Cafestol: a multi-faced compound

Kinetics and metabolic effects of cafestol in mice

Saskia T.J. van Cruchten

Thesis committee

Thesis supervisors

Prof. Dr. Renger F. Witkamp

Professor of Nutrition and Pharmacology

Wageningen University

Prof. Dr. Michael M. Müller

Professor of Nutrition, Metabolism and Genomics

Wageningen University

Thesis co-supervisor

Dr. ir. Guido J.E.J. Hooiveld

Assistant professor, Human Nutrition

Other members

Prof. Dr. Ivonne Rietjens, Wageningen University

Prof. Dr. Jaap Keijer, Wageningen University

Prof. Dr. Bert Groen, University Medical Center Groningen

Dr. Baukje de Roos, University of Aberdeen, Scotland

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Saskia T.J. van Cruchten

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Saskia T.J. van Cruchten

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Abstract

Cafestol and kahweol are two diterpenes present in unfiltered coffees such as Scandinavian-style boiled coffee, French press coffee and espresso. The health effects of cafestol and kahweol can be positive but also potentially harmful. On the one hand cafestol shows chemo-preventive properties, which might contribute to a reduction of the risk for certain cancers, especially colorectal cancer. On the other hand cafestol is known as the most potent cholesterol-raising compound present in the human diet and also causes a temporally rise in plasma ASAT and ALAT enzymes. This apparently 'two-faced' behavior of cafestol was the starting point of this thesis. It was demonstrated that in mice cafestol is extensively metabolized by the liver. Metabolism was found to be associated with Nrf2 activation, causing an induction of biotransformation enzymes and cellular antioxidant defense. This mechanism might explain the proposed anti-carcinogenic effects of cafestol. Furthermore, distribution studies indicated that in mice cafestol accumulates almost exclusively in liver and intestine, and suggested that cafestol undergoes enterohepatic cycling. Further studies showed that cafestol prevents the development of diet-induced obesity, its metabolic complications, and the development of hepatic steatosis in mice. Modulation of the dietary fat content was also used to study the hepatic and intestinal response to cafestol at a transcriptomic level. We showed that dietary fat content is an important determinant of the effects of cafestol. This has been evaluated for several processes already known to be influenced by cafestol, such as bile acid metabolism and Nrf2-mediated biotransformation. Furthermore it was shown that cafestol activated Nrf2-mediated biotransformation both in liver and small intestine.

It is concluded that cafestol behaves as a hormetic compound. It elicits a combination of mechanisms which together determine the balance between positive and negative health

outcomes. Although for some mechanisms, *i.e.* the induction of biotransformation enzymes and acute liver toxicity, a connection seems plausible, several questions remain regarding their interrelations. This thesis has generated new mechanistic insights in the multi-faced behavior of cafestol. More studies, including in humans, are needed to study its dose-effect relations and interactions with dietary compounds. For the time being it is advisable to keep cafestol under scrutiny.

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Chapter 1

General Introduction

Cafestol and kahweol are two diterpenes that are found in unfiltered coffees such Scandinavian-style boiled coffee, French press coffee and espresso (Urgert, Schulz et al. 1995; Urgert and Katan 1997). Cafestol is regarded as the most potent cholesterol raising compound present in human diet (Urgert and Katan 1997; Ricketts, Boekschoten et al. 2007). Although cafestol has been subject of several studies during the last decade, many questions regarding its molecular mechanisms and metabolic fate have remained.

Consumption of unfiltered coffee increases serum cholesterol

During the last 30 years numerous studies have been performed that link consumption of unfiltered coffees with an increased incidence of cardiovascular disease (Thelle 1995; Urgert and Katan 1997). In 1990, it was shown that increased coffee consumption was associated with increased coronary death in Scandinavian countries (Tverdal, Stensvold et al. 1990). Pietinen et al. showed that consumption of boiled coffee correlated with increased serum cholesterol in Finland (Pietinen, Aro et al. 1990).

Elucidation of the identity of the cholesterol raising compound present in unfiltered coffees

In several studies it was shown that serum cholesterol in humans only raises with coffee consumption when this coffee was not prepared by filtration (Weusten-Van der Wouw, Katan et al. 1994; van Rooij, van der Stegen et al. 1995). This led to the discovery by Zock et al. (Zock, Katan et al. 1990) that a lipid fraction, which is removed by filtration, is responsible

for the increased serum cholesterol in humans. Simultaneously, two groups independently identified these cholesterol raising factors as the diterpenes cafestol and kahweol (Figure 1) (Heckers, Gobel et al. 1994; Weusten-Van der Wouw, Katan et al. 1994).

