine (2-E-3-MP)

Figure 2-9: Chemical structure of some pyrazines present in coffee [Czerny and Grosch, 2000; Grosch, 1996, 2001; Richling et al., 2005].

Pyrazines may also be formed by heating of food in the Maillard reaction [Amrani-Hemaimi et al., 1995; Low et al., 2007]. The greatest amounts of pyrazines are formed at temperatures between 120 and 150 °C, but pyrazine formation has also been observed at 70 °C. The nitrogen atoms are derived from amino acids and the carbon atoms from sugars. Amino acids influence the substitution patterns of the pyrazines formed and fructose yields more pyrazines than glucose. Low *et al.* showed [Low et al., 2007] that the formation of alkylpyrazines depends on the amino acid composition.

Several routes have been published for the chemical synthesis of pyrazines. The total annual volume of use of pyrazine and quinoxaline derivatives as flavouring ingredients has been estimated to be 2135 kg in the USA. The production in 2002 was 347 kg of 2,3,5-TriMP, 144 kg of TMP and 923 kg of acetylpyrazine. The daily uptake of pyrazines added as flavouring agents has been estimated to be 3 µg/kg body weight [Adams et al., 2002].

Pyrazines are important contributors to the flavour of various roasted, toasted, or similarly heated foods. They are thought to arise primarily from a heat-induced condensation between amino acids and sugars (α-dicarbonyl compounds) through the Strecker degradation. Their

concentrations in foods are in the range from approximately 0.001 to 40 ppm [CIVO-TNO, 1999].

Formed usually at temperatures above 100 °C, alkylpyrazines are important products of the Maillard reaction, or nonenzymatic browning. This reaction, initiated with the condensation of a reducing carbohydrate with an amino compound, is mostly responsible for the formation of flavour and color in processed food products. On the sugar side, the reactants are mainly glucose, fructose, maltose, lactose and, to a smaller extent, reducing pentoses, e. g., ribose "On the side of the amino component, amino acids with a primary amino group are more important than those with a secondary because their concentration in foods is usually higher. The reactive carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid, and forms a complex mixture of molecules responsible for a range of odors and flavours. This process is accelerated in an alkaline environment, as the amino groups are deprotonated and, hence, have an increased nucleophilicity. The type of the amino acid determines the resulting flavour" cited from Belitz *et al.* [Belitz et al., 2009].

The most accepted mechanism for pyrazine formation involves the condensation reaction of two α-aminocarbonyl compounds with the formation of a dihydropyrazine, which oxidizes spontaneously to the corresponding pyrazines [Adams et al., 2008].

Nucleophilic compounds like amino acids or amines easily add to the carbonyl function of reducing carbohydrates and form imines (*Schiff bases*). The rearrangement of the imines leads to an aminoketose called Amadori compound (1-amino-1-deoxyketose). Amadori products are only intermediates formed in the course of the Maillard reaction. In spite of their limited stability, these products can be used under certain conditions as an analytical indicator of the extent of the heat treatment of food. Amadori compounds are degraded to 1-, 3-, and 4-deoxydicarbonyl compounds (deoxyosones) in the pH range 4–7 (see Figure 2-10). "The concentrations of Amadori and deoxyosones compounds vary, depending on the reaction conditions (pH value, temperature, time, type and concentration of the educts). As a result, there is a change in the product spectrum and, consequently, in the color, taste, odor, and other properties of the food in each case" cited from Belitz *et al.* [Belitz et al., 2009].

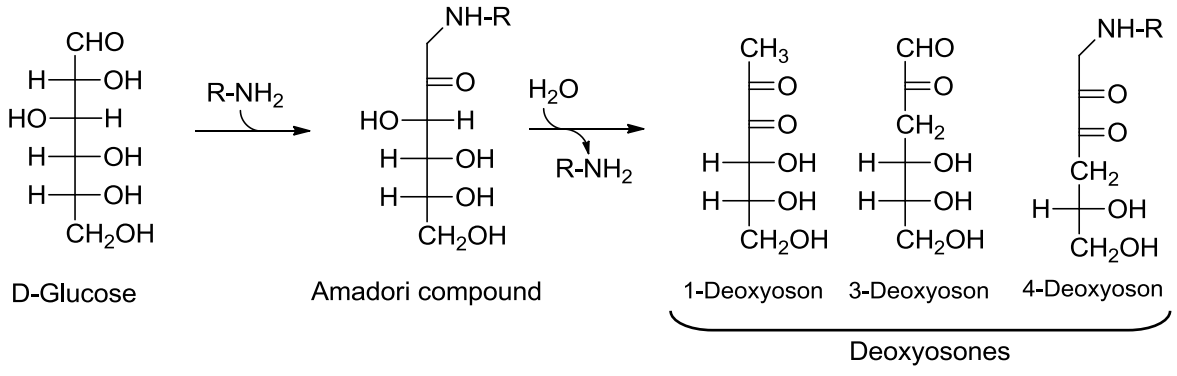


Figure 2-10: Formation of deoxyosones [Belitz et al., 2009]

The reactions between α-dicarbonyl compounds, like the deoxyosones obtained in the Maillard reaction, and amino acids are classed under the term Strecker reaction (see Figure 2-11). This reaction leads to the formation of aldehydes (Strecker aldehydes), CO_2 and α-aminoketones on oxidative decarboxylation of the α-amino acids [Adams et al., 2008].

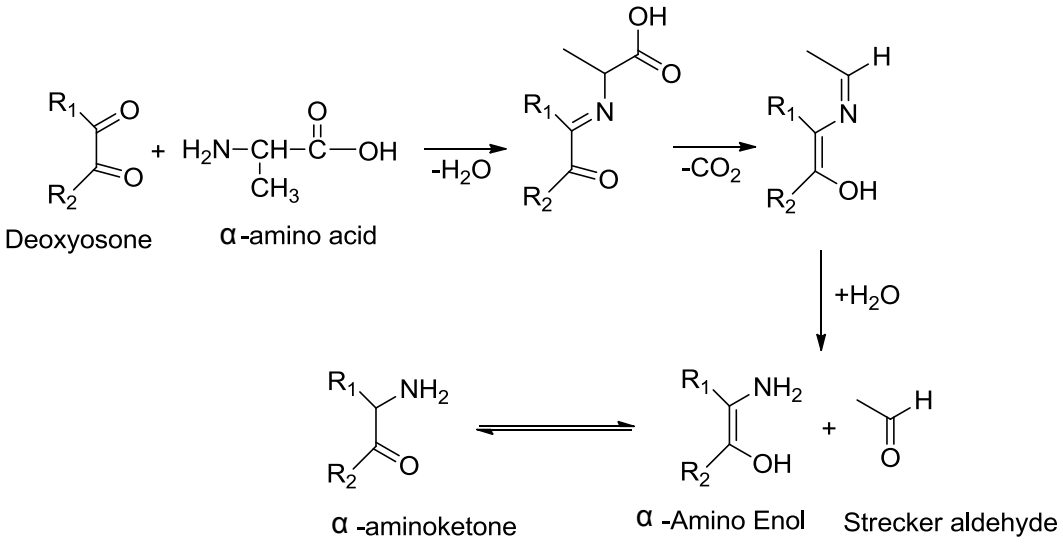


Figure 2-11: Strecker reaction and formation of α-aminoketones.

The condensation of two α-aminoketones and subsequent oxidation to dihydropyrazine results in the formation of pyrazines (see Figure 2-12). When the intermediate dihydropyrazine reacts with a carbonyl compound, an alkylpyrazine with an additional substituent is formed and the oxidation step is not necessary [Shibamoto et al., 1979]. The many studies performed so far show that there is more than one chemical pathway involved in pyrazines formation in complex Maillard systems. Even a very simple model system produces a large number and variety of alkylpyrazines. The availability of various precursors will determine which route predominates [Adams et al., 2008].

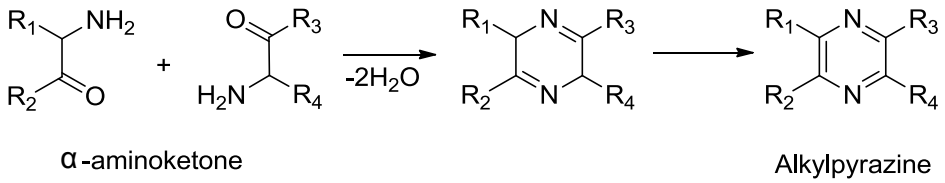


Figure 2-12: Alkylpyrazines formation from α-aminoketones [Shibamoto et al., 1979].

In humans and laboratory rodents, orally administered substituted pyrazines are rapidly absorbed from the gastrointestinal tract and excreted. Approximately 90% of the dose (100 mg/kg) of 2-MP, 2,5-DMP, 2,6-DMP or methoxypyrazine administered to male Wistar rats by a gastric feeding tube was excreted in the urine as polar metabolites within 24 h. More than

50% of the administered dose (100 mg/kg) of 2,3-DMP was recovered in the urine within 24 h [Hawksworth and Scheline, 1975].

In humans and animals, two basic reactions for the conversion of pyrazines have been described. Either an alkyl group is oxidized by P450 type enzymes to form the corresponding alcohols or carboxylic acids or the ring is hydroxylated by molybdenum-containing oxidases of the xanthin oxidase type [Hawksworth and Scheline, 1975]. Carboxylic acids and the hydroxylated pyrazines can then be excreted directly or conjugated with glucuronic acid or glutathione via the kidney [Adams et al., 2002]. There are no reports describing the degradation of the pyrazine ring itself in higher organisms [Muller and Rappert, 2010].

The acute toxicity of the pyrazines that are used as flavouring additives has been found to be very low. The oral LD_{50} in rats ranged from 158 mg/kg for 2-pyrazinethanthiol to more than 4,000 mg/kg for 2-isobutyl-3-methoxypyrazine. In short- and long-term subacute chronic studies, no adverse effects were found, and in *in vitro* and in *in vivo* carcinogenicity, mutagenicity and genotoxicity tests, the influence of pyrazines was also negative. Based on all these findings, the pyrazines have received the GRAS (generally regarded as safe) status by The Flavor and Extract Manufacturers Association for the use as flavouring agents in food [Adams et al., 2002].

Some substituted pyrazines, especially tetramethylpyrazine (TMP) an active ingredient of a commonly used Chinese herb named “Chung Chong” (Ligusticam Wallichii Franch), have been found to have pharmacological effects, such as diuretic [Jones et al., 1967], hepatoprotective [Chen et al., 2011; Kim et al., 1997], antithrombogenic [Sheu et al., 1997; Wang et al., 2008], and tuberculostatic effects [Abdel-Aziz and Abdel-Rahman, 2010; Milczarska et al., 1999]. There is some evidence that TMP scavenges superoxide anions dose-dependently, and decreases the production of nitric oxide significantly [Zhang et al., 2003], has antiinflammatory properties and regulates inflammatory responses at atherosclerotic lesions [Li et al., 2009] and possess anti-apoptotic and neuroprotective effects [Fan et al., 2006].

In 2006, Chen *et al*. [Chen et al., 2006] showed that TMP suppresses ischemia-induced ventricular arrhythmias and reduces the infarct size resulting from ischemia/reperfusion injury *in vivo*. They associated the cardioprotective activity of TMP with its antioxidant activity via induction of HO-1 and with its capacity for neutrophil inhibition (see Chapter 2.4.4).

Methylpyrazines and their metabolites (pyrazinoic acids) exhibited blood lipid-lowering effects in rats [Kagami et al., 2008].

2.3.7 Coffee compounds with undesirable effects

2.3.7.1 Polycyclic aromatic hydrocarbons (PAHs)

Roasting is a crucial step for the production of coffee, as it enables the development of color, aroma, and flavour, which are essential for the characterization of the coffee quality. At the same time, roasting may lead to the formation of not desirable compounds, such as polycyclic aromatic hydrocarbons (PAHs) [Oosterveld et al., 2003; Orecchio et al., 2009].

PAHs are a group of chemicals that are formed by the incomplete combustion of organic matter and are thus generated whenever fossil fuels or vegetation is burned, and they are one of several classes of carcinogenic chemicals present in tobacco smoke. PAHs are also ubiquitous environmental contaminants [Rey-Salgueiro et al., 2008]. Benzo[*a*]pyrene, a five-ring PAH (see Figure 2-13) whose metabolites are mutagenic and highly carcinogenic, usually serves as a lead compound [Eisenbrand and Schreier, 2006].

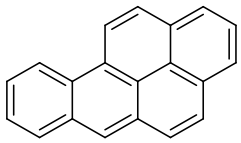


Figure 2-13: Chemical structure of Benzo[*a*]pyrene.

The presence of polycyclic aromatic hydrocarbons (PAHs) in coffee samples has also been reported and may be attributed to either contamination of the initial green beans [Houessou et al., 2006; Houessou et al., 2007] or formation of these compounds during the roasting step [Rey-Salgueiro et al., 2008]. Formation of phenanthrene, anthracene and benzo[*a*] anthracene in coffee beans was observed at temperatures above 220 °C, whereas formation of pyrene and chrysene required 260 °C. The PAH transfer to the infusion was quite moderate (<35%), with a slightly lower extractability for dark-roasted coffee as compared to light-roasted coffee [Houessou et al., 2007].

In 2009, Orecchio *et al*. [Orecchio et al., 2009] have shown that coffee contributes with very insignificant quantities to the daily human intake of carcinogenic PAHs and that the PAHs identified in most of the coffee samples originate from high temperature processes.

2.3.7.2 Furan

Furan is an organic compound with high volatility and lipophilicity. It is formed during commercial or domestic thermal treatment of food. In 2009, European authorities revealed the presence of relatively high furan levels in coffee (see Table 2-5) [EFSA, 2009]. The furan concentration in coffee drink depends on the content of the coffee powder used for brewing, as well as on the type of the brewing procedure. Coffee brews from espresso-type machines

have considerably higher amounts of furan than others, reaching concentrations of 88 µg/L [Kuballa et al., 2005]. According to US-FDA data, an average furan concentration of 52 µg/kg was found in ground coffee, brewed using automatic drip coffee makers [Morehouse et al., 2008]

Table 2-5 Coffee powder furan levels [EFSA, 2009]

Type of roasted coffee	Furan (µg/kg)	
	Average	Maximum
Beans or ground	1691	6500
Beans	2271	4895
Ground	1112	5749
Instant (powder)	588	2200

Furan induces hepatocellular tumors in rats and mice and cholangiocarcinomas in rats with a high incidence. It is assumed that *cis*-2-butene-1,4-dial, the reactive metabolite of furan, is the causative agent leading to toxicity and carcinogenicity. Based on this data, furan is classified as a possible human carcinogen. The initial exposure estimates revealed a relatively small margin between human exposure and those furan doses, which induce liver tumors in experimental animals.

The liver possess high capacity to eliminate furan from the bloodstream by first-pass metabolism. Furan is rapidly metabolized by cytochrome P450 (CYP) enzymes to the *α,β*-unsaturated dialdehyde, *cis*-2-butene-1,4-dial, identified as the major primary metabolite (see Figure 2-14) [Bakhiya and Appel, 2010]. *Cis*-2-butene-1,4-dial is a highly reactive compound and is considered to be the ultimate (geno)toxic intermediate in furan-treated animals. It can bind covalently to cellular proteins and nucleosides. The biotransformation of furan to a protein-binding intermediate was observed in earlier studies both *in vivo* and *in vitro* [Parmar and Burka, 1993].

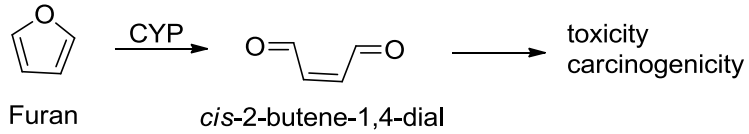


Figure 2-14: Bioactivation of furan to cis-2-butene-1,4-dial.

The margin of exposure (MOEs) for furan was calculated at dietary exposures of 0.001 mg/kg body weight per day, to represent the average dietary exposure to furan for the general population, and 0.002 mg/kg bw per day, to represent the dietary exposure to furan for consumers with high exposure. This estimate will also cover dietary exposure of children. Comparison of these dietary exposures with the lower limit on the benchmark dose for a 10%

induction of hepatocellular adenomas and carcinomas in female mice ($BMDL_{10}$) of 0.96 mg/kg bw per day, gives MOEs of 960 and 480 for average and high dietary exposures, respectively. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered that these MOEs indicate a human health concern for a carcinogenic compound that might act via a DNA-reactive genotoxic metabolite. The furan levels can be reduced in some foods through volatilization (e.g. by heating and stirring canned or jarred foods in an open saucepan). However, there is currently a lack of quantitative data for all foods, and no information is available on other mitigation methods [JECFA, 2010]

2.3.7.3 Acrylamide

Acrylamide (2-propenamide) is by room temperature (RT) a white odourless crystalline solid, soluble in water, ethanol, ether, and chloroform. Due to its α,β-unsaturated carbonyl structure tends acrylamide for polymerization and 1,4-Michael-like additions to nucleophiles [Eisenbrand and Schreier, 2006].

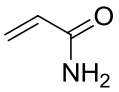


Figure 2-15: Chemical structure of acrylamide (AA).

The main route for the formation of acrylamide in coffee (as in most other concerned foods) is the Maillard reaction, initiated by the condensation of asparagine and reducing carbohydrates, such as fructose or glucose, or, alternatively, reactive carbonyls (including dicarbonyls, n-aldehydes, 2-oxo acids). However, coffee beans are subject to relatively higher temperatures than other foods because of the roasting at 220–250 °C. Under such conditions, more than one pathway, beyond the commonly accepted asparagine/sugar (or carbonyl) condensation, may be expected to furnish acrylamide via the Maillard reaction [Guenther et al., 2007].

Several thermally reactions for the formation of acrylamide are feasible and the salient intermediates are acrolein, acrylic acid and 3-aminopropionamide. Acrolein can be formed by different pathways, including the oxidative degradation of lipids, and could react further via acrylic acid to form acrylamide lipids, and could react further via acrylic acid to form acrylamide. Under certain conditions, acrolein together with asparagine may generate appreciable amounts of acrylamide [Yasuhara et al., 2003]. Acrylic acid can also react with ammonia (released during the thermolysis of amino acids) to furnish acrylamide by aminodehydroxylation, which is a well-known reaction of acids leading to amides [Stadler et al., 2002b; Yaylayan and Stadler, 2005].

Levels of reducing sugars [Guenther et al., 2007] and moisture [Lantz et al., 2006] in green coffees showed no correlation with acrylamide levels after roasting. A factor that could

contribute to relatively higher acrylamide levels is the number of immature beans used in production. Such beans, are characterized by significantly higher amounts of free asparagines (>2-fold) versus mature beans [Mazzafera, 1999].

Studies in rats indicate that AA orally administered via drinking water is rapidly and extensively absorbed from the gastrointestinal tract. AA and AA-related metabolites are widely distributed among tissues with no evidence for accumulation [Doerge et al., 2005].

Acrylamide is in part biotransformed to the epoxide glycidamide (GA). GA is considered to be responsible metabolite for the genotoxic and carcinogenic effect of AA [Doroshyenko et al., 2009]. In mice and humans, the conversion of AA to GA is mediated by cytochrome P450 2E1 (CYP2E1) [Settels et al., 2008; Sumner et al., 1999].

AA and GA are conjugated to glutathione, which is the main pathway of AA metabolism in both rodents and humans [Bjellaas et al., 2007]. Glutathione adducts are converted to the respective mercapturic acid (MA) metabolites N-acetyl-S-(2-carbamoylethyl)-cysteine (AAMA); N-acetyl-S-(2-carbamoylethyl)-cysteine-S-oxide; N-(R,S)-acetyl-S-(2-hydroxy-2-carbamoylethyl)-cysteine (glycidamide mercapturic acid -GAMA-); and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-cysteine to be excreted in the urine. Of these, AAMA and GAMA have been unequivocally confirmed in humans [Fuhr et al., 2006; Watzek et al., 2012].

Acrylamide and glycidamide have the capability to bind covalently to nucleophilic sites of proteins and nucleic acids [Ghanayem et al., 2005]. AA has been classified as class 2A carcinogen (probably carcinogenic to humans) by IARC [IARC, 1994.]. Foods found to contain substantial amounts of AA are potato products, such as fried potatoes or French fries (median content 240 µg AA/kg), gingerbread (300 µg AA/kg) and bread (15 µg AA/kg); where the main part of AA is located in the crust [Boon et al., 2005].

Another mode of interaction with biomolecules concerns binding of AA and GA to nucleophilic regions in proteins and peptides, such as amino and sulfhydryl groups. Hemoglobin (Hb) adducts are often used as validated biomarkers to monitor internal exposure to electrophilic xenobiotics [Ehrenberg and Tornqvist, 1991].

AA is presumed to exert its genotoxic activity via metabolic transformation into the genotoxic metabolite GA which is known to be mutagenic in bacteria and mammalian cells [Baum et al., 2005; Berger et al., 2011], without metabolic activation AA has not been found to be mutagenic or genotoxic at biologically relevant concentrations [Hashimoto and Tanii, 1985]. Carcinogenicity studies in rats indicate that chronic AA exposure increased the incidence of various tumor types from the dose of 0.5 mg/kg/d, which would correspond to a human equivalent dose of 7.6 µg/kg/d [Tyla et al., 2000].

In humans, there is some evidence about the relationship between dietary AA and cancer incidence/mortality in epidemiologic studies [Hogervorst et al., 2007, 2008]. Olesen *et al*. showed a statistically significant correlation between levels of AA adduct to Hb and breast

cancer with an estimated incidence rate ratio of 2.7 (95% confidence interval) per 10-fold increase in adduct level [Olesen et al., 2008].

2.4 Antioxidant activity of coffee

Antioxidant activity of foods and beverages is one of the properties that has generated much interest within the scientific community. Coffee brew, a complex mixture of numerous bioactive compounds, is known to exhibit distinct antioxidant activity [del Castillo et al., 2005]. As mentioned before, antioxidative activity in coffee is generally associated with its content of phenolic compounds as well as Maillard reaction products [Bakuradze et al., 2010; Gomez-Ruiz et al., 2007; Nenadis et al., 2004].

2.4.1 Oxidative stress

Oxidative stress is a disturbance in the oxidant-antioxidant balance leading to potential cellular damage. The imbalance can result from a lack of antioxidant capacity caused or by an overabundance of reactive oxygen species (ROS). ROS are potential carcinogens because of their roles in mutagenesis, tumor promotion, and progression [Droge, 2003].
If not regulated properly, the excess ROS can damage lipids, proteins or DNA, impeding their normal function. ROS alterations in different signaling pathways may modulate gene expression, cell adhesion, cell metabolism, cell cycle and cell death. These events may induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation. ROS may also lead to activation of cellular signal pathways, such as those mediated by mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K), p53, *β*-catenin/Wnt and pathways associated with angiogenesis [Ha et al., 2010; Martindale and Holbrook, 2002].

2.4.2 Reactive oxygen species (ROS)

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (see Table 2-6). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical [Halliwell and Gutteridge, 1999].

Table 2-6 Radical and non-radical reactive oxygen species [Halliwell and Gutteridge, 1999]

Radicals	Non-radicals
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1O_2$
Hydroperoxyl, $HO_2^{\bullet}$	Peroxynitrite, ONOO

Several basic cellular processes lead to the production of ROS within a cell. The mitochondrial electron transport chain is the main source of ATP in the mammalian cells. Cellular respiration involves the reduction of molecular oxygen (O_2) to water in the electron transport chain. This reduction occurs through four one-electron reductions (see below) resulting in the formation of reactive, partially reduced intermediates such as the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical that may act as prooxidants [Kelly et al., 1998].

(1) $O^2 + e^- \rightarrow {}^{\bullet}O_2^-$

(2) ${}^{\bullet}O_2^- + e^- + 2H^+ \rightarrow H_2O_2$

(3) $H_2O_2 + e^- + H^+ \rightarrow {}^{\bullet}OH + H_2O$

(4) ${}^{\bullet}OH + e^- + H^+ \rightarrow H_2O$

The addition of one electron to dioxygen forms the superoxide radical anion ($O_2^{\bullet-}$). Superoxide anion, arising either through metabolic processes or following oxygen “activation” by physical irradiation, is considered the “primary” ROS, and can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme- or metal-catalysed processes [Valko et al., 2007]. Once produced, ROS as $O_2^{\bullet-}$ and $OH^{\bullet}$ (two of the most studied ROS) may damage cellular components and tissues. Superoxide oxidizes catecholamines, tocopherols, ascorbate, and thiols. It can also inactivate enzymes such as catalase and peroxidases.

The hydroxyl radical is highly reactive, making it a very dangerous radical with a very short *in vivo* half-life of approx. 10^{-9} s. Thus, when produced *in vivo* ${}^{\bullet}OH$ reacts close to its site of formation [Pastor et al., 2000].

The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation ensures that there is no free intracellular iron; however, *in vivo*, under stress conditions, an excess of superoxide releases “free iron” from iron-containing molecules. The released Fe^{2+}

can participate in the Fenton reaction (5), generating highly reactive hydroxyl radicals [Valko et al., 2007].

$$(5)\ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

Under stress conditions, $O_2^{\bullet-}$ acts as an oxidant of [4Fe–4S] cluster-containing enzymes (dehydratase-lyase family enzymes) and facilitates $^{\bullet}OH$ production from H_2O_2 by making Fe^{2+} available for the Fenton reaction. The superoxide radical participates in the Haber–Weiss reaction (6) which combines a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen [Halliwell and Gutteridge, 1999].

$$(6)\ Fe^{3+} + {}^{\bullet}O_2^{-} \rightarrow Fe^{2+} + O_2$$

Fenton chemistry is known to occur *in vitro*, but its significance under physiological conditions is not clear, under non physiological conditions e.g. hemochromatosis, *β*-thalassemia, or hemodialysis, higher amounts of “free available iron” can have deleterious effects. “Free-iron” is transported into an intermediate, labile iron pool (LIP), which represents a steady state exchangeable, and readily chelatable iron compartment [Kakhlon and Cabantchik, 2002].

2.4.3 Antioxidant defenses

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms. Defense mechanisms against free radical-induced oxidative stress involve preventative mechanisms, repair mechanisms, physical defenses, and antioxidant defenses. The composition of antioxidant defenses differs from tissue and cell-type to cell type. Extracellular fluids have different protective mechanisms from the intracellular environment. In general, antioxidant defenses comprise: agents that catalytically remove free radicals and other “reactive species” (e.g. antioxidative enzymes like superoxide dismutase, glutathione peroxidase, catalase); proteins that minimize the availability of pro-oxidants such as iron, copper ions and haem (e.g. transferrins, metallothionein, ceruloplasmin); proteins that protect biomolecules against damage (e.g. heat shock proteins) and low-molecular-mass agents or non-enzymatic antioxidants that scavenge ROS and reactive nitrogen species (RNS) represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), uric acid, bilirubin, carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health [Halliwell and Gutteridge, 1999].

One of the best studied models of redox regulation in mammalian cells is the redox control of HO-1. HO-1 induction in skin fibroblasts may serve as an inducible defense pathway to remove heme liberated by oxidants. The mRNA and HO-1 protein are strongly induced by ROS, UVA irradiation and various stressors; thus the inducibility of HO-1 mRNA in many tissues and various mammalian species has rendered HO-1 mRNA a useful marker for cellular oxidative stress at the mRNA level [Keyse and Tyrrell, 1989].

2.4.4 Heme oxygenase (HO)

Heme oxygenases (HOs) are the rate-limiting enzymes in catabolism of heme, converting it to carbon monoxide (CO), biliverdin (BV), and ferrous iron [Maines, 1997]. The HOs play critical roles in physiological iron homeostasis, antioxidant defense and in signaling pathways that employ CO as a messenger. Three mammalian isoforms of HO have been identified: HO-1, an inducible enzyme that is most highly concentrated in tissues that are involved in the catabolism of heme proteins; HO-2, a non-inducible isoform that is present in highest concentrations in the brain and testes and is thought to be particularly involved in signaling pathways; and HO-3, an isoform with low catalytic activity and uncertain physiological role. HO isoforms are also found in plants and most other species, including bacteria [Montellano, 2000; Ravanelli and Branco, 2008; Was et al., 2011].

HO-1 is a member of the heat shock protein family (HSP-32), and its expression is triggered by diverse stress-inducing stimuli including hypoxia, heavy metals, UV irradiation, ROS and reactive nitrogen species (RNS). It is believed that induction of HO-1 protects cells from these toxic stimuli by multiple mechanisms: a) decreasing the prooxidant level (heme); b) increasing the antioxidant level (bilirubin); c) producing the antiapoptotic molecule CO; d) inducing ferritin, which removes and detoxifies free ferric ion; and e) preventing overstimulation of the immune response [Fang et al., 2004].

HO catalyzes the first and rate-limiting step in the oxidative degradation of heme *b* (Fe-protoporphyrin-IX) to form the open-chain tetrapyrrole biliverdin-IXα (see Figure 2-16). The HO reaction displays regiospecificity for the heme molecule, such that only the α-isomer of biliverdins produced. Biliverdin-IXα (BV) is subsequently converted to bilirubin-IXα (BR) by an NAD(P)H-dependent reductase. HO catalyzed heme cleavage releases the heme iron in the ferrous form Fe (II) and eliminates the α-methene bridge carbon of the heme as CO. The HO enzymatic activity requires three moles of molecular oxygen (O_2) per heme molecule oxidized, and reducing equivalents (seven electrons) from NADPH-cytochrome-P450-reductase [Ryter et al., 2006a].

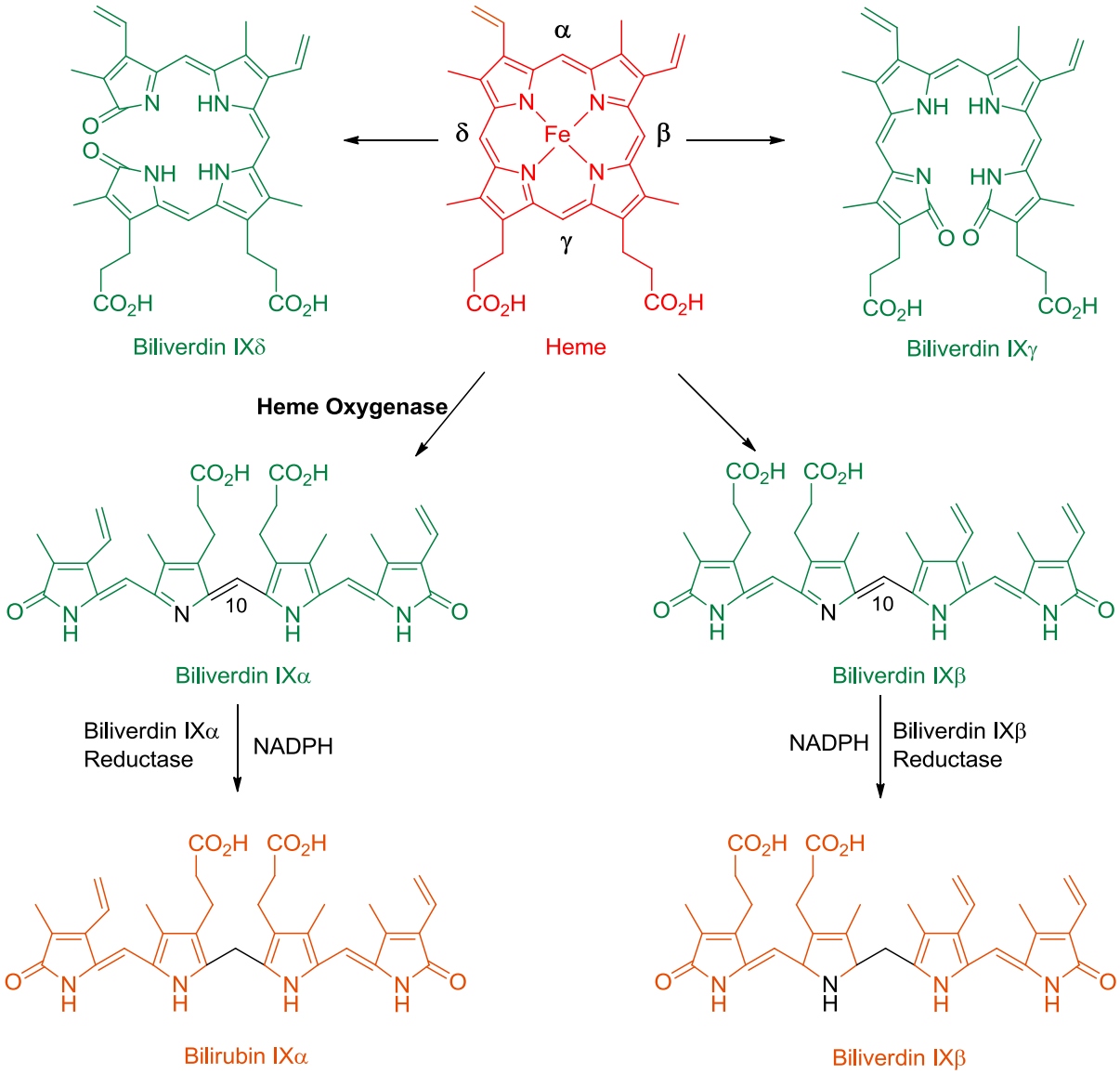


Figure 2-16: Ring opening of heme to give four isomeric biliverdins (IXα, IXβ, IXγ and IXδ) and reduction of the IXα and IXβ isomers to the corresponding bilirubins. Modified from McDonagh [McDonagh, 2001].

In more detail, the heme is first oxidized to α-*meso*-hydroxyheme, which in the deprotonated state has free radical character and reacts with oxygen to produce verdoheme and CO (see Figure 2-17). No exogenous reduction equivalents are required for this reaction. The verdoheme is subsequently converted to BV and free iron in a reaction that requires NADPH–cytochrome-P450-reductase and O_2 [Montellano, 2000]. A recent single-turnover kinetic analysis, in which the rate constants for the various catalytic steps were determined, has shown that product release involves sequential reduction of the ferric to the ferrous BV complex, release of the ferrous iron, and finally dissociation of the biliverdin from the protein [Liu and Ortiz de Montellano, 2000]. The slow step in the single-turnover sequence is BV release. However, BV release is accelerated in the presence of biliverdin reductase, the

enzyme that converts BV to BR, and reduction of ferric to ferrous verdoheme becomes rate-limiting.

Given the established reaction intermediates, the results of the kinetic analysis, and the fact that the oxygens in BV are derived from different molecules of oxygen, hypothetical mechanisms have been formulated for the conversion of α-*meso*-hydroxyheme to verdoheme and verdoheme to BV [Montellano, 2000; Unno et al., 2007; Wilks, 2002].

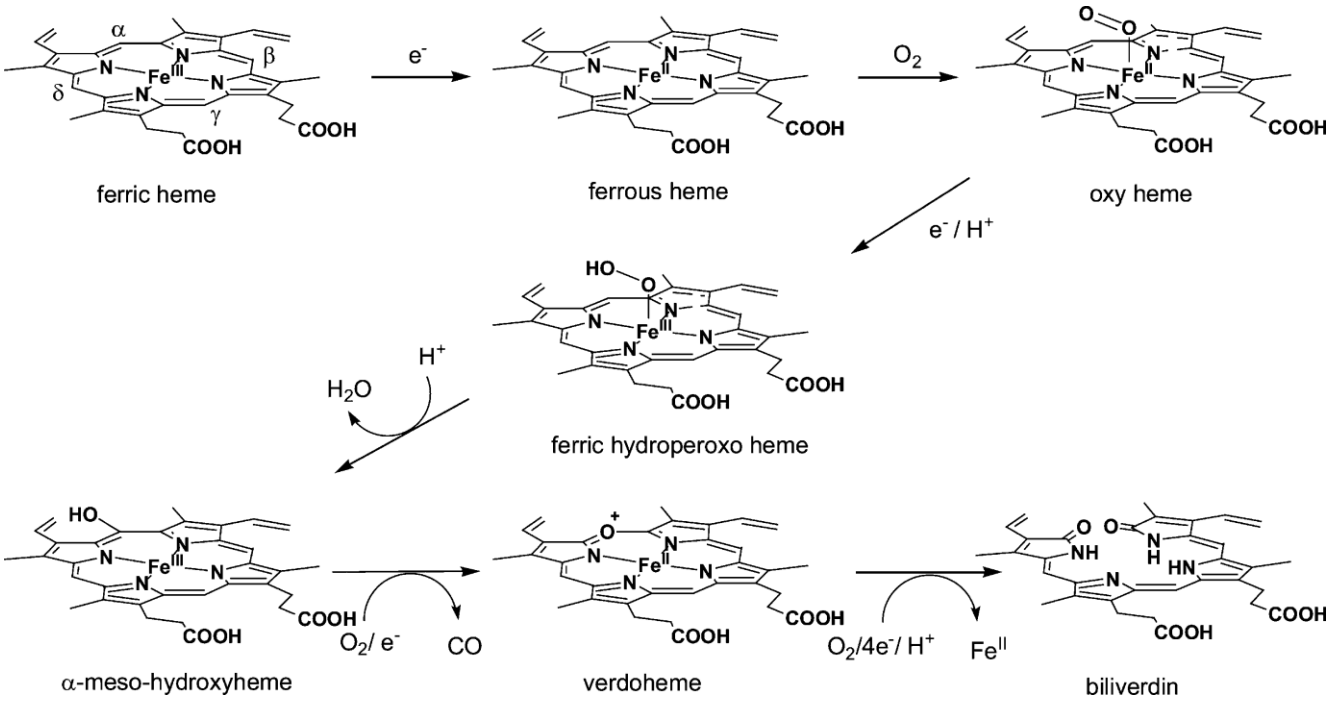


Figure 2-17: Schematic of HO catalytic intermediates [Unno et al., 2007].

The inducible form of HO, HO-1, occurs at a high level of expression in the spleen and other tissues that degrade senescent red blood cells, including specialized reticuloendothelial cells of the liver and bone marrow [Tenhunen et al., 1969]. HO is present in:

- hematopoietic stem cells of the bone marrow, where it may inhibit cellular differentiation by lowering the intracellular concentration of heme, a differentiation factor for these cells [Abraham, 1991]
- liver parenchyma, which is the site of uptake and degradation of plasma heme, hemoglobin, and methemalbumin. Under conditions of hemolysis, HO-activity dramatically increases in liver parenchyma, kidney, and macrophages a result of increased levels of circulating hemoglobin [Tenhunen et al., 1970]
- most other tissues not directly involved in erythrocyte or hemoglobin metabolism, HO-1 typically occurs at low to undetectable levels under basal conditions, but responds

to rapid transcriptional activation by diverse chemical and physical stimuli [Ryter et al., 2006a].

Highest expression of HO-2, the constitutively expressed isozyme, occurs in the testes, but the protein is also found abundantly and ubiquitously in other systemic tissues including, brain and central nervous system, vasculature, liver, kidney, and gut. In rats, HO-2 does not respond to transcriptional activation by environmental stress but may respond to developmental regulation by adrenal glucocorticoids in the brain [Raju et al., 1997].

There is some evidence about HO-1 gene expression and total HO-activity modulation in some cultured cells like liver carcinoma (HepG2) [Ghattas et al., 2002; Lavrovsky et al., 2000; Takahashi et al., 1999], and colo-rectal adenocarcinoma (Caco-2) [Follett et al., 2002; Uc and Britigan, 2003] and HT29 cell lines [Lee et al., 2007; Paul et al., 2005].

In the cell, HO-1 enzymes have been characterized as endoplasmic reticulum (ER) associated proteins, due to the abundant detection of HO-activity in microsomal (104,000 g) fractions. Both HO-1 and HO-2 contain a COOH-terminal hydrophobic domain segment that suggests a general membrane compartmentalization. Kim et al. described a functional association of HO-1 with plasma membrane caveolae, small invaginations of the plasma membrane, in endothelial cells [Kim et al., 2004].

HO-1 activity has been measured using several techniques. Although BV is a primary metabolite of the HO-1/heme reaction, it is rarely used as an indicator because of poor spectral properties, extinction coefficient (ε) of only 8 to 10 mM^{-1} cm^{-1}[Kutty and Maines, 1981]. Thus, one of the most common HO-1 activity assays relies on the reduction of biliverdin to bilirubin, as originally described by the group of Tenhunen [Tenhunen et al., 1969]. In this method, BR formation is monitored spectrophotometrically by the increase in absorbance at 468 nm (ε_{468}= 43.5 mM^{-1} cm^{-1}), which is approximately 5-fold higher than that of BV [Huber et al., 2009]. Thus, HO-1 activity is measured by monitoring BR formation using the diference in absorbance at 464 to 530 nm [McNally et al., 2004; Motterlini et al., 1995; Tenhunen et al., 1970].

HO-1 gene is commonly induced by agents and chemicals that produce an oxidative cellular stress involving ROS generation. Known examples of ROS generating systems that activate the expression of the *ho-1* gene include hydrogen peroxide (H_2O_2), quinines (e.g, menadione) which generate $O_2^{\bullet-}$ and/or H_2O_2 through redox cycling process, photosensitizers and UVA radiation [Gong et al., 2003].

Heavy metals (cadmium, cobalt, zinc, tin, lead and mercury) potently activate the *ho-1* gene in cell culture systems and produce tissue-specific effects *in vivo* depending on the compound and route of administration [Ryter et al., 2006a]. Heavy metals form complexes with thiols, such as reduced glutathione (GSH). *In vivo,* heavy metals cause a characteristic depletion of hepatic GSH followed by a rebound increase from *de novo* synthesis.

The complexation of metals with protoporphyrin IX (PPIX), the natural precursor of heme, generates metalloporphyrins. Several metals such as cobalt, zinc, copper and iron can serve as substrates for ferrochelatase, the enzyme that incorporates iron into PPIX in the final step of heme synthesis. The substrate and catalytic cofactor of HO-1, heme (iron-protoporphyrin-IX), acts as an inducer of *ho-1* gene expression and activity [Tenhunen et al., 1970]. Several metalloporphyrins, including SnPPIX, CoPPIX and ZnPPIX, induce HO-1 transcription but also act as competitive inhibitors of HO-activity both *in vitro* and *in vivo* [Sardana and Kappas, 1987].

Many phenolic compounds activate *ho-1* through the Nrf-2/Keap1 pathway, which plays a major role in the transcriptional activation of the *ho-1* gene by electrophilic compounds. A broad class of antioxidants, has recently been identified as potent *ho-1* inducing agents, including caffeic acid phenethyl ester [Scapagnini et al., 2002], carnosol [Martin et al., 2004], curcumin [Balogun et al., 2003b; Motterlini et al., 2000; Scapagnini et al., 2002], resveratrol [Juan et al., 2005], chalcones, rosolic acid [Foresti et al., 2005] and garlic organosulfur compounds [Chen et al., 2004a].

In 2008, Cavin *et al*. reported a significant induction of HO-1 mRNA expression in rat liver and primary hepatocytes treated with 800 µg/mL of standard instant coffee for 24 h [Cavin et al., 2008]. It is worth noting that HO-1-transcription *in vitro* was increased by 100 mg/mL coffee extract of a low-roast coffee rich in CGA (see chapter 2.3.5), while *in vivo*, only the extract of a strongly roasted coffee, low in CGA but containing high levels of NMP, increased HO-1-transcript levels [Boettler et al., 2011b].

The functional consequences of HO-activity are related to the three principle reaction products: BR, iron, and carbon monoxide (CO) see Figure 2-18. Iron released from HO-activity triggers pathways involving the activation of the iron regulatory protein (IRP), leading to its sequestration and recycling. Competing pathways involve its extracellular efflux and its potential participation in deleterious cellular reactions. BV and BR have been well documented as an initial defense mechanism against cellular oxidative stress and tissue inflammation in response to multiple stimuli. The third metabolite, CO, is known to act as a potent vasodilator and anti-inflammatory molecule via cellular signaling cascades like NO [Huber et al., 2009].

The **iron** released from heme by HO-activity potentially enters a pool of “labile” or “chelatable” iron, where it may be available for cellular iron depending processes included the promotion of intracellular ROS production (see Haber–Weiss reaction, Chapter 2.4.2). Free heme is also a potentially dangerous pro-oxidant compound, which may promote membrane lipid peroxidation [Ryter et al., 2002].

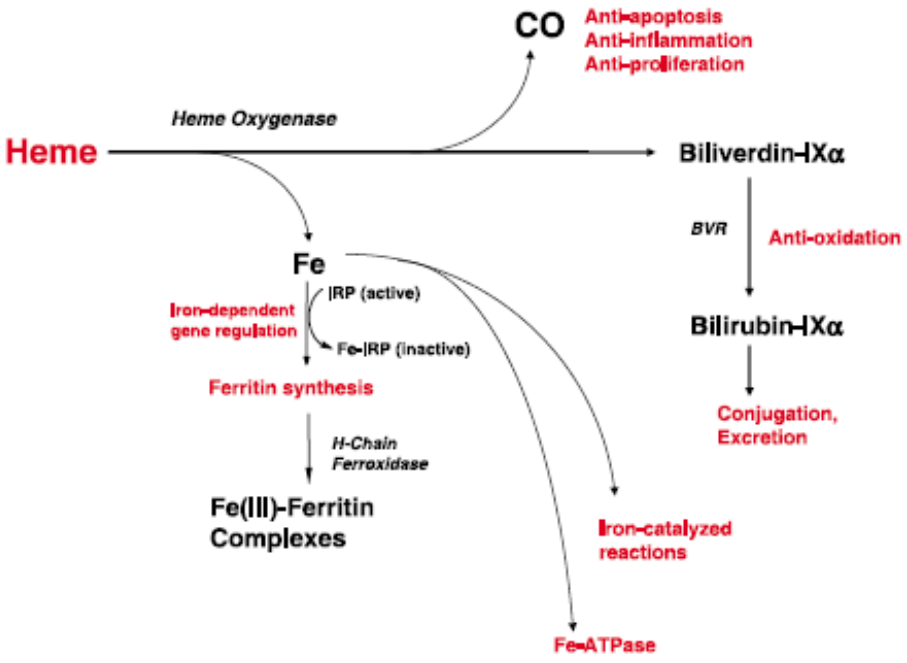


Figure 2-18: Functional consequences of the three principle reaction products of HO-activity: bilirubin, iron, and carbon monoxide (CO) [Ryter et al., 2006a].

The antioxidative protection afforded by HO-activity cannot be explained alone by the conversion of heme in exchange for increased intracellular iron. The antioxidant protection of HO with respect to iron metabolism, is facilitated by further coupling with proteins that either promote the sequestration or export of the liberated iron e.g. IRPs and ferritin. Ferritin has been implicated as a cytoprotective molecule in *in vitro* models [Balla et al., 1992; Lin and Girotti, 1998].

BR and **BV** are water-insoluble pigments derived in vertebrates from heme catabolism. In mammals and some other vertebrates BV is reduced promptly to BR by NADPH/biliverdin reductase (BVR). Only trace amounts of BV are detectable *in vivo* during normal human heme metabolism, demonstrating that the reduction is quantitative and rapid. Although green pigments, thought to be BV, have been observed under pathological conditions, there is no physiological evidence for accumulation of BV during normal human heme metabolism [McDonagh, 2010a].

Once formed, BR is transported in bloodstream in a reversible complex with serum albumin and absorbed into the liver where it is transformed into three different glucuronide derivatives by a specific glucuronosyl transferase enzyme, UGT1A1 [Blake et al., 2005]. The polar glucuronide derivatives are secreted into bile by the canalicular ATP-dependent transport protein MRP2 (multidrug resistanceassociated protein 2, ABCC2). Unlike BV, bilirubin needs

to be glucorodidated for efficient excretion and prevention of its intestinal reuptake. Conjugated BR is secreted by bile into the small intestine during the passage of the large intestine in to the feces, where it is further degraded to urobilinogens by the reductive processes of intestinal microorganisms. When glucoronidation is deficient, as in neonates or people with the Gilbert syndrome, unconjugated bilirubin accumulates in the circulation and extravascular tissue. Why humans and many other vertebrates reduce biliverdin to bilirubin, which is toxic and requires further energy-requiring steps to be eliminated, is not known [McDonagh, 2010b].

The reason why BR requires glucuronidation for elimination, whereas BV and BR conjugates do not, is that BV and BR are both flexible molecules that can assume many different conformations in solution. BV but not BR, this allows formation of particular conformations (stereoisomers) in which the acidic carboxyl side-chains are internalized by hydrogen bonding to other groups in the molecule (see Figure 2-19). This has the effect of 'neutralizing' the polar groups and accounts for the surprising lipophilicity of bilirubin [McDonagh, 2002]. Structure and solubility of BR in membranes and aqueous environments are still poorly characterised, as is the structure of the crucially important bilirubin–albumin complex that transports bilirubin in the circulation.

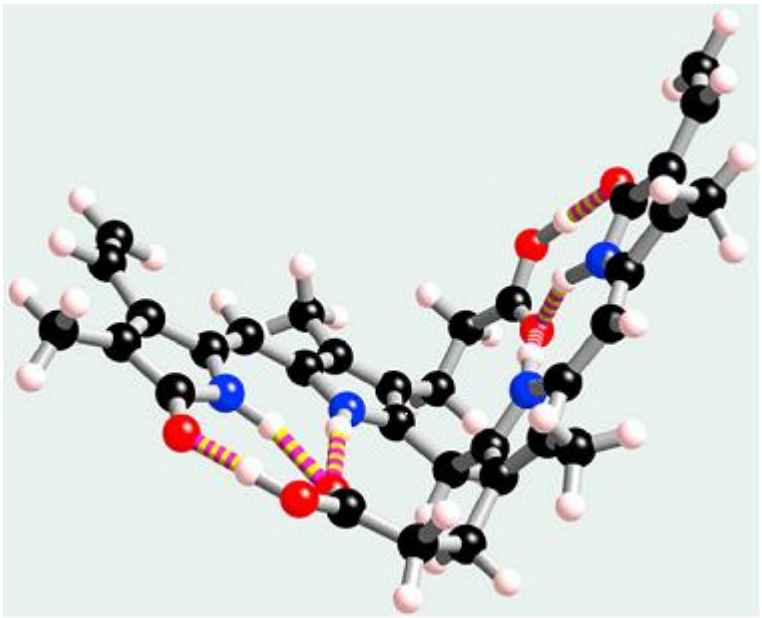


Figure 2-19: Constitutional structure and preferred conformation of bilirubin (BR). Striped bonds represent intramolecular hydrogen bonds [McDonagh, 2010b].

Physiological antioxidant benefit of the metabolic conversion of heme to the elimination product BR has been suggested to occur in the circulating plasma, which is supported by oxidative stress resistance of hyperbilirubinemic rats [Dennery et al., 1995]. Despite its elimination from cells and tissues, current evidence supports a beneficial role for

endogenously produced BR at a cellular level. This mechanism may underlie, the cytoprotective properties of HO-1 in certain models [Clark et al., 2000; Motterlini et al., 2000]. Currently, BV and BR remain viable experimental therapeutic agents for the treatment of several inflammatory disease states including transplant-associated ischemia/reperfusion (I/R) injury, i.e. tissue damage caused when blood supply returns to the tissue after a period of ischemia or lack of oxygen, and vascular injury [Adin et al., 2005; Fondevila et al., 2004].

CO has effects on intracellular signaling processes, which culminate in anti-inflammatory, antiproliferative, antiapoptotic, and anticoagulative effects. The physiological effects of CO have been related to its endogenous production from basal and inducible HO-activity. The physiological signaling effects of CO known to date involve relatively few defined mechanisms; the modulation of soluble guanylate cyclases (sGC) activity and subsequent stimulation of cGMP production are the most commonly observed. Like NO, CO binds directly to the heme iron of sGC, leading to stimulation of enzymatic activity. The stimulation of sGC and subsequent elevation of cGMP levels confers to CO its vasoactive properties [Furchgott and Jothianandan, 1991]. CO exerts a variable and multimodal effect on vasodilation, which involves several mechanisms. The dilatory effects of CO are attributed to direct endothelium-independent effects on vascular smooth muscle cells (SMC), including the modulation of cGMP and K^+ channel activity in the same model, and indirect effects on the expression of endothelial-derived vasoconstrictors and myogenic factors [Ryter et al., 2002].

CO exerts also potent anti-inflammatory effects and mediates much of the anti-inflammatory effects seen with HO-1. CO selectively inhibits the expression of the pro-inflammatory cytokines like tumor necrosis factor alpha (TNFα), IL-1β and MIP-1β and increases production of the anti-inflammatory cytokine IL-10. There is evidence that CO mediates these anti-inflammatory effects specifically through MAP kinases [Otterbein et al., 2000].

Furthermore, increasing evidence suggests possible roles for HO-derived CO as a signaling intermediate in the brain and central nervous system (CNS) in a number of neurological processes that include olfactory signal transduction, long-term potentiation (LTP), nonadrenergic noncholinergic (NANC)-dependent relaxation, cholinergic regulation of circadian rhythms, the autonomic regulation of cardiovascular function, and oxygen-sensing processes [Artinian et al., 2001; Stevens and Wang, 1993; Williams et al., 2004].

HO-1 has been implicated in the pathologies of a number of diseases associated with oxidative stress including Alzheimer's disease [Schipper, 2011], DM [Turkseven et al., 2005] and atherosclerosis [Ishikawa et al., 2001]. On the other hand, it should be noted that expression of HO-1 is usually higher in cancer cells than in surrounding healthy tissues, as shown for lymphosarcoma, prostate cancers, brain tumors, adenocarcinoma, hepatoma, squamous carcinoma, glioblastoma, melanoma, Kaposi sarcoma, or pancreatic carcinoma

[Doi et al., 1999; Goodman et al., 1997; Hara et al., 1996; Maines and Abrahamsson, 1996; McAllister et al., 2004; Torisu-Itakura et al., 2000; Was et al., 2010].

Expression of HO-1 in tumors can be further elevated in response to chemo-, radio-, or photodynamic therapies, possibly as a result of oxidative stress. There are several lines of evidence showing that HO-1 exerts potent and comprehensive protumoral effects in growing tumors [Was et al., 2011]. Its upregulation improves the survival of various tumor cell lines exposed to stressful agents both *in vitro* [Busserolles et al., 2006; Chen et al., 2004b; Liu et al., 2004] and *in vivo* [Hirai et al., 2007]. Downregulation of HO-1 has been shown to inhibit the growth of a pancreatic tumor cell line [Berberat et al., 2005], whereas overexpression of HO-1 in human and murine melanoma cells increased significantly their proliferation [Was et al., 2006]. These effects seem to be strongly cell-type-dependent, as HO-1 activity exhibited antiproliferative actions in human and rat breast cancer cell lines [Hill et al., 2005]. HO-1 can also promote tumor angiogenesis and metastasis as demonstrated in humans and in animal models. Its overexpression in melanoma cells and pancreatic cancer cells increased colonization of lungs, whereas inhibition of HO-activity completely inhibited formation of pulmonary metastases [Sunamura et al., 2003; Was et al., 2011].

2.5 The cAMP-Pathway

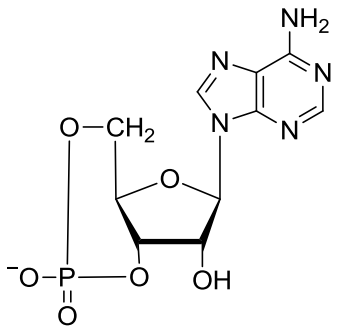
Despite the large number of known hormones that stimulate second-messenger formation, the most important second messengers belong only to three distinct groups: **cyclic nucleotide monophosphates** such adenosine 3',5'-cyclic monophosphate (cAMP) and the guanosine 3',5'-cyclic monophosphate (cGMP); **inositol phospholipids** such inositol trisphosphate (IP_3) and diacylglycerol (DAG) and **Ca^{2+} ions** (see Figure 2-20).

Binding of an external signal (first messenger) to a specific receptor from the outer surface of the target cell membrane activates a transducer protein that carries the signal through the membrane. The transducer protein then activates an amplifier that catalyzes the formation of a second messenger. The second messenger binds to an internal regulator that controls various effectors, leading to a cellular response(s) [Randall et al., 1997].

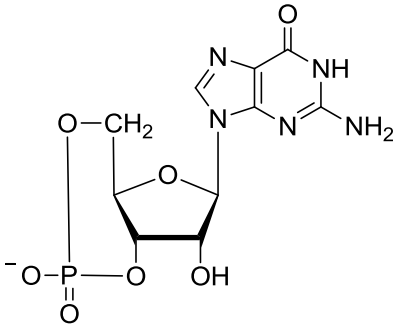
The hydrolysis of ATP into cAMP by adenylate cyclase requires Mg^{2+} and a trace amount of Ca^{2+}. As cAMP is produced, it binds to an inhibitory regulatory subunit of protein kinase A (PKA), causing the subunit to dissociate from the catalytic subunit of PKA. This free and active catalytic subunit can phosphorylate effectors proteins using ATP. Phosphorylation of these effector proteins may either increase or inhibit their activity, thereby inducing cellular response(s). Some effector proteins are enzymes, catalyze further chemical reactions; others are nonenzymatic proteins such as membrane channels, structural proteins, or regulatory proteins.

The cAMP-dependent pathway, also known as the adenylate cyclase pathway, is a G protein-coupled receptor-triggered signaling cascade used in cell communication. A central element of this signaling cascade is the second messenger cAMP (3',5'-cyclic adenosine monophosphate) that activates PKA [Alberts et al., 2004].

CYCLIC NUCLEOTIDES

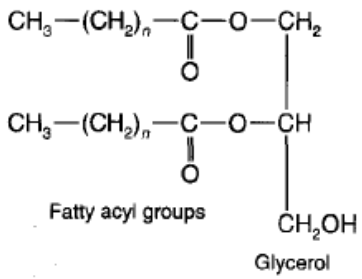


3´,5´-Cyclic AMP (cAMP)

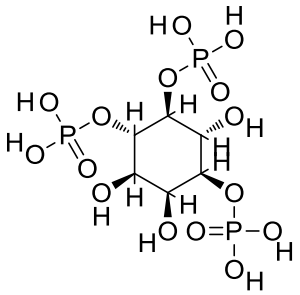


3´,5´-Cyclic GMP (cGMP)

INOSITOL PHOSPHOLIPIDS



1,2-Diacylglycerol (DAG)



Inositol triphosphate (IP_3)

Figure 2-20: Chemical structures of second messengers.

Many different cell responses are mediated by cAMP. These include increase in heart rate, cortisol secretion, and breakdown of glycogen and fat. This pathway can activate enzymes and regulate gene expression. The activation of preexisting enzymes is a fast process, whereas effects of the regulation of gene expression can take up to hours. The cAMP pathway was studied through loss of function (inhibition) and gain of function (increase) of cAMP [Devlin, 2002].

Signaling pathways employing cAMP or cGMP play a critical role controlling many processes across a broad spectrum of organisms. The actions of many physiological signals (see Table 2-7) are mediated by very specific and highly controlled changes in the cellular levels of these second messengers. These changes in either global cyclic nucleotide content or that constrained to specific cellular compartments within the cell are essential for a wide range of physiological processes like vision, olfaction, synaptic function, muscle contraction, water

and electrolyte homeostasis, immune responses, hemostasis, carbohydrate and lipid metabolism. The amplitude and duration of the cyclic nucleotide responses are determined by the balance between their rates of synthesis (by adenylate or guanylate cyclases) and degradation (by cyclic nucleotide phosphodiesterases). The key to the specificity of hormonal effects lies in the tissue distribution of effector proteins that that can be phosphorylated by cAMP dependent PKA [Beavo et al., 2007].

Table 2-7 Hormone-induced responses mediated by cAMP pathway* [Alberts et al., 2004; Berridge, 1985; Randall et al., 1997]

Signal	Tissue	Cell response
Stimulatory		
Epinephrine (β-receptors) (+ ACTH and glucagon)	Skeletal muscle	Breakdown of glycogen
	Fat cells	Increased breakdown of lipids
	Heart	Increased heart rate and force of contraction
	Intestine	Fluid secretion
	Smooth muscle	Relaxation
Thyroid-stimulating hormone (TSH)	Thyroid gland	Thyroxine secretion
Vasopressin	Kidney	Resorption of water
Glucagon	Liver	Breakdown of glycogen
Serotonin	Salivary gland (blowfly)	Fluid secretion
Prostaglandin I_1	Blood platelets	Inhibition of aggregation and secretion
Inhibitory		
Epinephrine ($\alpha_2$receptors)	Blood platelets	Stimulation of aggregation and secretion
	Fat cells	Decreased lipid breakdown
Adenosine	Fat cells	Decreased lipid breakdown

ACTH (Adrenocorticotropic hormone)

* Some responses to local mediators and to neurotransmitters are also mediated by cAMP

2.5.1 The cAMP signalling cascade

cAMP is involved in a multitude of cellular processes including cell proliferation and differentiation. The second messenger cAMP mediates the transcriptional regulation of various genes and activates target enzymes, especially PKA. cAMP homeostasis is primarily regulated by its formation through adenylate cyclases (AC) and by its degradation by phosphodiesterases (PDE).

The pathway employing cAMP as the second messenger has a stimulatory receptor (R_s) and an inhibitory one (R_i), which both communicate with the amplifier AC by way of transducer G proteins; stimulatory G protein (G_s) and inhibitory G protein (G_i) see Figure 2-21.

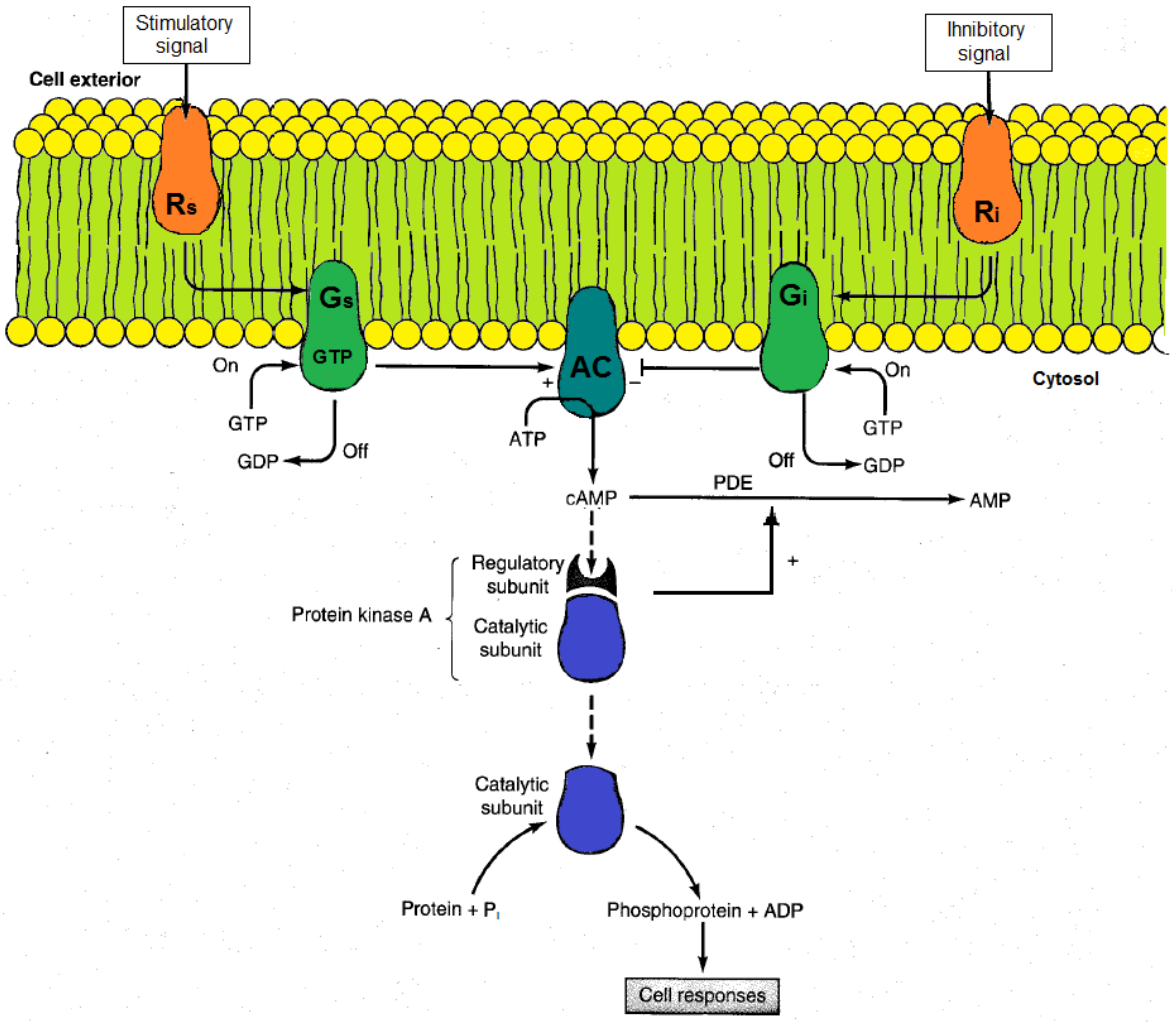


Figure 2-21: The cAMP pathway. Hormone-stimulated regulation of AC within the membrane leads to an increase or decrease in cytosolic cAMP level. Binding of hormones or other ligands to their stimulatory or inhibitory receptors (R_s and R_i respectively) induce binding of GTP to the respective transducer proteins, $\mathbf{G_s}$ and $\mathbf{G_i}$. The GTP-activated G proteins are then able to either activate or inhibit the **AC** until the GTP is hydrolysed to guanosine diphosphate (**GDP**). Activated AC catalyzes the conversion of ATP to cAMP, which binds to the regulatory subunit of protein kinase A **(PKA)**. The catalytic subunit can phosphorylate various intracellular effector proteins, yielding activated phosphoproteins that mediate cellular responses. In time, cAMP is degraded to AMP by a phosphodiesterase (**PDE**), and the phosphorylated effector proteins often are dephosphorylated to their inactive forms. Both of these mechanisms reduce or terminate the effects of the external signal. Adapted from Randall *et al.* [Randall et al., 1997].

G proteins are composed of three protein subunits (α, β and γ) two of which are bound to plasma membrane by short lipid tails. In the unstimulated state, the α-subunit has GDP bound to it, and the G protein is idle. When an extracellular ligand binds to its receptor, the altered receptor activates a G protein by causing the α-subunit to lose some of its affinity for GDP, and recruits a molecule of GTP. This activation breaks up the G protein subunits: the "switched-on" α subunit, clutching its GTP, detaches from the βγ complex, giving rise to two separate molecules that now roam independently along the plasma membrane. The two activated parts of a G protein (the α-subunit and the βγ complex) can both interact directly

with target proteins located in the plasma membrane, which in turn may relay the signal to yet other destinations. The longer these target proteins have an α or a βγ subunit bound to them, the stronger and more prolonged the relayed signal will be (see Figure 2-22). The amount of time that the α- and βγ-subunits remain dissociated (and hence available to relay signals) is limited by the behavior of the α-subunit. The α-subunit has an intrinsic GTP-hydrolyzing (GTPase) activity, and it eventually hydrolyzes its bound GTP to GDP; the α-subunit then reassociates with a βγ complex and the signal is shut off. This reunion generally occurs within seconds after the G protein has been activated. The reconstituted G protein is now ready to be reactivated by another activated receptor [Alberts et al., 2004] .

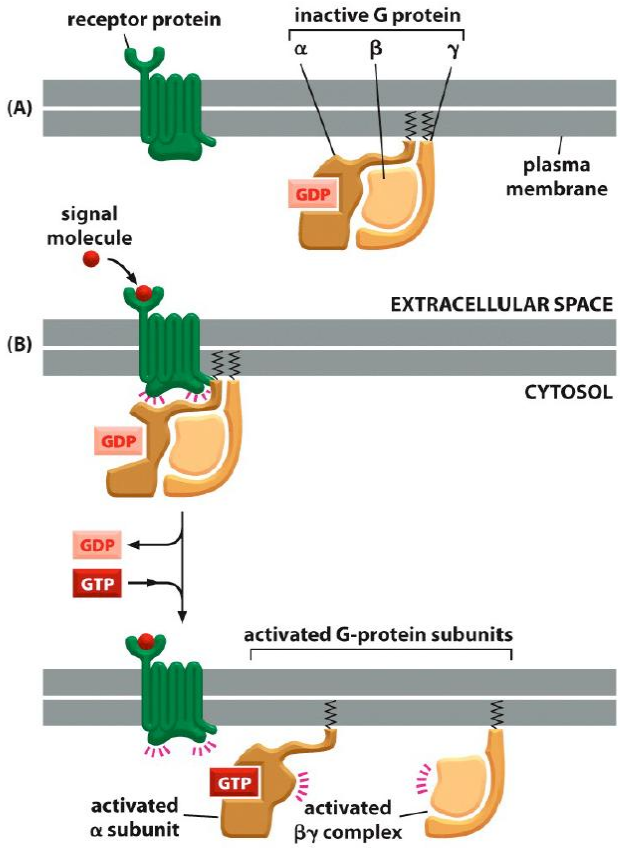


Figure 2-22: Stimulated G-protein-linked receptors activate G-protein subunits. From Alberts *et al.* [Alberts et al., 2004].

2.5.2 Adenosine and its receptors

2.5.2.1 Adenosine

Adenosine, a purine nucleoside (see Figure 2-23) generated extracellularly from adenine nucleotides, is produced by cells and tissues in response to a variety of physical and metabolic conditions and mediates physiological activities that include sedation, inhibition of platelet aggregation and vasodilatation [Ralevic and Burnstock, 1998]. As aforementioned, the effects of adenosine are mediated by a family of four G protein-coupled receptors, A_1,

A_{2A}, A_{2B} and A_3. Engagement of the adenosine A_{2A} receptor promotes the resolution of inflammation [Chan and Cronstein, 2002], which accounts for at least some of the anti-inflammatory actions of many commonly used drugs, including salicylates, methotrexate and sulfasalazine [Chan et al., 2006]. Adenosine A_{2A} receptors also promote tissue repair, wound healing and matrix production [Montesinos et al., 2002; Victor-Vega et al., 2002]. Subsequently, adenosine and its receptors have also been found to promote fibrosis (excess matrix production) in the skin, lungs, and liver [Chan et al., 2006].

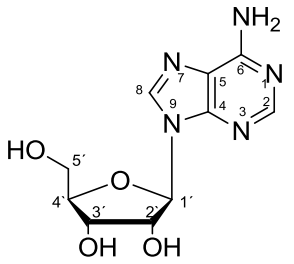


Figure 2-23: Chemical structure of adenosine (ADO).

Since many of the functions of adenosine are homeostatic and protective in nature, and in principle, equalize local energy requirements with energy supply, it has also been proposed to be a “retaliatory metabolite” [Spychala, 2000].

There are several independent sources of adenosine, including cell death and nucleotide degradation, ischemia and ATP breakdown, ATP/ADP/AMP release and subsequent dephosphorylation, and *S*-adenosylhomocysteine hydrolysis. Extracellular nucleotides may also originate from cytosolic sources by vesicular release exocytosis, passage through channels, and cell lysis, which would individually or in combination provide a steady-state or acute supply of adenosine. This constant release of adenosine is counteracted by its metabolism, which may proceed either by phosphorylation to form AMP, catalyzed by adenosine kinase (AK), or by deamination to inosine, catalyzed by adenosine deaminase (ADA). AK, due to its low K_m (40 nM), phosphorylates adenosine at physiological concentrations. ADA has a K_m value 3 orders of magnitude higher (70 mM) and specific activity 10-fold higher than AK, and in most tissues, functions as a high volume/low affinity system that turns on when available adenosine concentrations increase above a certain level. However, since AK and ADA have vastly different kinetic properties, the fate of adenosine is largely determined by its local concentration. At physiological levels of adenosine (below 1.0 mM), phosphorylation is the predominant route of its metabolism [Snyder and Lukey, 1982]. Overall, under normal conditions of low adenosine levels, AK is the major physiological regulator of adenosine concentrations [Lloyd and Fredholm, 1995] and characteristically, its K_m of 40 nM is physiologically positioned between the high-affinity binding of adenosine to A_1 (3–30 nM), intermediate-affinity binding to A_{2A} (20- 200 nM), and low-affinity binding to A_{2B} (5 mM) adenosine receptors [Spychala, 2000].

Under normal conditions, adenosine is continuously formed intracellularly as well as extracellularly. Adenosine is produced mainly by dephosphorylation of AMP catalyzed by 5´-nucleotidase (5´-NT) see Figure 2-24. Several forms of this enzyme have been described, but only two of them (cytosolic 5´-NT-I [cN-I] and ecto-5´-NT) seem to participate in the regulation of the adenosine homeostasis. In the heart, adenosine may be generated by AMP-selective (cN-I) 5´-NT, which is restricted to cardiac and breast muscle and, to some extent, brain tissue. This cytosolic enzyme produces adenosine that must be released from the cell to have pharmacological effects [Sala-Newby et al., 1999].

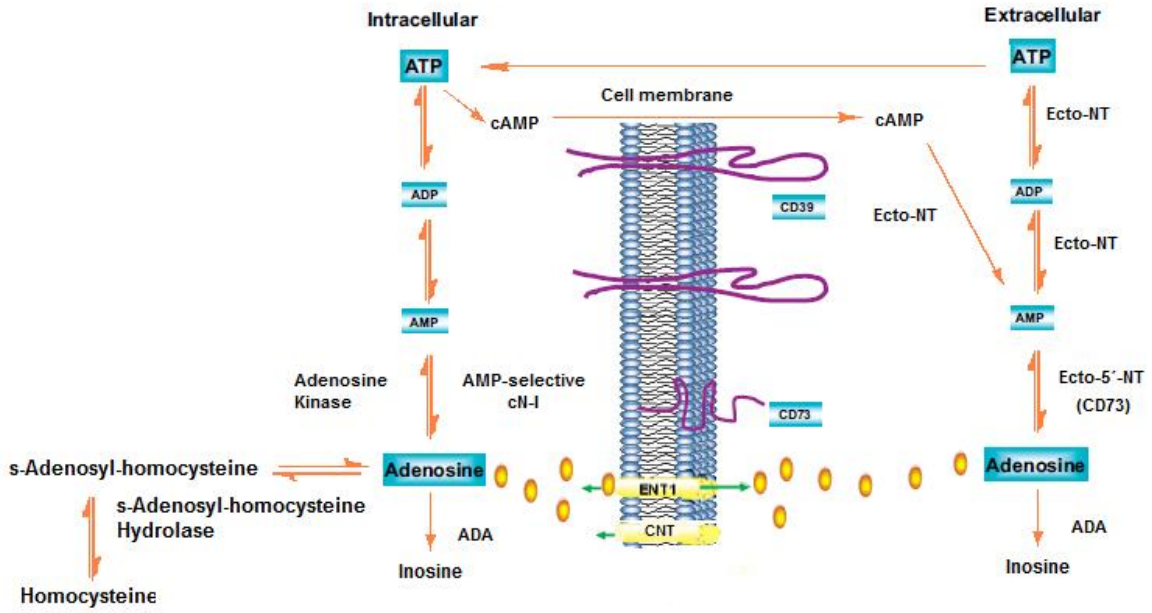


Figure 2-24: Interconversion of extracellular and intracellular adenine nucleotides and adenosine. Adenosine is formed both intracellularly and extracellularly from adenine nucleotides, which are sequentially dephosphorylated to adenosine. Intracellular adenosine may be transported into the extracellular space via facilitated transport by ENT1. Extracellular adenosine is also taken up by cells through the same transporter. Two cell surface molecules, CD39 and CD73, catalyze the dephosphorylation of adenine nucleotides to adenosine in the extracellular space. Adapted from Cronstein [Cronstein, 2011]. ENT1: equilibrative nucleoside transporter 1; CD39: nucleoside triphosphate phosphohydrolase; CD73: ecto-50-nucleotidase.

There is good evidence that intracellular formation of adenosine is at least as important as adenosine formation from breakdown of extracellular ATP. Intracellular formation predominantly occurs as a consequence of activity of intracellular 5´-nucleotidases, of which two forms, cN-I and cN-II, have been reported. These two enzymes may play different roles, cN-I breaking down AMP to adenosine and cN-II breaking down IMP and GMP to inosine and guanosine, respectively [Sala-Newby et al., 1999]. Adenosine generated intracellularly is transported into the extracellular space mainly via specific bi-directional transporters through facilitated diffusion that efficiently evens out the intra- and extracellular levels of adenosine

(see Figure 2-24). These transport proteins were termed ENT1 and ENT2 (for the equilibrative transport proteins) and CNT1 and CNT2 (for the concentrative types). When the activity of transporters is decreased, e.g., by drugs or by reducing temperature, extracellular biologically active levels of adenosine increase [Fredholm et al., 2001].

The dephosphorylation of extracellular AMP to adenosine is mediated by ecto-5´-nucleotidase, also known as CD73 [Zimmermann, 1992]. This is a ubiquitous enzyme expressed in many tissues at variable levels, ranging from very high in fibroblasts and some carcinomas to very low or absent in cells of hematopoietic origin. Ecto-5´-nucleotidase is linked to the plasma membrane by glycosyl phosphatidylinositol, and produces adenosine from AMP, which may be actively released from some cells or from adenine nucleotides that accumulate extracellularly as a consequence of either active release or passive efflux secondary to ischemia and necrosis in solid tumors [Spychala, 2000].

Overexpression of CD73 has been proposed to protect organs under stress by the formation of cytoprotective adenosine [Beldi et al., 2008]. As mentioned earlier, the adenosine produced extracellularly is also subject to metabolic breakdown by adenosine deaminase to produce inosine or (re)phosphorylation by adenosine kinase to produce AMP. Therefore, when an organ is under stress there is a highly complex and time-dependent interplay of the activation of many receptors in the same vicinity. In addition to the direct activation of ARs by selective agonists or their blockade by selective antagonists, inhibition of the metabolic or transport pathways surrounding adenosine is also being explored for therapeutic purposes [Wilson and Mustafa, 2009].

Most tissues in the body and cells in culture release adenosine to the extracellular medium, from where it can feed back and act as an autocoid on the ARs present locally. The basal levels of extracellular adenosine have been estimated near to 100 nM in the heart and 20 nM in the brain [Fredholm et al., 2005] whereas in human plasma concentrations ranging from 10 to 70 nM under normal physiological conditions (normoxia) and depending on the sampling site (radial artery, median cubital vein and internal jugular vein) have been found [Dolezalova et al., 2005; Saito et al., 1999].

In the case of severe ischemic stress, levels of adenosine can rapidly rise to the micromolar range, which would cause a more intense and generalized activation of the four subtypes of ARs. Nevertheless, it is thought that the exogenous administration of highly potent and selective AR agonists in such cases of severe ischemic challenge might still provide additional benefit beyond that offered by the endogenous adenosine generated [Wilson and Mustafa, 2009].

Ethanol, one of the most important causes of hepatic fibrosis/cirrhosis in the Western world, stimulates increased extracellular adenosine levels *in vitro* through its action on the nucleoside transporter and ethanol ingestion increases purine release into the bloodstream

and urine in normal volunteers. The released adenosine binds to its receptors in the liver to promote fibrosis, a form of sustained wound healing in response to injury [Chan et al., 2006]. There is some evidence that caffeine blocks the development of hepatic fibrosis, an effect that could explain the epidemiologic finding that coffee drinking reduces the likelihood of death from liver disease in a dose-dependent manner [Chan et al., 2006].

A number of observations strongly suggest that adenosine may have similar functions in cancer. Due to rapid growth, large solid tumors routinely experience severe hypoxia and necrosis, which causes adenine nucleotide degradation and adenosine release. The released adenosine would then exert a broad range of effects that benefit malignancy by providing a supportive environment for the accelerated growth. This supportive environment may include protection against ischemia, stimulation of growth and angiogenesis, and suppression of immune responses [Spychala, 2000].

The alkylxanthines caffeine and theophylline are the prototypical antagonists of adenosine receptors (ARs), and their stimulant actions are produced primarily through blocking the depressant actions of the adenosine through the A_1 and A_{2A} ARs. The stimulant actions of the alkylxanthines were thought to occur as a result of inhibition of phosphodiesterases. Although this inhibition may contribute to the actions of caffeine, there is growing evidence that most pharmacological effects of this xanthine result from antagonism of adenosine receptor [Varani et al., 2000] and these non-AR-mediated actions require higher concentrations of caffeine than are typically ingested in the human diet [Fredholm and Jacobson, 2009; Stanley et al., 1989; Wilson and Mustafa, 2009].

Conlay *et al.* [Conlay et al., 1997] showed that adenosine antagonists such as caffeine influences rat plasma adenosine concentrations, by an unknown mechanism, at caffeine doses approximating those provided to humans by 3–6 cups of coffee per day.

2.5.2.2 Adenosine receptors (ARs)

Extracellular adenosine acts on a family of cell surface receptors termed adenosine receptors (ARs). To date four subtypes of ARs are known: A_1, A_{2A}, A_{2B}, and A_3 (see Table 2-8). The ARs are G protein-coupled receptors (GPCRs) and consist of a single polypeptide chain with the N terminus located at the extracellular side and seven transmembrane helices. The A_1 and A_3 receptors preferentially couple to G_i protein to inhibit AC and consequently the production of cAMP, whereas the A_{2A} and A_{2B} subtypes stimulate the production of cAMP by coupling to G_s or G_o proteins [Muller and Jacobson, 2011a]. Nevertheless, the receptors can couple also to other G protein pathways, especially when over expressed. The natural ligand is adenosine, but at A_1 and A_3 receptors inosine can also act as a partial agonist [Fredholm et al., 2001]. Furthermore, the A_{2B} subtype, which has the lowest affinity *(*K_i > 1µM**)** of all the subtypes for native adenosine, is also coupled to G_q (subunit that activates phospholipase

C). Adenosine has the highest affinity at the A_1 and A_{2A} ARs (K_i values of 10-30nM), and the affinity of adenosine at the A_3 receptors is intermediate (approx. 1 μM for the rat A_3 AR) [Wilson and Mustafa, 2009].

Table 2-8 Expression, G protein-coupling and major functions of adenosine receptors [Fredholm et al., 2001]

Tissue distribution/ function	Receptor			
	A_1	**A_{2A}**	**A_{2B}**	**A_3**
Hight expression	Brain, spinal cord, eye, adrenal gland and atria	Spleen, thymus, leukocytes (lymphocytes and granulocytes),**blood platelets**.GABA-ergic neurons, olfactory bulb	Cecum, colon, bladder	Testis (rat), mast cells (rat).
Intermediate levels	Skeletal muscle, liver, kidney**, adipose tissue**, salivary glands, esophagus, colon, antrum and testis	Heart, lung, blood vessels	Lung, blood vessels, eye, and mast cells	Cerebellum, hippocampus, lung, spleen (sheep), pineal
Low levels	Lung (but probably higher In bronchi), pancreas	Other brain regions	**Adipose tissue**, adrenal gland, brain, kidney, liver, ovary, pituitary gland	Thyroid, adrenal gland, spleen, liver, kidney, heart, intestine, testis
Fuctions	Bradycardia; **inhibition of lipolysis**; reduced glomerular filtration, antinociception; reduction of sympathetic and parasympathetic activity; presynaptic inhibition; neuronal hyperpolarization; ischemic preconditioning	Regulation of sensorimotor integration in basal ganglia; **inhibition of platelet aggregation** and polymorphonuclear leukocytes; vasodilatation, protection against ischemic damage, stimulation of sensory nerve activity	Relaxation of smooth muscle in vasculature and intestine; inhibition of monocyte and macrophage function, stimulation of mast cell mediator release	Enhancement of mediator release from mast cells preconditioning
G Protein coupling*	$G_{i1/2/3}$	G_o G_s G_{olf} $G_{15/16}$	G_s $G_{q/11}$	$G_{i2,3}$ $G_{q/11}$
Effects of G Protein coupling	↓ cAMP ↑ IP_3/DAG	↑ cAMP ↑ IP_3	↑ cAMP ↑ IP_3/DAG	↓ cAMP ↑ IP_3/DAG

*The Gα subunits G_s, G_i, $G_{12/13}$, and G_{q11} regulate various enzymes. For example, **G_s** stimulates, whereas **G_i** inhibits AC to increase or decrease cAMP. **$G_{q/11}$** activates phospholipase Cp (PLCP) to generate inositol trisphosphate (IP_3), which mobilizes Ca^{2+}, and diacylglycerol (DAG), which activates protein kinase C (PKC).

ARs are well conserved and widely expressed. The endogenous agonist, adenosine, has a minimal concentration that is sufficient to slightly activate the receptors where they are very highly expressed as in the basal ganglia, on fat cells and in the kidney. Adenosine has an important physiological role in these tissues and here antagonists such as caffeine may exert effects in healthy individuals. Adenosine levels rise in stress and distress (up to 30 µM in ischemia) and tend to minimize the risk for adverse outcomes by increasing energy supply and decreasing cellular work, by stimulating angiogenesis, mediating preconditioning and having multiple effects on immune competent cells [Fredholm, 2010; Fredholm et al., 2001].

When given in very high amounts, adenosine can affect intracellular nucleotide pools and even provide a source of metabolizable energy. In addition, it was reported that the human growth hormone secretagogue receptor (GHS-R) could also be activated by adenosine and not only by the endogenous agonist ghrelin [Tullin et al., 2000]. However, most effects of adenosine are due to activation of adenosine receptors.

The effects of ARs activation tend to be cytoprotective in many organs and tissues under a wide variety of physiological conditions. The increase of extracellular adenosine in response to stress (e.g. hypoxia) and the resulting activation of ARs are supposed to be a stress adaptation mechanism [Muller and Jacobson, 2011b] . These protective responses may take the form of decreased energy demand (e.g. bradycardia), increased energy supply (e.g. vasodilation or angiogenesis), ischemic preconditioning (e.g. in the heart or brain), inhibition of the release of excitotoxic neurotransmitters, suppression of cytokine-induced apoptosis, or reduced inflammatory response [Fredholm et al., 2001].

The half-life of adenosine in circulation is very short, due to its rapid conversion to inosine (adenosine deaminase) or phosporylation to 5′-AMP (adenosine kinase), or its uptake through nucleoside transporters (such as the equilibrative transporter ENT1). Therefore, analogues of adenosine for selective activation of ARs tend to prevent these processes and thereby lengthen the half-life. For example, the A_3-selective agonist IB-MECA has a half-life of 8–9 h in man [van Troostenburg et al., 2004].

Similar to the function and regulation of other GPCRs, both activation and desensitization of the ARs occur after agonist binding. Interaction of the activated ARs with G protein-coupled receptor kinases leads to their phosphorylation. AR responses desensitize rapidly, and this phenomenon is associated with receptor downregulation, internalization and degradation. The most rapid downregulation among the AR subtypes is generally seen with the A_3 AR (often a matter of few minutes), due to phosphorylation by GRKs. The A_{2A} AR is only slowly (shorter than 1 h) desensitized and internalized as a result of agonist activation, whereas A_1 AR shows a typical half-life of several hours [Klaasse et al., 2008; Wilson and Mustafa, 2009].

Synthetic adenosine antagonists have also been explored for potential therapeutic applications. Various early analogues of the xanthines, which greatly increased the AR subtype-selectivity over the naturally occurring alkylxanthines, tended to be hydrophobic and poorly water-soluble and consequently of low bioavailability. Recently introduced AR antagonists and prodrug approaches have overcome some of these issues [Muller and Jacobson, 2011a; Vollmann et al., 2008].

The structure–activity relationship of adenosine derivatives as AR agonists has been exhaustively studied. Nearly all of the known AR agonists are derivatives of purine nucleosides, either adenosine or xanthosine [Muller and Jacobson, 2011a]. The prototypical AR antagonists are alkylxanthine derivatives. The natural plant alkaloids caffeine and theophylline were the first adenosine receptor (AR) antagonists described in the literature. They exhibit micromolar affinities and are non-selective. A large number of derivatives and analogues were subsequently synthesized and evaluated as AR antagonists. Very potent antagonists have thus been developed with selectivity for each of the four AR subtypes [Muller and Jacobson, 2011b].

The major caffeine and theophylline metabolites in humans, paraxanthine and 1-methyl xanthine, are as potent as their parent compounds and therefore may contribute to the observed effects on ARs. These simple alkylxanthines show affinity to the ARs at nanomolar concentrations with slight variations between different species (see Table 2-9).

Table 2-9 Adenosine receptor affinities of natural xanthine derivatives [Muller and Jacobson, 2011b]

Xanthine	K_i (nM)			
	A_1	A_{2A}	A_{2B}	A_3
Caffeine	10.7 – 44.9 (*h*) 41.0 – 44.0 (*r*) 47.0 (*gp*)	9.56 – 23.4 (*h*) 32.5 – 48.0 (*r*)	10.4 – 33.8(*h*) 30.0 (*r*) 13.0 (*m*)	13.3(*h*) >100.0 (*r*)
Theophylline	6.7 (*h*) 8.74 – 14.0 (*r*) 7.06 (*gp*) 4.71 (*rb*) 6.3 (*c*)	1.7 – 6.7 (*h*) 22.0 – 25.3 (*r*)	6.07 – 74 (*h*) 15.1 (*r*) 5.63 (*m*) 11.0 (*gp*) 17.7 (*rb*) 37.8 (*d*)	22.3 – 86.4 (*h*) 85.0 – >100.0 (*r*) >100.0 (*d*)
Theobromine	83.4 – 105.0 (*r*)	>250.0 (*r*)	130.0 (*h*)	>100.0 (*r*)
Paraxanthine	21.0 (*r*)	32.0 (*r*)	4.5 (*h*)	>100.0 (*r*)

Human *(h)*, cow (*c*), dog (*d*), guinea pig (*gp*), mouse (*m*), rat (*r)*, rabbit (*rb*).

The present work mainly focuses on effects on platelets and adipose tissue, both expressing mainly A_1 and A_{2A} AR. Therefore, these two receptor subgroups and their physiological function will be discussed in more detail.

2.5.2.3 Adenosine A_1 receptor

The A_1 R is coupled to the inhibitory G_i and G_o proteins and stimulation of the receptor can therefore result in inhibition of AC, activation of several types of K^+ channels, inactivation of Ca^{2+} channels and activation of phospholipase Cβ [Fredholm et al., 2001]. Previous studies, where pharmacological tools have been utilized for the evaluation of metabolic roles of adenosine, have proposed that the A_1 R is of major importance [Dong et al., 2001].

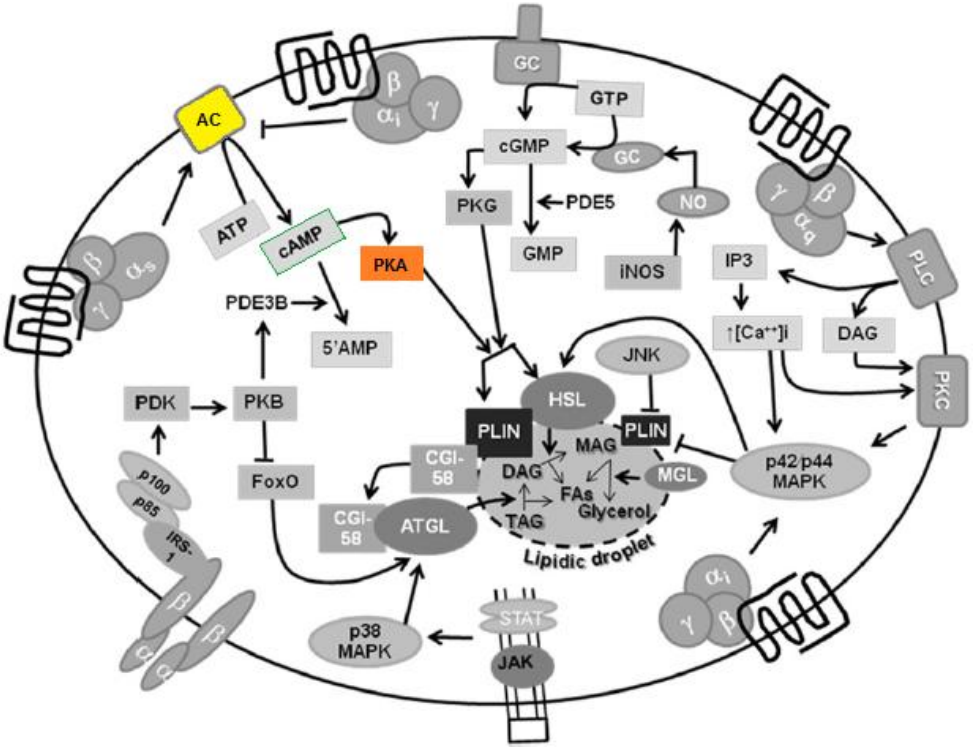


Figure 2-25: Summary of possible intracellular signalling pathways involved in the regulation of lipolysis in adipocytes. AC, adenylate cyclase; ATGL, adipose triglyceride lipase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CGI-58, comparative gene identification-58; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol FAs, fatty acids; FoxO1, forkhead box class O 1; GC, guanylate cyclase; GTP, guanosine triphosphate; HSL, hormone-sensitive lipase; iNOS, inducible nitric oxide; IP3, inositol triphosphate; IRS-1, insulin receptor substrate 1; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; NO, nitric oxide; PDE, phosphodiesterase; PDK, phosphoinositide-dependent kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PKG, cGMPdependent protein kinase; PLC, phospholipase C; PLIN, perilipin; STAT, signal transducers and activators of transcription; TAG, triacylglycerol. Modified from Chaves *et al* [Chaves et al., 2011].

There is evidence that adenosine can regulate several aspects of adipose tissue function including lipolysis. Adipose tissue is the only tissue capable of hydrolyzing its stores of triacylglycerol (TAG) and of mobilizing fatty acids and glycerol in the bloodstream so that they can be used by other tissues. The full hydrolysis of TAG depends on the activity of three enzymes, adipose triglyceride lipase (ATGL), hormonesensitive lipase (HSL) and monoacylglycerol lipase, each of which possesses a distinct regulatory mechanism [Chaves et al., 2011]. It has recently been shown that HLS and ATGL, together responsible for

approx. 95% of TAG hydrolase activity in adipocytes, can be activated simultaneously [Schweiger et al., 2006].

The main pathway leading to lipolysis is the cAMP-dependent proteinkinase (PKA) pathway (see Figure 2-25). The production of cAMP is modulated by G-protein-coupled receptors of the G_s/G_i family and cAMP degradation is regulated by PDE. Activation of AC in adipocytes subsequently increases intracellular cAMP levels leading to the activation of PKA, resulting in phosphorylation and translocation of HSL to fat droplets. However, other pathways that activate TAG hydrolysis are under investigation. Lipolysis can also be started by G-protein-coupled receptors of the G_q family, through molecular mechanisms that involve phospholipase C, calmodulin and protein kinase C. There is also evidence that increased lipolytic activity in adipocytes occurs after stimulation of the mitogen-activated protein kinase (MAPK) pathway or after cGMP accumulation and activation of protein kinase G [Greenberg et al., 2001]. Several agents, such as adenosine, atrial natriuretic peptide, β-hydroxybutyrate, catecholamines, endothelin-1, insulin, glucocorticoids, growth hormone, lactate, leptin, melanocortins, neuropeptide Y, peptide YY, prostaglandin E_2, thyroidstimulating hormone and TNFα, contribute to the control of lipolysis in adipocytes by modulating the activity of HSL and ATGL. However, some agents stimulate more than one intracellular transduction pathway [Chaves et al., 2011].

Secreted adenosine by adipocytes binds to the G-protein coupled adenosine A_1 receptor and reduces AC activity and induces the inhibition of lipolysis in adipose tissue [Dhalla et al., 2009]. Insulin also reduces lipolysis and intracellular cAMP levels, and both adenosine and insulin mediate additive antilipolytic effects in mouse adipocytes; however, the mechanisms of action are different, and the effects are not synergistic [Johansson et al., 2008]. Increases in cAMP accumulation and lipolysis in adipose tissue have been observed when adenosine deaminase is added to degrade endogenous adenosine or after treatment with adenosine receptor antagonists. Recently, it was shown that removal of endogenous adenosine with adenosine deaminase causes lipolysis in adenosine A_1 receptor (+/+) but not in adenosine A_1 receptor (-/-) adipocytes [Johansson et al., 2008] and that incubation of isolated rat adipocytes with an adenosine A_1 receptor antagonist induces an increase in lipolytic activity. Moreover, the adenosine A_1 receptor antagonist enhances the lipolytic response to epinephrine and increases cAMP levels in adipocytes. These findings provide strong evidence that the antilipolytic effect of adenosine involves impairment of PKA activation [Szkudelski et al., 2009].

The possibility that adenosine and insulin interact with each other in adipose tissue has potential clinical relevance in view of the growing concern for T2D secondary to obesity [Wild et al., 2004]. There is also considerable evidence that long-term coffee consumption is associated with a decreased risk for T2D, possibly via antagonistic actions of caffeine on adenosine A_1 receptors [van Dam and Hu, 2005].

2.5.2.4 Adenosine A_{2A}/A_{2B} receptors

The A_{2A} R are mainly coupled to stimulatory G proteins (G_s and G_{olf}) but can also couple to $G1_{5/16}$ [Kull et al., 2000; Offermanns and Simon, 1995] are present in the central nervous system as well as in peripheral organs (see Table 2-8) It has been shown that A_{2A} R antagonists can be of therapeutic value for the treatment of Parkinson's disease [Schwarzschild et al., 2006]. This receptor family is also involved in sleep regulation, shown by using A_{2A} R knock out mice where caffeine induced wakefulness [Huang et al., 2005]. Furthermore, A_{2A} R are important for regulating of blood pressure, vasodilation, ischemic damage, platelet aggregation, inflammation and pain [Fredholm, 2010; Jacobson and Gao, 2006].

Platelets are small anuclear circulating cell fragments which are critical for the process of thrombosis and the formation of hemostatic plugs [Watson, 2009]. Platelets are formed from the cytoplasm of large bone marrow megakaryocytes (Mks) by a process called megakaryocytopoiesis. A normal platelet count ranges between 150,000 and 350,000 per µL blood. The formed platelets are all of different shapes, have different compositions, and are all different sized. Despite lacking a nucleus, platelets are still highly active ‘cells’ retaining many intracellular organelles including mitochondria, an extensive cytoskeletal and tubule system, and specific alpha and dense granules which store platelet-specific proteins and coagulation factors which are released upon platelet activation, and enzymes such as cyclo-oxygenase and phospholipase A_2 which is activated in a Ca^{2+} dependent manner. Although they contain no nucleus, since platelets are formed from Mks, newly formed platelets contain small amounts of mRNA and mRNA processing enzymes [Kaushansky, 2008]. The platelet has a lifespan in the circulation of 7–10 days and the Mks-derived mRNA disappears from platelets over the first 24 h, therefore platelets containing mRNA (called reticulated platelets) only account for 5–15% of the total platelet population [Bishop-Bailey, 2010; Michelson, 2007]. Platelets function by adhering to the walls of injured blood vessels. They clump together or aggregate to form platelet plugs necessary for coagulation.

Atherothrombosis is a leading cause of mortality in the Western world. Platelet accumulation at sites of vascular injury is the primary event in arterial thrombosis, and the activation of platelets is a critical component of atherothrombosis [Ruggeri, 2002]. After the initial adhesion of platelets to extracellular matrix, such as to collagen at sites of vascular injury, autocrine and paracrine factors, including ADP, thrombin, epinephrine, and thromboxane A_2, amplify and sustain the initial platelet response, and recruit circulating platelets to form a hemostatic plug. Many agonists, such as ADP, thrombin, and epinephrine, directly activate platelet G_i receptors and decrease the cAMP level. Other agonists, including collagen and thrombin, also indirectly decrease their cAMP level by release of ADP [Davi and Patrono, 2007; Yang et al., 2010b; Yang et al., 2002].

It is well established that cAMP is a critical cytosolic regulator of platelet aggregation. cAMP inhibits platelets by activating PKA, which phosphorylates several substrates, such as inositol trisphosphate receptors, contributing to inhibition of cytoskeletal reorganization, integrin activation, and granule secretion [Sim et al., 2004].

Adenosine is released from vascular wall cells and platelets into the extracellular space as a breakdown product of ATP. Adenosine, acting through adenosine receptors, stimulates G-protein coupled AC in platelets and increases intracellular levels of cAMP, resulting in inhibiton of platelet activation [Yang et al., 2010b]. Platelets are rich in A_{2A} adenosine receptors and, it has been speculated that the A_{2A} AR is the only adenosine receptor that significantly affects platelet activation in response to adenosine [Varani et al., 1999]. A_{2A} AR knockout (KO) mice show higher platelet aggregation activity in response to ADP [Ledent et al., 1997].

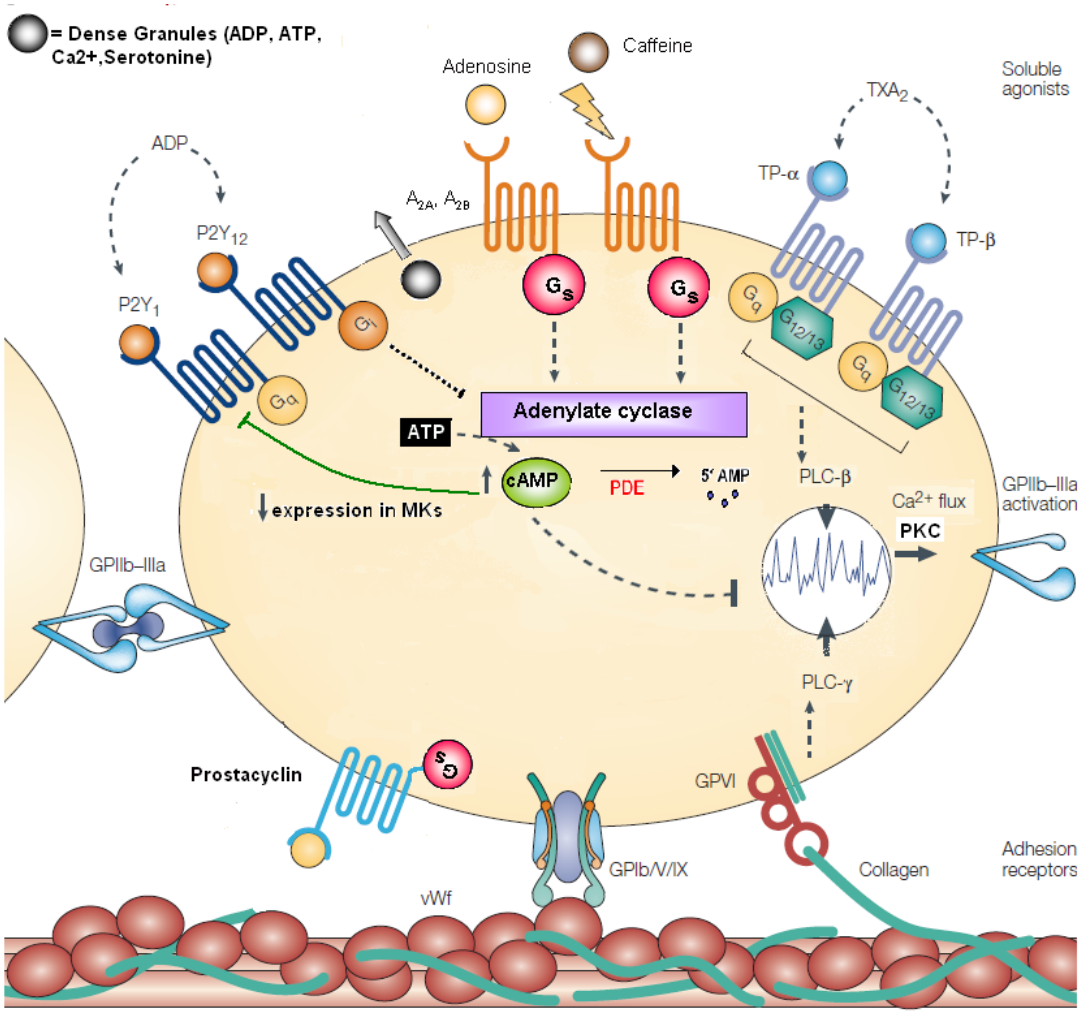


Figure 2-26: Regulation and action of cAMP in human platelets. PDE, phosphodiesterase; TXA_2, thromboxane A_2; GP, glycoprotein; PLC, phospholipase C; TP, thromboxane/prostanoid receptors; vWf, von Willebrand factor, MK, megakaryocyte; ATP, adenosine triphosphate; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; 5´AMP, Adenosin-5'-monophosphat; $A_{2A,}$ Adenosine A_{2A} receptor; $A_{2B,}$ Adenosine A_{2B} receptor; $P2Y_1$, P2Y purinoceptor 1; $P2Y_{12}$, P2Y purinoceptor 12. Adapted from Jackson and Schoenwaelder [Jackson and Schoenwaelder, 2003].

Amisten *et al.* [Amisten et al., 2008] recently showed that mRNA of the A_{2B} AR is expressed in human platelets at similar levels to A_{2A} AR mRNA. A_{2B} AR display a higher aggregation response controlling the expression of ADP receptors and ADP-induced platelet aggregation, where cAMP downregulates P2Y1R expression in megakaryocyte (MKs). It is, then, conceivable that the lifelong lower basal cAMP level in A_{2B} AR KO platelets could contribute to greater basal expression of ADP receptors, which would induce higher aggregation activity [Yang et al., 2010b].

There is evidence that repeated intake of caffeine alters the response of platelets to adenosine. After chronic caffeine consumption, platelet aggregability may be reduced due to upregulation of A_{2A} receptors present on the platelet surface. The antiaggregatory effects are associated with a rise in intracellular cAMP levels due to activation, by a selective A_{2A} agonist, of AC [Varani et al., 1999]. Caffeine acts most potently at A_{2A} receptors, followed closely by A_1 receptors, then A_{2B} receptors, and as a weak antagonist at human A_3 receptors [Klotz et al., 1998]. Blockade of ARs by caffeine, namely the A_1 and the A_{2A} receptor types, inhibits the action of endogenous adenosine in a variety of physiological processes. Under normal conditions, blood levels of adenosine appear to be sufficient to tonically activate A_{2A} receptors in platelets. It is therefore conceivable that caffeine could block these tonically activated A_{2A} receptors in platelets and alter their functions modulated by adenosine [Varani et al., 2000]. Biaggioni *et al.* [Biaggioni et al., 1991] found that a repeated dosing regimen of caffeine leads to significant changes in human platelets in the functional responses to the adenosine receptor agonist 5´-*N*-ethylcarboxamidoadenosine (NECA). Caffeine withdrawal produced a significant leftward shift of the NECA-induced inhibition of aggregation.

Repeated administration of caffeine (750 mg/d for 1 week) to human volunteers revealed an increase in A_{2A} receptor density in platelets, accompanied by sensitization of platelet responses, such as an increase in cAMP accumulation and decrease in platelet aggregation [Varani et al., 1999]. The treatment with 400 mg/d caffeine for 1 week did not modify A_{2A} receptor binding and functional parameters. However, treatment with 400 mg/d for 2 weeks or 600 mg/d for 1 week resulted in a significant increase of adenosine A_{2A} binding sites, a rise in cAMP accumulation, an increase of antiaggregatory effects, and a decrease in calcium levels elicited by the A_{2A} receptor agonist 2-hexynyl-5´-*N*-ethylcarboxamidoadenosine (HE-NECA) [Varani et al., 2000].

The upregulation of A_{2A} receptors can probably be ascribed to the synthesis of new receptors during differentiation of precursor cells (MKs). The increase of adenosine A_{2A} receptors found at 1 hour after the last dose of caffeine treatment was similar to that obtained at 12 or 60 hours after caffeine withdrawal, showing that the withdrawal was not necessary for the upregulation of A_{2A} receptors. This study also found that platelet aggregation was associated

with activation of AC and with a rise in intracellular calcium concentrations [Varani et al., 2000].

2.5.3 Cyclic nucleotide phosphodiesterase superfamily (PDEs)

PDEs comprise a group of enzymes that degrade the phosphodiester bond in the second messenger molecules cAMP and cGMP thereby hydrolyzing the 3´ bond of either cAMP or cGMP and converting it to its 5' counterpart (see Figure 2-27). While both cyclic nucleotides can be transported out of the cell at a low rate, evidence indicates that the catalytic action of PDEs provides the major pathway to rapidly lower cellular cyclic nucleotide content [Beavo et al., 2007].

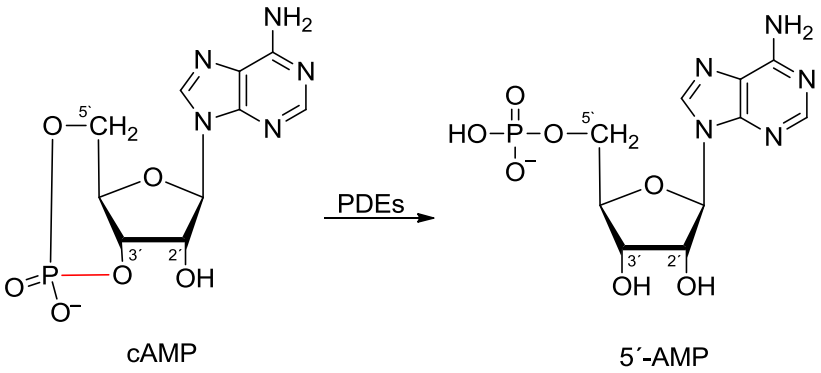


Figure 2-27: Degradation of cAMP to 5`-AMP by phosphodiesterases (PDEs).

As the cyclic phosphate structure is strained, this reaction is highly exothermic (>10 kcal/mol), making it essentially irreversible in the cell. This rapid and essentially irreversible reaction provides an effective mechanism for the control of a high-affinity second messenger, particularly, when levels of the messenger must oscillate rapidly [Greengar et al., 1969].

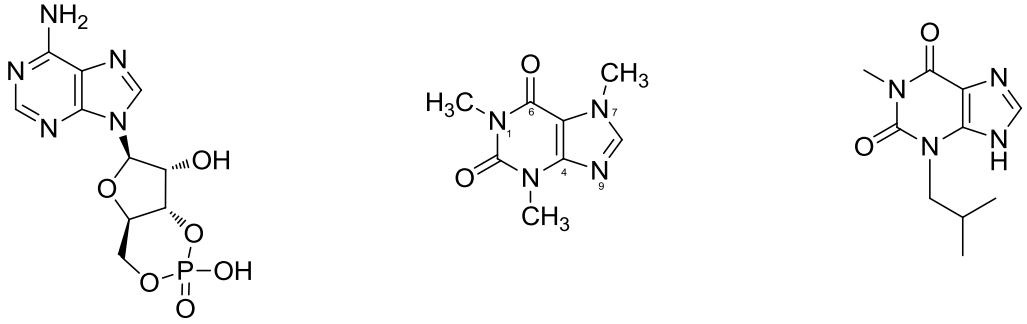
Two classes of PDEs (class I and class II) that can hydrolyze cAMP and cGMP are known. In mammals and flies, only class I enzymes have been identified whereas yeast and protozoans contain both class I and class II PDEs. It is interesting to note that multiple genes encoding cyclic nucleotide PDEs are present in nearly all eukaryotic species. However, in general, the complexity and number of different gene products increase as the complexity of the species increases [Bender and Beavo, 2006].

Class I PDEs are the most extensively studied groups of PDEs. In mammalian tissues, three of the eleven PDE families selectively hydrolyze cAMP, three families selectively hydrolyze cGMP and five families hydrolyze both cyclic nucleotides with varying efficacies (Table 2-10).

Table 2-10 PDE Isoenzyme Families [Bender and Beavo, 2006; Dousa, 1999; Francis, 2005; Jeon et al., 2005; Marko et al., 2000]

Enzyme family	Properties	Inhibitory compounds
PDE1	Ca^{2+}/calmodulin-stimulated, hydrolyzes cAMP and cGMP	Vinpocetine, 8-MeoM-IBMX, IC224, SCH51866
PDE2	cGMP-stimulated, hydrolyzes cAMP and cGMP	EHNA, PDP, IC933
PDE3	cGMP-inhibited, hydrolyzes cAMP and cGMP	Milrinone, Cilostamide, Trequinsin, Cilastazol,
PDE4	cAMP-specific, rolipram-sensitive	Rolipram, Ro-20-1724, Roflumilast, Cilomilat, Denbufyllin
PDE5	cGMP-specific, zaprinast-sensitive	Zaprinast, Dipyridamole, Sildenafil, Vardenafil, Tadalafil
PDE6	cGMP-specific, PDE of the photoreceptor	Zaprinast, Dipyridamole
PDE7	cAMP-specific, rolipram-insensitive	IC242, BRL 50481
PDE8	cAMP-specific, IBMX-insensitive	Dipyridamole
PDE9	cGMP-specific, IBMX-insensitive	SCH 51866, Zaprinast
PDE10	cAMP-inhibited, IBMX-sensitive, hydrolyzes cGMP and cAMP	Zaprinast, Dipyridamole
PDE11	hydrolyzes cAMP and cGMP	Tadalafil, Zaprinast, Dipyridamole

Is worth noting that the general structures for cyclic nucleotides and the derivatized alkylxanthine inhibitors, which are competitive inhibitors, are similar (see Figure 2-28).



cAMP **Caffeine** **IBMX**

Figure 2-28: Chemical structures of cAMP, caffeine and 3-isobutyl-1-methylxanthine (IBMX) a synthetic alkylxanthine nonspecific inhibitor of PDEs.

The heterocyclic ring of these inhibitors, which mimics the purine of cyclic nucleotides, comprises a six-membered pyrimidine ring conjoined with a five-membered ring containing two or more nitrogens. The affinity of a particular PDE for the various inhibitors is dictated by (1) the chemical characteristics of the particular heterocyclic ring, (2) groups appended to the ring, (3) differences in the distribution of electrons within the ring, and (4) structural restrictions of PDE catalytic sites for entry and binding of derivatized alkylxanthines with particular molecular structures. Substituents appended to the ring can impact affinity for the various PDE catalytic sites by forming new contacts with regions in and around the catalytic sites of PDEs or by interference with these regions. The distribution and number of carbons and nitrogens within the ring system can alter the electron distribution, thereby impacting the strength of the interactions [Beavo et al., 2007; Corbin et al., 2006; Francis et al., 2011].

The present work was focused on the study about the role of PDE3 and PDE4 isoenzymes in adipocytes and platelets as surrogate biomarker. For a better understanding of the problem, additional information about these isoenzymes will be described in more detail in the following chapters.

2.5.3.1 Phosphodiesterase 3 (PDE3)

The PDE3 has been extensively studied, especially in regard to its usefulness as drug targets. One unique feature of the PDE3 family is their ability to hydrolyze both cAMP and cGMP, but in a manner suggesting that *in vivo* the hydrolysis of cAMP is inhibited by cGMP. Thus, they have earned the title “the cGMP-inhibited PDE”. They also are characterized by their ability to be activated by several phosphorylation pathways including the PKA and PI3K/PKB pathways. Two PDE3 genes, *PDE3A* and *PDE3B,* have been identified, but splice/start variants have been conclusively demonstrated only for the PDE3A isoform [Bender and Beavo, 2006].

PDE3s were initially purified and described as enzymes that hydrolyze both cAMP and cGMP with relatively high affinities ($K_{m(cAMP)}$ <0.4 µM; $K_{m(cGMP)}$ <0.3 µM). However, the V_{max} for hydrolysis of cAMP is nearly 10-fold higher than the V_{max} for cGMP. Therefore, *in vitro* cGMP can act as an inhibitor of cAMP hydrolysis with an apparent K_i of 0.6 µM. This inhibition also occurs in platelets [Maurice and Haslam, 1990].

The PDE3A and PDE3B isoforms have a high degree of amino acid identity (>80% of the catalytic region) and very similar kinetic properties. The activity of both isoforms in several cell types is regulated by phosphorylation (e.g. by PKA) in response to hormonal stimulation. In platelets prostaglandins and epinephrine showed a feedback response through PKA to activate PDE3A [Shakur et al., 2001]. Similarly, first-messenger signals such as insulin, insulin-like growth factor 1 (IGF1), and leptin acting through the PI3K/PKB pathway can induce a phosphorylation of PDE3B and probably also PDE3A to stimulate their activity. It is thought that these phosphorylation events are important to many of the physiological processes controlled by these hormones [Bender and Beavo, 2006].

PDE3A and PDE3B are expressed in a variety of tissues and have distinct but overlapping localizations [Bender and Beavo, 2006] (see Table 2-11). PDE3B is a major PDE in adipose tissue, liver, and pancreas, as well as in several cardiovascular tissues [Shakur et al., 2001]. In contrast, PDE3A is distinctly expressed in platelets and oocytes, whereas PDE3B is unique to T lymphocytes, macrophages, *β*-cells, and adipocytes. In tissues, PDE3B is almost always found to be membrane bound, whereas PDE3A has been found to be both cytosolic and membrane bound [Shakur et al., 2001].

Table 2-11 PDE3 isoform localization and function [Beavo et al., 2007; Bender and Beavo, 2006; Shakur et al., 2001]

Isoform	Localization		Function
	Tissue/Cellular	Intracellular	
PDE3A	Heart, vascular smooth muscle, platelets, oocyte, kidney; PDE3A variants have differential expression in cardiovascular tissues	Can be either membrane associated or cytosolic, depending on the variant and the cell type	Regulates cardiac contractility, platelet aggregation, vascular smooth muscle contraction, oocyte maturation and regulation of renin release
PDE3B	Vascular smooth muscle, adipocytes, hepatocytes, kidney, β-cells, developing sperm, T lymphocytes, macrophages	Predominantly membrane associated; Localized to endoplasmic reticulum and microsomal fractions	Mediates insulin signaling, especially its antilipolytic effects. Regulates cell cycle/ proliferation and mediates the inhibitory effects of leptin and other signals on insulin secretion and renin release

Selective PDE3 inhibitors include amrinone, milrinone, cilostamide, and cilostazol. The most potent is trequinsin. Amrinone was the first recognized but possesses only modest affinity and selectivity [Bender and Beavo, 2006]. To date there have been no inhibitors described that clearly distinguish between PDE3A and PDE3B, although OPC-33450 has been reported to show some selectivity [Sudo et al., 2000].

PDE3 inhibitors antagonize platelet aggregation, block oocyte maturation, increase myocardial contractility, and enhance vascular and airway smooth muscle relaxation. In platelets, aggregation is highly regulated by cyclic nucleotides. PDE3A is a regulator of this process and PDE3 inhibitors effectively prevent platelet aggregation [Shakur et al., 2001]. In fact one drug, cilastazol (Pletal®), is approved for treatment of intermittent claudication. Its mechanism of action is thought to involve inhibition of platelet aggregation along with inhibition of smooth muscle proliferation and vasodilation. There is also evidence that cGMP, acting as a competitive inhibitor of PDE3A, exerts most of its antiplatelet effects by increasing cAMP via inhibition of PDE3A [Maurice and Haslam, 1990].

PDE3 enzymes are also involved in regulation of cardiac contractility and vascular smooth muscle. Interestingly, both PDE3A and PDE3B are expressed in vascular smooth muscle cells and are likely to modulate contraction. Their expression in vascular smooth muscle cells is altered under several conditions such as elevated cAMP, the switch from contractile to proliferative phenotype, and hypoxia [Maurice et al., 2003].

One of the most intensely studied roles for PDE3B have been in the areas of insulin, IGF1, and leptin signaling. Activation of PDE3B is thought to be important for the antilipolytic and antiglycogenolytic actions of insulin [Shakur et al., 2001], as well as for IGF1 and leptin inhibition of glucagon-like peptide-1 (GLP-1)-stimulated insulin release from pancreatic islets. This idea has now been expanded to include at least part of the effects of leptin on food

intake and body weight [Zhao et al., 2002]. At the molecular level it is thought that leptin, IGF1, and insulin activation of phosphatidylinositol 3-kinase (PI3K) in turn stimulates PKB phosphorylation of PDE3B triggering activation of the enzyme. In pancreatic β-cells, leptin causes the activation of PDE3B, which leads to marked inhibition of GLP-1-stimulated insulin secretion. The effect of leptin is abolished when insulin secretion is induced with cAMP analogues that cannot be hydrolyzed by PDE3B. Selective inhibitors of PDE3B and PI3K completely prevent the leptin effect on insulin secretion and cAMP accumulation [Zhao et al., 1998]. The involvement of PDE3B in regulation of these important metabolic pathways has encouraged researchers to begin exploring the possible roles of this enzyme in disorders such as obesity and DM [Bender and Beavo, 2006].

2.5.3.2 Phosphodiesterase 4

PDE4 has been one of the most studied PDEs, and there is a large body of literature on its biochemistry, genetics, and physiological function. It is now recognized that there are four genes (*PDE4A–PDE4D*) that make up this family. Moreover, each gene has multiple variants (>20 have been described). PDE4 is expressed in a multitude of tissues and cell types and plays a role in a large number of physiological processes (see Table 2-12) [Beavo et al., 2007; Bender and Beavo, 2006].

The PDE4 enzymes are universally selective for cAMP with K_m values typically between 1 and 10µM. V_{max} values of the PDE4 enzymes for cAMP, although lower than those for PDE1s and PDE2s, are comparable with those for most other PDEs [Beavo et al., 2007].

Several recent studies have shown links between PDE4 expression and disease conditions. For example, one region of the *PDE4D* gene was found to confer increased risk of ischemic stroke [Gretarsdottir et al., 2003] as well as a link between the *PDE4D* gene and bone density was found [Reneland et al., 2005].

This family of PDEs has been the most widely studied regarding its subcellular distribution and how this distribution is important to cellular functions and targets.

Many tumor cells exhibit significantly decreased cAMP levels as a consequence of overexpression of cyclic nucleotide phosphodiesterases. Marko *et al.* [Marko et al., 2000] found that in the solid large cell lung carcinoma LXFL529, both cytosolic and particulate PDE activities were much higher than in the respective cell lines. Furthermore, in both the tumor tissue and cell lines, Rolipram-sensitive PDE4 was found to represent the highest cAMP hydrolyzing activity [Marko et al., 2002].

The long forms of each PDE4 subtype can be phosphorylated by PKA. Phosphorylation of a site on the upstream conserved region 1 (UCR1) module causes a conformation change and a ~60 to 250% increase in activity of the catalytic domain. Functionally, phosphorylation also

leads to an increased sensitivity to Mg^{2+}, increased affinity for Rolipram, and an altered effect of extracellular signal-regulated kinase (ERK) phosphorylation [Sette and Conti, 1996].
Physiologically, it is thought that activation of the mitogen activated protein kinase (MAPK) pathway will initially lead to local increases in cAMP. This increase in turn will activate PDE4 phosphorylation by PKA that will cause a return of cAMP to a lower level [Houslay and Adams, 2003].
Nearly all of the PDE4 isozymes show both low (micromolar)- and high (nanomolar)-affinity Rolipram binding sites. These two different types of sites represent two or more different "states" of the catalytic domain of the PDEs. At least part of the high-affinity state seems to involve the bivalent metal ion sites in the catalytic domain [Bender and Beavo, 2006].

Table 2-12 PDE4 isoform localization and function [Beavo et al., 2007; Bender and Beavo, 2006; Shakur et al., 2001]

Isoform	Localization		Function
	Tissue/Cellular	Intracellular	
PDE4A	Widely expressed with mRNA found in many tissues; olfactory system, immune cells, and testis; high levels of several variants in the brain	PDE4A5 is localized to membrane ruffles through its Src homology domain; the supershort variant PDE4A1 is entirely membrane-associated; PDE4A4 associates with Src family kinases; PDE4A5 is localized by AKAP binding; PDE4A is recruited to a lipid raft fraction in activated T cells	At least one form is expressed in most cells, and PDE4s play roles in a wide array of processes, including brain function, monocyte and macrophage activation, neutrophil infiltration, vascular smooth muscle proliferation, fertility, vasodilation, and cardiac contractility
PDE4B	High levels of mRNA detected in a variety of tissues; notable expression in immune cells and the brain	PDE4s can be recruited to interact with β-arrestin; is recruited to a lipid raft fraction in activated T cells	
PDE4C	More restricted expression compared with other PDE4 isoforms; mRNA found in lung, testis, and several cell lines mainly of neuronal origin	Predominantly cytosolic; PDE4s can be recruited to interact with β -arrestin	
PDE4D	mRNA widely distributed and found in a variety of tissues; high protein levels in the brain; expression of variants seems to be localized to specific tissues and regions; variants are found in many commonly used cell lines (HEK293, COS) and in inflammatory cells	Variants can be found in cytosolic or particulate fractions; PDE4D3 is localized by binding to mAKAP and AKAP450; PDE4D3 is a part of the cardiac RyR2 channel complex; PDE4D5 interacts with RACK1; can be recruited to interact with β-arrestin	

mRNA (messenger RNA*);* RACK1, (receptor for activated C-kinase 1); AKAP (A-kinase anchoring proteins), RyR2 (ryanodine receptor 2), Src (Proto-oncogene tyrosine-protein kinase); mAKAP(muscle specific A-kinase anchoring protein).