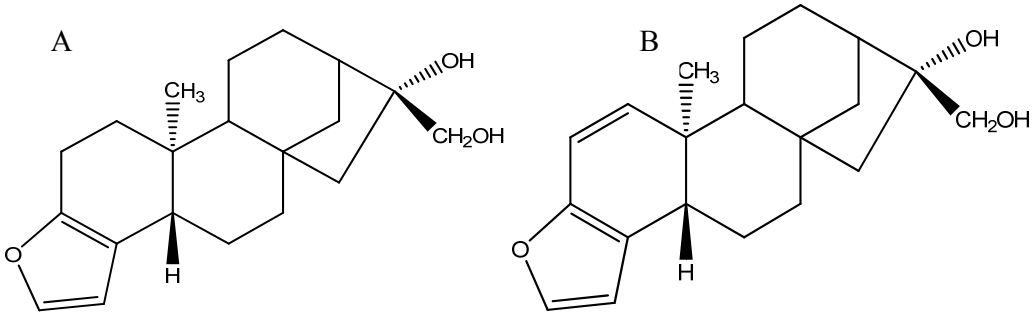

Figure 1: ***Structural formulas of A: cafestol and B: kahweol.***

Cafestol and kahweol increase serum cholesterol and liver enzymes

After the discovery of cafestol and kahweol as being responsible for the cholesterol raising effect of unfiltered coffee this was confirmed in a number of studies. It was shown that for every 10mg of cafestol consumed per day, which is the equivalent of 3 cups of French press coffee, serum cholesterol increases on average by 0.13mmol/l (Urgert and Katan 1997). Filtered or instant coffees, or coffee prepared by a Senseo® coffee machine do not contain cafestol or kahweol because these diterpenes are retained by a paper filter (van Dusseldorp 1991; Boekschoten, van Cruchten et al. 2006).

Interestingly, cafestol and kahweol were also found to increase serum levels of the liver enzymes serum alanine aminotransferase (ALAT) and to a lesser extent aspartate aminotransferase (ASAT) (Urgert, Essed et al. 1997). These liver enzymes are clinical

indicators of hepatocellular damage. In hepatocytes, ALAT is only found in cytoplasm, whereas ASAT is predominantly found in the mitochondria. These aminotransferases are released from cells as a consequence of increased membrane permeability as well as membrane damage. However, these effects seem to be temporally since life-long consumers of unfiltered coffees containing cafestol do not have increased ALAT levels (Casiglia, Spolaore et al. 1993).

Cholesterol-raising effects of cafestol – underlying mechanisms

Following the discovery of the effects of cafestol and kahweol on cholesterol levels, several studies have been undertaken to elucidate the underlying mechanisms. However, to date several questions have remained open. In general, the formation and turnover of cholesterol offer a number of regulatory steps that could be affected by cafestol. These will be briefly discussed in this introductory chapter.

Cholesterol homeostasis results from a balance of i) the absorption of dietary cholesterol , ii) endogenous cholesterol synthesis, and iii) elimination of cholesterol by excretion in bile followed by partial re-absorption (Figure 2). These processes are tightly regulated by several feedback mechanisms. At the molecular level multiple transcription factors play a role, of which the sterol regulatory element-binding proteins (Srebps) and nuclear receptors LXR and FXR are most relevant.

The average Western diet contains 400-500 mg cholesterol, but a much larger amount (800-1200 mg) enters the small intestine via bile. After solubilization and hydrolysis, cholesterol is thought to be transported over the brush border membrane into the enterocyte by the protein Niemann-Pick C1 like 1 (Npc1l1). After uptake in the enterocyte, cholesterol is packaged into

chylomicrons, and put into circulation where it is utilized throughout the body (Hui, Labonte et al. 2008). Currently it is not known how this is regulated at a molecular level and whether these processes are influenced by cafestol.