A significant number of PDE4 inhibitors have been developed, and a great deal of investigation is ongoing to explore their use as therapeutic agents. The prototypical PDE4

inhibitor is Rolipram, which was originally developed as a possible antidepressant agent. This compound and now many others (see Table 2-10) can have >100-fold selectivity for inhibition of PDE4 versus other PDE family members. One problem with PDE4 inhibitors as therapeutic agents has been their propensity to promote emesis. It is thought that most of these effects are mediated, at least in part, via actions in the CNS, and so far it has been difficult to separate the effects on emesis from more desirable effects. Recently it has been suggested that inhibition of PDE4D in the brain is responsible for the emetic side effects of PDE4 inhibitors [Lipworth, 2005]. Thus, development of PDE4B specific inhibitors is being undertaken as a possible means of maintaining anti-inflammatory activity without causing the emetic side effect of nonspecific PDE4 inhibition.

In addition to chronic obstructive pulmonary disease, other inflammatory diseases for which PDE4 inhibitors currently being developed include use as drugs against asthma, arthritis and psoriasis. It is well established that PDE4 inhibitors can effectively suppress release of inflammatory mediators and immune cell infiltration [Castro et al., 2005]. Major dampening effects have been seen on neutrophil, monocyte, and T-lymphocyte function. However, PDE4 is a major cAMP hydrolyzing activity in a large number of proinflammatory and inflammatory cells, and PDE4 inhibitors probably target multiple cell types [Abrahamsen et al., 2004].

Finally, in animal models, treatment with rolipram or other PDE4 inhibitors seems to enhance several models of learning and memory [Siuciak, 2008; Zhang et al., 2005].

3 Aims

This work was conceived as part of the project “Identifizierung, Prüfung und Optimierung der gewichtsreduzierenden Eigenschaften von Kaffee“ (Identification, analysis and optimization of coffee weight-reducing properties) as a follow-up project to ***Coffee Prevention*** (grant No. 0313843) within the initiative "Funktionelle Ernährungsforschung” (functional nutrition research) of the BMBF (German Federal Ministry of Education and Research). The principal aim of this research project was to provide the scientific basis for the development and reproducible production of a functional coffee beverage with specific weight-regulating effect, providing scientific evidence for the functionality of the product in biological systems. The project was carried out with the following research groups: workgroup (WG) **Marko**, Department of Food Chemistry and Toxicology, University of Vienna, Austria; WG **Hofmann**, Food Chemistry and Molecular Sensory Science at the Technical University of Munich, Germany; WG **Somoza**, Department of Nutritional and Physiological Chemistry, University of Vienna, Austria and WG **Eisenbrand/Richling**, Department of Food Chemistry and Toxicology/Molecular Nutrition, Technical University of Kaiserslautern, Germany.

Earlier studies have shown that compounds generated during the so called Maillard reaction are potent inhibitors of cAMP phosphodiesterases. This applies, e.g. for TMP, which is found in trace amounts in roasted coffee along with other pyrazines. TMP exhibited a strong relaxation endothelium-independent effect in rat aorta, TMP causes cAMP accumulation and blockade of the release of calcium from internal stores. Moreover, summation of the vasodilatation was found when TMP was combined with theophylline, suggesting that the elevation of cAMP by TMP was similar to that caused by theophylline, a known inhibitor of phosphodiesterase. Thus, an increase in cAMP level may be responsible for the vasodilator effect of TMP on rat aorta. Additionally, release of calcium from internal stores can also play an important role [Wu et al., 1989]. Similar mechanisms have also been attributed to the demonstrated inhibitory effect on platelet function. TMP inhibits platelet aggregation by blocking intracellular Ca^{2+} mobilization, inhibiting PDE, and preventing exposure of GP IIb/IIIa receptors to the outer surface of platelets following activation [Liu and Sylvester, 1994]. A further effect of TMP that may have health significance is its ability to induce antioxidant enzymes such as HO-1 [Chen et al., 2006]. The consumption of coffee has previously been shown by our group to reduce oxidative damage in lymphocytes [Bakuradze et al., 2011a]. Therefore, the effect of coffee consumption on HO-1 activity should be investigated as a part of planned human intervention study within the BMBF research project.

The main objective of this doctoral thesis was to investigate whether substances other than caffeine in coffee may influence the homeostasis of intracellular cyclic nucleotides *in vitro* and *in vivo*. The interest was focused not only on the substances present in coffee before

roasting but also in those compounds generated during the roasting process which are found in brewed coffee.

Whithin this thesis, one goal was to evaluate the effect of coffee extracts and isolated coffee compounds on the enzymes involved in the cAMP signaling cascades and therewith the intracellular cAMP concentrations. The use of biomarkers and surrogate endpoints (blood cells) was necessary because they were easier, earlier, cheaper or more ethical to obtain, achieve or monitor than clinical endpoints (adipocytes). Therefore, potential target cells in blood allowing to estimate the effect of coffee consumption on nucleotide homeostasis in humans were evaluated.

The present work was divided into an *in vitro* part, where the effect of coffee compounds, and in some experiments coffee extracts, on the HO-1 activity, PDE activity and cAMP concentration were comparatively investigated; and a second *in vivo* part, which included two human studies: a short term study to test and choose the appropriate biomarkers to be employed in the following cross over designed long term study where the effect of activity-optimized coffee drinks on the previously selected biomarkers should be determined.

- ✓ The aims of the ***in vitro*** experiments were (1) to determine if coffee or some coffee constituents may have a stimulatory effect on the HO-activity of intestinal colorectal adenocarcinoma (Caco-2), liver hepatocellular carcinoma (HepG2) and monocytic leukemia (Mono Mac 6/ MM6) cell lines, (2) to investigate whether substances other than caffeine in coffee may influence the homeostasis of intracellular cyclic nucleotides in a known cell model: the large cell lung tumour xenograft (LXFL529L) cells, (3) find an appropiate surrogate biomarker of adipocytes and characterize the effect of the previously mentioned coffee compounds and coffee extracts on its intracellular cyclic nucleotides homeostasis and (4) to evaluate the potential of preadipocytes (SGBS) cells, as a model for adipocyte differentiation, respect to these biomarkers.
- ✓ Furthermore, the aims of the ***in vivo*** experiments were (1) to find appropiate biomarkers for the modulation of the cAMP pathway, (2) to assess whether moderate consumption of regular coffee, caffeine reduced coffee and/or functional coffee can modulate the selected biomarkers, (3) to identify and characterize specific changes in circulating nucleosides (adenosine) and enzymes (adenosine deaminase) following exposure to coffee and (4) to attempt to interpret these changes in term of metabolic pathways.

4 Material and methods

4.1 Cell culture

To culture cells is a complex process by which cells are grown and maintained under controlled conditions. These conditions are appropriate temperature, air humidity and gas mixture (typically, 37 °C, 5% CO_2 for mammalian cells) in a cell incubator. Culture conditions may vary according to the cell type and variation of conditions for a particular cell type can result in different phenotypes being expressed.

The most commonly varied factor in culture systems is the growth medium. Recipes for growth media vary e.g. in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as calf serum.

Cells can be grown in suspensions or adherent cultures. Some cells need to be cultured in suspension without being attached to a surface, such as cells that naturally exist in the bloodstream (e.g. lymphocytes, monocytes etc). On the other hand, adherent cells require a surface, such as tissue culture plastic, which may be coated with extracellular matrix components to increase adhesion properties and provide other signals needed for growth and differentiation. Most cells derived from solid tissues are adherent.

4.1.1 Cell lines

HEK-293 cell line

HEK-293 cells also commonly known as 293 are fibroblastoid semi-adherent human primary embryonal kidney cells that grow as a monolayer with a doubling time of 20-24 h. They were established in 1977 from a human primary embryonal kidney and transformed using the adenovirus type 5. They are rated “risk category 1” by the German Central Commission for Biological Safety [DSMZ, 2004].

HEK-293 cells were grown in Dulbecco's Modified Eagle Medium (with 4500 mg/l Glucose, and pyridoxin HCl, without Natrium-Pyruvat) containing 1% (v/v) penicillin/streptomycin (10,000 units/10,000µg per ml) and 10% (v/v) heat-inactivated fetal calf serum (FCS) in a humidified incubator. HEK-293 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH –DSMZ).

HT29 cell line

HT29 cells are epithelial-like adherent cells that grow as monolayer in large colonies with a doubling time of 40-60h. They were first established in 1964, originally from a primary colonic tumour of a 44-year old Caucasian female. They form a well-differentiated adenocarcinoma

consistent with colony primary grade I and possess a hyper-triploid karyotype with a 17.5% polyploidy [DSMZ, 2004].

HT29 cells were cultured in Dulbecco's Modified Eagle Medium (with 4500 mg/l Glucose, and pyridoxin HCl, without sodium pyruvate) containing 1% (v/v) penicillin/streptomycin (10,000 units/10,000µg per ml) and 10% (v/v) heat-inactivated FCS in a humidified incubator. HT29 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH –DSMZ).

HepG2 cell line

HepG2 cells are human hepatoma cancer cells which were established from the tumour of a 15 year old Argentinian harboring a hyperploid kayrotype [DSMZ, 2004]. They are adherent cells which grow in a monolayer.

HepG2cells were cultured in RPMI 1640 medium containing L-glutamine, 1% (v/v) penicillin/streptomycin (10,000 units/10,000 µg per ml) and 10% (v/v) heat-inactivated FCS in a humidified incubator. Cells were kindly provided by Prof. Dr. Dr. med. D. Schrenk (University of Kaiserslautern, Germany).

Caco-2 cell line

The Caco-2 cell line is a continuous human epithelial colorectal adenocarcinoma cell line, established from the primary colon tumor (adenocarcinoma) of a 72-year-old Caucasian man in 1974 [DSMZ, 2004]. Although derived from a colon (large intestine) carcinoma, when cultured under specific conditions the cells become differentiated and polarized such that their phenotype, morphologically and functionally, resembles the enterocytes lining the small intestine.

Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium, 1% (v/v) penicillin/streptomycin (10,000 units/10,000µg per ml) and 20% (v/v) heat-inactivated FCS in a humidified incubator. Caco-2 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH –DSMZ).

MonoMac 6 cell line (MM6)

The MonoMac cell line is a human acute monocytic leukemia cell line established from the peripheral blood of a 64-year-old man with relapsed acute monocytic leukemia in 1985 following myeloid metaplasia.

MonoMac 6 cells were grown in RPMI 1640 containing L-glutamine, 1% (v/v) penicillin/streptomycin (10,000 units/10,000 µg per ml),10% (v/v) heat-inactivated FCS and

25 mM HEPES in a humidified incubator [DSMZ, 2004; Ziegler-Heitbrock, 2006]. Cells were kindly provided by Prof. Dr. Dr. med. D. Schrenk (University of Kaiserslautern, Germany).

LXFL 529L

The large cell lung tumour xenograft LXFL 529L was established in serial passage onto nude mice, NMRI genetic background (from which a permanent cell line was developed [Berger et al., 1992]. This cell line was cultured at 37 °C (5% CO_2 and 95% humidity) in RMPI 1640 medium with addition of 1% penicillin/streptomycin and 10% fetal calf serum (FCS).

In general, cells were subcultured once or twice weekly and were routinely tested for the absence of mycoplasm contamination. Cells were kindly provided by Prof. Dr. H. H. Fiebig(University of Freiburg, Germany).

SGBS Cells

The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell strain originates from an adipose tissue specimen of a patient with SGBS. They are neither transformed nor immortalized, and provide an almost unlimited source due to their ability to proliferate for up to 50 generations with retained capacity for adipogenic differentiation [Fischer-Posovszky et al., 2008]. Cells were kindly provided by Prof. Dr. M. Wabitsch (University of Ulm, Germany) [Wabitsch et al., 2001].

Adipogenic differentiation of SGBS cells

The optimized differentiation scheme is depicted in Figure 4-1. The cells were grown in plates for 3 days to near confluence in DMEM (Dulbecco's Modified Eagle Medium)/F12 containing 10% fetal calf serum (FCS). Differentiation is started by washing cells 3 times with phosphate buffered saline (PBS) and then changing to a serum- and albumin-free differentiation medium (DMEM/F12 supplemented with 2 µmol/l Rosiglitazone, 25 nmol/l Dexamethasone, 0.5 mmol/l methylisobuthylxantine, 0.1 µmol/l cortisol, 0.01 mg/ml transferrin, 0.2nmol/l triiodotyronin, and 20 nmol/l human insulin). After 4 days, medium is changed, and cells are further cultured in DMEM/F12 supplemented with 0.1 µmol/L cortisol, 0.01 mg/ml transferrin, 0.2 nmol/l triiodotyronin, and 20 nmol/lL human insulin [Wabitsch et al., 2001]. Within a few days, the cells start to accumulate lipids,and small lipid droplets are visible after approximately 1 week [Fischer-Posovszky et al., 2008], for functional cAMP studies the cells were differentiated until day 17 and for lipolysis assays until day 20.

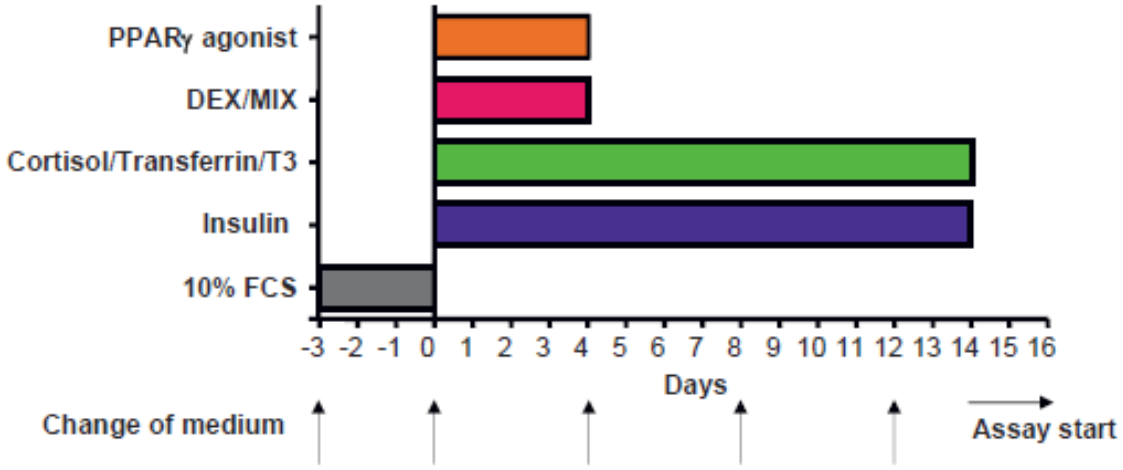


Figure 4-1. **Optimal differentiation protocol for human SGBS cells [Fischer-Posovszky et al., 2008]**

4.1.2 Changing Cell Medium

The culture medium is the most important component of the culture environment, because it provides the necessary nutrients and growth factors for cell growth, as well as regulating the pH and the osmotic pressure of the culture.

The medium in which cells are grown not only provides essential nutrients for cell growth, but also collects excretory products of cell metabolism. Increasing accumulation of cellular metabolic products as protons, leads to an increasing acidity of the growth medium, which can be monitored by the gradual change in the colour (from red to yellow) of the phenol red indicator contained in most growth medium.

To change the growth medium, old medium was removed and the cells were rinsed once with PBS (37°C) to wash out dead cells. Subsequently, pre-warmed (37°C) adequate growth medium was added and the flask is placed in the humidified incubator (37°C, 5% CO_2, 95% moisture).

Table 4-1: PBS Composition (1x)

Reagent	Final concentration (mM)
NaCl	171.0
Na_2HPO_4	10
KCl	3.4
KH_2PO_4	1.8

pH 7.4, autoclaving.

4.1.3 Subculture of Cells

Subculturing, also referred as passaging, is the removal of the medium and transfer of a fraction of cells from a previous culture into fresh medium, a procedure that enables further propagation of the cell line or cell strain.

Many adherent cell lines grow optimally as monolayer in cell culture flasks. On attaining a confluent population, some cell lines transcend to multilayer growth by adding multiple layers over the first, resulting in suppressed cell growth (contact inhibition) and a change in metabolism. To maintain monolayer cell growth, cells were passaged when they had reached a semi-confluent population.

The growth medium was aspirated and the cells were rinsed with pre-warmed and sterile PBS to remove dead cells and residual growth medium (that might inactivate trypsin/EDTA and hinder cell detachment). Then pre-warmed (37 °C) trypsin/EDTA was added and the culture was placed in a humidified incubator (HT29 and SGBS: 3 min, HEK-293: 10 s, Caco-2: 5 min). The culture flask was gently clapped to detach the cells. Pre-warmed (37 °C) serum-supplemented medium was added (inhibits the lysing activity of trypsin/EDTA) and the cells were re-suspended by pipetting up and down. The required volume of the cell suspension was transferred to a new culture flask, pre-warmed serum and antibiotic supplemented medium was added, and the cells were further cultivated under normal conditions.

For suspension cells like MonoMac6, an aliquot of the cell suspension with uniformly dispersed free cells and cell aggregates was transferred to a new flask with fresh medium.

Table 4-2: Trypsin-EDTA solution composition

Reagent	Amounts
Trypsin	500 mg
EDTA	250 mg
PBS (10x)	100 ml

Stirred overnight on ice, set to pH 7-7.4 and filled to 1000 ml with re-distilled water, steril filtered (0.2 µm) and aliquoted à 5 ml and stored at -20 °C.

4.1.4 Cell quantification

Measuring total cell numbers (by haemocytometer counts of whole cells) is easily achieved. The counting chamber consists of nine squares, each of these squares have a defined area of 1 mm^2 and a depth of 0.1 mm. Thus arises a volume of 0.1 µl per square. Shortly before the cells are passaged, the counting chamber was prepared: the cover was cleaned with 70% Ethanol and lightly moistened, then was gently slid onto the counting chamber .The

formation of Newton rings between the external support and the cover glass indicates correct positioning of the cover glass.

For cell counting, an aliquot of cell suspension (see 4.1.3) was diluted 1:1 with trypan blue. This dye stains dead cells due to their membrane permeability to blue. These blue-stained cells were not taken into account during subsequent counting. A defined part of the stained cell suspension was then pipetted under the coverslip by capillary action of the hemocytometer. Only living cells in the four corner squares of the chamber were counted. The mean of the cell number was multiplied by the dilution factor of 2 and 10^4 to obtain the number of cells per milliliter [Lindl, 2000].

4.1.5 Mycoplasma detection

Cultures can become infected by mycoplasma from media, sera, trypsin or the operator. They are not visible to the naked eye, and, while they can affect cell growth, their presence is often not obvious. It is important to test for the absence of mycoplasma at regular intervals (every 1-3 months) as they cab seriously affect cellular biochemistry, antigenicity and growth characteristics [Freshney, 1994].

To test for mycoplasma infection, cells were plated on a glass slide and allowed 24 h for regeneration and growth. Thereafter, medium was washed off the slide with cold methanol (-20°C) and the slide was placed in methanol (-20 °C) for at least 15 min to affix the cells. Subsequently, the cells were stained with DAPI/SR101 solution (4'-6-diamidino-2-phenylindol-di-hydrochloride/sulforhodamine 101) and scrutinized under a fluorescence microscope (excitation wavelength of 450 - 490 nm). Nucleic acid appears blue (DAPI) and plasma proteins appear red (SR101). Scattered small bluish dots seen over the red regions of the plasma are a positive sign for mycoplasma infection [Lindl, 2000].

4.1.6 Storage of Cells

Cellular damage induced by freezing and thawing is generally believed to be caused by intracellular ice crystals and osmotic effects. The addition of a cryoprotective agent, such as DMSO or glycerol, and the selection of suitable freezing and thawing rates, minimizes cellular injury [Freshney, 1994].

The cells were trypsinized as described in Section 6.1.1.4. Trypsin was inactivated by addition of 8 ml of fresh serum-containing medium. The cell suspension was divided into 1 ml aliquots and mixed with 100 µl DMSO. The cells are then frozen at -20 °C and further stored at -80 °C in the biofreezer. Long-term storage takes place in liquid nitrogen. About $1x10^6$ SGBS cells were suspended per freezing vial and then placed in a freezing container filled with isopropanol and stored at -80 °C overnight. Next day cells were placed in liquid nitrogen storage tank.

4.1.7 Re-culturing Cells

During cell culture, it is of utmost importance to be mindful because some cell lines, while in culture, change their physiological properties with increasing passage number. It is therefore necessary to replace old cells from stocks to maintain reproducible results (Lindl 2000).
The frozen cell suspension was thawed quickly in a water bath at 37 °C, rapidly taken up with 10 ml medium (10 % FCS, v/v) and centrifuged at room temperature at 200g for 4 min. The supernatant was discarded and the cell pellet was re-suspended in 5ml medium (20%, FCS v/v). Finally, the entire cell suspension is transferred to a small tissue culture flask (25 cm^2) and incubated at 37° C, 5% CO_2 and 95% humidity. Upon achieving high confluency, the cells are passaged into a medium culture flask (80 cm^2). Cells were maintained for at least two weeks in culture before attaining experimental competence [Lindl, 2000].

4.1.8 Trypan blue viability test

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm [Strober, 1997].
The mix of cell suspension and trypan blue was made as previously described, unstained (viable) and stained (nonviable) cells were count separately in the hemacytometer. To obtain the total number of viable cells per ml of aliquot, the total number of viable cells was multiplied by 2 (the dilution factor for trypan blue). Calculations of the percentage of viable cells were made as follows:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{Total number of cells per ml of aliquot}} \times 100$$

4.1.9 Cell culture materials

Equipment

Analytical balance	Sartorius
Autoclave-steam sterilizer	Tuttnauer Systec, 5075 EL
Biofreezers	Heraeus, Herafreeze
	Angelantoni Industrie, Irilab 800

Centrifuges	Heraeus Instruments Multifuge 1 L-R
	Eppendorf, Centrifuge 5417R
CO_2 incubators	Binder, Tuttlingen
	wtbBinder, Tuttlingen
Counting chamber	Marienfeld, Neubauer-improved
Drying cabinet	Heraeus, KTG 800
Electronic single-channel pipette	Research® pro, Eppendorf
Fluorescence microscope	Axioscop2 (HB 50/AC), Zeiss
Graduated and pasteur pipettes	Hirschmann Laborgeräte
Laminar flow cabinets	Heraeus Instruments Laminair HLB 2448 GS
	Kojair, BIOWIZARD Golden Line
Microscope	Leitz Labovert inverted microscope 090-122.01
Multichannel pipettes	Abimed HT
	Research® pro Electronic Pipette, Eppendorf
Multipette	Eppendorf, Abimed
NALGENE® freezing container	Thermo Fisher Scientific
pH meter	Hanna Instruments HI2210
Pipetus® (pipetting aid)	Hirschmann Laborgeräte
Thermomixer	Eppendorf
Ultrasonic bath	Bandelin, Sonorex RK 106
Water Bath	Julabo SW-20C
Vortex *Genie 2TM*	Bender & Hobein AG

Chemicals and culture media

Apo-transferrin	Sigma-Aldrich
Biotin	Sigma-Aldrich
DAPI/SR101 solution	Partec
Dexamethasone	Sigma-Aldrich
DMEM/F-12	Gibco/Invitrogen
DMSO for UV-spektroskopie	Fluka
Dulbecco´s MEM/Nutrient-Mix F12 (DM)	Gibco/Invitrogen
Dulbecco´s Modified Eagle Medium (DMEM)	Gibco/Invitrogen
EDTA	Merck
Ethanol, p.A.	Merck
Fetal calf serum (FCS)	Gibco/Invitrogen
Glukose	Merck
HBSS	Gibco/Invitrogen

HEPES	Merck
Hydrocortisone	Sigma-Aldrich
IBMX	Sigma-Aldrich
Insulin	Sigma-Aldrich
KH_2PO_4	Merck
KCl	Merck
$MgSO_4 * 7 H_2O$	Merck
Methanol	Merck
$NaHCO_3$	Merck
Na_2HPO_4	Merck
NaCl	Merck
NaOH	Merck
Pantothenic acid	Sigma-Aldrich
Penicillin-Streptomycin	Gibco, Invitrogen
2-Propanol	Merck
RPMI 1640	Gibco, Invitrogen
Rosiglitazone	Sigma-Aldrich
Triiodo-L-thyronine	Sigma-Aldrich
Trypan Blue solution (0.4%)	Sigma-Aldrich
Trypsin, 3,5 U/mg	Serva
Versene	Gibco, Invitrogen

Consumables

Cell culture dishes (35, 60 94mm)	Greiner
Cell scrapers	Greiner
Centrifuge tubes (Falcon tubes)	Greiner
Cryogenic storage vials (1ml)	Greiner
Microplates (6,12, 24, 48, 96 well formats)	Greiner
Pipette tips	Greiner
Reaction tubes (1,5 and 2 ml)	Greiner
Sterile filters (0,2µm, cellulose acetate, PVDF)	Sartorius, Roth
Suspension culture flasks (25cm^2, 75cm^2, 175cm^2)	Greiner
Syringes and needle	Braun
Tissue culture flasks (25 cm^2, 75 cm^2, 175 cm^2)	Greiner, Nunc, BD Falcon

4.2 Heme oxygenase assay (HO-assay)

4.2.1 Principles of the assay

The assay for determination of total HO-activity is based on the enzymatic conversion of heme to bilirubin via biliverdin (see Figure 4-2). Heme is oxidatively metabolized to biliverdin, which dissociates from the HO-1 and is rapidly converted to the hydrophobic molecule, bilirubin, in an NADPH-dependent reaction catalyzed by cytosolic biliverdin (BVR). Liver cytosol has been used as a source of BVR. This method has been described by Tenhunen et al. [Tenhunen et al., 1970] and modified by Motterlini et al. [Motterlini et al., 1995] and recently by McNally *et al.* [McNally et al., 2004].

HO-activity was assessed in the cell lysate by measuring formation of the HO metabolite bilirubin. Therefore cells were disrupted after incubation to achieve a complete release of the HO enzyme. Hemin converted by HO to bilirubin was extracted with chloroform. The amount of extracted, yellow-colored bilirubin is determined with a UV spectrometer and correlated with the enzymatic activity of HO.

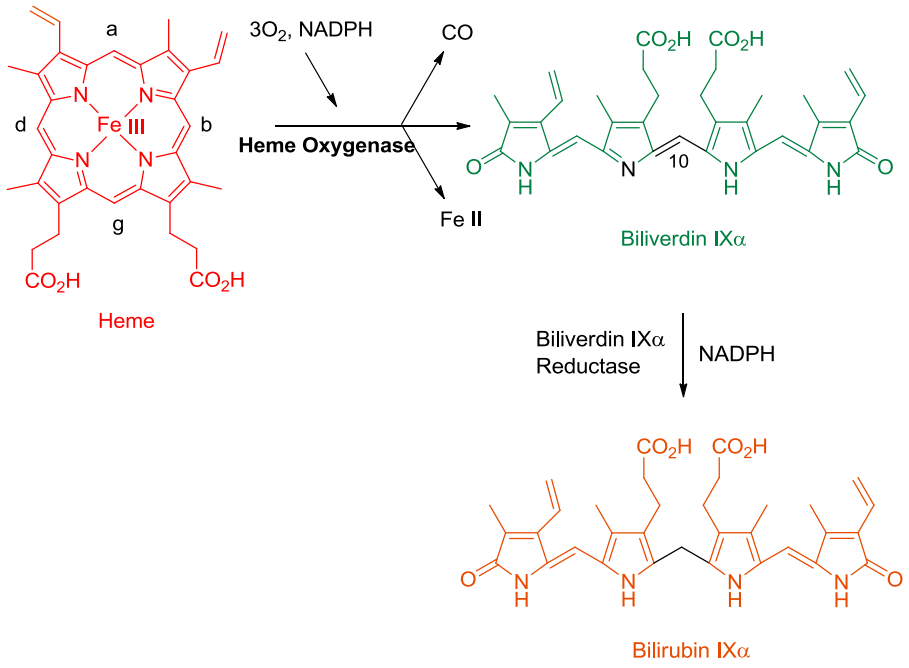


Figure 4-2: Pathway of heme metabolism. HO enzymes catalyze the rate-limiting step in heme metabolism. Both HO isoenzymes (HO-1 and HO-2) oxidize heme to the bile pigment biliverdin-IXα. The reaction requires 3 mol of molecular oxygen and NADPH as well as cytochrome *P*-450 reductase as a source of electrons. The cleavage of the heme ring releases the coordinated iron, as well as the α-methene bridge carbon as carbon monoxide (CO). The principal HO reaction product, biliverdin-IXα, is further metabolized to bilirubin-IXα by NAD(P)H and biliverdin reductase. Modified from Ryter *et al.* [Ryter et al., 2006b].

4.2.2 Cell lysate and sample preparation

$3x10^6$ HepG2, Caco2 cells or $1x10^6$ MonoMac6 cells were seeded in Petri dishes (∅ 92 mm) or in small tissue culture flask. Confluent cells were then incubated with the test substance for 24h in medium without FCS under normal growth conditions (37 °C, 5% CO_2 and 95% humidity).

Briefly, after the incubation, cells were washed twice with cold PBS, gently scraped off the dish, and centrifuged (1000*g* for 10 minutes at 4°C). The cell pellet was suspended in 500 µl buffer (2 mM $MgCl_2$ and 100 mM KH_2PO_4, pH 7.4), frozen in liquid nitrogen and thawed for 3 times, and finally sonicated on ice before centrifugation at 18 000*g* for 10 minutes at 4 °C. 400 µL of the supernatant was added to 200 µl NADPH-test system (see Table 4-3).

The reaction was conducted for 1 hour at 37 °C in the dark and terminated by the addition of 800 µl chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm with use of a quartz ultra microcuvette (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO-activity was expressed as picomoles of bilirubin formed per milligram protein per hour [Foresti et al., 1997; Polte et al., 2000]

Table 4-3: NADPH-test system Composition

Reagent	Concentration
ß-NADPH	0.8 mM
Glucose-6-phosphate	2 mM
Glucose-6-phosphate dehydrogenase	0.2 U
Hemin	50 µM
Rat liver cytosol (see 6.3)	500 µg Protein

Final volume: 200 µl

.

4.2.3 HO assay materials

Equipment (see 4.1.9 Cell culture)

Precision ultra micro Cell	Hellma
Ultrasonic homogenisor	Braun, Labsonic 2000
UV-VIS spectrophotometer	Shimadzu, UVmini-1240

Chemicals (see 4.1.9 cell culture)

Ammonium pyrrolidinedithiocarbamate	Sigma-Aldrich
Chloroform p.A	Fisher Scientific
D-Glucose 6- phosphate	Sigma-Aldrich
Glucose-6-phosphat-dehydrogenase *(S. cerevisiae)*	Sigma-Aldrich

Hemin	Sigma-Aldrich
KH_2PO_4	Merck
$MgCl_2$	Merck
β-NADPH	Sigma-Aldrich

Consumables (see 4.1.9 Cell culture)

4.3 Rat liver cytosol preparation

4.3.1 Rat liver cytosol preparation Method

The livers of three albino rats (120-180 g body wt.) were used for the preparation of the cytosol. Animals were starved for 24 h before being killed by decapitation.

4.3.1.1 Preparation of homogenate

After decapitation of rats, livers were dissected out rapidly and placed on ice in a beaker glass. The livers were washed three times with MIC-I-Buffer (see Table 4-4). The washed livers were blotted dry with filter paper and broken up by mincing. Portions of the mince were transferred with 9 ml of MIC-I-Buffer into a glass homogenizer (Potter-Elvehjem Tissue Grinder) and homogenized with 5 passes of a PTFE-pestle.

Table 4-4: MIC-I-Buffer Composition

Reagent	Concentration
Sucrose	0.25 M
EDTA	5.5 mM
Tris	0.2 M

pH: 7.5

4.3.1.2 Isolation of cytosol.

The homogenate was centrifuged at 100 g for 10min at 4 °C, the supernatant then centrifuged at 9000 g for 10 min at 4 °C. The resulting supernatant was ultracentrifuged at 105000 g for 1h at 4 °C to obtain the cytosolic fraction (supernatant) and a microsome pellet. The cytosolic fraction was aliquoted, frozen in liquid nitrogen and then stored at -80 °C.

4.3.2 Rat liver cytosol preparation materials

Equipment

Potter-Elvehjem tissue grinders	Wheaton Industries Inc
PTFE pestles	Wheaton Industries Inc
Tissue homogenizer	Heidolph-Laboratory RZR-2100
Ultracentrifuge	Beckman, Optima™ TL

Chemicals

EDTA	Serva
HCl	Roth
Sucrose	Fluka
Tris	Roth

Consumables (see 4.1.9 Cell culture)

4.4 Protein determination by Bradford

The Bradford assay is a colorimetric protein assay based on an absorbance shift of the dye Coomassie® Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its blue form that binds to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 365 to 595 nm and it is the increase in absorption at 595 nm which is monitored. The amount of the complex present in solution is a measure for the protein concentration and can be estimated by use of an absorbance reading [Bradford, 1976].

4.4.1 Procedure

A sequential standard row from 0.1-1.0 mg/ml protein was prepared with bovine serum albumin (BSA, Fraktion V, 2 mg/ml, Bio-Rad). If required, the samples were diluted with re-distilled water. 10µl of each probe, blank (re-distilled water or buffer in which the sample was processed) and standard was pipetted in triplicates into a 96-well microplate on ice. Then 190 µl of Bradford solution was added to each well and after 5 min incubation at RT, the plate was measured at λ = 595 nm in a microplate reader. The standard curve was used to determine the protein concentration for each unknown sample.

4.4.2 Protein determination by Bradford Materials

Equipment

Multidetection microplate reader	BioTek, Synergy™ 2

Chemicals

Bradford reagent	Bio-Rad
Bovine serum albumine (2 mg/ml)	Bio-Rad

Consumables

96-well microplates (F-bottom, PS)	Greiner
Pipette tips	Greiner
Reaction tubes	Greiner

4.5 Phosphodiesterase (PDE) activity determination

4.5.1 PDE assay method

PDE activity was determined according to the method of Pöch [Poch, 1971] with slight modifications. It is used to determine the turnover of cAMP (cGMP) to 5'AMP (5'GMP) by phosphodiesterases. A definite amount of tritiated cyclic nucleotide was added to the cell lysate and depending on its PDE activity cAMP (cGMP) will be converted at different rates to the corresponding 5'-nucleotide. This will be precipitated after a definite period of incubation (≈ 20% conversion of cAMP to 5´AMP). The remaining radioactive cAMP (cGMP) in the supernatant was measured using a scintillation counter, its value is inversely proportional to cAMP (cGMP) concentration in the sample.

4.5.1.1 Preparation of the cell lysates (*in vitro* assay)

The cells were trypsinized and counted as described in 6.1.1.4 and 6.1.1.5. Per cell line, three petri dishes (∅ 92 mm) with $1x10^6$ cells (LXFL or SGBS) were seeded.

After 24h of incubation (cells should have 60-80% confluency) the cells were processed, medium was removed and the cell layer rinsed twice with 1-2 ml ice-cold PBS (see Table 4-1). Thereafter, the cells were scraped in 2 × 150 µL RunIII buffer (Table 4-5) per petri dish and then the cell suspension was homogenized using a Wheaton glass tissue grinder („tight“ Pistill, 40 strokes). The supernatant of a first centrifugation (100 000 *g*, 4 °C, 40 min) corresponds to the so-called cytosolic fraction. All of the above steps were performed with cool solutions and over ice. The RunIII buffer was freshly prepared just before testing.

Table 4-5. RunIII Buffer Composition

Reagent	Final concentration
Tris/HCl pH 7.4	50 mM
$MgCl_2 \times 6\ H_2O$	10 mM
EDTA	0.1 mM
Benzamidine	5 mM
Pepstatine A	1 µM
Leupeptine	1 µM
Soybean trypsin inhibitor	0.5 µM
PMSF	0.5 mM
β-Mercaptoethanol	0.5 mM

Addition of Triton® X-100 (final concentration 0.5%, v/v) in the RunIII/Triton buffer for the membran fraction test.

4.5.1.2 Preparation of platelet suspensions (*in vivo*)

Preparation of platelet-rich plasma (PRP)

The preparation of PRP from whole blood was achieved by centrifugation by 800 *g* for 5 min at RT. Three distinct phases were seen:

a) Packed red cells
b) Buffy coat (thin band of white blood cells)
c) Straw-coloured PRP.

The PRP was aspirated taking care not to disturb either the buffy coat or red cells to prevent contamination of the platelet preparation and was then transferred to a fresh eppendorf tube. Platelet counts in PRP are normally 350 000 to 500 000 platelets/µl [Watson and Authi, 1996].

4.5.1.3 Isolation of platelets from PRP

Platelets were isolated from PRP by centrifugation at *800 g* for 15 min at room temperature to yield a platelet pellet. Then the supernatant (Platelet poor plasma) was aspirated and stored for the ADA experiments and the pellet was gently resuspended in buffer (for the PDE assay in Run III buffer und for cAMP assay in Stimulation buffer). The presence of platelet clumps at this stage represents a large degree of platelet activation and, if present, the preparation should be discarded. The platelet suspensions were stored at -80°C and before the test thawed and sonicated on ice.

4.5.1.4 Isolation of platelets from platelet concentrate (*in vitro*)

Plateletconcetrates were obtained from the DRK-Blutspendedienst (Red Cross Blood Transfusion Service) Baden Württemberg-Hessen in Mannheim. Each platelet concentrate was a suspension of human platelets (pooled from four donors) in 255 ml of a platelet additive solution and CPD (citrate-phosphate-dextrose) plasma, containing approximately 2.0-4.5 x 10^{11} thrombocytes/preparation.

Concentrates were stored for max. 5 days at 25 °C under constant agitation. To isolate the platelets from the concentrate, 50 ml of this suspension were decanted in 50 ml tubes, then EDTA was added to a final concentration of 5 mM, mixed thoroughly and centrifuged at 1500 *g* for 15 min at room temperature. The supernatant was discarded (taking into account that this is potentially biohazardous) and the pellet was resuspended in RunIII buffer at a volume equivalent to that of the original concentrate.

4.5.2 cAMP-PDE assay

Using the cAMP-PDE assay, the cAMP to 5´AMP hydrolyzing activity of PDE isoenzymes (cellular fraction) can be measured. In particular enzyme activities of the isoforms that work best with substrate concentrations of 1 µM in the assay are determined. Furthermore,

influence of different modulators on the PDE activity can be tested by partial replacement of buffer with the appropriate test compound (see Table 4-7). All solutions were stored on ice and the assay was pipetted and stopped in an ice bath.

4.5.2.1 Procedure

The cAMP [^{3}H]-mix was prepared by addition of 13 µl of tritiated cAMP[^{3}H] (≈13 µCi) to 5 ml cAMP-mix (see cAMP-mix composition) which is then mixed thoroughly.

Table 4-6: cAMP-mix composition

Reagent	Final concentration
Tris/HCl (pH 7.4)	30 mM
MgCl2 × 6 H_2O	9 mM
5'AMP	3 mM
cAMP	3 µM

Aliquot of 5 ml were stored at -20 °C. Just before starting the test: addition of 13 µl cAMP [^{3}H] (≡ 13 µCi) per 5 ml → cAMP [^{3}H]-mix.

Assay tubes were prepared according to the following pipetting scheme. PDE activity of each sample was always performed in triplicate.

Table 4-7: Pipetting scheme for PDE assay

	Components
Blank	100 µl RunIII-Buffer 50 µl cAMP[^{3}H]-mix
Basal activity	50 µl RunIII-Buffer 50 µl cell lysate 50 µl cAMP[^{3}H]-mix
Modulator	50 µl Modulator 50 µl cell lysate 50 µl cAMP[^{3}H]-mix

After the addition of cAMP [^{3}H] samples were mixed using a vortex mixer. The reaction started by incubating the tubes in a water bath at 37 °C temperature. The incubation period depends on the cAMP turnover which should not exceed about 20%, so the enzyme kinetics is guaranteed to be in a steady state. The corresponding incubation period was determined by a pre-test at different incubation times. The reaction was stopped by adding 250 µL 0.266 M $ZnSO_4$ on ice. The 5´AMP[^{3}H] was precipitated by addition of 250 µL 0.266 M $Ba(OH)_2$ and separated by centrifugation at *20 000 g* for 5 min. From the supernatant, which contains the nonhydrolyzed cAMP [^{3}H], 450 µL were transferred into a tube containing 4.5 ml scintillation cocktail and after vortex, the samples were measured for 2 min in a scintillation counter.

4.5.2.2 Results interpretation

The measured [^{3}H]-cAMP activity is proportional to the remaining cAMP[^{3}H]-concentration, i.e. the turnover corresponds to the difference between the original and remaining [^{3}H] activity. To compare data the PDE activity was normalized to protein content. The determination of protein content was performed according to Bradford [Bradford, 1976]. The PDE activity is given in (cAMP pmol / min × mg protein).

4.5.2.3 PDE assay materials

4.5.2.4 Equipment

Ultrasonic Homogenisor	Braun, Labsonic 2000
Incubator shaker	Edmund Bühler, Hood TH 15
Liquid Scintillation Counter	Beckman, LS-5801
Microcentrifuge	Eppendorf, 5417R

Chemicals

Adenosine 5′-monophosphate	Alexis, Sigma
Adenosine 3′,5′-cyclic monophosphate	Sigma
Adenosine 3,5,cyclic phosphate ammoniumsalt (2,8-^{3}H)	Hartmann Analytik, 250 µCi/ml
$Ba(OH)_2$	Merck
Benzamidine	Sigma-Aldrich
Caffeine	Sigma-Aldrich
EDTA	Serva
Hydrochloric acid (37%)	J.T. Baker
IBMX	Calbiochem
Leupeptin	Alexis
β-Mercaptoethanol	Fluka
$MgCl_2$ x $6H_2O$	Merck
Paraxanthine	Sigma-Aldrich
Pepstatin A	Alexis
PMSF	Alexis
Pyrazines	Aldrich, SAFC
Pyridine	Merck
Rolipram	Calbiochem
Scintillation Cocktail	Roth, Rotiszint eco plus
Soybean trypsin inhibitor	Roche
Tris/HCl	Roth
Triton® X-100	Serva
Zaprinast	Calbiochem
$ZnSO_4$	VWR-prolabo

Consumables (see 4.1.9 cell culture)
Measuring vials for scintillation counter Roth

4.6 Blood adenosine quantification

4.6.1 Samples preparation

The adenosine quantification in blood was performed by HPLC-ESI-MS/MS as described previously by Doležalová *et al.* [Dolezalova et al., 2005] with some modifications. For this purpose, the venous blood samples were deproteinized immediately after collection with an equal volume of 1 M perchloric acid (PCA) to prevent potential enzymatic degradation or production of adenosine. The samples were cooled in ice-water and centrifuged at *4 000 g* for 5 min at 4 °C. 300 µl of the clear supernatant was transferred to a test tube and 20 µl of internal standard containing 1.6 µM [1´,2´,3´,4´,5´-$^{13}C_5$]-Adenosine (Omicron, USA) was added. The solution was neutralized by addition of 20 µl buffer containing 2.5 M K_3PO_4 and 1.3 M KOH. After a short centrifugation step (*4 000 g*, 5 s) the potassium perchlorate precipitate was removed and the supernatant was diluted with 250 µl of re-destilled water and applied on a preconditioned StrataX 33u Polymeric Reversed Phase-solid-phase extraction (SPE) column (30 mg/ml, Phenomenex). After the sample had passed through the column, the column was rinsed with 1.5 ml of re-destilled water. Adenosine was eluted with 1000 µl (2 times 500 µl) of methanol [Dolezalova et al., 2005].
The eluate was concentrated to a volume of about 100 µl using a vacuum centrifuge, then 1:4 diluted with 0.02 % acetic acid. Aliquots (10 µl) were measured using HPLC-ESI-MS/MS. The chromatographic separation of adenosine was performed with an Aqua C18 column (250 × 4.6 mm, 5 u, Phenomenex) using an isocratic solvent system of 0.1% formic acid in water (v / v) and methanol (85:15; v / v) at a flow rate of 0.5 ml / min.
The detection of the analyte and the internal standard was performed using a triple-quadrupole tandem mass spectrometer API 3200™ (Applied Biosystems, Darmstadt, Germany) with positive electrospray ionization (ESIpos). The quantification of adenosine was performed by determining the ratio of the peak area of adenosine with the peak area of isotope-labeled standard.

4.6.2 Tuning and calibration of the mass spectrometer

In a mass spectrometer, the ion source, mass filter, and detector operational parameters influence the produced mass spectra. The ion source parameters affect how many ions are produced, how many ions are directed toward the mass filter, and the relative amount of a given mass ion that is directed into the mass filter. The mass filter parameters in a quadrupole instrument affect the peak widths, mass assignments, mass resolution, and sensitivity and the detector parameters the magnitude of the signal and the sensitivity of the

system. All of the tuning parameters influence the quality of the mass spectra and the ability of a given mass spectrum to be matched with a spectral data base.

Tuning and calibration of the instrument ensures the best results of the experiments. Tuning is performed to check the mass spectrometer or to ensure that the spectra produced resemble a previously determined standard. On the other hand, calibration is the process of calibrating the mass scale using a compound or mixture that showed peaks with accurately known masses. Tuning and calibration of the mass spectrometer were carried out with polypropylene (PPG) 1:10 standards for ESIpos polarization as indicated by Applied Biosystems [Applied-Biosystems, 2008]**.**

4.6.3 Optimization of the substance and source specific parameters

The mass spectrometer has several parameters (source and substance-specific parameters) that can be specifically optimized for each analyte to achieve a high sensitivity of the device (see Figure 4-3)

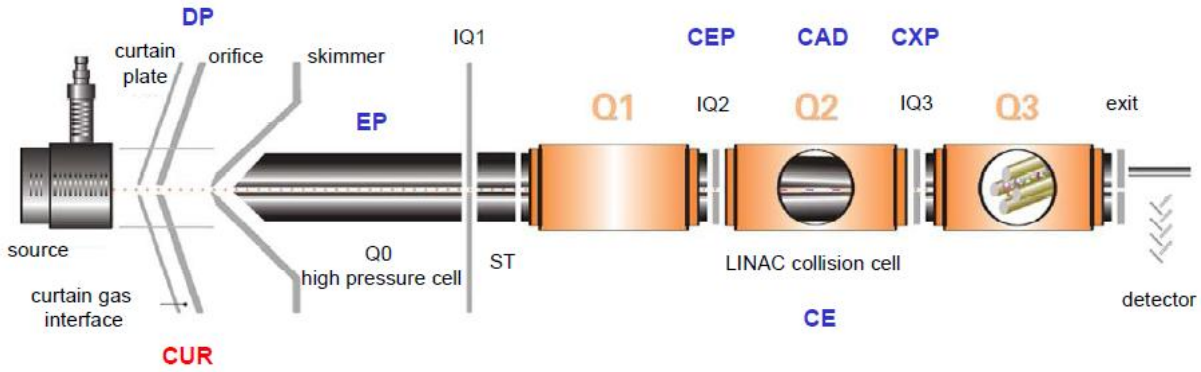


Figure 4-3: **Schematic structure of a triple quadrupole mass spectrometer.** Source-specific parameters, substance-specific parameters, CUR: Curtain Gas, DP: Declustering Potential, EP: Entrance Potential, CEP: Collision Cell Entrance Potential, CE: Collision Energy, CAD: Collision Activated Dissociation, CXP: Collision Cell Exit Potential [Applied-Biosystems, 2008].

The source-specific parameters include ion spray voltage, nebulizer gas, turbo gas, temperature and curtain gas. Their function is explained in Table 4-8.

Table 4-8: Source-specific parameters of a Triple quadrupole mass spectrometer

Parameter	Abbreviation	Function
Ion Spray Voltage	IS	Voltage applied between the needle and orifice plate that "ionizes" and nebulizes the liquid flow. Polarity determines what type of ions will reach MS. In positive mode typically 4000 and 5500V; In negative mode –3000 to –4000V
Nebulizer Gas (N_2)	GS1	Facilitates droplet formation. Higher flow, higher GS1
Turbo Gas (N_2)	GS2	Aids in solvent evaporation, increasing ion efficiency. Heated gas stream intersects nebulized liquid stream at about 90o right in front of the curtain plate

Temperatur	TEM	Temperature of GS2: Promotes desolvation, thereby forming Analyte ions in the gas phase
Curtain Gas	CUR	High purity N_2 that flows between the orifice and the curtain plate. It repulses large droplets and neutrals keeping the Q0 clean

To optimize the source-specific parameters, first the composition of the mobile phase and the retention time of the analyte were determined. This was then adjusted with the corresponding isocratic flow rate of the HPLC. Then the analyte was injected by loop injection (no column separation) under changing source parameters with the help of the FIA (Flow Injection Analysis) tools of the Analyst 1.4.2 Software.

The substance-specific parameters include declustering potential, entrance potential, collision cell entrance potential, collision energy, CAD gas and collision cell exit potential. These parameters were optimized using a syringe pump (flow: 10 µl/min) and the "Quantitative Optimization" tool of the Analyst 1.4.2 software. In Table 4-9 the substance specific parameters to be optimized and their functions are listed.

Table 4-9: Substance-specific parameters of a Triple quadrupole mass spectrometer

Parameter	Abbreviation	Function
Declustering Potential	DP	Potential difference between the skimmer and the Orifice: minimizes the formation of solvent cluster ions, reduces chemical noise.
Entrance Potential	EP	The voltage between the skimmer (ground) and the entrance to Q0. Focusing of the ions in Q0
Collision Cell Entrance Potential	CEP	Potential difference between Q0 and the entrance to Q2. Facilitates ion transmission to the collision cell. Is the most mass dependent parameter
Collision Energy	CE	Potential difference between the Q0 and Q2. Determines the degree of fragmentation in Q2
Collisionally Activated Dissociation gas	CAD-gas	Pressure of the collision gas (N_2) in Q2
Collision Cell Exit Potential	CXP	Potential difference between Q2 and IQ3. Always 4V

4.6.4 Quantification by stable isotope dilution analysis (SIDA)

The adenosine quantification in blood was carried by stable isotope dilution analysis (SIDA). A stable isotope [1´,2´,3´,4´,5´-$^{13}C_5$] (100nM)-Adenosine was used as internal standard. This method has the advantage that the isotope standard can already be added before sample preparation and treated analogously to the analyte. Due to the structural similarity of the analyte with the internal standard, its physical and chemical properties are almost identical. For the sample preparation, chromatography, ionization and detection can be assumed that the analyte and the internal standard behave similar. As shown in Figure 4-4, the internal standard (IS) also showed the same retention time as the analyte.

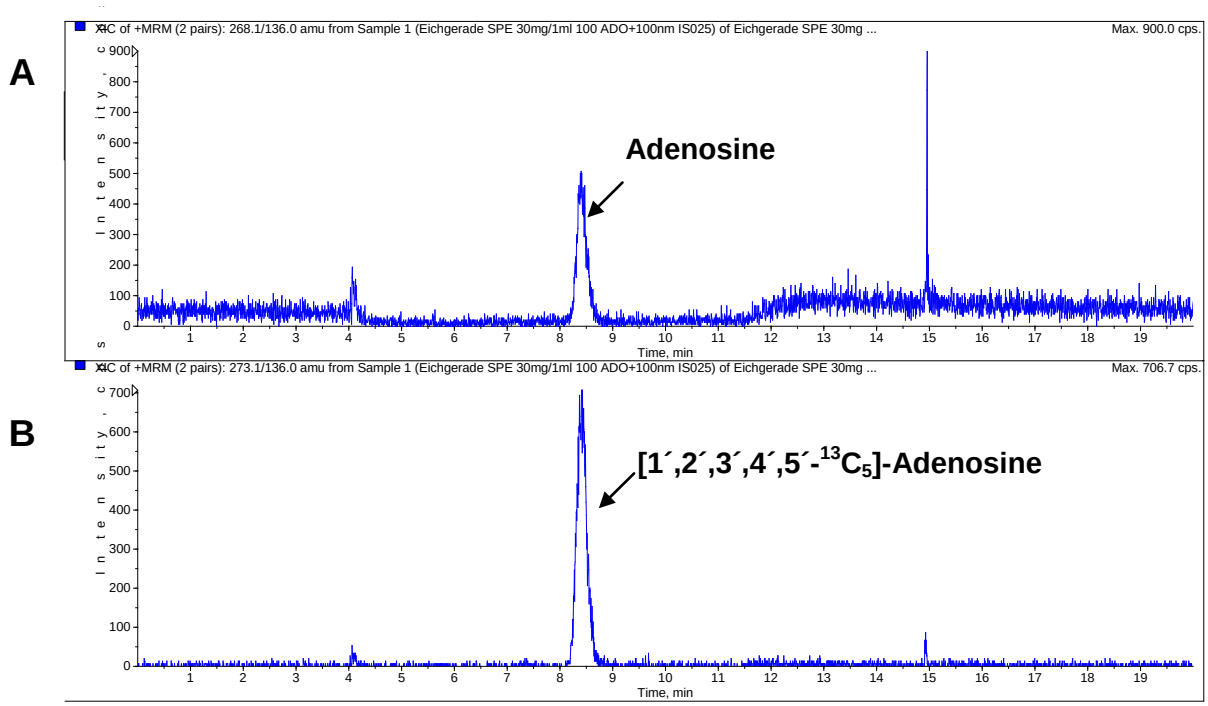


Figure 4-4: HPLC-MS/MS chromatogram of the analyte Adenosine (100 nM) (A) and the internal standard [1´,2´,3´,4´,5´-$^{13}C_5$]-Adenosine (100 nM) (B) performed with an Aqua C18 column (250 × 4.6 mm, 5 u, Phenomenex) under isocratic conditions (0.1% formic acid in water (v/v) and methanol (85:15; v/v) 0.5 ml / min).

4.6.5 Adenosine quantification by mass spectrometry

The detection of adenosine was performed by HPLC-ESI-MS/MS (API 3200) by positive ionization (electrospray ionization, ESI) in multiple reaction monitoring (MRM) mode. The transition of protonated [1´,2´,3´,4´,5´-$^{13}C_5$]-adenosine (*m/z* 273) and adenosine (*m/z* 268) to fragment *m/z* 136, which corresponds to the mass protonated adenine, were monitored (see Figure 4-5 and 4-6).

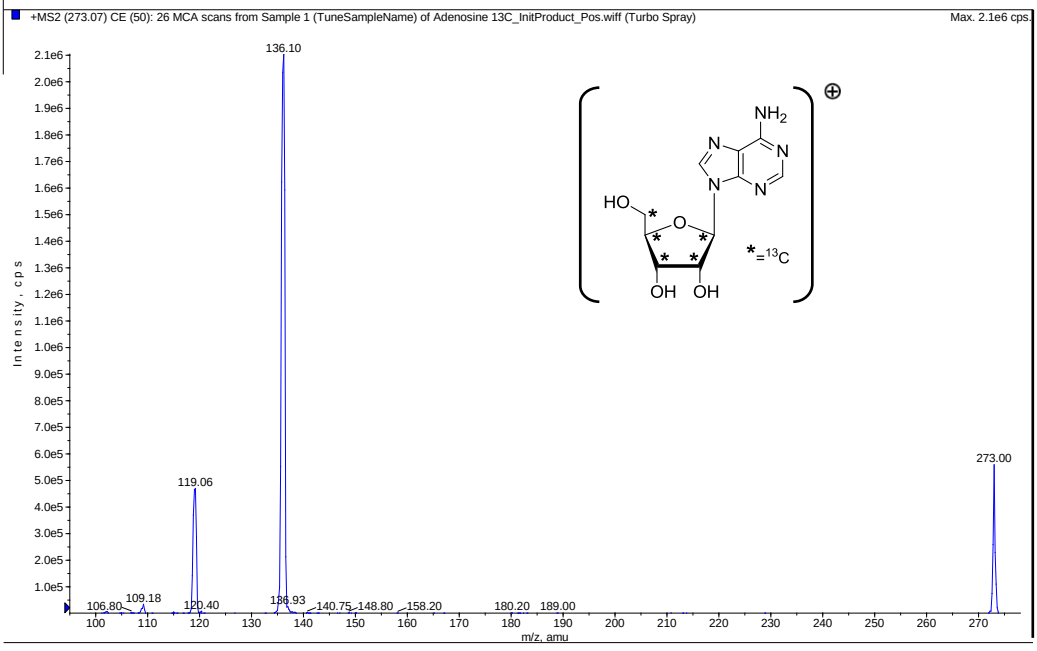


Figure 4-5: Mass scan in positive mode of [1´,2´,3´,4´,5´-$^{13}C_5$]-adenosine at Q1(*m/z* 100-300)

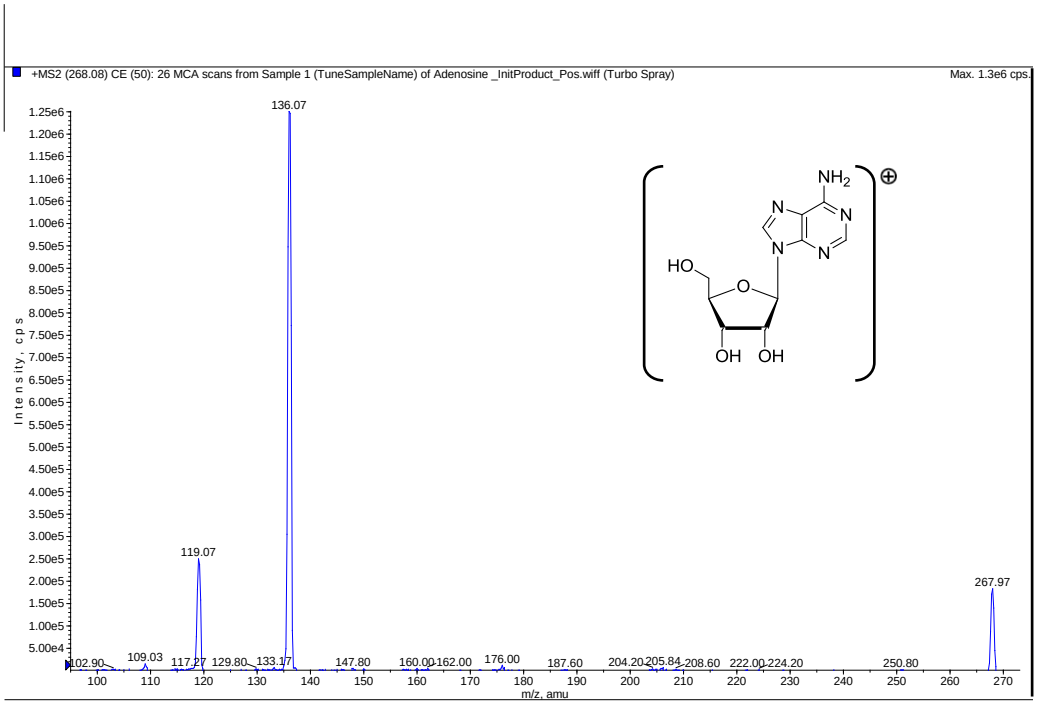


Figure 4-6: Mass scan in positive mode of [1´,2´,3´,4´,5´-$^{13}C_5$]-adenosine at Q1(*m/z* 100-300)

In Table 4-10, the calculated mass-specific parameters used for the measurement of adenosine are listed. Due to the strong structural similarity of the analyte and the internal standard no differences in the optimized parameters were determined.

Table 4-10: Mass-specific parameters for adenosine quantification

Parameter	Adenosin	[1´,2´,3´,4´,5´-$^{13}C_5$]-adenosine
Q1 [amu]	268	273
Q3 [amu]	136	136
Dwell time [ms]	150	150
Source specific parameters		
CUR [psi]	10	10
CAD [psi]	5	5
IS [kV]	5500	5500
TEM [°C]	390	390
GS 1 [psi]	40	40
GS 2 [psi]	80	80
Substance-specific parameters		
DP	36	41
EP	3	4.5
CEP	25	25
CXP	4	4

CUR: curtain gas, **CAD**: Collisionally Acivated Dissociation Gas, **IS**: Ion Spray Voltage, **TEM**: Temperatur, **GS 1**: Nebulizer Gas, **GS 2**: Turbo Gas, **DP**: Declustering Potential, **EP**: Entrance Potential, **CEP**: Collision Cell Entrance Potential, **CXP**: Collision Cell Exit Potential

4.6.6 Chromatography

The chromatographic separation of adenosine was carried out with an Aqua C18 column (250 × 4.6 mm, 5 u, Phenomenex) at a flow of 0.5 ml /min. The elution was achieved using an isocratic solvent system of 0.1% formic acid in water (v/v) and methanol (85:15; v/v) see Table. 4-11, at 8.5 min. A fully automated software controlled the Valco two position valve to divert the eluent fractions that contain the analyte in to the mass spectrometer. The early eluting components (before the first 4 minutes) as well as the components after 15 min were deviant to waste, thereby reducing contamination of the ion source. 10 µl of sample were injected for each measurement.

Table 4-11: Solvent composition for gradient elution of adenosine

Time (min)	Flow Rate (%)	Eluent A (%)	Eluent B (%)
0.05	0.5	85	15
11.5	0.5	85	15
11.7	0.8	1	99
15.7	0.8	1	99
16	0.5	85	15
20	0.5	85	15

Eluent A: 0.1% formic acid in water (v/v), Eluent B: methanol

4.6.7 Calibration curve for the quantitative analysis of adenosine

The standard addition method (often referred to as "spiking" the sample) is commonly used to determine the concentration of an analyte in a complex matrix such as biological fluids. The idea is to add analyte to the sample ("spike" the sample) and monitor the change in instrument response [Harris, 1991]. For this purpose (100 nM -final concentration-) of the [1´,2´,3´,4´,5´-$^{13}C_5$]-adenosine and known quantities (0, 50, 100, 150 and 200 nM –final concentration-) of the analyte were added to the plasma samples (300 µl each sample). Thereafter the samples preparation was performed as described in 4.7.1. The instrument response was measured for samples (each concentration in triplicate) and data were plotted as seen in Figure 4-6. The recovery rate for the analyte in spiked plasma was 102.7±12.8%

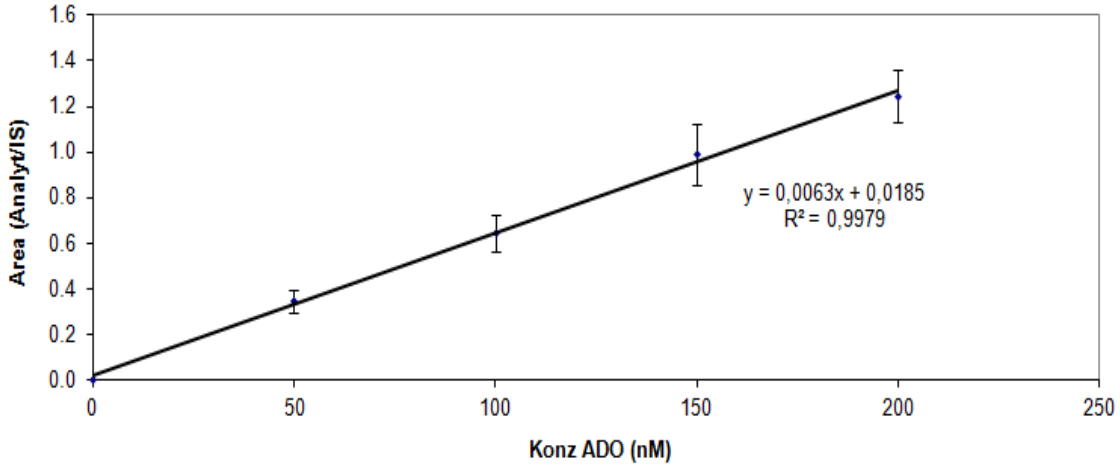


Figure 4-7: **HPLC-ESI-MS/MS Calibration curve (Adenosine 0 – 200 nM). Internal Standard (IS) 1´,2´,3´,4´,5´-$^{13}C_5$]-Adenosin(100 nM).** Concentrations are given in nM and the Areas in cps/min.

4.6.8 Detection and quantification limits

The limit of detection (LOD) expressed as the concentration of a compound is derived from the smallest measure that can be detected with reasonable certainty for a given analytical procedure based on a signal/ noise ratio of 3:1 [IUPAC, 1997].

The limit of quantification (LOQ) is usually defined as twice the limit of detection and gives the lowest value that can be quantified reproducibly. The determination of detection and quantification limits for adenosine was based on a calibration curve where the peak height versus concentration was plotted.

Based on a background noise of 35 cps the calculated the LOD was 30.6 fmol and the LOQ was 61.1 fmol (absolute amount).

4.6.9 Day-to-day variability

As previously described by Doležalová et al. adenosine concentrations in deproteinized blood samples are not stable [Dolezalova et al., 2005]. The inter-assay (between-day) variation was determined by replicate analyses of the same sample on three separate days. For this purpose, deproteinized blood samples were extracted within 6 h of collection and analyzed on HPLC-ESI-MS/MS immediately and after storage at −80°C for 1, 2, 4 and 6 days. Results are shown in results and discussion section.

4.6.10 Blood adenosine quantification materials

Equipment

Aqua C18 column (250 × 4.6 mm, 5 u)	Phenomenex
Binary HPLC-Pump	PU-2080 Plus Intelligent HPLC Pump (Jasco, Groß Umstadt)
Interface	LC-Net II/ADC (Jasco, Groß-Umstadt)
Mass spectrometer with valco valve	API 3200 Triple Quadropole (Applied Biosystems, Darmstadt)
Oil rotary vacuum pump	Univac (Uniequip)
Sampler	AS-2057 Plus Intelligent Sampler (Jasco, Groß Umstadt), 4 °C
Software	Analyst 1.4.2 (Jasco, Groß-Umstadt)
Vacuum Concentrator Centrifuge	UniVapo 150H (Uniequip)

Chemicals

[1´,2´,3´,4´,5´-$^{13}C_5$]-adenosine	Omicron Biochemicals, Inc.
Acetic Acid p.a.	Roth
Formic Acid	Chemsolute
K_3PO_4	Sigma-Aldrich
KOH	Sigma-Aldrich
MeOH (HPLC grade)	J.T. Baker
Perchloric Acid p.a.	Fluka

Consumables

HPLC-Vials	Buddeberg
Inserts for Vials	Wagner & Munz
Strata-X 33u polymeric reversed phase SPE Tubes (30 mg/ml)	Phenomenex
Pipette tips	Greiner
Reaction tubes	Greiner

4.7 Intracellular cAMP quantification

cAMP is one of the most important second messengers, mediating diverse physiological responses of neurotransmitters, hormones and drugs. Intracellular concentration of cAMP is tightly regulated by two membrane-bound enzymes, AC and PDE. AC activity promotes the synthesis of cAMP from ATP while PDE degrades cAMP to 5´-AMP. The measurement of

intracellular cAMP could be used as a marker for measuring the effect of test compounds on AC activation or inhibition. Measurement of intracellular cAMP concentration was carried out using the PerkinElmer´s LANCE® Kit. This kit is intended for the quantitative determination of adenosine 3',5'-cyclic monophosphate (cAMP) in cell culture and cell membrane samples [PerkinElmer and Inc., 2007].

4.7.1 Principles of the assay

The LANCE cAMP assay is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay designed to measure cAMP produced upon modulation of AC activity by G protein-coupled receptors (GPCRs). The assay is based on the competition between an europium-labeled cAMP tracer complex and sample cAMP for binding sites of cAMP-specific antibodies labeled with the Alexa Fluor®647 dye. The europium-labeled cAMP tracer complex is formed by the tight interaction between Biotin-cAMP (b-cAMP) and streptavidin labeled with Europium-W8044 chelate (Eu-SA).

When antibodies are bound to the Eu-SA/b-cAMP tracer, a light pulse at 340 nm excites the Eu-chelate molecules of the tracer. The energy emitted by the Eu-chelate is transferred to an Alexa molecule on the antibody, which in turn emits light at 665 nm (see figure 4-6). The fluorescence intensity measured at 665 nm will decrease in the presence of cAMP from test samples and resulting signals are inversely proportional to the cAMP concentration of a sample [PerkinElmer and Inc., 2007].

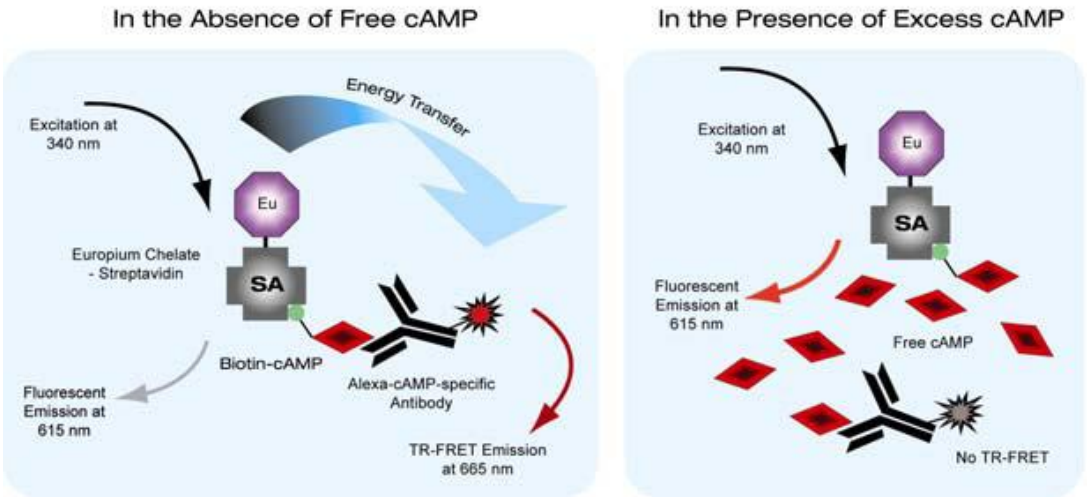


Figure 4-8: LANCE cAMP assay principle. From PerkinElmer [PerkinElmer and Inc., 2007]

4.7.2 Standard curve assay protocol

The standard curve was carried out according to the manual of PerkinElmer in a final assay volume of 24 µl [PerkinElmer and Inc., 2007].

a. 6 µL of each cAMP Standard dilution was added in a well of the half-area Platte (measurements were performed in triplicate). The preparation of the standard serial dilutions was made according to the Table 4-12.

Table 4-12: Preparation of cAMP Standard serial dilutions

Dilution number	Final concentration in the assay (M)	Volume of dilution	Volume of stimulation buffer
1	$1x10^{-6}$	8 µL of cAMP Standard	92 µL
2	$3x10^{-7}$	30 µL of dil. 1	70 µL
3	$1x10^{-7}$	30 µL of dil. 2	60 µL
4	$3x10^{-8}$	30 µL of dil. 3	70 µL
5	$1x10^{-8}$	30 µL of dil. 4	60 µL
6	$3x10^{-9}$	30 µL of dil. 5	70 µL
7	$1x10^{-9}$	30 µL of dil. 6	60 µL
8	$3x10^{-10}$	30 µL of dil. 7	70 µL
9	$1x10^{-10}$	30 µL of dil. 8	60 µL
10	$3x10^{-11}$	30 µL of dil. 9	70 µL
11	$1x10^{-11}$	30 µL of dil. 10	60 µL
12	0	0	70 µL

The dilutions were prepared in stimulation buffer 1X HBSS containing 5 mM HEPES, 0.1% BSA and 0.5 mM IBMX at pH 7.4.

b. then 6 µL of antibody solution (1/100 dilution of the Alexa Fluor®647-anti cAMP antibody solution in stimulation buffer) were added
c. Incubation for 60 min at room temperature
d. addition of 12 µL of detection mix (Eu-W8044 labeled streptavidin plus Biotin-cAMP in detection buffer)
e. coverage of microplate with TopSeal-A film and incubation for 60 min at room temperature
f. read on a TRF detection instrument.

A representative cAMP standard curve is presented in Figure 4-8. The EC_{50} was 3.76 nM ± 0.71 for all repetitions with a detection limit of 1 fmol/well approximately.

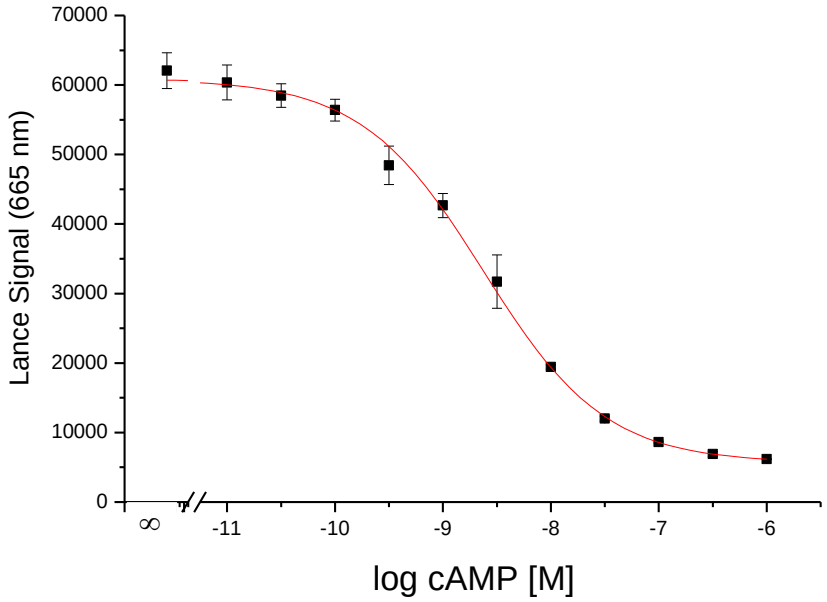


Figure 4-9: Example of a typical Lance cAMP standard curve measured on EnVision instrument after 1 hour incubation in a half-area microplate.

4.7.3 Cell based assay

4.7.3.1 Cell preparation (*in vitro*)

It was necessary to generate Forskolin dose-response curves in order to establish the optimal cell number per well. The optimal cell number was the one for wich the Forskolin dose-response curves fits most of the linear region of a cAMP standard curve (see results and discussion chapter). For this purpose HEK-293 and SGBS cells (from day 1 to 7 after differentiation) were cultured as indicated and then harvested with versene, a non enzymatic cell dissociation solution. Thereafter cells were washed with 1x HBSS and resuspended in stimulation buffer at a concentration of $2x10^6$ cells per ml (12000 cells per 6 µl) and further diluted to obtain the needed cell number. It is recommended to test 12000, 6000, 3000 and 1000 cells per 6 µl [PerkinElmer and Inc., 2007]. A “no cell” control and a cAMP Standard curve were always run in parallel. The Alexa Fluor® 647-labeled antibodies were added to the final cell suspension by making a 1/100 dilution of the stock solution in the cell suspension.

The assay was performed in white half-area microplates in a final assay volume of 24 µl, following the same protocol as the standard curve. The preparation of the Forskolin serial dilutions was made using a stock solution 500 µM Forskolin according to the Table 4-13.

Table 4-13: Preparation of Forskolin serial dilutions

Dilution number	Forskolin (M)	Final concentration in 12 µl reaction (M)	Volume of dilution	Volume of stimulation buffer
1	$2x10^{-4}$	$1x10^{-4}$	120 µl of Stock solution	180 µl
2	$6x10^{-5}$	$3x10^{-5}$	30 µL of dil. 1	70 µL
3	$2x10^{-5}$	$1x10^{-5}$	30 µL of dil. 2	60 µL
4	$6x10^{-6}$	$3x10^{-6}$	30 µL of dil. 3	70 µL
5	$2x10^{-6}$	$1x10^{-6}$	30 µL of dil. 4	60 µL
6	$6x10^{-7}$	$3x10^{-7}$	30 µL of dil. 5	70 µL
7	$2x10^{-7}$	$1x10^{-7}$	30 µL of dil. 6	60 µL
8	$6x10^{-8}$	$3x10^{-8}$	30 µL of dil. 7	70 µL
9	$2x10^{-8}$	$1x10^{-8}$	30 µL of dil. 8	60 µL
10	$6x10^{-9}$	$3x10^{-9}$	30 µL of dil. 9	70 µL
11	$2x10^{-9}$	$1x10^{-9}$	30 µL of dil. 10	60 µL
12	0	0	0	70 µL

The dilutions were prepared in stimulation buffer 1X HBSS containing 5 mM HEPES, 0.1 % BSA and 0.5 mM IBMX at pH 7.4.

To determine the EC_{50}, the Forskolin signal inhibition curve should be transformed to a cAMP production curve, using the cAMP standard curve that was measured in parallel.

Agonist concentration

The presence of an agonist is required to produce G_s alpha ($G_{\alpha s}$) and G_i alpha subunit ($G_{\alpha i}$) antagonist dose-response curves. To determine the optimal concentration of the agonist, two agonists dose-response curves were generated: one in the absence of antagonist and the other with excess (10µM) of a reference antagonist. The curves were plotted in the same graph. The agonist concentration at wich the difference between the two curves is the largest was selected. DMSO at concentrations up 2% during cell stimulation does not affected assay performance [PerkinElmer and Inc., 2007].

4.7.3.2 Platelet suspensions preparation (*in vivo*)

Platelets suspensions were prepared as described in 6.5.1.2, concentrations were measured and data were normalized to protein concentrations. The determination of protein concentration was performed according to Bradford [Bradford, 1976]. The cAMP content is given in [cAMP nM × mg protein].

4.7.4 Intracellular cAMP quantification materials

Equipment

EnVision® multilabel reader	PerkinElmer
Optical filters 665 and 615nm	PerkinElmer

Chemicals

BSA	Sigma Aldrich
Caffeine	Sigma-Aldrich
DMSO	Fluka
Forskolin	Sigma-Aldrich
HBSS	Invitrogen
Hepes	Roth
IBMX	Sigma Aldrich
Lance® cAMP 384 Kit	PerkinElmer, Massachusetts, USA
Paraxanthine	Sigma Aldrich
Pyrazines	Aldrich, SAFC
Versene	Invitrogen

Consumables

Pipette tips	Greiner
Reaction tubes	Greiner
Top Seal-A 384	PerkinElmer
White, Half-area 96-well microplates	PerkinElmer

4.8 Adenosine deaminase measurements (*in vivo*)

Adenosine deaminase (ADA) levels in the plasmas of subjects were measured using a commercially available assay kit from Diazyme Laboratories. The ADA assay (see figure 4-8) is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase (XOD). H_2O_2 further reacted with N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generate a quinone dye which is monitored in a kinetic manner [Diazyme and Laboratories, 2010].

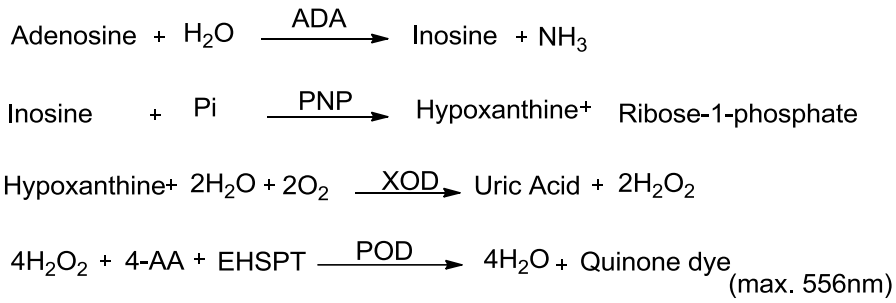


Figure 4-10. **Adenosine deaminase assay principle**. Adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), hydrogen peroxide (H_2O_2), xanthine oxidase (XOD), N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT), 4-aminoantipyrine (4-AA), peroxidase (POD) [Diazyme and Laboratories, 2010].

4.8.1 Plasma collection and handling

Plasma from venous blood was assayed. After the obtention of the platelets pellet, the PPP (platelet poor plasma) was stored at -80°C.

4.8.2 Assay procedure

The assay was performed in 96 well non-sterile microplates in a final assay volume of 275 µl, following the indications of the protocol [Diazyme and Laboratories, 2010].

a) 180 µl of the Reagent 1 (see composition in Table 4-14) and 5 µl of Sample where added in the microplate
b) After three minutes at RT in a dark place, 90 µl of Reagent 2 (see composition in Table 4-14) were added and the microplate was incubated at 37 °C within the microplate reader
c) Finally, the absorbance at 550 nm was measured after five and eight minutes consecutively

Table 4-14: Reagent composition of the ADA asay kit [Diazyme and Laboratories, 2010]

Reagent	Concentration
Reagent 1 pH 8.0	
Tris HCl	50mM
4-AA	2mM
PNP	0.1U/ml
XOD	0.2U/ml
Peroxidase	0.6U/ml
Stabilizers	
Reagent 2 pH 4.0	
Tris HCl	50mM
Adenosine	10mM
EHSPT	2mM

4.8.3 Calibration and results interpretation

To calibrate the procedure two standard ADA controls (adenosine deaminase from bovine liver and BSA) 10.3 and 47.2 U/L from Diazyme were used along with 0.9% saline as zero reference.

To calculate the ADA activity in Plasma, first the average rate of the absorbance change ΔA/min was estimated. Then ADA activity (U/L) in the plasma sample were calculated by using the following formula:

$$ADA\ (U/L) = \Delta A/min * Tv\ /\ \varepsilon \times Sv \times L$$

ε: μmolar extinction coefficient of quinone dye ($\varepsilon = 32.2 \times 10^{-3}$ μM-1cm^{-1})

Tv: Total reaction volume (ml)

Sv: Sample volume (ml)

L: Cuvette light path length (1.0cm)

4.8.4 Adenosine deaminase materials

Equipment

Multidetection microplate reader	BioTek, Synergy™ 2

Chemicals

Adenosine deaminase assay kit	Diazyme Laboratories, Poway CA, USA
NaCl	Merck

Consumables

96-well microplates (F-bottom, PS)	Greiner
Pipette tips	Greiner
Reaction tubes	Greiner

4.9 Statistical analysis

The results are expressed as arithmetic mean ± standard deviation of mean (SD) or median and interquartile range (IQR: from lower quartile, Q1 to upper quartile, Q3). The Anderson Darling test was used to verify whether the data were normally distributed.

The significance of differences was determined with ANOVA for repeated measures followed by either the paired Student's t-test for parametric data. Differences without normal distribution were analyzed by the two-sided paired Wilcoxon test or by the unpaired Mann-Whitney test.

5 Results and Discussion

The present work investigated the question if coffee or coffee constituents may have a stimulatory effect on HO-activity in different cell lines such as liver hepatocellular carcinoma (HepG2), intestinal colorectal adenocarcinoma (Caco-2) and the monocytic leukemia (MM6) cells. Here previously published data showed effects depending on the degree of roasting. Therefore two coffee extracts, a slightly (K2) and an intensively roasted coffee (K5), as well as the coffee constituents: caffeine, NMP and several alkyl-pyrazines were studied. The present work was performed within the project „Coffee Prevention: Charakterisierung, Optimierung und Prüfung antioxidativer Effektivität" (Health and Coffee: Characterization, optimization and testing of antioxidant effectiveness) funded by the German Federal Ministry of Education and Research (BMBF) grant No. 0313843.

In a follow up project entitled „Slim Down Coffee": Identifizierung, Prüfung und Optimierung der gewichtsreduzierenden Eigenschaften von Kaffee (Identification, analysis and optimization of the weight-reducing properties of coffee) the aim was to determine whether substances other than caffeine in coffee may influence the homeostasis of intracellular cyclic nucleotides *in vitro* and *in vivo*. The study was also focused on substances present in coffee before roasting but also compounds generated during the roast process which are found in brewed coffee were of interest.

5.1 HO-activity

Mammalian cells have developed several protective mechanisms to prevent ROS formation or to detoxify ROS. These mechanisms involve both antioxidants and protective enzymes. Among the various cytoprotective enzymes, the protective functions of HO-1 are described in chapter No 2.4.4.

As increasing evidence indicates that HO-1 provides protection, modulation of HO-1 expression may represent a novel target for therapeutic intervention. In particular, a non-cytotoxic pharmacological inducer of HO-1 may increase the intrinsic antioxidant potential of cells.

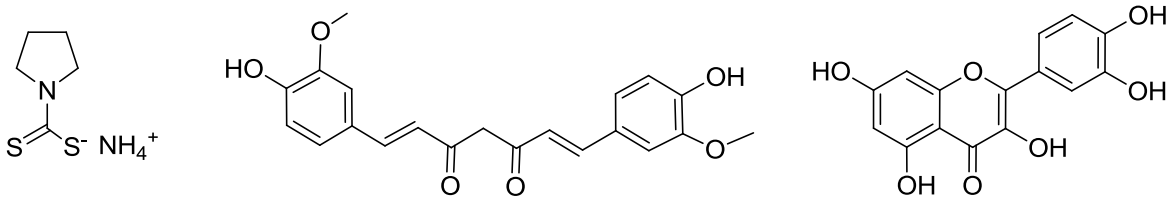
One of the goals of this work was to determine if coffee or some coffee constituents may have a stimulatory effect on HO-activity in different cell lines like Caco-2, HepG2 or MonoMac 6 (MM6). Two coffee extracts, a slightly (AB1) and a intensively roasted coffee (AB2), caffeine, NMP and some aroma compounds like pyrazines were investigated.

5.1.1 HO-activity assay optimization and screening of positive controls

The influence of coffee constituents in the total HO-activity was measured using a method first described by Tenhungen *et al.* [Tenhunen et al., 1970] with modifications described by Motterlini *et al.* [Motterlini et al., 1995] and McNally *et al.* [McNally et al., 2004]. Here the

determination of total HO-activity is based on the enzymatic conversion of heme to bilirubin via biliverdin (see chapter 4.2). Heme is oxidatively metabolized to biliverdin, which dissociates from the HO-1 and is rapidly converted to the hydrophobic molecule, bilirubin, in an NADPH-dependent reaction catalyzed by cytosolic biliverdin (BVR). Liver cytosol has been used as a source of BVR. HO-activity was assessed in the cell lysate by measuring formation of the HO metabolite bilirubin. The amount of extracted, yellow-colored bilirubin was determined with a UV spectrometer and correlated with the enzymatic activity of HO.

For positive evaluation of the newly established method three compounds previously described as HO-1 inducers, namely pyrrolidine dithiocarbamate (PDTC), quercetin and curcumin (see Figure 5-1) were tested.



PDTC Curcumin Quercetin

Figure 5-1: Chemical structures of HO-1 inducers: pyrrolidine dithiocarbamate (PDTC), curcumin und quercetin.

PDTC is a thiol-containing agent, which mediates a variety of effects on cell physiology. There is some evidence that PDTC induces expression of HO-1 *in vitro* in DLD-1 colon carcinoma and endothelial cells [Hellmuth et al., 2002; Stuhlmeier, 2000] and *in vivo* [Hata et al., 2003]. Curcumin a yellow colouring ingredient of the spice turmeric obtained from the rhizome of Curcuma longa could induce HO-1 expression in dimethyl nitrosamine injured liver of rats [Farombi et al., 2008] and renal ephitelial cells [Balogun et al., 2003a]. Furthermore, quercetin, a member of the flavonoid family, has been reported to mediate cytoprotection through induction of HO-1 in lung epithelial cells [Hayashi et al., 2011] and fibroblasts [Nakamura et al., 2011].

Three different human neoplastic cancer cell lines were used for this experiment: human hepatoma cancer cells (HepG2), human epithelial colorectal adenocarcinoma cells (Caco-2) and human acute monocytic leukemia cells (MonoMac6). To determine if these compounds provide a high amount of enzyme activity (positive control) cells were exposed to various concentrations of the above mentioned inducers (10–1000µM) for 12, 24 and 48 h. Results are shown in Figure 5-2, 5-3 and 5-5.

Incubation of the three cell lines for 12 hours with various concentrations of selected inductors shows no significant differences (see Figure 5-2), except for 1000 µM PDTC in HepG2 cells .The basal total HO-activity for the HepG2 and the Caco-2 cell lines was similar, whereas MonoMac 6 cells showed almost three times more background activity.

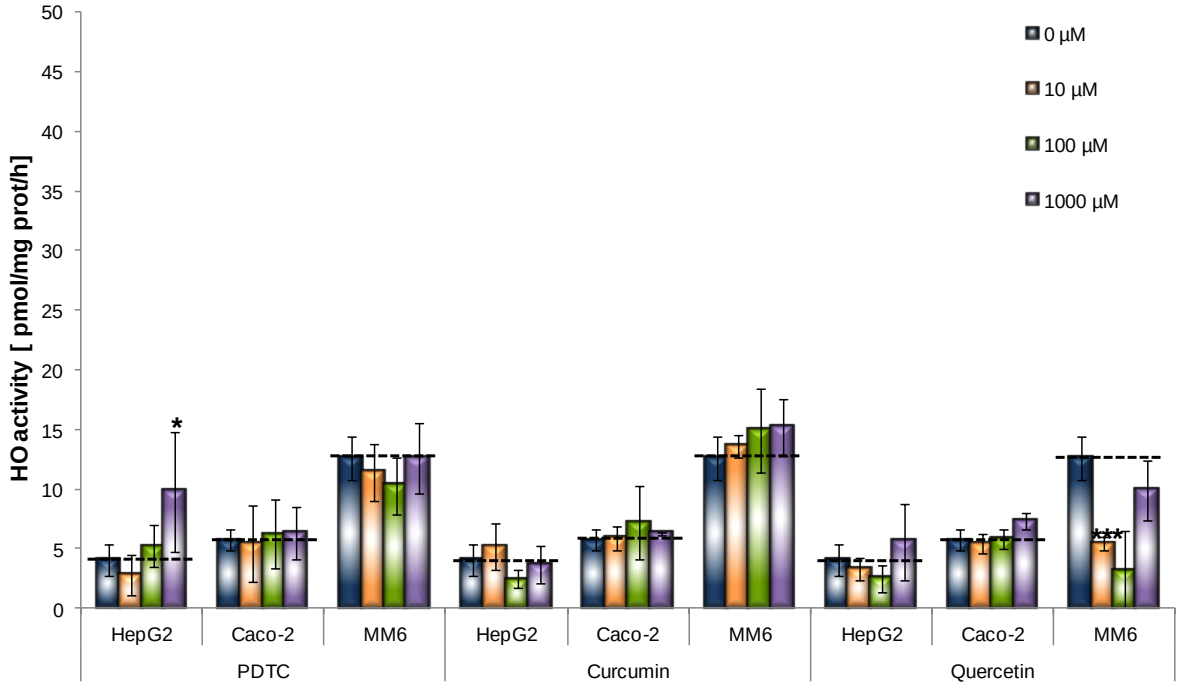


Figure 5-2: Effect of PDTC, curcumin and quercetin on HO-activity in HepG2, Caco-2 and MM6 cells. Cells were exposed for 12 h to medium or medium supplemented with various concentrations of compounds (0-1000 µM). Data are expressed as mean ±SD of four to five independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. untreated cells).

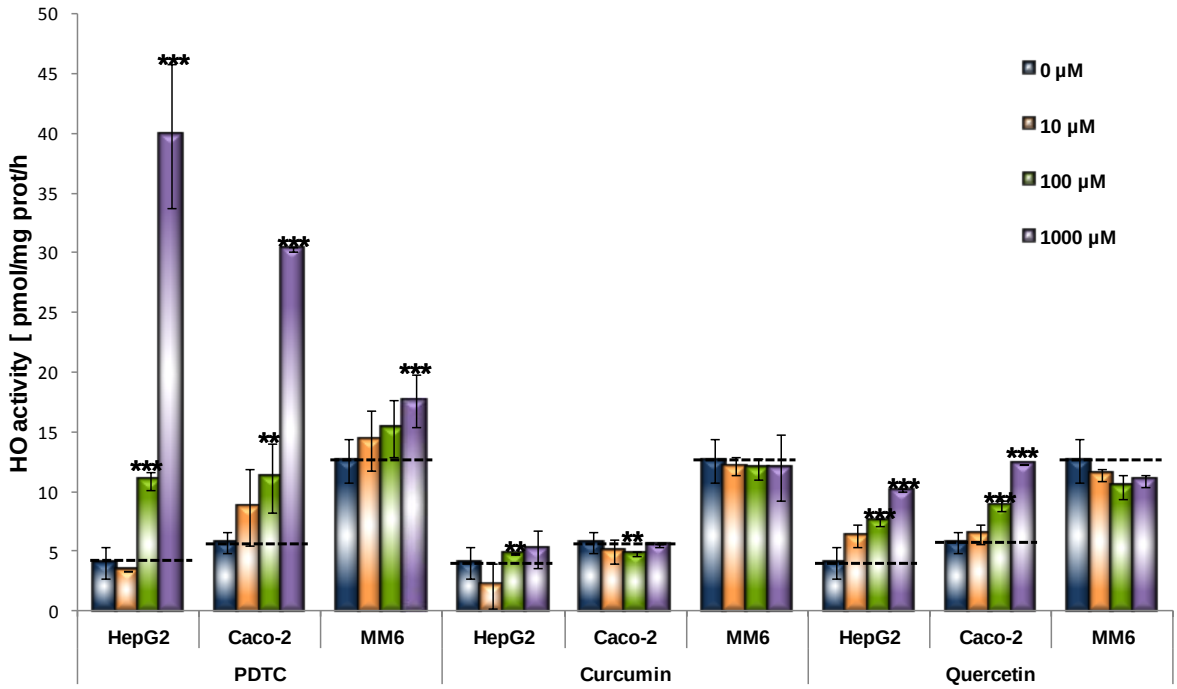
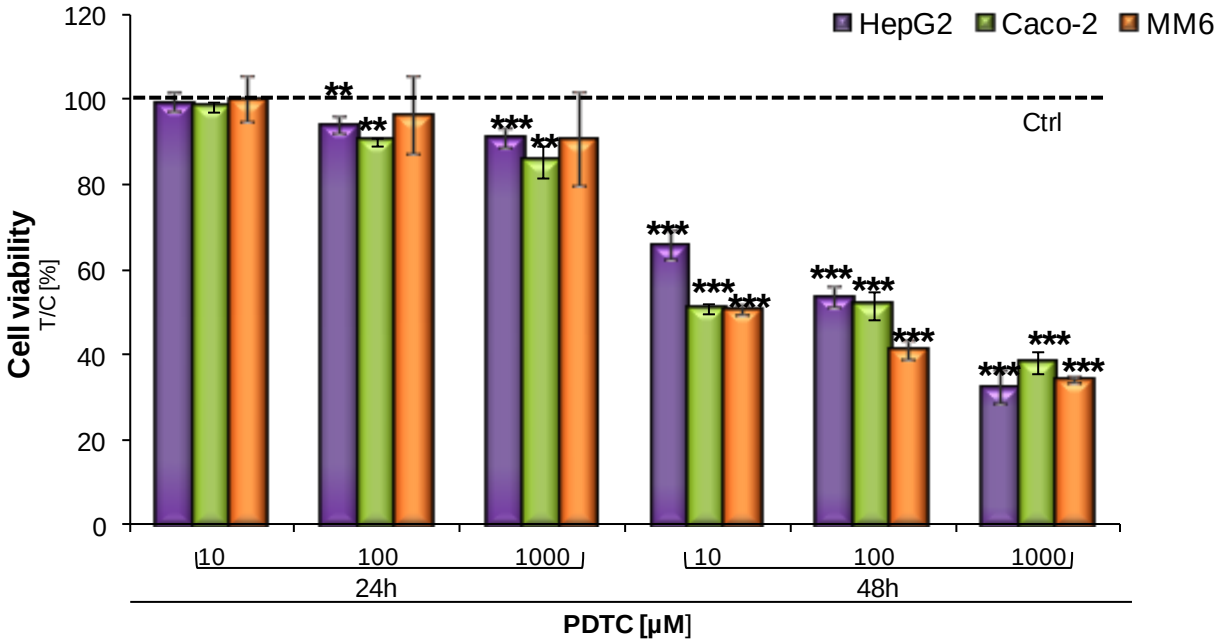


Figure 5-3: Effect of PDTC, curcumin and quercetin on HO-activity in HepG2, Caco-2 and MM6cells. Cells were exposed for **24 h** to medium or medium supplemented with various concentrations of compounds (0-1000 µM). Data are expressed as mean ±SD of four to five independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. untreated cells).

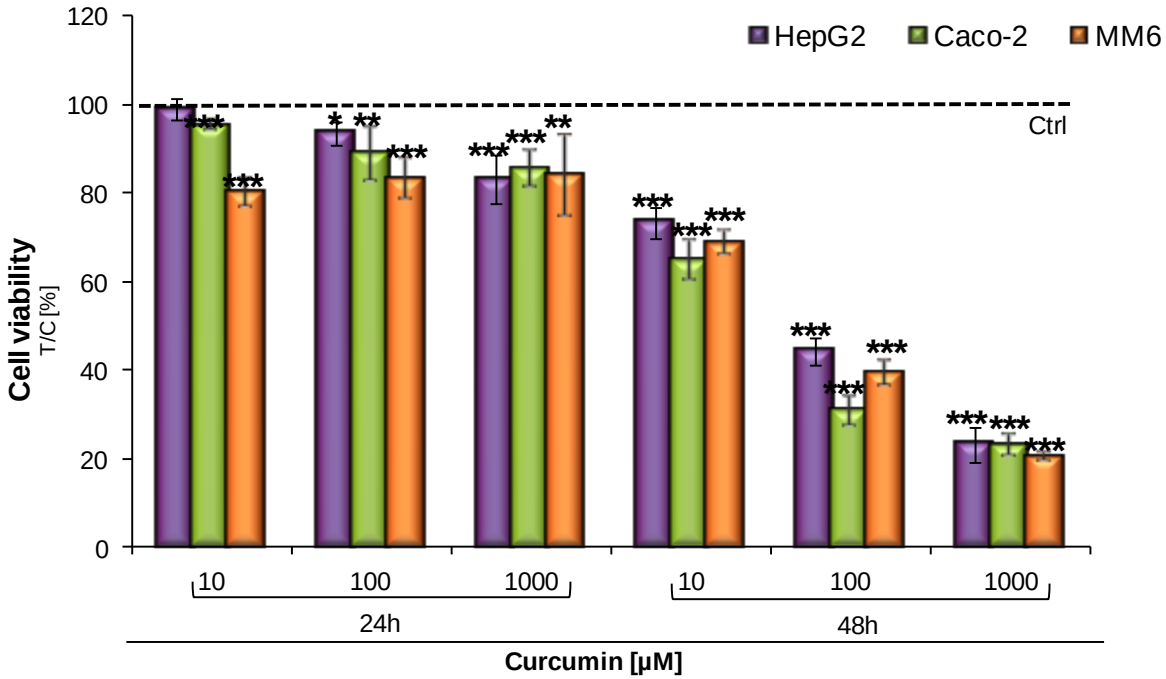
After 24 hours incubation, HO-activity analysis showed an inhomogeneous and complex dose response effect among all cell lines under study. HepG2 and Caco-2 cells showed the most potent induction of HO-activity after 24 h upon treatment with PDTC 1000 µM together with a high cell viability 90.6% ± 2.2% (HepG2) and 85.4% ± 3.9% (Caco-2) viable cells. HO-activity induction by 1000 µM PDTC for 24h was about 10-fold in HepG2 cells, 5-fold in Caco-2 and only 1.4-fold compared to basal HO-activity (see Figure 5-4 A).

PDTC and quercetin have shown a dose related effect in HepG2 and Caco-2 cells. PDTC and quercetin concentrations (100 µM and 1000 µM) caused a significant induction of the HO-activity without a substantial reduction of viable cells compared to control treatment. MM6 cell line showed no induction of the HO-activity after 24 h of treatment with curcumin and quercetin (see Figure 5-4 B+C).

A



B



C

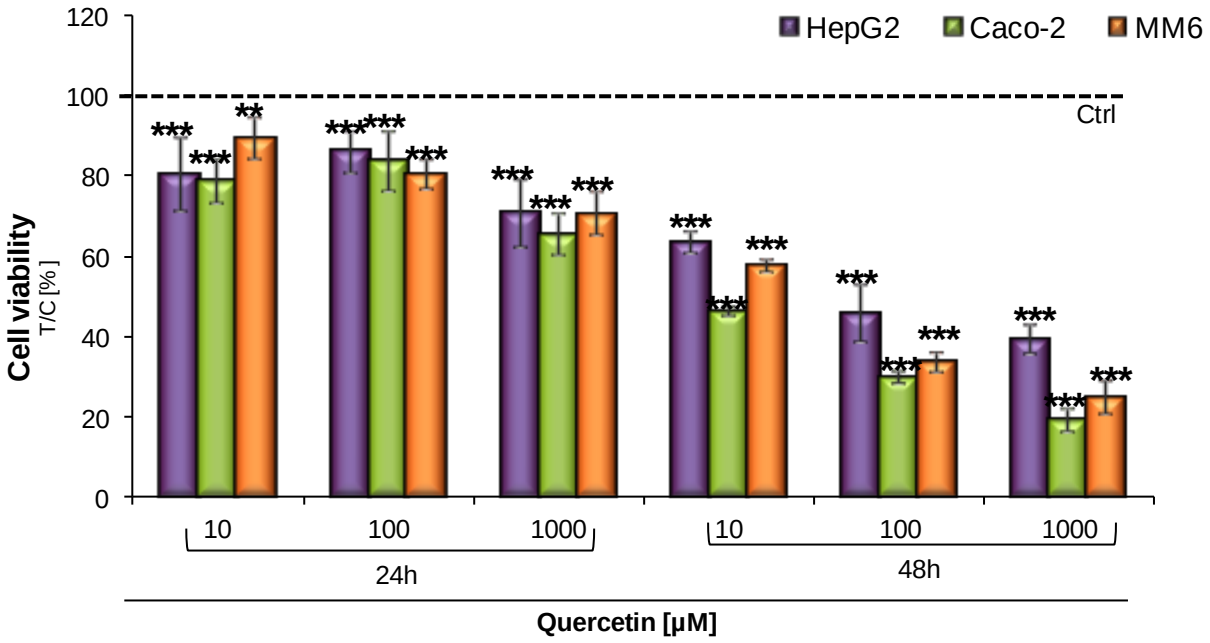


Figure 5-4: Viability of cells exposed to PDTC (A), curcumin (B) and quercetin (C) at 37°C. HepG2, Caco-2 and MM6 cells were exposed for **24** and **48 h** to medium or medium supplemented with various concentrations of compounds (0-1000µM). Cell viability was measured using the trypan blue viability test (see Materials and methods). Results represent means ±SD of 6 independent experiments (triplicates). Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

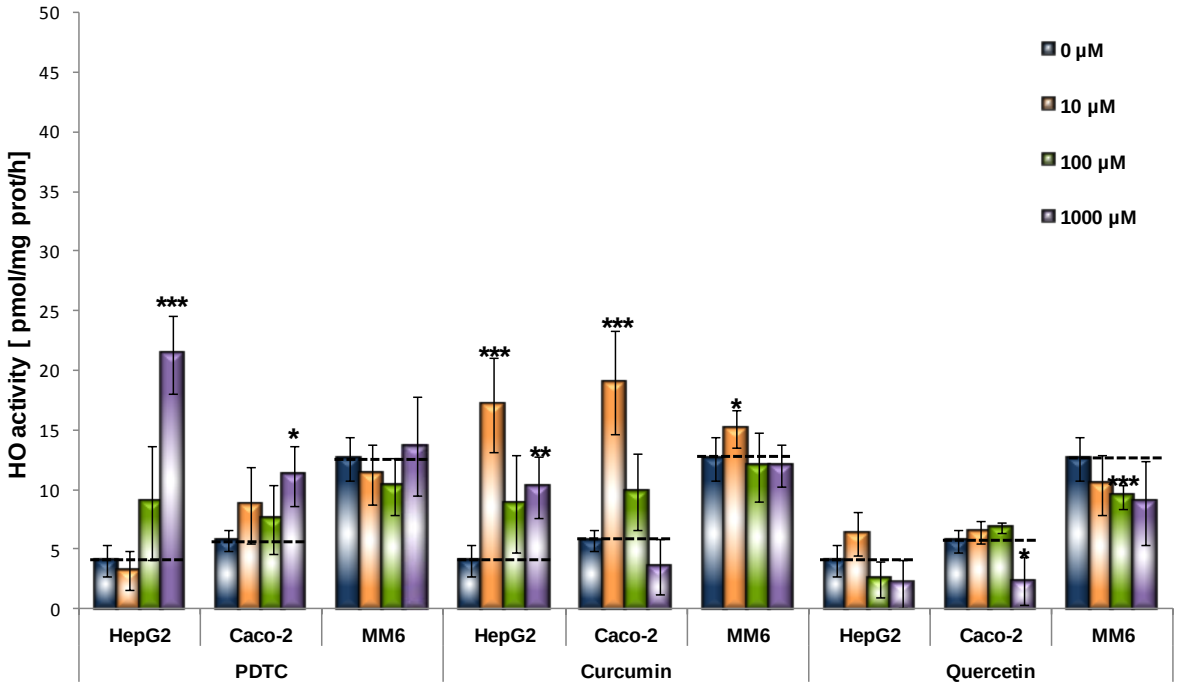


Figure 5-5: Effect of PDTC, curcumin and quercetin on HO-activity in HepG2, Caco-2 and MM6 cells. Cells were exposed for **48 h** to medium or medium supplemented with various concentrations of compounds (0-1000µM). Data are expressed as mean ±SD of four to five independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. untreated cells)

Concentration response effects after 48 h were neither straight proportional nor uniform among different cell lines (see Figure 5-5). In general a large reduction in cell viability below 60% for all cell lines of all tested substances was observed from 100 µM (see Figure 5-4). Therefore, the following experiments were incubated for 24 h with inducers.

PDTC 1 mM was selected as positive control due to its ability to induce a large amount of HO-activity and its reproducibility, without drastically affecting the cell viability in comparison to curcumin and quercetin.

Artifactual H_2O_2 formation in DMEM (Dulbecco's Modified Eagle Medium) media has been previously reported [Bellion et al., 2009; Long et al., 2007]. From the cell lines under study, only Caco-2 cells were incubated in DMEM media, where sodium bicarbonate (a constituent of DMEM cell culture media) was identified as a strong generator of H_2O_2 in cell culture [Bellion et al., 2009]. Considering the evidence that artifactual H_2O_2 formation potentially influences *in vitro* antioxidant biomarkers and that quercetin acts as a moderate H_2O_2 generator [Bellion et al., 2009], control incubations were performed in presence or absence of 100 U/mL catalase, results are shown in Figure 5-6.

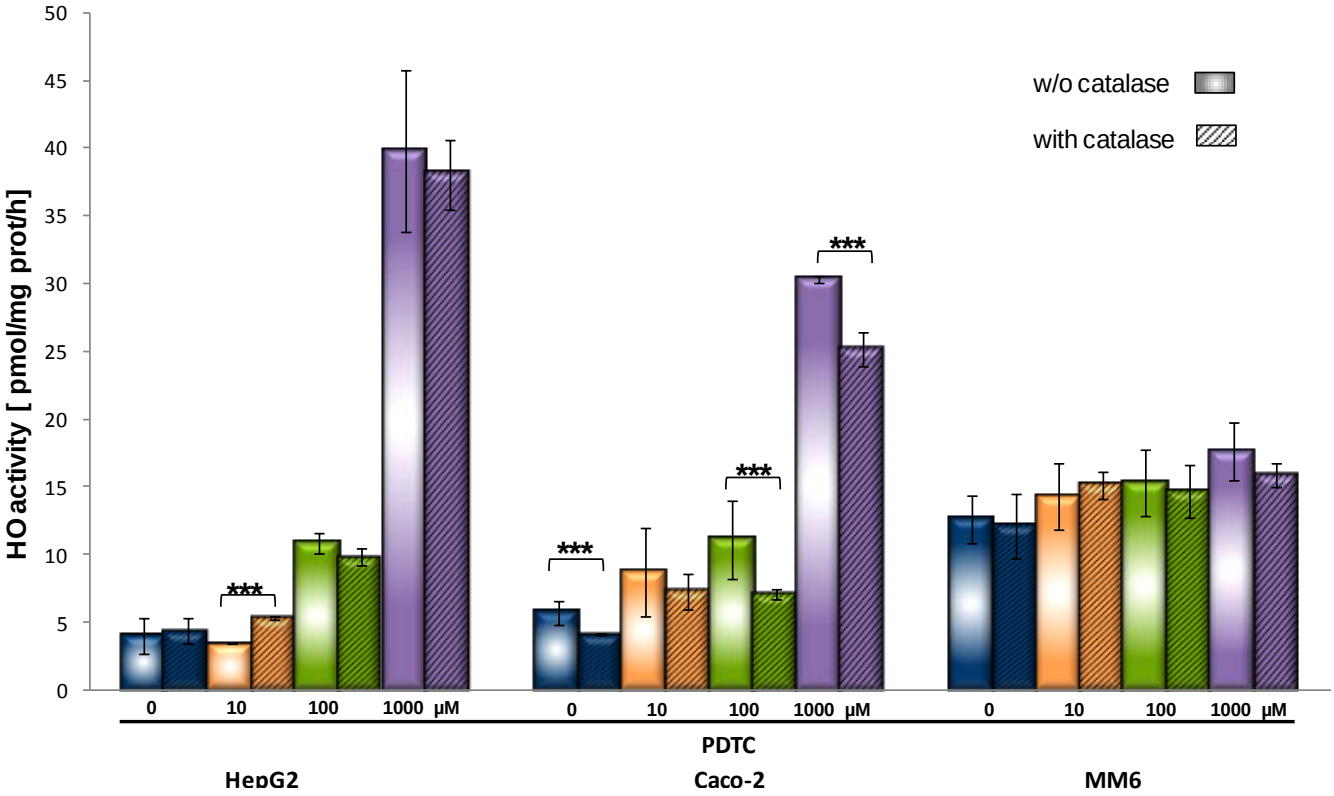


Figure 5-6: Modulation of HO activity in HepG2, Caco-2 and MM6 cells after 24 h incubation with PDTC (0 –1000 µM with/without catalase 100 U/mL). Data are expressed as mean ±SD of 3 - 5 independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances of untreated vs. catalase-treated cells were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001).

The results show almost no difference between the HO-activities in HepG2 and MM6 cells incubated with or without catalase. Furthermore, HO-activity after incubation of Caco-2 cells with catalase at 0, 100 and 1000 µM PDTC was slight but significantly lower compared to cells incubated without catalase. Evidence shows that H_2O_2 decreased cell viability but increased HO-1 expression in a concentration- and time-dependent manner in human pulp cells [Min et al., 2008] and that upregulation of HO-1 protects PC12 (rat adrenal medulla) and L02 (normal human hepatic) cells against H_2O_2 induced cell damage via induction of Nrf2 pathway [Chen et al., 2005; Chen et al., 2010].

5.1.2 Influence of coffee constituents on the total HO-activity

As already mentioned, coffee is a complex mixture consisting of several compounds. Recently, kahweol was reported as upregulator of the expression and activity of HO-1 in human neuroblastoma SH-SY5Y cells [Hwang and Jeong, 2008]. Likewise, Cavin et al. reported a marked induction of mRNA expression of HO-1 in rat liver and primary hepatocytes treated with 800 µg/mL of standard instant coffee for 24 h [Cavin et al., 2008]. Boettler *et al*. reported that 100 µg/mL of an extract from NMP enriched coffee elevated the HO-1 gene expression in human PBL (peripheral blood leukocytes) after 3 h incubation and in HT29 cells after 3 h and 24 h incubation with 100 µg/mL of the same coffee extract. In parallel, a pilot intervention trial with 27 healthy moderate coffee drinkers showed an increase of HO-1 gene expression after consumption of (500 mL/day) dark roast coffee for 4 weeks [Boettler et al., 2011b].

The effect of various concentrations (1µM – 1mM) of NMP, caffeine, TMP, 2E-3,5 DMP, 2-E-3-MP, 2MP and two coffee extracts (K2 and K5) on HO-activity of HepG2, Caco2 and MM6 cells were tested. For each independent experiment the positive control (PDTC 1mM) and the negative control (water or DMSO) were run in parallel. Results are shown in Figure 5-7 to Figure 5-11

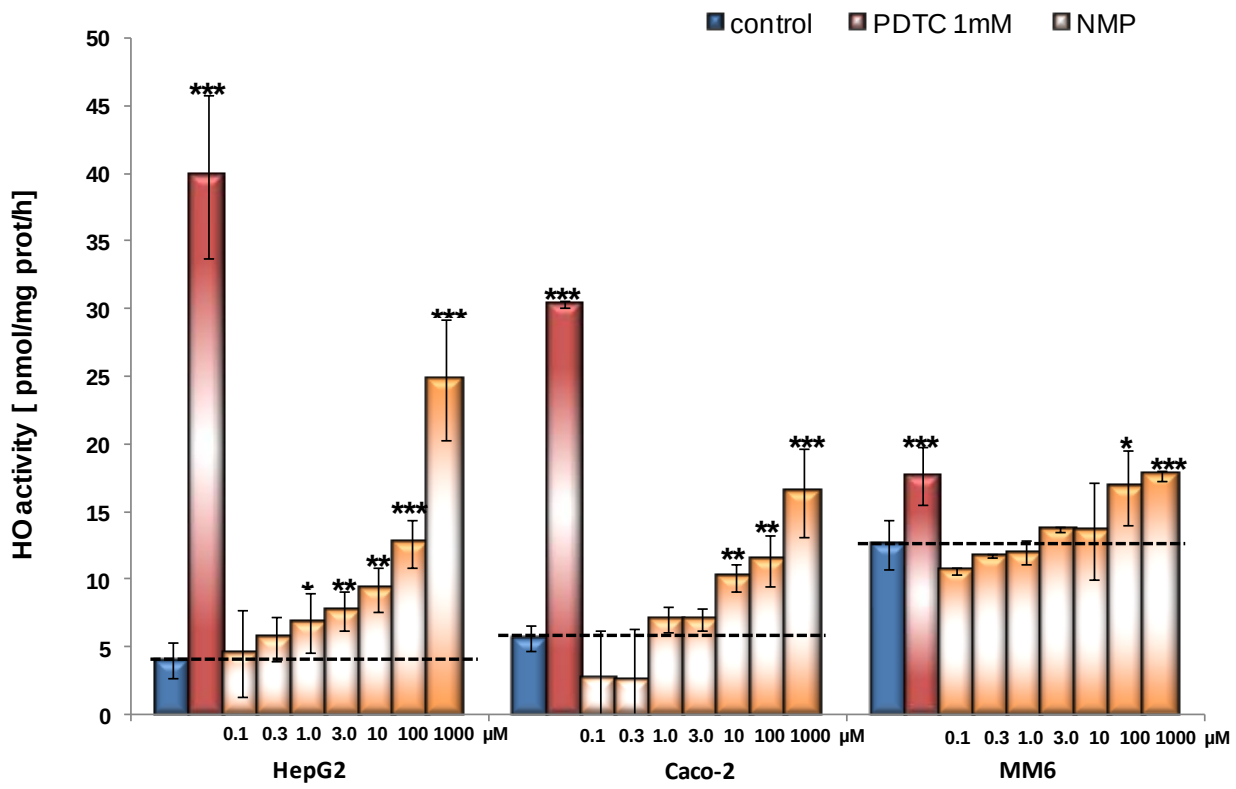


Figure 5-7: Effect of NMP (N-methylpyridinium) on HO-activities in HepG2, Caco-2 and MM6 cells. Cells were exposed for 24h to medium or medium supplemented with various concentrations of NMP (0-1000µM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

Results about the NMP induction of the HO-activity in HepG2 (from 0.3 µM) and Caco-2 cells (from 10 µM) were consistent with the potential of this coffee compound to elevate HO-1 gene expression [Boettler et al., 2011b]. It is worth noting that Hwang *et al.* estimated a positive correlation between time-dependent increases in HO-1 mRNA, protein expression and enhancement of HO-1 activity in human neuroblastoma cells treated with kahweol [Hwang and Jeong, 2008]. This might suggest that a part of the protective effects of NMP may be due its potential to stimulate gene and protein expression resulting in increased HO-1 activity.

Caffeine, along with its catabolic products theobromine and xanthine, is a key component of coffee. Caffeine and its metabolites may also contribute to the overall antioxidant and chemopreventive properties of caffeine-containing beverages, such as coffee [Azam et al., 2003]. The determination of the influence of caffeine on the HO-activity in HepG2 cells were performed in the working group as part of a four-weeks training in research work by Y. Diener. Results are shown in the Figure 5-8.

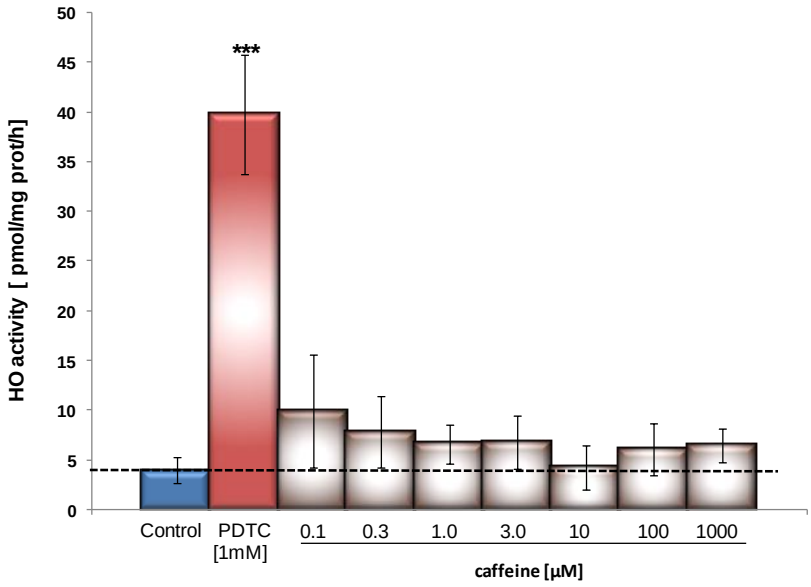


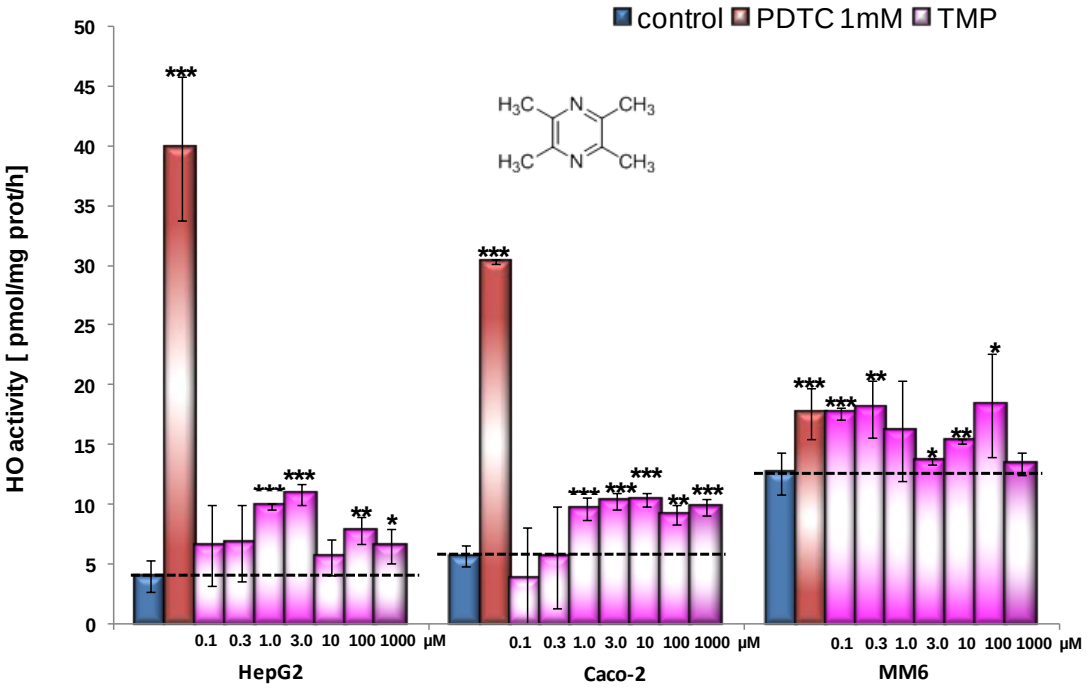
Figure 5-8: Effect of caffeine on HO-activities in HepG2 cells. Cells were exposed for 24 h to medium or medium supplemented with various concentrations of caffeine (0-100µM). Data are expressed as mean ±SD of three independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

As shown in Fig. 5-8 caffeine up to 1000 µM exhibited no inductive effect on HO-activity of HepG2 cells. In fact, Takakusa et al. [Takakusa et al., 2008] reported that HO-1 levels in human hepatocytes exposed for 24h to increasing concentrations of caffeine (30, 100, and 300 µM) were not different from those of the control.

Tetramethylpyrazine (TMP), an active ingredient of a traditional Chinese herb (Ligusticum wallichii Franch) has been widely used especially in the treatment of patients with cerebral and cardiac ischemic diseases. In 2006, Chen *et al*. showed in a rat model of myocardial ischemia/reperfusion injury that TMP suppresses ischemia-induced ventricular arrhythmias and reduces the infarct size resulting from ischemia/reperfusion (I/R) injury *in vivo* [Chen et al., 2006]. They associated the cardioprotective activity of TMP with its antioxidant activity via induction of HO-1 and its capacity to inhibit neutrophils. The authors showed that treatment with TMP (10 mg/kg) in non-ischemic myocardium for 2 h strongly induced HO-1 expression. The level of HO-1 protein in the control group was quite low, whereas pretreatment with TMP 5 and 10 mg/kg significantly increased HO-1 protein expression in ischemic.

To determine if TMP and other alkyl pyrazines with different substitution patterns like 2E-3,5-DMP, 2E-3-MP and 2-MP have any effect on HO-activity in our model, cells were treated for 24 h with various concentrations of pyrazines (0-1000 µM). Incubations with 2-E-3-MP were performed by the student M. Hiery as part of a four-weeks training research work.

A



B

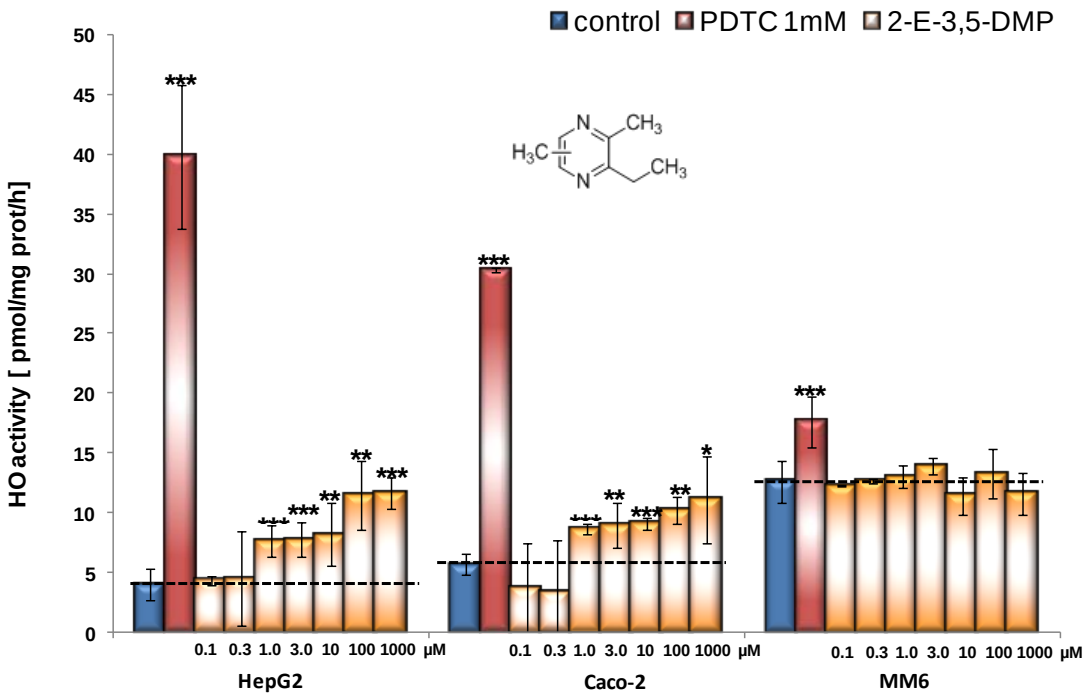


Figure 5-9: Effect of TMP (A) and 2-E-3,5-DMP on HO-activities in HepG2, Caco-2 and MM6 cells. Cells were exposed for 24 h to medium or medium supplemented with various concentrations of TMP or 2-E-3,5-DMP (0-100µM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

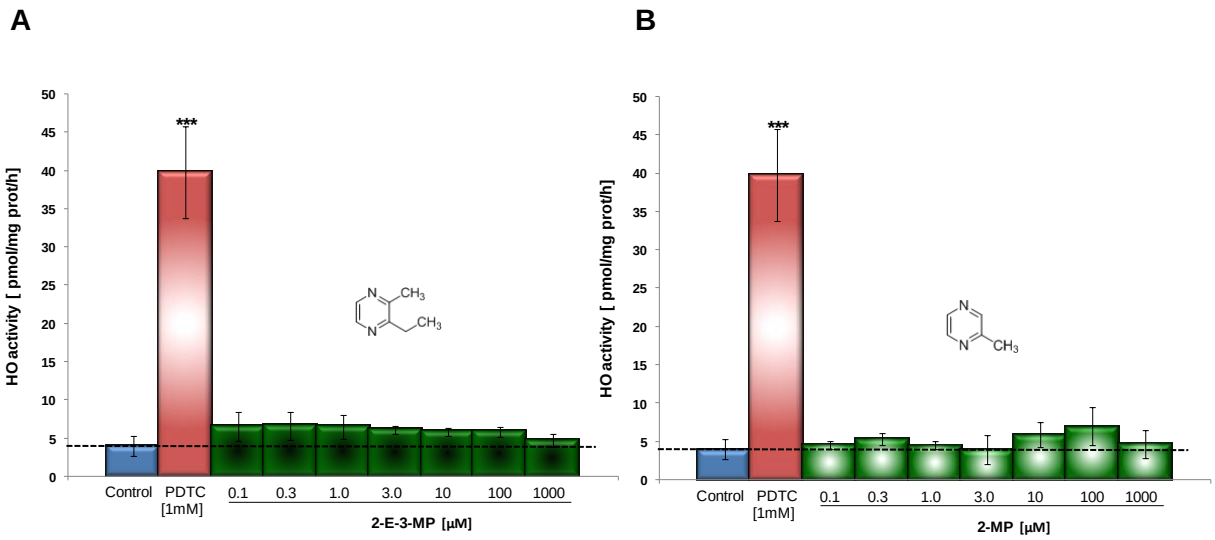


Figure 5-10: Effect of 2-E-3-MP (A) and 2-MP (B) on HO-activities in HepG2 cells. Cells were exposed for 24 h to medium or medium supplemented with various concentrations of 2-E-3-MP or 2MP (0-100µM). Data are expressed as mean ±SD of three independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

In general, after 24 hours incubation the effects of the studied pyrazines show a nonhomogeneous and complex dose response effects among the studied cell lines. It is important to note that highly substituted pyrazines, like TMP and 2-E-3,5-DMP, significantly induced the HO-activity in HepG2 and Caco-2 cells at concentrations >1 µM but without a clear dose-response relationship. In contrast, 2-E-3-MP and 2-MP showed no effect.

To achieve an estimation of the potential of coffee as inducer of HO-activity two coffee extracts prepared from coffee beans, derived from the same batch of green coffee beans (Arabica Brazil, Santos, unwashed) which were roasted in a fluidized bed roaster (RFB Neuhaus Neotec) at the Institute of Thermal Separation Processes (Technische Universität Hamburg-Harburg) were tested by D. Liesenfeld in his training research. The resulting coffees were: a light roast (**AB1**; 2 min of roasting time) comprising a high CGA content (819.2 mg/L) and a dark roast (**AB2**; 5 min of roasting time) that was extremely high in NMP (73.7 mg/L) compared to standard coffees [Bakuradze et al., 2011a; Boettler et al., 2011b].

Coffee brews were produced from ground coffee beans as described by Lang et al. [Lang et al., 2008]. Briefly, 48 g of coffee powder was placed onto the filter of a conventional coffee machine and extracted with 900 mL of (boiling) water; the resulting coffee brews were immediately cooled (ice bath) and lyophilized. NMP and CGA were quantified as described by Lang *et al.* and Weiss *et al.* [Lang et al., 2006; Lang et al., 2008; Weiss et al., 2010].

For HO-activity experiments, coffee extracts were dissolved in double distilled water; results are shown in Figure 5-11.

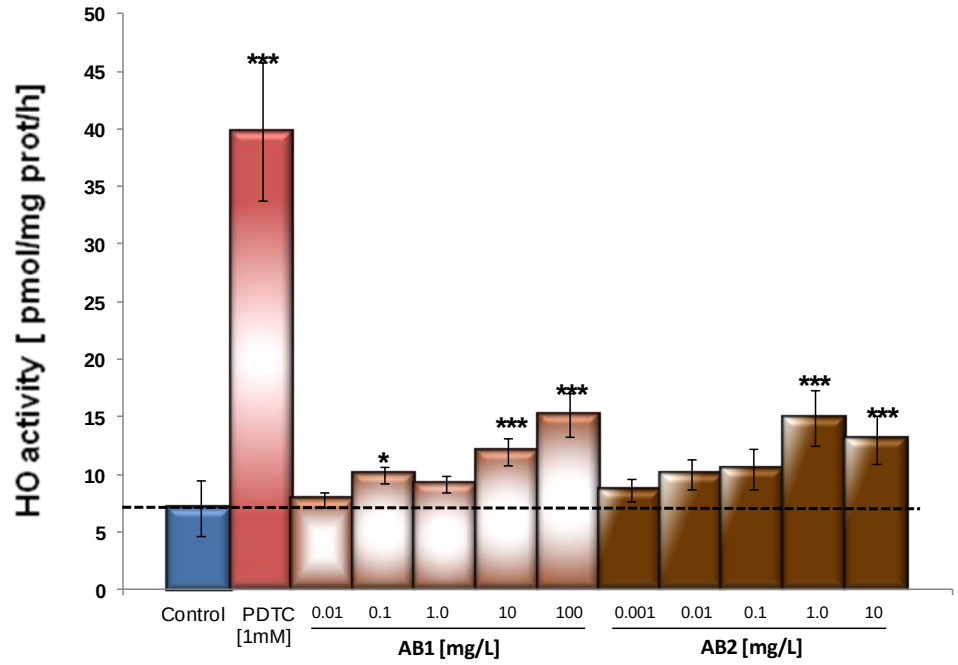


Figure 5-11: Effects of coffee extracts from a light (AB1) and a dark (AB2) roasted coffee on HO-activity in HepG2 cells. Cells were exposed for 24 h to medium or medium supplemented with various concentrations of AB1 or AB2 coffee extracts (0.001–100 mg/L) w/o catalase. Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed as picomoles of bilirubin formed per milligram protein per hour. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

Coffee extracts showed a significant induction of HO-activity at high concentrations (from 10 mg/L for AB1 and 1 mg/mL for AB2). Is worth noting that 100 mg/L AB1 showed similar increase in HO-activity as 1 mg/L AB2 extract. These results indicate that the degree of roasting and thereby the composition of the coffee have a major influence on HO-activity stimulation.

The stimulatory effect of NMP and substituted pyrazines on the HO-activity in HepG2 and Caco-2 cells has been previously shown (see Figure 5-7 and Figure 5-9) correlates with the observed effect of the coffee extract AB2 containing high concentrations of NMP. Furthermore, the results obtained by Bottler at al. [Boettler et al., 2011b] where NMP was identified as a strong modulator of ARE-dependent gene transcription in *vitro*, HO-1 gene expression *ex vivo* and *in vivo*, are in agreement with the findings presented in this work.

The possibility that other coffee constituents may also play a role for the HO-stimulation must be considered, e.g. caffeic acid esters have alredy been described to activate HO-1 gene expression in mouse macrophage cells [Suzuki et al., 2006]. Although kahweol was reported as upregulator of the expression and activity of HO-1 [Hwang and Jeong, 2008] it is important to take into account that this natural component in coffee is degraded by the roasting process and largely trapped by the use of a paper filter in coffee preparation. Until now CGA have not shown HO-1 gene expression *in vitro* and *ex vivo* [Boettler et al., 2011b; Suzuki et al., 2006].

5.2 PDE activity

Cyclic nucleotide phosphodiesterases comprise a group of enzymes that degrade the phosphodiester bond in the second messenger molecules cAMP and cGMP. They regulate the localization, duration, and amplitude of cyclic nucleotide signaling within subcellular domains. PDEs are therefore important regulators of signal transduction mediated by these second messenger molecules [Randall et al., 1997]. While both cyclic nucleotides can be transported out of the cell at a low rate, evidence indicates that, quantitatively, the catalytic action of PDEs provides the only pathway to rapidly lower cellular cyclic nucleotide content at least in the majority of cell types [Beavo et al., 2007].

The isoenzyme PDE3 has been shown to participate in a complex interplay between insulin and cAMP signalling networks, important for the regulation of insulin-induced glucose uptake and lipogenesis as well as insulin-induced inhibition of lipolysis in adipocytes [Beavo et al., 2007].

The stimulatory actions of the alkylxanthines were thought to occur as a result of inhibition of phosphodiesterases. Although this inhibition may contribute to the actions of caffeine (e.g. influence on thermogenesis, lipolysis, fat oxidation and insulin secretion) there is growing evidence that most pharmacological effects result from antagonism of adenosine receptors [Stanley et al., 1989; Varani et al., 2000] and these non-AR-mediated actions require higher concentrations of caffeine than those typically ingested with the human diet [Fredholm and Jacobson, 2009; Stanley et al., 1989; Wilson and Mustafa, 2009].

An intervention study comprising 33 healthy volunteers showed that daily consumption of 3–4 cups of a special Arabica coffee brew exerts health beneficial effects, such as reduced oxidative damage, body fat mass and energy uptake [Bakuradze et al., 2011a].

One of the main objectives of the present work was to investigate whether substances other than caffeine in coffee may influence the homeostasis of intracellular cyclic nucleotides *in vitro* and to determine whether it is possible to modulate the phosphodiesterase activity through coffee consumption in potential human target cells (*in vivo*). The interest was focused not only on substances present in coffee before roasting but also in those compounds generated during roasting which are found in coffee brews.

5.2.1 PDE activity *in vitro*

5.2.1.1 PDE experiments in LXFL529L cell lysates

The assay to measure cAMP-phosphodiesterase activity was performed using lysates of large cell lung tumor xenograft cell line LXFL529L [Fiebig et al., 1992]. Previous studies in our department showed that Rolipram-sensitive PDE4 isoenzyme represent the highest

cAMP hydrolysing activity in this cell line, and 85% of the total activity was localized in the cytosol [Kunz, 2000; Marko et al., 2002; Marko et al., 2000].

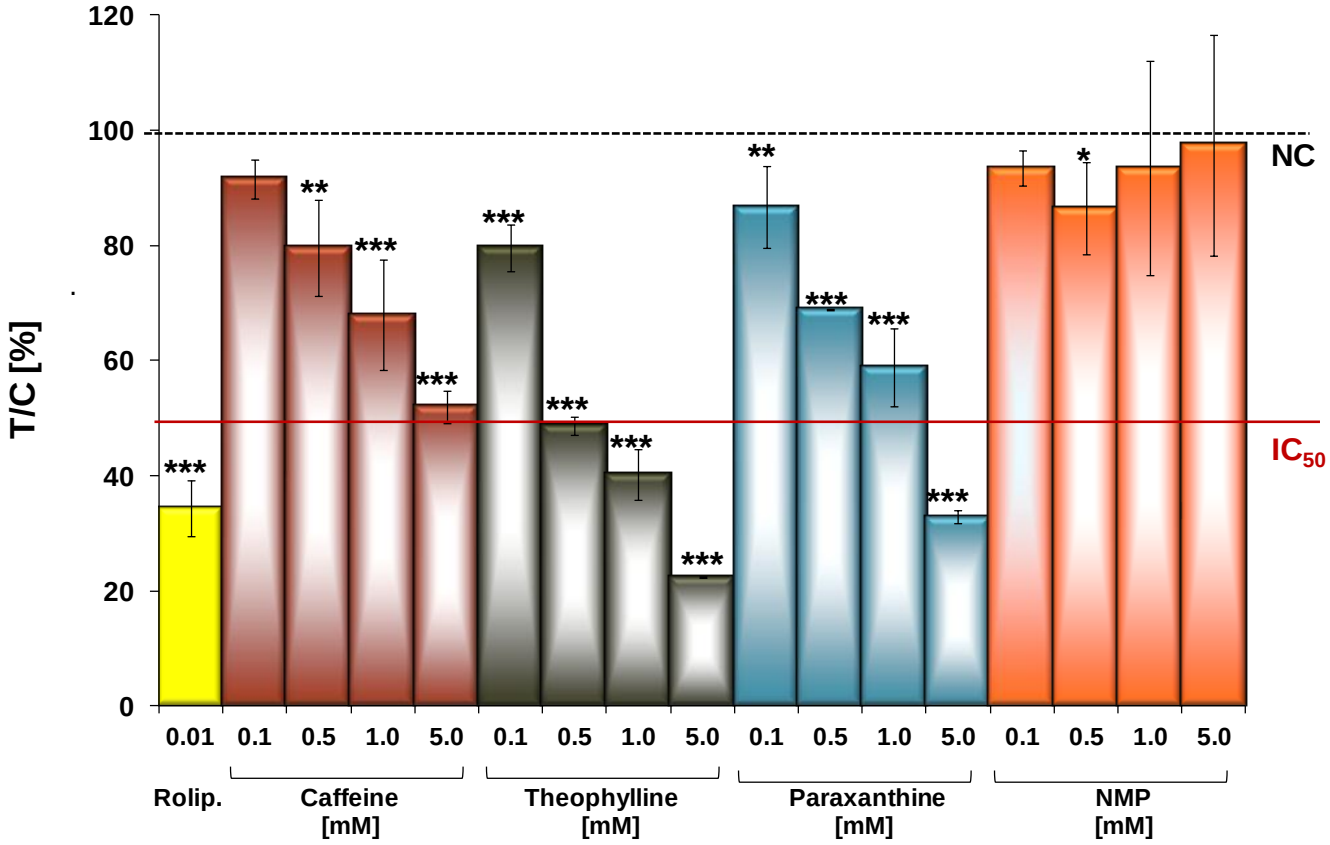


Figure 5-12: Modulation of cAMP-phosphodiesterase activity by coffee constituents and caffeine metabolites in LXFL529L cell lysates. Lysates were directly incubated with various concentrations of NMP, caffeine, theophylline or paraxanthine (0.1–5 mM). Data are expressed as mean ±SD of four to five independent experiments (triplicates); normalized to protein content and expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Rolipram (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression.

NMP shows no inhibition of cAMP-hydrolysis in LXFL529L cells. Nevertheless, caffeine, paraxanthine (the principal metabolite of caffeine) and theophylline showed a half maximal inhibitory concentration (IC_{50}) of 4.8, 1.9 and 0.5 mM respectively.

Alredy in 1962, Butcher and Sutherland [Butcher and Sutherland, 1962] demonstrated that theophylline and theobromine inhibit PDE. Inhibitory potencies of caffeine and theobromine were comparable, whereas theophylline was approximately six-fold more potent. Similarly, theophylline showed a stronger inhibitory effect on cAMP hydrolysis of LXFL529L cells (approx. 9 times higher compared to caffeine) in our test system.

An important object of this study was to determine whether some aroma compounds generated during the roasting process, show effects on the activity of PDEs. Therefore, a total of 12 pyrazines with different substitution patterns, all present in coffee were studied (see Figure 5-13).

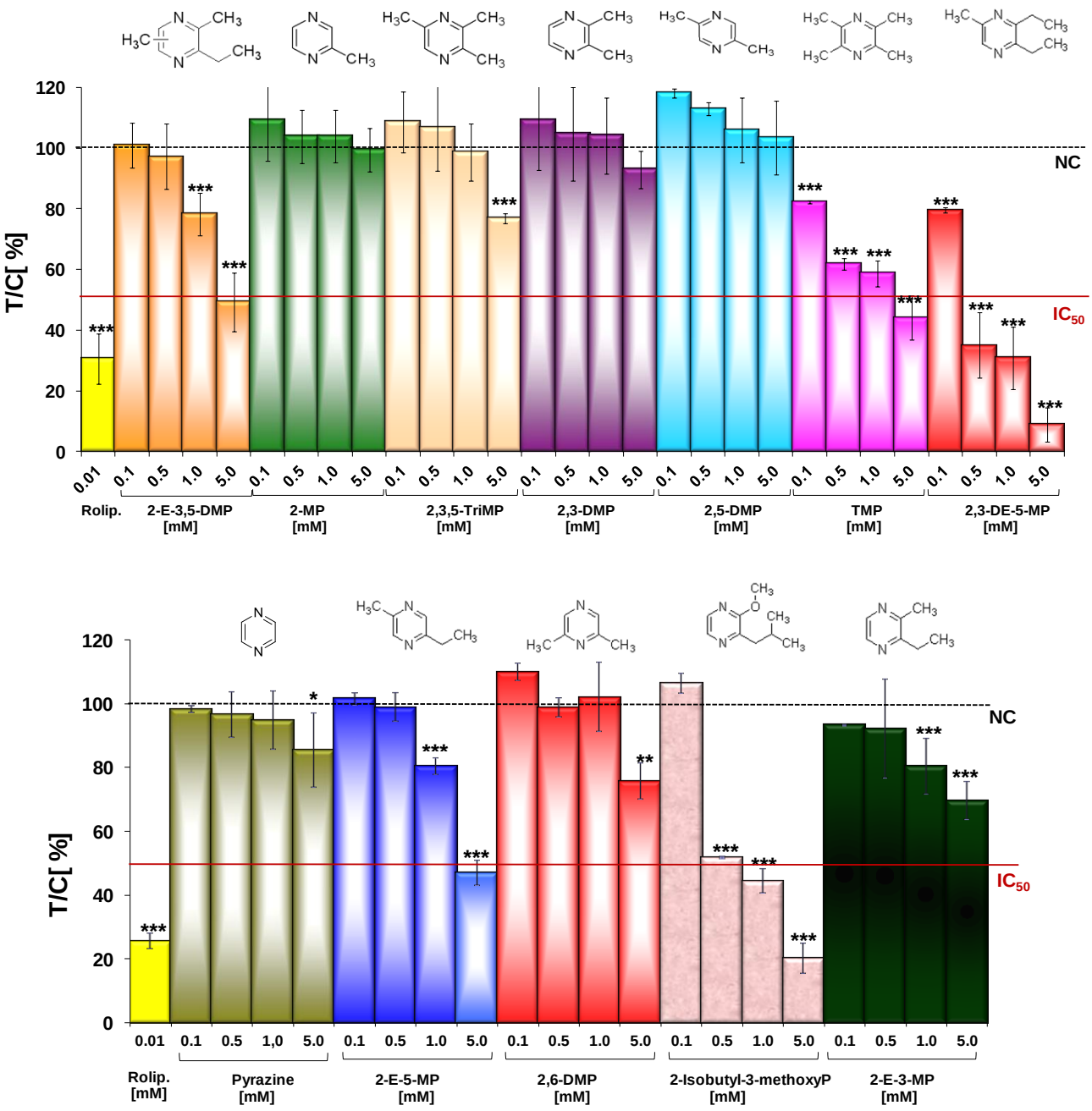


Figure 5-13: Modulation of cAMP-phosphodiesterase activities by twelve pyrazines in LXFL529L cell lysates. Lysates were directly incubated with various concentrations of pyrazines (0.1–5 mM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Rolipram (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression.

Depending on their substitution pattern, pyrazines show either weak or no effect on PDE activity. In general, unsubstituted pyrazines and pyrazine derivatives containing only methyl group substituents (with the exception of TMP) showed no effect, whereas, pyrazines substituted with ethyl groups like 2-E-3,5-DMP, 2-E-5-MP and 2-E-3-MP were able to inhibit cAMP-hydrolysis in LXFL529 cell lysates from approx. 1mM. The lowest IC_{50} values were

observed for pyrazine derivatives containing more than one ethyl group like 2-E-3-MP or a methoxy functional group as 2-Isobutyl-3-methoxyP (see Figure 5-13)**.**

To get more profound estimation of the potential of coffee as cAMP hydrolysis inhibitor, six coffee extracts were examined. The **AB1** and **AB2** extracts were previously described in chapter 5.1.2. Furthermore, coffees from the human trials (Caffè Crema, reduced caffeine coffe, SC and RC) are described in the design of each study.

Coffee brews were produced from coffee pods (pre-packaged ground coffee beans in their individual filter) as indicated to volunteers who participated in the short term study (Cafè Crema and caffeine reduced) and in the long term study (reference (RC) and study coffee (SC)). From each coffee pod (content 7.8 ± 0.2 g coffee) 110 to 130 mL of coffee were brewed, immediately cooled (ice bath) and lyophilized. After lyophilization 14 g/L extract for the caffeine reduced coffee, 16 g/L for Caffè Crema and 12 g/L for the RC and SC were obtained.

For PDE activity experiments, coffee extracts were dissolved in double distilled water, results are shown in Figure 5-14.

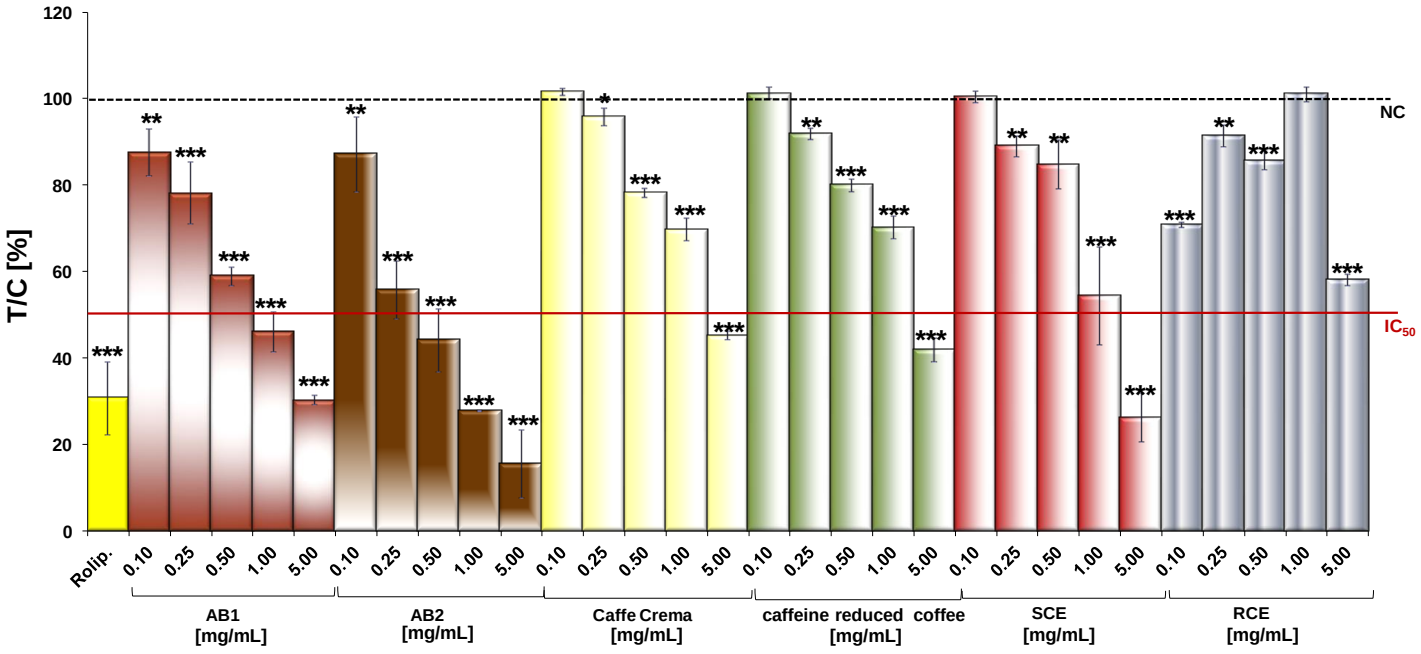


Figure 5-14: Modulation of cAMP-phosphodiesterase activity by different coffee extracts in LXFL529L cell lysates. Lysates were directly incubated with various concentrations of coffee extracts (0.1–5 µg/mL). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Rolipram (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression. SCE: study coffee extract; RCE: reference coffee extract.

It is well-known from literature that caffeine is a nonselective inhibitor of phosphodiesterases [Essayan, 2001]. The observed PDE inhibition effects of coffee extracts AB1 and AB2 (IC_{50}= 0.42 and and 0.47 mg/mL respectively) can not be attributed solely to caffeine (IC_{50}= 4.8 mM ≈ 0.9 mg/mL in LXFL529L lysates) see Table 5-1 (see page No. 125). Additionally, the inhibitory effect of a extract from a commercial coffee (Caffè Crema), caffeine reduced coffee and the coffee extract (SCE) from the SC coffee were similar; with a half maximal inhibitory concentration of 2.6, 3.3 and 2.7 mg/mL respectively. For the reference coffee extract (RCE) no concentration-response relationship was found.
In general the coffee extracts AB1 and AB2, rich in pyrazines and NMP, showed potent PDE inhibition compared to other investigated coffee extracts samples.

5.2.1.2 Biomarker evaluation

The used biomarkers and surrogate endpoints (blood cells) were necessary because they were easier, more ethical to obtain and monitored than clinical endpoints (adipocytes). Therefore, potential target cells in blood allowing the estimation of the effect of coffee consumption on nucleotide homeostasis in humans were preferred.
It is known from the literature that phosphodiesterase 3 (PDE3) is present in rabbit and human erythrocyte membranes and its inhibition by Iloprost induced cAMP [Hanson et al., 2008]. However phosphodiesterase activity in the particulate fraction of erythrocytes by the used method (see Material and Methods chapter) could not be detected. Such results could be explained with findings of Nakagawa et al. [Nakagawa et al., 1984], showing that the stabilization of PDE during the isolation of erythrocyte membranes is critical to maintain enzyme activity, or because, the major PDE activity present in mammalian (human) erythrocytes has been shown to be PDE1 that hydrolyzes cGMP, not cAMP [Petrov et al., 1998].
In contrast, monocytes and lymphocytes isolated following the protocol of Gantner *et al*. [Gantner et al., 1997] showed a robust cAMP hydrolysing activity, both in the particulate (45 ± 2 for monocytes and 15 ± 6 pmol/10^8 cells x min) and in the cytosolic fraction (60 ± 3 for monocytes and 30 ± 5 pmol/10^8 cells x min). Of note, for each experiment 250 mL peripheral blood were needed to isolate the required cell number and that PDE4 was the predominant phosphodiesterase in monocytes and lymphocytes (PDE3 is a major PDE in adipose tissue). For these reasons these potential target cells were not of use in the long term study.

5.2.1.3 PDE experiments in platelet cell lysates

cAMP and cGMP are two critical intracellular second messengers with strong inhibitory activity on fundamental platelet functions. PDEs, by catalysing the hydrolysis of cAMP and cGMP, limit the intracellular levels of cyclic nucleotides, thus regulating platelet function. The

inhibition of PDEs may therefore exert a strong platelet inhibitory effect. Platelets possess three PDE isoforms, PDE2, PDE3 and PDE5, with different selectivity for cAMP and cGMP [Gresele et al., 2011].

For the *in vitro* experiments corresponding to cAMP hydrolysing activity, platelets were isolated from PRP plasma (see Material and Methods chapter) or from platelet concentrates. Platelet-concentrates were obtained from the DRK-Blutspendedienst (German Red Cross Blood Transfusion Service) Baden Württemberg-Hessen in Mannheim. Each platelet concentrate was a suspension of human platelets (pooled from four donors) in 255 ml of a platelet additive solution and CPD (citrate-phosphate-dextrose) plasma, containing approximately 2.0-4.5 x 10^{11} thrombocytes per preparation.

A previous characterization of the phosphodiesterases in each batch of platelets was always performed using Rolipram (PDE4), Milrinon (PDE3), and Zaprinast (PDE5) as specific inhibitors and Ca^{2+}/calmodulin (PDE1) as stimulator. In general, the major effect was shown by the selectively PDE3 inhibitor Milrinon (10µM) which inhibited 61.4 ± 2.1% of the total PDE activity in platelet lysates (see Figure 5-15). The results are in accordance with previous observations of Sun *et al.* [Sun et al., 2007], showing that PDE3 is the main PDE expressed in platelets and cardiac ventricular myocytes, and is responsible for the functional changes caused by PDE inhibition.

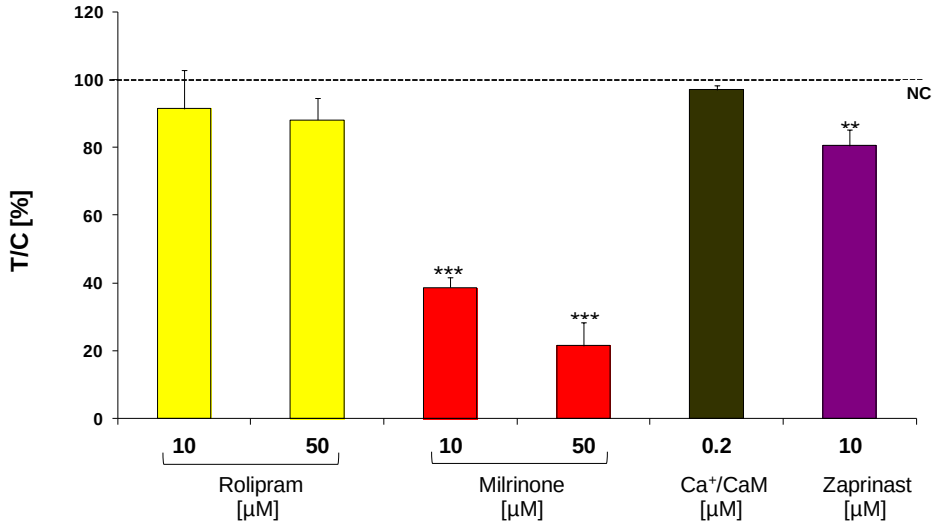


Figure 5-15: Profiles of phosphodiesterase activities in human platelets lysates. Lysates were directly incubated with Rolipram, Milrinone, Ca^{+}/CaM and Zaprinast. Data are expressed as mean ±SD of eleven independent experiments (triplicates); normalized to protein content and expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC).

As aforementioned naturally occurring methylxanthines were the earliest PDE inhibitors to be discovered, and among them, caffeine was the first [Sutherland and Rall, 1958]. Ardlie and

co-workers documented inhibition of platelet aggregation by caffeine and theophylline in 1967 [Ardlie et al., 1967]. In 1971, Mills and Smith [Mills and Smith, 1971] showed that adenosine increases cAMP in platelets and that methylxanthines prevent the conversion of cAMP to AMP, acting as PDE inhibitors, thus greatly increasing the inhibitory effects of adenosine on platelet aggregation. Caffeine and theophylline also act as nonselective adenosine receptor antagonists [Beavo et al., 2007; Gresele et al., 2011]. Results about the effect of coffee compounds and coffee extracts on the PDE activity of platelet cell lysates are shown in Figure 5-16 to 5-20.

In general, effects in platelet lysates are comparable to those obtained from LXFL529L cell lysates. Tested coffee constituents (except NMP) showed various inhibitory effects on PDE activity in platelets (see Figure 5-16). The PDE-inhibitory effect of caffeine was more pronounced in lysates of platelets (IC_{50}=0.7 mM) than in LXFL529L cell lysates (IC_{50}=4.8 mM). Paraxanthine, its mammalian metabolite, and theophylline exhibited similar IC_{50} values (see Table 5-1) in platelet and LXFL529L cell lysates.

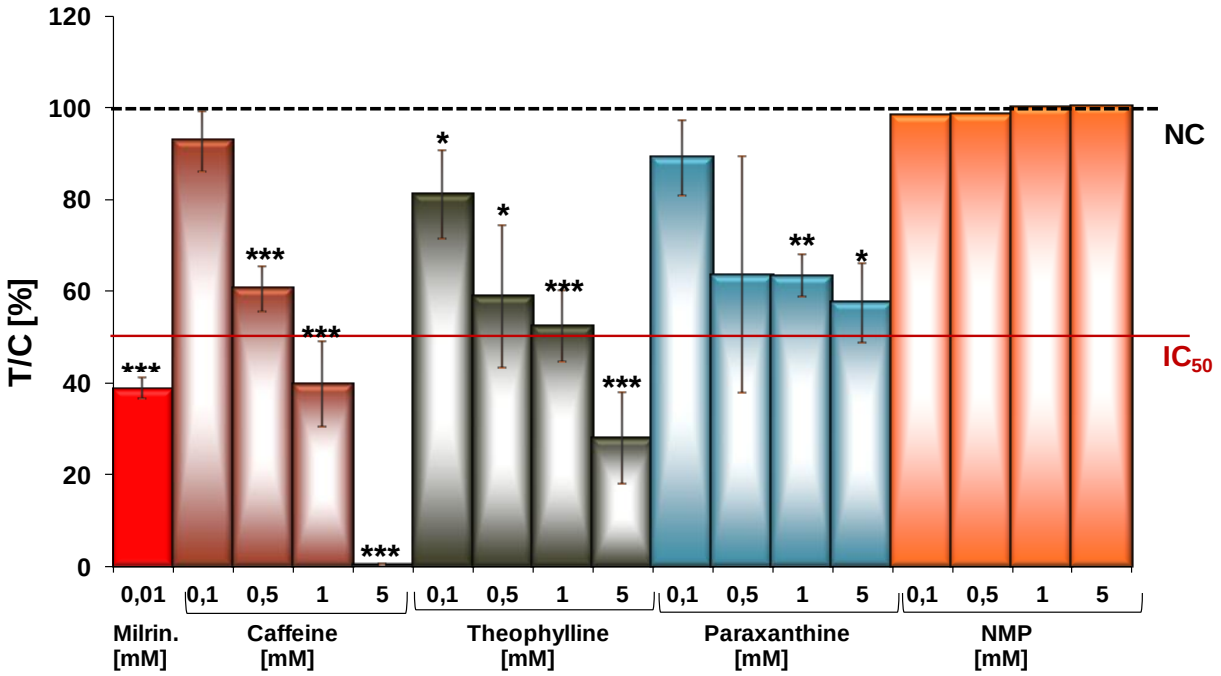


Figure 5-16: Modulation of cAMP-phosphodiesterase activities by coffee constituents (caffeine, theophylline, paraxanthine and NMP) and caffeine metabolites in platelet lysates. Lysates were directly incubated with various concentrations of NMP, caffeine, theophylline or paraxanthine (0.1–5 mM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Milrinone (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression.

Pyrazines showing a good PDE inhibitory effect on LXFL529L cell lysates were chosen to be studied for inhibition of PDE activity from platelet lysates. Tested pyrazines showed PDE inhibitory effects similar to caffeine (see

Figure 5-17) and were partly cell type dependent Table 5-1 shows for comparison the IC_{50} PDE inhibition values for pyrazines in LXFL529L and platelet lysates.

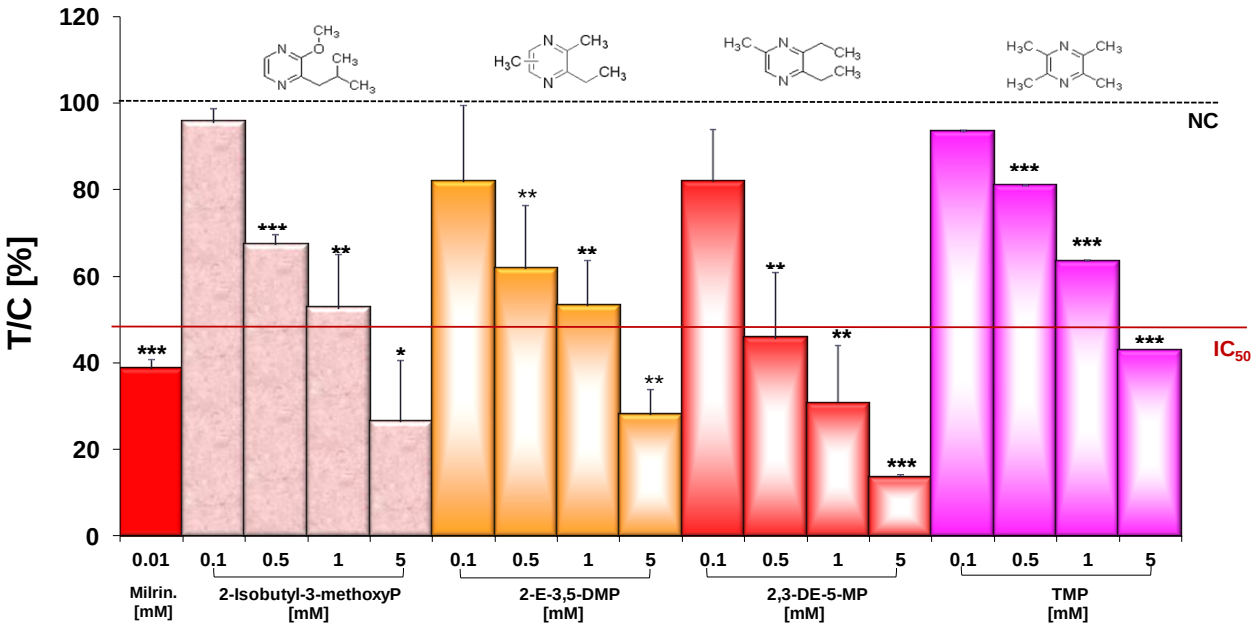


Figure 5-17: Modulation of cAMP-phosphodiesterase activities by selected pyrazines (2-Isobutyl-3-methoxyP, 2-E-3,5-DMP, 2,3-DE-5-MP, TMP) in platelet lysates. Lysates were directly incubated with various concentrations of pyrazines (0.1–5 mM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Milrinone (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression.

The coffee extracts previously studied in LXFL529L cell lysates were used to determine their effects on cAMP hydrolysing activity of platelets lysates, results are shown in Figure 5-18.

In platelets PDE inhibition by coffee extracts was more efficient by intensively roasted coffee, the half maximal inhibitory concentrations of AB1 was 0.5 mg/mL and 0.25 mg/mL for AB2. Thus AB2 showed in platelets almost twice as much inhibitory activity as the AB1 coffee extract. These results indicate that the degree of roasting has a major influence on PDE inhibition since both extracts have similar caffeine content.

Extracts from Caffè Crema and caffeine reduced coffee showed in the investigated concentration range (0.1-5 mg/ml) showed a significant inhibitory effect of the platelets PDE activity. For the reference coffee extract (RCE) again no concentration-response relationship

was found, though it showed a more potent inhibition of PDE activity when compared with LXFL529L results.

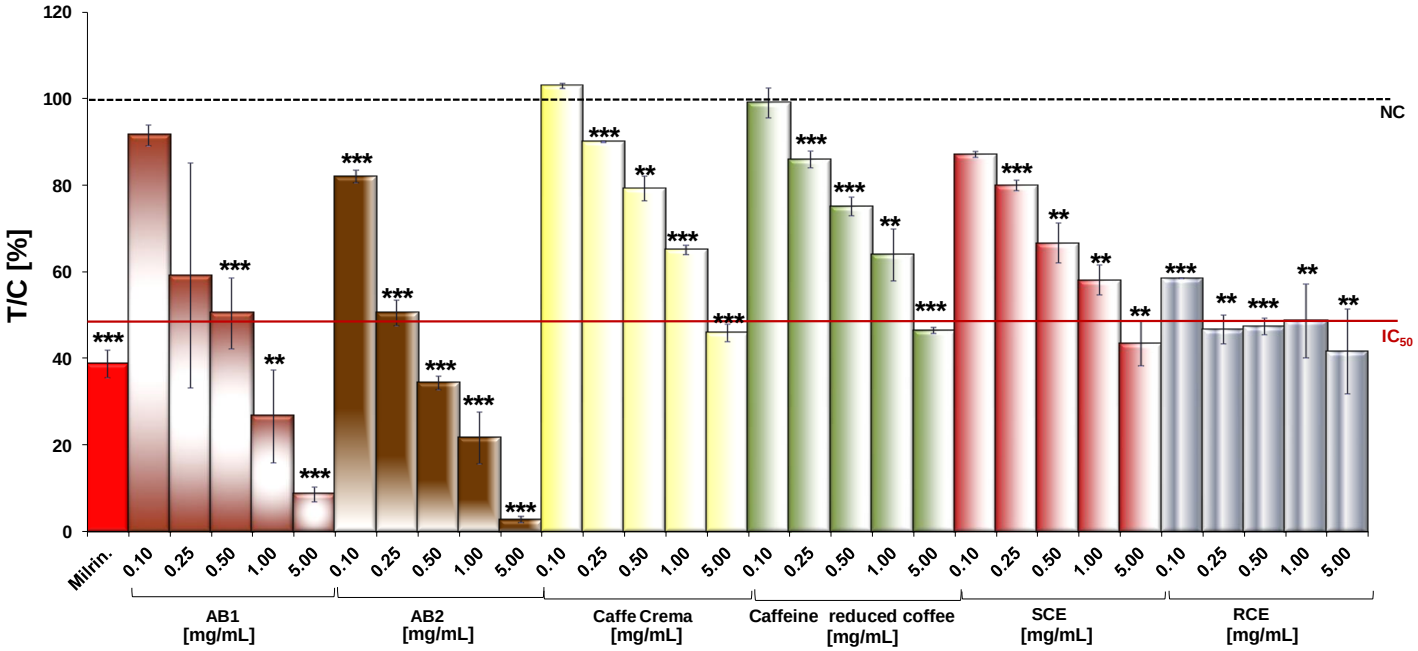


Figure 5-18: Modulation of cAMP-phosphodiesterase activities by coffee extracts from AB1, AB2, Caffè Crema, caffeine reduced, SCE and RCE coffees in platelet lysates. Lysates were directly incubated with various concentrations of coffee extracts (0.1–5 µg/mL). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Milrinone (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression. SCE: study coffee extract; RCE: reference coffee extract.

Platelet lysates were for PDE activity further analyzed to determine whether the PDE activity might be affected by other natural substances present in coffee such as polyphenols. Preliminary results are shown in Figure 5-19.

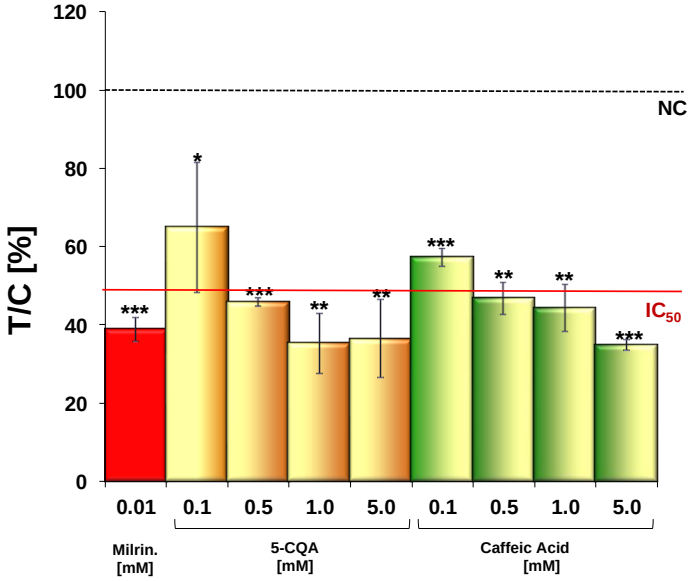


Figure 5-19: Modulation of cAMP-phosphodiesterase activities by coffee polyphenols in platelet lysates (preliminary data). Lysates were directly incubated with various concentrations of 5-CQA and caffeic acid (0.1–5 mM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content and expressed expressed as a percentage of the NC. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. NC). Positive control: Milrinone (10µM); negative control (NC): 1% DMSO. IC_{50} (half maximal inhibitory concentration) were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear regression. SCE: study coffee extract; RCE: reference coffee extract.

5-CQA and caffeic acid showed IC_{50} values of 0.49 and 0.48 mM respectively, which are comparable to pyrazines. Is worth noting that the pH of these incubation batch could have an artifactual inhibitory effect on PDE activity, therefore, it is recommended to verify these data. The results show that different coffee constituents, exhibit caffeine-like PDE inhibition in human platelet lysates and that caffeine, polyphenols and some pyrazines might represent the main modulating constituents.

Table 5-1: IC_{50} values for coffee constituents and coffee extracts in LXFL529L and platelet concentrate lysates

Tested compound or Extract	IC_{50} values*	
	LXFL529L	Platelets concentrate
Caffeine	4.8 ± 0.6 mM (≈0.9 mg/mL)	0.7 ± 0.003 mM(≈0.14 mg/mL)
Paraxanthine	1.9 ± 0.8 mM (≈0.4 mg/mL)	8.5 ± 1.3 mM (≈1.5 mg/mL)
Theophylline	0.5 ± 0.07 mM (≈0.09 mg/mL)	0.9± 0.06 mM (≈0.2 mg/mL)
AB1 Extract	0.42 ± 0.04 mg/mL	0.5 ± 0.03mg/mL
AB2 Extract	0.47 ± 0.06mg/mL	0.25± 0.09 mg/mL
Caffè Crema Extract	3.3 ± 0.8 mg/mL	4.1 ± 0.5 mg/mL
Caffeine reduced coffee	2.6 ± 0.5 mg/mL	4.2± 1.6 mg/mL
SCE**	2.7 ± 0.3 mg/mL	3.2± 1.5. mg/mL
2-E-3,5 DMP	2.0± 0.2 mM (≈0.3 mg/mL)	1.0 ± 0.3mM (≈0.1 mg/mL)
TMP	1.4± 0.1 mM (≈0.2 mg/mL)	1.6 ± 0.05 mM (≈0.2 mg/mL)
2,3-DE-6-MP	0.3± 0.02 mM (≈0.1 mg/mL)	0.4± 0.03mM (≈0.1 mg/mL)
2-E-5-MP	1.7 ± 0.2mM (≈0.2 mg/mL)	nd***
2-Isobutyl-3-methoxyP	0.6 ± 0.01 mM (≈0.1 mg/mL)	1.0± 0.005 mM (≈0.2 mg/mL)
5-CQA	nd***	0.49 ± 0.01 mM (≈0.17 mg/mL)[a]
Caffeic acid	nd***	0.48 ± 0.09 mM (≈0.09 mg/mL)[a]

* IC_{50} (half maximal inhibitory concentration) values were determined as the mean of the IC_{50} values of the different experiments, each calculated by linear or sigmoidal regression.
** SCE: study coffee extract
*** nd: no determined

It is known from the literature that caffeine inhibition of PDEs requires high levels (100 –1000 µM in mouse and pigs cerebral cortex slices) than for interaction with adenosine receptors (10 – 100 µM), but the potencies of the metabolites paraxanthine and theobromine respect to inhibition of most known PDEs have not been studied [Francis et al., 2011]. Depending on the serving size, the caffeine content of beverages and/or food, and the variation in clearance times, it is plausible that plasma and cellular caffeine levels could be within the range for pharmacological actions on PDEs [Chou and Bell, 2007]. It is also well established that the intracellular concentration/action of a PDE inhibitor cannot be confidently predicted strictly on the basis of its extracellular concentration [Francis et al., 2011; Thompson, 1991].

5.2.1.4 PDE experiments in SGBS cell lysates

As aforementioned, the clinical endpoint in focus of this work are adipocytes, where the production of cAMP is modulated by G-protein-coupled receptors of the G_s/G_i family and cAMP degradation is regulated by PDEs. The activation of AC and the subsequent increase in intracellular cAMP levels leads to the activation of PKA and the phosphorylation and translocation of HSL to fat droplets, thus activating lipolysis.

As shown in LXFL529L and platelet lysates, caffeine and theophylline weakly inhibit cyclic nucleotide PDEs, and additionally show little selectivity among PDE families. However, there is evidence that very modest (less than 20%) inhibition of crude PDE activity by alkylxanthines resulted in a significant increase in lipolysis [Beavo et al., 2007; Beavo et al., 1970].

For the experiments with adipocytes, the human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell strain was used. Cells were obtained from Prof. Dr. M. Wabitsch (University of Ulm, Germany) [Wabitsch et al., 2001]. SGBS preadipocytes were seeded at a concentration of 5×10^5 cells/petri dishes (in ∅ 92 mm) and cultured until 80 to 90% confluence in 10% FBS-containing DMEM/F12 medium supplemented with biotin, pantothenic acid and penicillin/streptomycin antibiotics. Every 2 to 3 days cells were washed with PBS buffer and the medium was changed (see Material and Methods chapter).

To induce differentiation, confluent preadipocytes were incubated in DMEM/F12 medium containing human transferrin, insulin, cortisol and triiodothyronine, IBMX, Dexamethasone and Rosiglitazone for the first 96 h. Subsequently, cells were further differentiated in serum free DMEM/F12 medium containing human transferrin, insulin, cortisol and triiodothyronine. Every 2 to 3 days the medium was refreshed without washing the cells. After 12 days 65−85% of the preadipocytes were differentiated into mature adipocytes. The number of adipocytes per Petri dish was $1.57 \pm 0.56 \times 10^6$ (mean ± SD, $n = 9$). Small lipid droplets were visible after approximately 1 week (see Figure 5-20).

For PDE activity experiments, cells were tested at different stages of differentiation.

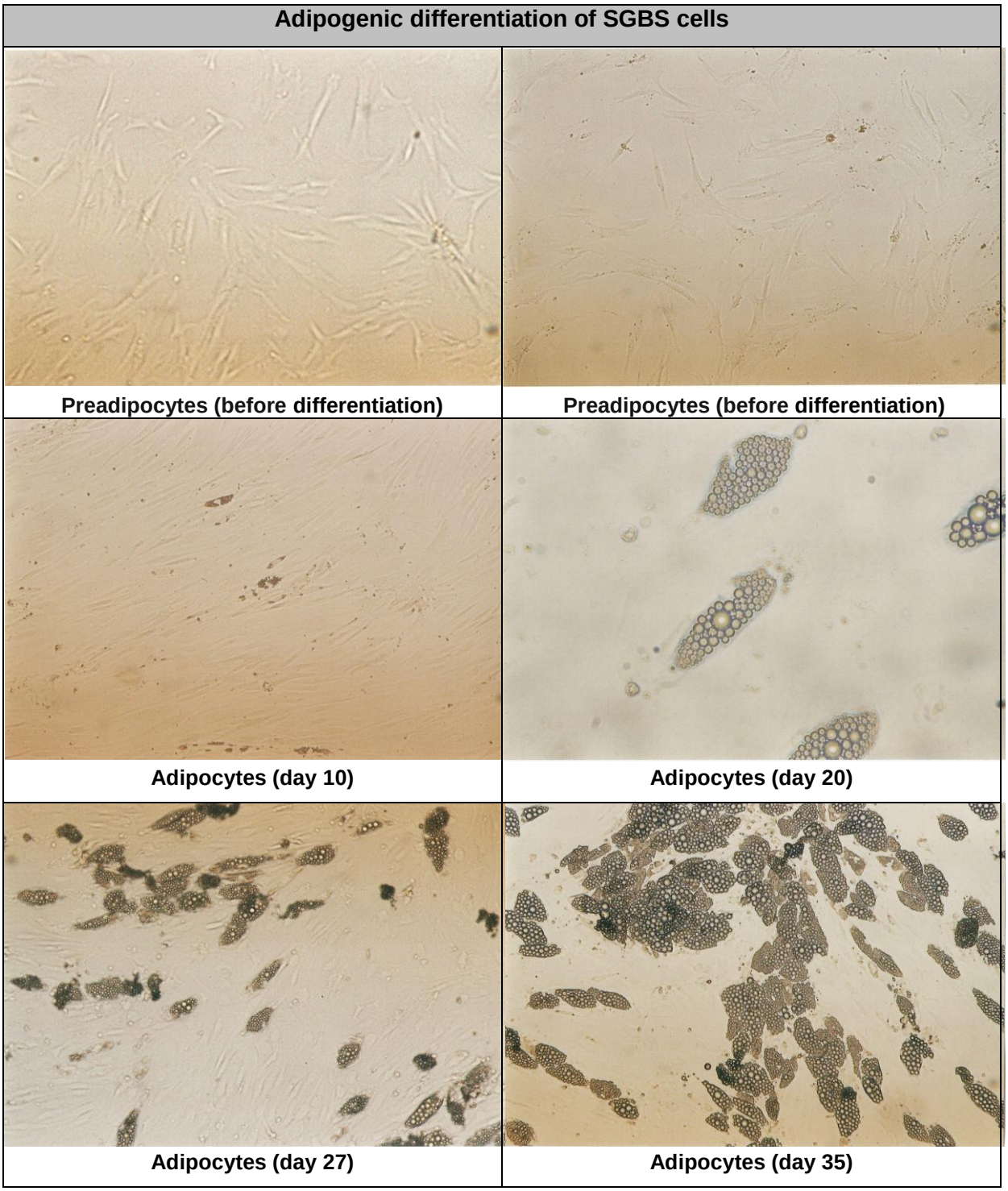


Figure 5-20: Adipogenic differentiation of SGBS cells. Microphotographs of SGBS cells during adipose differentiation cultured acocording to the differentiation protocol [Fischer-Posovszky et al., 2008]. Cells were viewed under phase contrast using a Zeiss Axiovert 25 inverted microscope and images captured using a mounted Canon EOS 33 camera. Intracellular lipid droplets were visible on culture day 7 (magnification x 80). On culture day 20 (magnification x 80), the cells are filled with high amounts of intracellular lipids and after 35 days aprox. 100% of the preadipocytes were differentiated into mature adipocytes (magnification x 25).

Very low levels of PDE activity was found in adipocytes during differentiation < 2 pmol cAMP (^{3}H)/min*mg protein (see Figure 1-22) compared to e.g. ≈ 200 pmol cAMP (^{3}H)/min*mg protein in platelet lysates. It must be noted that elevation of cellular cAMP concentrations is a crucial event in the adipocytes differentiation and that factors increasing cellular cAMP, such as IBMX or Forskolin, strongly accelerate the initiation of the differentiation program. Thus, it was observed that the use of a nonspecific inhibitor of PDEs (IBMX) during the first 4 days of differentiation dramatically affected the cAMP-hydrolysis capacity in SGBS cells. Though the unspecific PDE inhibitor IBMX is removed after day 4 of differentiation, the cAMP hydrolyzing activity in adipocytes, up to day 30, still remains at very low level.

Additionally, IBMX withdrawal or the use of dibutyryl cAMP (a cell permeable cAMP analog which mimics the action of endogenous cAMP) in µM concentrations did not induce adipocyte differentiation.

Likewise, a weak PDE activity was detected in SGBS adipocytes and adipocytes from epididymal fat pads isolated according to protocol of Rodbell [Rodbell, 1964] (see Figure 5-21), that could be significantly inhibited by the PDE3 inhibitor Milrinone.

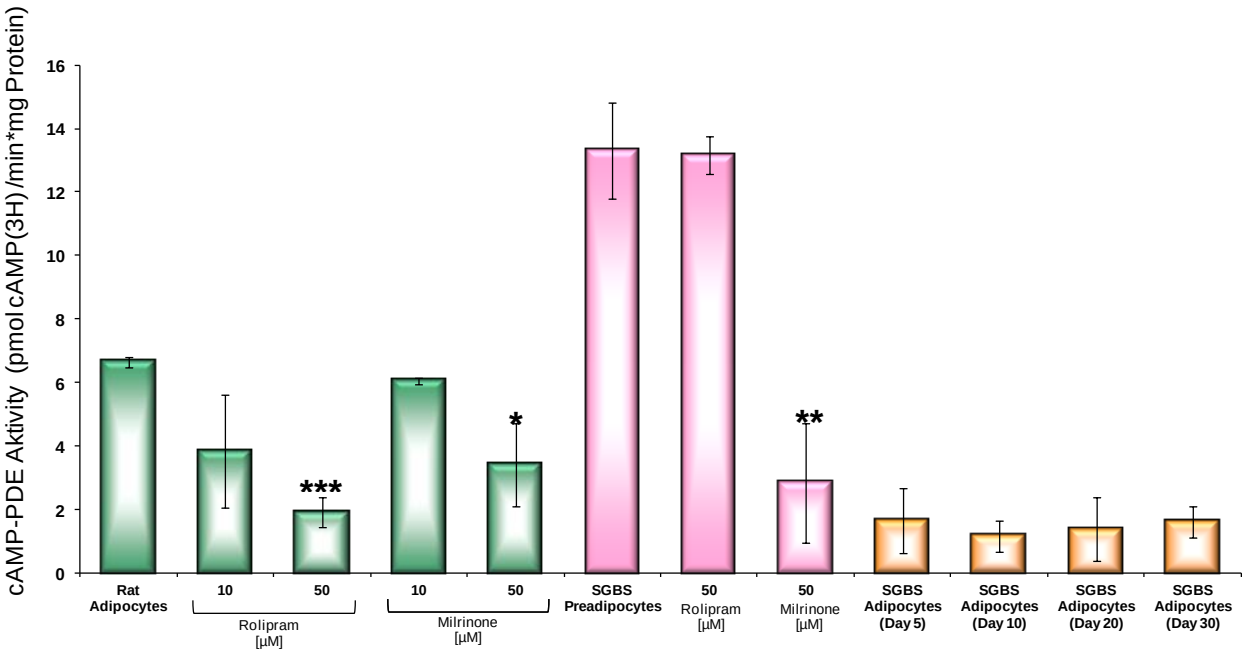


Figure 5-21: cAMP hydrolysis activity of rat adipocytes and SGBS cells. Lysates were directly incubated with Rolipram and Milrinone (10 and 50 µM). Data are expressed as mean ±SD of five independent experiments (triplicates); normalized to protein content. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. untreated cells).

5.2.2 PDE activity *in vivo*

5.2.2.1 Short term study with human volunteers

Ten male and female healthy probands were recruited within the University of Kaiserslautern. The inclusion criteria were as follows: age 20–44 years, no known active ongoing disease

(apparent good health), nonsmoking status (to avoid contributory effects of nicotine or other tobacco alkaloids to caffeine effects or tolerance), average coffee consumers. The exclusion criteria were as follows: treatment with any drug (to avoid any interference with the autonomic nervous system or with the effects of caffeine; this included therapy with sympathomimetic drugs, *α*- or *β*-adrenergic receptor blockers, theophylline, and any antihypertensive therapy), high performance athletes, existing metabolic disorders/diseases and a BMI (in kg/m^2) ≥ 30. From 10 volunteers, 2 left the study left the study for private reasons.

This short term intervention was a seven-week human study divided into five phases (see Scheme 1). Throughout the study, the volunteers were asked to completely refrain from consuming coffee, tea, cola drinks, energy drinks, and other caffeine containing drinks or medicaments. A written list of such drinks was provided to the candidates at the time of screening. Written informed consent was obtained from each volunteer. On October 12th 2010, the short term study was approved by the local Ethics Committee of the Medical Association of Rhineland-Palatinate, Germany.

In this short term study, two different coffees were used. Café Crema coffee consisted in a commercial blend of 100% Arabica coffees from Brazil and Peru. The degree of roasting was 60 SKT for the Brazilian coffee in the Agtron scale (most coffee is roasted between scores of 25 and 75, with commercial scores primarily between 55 and 75) and 64 SKT for the Peru coffee. The degree of roasting of the blend was slightly above 60 SKT, this small difference with the caffeine reduced coffee were negligible. The caffeine reduced coffee was a mixture of Arabica Brazil (75% decaffeinated with 25% non-decaffeinated coffee), the specified degree of roasting for this coffee was 60 SKT.

Table 5-2: Caffeine concentration in coffees of the short term study

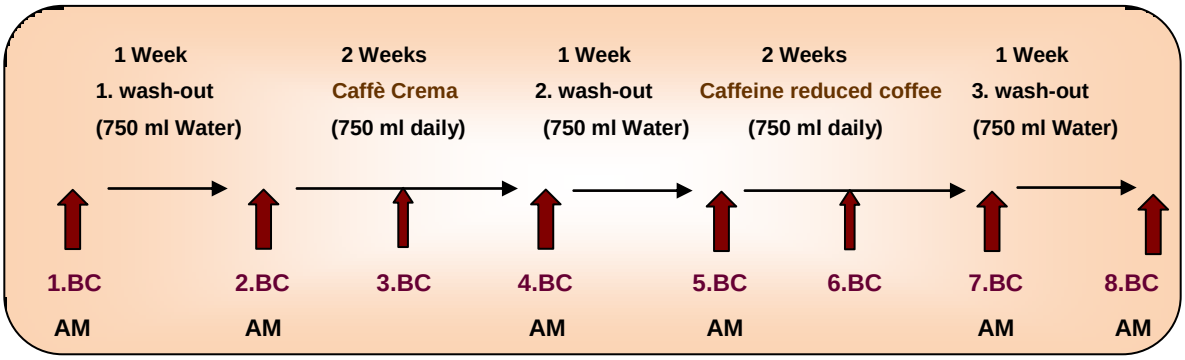
Content	Caffè Crema	Caffeine reduced coffee
Caffeine (% dry matter)	1.23	0.27
Moisture (%)	4.5	3.8

The 7-week intervention study was designed as follows:

- week 1, 1st wash-out
- weeks 2 & 3, coffee uptake (Caffè Crema)
- week 4, 2nd wash-out
- weeks 5 & 6, coffee uptake (caffeine reduced coffee)
- week 7, 3rd wash-out.

Probands daily consumed 750 ml of freshly brewed coffee (8 coffee pods, with/without sugar in three equal portions: morning, noontime, afternoon). In the wash-out phases, the coffee brew was replaced by equal volumes of water (750 ml). Anthropometric measurements were

performed in fasting during the morning at the beginning and at end of each study phase of the study (see Scheme 1). In addition blood sanples were collected each week throughout the study (1.BC to 8. BC).



Scheme 1: Study design of the short term human intervention. Blood samples were collected at 8 time points (red arrows). Caffè Crema coffee or the caffeine reduced coffee were administered alternatively on the first or second intervention phase of the study. BC: Blood collection; AM: Anthropometric measurements.

Additionally, body weight of subjects was assessed by a medical scales (Seca delta 707, Seca, Hamburg, Germany) and body height with a Seca 206 (Seca, Hamburg, Germany) measuring tape. The body composition of subjects was determined by a bioimpedance analyzer (Maltron-BF-906, Maltron International, UK) and skinfold measurements using a SlimGuide skinfold caliper (Creative health products, Michigan, USA) using the 7-folds formula of Jackson Pollock [Jackson et al., 1980].

The PDEs activities of platelets of volunteers were evaluated in the short term study. For this, platelets were isolated from the peripheral venous blood PRP of probands as described in the materials and methods chapter. Results of cAMP hydrolyzing activity in platelets of the 8 volunteers are shown Figure 1-23. The PDE activity significantly increases after the first wash-out phase. During the subsequent two-weeks intervention with coffee (Caffè Crema) a highly significant inhibition of PDE activity could be observed. This effect was even maintained until the next week wash-out phase (2nd wash-out, 5.BC), but returned to the level of the 1st wash-out phase (2. BC) during the two-weeks intervention with caffeine reduced coffee. A further slight increase in PDE activity was observed in the 2 study weeks with caffeine reduced coffee and the last wash-out phase (BC. 6 – 8).

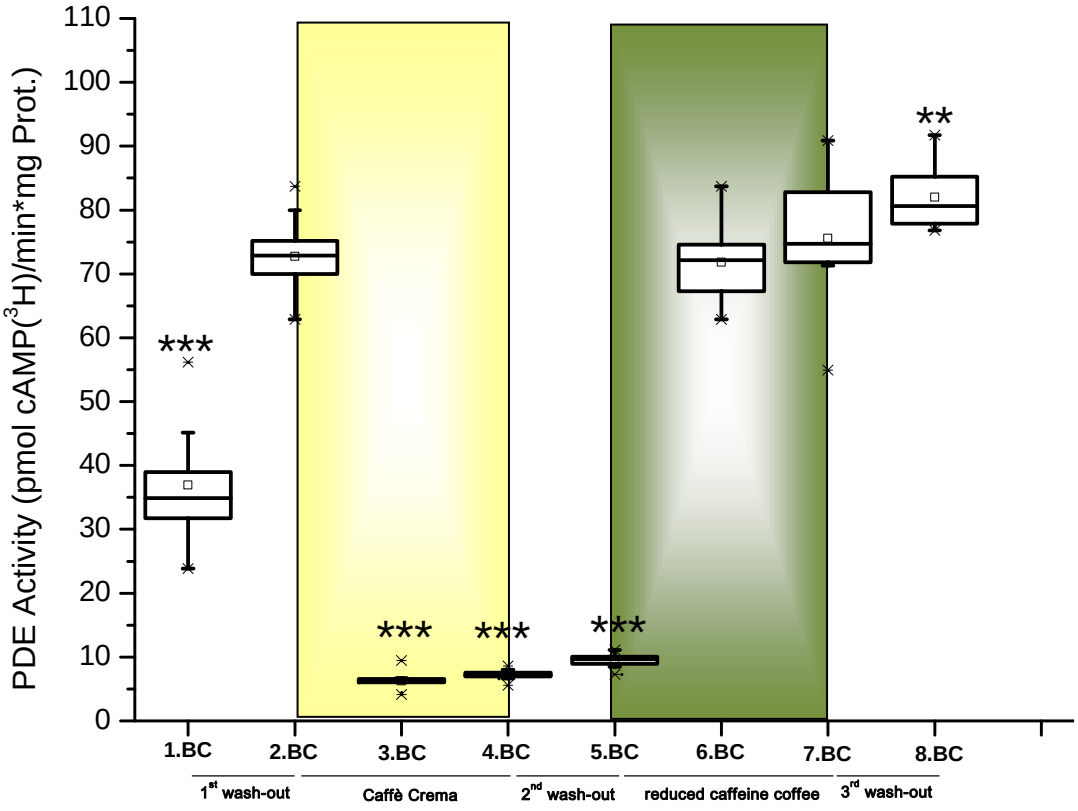


Figure 5-22: Modulation of cAMP-phosphodiesterase activities by coffee consumption in platelets of volunteers during the course of the short term trial. The data (Box diagrams) are normalized to protein content and expressed as PDE activity (cAMP pmol/min x mg protein). Significances were calculated using F-test followed by Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. 1st wash-out).Yellow background: 2 weeks Caffè Crema intervention, green background: 2 weeks caffeine reduced coffee intervention.(n=8)

It is possible that results during the coffee intervention may be attributed to a caffeine concentration-dependent effect on the platelets PDE activity. As mentioned above, caffeine is known as a nonselective inhibitor of phosphodiesterases and the intervention coffees have different concentrations of caffeine: Caffè crema (91.6 mg/pod) and caffeine reduced coffee (20.8 mg/pod). Furthermore, the possibility of the existence of an adaptative response of the platelet PDE to increased levels of intracellular cAMP as previously shown by Huang et al. in transgenic mice [Huang et al., 2002] could not be excluded. Nonetheless, in the present study we were able to show for the first time that moderate consumption of coffee can modulate the activity of platelet phosphodiesterases *in vivo*.

5.2.2.2 Long term study

Subjects

90 healthy volunteers (42 women and 48 men) were recruited within the University of Kaiserslautern, Germany. The inclusion criteria were: age 19–44 years, no known active

ongoing disease (apparent good health), BMI between 19-26 (kg/m^2), nonsmoking status, average daily coffee intake (1 to 3 cups/d).

The exclusion criteria were as follows: treatment with any medicament, smokers, high performance athletes, existing metabolic disorders/diseases, existing irregularity of the menstrual cycle, pregnancy, and BMI (in kg/m^2) ≤ 19 or ≥ 26.

The volunteers were asked to completely refrain from consuming additional caffeinated beverages such as coffee, tea, cola drinks, energy drinks, or other caffeine containing drinks or medicaments, dietary supplements as well as dark chocolate. A written list of products was provided to the candidates at the time of screening. Otherwise, subjects were asked to maintain their usual diet.

Candidates were invited to an information event where detailed information on study design, stage and duration of the study was provided. According to the vote of ethics, subjects underwent a medical examination to ensure that the health status met the predefined criteria. 92 study participants were selected on the basis of the medical examination. Written informed consent was obtained from each volunteer.

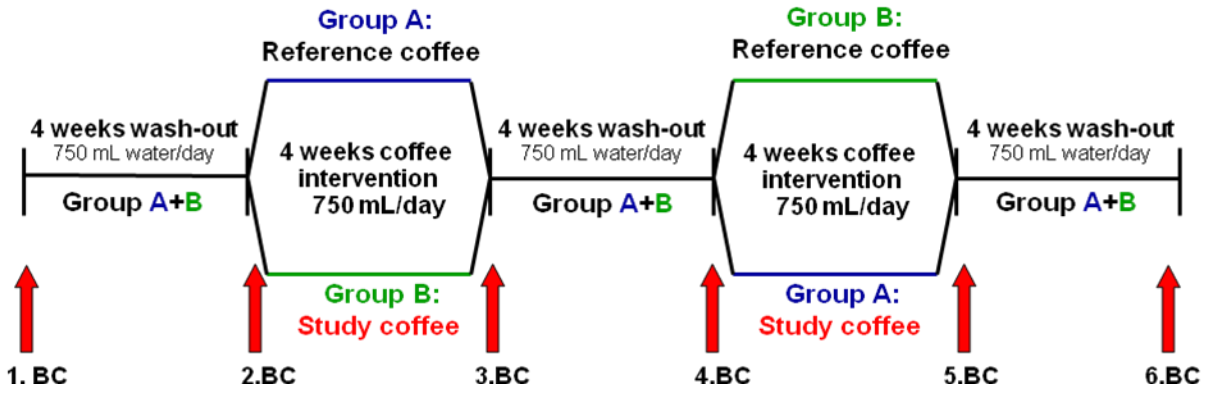
On December 14th 2010, the protocol was approved by the Ethics Committee of the Medical Association of Rhineland-Palatinate, Mainz (Germany). On January 8th 2011 the study was started in the Department of Food Chemistry and Toxicology of the University of Kaiserslautern with 90 participants who were randomly divided into two groups (A and B). From 90 volunteers, 6 did not complete the requirements in the second phase of the trial, which resulted in a total of 84 subjects.

Study design

This was a prospective, double-blind, randomized study with crossover design (see Scheme 2). The principal investigators and the study participants were kept unaware of which coffee group A and B were drinking.

The long term study comprised 5 phases each of 4 weeks duration (see Scheme 2). In the first phase (1st wash-out) both groups consumed at least 750 mL of water distributed through out the day. During the first 4 weeks coffee intervention phase, group A consumed the reference coffee (RC) while the group B consumed the study coffee (SC). After a further four-week "2nd wash-out phase" the RC and SC groups were switched (i.e. group A received SC and group B RC). The study was completed with a third phase of wash-out for 4 weeks.

At the beginning of each phase 40 mL of peripheral venous blood was collected from each volunteer by medical personnel from the Westpfalz-Klinikum, Kaiserslautern, as approved by the ethics committee. Additionally 50 mL urine was collected in a total of 6 sampling appointments.



Scheme 2: Crossover design used in the long term study. Blood and urine collection (BC) time points are indicated by red arrows. Reference coffee or the study coffee were provided alternatively on the first or second intervention phase of the study. Blood samples were collected before and after each phase. The 2.BC represents the baseline.

Coffee administration

In the four-week intervention phase, subjects consumed a total of 750 mL coffee (study or reference coffee) throughout the day (6 coffee cups/day, 125 mL each one). Subjects brewed their coffee themselves. Therefore, study participants were provided with an automatic pod coffee machine (from Tchibo Company). They also received the study or reference coffee pods (for 28 days) and instructions for preparing the coffee beverage. For each portion a coffee pod (approx. 7.2 g) was brewed with 130 mL of tap water. Finally, approx. 125 ml of coffee brew was consumed (without milk and optional addition of sugar). In the wash-out phases coffee was replaced by equal volumes of water.

The optimized study coffee (SC, 3012, 60 SKT) was produced by selection of four individual batches from eight industrially roasted coffees. Furthermore, the reference coffee (RC, 2601, 74SKT) was established from a blend of equal representation the five top-selling ground coffees of the German market. It is worth noting that the NMP concentration in the SC was more than twice as RC. Composition of the coffees is presented in Table 5-3.

Table 5-3 Concentration of selected constituents of coffees of the long term study (4thInterim report/Juli 2011/BMBF: Slim Down Coffee)

Content	Coffee powder / Coffee brews	
	Reference coffee (RC)	Study coffee (SC)
Caffeine (mg/L)	12.4 / 743.7	13.1 / 769.7
CQA (mg/L)	19.3/ 1185.8	10.2 / 600.5
NMP (mg/L)	0.4 / 23.6	1.5 / 72.8
Trigonelline (mg/L)	6.3 / 379.9	4.1 / 193.6

Volunteers filled out daily food intake records for one week prior each blood collection time point. Dietary protocols were analyzed using the PRODI® 5 Expert software package (Nutri-Science GmbH, Freiburg, Germany).

Additionally, at each visit, body weight of subjects was assessed by a medical scales (Seca delta 707, Seca, Hamburg, Germany) and body height with a Seca 206 (Seca, Hamburg, Germany) measuring tape. The body composition of subjects was determined by a bioimpedance analyzer BIA 101 (SMT medical GmbH, Würzburg) and skinfold measurements in a subpopulation (10 volunteers per group) were performed using a SlimGuide skinfold caliper (Creative health products, Michigan, USA) using the 7-folds formula of Jackson Pollock [Jackson et al., 1980].

The hydrolytic activity of PDEs in platelet lysates of volunteers from the long term study was also estimated by the Pöch method [Poch, 1971] (see Material and methods chapter). Platelets were isolated from EDTA-anticoagulated blood as previously described and then lysed.

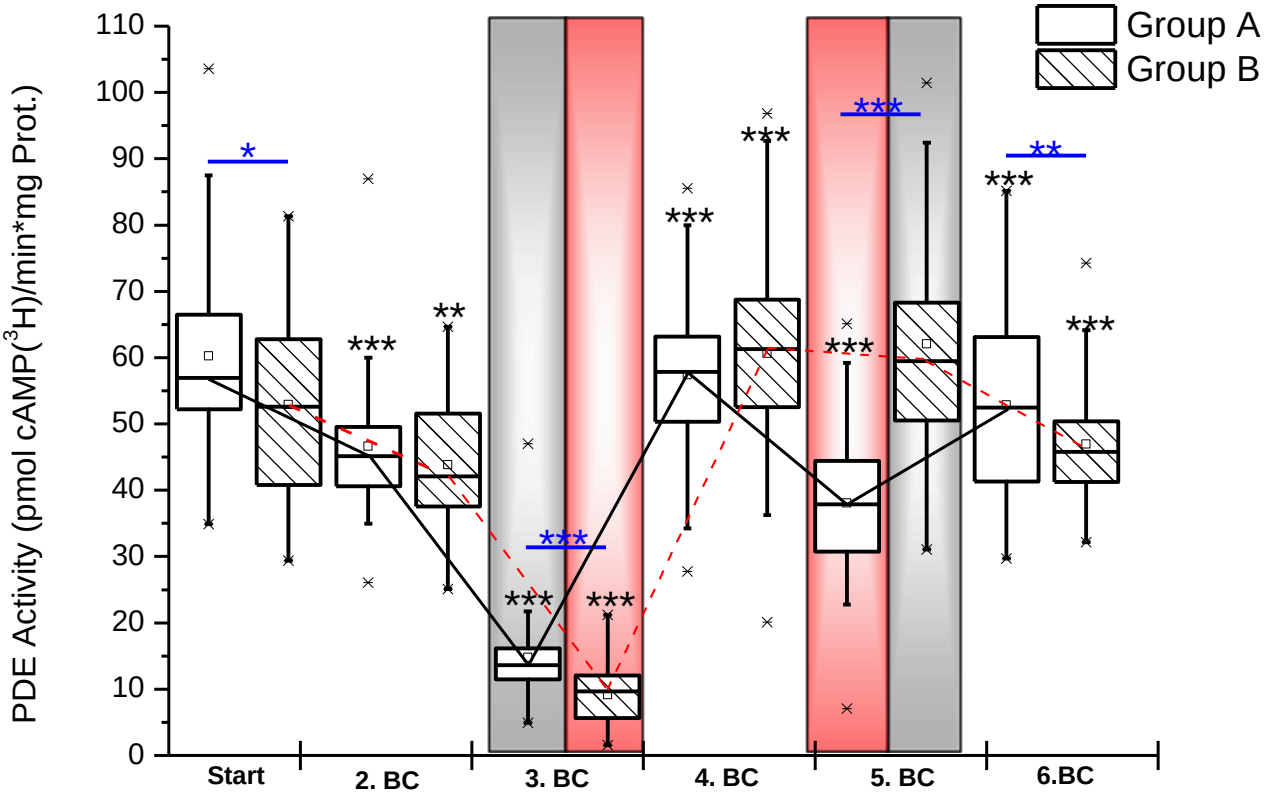


Figure 5-23: Modulation of cAMP-phosphodiesterase activities by coffee consumption in platelets of volunteers during the course of the long term trial. The data (Box diagrams) are normalized to protein content and expressed as PDE activity (cAMP pmol/min x mg protein). The Anderson Darling test was used for the analysis of normal distribution. Significances between time points were calculated using two-sided, paired Wilcoxon test (*p<0.05; **p<0.01; ***p<0.001 vs. the preceding phase) and the analysis of differences between groups using the two-sided, unpaired Mann-Whitney test (*p<0.05; **p<0.01; ***p<0.001 A vs.B). Silver background: 4 weeks reference coffee intervention, red background: 4 weeks study coffee consumption (n=84). Solid black trendline: group A, dash red trendline: group B. In blue: significances between groups.

In general, statistically significant differences were observed between the standard treatment (reference coffee) and the study coffee, where the study coffee inhibited more effectively the PDE activity in the platelets of probands. These results were noteworthy because in the *in vitro* experiments NMP showed no effect on phosphodiesterases activity, but one of the most important differences in the composition of the coffees lies in the NMP concentration (SC was more than twice as RC, see table 5-3). Remarkably, as indicated in Figure 5-23, the different responses between the first and the second exposure to coffee intervention, where the first showed a strong loss of the PDE activity in the platelet lysates of the participants while the second coffee phase showed no effect (for the group B). Coffee withdrawals (wash-out phases) were similar over the study.

It should be noted that the variability of responses of volunteers to coffee consumption may be due to genetic factors [Renda et al., 2012], making it plausible that the obtained results in the second coffee phase exposure might be related to an adaptive response of the PDEs to the cAMP levels in the platelets as discussed earlier. There is evidence in the literature demonstrating that elevating intracellular cAMP levels can provoke increases in PDEs that metabolize the cyclic nucleotide, as dampening effect [Huang et al., 2002]. Therefore it was of interest to know the intracellular cAMP concentrations in platelets after coffee consumption. The data will be presented and discussed in the following chapter.

5.3 Determination of cellular cAMP (3',5'-cyclic adenosine monophosphate) levels

cAMP is one of the most important second messengers, mediating diverse physiological responses of neurotransmitters, hormones, and drugs. Intracellular concentration of cAMP is tightly regulated by two membrane bound enzymes, AC and PDE. AC activity promotes the synthesis of cAMP from ATP while PDE degrades cAMP to AMP.

The measurement of intracellular cAMP is thus an ideal method for measuring the effect of test compounds on GPCR-mediated AC activation or inhibition that should be accompanied by determining effects on the cAMP hydrolytic activity of PDEs.

The alkylxanthines caffeine and theophylline are the prototypical antagonists of adenosine receptors (ARs) which are linked to GPCRs, and their stimulant actions are produced primarily through blocking the depressant actions of the adenosine through the A_1 and A_{2A} ARs. There is growing evidence that most pharmacological effects of xanthines result from antagonism of AR. Additionally, the activity of PDE is slowed by methyl xanthines, caffeine, or theophylline; thus increasing the intracellular concentration of cAMP. Varani et al. [Varani et al., 2000] have shown that treatment with caffeine for 2 weeks with 400 mg/d or 600 mg/d for 1 week resulted in a significant increase (upregulation) of adenosine A_{2A} binding sites, a rise in cAMP accumulation, an increase of antiaggregatory effects, and a decrease in

calcium levels elicited by the A_{2A} receptor agonist 2-hexynyl-5´-N-ethylcarboxamidoadenosine (HE-NECA).

Measurement of intracellular cAMP concentration was carried out using the PerkinElmer LANCE® Kit (see Principles of the assay in the Material and methods chapter). This kit is intended for the quantitative determination of cAMP in cell culture and cell samples [PerkinElmer and Inc., 2007].

To study the effects of coffee and its components on the intracellular concentrations of cAMP, the assay was performed *in vitro* using HEK293 and SGBS cells (at different stages of differentiation) and *in vivo* using platelets from the short term and the long term studies.

5.3.1 cAMP *in vitro* experiments

Using Forskolin (an AC activator) dose-response curves were generated in order to establish the optimal cell number per well. The optimal cell number was the one for which the Forskolin dose-response curves fitted most of the linear region of a cAMP standard curve (see Figure 5-24). For this purpose HEK-293 (cell model suggested by the assay producer, and therefore taken as reference for the experiments in this work) and SGBS cells (from day 1 to 20 after differentiation) were cultured, harvested and prepared for the assay as indicated in the material and methods chapter. A “no cell” control and a cAMP standard curve were always run in parallel. To determine the half maximal effective concentration (EC_{50}), the Forskolin signal inhibition curve was transformed to a cAMP production curve, using the cAMP standard curve that was measured in parallel. Cells were incubated parallel with IBMX to prevent enzymatic degradation of cAMP by PDEs.

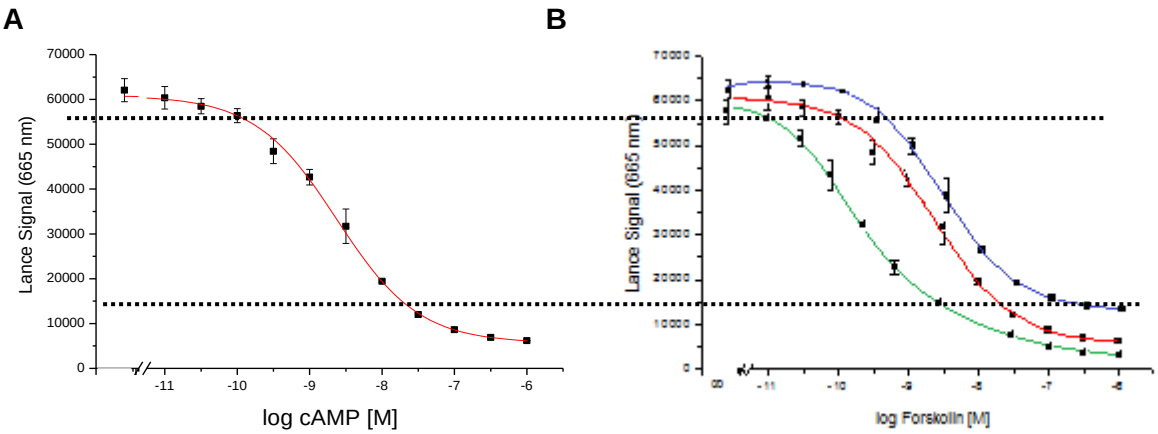


Figure 5-24: Determination of the optimal HEK-293 cell number. (A) Typical LANCE cAMP standard curve, (B) Forskolin dose-response curves for HEK-293 cells; blue curve with 1000 cells/well, red 3000 cells/well and green 6000 cells/well. EC_{50} for Forskolin in 3000 cells/well = 2.25 ± 0.7 µM.

The obtained forskolin dose-response with 3000 HEK293 cells/well provides a response that falls within the linear region of the cAMP curve and is in good accordance with data presented in the assay protocol. Similar experiments were conducted with SGBS cells at different time points of the adipose differentiation process. The Forskolin dose-responses for 6000 cells/well preadipocytes and adipocytes in the 17th day of differentiation are shown in the Figure 5-25.

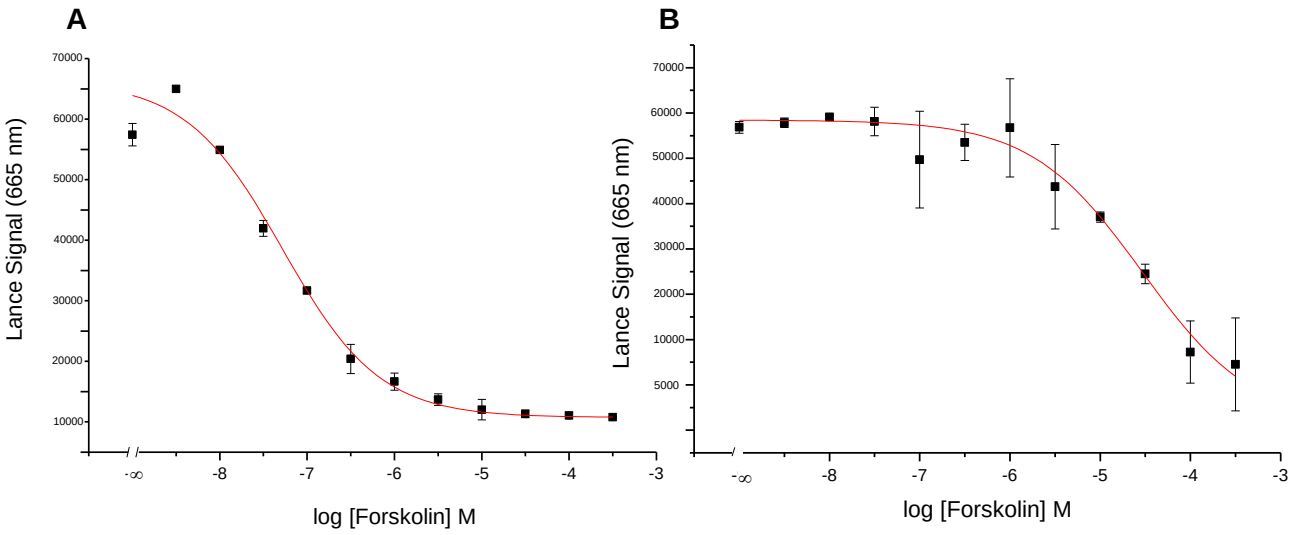


Figure 5-25: Forskolin dose-response of SGBS preadipocytes (A) and adipocytes at the 17th day of differentiation (B). 6000 cells/well were stimulated with various concentrations of Forskolin (0 – 0.1mM) for 1h in a 96 half-Area well plates (n=3).

Factors that increase cellular cAMP, such as IBMX or Forskolin, lead to the initiation of the differentiation program. There is some evidence that cAMP concentration in adipocytes is pivotal during the early stages of adipocyte differentiation [Petersen et al., 2008]. As shown observed in Figure 5-25, the Forskolin dose-responses for the SGBS cells at different time points of differentiation are diverse. Preadipocytes have an EC_{50} of 0.063 ± 0.01 µM while adipocytes on day 17 of differentiation have an EC_{50} of 19.9 ± 0.2 µM which is 316 times higher. It would mean that the stimulatory capacity of the potent activator of AC, Forskolin, may exert less stimulatory effect on adipocytes in advanced stages of differentiation.

These data show that the SGBS cell system is not stable, thus it was necessary to estimate the reproducibility of the assay at different stages of differentiation before performing the experiments with coffee and coffee compounds. The results showed that adipocytes on day 17th of differentiation showed similar Forskolin dose-response curves which EC_{50} between 10.7 - 20.1 µM (n=7).

It is well know that the administration of caffeine to fasted humans also causes an increase in FFA mobilization. It has been proposed that the mechanism of action of caffeine for the increased lipolysis is blockage of adenosine receptors. It has also been shown that it takes

much more caffeine to cause fat mobilization *in vitro* than *in vivo* [Winder, 1986], which suggests that there may be an active metabolite working with caffeine to bring about the increase in lipolysis. There is also evidence that the caffeine metabolite paraxanthine might play a role in increased lipolysis after caffeine administration to humans [Hetzler et al., 1990]. The effect of some coffee compounds like caffeine, NMP, chlorogenic acid and trigonellien on the cAMP concentration in the SGBS cell model at day 17th of differentiation was investigated. For each compound the signal inhibition curve was transformed to a cAMP production curve, using the cAMP standard curve that was run in parallel in the same plate. Additionally, cells were incubated parallel with IBMX to prevent enzymatic degradation of cAMP.

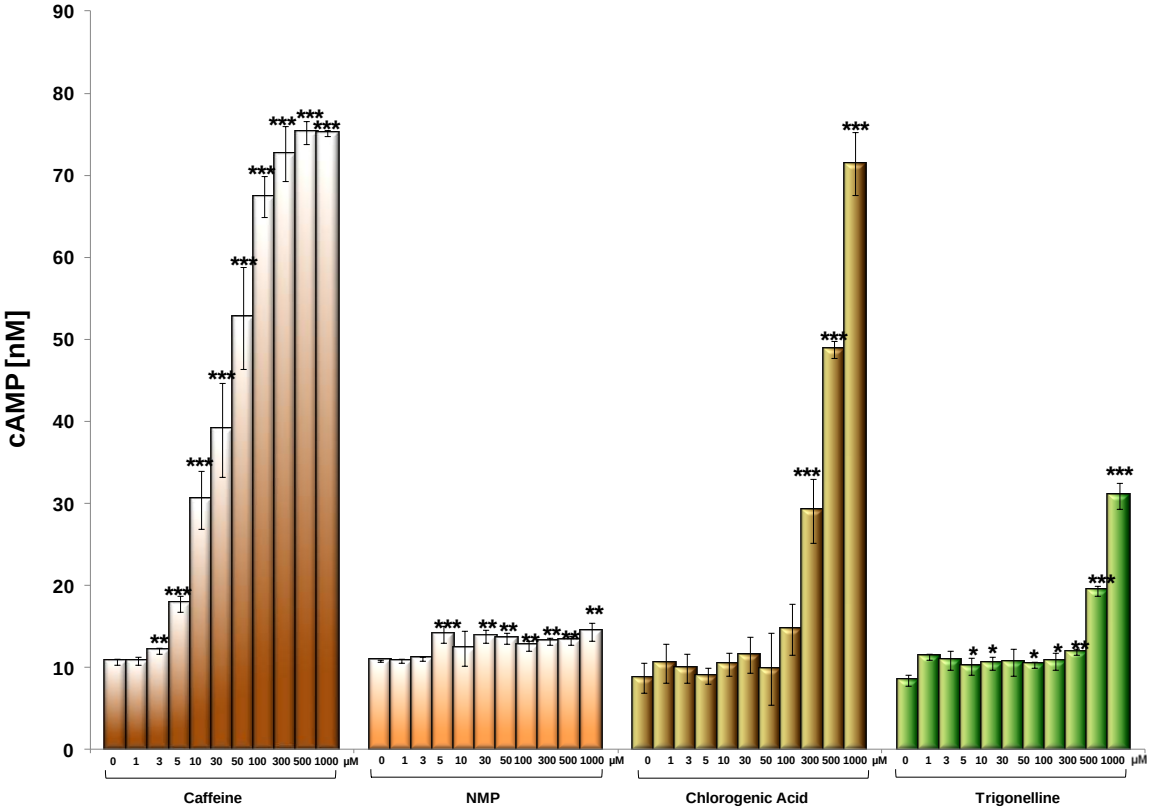


Figure 5-26: Modulation of the cAMP concentration in SGBS cells by caffeine, NMP, chlorogenic acid and trigonelline in concentrations between 1 µM and 1 mM. Cells were incubated with IBMX, Forskolin (EC_{50} concentration) and the respective substance in 96 half-area plate. Data are expressed as mean ±SD of five independent experiments (triplicates) and normalized to protein content. Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. untreated cells).

As shown in Figure 5-26, caffeine significantly increased cAMP concentration in SGBS adipocytes at concentrations ≥ 3 µM. A concentration of 6.5 µM caffeine is needed for doubling the initial cAMP content in the SGBS cells. Surprisingly, other substances present in coffee such as chlorogenic acid and trigonelline were also able to increase the cAMP

concentrations in the model with doubling effective concentrations of 0.15 and 0.4 mM respectively.

On the other hand, pyrazines (data not shown) and NMP did not show any effect up to 1mM. It is also important to note that the determination of the effect of coffee extracts was not possible due to interferences caused by the color of the extracts with the fluorescent signal of the immunoassay emitting at 665 nm.

In vitro experiments on the lipolysis in SGBS cells according to the protocol of *Hellmer et al.* and Lundin *et al*. [Hellmer et al., 1989; Lundin et al., 1989] were not successful because the luciferase enzyme was inactivated by the used microplate, generating a higher decay rate in the control experiment.

5.3.1.1 cAMP platelet concentrations in the short term study

cAMP concentration in platelets of participants in the short term study was also evaluated. As aforementioned, platelets were isolated of probands and data were normalized to protein concentrations. cAMP content is given in (cAMP nM per mg protein).

After the first wash-out phase a decrease of cAMP level in platelets was observed (see Figure 5-27). During the subsequent intervention with coffee the cAMP levels increased. A further increase was observed after the 2nd wash-out phase (4th week), which is in line with data from Borea et al. describing similar increases in cAMP concentrations during caffeine withdrawal [Borea et al., 1999]. After the next 1 week coffee intervention (6th BC), with caffeine reduced coffee, the cAMP levels decreased almost to the starting point (1st BC) and a week later (7. BC) increased again and remained at a similar level over the last wash-out period to the end of the study (8.BC).

As previously mentioned, intracellular concentration of cAMP is tightly regulated by two enzymes, AC and PDE. AC activity promotes the synthesis of cAMP from ATP while PDE degrades cAMP to AMP. Even though concentrations of cAMP in the platelets of subjects correlate with the PDE activities until the 6.BC (see), the observed phenomenon in the last two blood sampling points might be explained with the AC role in the intacellular cAMP synthesis. AC can be activated or inhibited by G proteins, which are coupled to membrane receptors.

Platelet maturation and development involve the expression of many receptors on the platelet cell surface. Of particular physiological relevance to platelet cAMP concentration are the adenosine receptors (A_{2A} and A_{2B}) and the prostacyclin receptors, mainly coupled to stimulatory G proteins; the P2Y nucleotide ADP receptors ($P2Y_{12}$ and $P2Y_1$), and thrombin receptors (PAR1), coupled to inhibitory G proteins [Yang et al., 2010b].

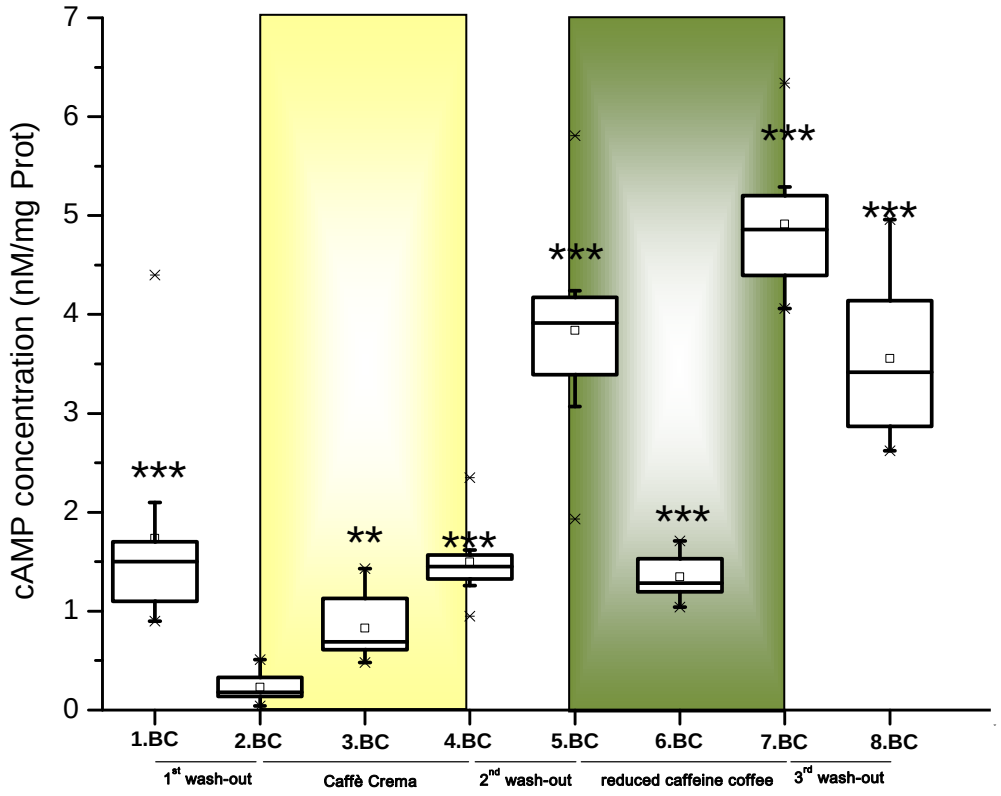


Figure 5-27: Modulation of cAMP concentration by coffee consumption in platelets of volunteers during the course of the trial. The data (Box diagrams) are normalized to protein content and expressed as cAMP concentration (nM/mg protein). Significances were calculated using F-test followed by Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. 1st wash-out). Yellow background: 2 weeks Caffè Crema intervention, green background: 2 weeks caffeine reduced coffee intervention (n=8).

There is only evidence about the effect of caffeine on adenosine receptors. Varani *et al*. [Varani et al., 2000] published that caffeine consumption (600 mg/d for 1 week or 400 mg/d for 2 weeks), upregulated platelet adenosine A_{2A} receptors, which was highly correlated with antiaggregatory effects, a rise in cAMP concentration and a decrease in intracellular calcium levels. In fact, the upregulation of adenosine A_{2A} receptors caused by intake of caffeine could be interpreted to indicate that endogenous adenosine has a tonic influence on human platelets, and the presence of the antagonist is counterbalanced by the upregulation of A_{2A} receptors [Varani et al., 2000].

It is important to mention that, although human platelets are anucleate fragments of megakaryocytes, they retain cytoplasmic mRNA that could be translated to proteins [Gnatenko et al., 2003; Williams et al., 2010].

5.3.1.2 cAMP platelet concentrations in the long term study

In order to evaluate the long-term effects of coffee consumption on cAMP, platelets of the participants in the long term study were isolated and analyzed using a time-resolved fluorescence immunoassay [PerkinElmer and Inc., 2007] as described in the Material and methods section. Results of the cAMP platelet concentration in the long term study are shown in the Figure 5-28.

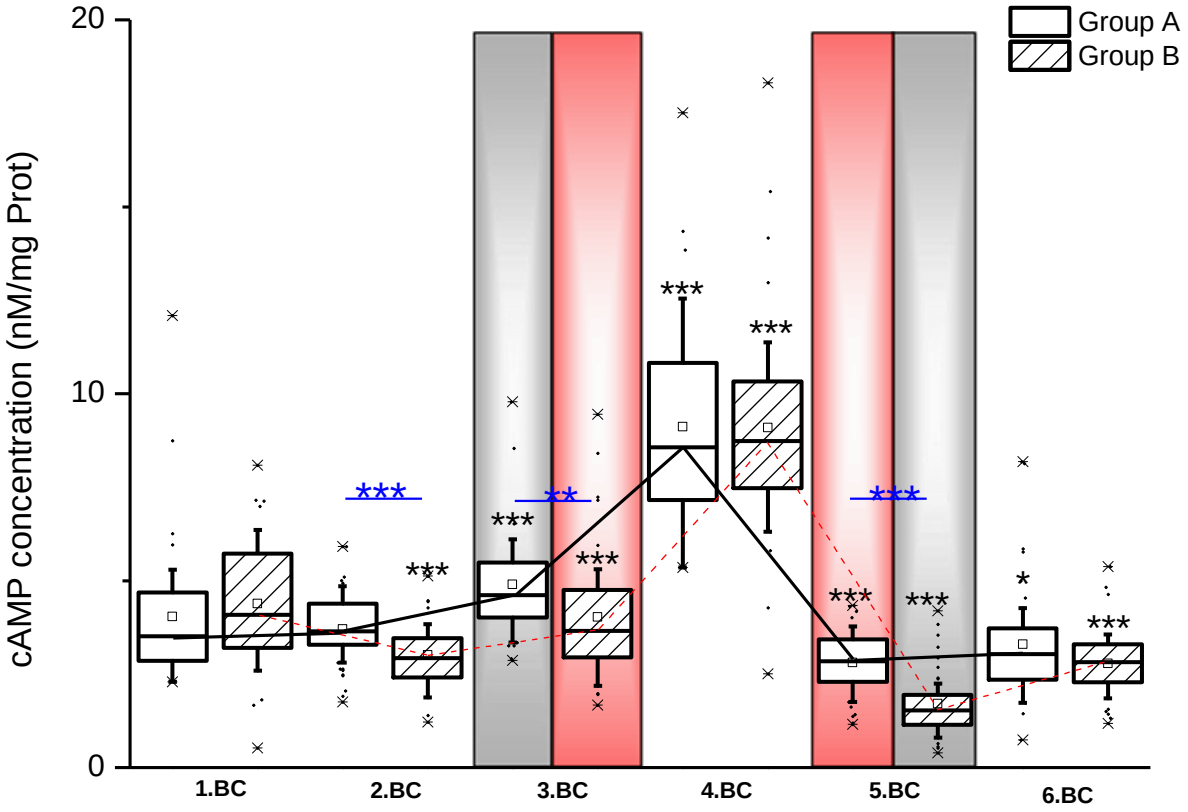


Figure 5-28: Modulation of the cAMP concentrations in platelets of volunteers during the course of the long term trial. The data (Box diagrams) are normalized to protein content and expressed as cAMP concentration (nM/mg protein). The Anderson Darling test was used for the analysis of normal distribution Significances between time points were calculated using two-sided, paired Wilcoxon test (*p<0.05; **p<0.01; ***p<0.001 vs. the preceding phase) and the analysis of differences between groups using the two-sided, unpaired Mann-Whitney test (*p<0.05; **p<0.01; ***p<0.001 A vs.B). Silver background: 4 weeks reference coffee intervention, red background: 4 weeks study coffee intervention (n=84). Solid black trendline: group A, dash red trendline: group B. In blue: significances between groups.

No changes in the cAMP concentrations of platelets were observed between the 1.BC and the 2.BC time points for the group A, while group B experimented first a decrease of the cAMP concentrations during the first wash-out phase followed by a significant increase in the first coffee phase. In common a significant cAMP increase after the first coffee phase (coffee withdrawal, 4.BC) for both groups was shown, despite that the activity of PDE increased considerably in this same phase of the study. This observation may be due to an effect mediated by platelet surface receptors as previously described in chapter No.2.5.24 . Then from 5.BC (second coffee intervention) the values trend to return to the initial cAMP

concentrations, phenomenon that correlates with the activity of enzymes phosphodiesterases in 5.BC. According to the PDE results, the group B which showed more PDE activity in this intervention point showed a stronger decrease of cAMP levels as group A.

It is important to emphasize, that despite both groups (A and B) behaved similarly over the course of the study, but significant differences during the coffee intervention and the first wash-out phase were observed. Group B showed a greater response to coffee intervention, regardless of whether it was SC or RC, suggesting a greater sensitivity of this group to the coffee. Variability in the physiologic responses to coffee is well documented, with subjects totally indifferent to even a large amount of coffee ingestion, and subjects deriving e.g. profound cardiovascular and neuropsychological consequences to coffee drinking. Renda et al. suggested that part of this variability may be explained by genetic factors [Renda et al., 2012].

Comparing the long and short-term effects of coffee consumption on the cAMP levels in platelets of volunteers from both studies, it can be stated that the first phase of coffee withdrawal led to an increase in the cAMP concentrations, as also previously described by Varani et al. [Varani et al., 2000; Varani et al., 1999]; this effect trends to be regulated rapidly by compensatory mechanisms, such as increased activity of PDEs, so that no apparent differences were observed within the long term study.

5.4 Adenosine

Adenosine is a key endogenous molecule that regulates tissue function by activating four G-protein-coupled adenosine receptors: A_1, A2A, A_{2B} and A_3. Since many of the functions of adenosine are homeostatic and protective in nature, and in principle, equalize local energy requirements with energy supply. The organ- and cytoprotective functions of adenosine also include stimulation of angiogenesis and inhibition of inflammatory reactions at the site of injury [Spychala, 2000].

Adenosine quantification in blood was performed by HPLC-ESI-MS/MS as described previously by Doležalová *et al.* [Dolezalova et al., 2005] with some modifications (see Material and Methods chapter). As described by these authors, concentrations of adenosine in deproteinized blood samples wer not stable. Our interassay (between-day) variation was established by replicate analyses of the same sample on three separate days. For this purpose, deproteinized blood samples were extracted within 6 h of collection and analyzed on LC-MS/MS immediately and after storage at −80°C for 2, 4 and 6 days.

Intra- (within-day) and inter-assay (between-day) experiments revealed that adenosine concentrations in deproteinized blood samples were not stable at RT, with a significant elevation of 20 ± 3.6% for the control, same sample after solid-phase extraction (SPE) and analyzed immediately on LC-MS/MS (see Figure 5-29). After solid-phase extraction of the

deproteinated samples elevated adenosine levels in samples stored at -80 °C for longer than 2 days (5 ± 4.5%), 4 days (13 ± 2.2%) and 6 days (27 ± 1.1%) were observed. Adenosine concentrations remained constant in the extracts stored at room temperature or -80 °C for up 24h. In the present work, deproteinized blood samples were extracted within 4h of collection and analyzed on LC-MS/MS immediately in order to avoid such quantification artefacts.

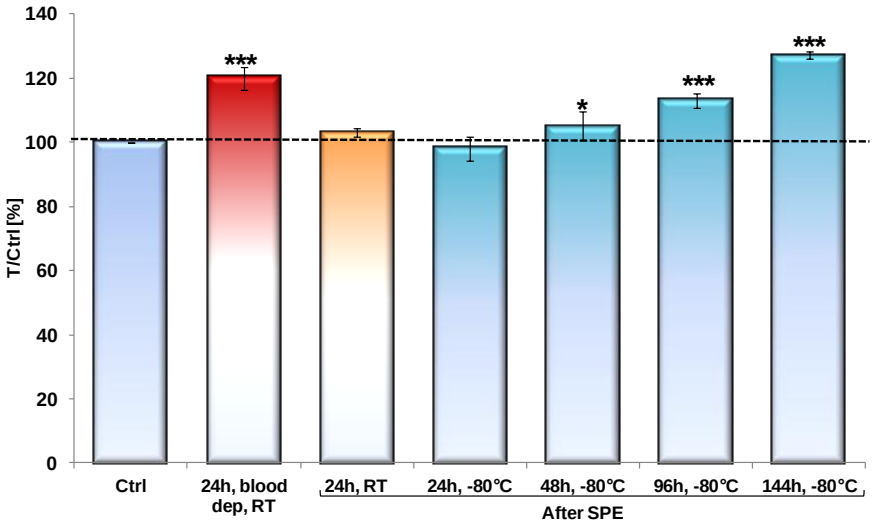


Figure 5-29: Intra-assay (within-day) and inter-assay (between-day) variations of the adenosine quantification in plasma samples by HPLC-ESI-MS/MS as described in the material and methods chapter. % of variation of the method was established by replicate of of three independent experiments (triplicates) vs. a deproteinized control blood sample wich was extracted (SPE) and immediately analyzed on LC-MS/MS. Samples were also analyzed after 1, 2, 4 and 6 days of storage at -80 ° C. Data are expressed as % of the control (mean ±SD). Significances were calculated using Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. control).

5.4.1 Effect of coffee consumption on adenosine (ADO) plasma concentrations in the platelets of participants in the short term study.

In 1997 Conlay *et al*. [Conlay et al., 1997] reported that caffeine, particularly after prolonged administration, increases dramatically the levels of adenosine in plasma in rats. They found an increase in adenosine concentration after antagonist administration and its reduction after antagonist withdrawal, suggesting a receptor-mediated regulation of the plasma adenosine (ADO) concentration. As suggested by Fredholm *et al.* [Fredholm et al., 1999], this finding clearly needs to be reproduced, especially in humans.

Taking into account the stimulatory effect of adenosine on its receptors in platelets, and understanding that this effect may affect the homeostasis of intracellular cAMP, the effect of coffee consumption on ADO plasma concentrations were determined following the protocol previously described in the material and methods chapter. For this purpose EDTA-

anticoagulated blood samples were deproteinized, extracted and analyzed immediately after collection.

Under normal physiological conditions, human plasma concentrations of ADO in the radial artery were 12.0 ± 0.9 nM, in the median cubital vein (17.7 ± 6.5 nM) and in the internal jugular vein (14.0 ± 2.8 nM) [Reid et al., 1991; Saito et al., 1999]. Extracellular concentrations of ADO are directly linked with the local energy balance. Once the balance between energy production (ie. ATP production) and its consumption is disturbed, bidirectional ADO transporters pump ADO from the cell. In fact, the level of ADO can increase dramatically to 10 µM or more following ischemia [Andine et al., 1990; Gunnar, 2010].

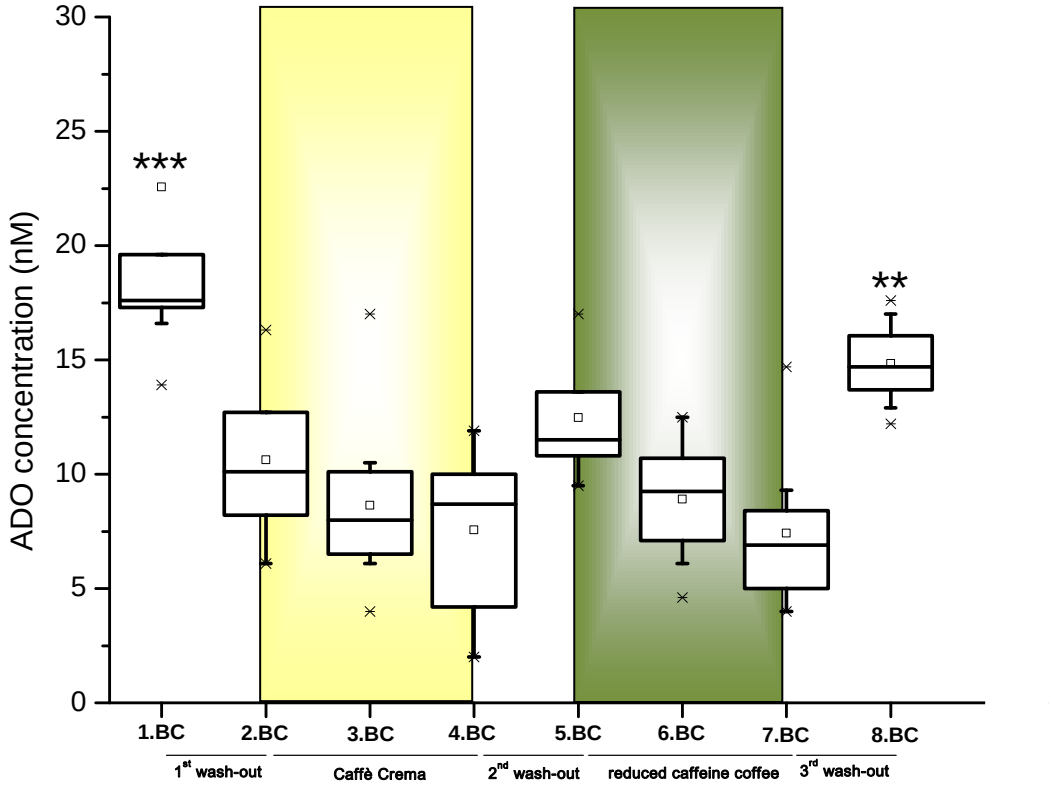


Figure 5-30: Modulation of adenosine (ADO) plasma concentrations by coffee consumption of volunteers during the course of the short term trial. The data (Box diagrams) are expressed as ADO concentration (nM). Significances were calculated using F-test followed by Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. 1st wash-out).Yellow background: 2 weeks Caffè Crema intervention, green background: 2 weeks caffeine reduced coffee intervention (n=8).

In contrast to the findings of Conlay *et al*., adenosine concentrations in plasma of the probands, showed no considerable changes during the study. It should be noted that in the first wash-out (2.BC) adenosine concentrations in plasma decreased approx. 50% when compared to baseline (1. BC), this effect correlates with the decrease in the concentration of cAMP in platelets at the same point. During both coffee interventions, values tended to

decrease compared with the previous phase wash-out, however, these changes were not significant. Additionally, no changes were observed between the responses to both coffees.
This observation could be supported by Gunnar, who stated that adenosine is subject of a quick and effective neutralization by phosphorylation (adenosine kinase) or deamination (adenosine deaminase) to keep the concentration of adenosine within a well balanced homeostasis [Gunnar, 2010; Spychala, 2000].
In order to determine whether changes in adenosine concentrations are detectable in short time period after caffeine consumption, as described by Conlay et al. [Conlay et al., 1997], sampling was also carried out at 0, 1 and 2 hours after coffee consumption during the first phase of intervention. Results are shown in Figure 5-31.

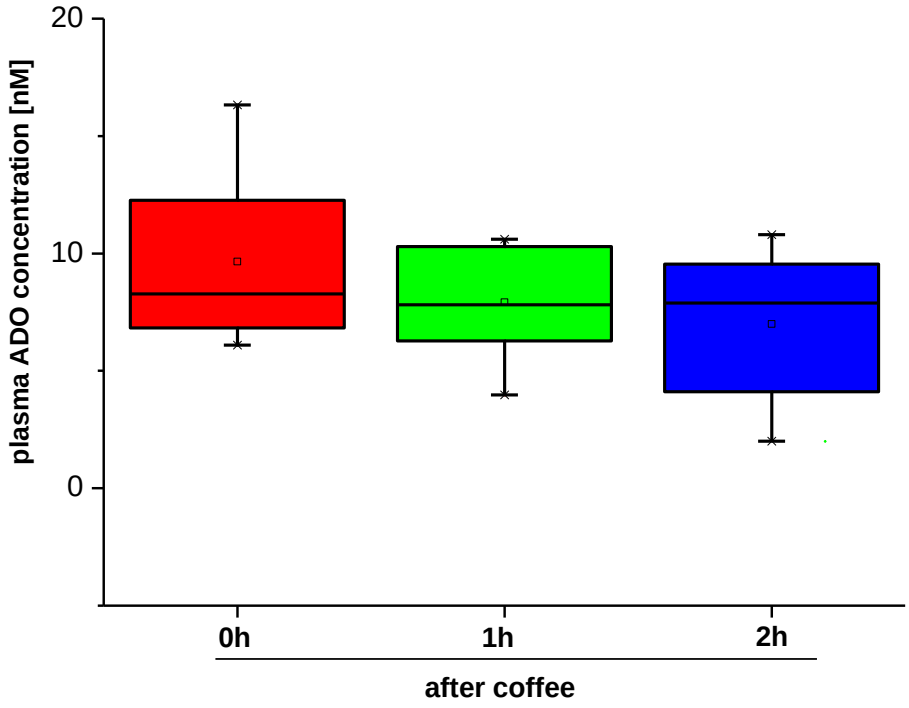


Figure 5-31: Modulation of adenosine plasma concentrations after (0, 1 and 2h) coffee consumption. Data (Box diagrams) are expressed as ADO concentration (nM). Significances were calculated using ANOVA followed by Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. 0h). (n=8).

Adenosine plasma concentrations of the participants of the short term study after 0, 1 and 2h showed no significantly changes. These results shown that short-term adenosine homeostasis is not affected by coffee consumption by the used amounts in this work.

5.4.2 Plasma concentrations of ADO in the long term study

Due to the intricate work methodology, the long-term effect of coffee consumption on plasma concentrations of adenosine was estimated in a randomly selected subpopulation of 19 participants (11 group A and 8 group B) from the long term study. As aforementioned the

extracellular concentrations of this purine nucleoside are well regulated by various intracellular and extracellular mechanisms. According to data obtained in the short term study, no significant changes from the first wash-out phase were observed. Results are shown in Figure 5-32.

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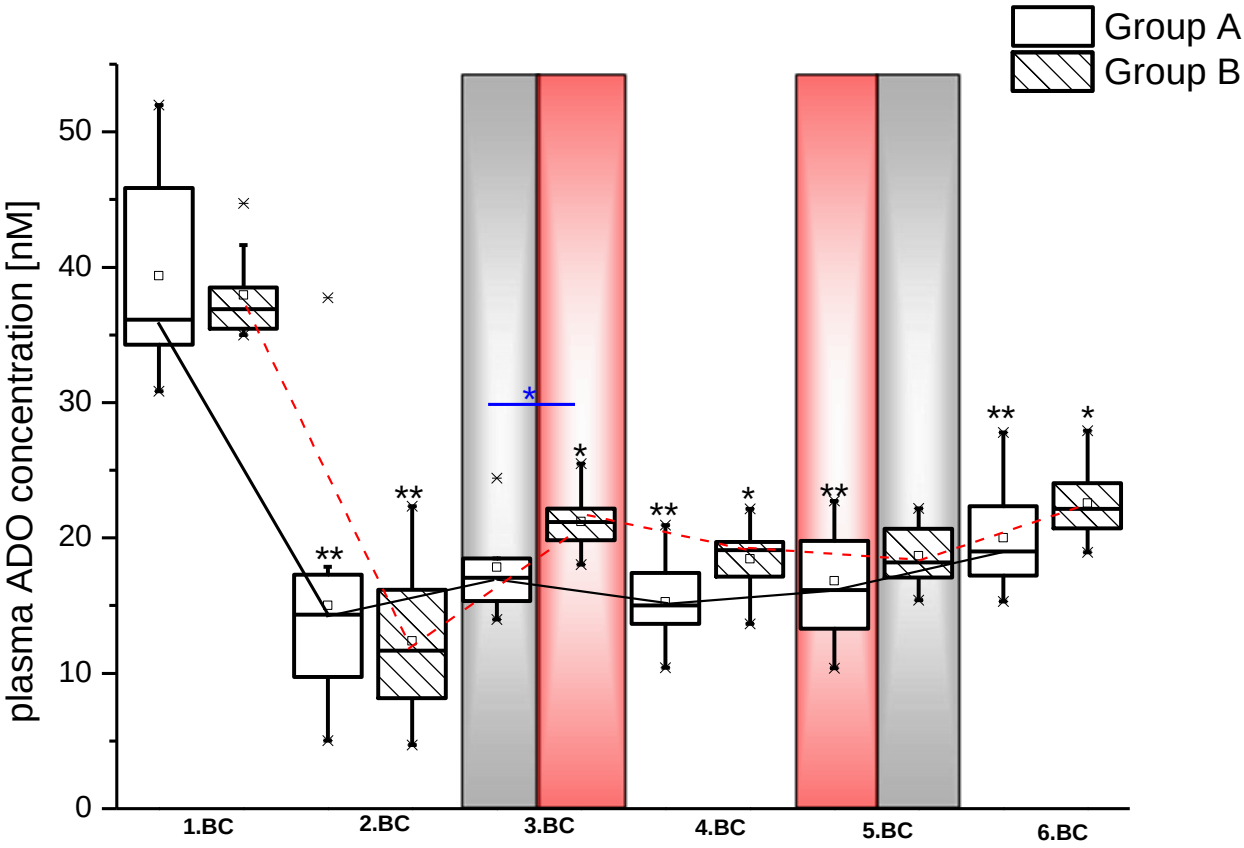


Figure 5-32: Modulation of plasma adenosine concentrations by coffee consum of volunteers during the course of the long term trial. The data (Box diagrams) are expressed as plasma ADO concentration (nM). The Anderson Darling test was used for the analysis of normal distribution Significances between time points were calculated using two-sided, paired Wilcoxon test (*p<0.05; **p<0.01; ***p<0.001 vs. the preceding phase) and the analysis of differences between groups using the two-sided, unpaired Mann-Whitney test (*p<0.05; **p<0.01; ***p<0.001 A vs.B). Silver background: 4 weeks reference coffee intervention, red background: 4 weeks study coffee intervention (n=19). Solid black trendline: group A, dash red trendline: group B. In blue: significances between groups.

Again a decrease of almost 50% in the first wash-out (2.BC) compared to the 1.BC was observed. Then the values remained in the same range of concentration until the end of the study, with a slight tendency to return to baseline. In general, no statistically significant differences below 1% (p<0.01) were observed between groups or treatments, considering that during the first coffee phase, group B showed a greater increase of the ADO concentration in plasma when compared with group A. There is evidence that modifications of ADO concentrations are associated with local changes in energy metabolism as indicated by increases in levels of pyruvate and lactate and increased phosphorylation of AMP-activated protein kinase. In fact, adenosine is the end-product of hydrolysis of the universal

“energy currency”, ATP, thus energy consumption and adenosine formation are directly related [Gunnar, 2010; Porkka-Heiskanen and Kalinchuk, 2011].

Although volunteers of the short term and the long term studies were asked to maintain their usual diet, the possibility cannot be ignore that food consumption records would have been useful to show or exclude the participation in a trial, and this may alter the dietary behaviors [Kohlmeier, 1994]. Such a change is not very probable given the guidance the participants received, but still may have contributed for the dramatic change observed at the time of initiating the study (2. BC).

5.5 Adenosine Deaminase (ADA)

As mentioned in the state of the art chapter, there are two enzymes that constitute the major pathways of adenosine removal: adenosine kinase and adenosine deaminase (ADA). The latter enzyme is present mostly intracellularly but is also found in some extracellular compartments. The K_m for adenosine is well above 5 mM and adenosine deaminase is therefore of particular importance when adenosine levels are high [Fredholm et al., 1999]. Adenosine kinase, by contrast, has a K_m level in the range of physiological intracellular adenosine concentrations. Indeed, blockade of adenosine kinase has a much larger effect on the rate of adenosine release than the blockade of ADA [Lloyd and Fredholm, 1995].

Other possible roles have been proposed for the ecto-ADA (although the location of the enzyme is mainly cytosolic, ADA is also found associated to membrane fractions so that its active site is available to the exterior environment of the cells), e.g. an interaction between adenosine deaminase and A_1 adenosine receptors in pig brain cortical membrane [Saura et al., 1996] suggesting that A_1R could be a second ecto-ADA binding protein and that ADA is needed for an effective coupling between A_1R and heterotrimeric G proteins [Cristalli et al., 2001].

Many constituents of dietary crude plant material can exert biological effects through ADA inhibition (i.e., garlic and onion extracts); among them compounds such as flavonoids and sapogenins show interesting biological properties. With respect to coffee compounds, a direct inhibitory effect of caffeic and ferulic acid on the enzyme, with IC_{50} values of 0.06 and 0.04 mg/mL respectively has been reported [Koch et al., 1992].

The effect of coffee consumption on the activity of ADA was determined in plasma samples of a human study conducted in 2008 [Bakuradze et al., 2011b]. In this 12-wk intervention study, 33 healthy male probands consumed daily 750 mL of freshly brewed black filtered coffee (with/without sugar) in three equal portions (morning, noontime, afternoon). In the wash-out phases, the coffee brew was replaced by equal volumes of water. The 12-wk intervention study was designed as follows: weeks1–4, 1st wash-out; weeks 5–8, coffee uptake; and weeks 9–12, 2nd wash-out. Results are shown in Figure 5-33 .

ADA levels in the plasmas of subjects were measured as described in the material and methods chapter. To calibrate the procedure, two standard ADA controls (adenosine deaminase from bovine liver and BSA) 10.3 and 47.2 U/L provided by the manufacturer were used along with 0.9% saline as zero reference.

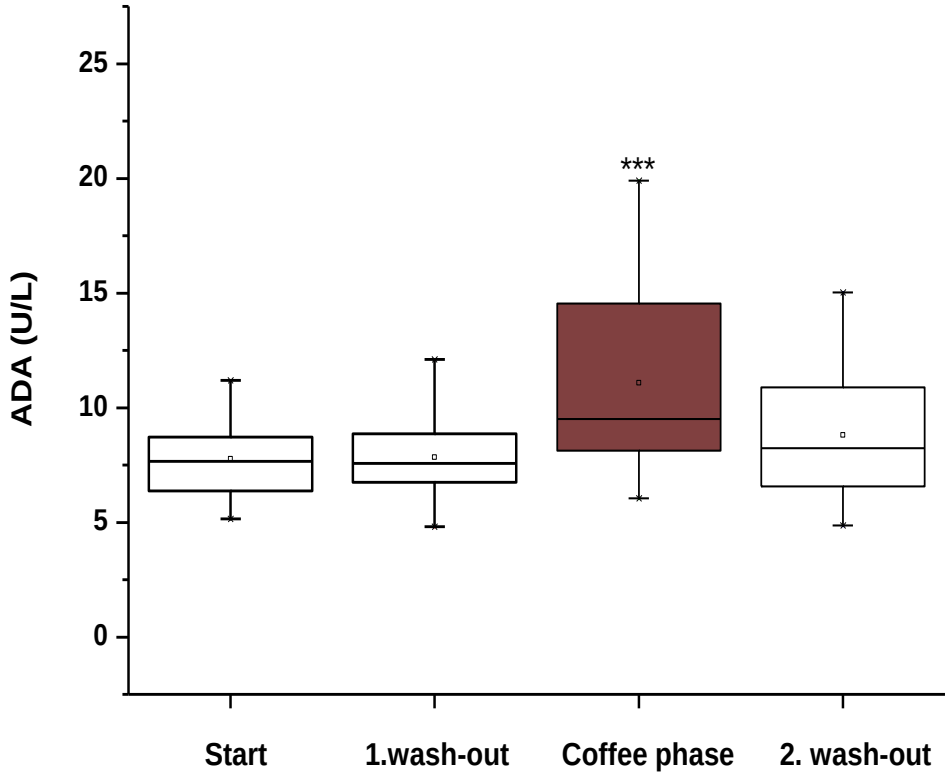


Figure 5-33: Modulation of the adenosine deaminase activity (ADA) by coffee consumption in plasma of volunteers during the course of the trial. The data (Box diagrams) are expressed as ADA activity (U/L). Significances were calculated using a f-test followed by Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. 1st wash-out). Brown box: 4-wk. coffee intervention phase. (n=32).

After the four-week trial intervention ADA significantly increased during the coffee treatment compared to the first wash-out phase. Based on these results it was important to clarify, whether the observed effect had an influence on the adenosine levels or adenosine metabolism.

For this purpose the short term study was designed to also study effects on ADA. Experiments about the ADA activity and uric acid concentrations in plasma were carried out by the diplom student Eirich, M.. Results show a significant increase (approx. 30% compared to the first wash-out phase) of the ADA enzyme activity during the Caffè Crema intervention lasting until the first week of the second coffee intervention with the caffeine reduced coffee. Then the activity decreased, returning to baseline levels. Plasmatic uric acid concentrations showed no cahnges within the short term study [Eirich, 2011].

5.5.1 Plasma ADA activity in the long term study

ADA activity in plasma of participants in the long term study was determined. Statistically significant differences between groups, from the start of the study and the second wash-out phase, and between coffes (SC and RC) in the first coffee intervention phase were observed. Results up the first wash-out phase correlate with the observations corresponding to adenosine concentrations in plasma (see Figure 5-34).

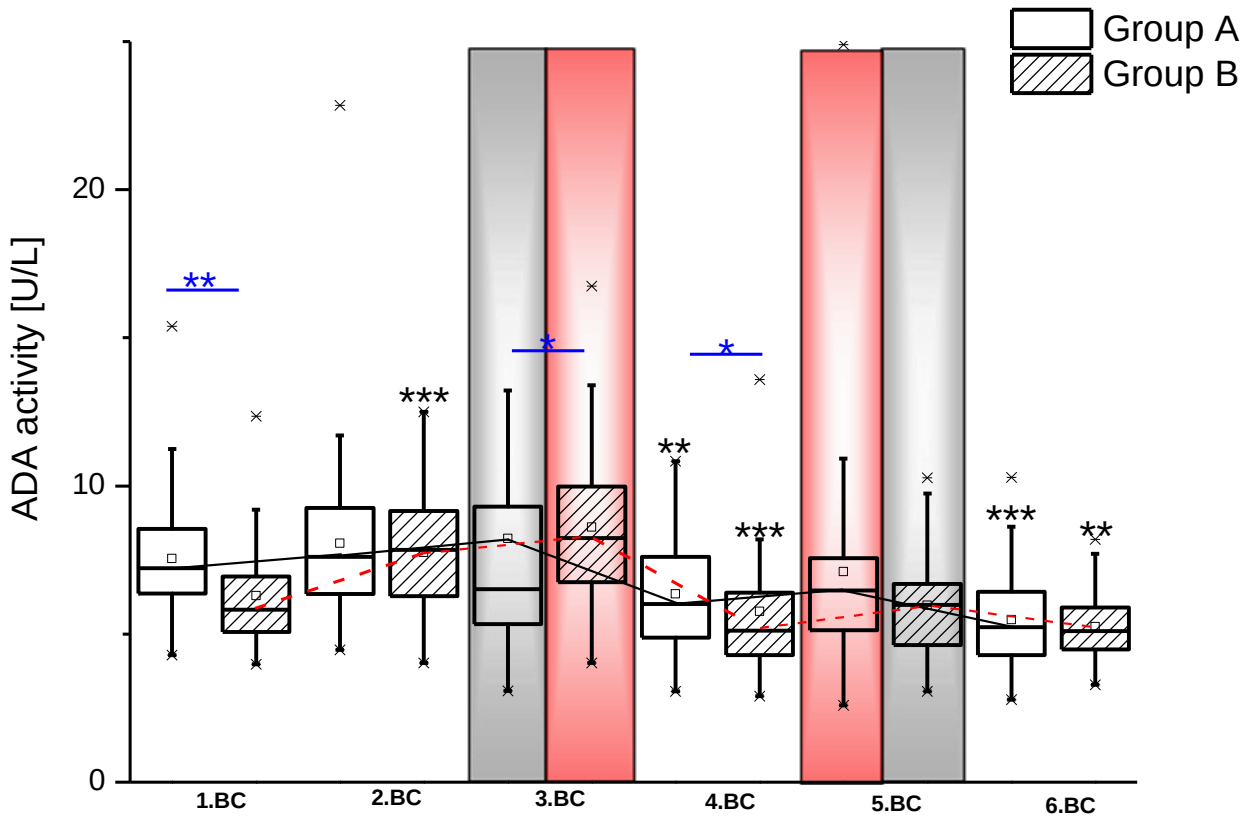


Figure 5-34: Modulation of plasma adenosine deaminase activity by coffee consumption of volunteers during the course of the long term trial. The data (Box diagrams) are expressed as plasma ADA activity (U/L). The Anderson Darling test was used for the analysis of normal distribution Significances between time points were calculated using two-sided, paired Wilcoxon test (*p<0.05; **p<0.01; ***p<0.001 vs. the preceding phase) and the analysis of differences between groups using the two-sided, unpaired Mann-Whitney test (*p<0.05; **p<0.01; ***p<0.001 A vs.B). Silver background: 4 weeks reference coffee intervention, red background: 4 weeks study coffee intervention (n=84). Solid black trendline: group A, dash red trendline: group B. In blue: significances between groups.

Healthy subjects show a plasma ADA activity in the range of 4 – 20 U/L [Diazyme and Laboratories, 2010], indicating that the enzymatic activity of ADA in plasma from 98.8% of the volunteers was normal. As expected, neither the ADA activity nor the adenosine concentrations in plasma were markedly modulated by coffee. This confirms that adenosine deaminase acquires particular importance only when adenosine levels are high [Fredholm et al., 1999; Gunnar, 2010; Spychala, 2000].

6 General Discussion

Coffee is one of the most widely consumed beverages in the world and has been linked with beneficial biological effects. Epidemiological evidence suggests coffee consumption to be associated with prevention or delay of degenerative diseases including T2D, Parkinson and Alzheimer disease, cardiovascular disease (CVD) and cancer [Deutscher-Kaffeeverband, 2011; Greenberg et al., 2006; Ranheim and Halvorsen, 2005; Zhang et al., 2009]. Many beneficial effects have been attributed to the antioxidant activity of coffee [Bakuradze et al., 2011a; Bakuradze et al., 2010; Hoelzl et al., 2010]. Additionally, moderate reduction in body weight was reported to correlate with regular coffee consumption [Thom, 2007; Tunnicliffe and Shearer, 2008]. Coffee beans contain thousands of constituents, including lipids, proteins, carbohydrates, vitamins, and minerals. In fact, addressing specific compounds responsible for the positive effects of coffee is quite difficult. To date, the majority of research on the biological activity of coffee has mainly been focused on caffeine. More recently, the acknowledgement that coffee and caffeine are not physiologically equivalent has increased the exploration of other coffee constituents [Boettler et al., 2011b; Farah et al., 2006; Johnston et al., 2003; Tunnicliffe and Shearer, 2008].

The HOs play critical roles in physiological iron homeostasis, antioxidant defense, and, as shown from accumulating evidence, in signaling pathways that employ CO as a messenger. Particularly, through its enzymatic products HO-1 mediates many cellular functions in protecting cells and tissues against inflammation and oxidative stress. Numerous studies have indicated the protective function of HO-1 in some diseases, especially in cardiovascular conditions [Wu et al., 2011].

One of the goals of this work was to determine if coffee or some coffee constituents may have a stimulatory effect on the HO-activity of intestinal colorectal adenocarcinoma (Caco-2), liver hepatocellular carcinoma (HepG2) and monocytic leukemia (Mono Mac 6/ MM6) cell lines. Two coffee extracts, a slightly (AB1) and an intensively roasted coffee (AB2), were studied along with selected individual compounds (see Table 6-1).

Caffeine and low substituted pyrazines showed no effect on the HO-actvity of HepG2 cells, while NMP and pyrazines with a greater substitution pattern such as TMP and 2-E-3,5-DMP significantly induced the HO-activity in HepG2, Caco-2 and in some instances in MM6 cells (see Table 6-1).

NMP, the compound formed during the coffee roasting from trigonelline, showed particularly high and reproducible inducing HO-activity in HepG2 and Caco-2 cell lines.

Table 6-1: Induction of HO-activity by coffee compounds and coffee extracts in in HepG2, Caco-2 and MM6 cells

Tested compound or extract	Cell line		
	HepG2	Caco-2	MM6
NMP	1 µM*	10 µM*	100µM***
Caffeine	no effect	nd	nd
TMP	1 µM***	1 µM*	0.1 µM ***
2-E-3,5-DMP	1 µM*	1 µM*	no effect
2-E-3-MP	no effect	nd	nd
2-MP	no effect	nd	nd
AB1 extract	10 mg/L*	nd	nd
AB2 extract	1 mg/mL*	nd	nd

***** lowest concentration showing a significant effect and concentration-response relationship
****** nd: no determined
*** no concentration-response relationship

The concentration of NMP in both coffee extracts varied considerably (AB1: 9.08 mg/L and AB2: 70.53 mg/L) when comparing the lowest effective concentration of both extracts in the HepG2 cells (10 mg/L and 1 mg/L respectively) they were in a similar range (0.03 µM and 0.02 µM) see Table 6-2.

Table 6-2 Lowest concentration of coffee extracts showing a significant effect on the HO-activity induction in HepG2 cells and its equivalent content of NMP

Content	LC*	corresponds to
AB1	10 mg/L	6.3 µg NMP (0.03µM)
AB2	1 mg/L	4.6 µg NMP (0.02µM)

***** lowest concentration showing a significant effect

An inductive effect of NMP as single compound became measurable from 1 µM in HepG2 cells. This suggests that in addition to NMP the complex matrix of coffee and presence of other substances may play a role.

Regarding the use of HO as a biomarker in clinical studies, the European Food Safety Agency (EFSA) [EFSA, 2011] published an opinion discounting induction of antioxidant enzymes (as SOD, catalase glutathione peroxidase and HO) as evidence for claims related to the “antioxidant defense system”. Modulation of these enzymes may indicate a biological response to oxidative stress of any origin, including diet, and as such is not considered specific. May even reflect a pro-oxidant effect of a dietary component.

The influence of coffee on the HO-1 gene expression has been previously discussed. A marked induction of mRNA expression of HO-1 in rat liver and primary hepatocytes treated with 800 µg/mL of standard coffee for 24 h was reported by Cavin *et al.* [Cavin et al., 2008].

Similarly, Boettler *et al*. reported that an NMP rich-coffee extract elevated the HO-1 gene expression in leukocytes and HT29 cells after 3 h and 24 h incubation with 100 µg/mL [Boettler et al., 2011b]. The experiments of Boettler *et al*. were carried out with the same coffee extracts i.e. NMP rich coffee extract corresponding to AB2. It may be therefore concluded, that considerably more AB2 extract was needed to stimulate gene expression in HT29 cells than to achieve the stimulation of HO-1 activity in HepG2 cell line.

Nrf2/ARE pathway has been proposed as the potential mechanism whereby coffee activates phase I and phase II enzyme activity in mice liver [Cavin et al., 2008]. Recently it has been shown that 5-CQA and NMP are potent activators of Nrf2-nuclear translocation and subsequent ARE-dependent gene transcription in human colon carcinoma cells (HT29) [Boettler et al., 2011a].

Cells have developed defense mechanisms against ROS-induced damage, such as the induction of the expression of Phase II detoxifying enzymes [Zhang and Gordon, 2004]. The expression of many Phase II detoxifying genes is regulated by the activation of antioxidant or electrophile response elements (ARE/EpRE) which are activated via binding of the transcription factor Nrf2. Increased nuclear levels of Nrf2 provoke gene transcription of phase II detoxifying enzymes. In quiescent cells, Nrf2 is sequestered in the cytoplasm associated with Keap1. Activation by ROS or upstream protein kinases induces the translocation of Nrf2 into the nucleus, its binding to ARE and the onset of the transcription of phase II enzymes such HO-1 [Itoh et al., 1997; Thimmulappa et al., 2002].

Relatively few studies have implied roles for protein kinases in *ho-1* transcriptional regulation. In several cell culture models (i.e., primary rat hepatocytes and vascular smooth muscle), treatment with dibutylated cAMP and other agonists of protein kinase A (PKA) activated *ho-1* transcription [Durante et al., 1997; Immenschuh et al., 1998; Ryter et al., 2006a]. However, this potential interplay between HO-1 and PKA was not evidenced in this work, because those substances (such as NMP) that stimulated the HO-1 activity did not show any stimulatory effect on intracellular cAMP concentrations and in contrast, those that increased intracellular cAMP levels (such as the caffeine) did not affect the activity of HO-1.

The precise mechanisms underlying the actions of coffee on a reduced risk of several chronic and degenerative diseases including cardiovascular disorders, DM, obesity and neurodegenerative disorders are incompletely understood. Many effects have been attributed to caffeine. However coffee is a mixture of many chemical substances, some of which, such as polyphenols, are pharmacologically active [Ferruzzi, 2010]. The main mechanism of action of caffeine is to antagonize adenosine receptors; a secondary effect is the inhibition of phosphodiesterases [Ribeiro and Sebastiao, 2010], with the subsequent accumulation of cyclic AMP and an intensification of the effects of catecholamines [Renda et al., 2012].

Although the inhibition of phosphodiesterases may contribute to the actions of caffeine, there is growing evidence that most pharmacological effects of this xanthine result from antagonism of adenosine receptors.

One of the main objectives of the doctoral thesis was to investigate whether substances other than caffeine in coffee may influence the homeostasis of intracellular cyclic nucleotides *in vitro* and *in vivo*. The influence of coffee and some coffee compounds on several components of the cAMP pathway (see red circles in Figure 6-1) have been investigated.

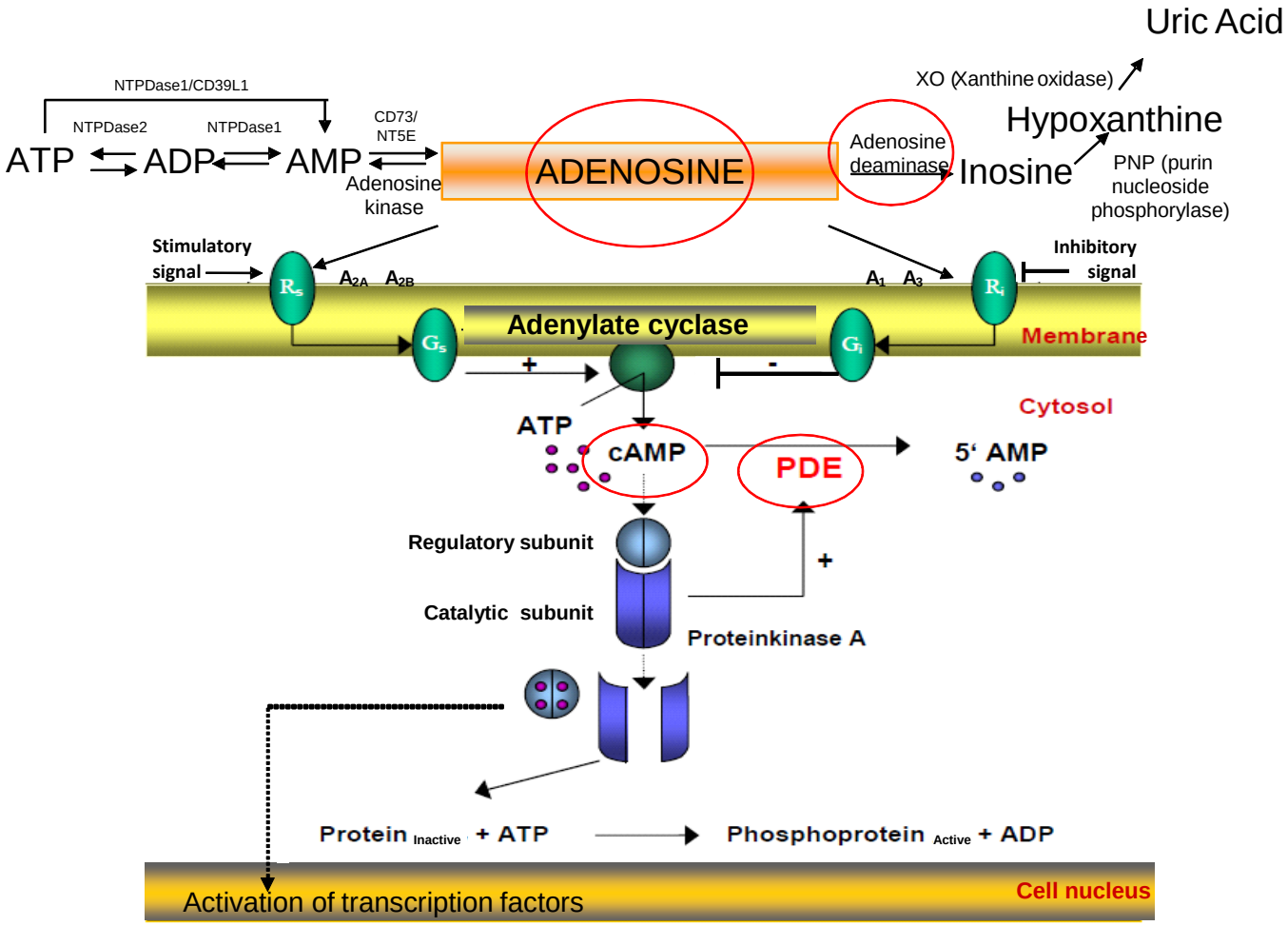


Figure 6-1: The cAMP Signaling Pathway: investigated parameters in human and partly in cell culture studies (in red circles). Adapted from Cronstein [Cronstein, 2011]. and Randall *et al*. [Randall et al., 1997].

In Table Table 6-3 the results during the coffee phases of two human studies are schematically summarized. Results are expressed compared to the first wash-out phase for the short term study and the corresponding wash-out phase for the long term study.

Table 6-3: Biomarker modulation by coffee consumption during the coffee intervention phases in both human studies

Biomarker	Short term Study (n=8)				Long term study (n=84)			
	Caffè Crema		Caffeine reduced coffee		Reference coffee (RC)		Study coffee (SC)	
	1st week	2nd week	1st week	2nd week	Group A	Group B	Group A	Group B
PDE activity in platelets	⇩ p<0.001	⇩ p<0.001	⇔	⇔	⇩ p<0.001	⇔	⇩ p<0.001	⇩ p<0.001
cAMP concentration in platelets	⇧ p<0.01	⇧ p<0.001	⇧ p<0.001	⇧ p<0.001	⇧ p<0.001	⇩ p<0.001	⇩ p<0.001	⇧ p<0.001
ADO concentration in plasma	⇔	⇔	⇔	⇔	⇔	⇔	⇧ p<0.01	⇧ p<0.05
ADA activity in Plasma	⇔*	⇧* p<0.001	⇧* p<0.01	⇔*	⇔	⇔	⇔	⇔

The Anderson Darling test was used for the analysis of normal distribution. Short term study significances were calculated using F-test followed by Student´s t-test (*p<0.05; **p<0.01; ***p<0.001 vs. 1st wash-out). For the long term study significances between time points were calculated using two-sided, paired Wilcoxon test (*p<0.05; **p<0.01; ***p<0.001 vs. the preceding phase) and the analysis of differences between groups using the two-sided, unpaired Mann-Whitney test (*p<0.05; **p<0.01; ***p<0.001 A vs.B).
⇔ = No changes; ⇩= decrease; ⇧= increase
* Data from [Eirich, 2011]

Caffeine was used for many years to block PDE in biochemical studies until more potent (theophylline) and specific (Rolipram) inhibitors became available [Belibi et al., 2002]. There is evidence that caffeine inhibition of PDEs *in vitro* requires higher levels (100 –1000 µM in mouse and pigs cerebral cortex slices) than for interaction with adenosine receptors (10 – 100 µM), but the potencies of its metabolites, paraxanthine and theobromine, for inhibition of most known PDEs have not been studied [Francis et al., 2011]. In the current work we found that caffeine, theophylline, coffee extracts (AB1 and AB2), volatile pyrazines (2,3-DE-6-MP, 2-Isobutyl-3-methoxyP)**,** the phenolic compounds 5-CQA and caffeic acid all significantly inhibited the basal cytoplasmatic PDE activity in lysates of LXFL529L cells and platelets. To a lesser somewhat extent a PDE inhibition was also found in experiments performed with paraxanthine, other coffee extracts (SCE, RCE, Caffè Crema and reduced caffeine coffee), and other pyrazines (2-E-3,5-DMP, TMP and 2-E-5-MP (see Table 5-1, results and discussion chapter).

The analysis of interaction of methylxanthines with cAMP homeostasis is complicated by the fact that, in addition to inhibiting PDE, they may block A_1 and A_2 receptors, thereby influencing the generation of cAMP [Dousa, 1994]. Binding of adenosine to A_1 receptors blocks AC, in adipocytes, through the activation of the G_i inhibitory mechanism previously

mentioned in the Chapter Nr. 2.5.2.3. By contrast, binding to the A_2 subtype, expressed by platelets, activates AC through G_s stimulatory protein.

There is evidence that Rolipram lacks adenosine receptor affinity and primarily blocks PDE (as also shown in this work in the LXFL529L cells), elevating basal levels of cAMP, in primary polycystic kidney disease cells strongly potentiating the effect of adenosine to increase intracellular levels of cAMP [Belibi et al., 2002]. By contrast, caffeine did not potentiate the action of adenosine, but at blood concentrations resulting from normal coffee consumption acts mainly as an adenosine receptor antagonist. As evidence of antagonism of caffeine, the *in vitro* experiments performed in this work with SGBS adipocytes have shown that caffeine at concentrations ≥ 3 µM increased significantly the cAMP levels in a concentration dependent manner (see Figure 5-26 in the Results and Discussion chapter). Similar effects were also observed with other coffee compounds, such as chlorogenic acid and trigonelline at concentrations ≥ 300 µM. NMP showed no effect on cAMP concentrations of SGBS cells.

Moreover, platelets express mainly A_2 receptors which are linked to stimulatory G proteins. Both *in vivo* studies (the short term and the long term) showed a significant increase in intracellular cAMP levels of platelets in the second phase of coffee withdrawal. This may be interpreted, at least in part to reflect the absence of the antagonist caffeine in connection with a presumed upregulation of A_{2A} receptors in platelets as a consequence of repeated coffee consumption as suggested from data of Varani *et al*. [Varani et al., 2000; Varani et al., 1999]. The latter effect has also been observed in mouse brain [Johansson et al., 1997].

Many authors indicate that although human platelets are anucleate fragments of megakaryocytes, they retain cytoplasmic mRNA and may translate proteins [Gnatenko et al., 2003; Williams et al., 2010]. Varani et al. suggested that the observed increase in A_{2A}-binding by its selective agonist (HE-NECA), as a response of continuous caffeine treatment and that it is unlikely to have been due to the synthesis of new A2A receptors of mature platelets. It may more likely occur during differentiation of precursor cells [Varani et al., 1999]. This would indicate that generation of platelets originating from precursor cells which had been exposed to caffeine/coffee may present enhanced adenosine binding density in comparison to previously unexposed generations. The same group reported increased adenosine A_{2A} receptors alredy at 1 hour after the last dose of caffeine treatment. The effect was similar to that obtained at 12 or 60 hours after caffeine withdrawal. Thus suggests that the withdrawal was not necessary for the upregulation of A_{2A} receptors [Varani et al., 2000]. Taken together, there is evidence that chronic intake of caffeine alters the response of platelets to the actions of adenosine and that chronic caffeine consumption may lead to a reduction in platelet aggregability as a result of upregulation of adenosine binding sites located on the platelet surface.

Additionally, cAMP concentrations changed significantly during the first phase of exposure to coffee compared with the first wash-out phase (2. BC). This increase is however distinct as that observed in the withdrawal phase. It is possible that this phenomenon, could be due to the rapid response of PDEs to the effect of the first coffee withdrawal (first wash-out phase/ 2.BC) as shown in the short term study (short-term effect). Alternatively the study participants may not have regularly consumed moderate to high amounts of coffee before entry to the study. Based on *in vitro studies*, many authors have reported that the modulation of the enzyme PDE with regular consumption of caffeine and/or coffee is unlikely [Fredholm and Jacobson, 2009; Stanley et al., 1989; Wilson and Mustafa, 2009] . Some authors even claim that to cause an effective inhibition of PDEs *in vivo* doses of caffeine near convulsant effects are required. Furthermore, they conclude that inhibition of PDEs by caffeine is unlikely to significantly contribute to the *in vivo* pharmacology of moderate doses of caffeine [Tarter et al., 1998]. Even though in the *in vitro* experiments of the present work, the required concentrations of caffeine and coffee extracts to obtain a half maximal inhibition were unphysiological, it was demonstrated for the first time *in vivo* that moderate consumption of coffee can modulate the activity of platelet phosphodiesterases in humans.

As aforementioned, in view of the serving size and the variation in clearance times, it cannot be excluded that plasma and cellular caffeine levels may come close to the range for inhibitory activity on PDEs [Chou and Bell, 2007]. It is also well established that the intracellular concentration/action of a PDE inhibitor cannot be confidently predicted strictly on the basis of its extracellular concentration [Francis et al., 2011; Thompson, 1991]. It is also possible that by inhibiting PDE in conjunction with receptor modulation, moderate consumption of coffee/caffeine might affect affect intracellular cAMP concentrations.

It is worth noting that not only caffeine showed an inhibitory effect in *in vitro* experiments. Other compounds present in coffee as pyrazines, theophylline, some polyphenols and to a lesser extent the major metabolite of caffeine, paraxanthine, also had an inhibitory effect on PDE activity of LXFL529L cells and platelets. Thus the degree of roasting has a considerable impact on constituents of influence for PDE activity. Out of the compounds under study caffeine, coffee polyphenols and some pyrazines represented the main modulating constituents.

To estimate the interaction of substances in the complex matrix coffee, concentration-dependent effects on PDE were determined for each compound in combination with fixed concentrations of one compound in LXFL529L cells. The interaction of two compounds was quantified by determining the combination index (CI), in accordance with the following classic isobologram equation [Chou and Talalay, 1984; Tallarida, 2006].

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

Where Dx is the dose of one drug alone required to produce an effect (in this case IC_{50}), and $(D)_1$ and $(D)_2$ are the doses of compounds 1 and 2, respectively, in combination that produce the same effect. From this analysis, the combined effects of the two coffee compounds can be summarized as follows: CI = 1 indicates summation (additive and zero interaction); CI < 1 indicates synergism; and CI> 1 indicates antagonism

According with the foregoing the combination of caffeine and paraxanthine with pyrazines (2E-3,5-DMP, TMP, 2,3-DE-6-MP, 2-E-5-MP and 2-Isobutyl-3-methoxyP) was not found synergistic. Rather the effect found was located in the line of additivity suggesting that each constituent contributes to the effect according to its own potency.

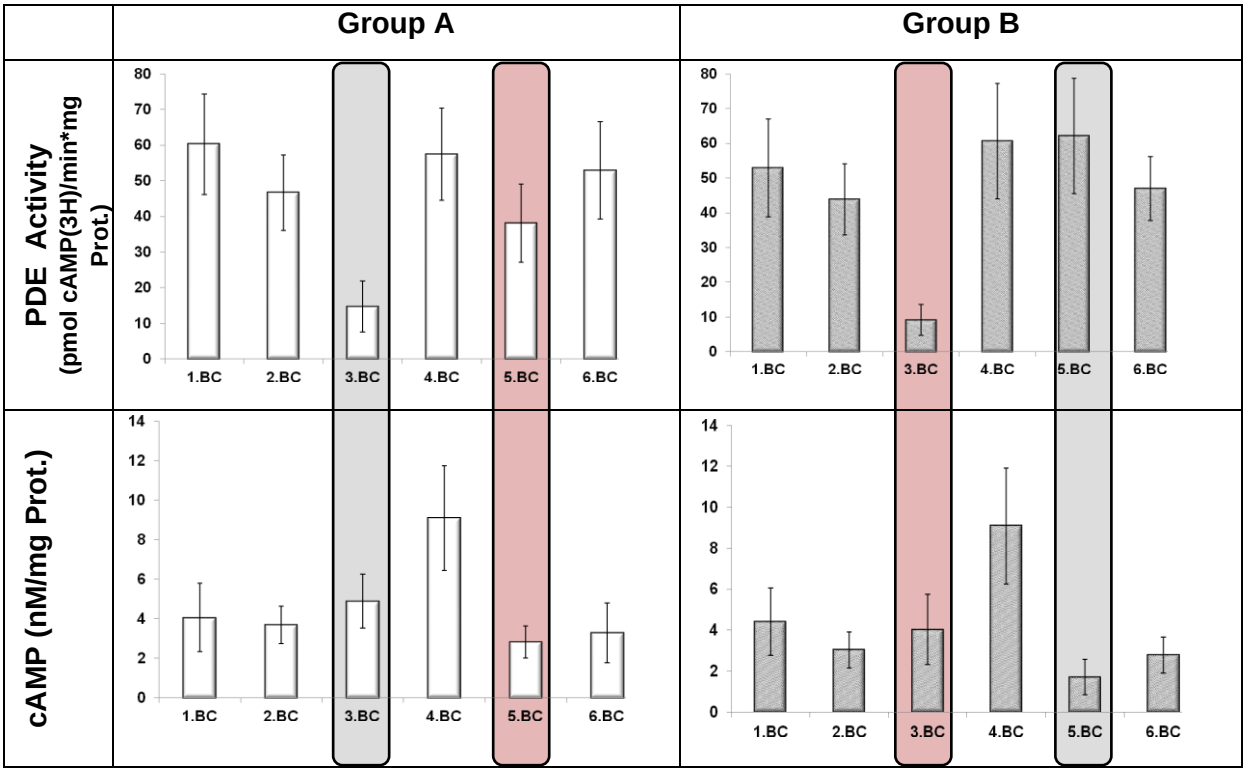
All known PDE catalytic domains interact to some extent with methylxanthines and related compounds in a very wide range of affinities. Methylxanthine-related compounds that are profoundly potent inhibitors for certain PDEs are ineffective for inhibiting other PDEs [Beavo et al., 2007]. Moreover, members of the PDE8 and PDE9 families are not significantly inhibited by methylxanthine inhibitors such as IBMX are commonly described as “nonspecific”. The affinities of the various PDEs for IBMX vary by more than 100-fold, for Zaprinast by more than 1,000-fold, for Sildenafil by more than 7,000-fold, and for Vardenafil by more than 300,000- fold [Francis et al., 2011]. In this work, similarly, PDE-inhibitory effect were partly cell type/PDE family dependent (PDE4 for LXFL529 and PDE3 the major PDE expressed by platelets). Caffeine was seven times more active in platelets than in LXFL529L cells, 2-E-3,5-DMP showed a two fold stronger inhibitory activity in platelet concentrates, compared to 2-isobutyl-3-methoxyP which was more effective in LXFL529L cells. Thus, despite the overall similarities in the catalytic pockets of these enzymes, there are elements that strongly discriminate among these compounds.

As indicated in the chapter Nr. 5.2.2, PDE responses between the first and the second coffee intervention phases in both *in vivo* studies were different. The first exposure to coffee (3.BC) showed a strong inhibition of the PDE activity with a simultaneous increase in cAMP concentrations in the platelet lysates of the participants (see Table 6-4) while the second coffee phase showed no (for group B) or only a slight (for group A) effect when compared with the first part of the cross-over intervention. There is ample literature to demonstrate that elevating intracellular cAMP levels can provoke increases in PDEs activity, as a so called dampening effect [Huang et al., 2002]. In both studies, there was a significant increase in intracellular cAMP concentrations during the wash-out phase (4. BC) preceding the second coffee phase (see Table 6-4).

cAMP elevation is known to lead to increased expression and activity of PDEs in other blood cells such as monocytes and lymphocytes [Schudt et al., 1996], cultured rat aortic vascular smooth muscle cells [Maurice, 1998] and adipocytes from transgenic mice [Huang et al.,

2002], and this may be the reason of the different responses observed between the first and the second coffee phase.

Table 6-4: PDE activity and cAMP modulation by coffee consumption during the long term study



Silver background: 4 weeks reference coffee intervention red background: 4 weeks study coffee intervention (n=84). Data are expressed as mean ±SD.

Conlay *et al*. [Conlay et al., 1997] reported that adenosine, particularly after prolonged administration of caffeine, increases dramatically in rat plasma. They found also an increase in adenosine concentration after antagonist administration and its reduction after antagonist withdrawal, suggesting a receptor-mediated regulation of the plasma adenosine concentration.

In contrast to the findings of Conlay *et al*., adenosine concentrations in plasma of the probands showed no dramatical changes in both human studies. It should be noted that during the first wash-out (2.BC) adenosine concentrations in plasma decreased approx. 50% when compared to baseline (1.BC), this effect correlates with the decrease in the concentration of cAMP in platelets at the same point. During both coffee interventions, values increased ($p<0.05$) compared with the previous phase wash-out. Adenosine concentrations in plasma of probands showed a significant increase in response to the study coffee intervention ($p<0.01$ for group A and $p<0.05$ for group B). However, in general it can be noted that the values remained within a narrow range. This may reflect the fact that adenosine is subject of a quick and effective neutralization by phosphorylation (adenosine kinase) or deamination (adenosine deaminase) to keep its concentration within a well

balanced homeostasis [Gunnar, 2010; Spychala, 2000]. Adenosine plasma concentrations of the participants of the short term study after 0, 1 and 2 h showed also no significant changes. Taken together**,** results show that adenosine homeostasis was not considerably affected by moderate coffee consumption in both studies. Likewise, adenosine desaminase activity did not show large fluctuations in both studies. However, by the long term trial a significant trend to decrease after the two coffee intervention phases, especially for group B, was observed.

As expected, neither the ADA activity nor the adenosine concentrations in plasma were markedly modulated by the coffee consumption. Adenosine deaminase acquires particular importance only when adenosine levels are high [Fredholm et al., 1999].

The precise long term mechanisms underlying the actions of coffee are incompletely understood. Many effects have been attributed to caffeine, neglecting that coffee is a mixture of many chemical substances, some of which, are pharmacologically active [Ferruzzi, 2010]. Some authors suggest that the main mechanism of action of caffeine is to antagonize adenosine receptors; a secondary effect is the inhibition of phosphodiesterases [Ribeiro and Sebastiao, 2010], with the subsequent accumulation of cAMP and an intensification of the effects of catecholamines [Renda et al., 2012].

However, it is also well known, that considerable variability exists in the responses to coffee drinking. In part, such variability is due to tolerance [Colton et al., 1968], but there is also evidence for a genetic background. Individual differences in responses to caffeine may occur at the metabolic (pharmacokinetic) or at the drug-receptor level (pharmacodynamic), and they can contribute to the quality and magnitude of direct effects as well as to consumption [Yang et al., 2010a].

Genetic variations which can affect pharmacokinetic and pharmacodynamic responses to coffee encompass CYP1A2 gene variants affecting caffeine pharmacokinetics. An A to C substitution at position 734 (CYP1A2*1F) decreases enzyme inducibility, which results in impaired caffeine metabolism [Renda et al., 2012]. As aforementioned, most pharmacologic effects of caffeine result from the antagonism to adenosine, mostly with respect to A_{2A} (ADORA2A), receptors [Ongini and Fredholm, 1996]. The C to T genetic variant of the ADORA2A gene has been shown to be related to susceptibility to anxiety and sleep changes [Renda et al., 2012; Yang et al., 2010a]. Moreover, the 34 C to T genetic mutation of AMP deaminase, which catalyzes the deamination of AMP to inositol monophosphate and thus reduces enzyme activity, may increase adenosine availability for its receptors [Feldman et al., 1999]. Genetic polymorphisms of adrenergic receptors may also contribute to the variability in platelets responses to coffee [Beres et al., 2008].

Altogether the data reported in this work provide evidence that moderate coffee consumption positively modulates platelet aggregation, a critical step involved in CVD and thrombosis, by

influencing cAMP homeostasis. However, further research and mechanistic in depth studies for further substantiation of benefitial health effects of coffee consumption is still needed.

7. References

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8. Publications

Montoya, G.A., Bakuradze, T., Eisenbrand, G., Baum, M., Richling, E. (2012), Modulation von Adenosin-Rezeptor vermittelten Signalwegen durch Kaffeekonsum in Human-Thrombozyten. Regional meeting of the german society of food chemist. Southwest Regional Association. **Oral presentation**. Kaiserslautern, Germany.

Montoya, G.A., Baum, M., Eisenbrand, G., Richling, E. (2012), Coffee constituents as modulators of cAMP phosphodiesterases activity of human platelets and lung carcinoma cells Regional meeting of the german society of food chemist. Southwest Regional Association. **Poster**. Kaiserslautern, Germany.

Montoya, G.A., Baum, M., Eisenbrand, G., Richling, E. (2011), Coffee polyphenols modulating the activity of cAMP phosphodiesterases activity in human platelets. The 5th International Conference on Polyphenols and Health, **Poster**, Sitges, Barcelona, Spain.

Montoya, G.A., Baum, M., Eisenbrand, G., Richling, E. (2011), Modulation of cAMP-phosphodiesterase activity by coffee constituents in human platelets and in lung carcinoma cells. 2nd Luxembourgish Int. Nutrition Conference „Dietary Composition, Pattern and Health“, **Poster**. Mondorf-les Bains, Luxemburg. **Best Poster Award.**

Montoya, G.A., Richling, E., Eisenbrand, G., Baum, M. (2010), Modulation der cAMP-Phosphodiesterase-Aktivität durch Kaffeeinhaltsstoffe. 39th annual meeting of the german society of food chemist. **Poster**. Stuttgart-Hohenheim, Germany.

Montoya, G.A., Richling, E., Eisenbrand, G., Baum, M. (2010), Modulation der Hämoxygenase-Aktivität durch Kaffeeinhaltsstoffe in Caco2, HepG2 und MonoMac6 Zellen. 38th annual meeting of the german society of food chemist. **Poster**. Berlin, Germany.

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Appendices

Data from Figure 5-2: Effect of PDTC, curcumin and quercetin on HO-activity in HepG2. Caco-2 and MM6 cells (12h incubation)

	PDTC			Curcumin			Quercetin		
MEAN*	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
0 µM	4.0	5.7	12.6	4.0	5.7	12.6	4.0	5.7	12.6
10 µM	2.8	5.4	11.4	5.2	5.9	13.6	3.3	5.4	5.4
100 µM	5.2	6.2	10.3	2.45	7.18	14.9	2.5	5.8	3.2
1000 µM	9.8	6.3	12.6	3.64	6.3	15.2	5.6	7.3	9.9

* 4-5 independent experiments (triplicates)

Standard Deviation								
PDTC			Curcumin			Quercetin		
HepG2	Caco-2	MM6	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
1.327	0.893	1.790	1.327	0.893	1.790	1.327	0.893	1.790
1.652	3.224	2.420	1.907	1.014	0.936	0.962	0.812	0.560
1.755	2.884	2.401	0.765	3.056	3.563	1.147	0.814	3.251
5.005	2.225	2.982	1.547	0.121	2.402	3.211	0.650	2.500

Data from Figure 5-3: Effect of PDTC, curcumin and quercetin on HO-activity in HepG2. Caco-2 and MM6cells (24h incubation)

	PDTC			Curcumin			Quercetin		
MEAN*	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
0 µM	4.0	5.7	12.6	4.0	5.7	12.6	4.0	5.7	12.6
10 µM	3.4	8.7	14.3	2.1	5	12.1	6.3	6.4	11.4
100 µM	10.9	11.15	15.3	4.8	4.8	11.9	7.5	8.8	10.4
1000 µM	39.8	30.3	17.6	5.2	5.5	12.0	10.1	12.3	10.9

* 4-5 independent experiments (triplicates)

Standard Deviation								
PDTC			Curcumin			Quercetin		
HepG2	Caco-2	MM6	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
1.327	0.893	1.790	1.327	0.893	1.790	1.327	0.893	1.790
0.001	3.224	2.501	1.907	1.014	0.767	0.962	0.812	0.560
0.755	2.884	2.401	0.079	0.173	0.849	0.315	0.467	0.988
6.005	0.225	2.150	1.547	0.121	2.785	0.149	0.002	0.520

Data from Figure 5-5: Effect of PDTC, curcumin and quercetin on HO-activity in HepG2. Caco-2 and MM6 cells (48 h incubation)

	PDTC			Curcumin			Quercetin		
MEAN*	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
0 µM	4.0	5.7	12.6	4.0	5.7	12.6	4.0	5.7	12.6
10 µM	3.2	8.7	11.3	17.1	19	15.1	6.3	6.4	10.4
100 µM	8.9	7.5	10.3	8.8	9.8	11.9	2.5	6.8	9.4
1000 µM	21.3	11.15	13.6	10.2	3.5	12	2.1	2.3	8.9

* 4-5 independent experiments (triplicates)

Standard Deviation								
PDTC			Curcumin			Quercetin		
HepG2	Caco-2	MM6	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
1.327	0.893	1.790	1.327	0.893	1.790	1.327	0.927	1.790
1.632	3.224	2.501	3.987	4.315	1.542	1.857	0.932	2.560
4.755	2.884	2.401	4.079	3.173	2.849	1.542	0.467	1.001
3.250	2.489	4.150	2.517	2.323	1.785	1.958	2.002	3.520

Data from Figure 5-4: Viability of cells exposed to PDTC, curcumin and quercetin at 37°C (24 and 48h incubation).

	PDTC		
MEAN*	HepG2	Caco-2	MM6
Control	100	100	100
10	98.9	98.3	99.6
100	93.5	90.2	95.9
1000	90.6	85.4	90.3
10	65.4	50.9	50.2
100	53.2	51.7	40.8
1000	32.2	38.2	33.9
SD			
10 (24h)	1.987	0.988	5.155
100 (24h)	1.855	0.820	8.862
1000 (24h)	2.199	3.898	10.890
10 (48h)	3.257	1.357	0.983
100 (48h)	2.257	3.154	2.141
1000(48h)	3.587	2.495	0.584
	Curcumin		
MEAN*	HepG2	Caco-2	MM6
Control	100	100	100
10	98.9	95.1	80
100	93.5	88.6	83.2
1000	83.2	85.4	83.8
10	73.2	64.5	68.6
100	44.2	30.6	39.2
1000	23.2	22.9	20.3
SD			
10 (24h)	2.570	0.988	3.155
100 (24h)	2.649	5.923	4.236
1000 (24h)	5.549	3.898	8.955
10 (48h)	3.459	4.274	2.419
100 (48h)	2.983	3.219	2.574
1000(48h)	3.954	2.275	0.887
	Quercetin		
	HepG2	Caco-2	MM6
Control	100	100	100
10	80.1	78.4	89.2
100	85.9	83.5	80.2
1000	70.6	65.4	70.3
10	63.3	45.9	57.2
100	45.4	29.2	33.2
1000	38.9	18.6	24.5
SD			
10 (24h)	8.741	5.219	4.854
100 (24h)	4.894	7.146	3.590
1000 (24h)	8.237	4.897	5.190
10 (48h)	2.544	0.983	1.549
100 (48h)	6.875	1.366	2.302
1000(48h)	3.257	2.545	3.824

* 6 independent experiments (triplicates)

Data from Figure 5-6: Modulation of HO-activity in HepG2, Caco-2 and MM6 cells after 24 h incubation with PDTC (0 –1000 µM with/without catalase 100 U/mL).

	PDTC (Mean*)			SD		
	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
0 µM	4.0	5.7	12.6	1.327	0.893	1.790
0 µM + cat**	4.4	4.1	12.1	0.985	0.057	2.385
10 µM	3.4	8.7	14.3	0.001	3.224	2.501
10µM + cat**	5.3	7.3	15.1	0.124	1.337	0.985
100 µM	10.9	11.2	15.3	0.755	2.884	2.401
100 µM + cat**	9.8	7.1	14.7	0.636	0.366	1.987
1000 µM	39.8	30.3	17.6	6.005	0.225	2.150
1000 µM + cat**	38.1	25.2	15.9	2.569	1.269	0.895

***** 5 independent experiments (triplicates)
** Catalase-treated cells

Data from Figure 5-7: Effect of NMP (N-methylpyridinium) on HO-activity in HepG2, Caco-2 and MM6 cells.

	NMP (Mean*)			SD		
	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
Control	4.0	5.7	12.6	1.327	0.893	1.790
PDTC 1mM	39.8	30.3	17.6	6.005	0.225	2.150
0.1 µM	4.6	2.6	10.6	3.181	3.538	0.250
0.3 µM	5.6	2.5	11.7	1.668	3.805	0.121
1 µM	6.8	7.0	12.0	2.166	0.962	0.849
3 µM	7.6	7.0	13.7	1.429	0.767	0.173
10 µM	9.2	10.2	13.6	1.666	1.019	3.547
100 µM	12.6	11.4	16.8	1.729	1.907	2.785
1000 µM	24.8	16.4	17.7	4.479	3.269	0.359

***** 5 independent experiments (triplicates)

Data from Figure 5-8: Effect of caffeine on HO-activity in HepG2 cells

	Caffeine (Mean*)	SD
Control	4.0	1.327
PDTC 1mM	39.8	6.005
0.1 µM	9.9	5.665
0.3 µM	7.8	3.564
1 µM	6.6	1.920
3 µM	6.8	2.655
10 µM	4.2	2.248
100 µM	6.1	2.583
1000 µM	6.5	1.703

***** 3 independent experiments (triplicates)

Data from Figure 5-9: Effect of TMP and 2-E-3,5-DMP on HO-activity in HepG2, Caco-2 and MM6 cells

	TMP(Mean*)			SD		
	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
Control	4.0	5.7	12.6	1.327	0.893	1.790
PDTC 1mM	39.8	30.3	17.6	6.005	0.225	2.150
0.1 µM	6.5	3.8	17.6	3.367	4.331	0.482
0.3 µM	6.7	5.6	18.0	3.186	4.298	2.346
1 µM	9.8	9.7	16.2	0.224	0.963	4.172
3 µM	10.8	10.3	13.6	0.886	0.671	0.218
10 µM	5.6	10.4	15.3	1.553	0.560	0.197
100 µM	7.8	9.1	18.3	1.120	0.812	4.319
1000 µM	6.5	9.8	13.4	1.386	0.695	0.982
	2-E-3,5-DMP (Mean*)			**SD**		
	HepG2	Caco-2	MM6	HepG2	Caco-2	MM6
Control	4.0	5.7	12.6	1.327	0.893	1.790
PDTC 1mM	39.8	30.3	17.6	6.005	0.225	2.150
0.1 µM	4.3	3.7	12.3	0.398	3.767	0.058
0.3 µM	4.5	3.4	12.6	3.951	4.283	0.171
1 µM	7.6	8.6	13.0	1.299	0.430	0.923
3 µM	7.7	8.9	13.9	1.398	1.868	0.672
10 µM	8.2	9.1	11.4	2.606	0.547	1.570
100 µM	11.4	10.2	13.3	2.883	1.157	2.043
1000 µM	11.6	11.1	11.6	1.327	3.614	1.790

***** 5 independent experiments (triplicates)

Data from Figure 5-10: Effect of 2-E-3-MP and 2MP on HO-activity in HepG2 cells

	2-MP (Mean*)	SD
Control	4.0	1.327
PDTC 1mM	39.8	6.005
0.1 µM	4.53	0.520
0.3 µM	5.27	0.842
1 µM	4.45	0.536
3 µM	3.89	1.862
10 µM	5.83	1.613
100 µM	6.94	2.502
1000 µM	4.55	1.859

	2-E-3-MP (Mean*)	SD
Control	4.0	1.327
PDTC 1mM	39.8	6.005
0.1 µM	6.54	1.862
0.3 µM	6.60	1.859
1 µM	6.45	1.613
3 µM	6.10	0.520
10 µM	5.83	0.536
100 µM	5.80	0.625
1000 µM	4.65	0.842

***** 5 independent experiments (triplicates)

Data from Figure 5-11: Effect of AB1 and AB2 coffee extracts on HO-activity in HepG2 cells

	(Mean*)	SD
Control	4.0	1.327
PDTC 1mM	39.8	6.005
AB 1 10µg/l	7.8	0.604
AB 1 100µg/l	10.0	0.715
AB 1 1mg/l	9.2	0.684
AB 1 10mg/l	12.0	1.203
AB 1 100mg/l	15.2	1.920
AB 2 1µg/l	8.6	0.986
AB 2 10µg/l	10.0	1.286
AB 2 100µg/l	10.5	1.792
AB 2 1mg/l	14.9	2.384
AB 2 10mg/l	13.0	2.118

***** 3 independent experiments (triplicates)

Data from Figure 5-12: Modulation of cAMP-phosphodiesterase activity by coffee constituents and caffeine metabolites in LXFL529L cell lysates.

Concentration (mM)	(Mean*)	SD	p-value	Compound
Control (Water)	100.0	0		
0.01	34.3	4.808	1.17935E-13	Rolipram
0.1	91.7	3.374	0.069715851	Caffeine
0.5	79.6	8.342	9.61E-03	
1.0	68.0	9.607	4.00806E-07	
5.0	52.0	2.800	6.20199E-14	
0.1	79.6	4.009	1.24106E-24	Teophylline
0.5	48.7	1.511	6.66436E-12	
1.0	40.1	4.383	1.16802E-16	
5.0	22.4	0.067	1.82368E-14	
0.1	86.7	7.129	0.001984479	Paraxanthine
0.5	68.9	0.061	0.000768775	
1.0	58.9	6.856	1.04194E-09	
5.0	32.8	1.136	8.23519E-05	
0.1	93.5	3.047	0.070380067	NMP
0.5	86.4	8.007	0.05382831	
1.0	93.4	18.647	0.375962848	
5.0	97.4	19.199	0.605257644	

***** 5 independent experiments (triplicates)

Data from Figure 5-13: Modulation of cAMP-phosphodiesterase activity by pyrazines in LXFL529L cell lysates.

Concentration (mM)	(Mean*)	SD	Compound
0.01	30.5	8.208	Rolipram
0.1	100.9	7.475	2-E-3,5-DMP
0.5	97.2	10.782	
1.0	78.3	7.096	
5.0	49.2	9.643	
0.1	109.0	13.268	2-MP
0.5	103.8	8.856	
1.0	103.8	8.610	
5.0	99.4	7.219	
0.1	108.6	10.069	2,3,5-TMP
0.5	106.9	14.524	
1.0	98.6	9.477	
5.0	76.9	1.604	
0.1	109.4	16.799	2,3-DMP
0.5	104.8	15.412	
1.0	104.0	12.586	
5.0	93.0	6.155	
0.1	118.1	1.461	2,5-DMP
0.5	113.0	2.199	
1.0	105.9	10.728	
5.0	103.4	12.063	
0.1	82.2	0.394	TMP
0.5	61.8	1.924	
1.0	58.6	4.204	
5.0	44.0	7.281	
0.1	79.5	0.892	2,3-DE-6-MP
0.5	35.0	10.762	
1.0	30.8	10.327	
5.0	8.9	5.676	
0.1	98.2	0.876	Pyrazine
0.5	96.5	7.006	
1.0	94.8	8.814	
5.0	85.4	11.424	
0.1	101.6	1.700	2-E-5-MP
0.5	98.8	4.300	
1.0	80.4	2.419	
5.0	46.9	3.741	
0.1	109.9	2.399	2,6-DMP
0.5	98.8	2.777	
1.0	102.0	10.606	
5.0	75.7	5.397	
0.1	106.4	2.907	2-Isobutyl-3-methoxyP
0.5	51.6	0.217	
1.0	44.2	3.583	
5.0	20.0	4.449	
0.1	93.2	0.100	2-E-3-MP
0.5	92.1	15.269	
1.0	80.3	8.571	
5.0	69.5	5.733	

***** 4 to 5 independent experiments (triplicates)

Data from Figure 5-14: Modulation of cAMP-phosphodiesterase activity by coffee extracts in LXFL529L cell lysates.

Concentration (µg/mL)	(Mean*)	SD	
Rolip.	30.5	8.200	Rolipram
0.10	87.4	5.320	
0.25	78.0	7.012	
0.50	58.8	1.939	
1.00	45.9	4.551	
5.00	30.1	0.929	AB1
0.10	87.0	8.451	
0.25	55.6	6.543	
0.50	44.0	7.137	
1.00	27.6	0.081	
5.00	15.3	7.656	AB2
0.10	101.4	0.627	
0.25	95.6	1.843	
0.50	78.1	0.857	
1.00	69.6	2.427	
5.00	45.1	0.860	Caffè crema
0.10	101.1	1.237	
0.25	91.7	1.132	
0.50	79.9	1.347	
1.00	70.1	2.448	
5.00	41.6	2.532	Caffeine reduced coffee
0.10	100.4	1.207	
0.25	88.9	2.331	
0.50	84.7	5.452	
1.00	54.2	11.194	
5.00	26.2	5.490	ECE
0.10	70.6	0.489	
0.25	91.3	2.447	
0.50	85.5	2.006	
1.00	100.9	1.540	
5.00	58.0	1.187	RCE

***** 4 to 5 independent experiments (triplicates)

Data from Figure 5-15: Profile of phosphodiesterase activity in human platelets lysates

Concentration (µM)	(Mean*)	SD	Modulator
10	91,4	11,442	Rolipram
50	88,1	6,444	Rolipram
10	38,6	2,909	Millrinone
50	21,4	7,002	Ca^{+}/CaM
0.2	97,0	1,387	Zaprinast

***** 11 independent experiments (triplicates)

Data from Figure 5-16: Modulation of cAMP-phosphodiesterase activity by coffee constituents and caffeine metabolites in platelet lysates

Concentration (mM)	(Mean*)	SD	p-value	Compound
0.01	38.6	2.199	7.17001E-08	Milrinone
0.1	92.6	6.367	0.116167061	
0.5	60.4	4.672	0.000522629	
1	39.7	9.081	0.001076313	
5	0.2	0.109	4.18809E-10	Caffeine
0.1	80.96	9.248	0.023479704	
0.5	58.74	15.399	0.02434035	
1	52.21	7.659	0.001295146	
5	27.79	9.818	0.000796972	Teophylline
0.1	88.96	7.951	0.073914262	
0.5	63.48	25.537	0.068408599	
1	63.22	4.408	0.014043106	
5	57.40	8.440	0.025672167	Paraxanthine
0.1	98.1	2.453	0.243007458	
0.5	98.5	2.133	0.301717985	
1	100.1	5.556	0.981927881	
5	100.3	7.438	0.948172101	NMP

***** 5 independent experiments (triplicates)

Data from Figure 5-17: Modulation of cAMP-phosphodiesterase activity by pyrazines in platelet lysates

Concentration (mM)	(Mean*)	SD	p-value	Compound
0.01	38.6	2.910	7.17001E-08	
0.1	95.6	1.998	0.158044690	
0.5	67.4	2.863	0.001276213	
1	52.6	2.031	0.001831163	
5	26.4	12.005	0.025622810	2-Isobutyl-3-methoxyP
0.1	81.8	13.882	0.320735203	
0.5	61.7	17.361	0.015817001	
1	53.2	14.364	0.008274873	
5	28.0	10.279	0.019791668	2-E-3,5 DMP
0.1	81.8	5.531	0.081618549	
0.5	45.7	11.758	0.038962693	
1	30.7	14.838	0.028843090	
5	13.6	12.922	0.001660515	2,3-DE-5-MP
0.1	93.3	0.400	0.871446791	
0.5	80.8	0.180	0.000423986	
1	63.4	0.150	0.001226800	
5	42.8	0.200	0.001340374	TMP

***** 5 independent experiments (triplicates)

Data from Figure 5-18: Modulation of cAMP-phosphodiesterase activity by coffee extracts in platelet lysates.

Concentration (µg/mL)	(Mean*)	SD	Compound
Milrin.	38.6	2.910	Milrinone
0.10	91.4	2.295	
0.25	59.0	25.731	
0.50	50.3	8.148	
1.00	26.5	10.507	
5.00	8.5	1.586	AB1
0.10	81.8	1.336	
0.25	50.3	2.777	
0.50	34.2	1.427	
1.00	21.5	5.667	
5.00	2.6	0.636	AB2
0.10	102.8	0.461	
0.25	89.9	0.106	
0.50	79.2	2.672	
1.00	64.9	0.932	
5.00	45.7	1.831	Caffé crema
0.10	99.0	3.357	
0.25	85.8	1.792	
0.50	75.1	2.007	
1.00	63.8	5.942	
5.00	46.3	0.565	Caffeine reduced coffee
0.10	86.9	0.556	
0.25	79.9	1.076	
0.50	66.5	4.547	
1.00	57.9	3.283	
5.00	43.3	4.862	SCE
0.10	58.4	0.032	
0.25	46.5	3.198	
0.50	47.3	1.733	
1.00	48.5	8.340	
5.00	41.4	9.577	RCE

***** 4 to 5 independent experiments (triplicates)

Data from Figure 5-19: Modulation of cAMP-phosphodiesterase activity by coffee polyphenols in platelet lysates.

Concentration (mM)	(Mean*)	SD	p-value	Compound
0.01	38.6	2.910	0.000134393	
0.1	64.8	16.473	0.054527628	
0.5	45.7	0.897	1.04365E-07	
1.0	35.2	7.528	7.95255E-05	
5.0	36.3	9.828	0.006396169	Chlorogenic Acid
0.1	57.1	2.094	3.23322E-06	
0.5	46.6	3.904	0.001498772	
1.0	44.3	5.884	0.003050773	
5.0	34.7	1.221	1.08757E-07	Caffeic acid

***** 3 independent experiments (triplicates)

Data from Figure 5-21: cAMP Hydrolisis activity of Rat adipocytes and SGBS cells.

	(Mean*)	SD
Rat Adipocytes (ground activity)	6.6	0.159
10 µM Rolipram	3.8	1.779
50 10 µM Rolipram	1.9	0.487
10 µM Milrinone	6.1	0.099
50 µM Milrinone	3.4	1.306
SGBS Preadipocytes (ground activity)	13.3	1.520
50 µM Rolipram	13.1	0.595
50 µM Milrinone	2.8	1.890
SGBS Adipocytes (Day 5)	1.6	1.005
SGBS Adipocytes (Day 10)	1.2	0.500
SGBS Adipocytes (Day 20)	1.4	1.000
SGBS Adipocytes (Day 30)	1.6	0.500

***** 5 independent experiments (triplicates)

Data from Figure 5-22. Modulation of cAMP-phosphodiesterase activity by coffee consumption in platelets of volunteers during the course of the trial.

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*	7.BC*	8.BC*
P1	45.13	64.62	6.15	7.45	9.90	73.99	76.05	78.06
P3	38.95	75.16	6.03	6.95				
P4	56.15	71.44	6.73	7.08	7.30	75.16	54.92	83.51
P5	34.92	72.90	4.44	7.54	9.45	64.62	73.44	86.93
P6	31.25	69.97	6.26	8.59	11.10	62.87	72.32	77.72
P7	36.13	73.99	4.15	6.62	10.16	69.97	86.33	76.81
P8	31.71	83.72	6.46	8.03	8.48	71.44	71.32	80.82
P9	34.06	79.96	6.60	5.57	9.79	72.90	90.86	91.71
P10	23.85	62.87	9.43	7.16	9.49	83.72	79.30	80.42
P11	57.29	72.22						

* Values are means of triplicate measurements

Data Summary	1. BE	2.BE	3. BE	4. BE	5.BE	6.BE	7.BE	8.BE
Mean	38,9	72,7	6,3	7,2	9,5	71,8	75,6	82,0
SD	9,250	6,641	1,509	0,856	1,141	6,482	10,845	5,150
F-test	0,05876398		0,00025333	3,4309E-06	8,7766E-05	0,45082132	0,06408611	0,309816449
Student´s t-test p-Value vs. 2.BC	6,5253E-07		2,1799E-16	2,1027E-16	0,00034514	0,78247733	0,48865774	0,003784551
	***		***	***	***			**

Data from Figure 5-23. Modulation of cAMP-phosphodiesterase activity by coffee consumption in platelets of volunteers during the course of the long term trial.

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*
P1	48,8	70,9	7,1	35,2	44,5	70,5
P2	46,5	47,5	15,6	56,6	39,7	68,5
P3	53,8	43,3	11,5	38,5	31,0	44,1
P4	52,2	42,2	16,4	58,0	30,8	58,3
P5	47,6	87,0	8,6	57,6	44,3	85,2
P6	56,1	44,4	14,6	58,4	37,9	29,7
P7	34,9	43,4	12,8	67,3	34,1	45,5
P8	57,1	53,8	12,2	58,1	30,4	40,7
P9	87,5	45,1	20,4	55,4	59,2	63,1
P10	56,6	37,9	11,4	57,9	35,4	56,5
P11	68,1	40,6	13,6	47,8	25,1	60,6
P12	61,6	45,0	12,2	56,8	42,7	67,6
P13	64,9	36,8	-	-	-	-
P14	54,5	43,7	13,7	61,3	35,7	51,2
P15	57,4	48,4	19,9	71,4	44,6	42,9
P17	56,8	42,5	12,8	50,7	43,4	66,7
P18	54,3	37,8	18,9	78,3	52,3	60,8
P19	64,1	48,4	14,8	50,3	55,2	62,7
P20	65,2	51,2	47,0	47,8	41,6	66,3
P21	53,0	38,7	16,1	59,3	43,2	33,0
P22	56,8	52,9	30,2	69,2	49,5	38,1
P23	70,2	46,5	21,7	50,3	43,0	67,9
P24	79,3	60,0	15,3	46,4	28,8	65,0
P25	61,2	46,8	16,1	27,8	38,2	49,4
P26	45,6	49,6	11,5	58,5	29,2	41,3
P27	45,8	-	-	-	-	-
P28	71,7	50,1	15,4	38,4	45,3	40,0
P29	58,1	35,0	12,9	59,3	26,5	49,4
P30	58,0	47,2	29,4	62,9	27,3	56,0
P31	49,4	46,0	10,1	80,0	7,0	58,6
P32	52,6	55,2	12,1	20,9	38,2	74,3
P33	72,0	42,3	8,1	53,2	34,7	59,8
P34	66,5	45,8	11,9	51,6	32,2	77,2
P35	93,0	41,0	-	-	-	-
P36	50,2	35,7	11,8	84,9	31,4	62,4
P37	53,0	57,5	13,0	39,8	22,8	44,5
P38	47,8	26,1	11,6	85,6	26,9	44,6
P39	78,3	49,9	14,4	34,2	30,0	59,0
P40	54,1	45,3	10,4	58,4	38,7	39,0
P41	68,0	38,5	13,8	55,6	31,7	48,7
P42	56,8	63,1	8,7	54,9	32,8	32,2
P43	90,1	41,2	16,2	67,9	40,2	66,4
P44	57,8	35,5	11,0	56,2	65,1	52,4
P45	35,6	47,3	13,8	63,2	31,3	50,9
P46	51,9	37,4	13,3	73,2	48,1	43,9
P47	78,3	-	-	-	-	-
P48	56,6	62,5	15,1	62,1	49,7	61,0
P49	57,3	39,8	10,3	59,1	92,4	59,1
P50	58,1	33,9	15,7	52,6	68,4	58,1

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*
P51	53,3	42,7	9,5	63,1	96,9	42,0
P52	47,7	68,3	17,9	60,3	58,5	33,0
P53	77,5	48,5	10,5	62,7	65,2	53,0
P54	51,9	40,0	-	-	-	-
P55	36,0	61,1	13,5	53,5	101,4	55,8
P56	63,3	37,7	4,5	40,9	46,4	46,7
P57	29,4	27,3	13,6	66,5	68,0	54,1
P58	36,2	63,9	12,1	67,6	59,5	38,1
P60	37,6	32,7	2,7	56,6	55,2	64,1
P61	37,6	45,6	21,2	51,7	85,1	60,1
P62	55,0	48,6	18,2	65,1	46,8	48,3
P63	46,4	54,2	4,9	52,6	99,0	32,6
P64	41,5	42,3	7,1	20,1	81,9	32,2
P65	62,8	50,5	10,6	51,1	61,9	39,2
P66	48,8	39,8	3,5	50,2	55,5	33,9
P67	49,4	37,6	7,8	36,3	55,5	45,1
P68	55,0	41,2	10,2	96,9	62,5	47,4
P69	73,3	53,6	1,6	60,1	68,8	48,9
P70	45,0	39,3	8,5	68,8	91,0	49,3
P71	39,8	55,2	9,9	70,2	55,2	45,8
P72	31,3	42,6	4,2	28,2	71,7	49,6
P73	32,5	39,8	12,9	75,4	64,8	50,4
P74	34,5	56,0	10,1	79,3	68,3	47,5
P75	33,9	46,9	7,6	67,1	59,9	45,8
P76	41,7	46,0	10,8	79,9	62,4	41,2
P77	40,8	64,7	9,4	48,0	48,2	36,8
P78	50,6	34,4	14,4	55,5	53,0	56,5
P79	63,2	33,1	9,7	60,7	59,3	44,8
P80	67,4	51,6	6,6	71,3	50,5	46,4
P81	103,6	40,3	5,7	76,0	47,5	32,7
P82	65,5	47,2	3,0	78,1	54,7	40,7
P83	58,6	35,1	4,5	61,3	48,5	50,1
P84	59,7	41,9	10,6	67,9	62,9	44,3
P85	61,9	38,0	9,8	92,7	56,9	41,8
P86	81,3	55,0	7,1	82,9	43,3	45,3
P87	60,0	38,8	5,9	60,2	52,9	43,4
P88	49,6	30,5	3,4	57,3	44,6	32,9
P90	68,6	25,3	2,9	49,6	31,1	36,0
P91	80,8	25,1	5,6	68,4	59,7	39,2
P92	61,4	37,5	4,9	67,3	46,4	31,0

* Values are means of triplicate measurements

Normal Distribution Test

Anderson Darling Test auf Normalverteilung

Anzahl Werte	504
Mittelwert:	45,40
Standardabw:	20,42
Sortierte Originaldaten	

Prüfgrösse AD:	4,0623
Schwellwert SW [95%]:	0,7509
Signifikant	keine Normalverteilung

Deskriptive Statistik

	Group			Statistik	Standardfehler
Value	1,00	Mittelwert		44,9729	1,19478
		95% Konfidenzintervall des Mittelwerts	Untergrenze	42,6201	
			Obergrenze	47,3257	
		5% getrimmtes Mittel		44,8008	
		Median		46,5000	
		Varianz		368,294	
		Standardabweichung		19,19098	
		Minimum		4,90	
		Maximum		103,60	
		Spannweite		98,70	
		Interquartilbereich		25,25	
		Schiefe		-,108	,152
		Kurtosis		-,253	,302
	2,00	Mittelwert		45,8557	1,38116
		95% Konfidenzintervall des Mittelwerts	Untergrenze	43,1352	
			Obergrenze	48,5762	
		5% getrimmtes Mittel		45,7342	
		Median		48,4000	
		Varianz		469,271	
		Standardabweichung		21,66267	
		Minimum		1,60	
		Maximum		101,40	
		Spannweite		99,80	
		Interquartilbereich		25,55	
		Schiefe		-,206	,155
		Kurtosis		-,175	,309

Tests auf Normalverteilung

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistik	df	Signifikanz	Statistik	df	Signifikanz
Value	1,00	,071	258	,003	,979	258	,001
	2,00	,078	246	,001	,967	246	,000

a. Signifikanzkorrektur nach Lilliefors

Wilcoxon test for the analysis of significances between time points (Group A)

Wilcoxon-Test

Ränge

		N	Mittlerer Rang	Rangsumme
SecBC - FirstBC	Negative Ränge	34[a]	23,69	805,50
	Positive Ränge	9[b]	15,61	140,50
	Bindungen	0[c]		
	Gesamt	43		
ThrBC - SecBC	Negative Ränge	43[d]	22,00	946,00
	Positive Ränge	0[e]	,00	,00
	Bindungen	0[f]		
	Gesamt	43		
FourBC - ThrBC	Negative Ränge	0[g]	,00	,00
	Positive Ränge	43[h]	22,00	946,00
	Bindungen	0[i]		
	Gesamt	43		
FifthBC - FourBC	Negative Ränge	37[j]	24,35	901,00
	Positive Ränge	6[k]	7,50	45,00
	Bindungen	0[l]		
	Gesamt	43		
SixBC - FifthBC	Negative Ränge	10[m]	11,95	119,50
	Positive Ränge	33[n]	25,05	826,50
	Bindungen	0[o]		
	Gesamt	43		

Statistik für Test[c]

	SecBC - FirstBC	ThrBC - SecBC	FourBC - ThrBC
Z	-4,015[a]	-5,712[a]	-5,711[b]
Asymptotische Signifikanz (2-seitig)	,0000594379	,0000000112	,0000000112

Statistik für Test[c]

	FifthBC - FourBC	SixBC - FifthBC
Z	-5,168[a]	-4,269[b]
Asymptotische Signifikanz (2-seitig)	,0000002363	,0000196727

a. Basiert auf positiven Rängen.
b. Basiert auf negativen Rängen.
c. Wilcoxon-Test

Wilcoxon test for the analysis of significances between time points (Group B)

Ränge

		N	Mittlerer Rang	Rangsumme
SecBC - FirstBC	Negative Ränge	27[a]	23,46	633,50
	Positive Ränge	14[b]	16,25	227,50
	Bindungen	0[c]		
	Gesamt	41		
ThrBC - SecBC	Negative Ränge	41[d]	21,00	861,00
	Positive Ränge	0[e]	,00	,00
	Bindungen	0[f]		
	Gesamt	41		
FourBC - ThrBC	Negative Ränge	0[g]	,00	,00
	Positive Ränge	41[h]	21,00	861,00
	Bindungen	0[i]		
	Gesamt	41		
FifthBC - FourBC	Negative Ränge	23[j]	19,39	446,00
	Positive Ränge	18[k]	23,06	415,00
	Bindungen	0[l]		
	Gesamt	41		
SixBC - FifthBC	Negative Ränge	32[m]	24,34	779,00
	Positive Ränge	9[n]	9,11	82,00
	Bindungen	0[o]		
	Gesamt	41		

Statistik für Test[c]

	SecBC - FirstBC	ThrBC - SecBC	FourBC - ThrBC	FifthBC - FourBC
Z	-2,631[a]	-5,579[a]	-5,579[b]	-,201[a]
Asymptotische Signifikanz (2-seitig)	,0085240698	,0000000242	,0000000242	,8408073530

Statistik für Test[c]

	SixBC - FifthBC
Z	-4,516[a]
Asymptotische Signifikanz (2-seitig)	,0000063008

a. Basiert auf positiven Rängen.
b. Basiert auf negativen Rängen.
c. Wilcoxon-Test

Mann-Withney test for the analysis of significances between groups

Mann-Whitney-Test

Ränge

	Type	N	Mittlerer Rang	Rangsumme
firstBC	1,00	43	47,91	2060,00
	2,00	41	36,83	1510,00
	Gesamt	84		
secondBC	1,00	43	45,58	1960,00
	2,00	41	39,27	1610,00
	Gesamt	84		
thirdBC	1,00	43	54,27	2333,50
	2,00	41	30,16	1236,50
	Gesamt	84		
fourBC	1,00	43	38,97	1675,50
	2,00	41	46,21	1894,50
	Gesamt	84		
fiveBC	1,00	43	25,57	1099,50
	2,00	41	60,26	2470,50
	Gesamt	84		
sixBC	1,00	43	47,91	2060,00
	2,00	41	36,83	1510,00
	Gesamt	84		

Statistik für Test[a]

	firstBC	secondBC	thirdBC	fourBC
Mann-Whitney-U	649,000	749,000	375,500	729,500
Wilcoxon-W	1510,000	1610,000	1236,500	1675,500
Z	-2,081	-1,186	-4,528	-1,360
Asymptotische Signifikanz (2-seitig)	,0374645866	,2357213280	,0000059438	,1737511768

Statistik für Test[a]

	fiveBC	sixBC
Mann-Whitney-U	153,500	649,000
Wilcoxon-W	1099,500	1510,000
Z	-6,515	-2,081
Asymptotische Signifikanz (2-seitig)	,0000000001	,0374674812

a. Gruppenvariable: Type

Data from Figure 5-24. Determination of the optimal HEK-293 cell number

cAMP concentration	Lance Signal (665nm) Mean*	SD
1,0E-06	5587	75,0
3,0E-07	6532	298,4
1,0E-07	8928	399,5
3,0E-08	16030	685,9
1,0E-08	26160	56,6
3,0E-09	39474	1364,0
1,0E-09	51684	1200,7
3,0E-10	58976	33,2
1,0E-10	60615	456,8
3,0E-11	63763	1877,4
1,0E-11	62907	2957,8
0,0E+00	62462	1300,4

* Values are means of triplicate measurements

Data from Figure 5-25. Forskolin Dose-Response of SGBS preadipocytes and adipocytes at the 17th day of differentiation.

Forskolin concentration (M)	Preadipocytes (6000 cells/well)			Mean	SD
3,00E-04	7938	7890	7818	7882	60,399
1,00E-04	8251	8054	7801	8035	225,580
3,00E-05	8494	7906	8071	8157	303,287
1,00E-05	8862	9122	7539	8508	848,903
3,00E-06	9847	8881	9296	9341	484,593
1,00E-06	11646	10493	10347	10829	711,586
3,00E-07	13765	11403	12928	12699	1197,584
1,00E-07	18366	18515	18140	18340	188,813
3,00E-08	24201	23298	22921	23473	657,766
1,00E-08	20493	30366	29520	29943	5472,333
3,00E-09	34987	28186	28612	34987	3809,543
1,00E-09	31759	31732	30152	31214	920,107
Forskolin concentration (M)	**Adipocytes at 17th day (6000 cells/well)**			**Mean**	**SD**
3,00E-04	43272	34947	55361	44527	10264,7
1,00E-04	39612	52914	49222	47249	6866,9
3,00E-05	62001	65612	65863	64492	2160,9
1,00E-05	77400	77950	75767	77039	1135,4
3,00E-06	73065	87692	90373	83710	9315,8
1,00E-06	108230	95202	86748	96727	10821,9
3,00E-07	96406	88941	95181	93509	4003,4
1,00E-07	78237	91546	99351	89711	10675,9
3,00E-08	94801	98469	101092	98121	3159,9
1,00E-08	98302	99829	99238	99123	770,0
3,00E-09	98999	96901	97694	97865	1059,4
1,00E-09	96692	95625	98209	96842	1298,5

Data from Figure 5-26. Modulation of the cAMP concentration in SGBS cells by caffeine, NMP, chlorogenic acid and trigonelline.

Adipos Tag 17	Mean*	SD	F-test	t-test
Caffeine (M)				
1,00E-03	75,13	0,329	0,44680715	2,25339E-09
5,00E-04	75,23	1,400	0,063823442	0,000167413
3,00E-04	87,69	26,225	0,000194271	0,007049088
1,00E-04	67,39	2,525	0,020528089	2,71784E-06
5,00E-05	52,61	6,215	0,003448144	0,000308473
3,00E-05	38,97	5,749	0,004027231	0,001046992
1,00E-05	30,45	3,525	0,010638843	0,000641464
5,00E-06	17,72	0,554	0,303027312	0,000351164
3,00E-06	12,00	0,385	0,474460421	0,012391297
1,00E-06	10,75	0,473	0,374079529	0,820635805
0,00E+00	10,67	0,366		
NMP (M)				
1,00E-03	14,36	1,098	0,005480879	0,004939244
5,00E-04	13,32	0,541	0,022166331	0,001315612
3,00E-04	13,19	0,448	0,031980517	0,000801967
1,00E-04	12,62	0,658	0,015093381	0,008795136
5,00E-05	13,52	0,714	0,012860341	0,002745785
3,00E-05	13,74	0,800	0,010255638	0,003133866
1,00E-05	12,28	2,128	0,001464156	0,291659916
5,00E-06	14,03	0,385	0,042877288	0,0001399
3,00E-06	11,03	0,280	0,078138659	0,283752154
1,00E-06	10,76	0,234	0,108462405	0,884927621
0,00E+00	10,79	0,081		
Chlorogenic Acid (M)				
1,00E-03	71,38	3,842	0,182872659	0,000131529
5,00E-04	48,73	1,031	0,243421304	6,01454E-05
3,00E-04	29,12	3,905	0,178029259	0,003774906
1,00E-04	14,64	3,061	0,260656196	0,062779737
5,00E-05	9,77	4,396	0,145976895	0,721074345
3,00E-05	11,50	2,178	0,410401461	0,161836827
1,00E-05	10,41	1,419	0,378805079	0,267732488
5,00E-06	8,93	1,944	0,466330973	0,883492185
3,00E-06	9,87	1,750	0,481145244	0,465575294
1,00E-06	10,45	2,397	0,365042677	0,368969939
0,00E+00	8,69	1,817		
Trigonelline (M)				
1,00E-03	30,93	1,570	0,137549199	0,000178708
5,00E-04	19,35	0,580	0,461103207	2,45277E-05
3,00E-04	11,85	0,363	0,251122914	0,003820679
1,00E-04	10,70	1,024	0,272885903	0,046260063
5,00E-05	10,30	0,352	0,239111502	0,020300965
3,00E-05	10,59	1,664	0,124425548	0,125002813
1,00E-05	10,52	0,822	0,368069621	0,024575484
5,00E-06	10,14	0,409	0,298371588	0,028505334
3,00E-06	10,85	1,113	0,241032519	0,045965925
1,00E-06	11,29	0,371	0,259486358	0,006491772
0,00E+00	8,42	0,627		

***** 5 independent experiments (triplicates)

Data from Figure 5-27. Modulation of cAMP concentration by coffee consumption in platelets of volunteers during the course of the trial

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*	7.BC*	8.BC*
P1	4,4	0,18	0,48	1,26	5,81	1,28	4,53	3,31
P3	1,2	0,51	1,13	--	--	--	--	--
P4	2,1	0,33	0,69	1,42	4,11	1,6	4,91	4,96
P5	1,7	0,14	1,43	1,48	3,07	1,29	5,11	4,69
P6	1,5	0,25	0,76	1,62	3,71	1,22	4,26	3,52
P7	1,1	0,04	0,61	0,95	4	1,71	4,81	2,72
P8	0,9	0,1	0,69	2,35	3,83	1,46	5,29	2,62
P9	1,1	0,33	1,18	1,39	4,24	1,04	6,34	3,59
P10	1,6	0,18	0,48	1,52	1,93	1,17	4,06	3,02
P11	1,3	0,20	0,24	0,44				

* Values are means of triplicate measurements

Data Summary	1. BE	2.BE	3. BE	4. BE	5.BE	6.BE	7.BE	8.BE
Mean	1,7	0,2	0,8	1,5	3,8	1,3	4,9	3,6
SD	1,009	0,137	0,337	0,399	1,095	0,225	0,711	0,860
F-test	9,2352E-07		0,007145477	0,001659066	6,02206E-07	0,138985611	0,043033767	3,06832E-06
Student´s t-test p-Value vs. 2.BC	0,000241188		7,22601E-05	1,97103E-07	1,56616E-08	1,18955E-07	3,13083E-14	9,92795E-09
	***		***	***	***	***	***	***

Data from Figure 5-28. Modulation of the cAMP concentration in platelets of volunteers during the course of the long term trial.

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*
P1	3,51	4,34	3,36	9,83	2,38	2,29
P2	3,40	2,64	5,78	10,13	2,84	3,01
P3	3,78	3,64	3,26	7,13	2,95	3,04
P4	2,29	5,10	3,40	14,35	3,72	5,77
P5	3,25	4,05	4,30	10,45	2,29	2,53
P6	2,43	3,49	6,10	6,51	1,37	1,44
P7	2,73	2,64	4,31	7,37	2,44	2,34
P8	2,31	3,36	4,79	10,57	1,94	4,99
P9	2,96	3,41	2,92	9,51	2,45	3,72
P10	4,69	3,67	4,61	8,20	2,24	0,74
P11	2,85	4,41	4,02	6,09	3,16	4,54
P12	4,41	4,38	4,33	11,36	4,34	8,14
P13	4,25	3,53	-	-	-	-
P14	2,58	3,91	3,95	7,24	4,02	3,34
P15	2,92	4,49	6,03	10,15	4,18	1,73
P17	4,59	2,06	4,54	10,43	2,43	3,93
P18	3,89	2,81	4,37	5,88	3,56	8,18
P19	4,91	5,01	5,44	10,50	2,36	2,21
P20	3,54	5,91	5,39	10,83	2,27	3,68
P21	3,94	4,73	5,22	7,31	3,63	2,34
P22	3,47	4,85	5,11	11,05	3,55	4,26
P23	2,82	3,69	5,46	5,66	2,56	2,46
P24	2,51	3,07	4,47	13,85	2,61	2,87
P25	2,63	3,98	3,34	6,10	2,87	3,23
P26	4,22	3,29	5,26	11,11	2,77	3,54
P27	4,69	-	-	-	-	
P28	3,31	3,57	3,26	7,50	2,04	2,54
P29	4,91	3,43	8,54	10,35	3,33	3,16
P30	3,19	3,44	4,64	8,25	3,43	2,73
P31	4,32	1,76	3,88	7,34	2,34	3,27
P32	5,67	3,11	2,21	7,56	1,89	3,01
P33	2,39	2,46	5,62	11,58	1,63	2,65
P34	6,26	4,93	4,18	17,51	1,42	2,80
P35	4,62	3,38	-	-	-	-
P36	4,29	3,57	4,58	8,57	3,22	4,25
P37	4,68	3,82	9,78	7,16	3,60	3,23
P38	2,80	2,49	6,52	6,04	2,84	2,08
P39	3,39	3,73	4,56	5,45	1,76	3,60
P40	4,34	4,65	6,09	8,02	3,09	2,53
P41	5,29	3,89	4,34	10,97	3,21	4,05
P42	5,14	4,12	5,48	8,71	3,24	2,39
P43	5,10	5,91	5,04	7,09	2,97	3,23
P44	12,09	2,60	6,11	5,35	3,39	3,20
P45	5,96	3,44	6,64	12,54	4,31	5,86
P46	6,03	3,83	4,91	7,47	4,19	2,35
P47	4,63	-	-	-	-	-
P48	5,72	5,13	5,95	6,91	2,25	4,64
P49	6,99	3,65	5,30	6,78	2,38	4,83
P50	3,46	2,93	7,16	7,26	3,83	3,03

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*
P51	3,75	2,25	8,41	9,67	2,40	2,29
P52	8,75	3,50	5,19	7,73	3,77	1,81
P53	7,16	4,47	7,24	9,94	3,22	3,53
P54	5,45	4,06	-	-	-	-
P55	3,58	4,44	4,48	11,34	1,54	5,38
P56	5,27	2,34	4,87	7,01	1,74	3,34
P57	5,95	3,03	4,62	8,80	1,39	2,83
P58	3,27	5,12	3,75	9,59	0,93	2,64
P60	3,20	2,70	2,26	9,19	1,40	2,82
P61	4,50	1,22	3,29	10,32	3,55	2,88
P62	7,13	2,89	3,59	18,32	2,94	3,38
P63	0,52	2,20	4,74	15,41	0,52	1,43
P64	5,20	2,74	3,02	2,51	1,54	1,87
P65	6,30	2,75	1,98	7,78	1,41	2,16
P66	3,12	2,40	9,45	8,39	1,53	2,04
P67	4,29	3,17	3,87	8,62	1,30	2,77
P68	1,67	2,87	3,13	5,81	0,40	2,24
P69	2,84	2,10	3,04	9,60	1,41	1,51
P70	4,65	3,08	4,83	8,71	1,67	3,48
P71	5,32	1,40	4,63	9,80	1,13	2,76
P72	3,40	3,14	2,19	8,73	2,13	3,09
P73	1,82	3,35	2,78	10,18	1,49	3,43
P74	6,07	1,93	1,96	6,31	1,81	2,94
P75	6,07	2,73	2,67	10,58	0,65	3,30
P76	3,94	3,79	4,19	11,03	1,38	2,66
P77	2,90	2,50	3,44	7,32	1,08	2,85
P78	8,09	2,62	4,16	11,37	1,38	1,33
P79	2,83	2,18	3,65	7,60	0,56	3,53
P80	3,04	2,38	3,34	7,54	0,82	2,85
P81	3,13	3,32	3,43	11,87	1,16	2,15
P82	3,29	3,45	3,04	12,97	1,93	2,65
P83	4,37	4,28	3,93	7,60	0,80	3,56
P84	5,10	3,51	2,94	9,22	1,11	1,56
P85	2,88	2,84	3,56	11,20	1,03	2,28
P86	4,09	3,65	4,60	4,28	2,69	2,43
P87	6,36	3,03	5,00	14,17	1,74	2,94
P88	3,67	3,27	2,40	10,73	1,67	2,40
P90	2,59	1,88	1,67	7,67	1,43	1,85
P91	3,89	2,98	2,85	7,43	1,78	1,18
P92	3,42	1,91	2,87	8,12	1,20	1,81

* Values are means of triplicate measurements

Normal Distribution Tests

Anderson Darling Test auf Normalverteilung

Anzahl Werte	504
Mittelwert:	4,41
Standardabw:	2,77
Sortierte Originaldaten	

Prüfgrösse AD:	237,0145
Schwellwert SW [95%]:	0,7509
Signifikant	keine Normalverteilung

Deskriptive Statistik

	Group			Statistik	Standardfehler
Value	1,00	Mittelwert		4,6421	,16517
		95% Konfidenzintervall des Mittelwerts	Untergrenze	4,3169	
			Obergrenze	4,9674	
		5% getrimmtes Mittel		4,3906	
		Median		3,8000	
		Varianz		7,039	
		Standardabweichung		2,65306	
		Minimum		,74	
		Maximum		17,51	
		Spannweite		16,77	
		Interquartilbereich		2,54	
		Schiefe		1,712	,152
		Kurtosis		3,299	,302
	2,00	Mittelwert		4,1676	,18312
		95% Konfidenzintervall des Mittelwerts	Untergrenze	3,8069	
			Obergrenze	4,5282	
		5% getrimmtes Mittel		3,9057	
		Median		3,2450	
		Varianz		8,249	
		Standardabweichung		2,87210	
		Minimum		,40	
		Maximum		18,32	
		Spannweite		17,92	
		Interquartilbereich		2,86	
		Schiefe		1,647	,155
		Kurtosis		3,326	,309

Tests auf Normalverteilung

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistik	df	Signifikanz	Statistik	df	Signifikanz
Value	1,00	,167	258	,000	,838	258	,000
	2,00	,179	246	,000	,850	246	,000

a. Signifikanzkorrektur nach Lilliefors

Wilcoxon test for the analysis of significances between time points (Group A)

Wilcoxon-Test

Ränge

		N	Mittlerer Rang	Rangsumme
SecBC - FirstBC	Negative Ränge	20[a]	24,65	493,00
	Positive Ränge	23[b]	19,70	453,00
	Bindungen	0[c]		
	Gesamt	43		
ThrBC - SecBC	Negative Ränge	11[d]	13,14	144,50
	Positive Ränge	32[e]	25,05	801,50
	Bindungen	0[f]		
	Gesamt	43		
FourBC - ThrBC	Negative Ränge	3[g]	6,67	20,00
	Positive Ränge	40[h]	23,15	926,00
	Bindungen	0[i]		
	Gesamt	43		
FifthBC - FourBC	Negative Ränge	43[j]	22,00	946,00
	Positive Ränge	0[k]	,00	,00
	Bindungen	0[l]		
	Gesamt	43		
SixBC - FifthBC	Negative Ränge	16[m]	18,03	288,50
	Positive Ränge	27[n]	24,35	657,50
	Bindungen	0[o]		
	Gesamt	43		

Statistik für Test[c]

	SecBC - FirstBC	ThrBC - SecBC	FourBC - ThrBC
Z	-,242[a]	-3,967[b]	-5,470[b]
Asymptotische Signifikanz (2-seitig)	,8091632372	,0000728875	,0000000450

Statistik für Test[c]

	FifthBC - FourBC	SixBC - FifthBC
Z	-5,712[a]	-2,228[b]
Asymptotische Signifikanz (2-seitig)	,0000000112	,0258838132

a. Basiert auf positiven Rängen.
b. Basiert auf negativen Rängen.
c. Wilcoxon-Test

Wilcoxon test for the analysis of significances between time points (Group B)

Wilcoxon-Test

Ränge

		N	Mittlerer Rang	Rangsumme
SecBC - FirstBC	Negative Ränge	35[a]	21,34	747,00
	Positive Ränge	6[b]	19,00	114,00
	Bindungen	0[c]		
	Gesamt	41		
ThrBC - SecBC	Negative Ränge	13[d]	13,27	172,50
	Positive Ränge	28[e]	24,59	688,50
	Bindungen	0[f]		
	Gesamt	41		
FourBC - ThrBC	Negative Ränge	3[g]	3,33	10,00
	Positive Ränge	38[h]	22,39	851,00
	Bindungen	0[i]		
	Gesamt	41		
FifthBC - FourBC	Negative Ränge	41[j]	21,00	861,00
	Positive Ränge	0[k]	,00	,00
	Bindungen	0[l]		
	Gesamt	41		
SixBC - FifthBC	Negative Ränge	7[m]	11,36	79,50
	Positive Ränge	34[n]	22,99	781,50
	Bindungen	0[o]		
	Gesamt	41		

Statistik für Test [c]

	SecBC - FirstBC	ThrBC - SecBC	FourBC - ThrBC
Z	-4,101[a]	-3,343[b]	-5,449[b]
Asymptotische Signifikanz (2-seitig)	,0000410646	,0008275943	,0000000506

Statistik für Test [c]

	FifthBC - FourBC	SixBC - FifthBC
Z	-5,579[a]	-4,548[b]
Asymptotische Signifikanz (2-seitig)	,0000000242	,0000054047

a. Basiert auf positiven Rängen.
b. Basiert auf negativen Rängen.
c. Wilcoxon-Test

Mann-Withney test for the analysis of significances between groups

Ränge

	Type	N	Mittlerer Rang	Rangsumme
firstBC	1,00	43	38,36	1649,50
	2,00	41	46,84	1920,50
	Gesamt	84		
secondBC	1,00	43	51,28	2205,00
	2,00	41	33,29	1365,00
	Gesamt	84		
thirdBC	1,00	43	50,69	2179,50
	2,00	41	33,91	1390,50
	Gesamt	84		
fourBC	1,00	43	42,27	1817,50
	2,00	41	42,74	1752,50
	Gesamt	84		
fiveBC	1,00	43	55,73	2396,50
	2,00	41	28,62	1173,50
	Gesamt	84		
sixBC	1,00	43	46,26	1989,00
	2,00	41	38,56	1581,00
	Gesamt	84		

Statistik für Test[a]

	firstBC	secondBC	thirdBC	fourBC
Mann-Whitney-U	703,500	504,000	529,500	871,500
Wilcoxon-W	1649,500	1365,000	1390,500	1817,500
Z	-1,593	-3,378	-3,150	-,089
Asymptotische Signifikanz (2-seitig)	,1111810757	,0007296242	,0016329596	,9286949743

Statistik für Test[a]

	fiveBC	sixBC
Mann-Whitney-U	312,500	720,000
Wilcoxon-W	1173,500	1581,000
Z	-5,092	-1,445
Asymptotische Signifikanz (2-seitig)	,0000003543	,1483733488

a. Gruppenvariable: Type

Data from Figure 5-29. Intraassay (within-day) and interassay (between-day) variation of the adenosine quantification in plasma samples by HPLC-ESI-MS/MS.

	Mean*	**SD**
Ctrl	100	0,000
24h, blood dep, RT	120	3,579
24h, RT	103	1,254
24h, -80°C	98	3,887
48h, -80°C	105	4,541
96h, -80°C	113	2,155
144h, -80°C	127	1,117

***** 5 independent experiments (triplicates)

Data from Figure 5-30. Modulation of cAMP concentration by coffee consumption in platelets of volunteers during the course of the trial

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*	7.BC*	8.BC*
P1	47,7	12,7	12,5	8,1	7,2	17,0	11,8	15,0
P3	17,3	10,1	9,5					
P4	19,6	6,1	16,1	9,3	6,0	17,6	11,2	20,0
P5	16,6	16,3	10,0	6,1	4,0	12,2	6,1	17,2
P6	18,0	11,8	17,0	9,3	9,3	15,1	8,9	14,6
P7	34,8	8,2	11,2	4,6	4,0	14,6	7,3	15,3
P8	13,9	8,4	10,8	12,5	6,6	12,9	7,8	12,9
P9	17,6	15,7	11,5	12,1	14,7	14,5		
P10	17,6	6,4	13,6	9,2	7,5	14,8	14,3	13,8
P11	21,1	7,2						

* Values are means of triplicate measurements

Data Summary	1. BE	2.BE	3. BE	4. BE	5.BE	6.BE	7.BE	8.BE
Mean	22,43	10,30	12,47	8,89	7,41	14,90	9,62	15,53
SD	10,54625	3,71010	2,62422	2,68946	3,43654	1,96128	2,91589	2,39021
F-test	0,085981183		0,080501819	0,204202025	0,060550928	0,067328592	0,287037581	0,14835775
Student´s t-test p-Value vs. 2.BC	0,005594817		0,156567849	0,364514701	0,10691213	0,005129718	0,681503553	0,003023187
	***							**

Data from Figure 5-31. Modulation of adenosine plasma concentrations after (0, 1 and 2h) coffee consumption

Volunteer Nr.	2. BC 0h	2. BE after 1h	2. BE after 2h
P1	12,7	6,1	10,0
P3	10,1	8,0	9,4
P4	6,1	10,5	4,2
P5	16,3	4,0	2,0
P6	11,8	7,1	7,1
P7	8,2	6,5	4,0
P8	8,4	8,5	10,8
P9	15,7	17,0	11,9
P10	6,4	10,1	8,7
P11	7,2	10,6	9,0

Data from Figure 5-32. Modulation of adenosine plasma concentrations by coffee consum of volunteers during the course of the trial.

Proband Nr.	1.BE	2.BE	3.BE	4.BE	5.BE	6.BE
P1	42,0	10,8	17,8	17,4	19,4	22,3
P2	45,8	14,3	18,5	15,3	19,0	21,0
P3	35,9	9,7	14,0	10,4	10,4	15,3
P4	50,4	17,0	24,0	19,8	20,6	25,8
P5	52,0	9,4	24,4	21,0	22,7	27,8
P6	35,4	5,0	18,3	15,4	15,5	18,6
P7	36,1	37,7	16,0	13,6	13,3	15,7
P9	34,3	17,9	16,5	15,0	19,8	19,0
P10	33,6	11,1	14,3	11,2	13,0	17,2
P30	36,7	14,8	17,1	14,7	16,2	19,5
P46	38,5	22,3	18,0	19,7	17,7	23,0
P47	44,7	-	-	-	-	-
P48	36,9	14,3	19,4	18,7	15,4	20,2
P49	35,0	16,0	22,6	22,2	22,2	25,1
P50	35,5	16,3	20,2	19,7	17,3	21,6
P51	41,6	4,7	25,5	19,5	22,2	21,2
P52	30,8	17,3	15,3	14,2	15,3	17,8
P53	36,9	9,0	21,7	17,3	16,9	27,9
P55	35,1	7,3	21,6	16,9	18,7	22,8
P84	37,4	9,0	20,8	13,7	19,1	18,9

Normal Distribution Tests

Anderson Darling Test auf Normalverteilung

Anzahl Werte	114
Mittelwert:	21,14
Standardabw:	9,30
Sortierte Originaldaten	

Prüfgrösse AD:	52,5550
Schwellwert SW [95%]:	0,7470
Signifikant	keine Normalverteilung

Wilcoxon test for the analysis of significances between time points (Group A)

Ränge

		N	Mittlerer Rang	Rangsumme
secondBC - firstBC	Negative Ränge	10[a]	6,50	65,00
	Positive Ränge	1[b]	1,00	1,00
	Bindungen	0[c]		
	Gesamt	11		
thirdBC - secondBC	Negative Ränge	3[d]	4,67	14,00
	Positive Ränge	8[e]	6,50	52,00
	Bindungen	0[f]		
	Gesamt	11		
fourthBC - thirdBC	Negative Ränge	11[g]	6,00	66,00
	Positive Ränge	0[h]	,00	,00
	Bindungen	0[i]		
	Gesamt	11		
fiveBC - fourthBC	Negative Ränge	1[j]	2,00	2,00
	Positive Ränge	9[k]	5,89	53,00
	Bindungen	1[l]		
	Gesamt	11		
sixBC - fiveBC	Negative Ränge	1[m]	1,00	1,00
	Positive Ränge	10[n]	6,50	65,00
	Bindungen	0[o]		
	Gesamt	11		

Statistik für Test[c]

	secondBC - firstBC	thirdBC - secondBC	fourthBC - thirdBC
Z	-2,845[a]	-1,690[b]	-2,936[a]
Asymptotische Signifikanz (2-seitig)	,0044390927	,0910009235	,0033300139

Statistik für Test[c]

	fiveBC - fourthBC	sixBC - fiveBC
Z	-2,599[b]	-2,845[b]
Asymptotische Signifikanz (2-seitig)	,0093441130	,0044390927

Wilcoxon test for the analysis of significances between time points (Group B)

Ränge

		N	Mittlerer Rang	Rangsumme
secondBC - firstBC	Negative Ränge	8[a]	4,50	36,00
	Positive Ränge	0[b]	,00	,00
	Bindungen	0[c]		
	Gesamt	8		
thirdBC - secondBC	Negative Ränge	1[d]	2,00	2,00
	Positive Ränge	7[e]	4,86	34,00
	Bindungen	0[f]		
	Gesamt	8		
fourthBC - thirdBC	Negative Ränge	7[g]	4,57	32,00
	Positive Ränge	1[h]	4,00	4,00
	Bindungen	0[i]		
	Gesamt	8		
fiveBC - fourthBC	Negative Ränge	4[j]	3,50	14,00
	Positive Ränge	3[k]	4,67	14,00
	Bindungen	1[l]		
	Gesamt	8		
sixBC - fiveBC	Negative Ränge	2[m]	1,50	3,00
	Positive Ränge	6[n]	5,50	33,00
	Bindungen	0[o]		
	Gesamt	8		

Statistik für Test[d]

	secondBC - firstBC	thirdBC - secondBC	fourthBC - thirdBC
Z	-2,521[a]	-2,240[b]	-1,960[a]
Asymptotische Signifikanz (2-seitig)	,0117186856	,0250618443	,0499499765

Statistik für Test[d]

	fiveBC - fourthBC	sixBC - fiveBC
Z	,000[c]	-2,100[b]
Asymptotische Signifikanz (2-seitig)	1,0000000000	,0356919001

Mann-Withney test for the analysis of significances between groups

Ränge

	Type	N	Mittlerer Rang	Rangsumme
firstBC	1,00	11	9,91	109,00
	2,00	8	10,13	81,00
	Gesamt	19		
secondBC	1,00	11	11,05	121,50
	2,00	8	8,56	68,50
	Gesamt	19		
thirdBC	1,00	11	7,45	82,00
	2,00	8	13,50	108,00
	Gesamt	19		
fourBC	1,00	11	8,00	88,00
	2,00	8	12,75	102,00
	Gesamt	19		
fiveBC	1,00	11	9,00	99,00
	2,00	8	11,38	91,00
	Gesamt	19		
sixBC	1,00	11	8,00	88,00
	2,00	8	12,75	102,00
	Gesamt	19		

Statistik für Test[b]

	firstBC	secondBC	thirdBC	fourBC
Mann-Whitney-U	43,000	32,500	16,000	22,000
Wilcoxon-W	109,000	68,500	82,000	88,000
Z	-,083	-,950	-2,312	-1,817
Asymptotische Signifikanz (2-seitig)	,9341628248	,3419012672	,0207763659	,0691578024
Exakte Signifikanz [2*(1-seitig Sig.)]	,968[a]	,351[a]	,020[a]	,075[a]

Statistik für Test[b]

	fiveBC	sixBC
Mann-Whitney-U	33,000	22,000
Wilcoxon-W	99,000	88,000
Z	-,909	-1,817
Asymptotische Signifikanz (2-seitig)	,3635118076	,0692798832
Exakte Signifikanz [2*(1-seitig Sig.)]	,395[a]	,075[a]

a. Nicht für Bindungen korrigiert.
b. Gruppenvariable: Type

Data from Figure 5-33. Modulation of the adenosine deaminase activity by coffee consumption in plasma of volunteers during the course of the trial.

Volunteer Nr.	Start	1st WO	Coffee phase	2nd WO
P1	9,03	8,38	14,22	8,70
P2	9,03	7,73	8,86	10,81
P3	8,05	7,56	8,70	7,89
P4	7,89	7,40	9,51	8,21
P5	7,55	9,03	17,03	10,97
P6	6,10	7,73	11,14	11,95
P7	6,10	7,40	8,05	6,43
P8	8,05	10,49	9,35	8,86
P9	7,40	9,35	8,21	7,40
P10	7,89	10,65	9,51	8,05
P11	7,201	7,245	9,615	7,636
P12	7,158	6,679	15,594	12,354
P14	8,245	6,897	9,984	8,767
P15	8,767	9,615	17,898	13,811
P16	7,940	8,701	9,419	8,245
P17	8,680	6,636	9,897	6,723
P18	11,14	8,65	18,82	8,11
P19	9,40	8,27	13,35	12,87
P20	7,78	7,24	14,87	15,03
P21	6,27	7,65	19,90	11,41
P22	11,19	12,11	18,06	12,76
P23	10,16	9,40	12,49	9,73
P26	6,19	7,57	8,28	8,86
P27	6,11	6,57	7,28	6,03
P28	6,82	5,40	7,36	5,32
P29	5,36	4,82	6,11	4,86
P30	6,90	5,07	6,19	6,07
P31	5,15	4,90	6,49	5,90
P32	6,15	6,57	6,57	6,99
P33	6,47	6,83	6,05	6,05
P34	7,50	7,34	8,84	5,38
P35	11,05	11,21	17,14	9,82
Mean	7,77	7,85	11,09	8,81
SD	1,609	1,764	4,238	2,698
Student´s t-test p-Value vs. 2.BC	0,00026309		0,000175919	0,013295476

Data from Figure 5-34. Modulation of plasma adenosine deaminase activity by coffee consumption of volunteers during the course of the long term trial.

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*
P1	6,6	5,6	3,8	5,8	10,1	5,6
P2	10,3	8,8	5,7	9,0	10,6	8,4
P3	4,8	6,5	7,3	5,8	5,4	4,3
P4	8,8	11,4	9,7	10,8	7,4	6,2
P5	6,8	22,9	41,8	10,0	12,0	7,4
P6	7,9	7,3	8,4	8,2	10,7	8,6
P7	7,7	7,6	8,4	5,6	5,1	5,5
P8	10,1	7,9	9,3	7,5	6,8	5,8
P9	8,5	6,5	6,4	6,0	24,9	4,7
P10	8,6	10,0	6,5	8,5	10,9	6,4
P11	8,5	9,5	6,9	6,7	6,6	7,5
P12	8,2	9,3	7,9	7,2	9,7	5,8
P13	7,2	7,5	-	-	-	-
P14	12,1	8,1	13,2	4,8	9,1	4,2
P15	4,3	4,5	4,6	3,7	4,3	2,8
P17	5,2	5,5	6,2	5,7	5,7	4,8
P18	5,2	7,3	4,4	5,3	5,5	4,0
P19	11,3	4,5	3,1	6,0	5,4	5,3
P20	6,5	7,3	6,4	6,3	6,7	6,0
P21	5,7	6,0	5,8	4,4	4,9	4,0
P22	6,6	7,6	10,1	7,6	7,5	6,6
P23	7,2	6,4	5,3	4,4	6,4	3,8
P24	9,5	8,8	20,4	8,0	7,4	10,3
P25	5,6	4,9	4,9	3,3	4,1	3,7
P26	9,1	9,8	9,9	8,5	7,6	5,6
P27	9,0	-	-	-	-	-
P28	9,2	11,7	6,5	6,8	6,8	7,1
P29	9,0	5,7	5,4	6,1	5,2	3,6
P30	7,9	7,4	5,2	6,0	7,7	3,1
P31	15,4	9,0	6,9	7,7	6,5	3,9
P32	5,7	5,8	5,8	4,6	6,6	5,2
P33	4,7	6,1	5,8	4,4	7,1	4,6
P34	6,7	9,0	6,4	10,5	6,3	7,8
P35	8,3	5,6				
P36	6,1	9,3	11,0	7,2	2,6	6,3
P37	4,3	8,2	9,2	6,4	4,8	4,5
P38	7,2	9,3	7,6	4,9	4,6	4,9
P39	4,5	6,3	5,0	5,1	4,0	4,9
P40	7,4	10,5	8,6	6,7	6,1	4,3
P41	6,5	5,5	4,3	6,1	6,5	5,8
P42	6,9	8,3	10,3	4,8	11,7	6,7
P43	6,6	9,3	5,5	5,9	3,1	5,2
P44	7,2	9,9	9,4	4,7	4,5	7,5
P45	6,2	7,2	9,2	8,5	6,0	4,3
P46	4,0	6,3	9,2	8,2	4,2	3,8
P47	5,7	-	-	-	-	-
P48	4,2	5,3	6,8	2,9	4,8	4,5
P49	5,4	8,4	11,0	3,1	6,9	5,7
P50	5,0	7,4	4,8	4,5	6,7	4,9

Volunteer Nr.	1. BC*	2.BC*	3. BC*	4. BC*	5.BC*	6.BC*
P51	6,8	8,9	5,4	6,4	5,9	5,3
P52	6,4	7,7	5,3	4,1	4,4	3,9
P53	5,0	7,8	8,2	3,4	3,8	7,7
P54	5,9	4,5	-	-	-	-
P55	9,2	8,2	6,4	10,6	10,3	4,7
P56	4,9	6,0	5,6	4,1	5,2	4,2
P57	4,5	6,3	9,9	6,0	4,4	4,1
P58	5,1	4,0	11,4	3,9	6,7	4,8
P60	6,9	5,8	8,4	4,6	6,6	5,1
P61	4,7	6,9	5,3	6,4	5,6	3,3
P62	6,8	6,2	6,4	4,1	5,7	5,2
P63	5,0	7,9	8,2	4,7	6,8	5,2
P64	7,9	8,6	15,2	5,1	6,5	6,7
P65	8,6	9,7	5,5	10,9	9,7	8,2
P66	6,5	9,5	16,7	6,5	7,6	5,0
P67	6,4	12,5	12,5	7,2	7,9	6,7
P68	5,1	7,8	7,2	5,8	7,5	5,7
P69	6,5	9,1	10,6	13,6	6,1	6,1
P70	5,0	7,2	9,2	5,4	4,8	4,2
P71	6,9	9,6	6,8	3,3	4,6	5,0
P72	5,7	8,0	11,0	4,5	4,0	5,5
P73	5,8	7,8	4,8	4,3	6,1	6,0
P74	7,2	9,5	8,1	5,4	7,4	5,2
P75	7,7	8,3	8,9	5,3	6,2	4,8
P76	6,9	7,7	7,9	5,1	3,1	4,3
P77	9,1	11,6	8,4	4,2	4,5	5,9
P78	5,2	9,5	10,9	13,2	6,0	4,3
P79	5,3	8,4	10,0	6,9	4,2	4,0
P80	12,4	6,9	7,9	4,6	4,8	3,9
P81	8,0	7,3	10,7	4,9	6,9	4,9
P82	4,9	5,0	10,0	7,3	6,7	4,2
P83	5,7	9,9	9,7	3,7	5,9	7,4
P84	5,6	4,7	8,0	5,6	4,4	6,0
P85	6,6	8,9	13,4	4,3	3,8	5,5
P86	7,2	8,0	7,2	3,3	10,3	6,6
P87	6,6	10,5	9,2	5,1	5,2	4,9
P88	7,4	7,6	7,2	4,6	4,4	5,1
P90	7,7	9,4	9,8	5,5	6,7	6,0
P91	5,8	4,2	4,0	8,2	6,6	4,5
P92	7,8	8,4	5,0	3,1	5,6	4,9

* Values are means of triplicate measurements

Normal Distribution Tests

Anderson Darling Test auf Normalverteilung

Anzahl Werte	504
Mittelwert:	6,88
Standardabw:	2,99
Sortierte Originaldaten	

Prüfgrösse AD:	236,5505
Schwellwert SW [95%]:	0,7509
Signifikant	keine Normalverteilung

Deskriptive Statistik

	Group			Statistik	Standardfehler
Value	1,00	Mittelwert		6,8834	,16156
		95% Konfidenzintervall des Mittelwerts	Untergrenze	6,5653	
			Obergrenze	7,2016	
		5% getrimmtes Mittel		6,7175	
		Median		6,5000	
		Varianz		6,604	
		Standardabweichung		2,56975	
		Minimum		1,81	
		Maximum		24,90	
		Spannweite		23,09	
		Interquartilbereich		3,00	
		Schiefe		2,219	,153
		Kurtosis		11,534	,305
	2,00	Mittelwert		6,5367	,15004
		95% Konfidenzintervall des Mittelwerts	Untergrenze	6,2411	
			Obergrenze	6,8322	
		5% getrimmtes Mittel		6,3965	
		Median		6,0000	
		Varianz		5,651	
		Standardabweichung		2,37715	
		Minimum		1,18	
		Maximum		16,70	
		Spannweite		15,52	
		Interquartilbereich		3,10	
		Schiefe		1,049	,154
		Kurtosis		1,705	,306

Tests auf Normalverteilung

		Kolmogorov-Smirnov [a]			Shapiro-Wilk		
	Group	Statistik	df	Signifikanz	Statistik	df	Signifikanz
Value	1,00	,090	253	,000	,863	253	,000
	2,00	,101	251	,000	,942	251	,000

a. Signifikanzkorrektur nach Lilliefors

Wilcoxon test for the analysis of significances between time points (Group A)

Wilcoxon-Test

Ränge

		N	Mittlerer Rang	Rangsumme
SecBC - FirstBC	Negative Ränge	16[a]	20,47	327,50
	Positive Ränge	27[b]	22,91	618,50
	Bindungen	0[c]		
	Gesamt	43		
ThrBC - SecBC	Negative Ränge	26[d]	21,88	569,00
	Positive Ränge	16[e]	20,88	334,00
	Bindungen	1[f]		
	Gesamt	43		
FourBC - ThrBC	Negative Ränge	29[g]	23,40	678,50
	Positive Ränge	14[h]	19,11	267,50
	Bindungen	0[i]		
	Gesamt	43		
FifthBC - FourBC	Negative Ränge	20[j]	18,10	362,00
	Positive Ränge	21[k]	23,76	499,00
	Bindungen	2[l]		
	Gesamt	43		
SixBC - FifthBC	Negative Ränge	33[m]	23,00	759,00
	Positive Ränge	10[n]	18,70	187,00
	Bindungen	0[o]		
	Gesamt	43		

Statistik für Test [c]

	SecBC - FirstBC	ThrBC - SecBC	FourBC - ThrBC
Z	-1,758[a]	-1,469[b]	-2,482[b]
Asymptotische Signifikanz (2-seitig)	,0788274597	,1417137663	,0130700412

Statistik für Test [c]

	FifthBC - FourBC	SixBC - FifthBC
Z	-,888[a]	-3,455[b]
Asymptotische Signifikanz (2-seitig)	,3745548386	,0005510426

a. Basiert auf negativen Rängen.
b. Basiert auf positiven Rängen.
c. Wilcoxon-Test

Wilcoxon test for the analysis of significances between time points (Group B)

Wilcoxon-Test

Ränge

		N	Mittlerer Rang	Rangsumme
SecBC - FirstBC	Negative Ränge	7[a]	15,07	105,50
	Positive Ränge	34[b]	22,22	755,50
	Bindungen	0[c]		
	Gesamt	41		
ThrBC - SecBC	Negative Ränge	16[d]	17,94	287,00
	Positive Ränge	23[e]	21,43	493,00
	Bindungen	2[f]		
	Gesamt	41		
FourBC - ThrBC	Negative Ränge	34[g]	21,62	735,00
	Positive Ränge	7[h]	18,00	126,00
	Bindungen	0[i]		
	Gesamt	41		
FifthBC - FourBC	Negative Ränge	17[j]	19,44	330,50
	Positive Ränge	24[k]	22,10	530,50
	Bindungen	0[l]		
	Gesamt	41		
SixBC - FifthBC	Negative Ränge	30[m]	20,65	619,50
	Positive Ränge	10[n]	20,05	200,50
	Bindungen	1[o]		
	Gesamt	41		

Statistik für Test [c]

	SecBC - FirstBC	ThrBC - SecBC	FourBC - ThrBC
Z	-4,213[a]	-1,438[a]	-3,946[b]
Asymptotische Signifikanz (2-seitig)	,0000251936	,1504702619	,0000793487

Statistik für Test [c]

	FifthBC - FourBC	SixBC - FifthBC
Z	-1,296[a]	-2,817[b]
Asymptotische Signifikanz (2-seitig)	,1949054772	,0048493492

a. Basiert auf negativen Rängen.
b. Basiert auf positiven Rängen.
c. Wilcoxon-Test

Mann-Withney test for the analysis of significances between groups

Ränge

	Type	N	Mittlerer Rang	Rangsumme
firstBC	1,00	43	49,12	2112,00
	2,00	41	35,56	1458,00
	Gesamt	84		
secondBC	1,00	43	41,49	1784,00
	2,00	41	43,56	1786,00
	Gesamt	84		
thirdBC	1,00	43	36,40	1565,00
	2,00	41	48,90	2005,00
	Gesamt	84		
fourBC	1,00	43	48,22	2073,50
	2,00	41	36,50	1496,50
	Gesamt	84		
fiveBC	1,00	43	45,40	1952,00
	2,00	41	39,46	1618,00
	Gesamt	84		
sixBC	1,00	43	42,30	1819,00
	2,00	41	42,71	1751,00
	Gesamt	84		

Statistik für Test[a]

	firstBC	secondBC	thirdBC	fourBC
Mann-Whitney-U	597,000	838,000	619,000	635,500
Wilcoxon-W	1458,000	1784,000	1565,000	1496,500
Z	-2,547	-,389	-2,350	-2,202
Asymptotische Signifikanz (2-seitig)	,0108577678	,6969875242	,0187748843	,0276525790

Statistik für Test[a]

	fiveBC	sixBC
Mann-Whitney-U	757,000	873,000
Wilcoxon-W	1618,000	1819,000
Z	-1,115	-,076
Asymptotische Signifikanz (2-seitig)	,2650279535	,9393284325

a. Gruppenvariable: Type

Curriculum vitae

Gina Alejandra Montoya Parra

Nutritionist and Dietitian

Academic Track Record

04/2007 – 04/2012	PhD in food chemistry at the Kaiserslautern University, supervisor: Prof. Dr. G. Eisenbrand. Faculty of Chemistry. Kaiserslautern, Germany. Doctoral thesis: "*In vitro* and *in vivo* biofunctional effects of selected coffee compounds, extracts and brews on key elements of adenosine receptor-mediated signaling pathways and on cellular heme oxygenase".
01/2003 – 04/2005	Specialist Degree in Food Science and Technology at the National University of Colombia, supervisor: Prof. Dr. A. Bermudez. Faculty of Chemistry. Bogotá D.C. Colombia; specialization thesis: "Development of a functional beverage type flavoured milk with addition of soluble fiber for type 2 diabetic patients" (Meritorious mention).
01/1997 – 12/2001	Nutritionist and Dietitian at the National University of Colombia. Faculty of Medicine. Bogotá D.C., Colombia; BSc. Work: "Osmolality, osmolarity and chemistry characterization of drinks in Bogotá-Colombia" (Meritorious mention).
01/1983 – 12/1996	Instituto Colsubsidio de Educación Femenina. High School. Bogotá D.C., Colombia

Professional Experience

02/2005 – today	Lecturer at the Nutrition Faculty. University of Antioquia. Medellín, Colombia. At present in full-time commission for doctoral studies.
01/2004 – 12/2004	Teacher at the Nutrition Faculty. National University of Colombia. Bogotá D.C., Colombia.
04/2004 – 12/2004	Clinical nutritionist (dietary assistance to patients with a variety of health conditions, member of the team of pediatric enteral and parenteral nutrition) at the David Restrepo Clinic, Bogotá D.C., Colombia.
06/2003 – 12/2003	Nutritionist and Dietitian (Marketing/customer consulting) by Mead Johnson Nutritionals, Bristol Myers Squibb, Bogotá D.C., Colombia.
01/2003 – 05/2003	Nutritionist and Dietitian by Merisant (Monsanto): Marketing/ customer advisory for SABRO®, NUTRASWEET® und SUCARYL®, Bogotá D.C., Colombia.
04/2002– 12/ 2002	Nutritionist and Dietitian (public health) by DAMA (Departamento Administrativo del Medio Ambiente – Ministry of Environment), Universidad Nacional de Colombia, Bogotá D.C., Colombia.

Projects and Temporary Jobs

02/2011 – 03/2012	HiWi (Hilfswissenschaftler - Research assistant) at the Kaiserslautern University. Faculty of Chemistry Kaiserslautern. Dept. Food Chemistry & Toxicology. Kaiserslautern. Germany.
05/2004 – 04/2005	Research project in food technology at the Faculty of Chemistry, National University of Colombia. Bogotá D.C., Colombia. Topic: "Development of a functional beverage type flavoured milk with addition of soluble fiber for type 2 diabetic patients".
01/2000– 12/2001	Research project at the Department of Nutritional Sciences. National University of Colombia. Bogota DC, Colombia, Topic: "Physical and chemical characterization of non-alcoholic beverages at the market in Bogotá DC".

01/2000 – 12/2000 HiWi (Hilfswissenschaftler - Research assistant) at the National University of Colombia. Bogotá D.C., Colombia. Update of the database for the program Epi Info™ (for the national nutrition survey).

Language skills

Spanish: native speaker
English: fluent (spoken and written)
German: fluent (spoken and written)
Portuguese: intermediate

Scholarships

10/2006 – today DAAD (German Academic Exchange Service) PhD scholarship

01/2003 – 04/2005 **ICETEX-Scholarship** (Colombian Institute of Credit and Abroad Technical Studies)

Publications

Original Article

- Z. Fonseca, G. Montoya, O. Cobos. La osmolalidad, osmolaridad y otros parámetros fisicoquímicos en la caracterización de bebidas no alcohólicas disponibles en el comercio de Bogotá D. C. *Perspectivas en Nutrición Humana.* ***2002****, 7, 42-50.*

Reviews und Book Chapter

- A. Bermudez, G. Montoya, L. Valderrama. Book Chapter: Carbohidratos en alimentos regionales iberoamericanos. **Capítulo No. 26 Elaboración de Bebida Láctea con Pectina. Experiencia de Colombia. 2006**, 637-646.

- G. Montoya, A. Suaterna. Review: Modificaciones por técnicas de cocción casera de compuestos nutritivos y no nutritivos de importancia para la salud. *Perspectivas en Nutrición Humana.* ***2005****, 14,* 61-72.

Oral and Poster Presentations

- Regional meeting of the german society of food chemist. Southwest Regional Association. **Oral presentation**: "Modulation von Adenosin-Rezeptor vermittelten Signalwegen durch Kaffeekonsum in Human-Thrombozyten". Kaiserslautern, Germany, March 2012.

- Regional meeting of the german society of food chemist. Southwest Regional Association. **Poster**: "Coffee constituents as modulators of cAMP phosphodiesterases activity of human platelets and lung carcinoma cells". Kaiserslautern, Germany, March 2012.

- The 5th International Conference on Polyphenols and Health, **Poster:** "Coffee polyphenols modulating the activity of cAMP phosphodiesterases activity in human platelets", Sitges, Barcelona, Spain, October 2011.

- 2nd Luxembourgish Int. Nutrition Conference „Dietary Composition, Pattern and Health", **Poster**: "Modulation of cAMP-phosphodiesterase activity by coffee constituents in human platelets and in lung carcinoma cells", Mondorf-les Bains, Luxemburg, May 2011. **Best Poster Award.**

- 39th annual meeting of the german society of food chemist. **Poster**: „Modulation der cAMP-Phosphodiesterase-Aktivität durch Kaffeeinhaltsstoffe", Stuttgart-Hohenheim. September 2010.

- 38th annual meeting of the german society of food chemist. **Poster**: „Modulation der Hämoxygenase-Aktivität durch Kaffeeinhaltsstoffe in Caco2, HepG2 und MonoMac6 Zellen", Berlin, September 2009.

- 10th national symposium on human nutrition. University of Antioquia**,** *Oral Presentation:* **"**Changes in the content and bioavailability of nutrients during cooking", Medellín, Colombia, November 2005.

- First international and second national symposium about Food and Nutrition Research. *Oral Presentation:* "Physical and chemical characterization of non-alcoholic beverages at the market in Bogotá DC". Medellín, Colombia, August 2002.

Trainings and Meetings

- 59. Meeting of Nobel Laureates, dedicated to Chemistry (worldwide young scientists competition), Lindau, July 2009.

- Course of Food Toxicology. Society of Toxicology (DGPT-Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie), Kaiserslautern, October 2009.

- EFSA (*European Food Safety Authority*) Meeting**:** "Technical meeting with stakeholders on recent developments related to health claims", Parma, Italien, October 2010.

Declaration

Hiermit erkläre ich an Eides statt, dass ich die eingereichte Dissertation selbstständig verfasst, die für die Arbeit benutzen Hilfsmittel genannt habe und die Ergebnisse beteiligter Mitarbeiter sowie anderer Autoren klar gekennzeichnet habe. Ich habe weder die Dissertation oder Teile der Dissertation als Prüfungsarbeit bei einem anderen Fachbereich eingereicht noch ein Promotionsverfahren bei einer Hochschule beantragt.

Gina A. Montoya Parra

Kaiserslautern