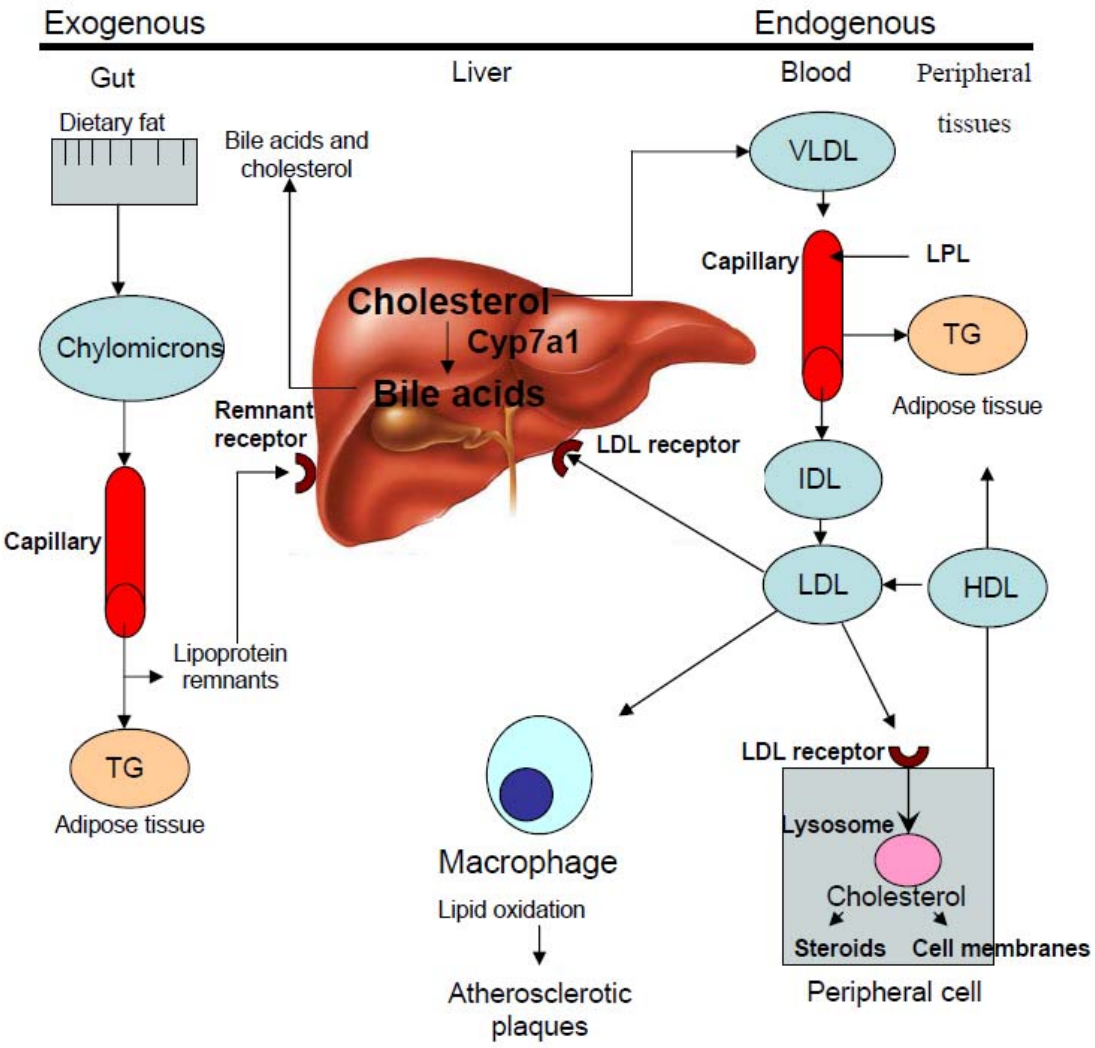

Figure 2: ***Steps in cholesterol homeostasis****.*

Possible regulatory steps in cholesterol synthesis, elimination and transport

The **endogenous synthesis of cholesterol** could represent a second candidate for interaction with cafestol and kahweol explaining the cholesterol raising effects of these compounds. Twenty to 25% of the endogenous synthesis of cholesterol takes place in liver. Cells maintain sterol homeostasis by multiple feedback controls that act through transcriptional and posttranscriptional mechanisms. The main regulatory mechanism is the sensing of intracellular cholesterol in the endoplasmatic reticulum by Srebp2. In short, upon activation Srebp2 is translocated from membrane to Golgi apparatus where it is cleaved. This part travels to the nucleus where it induces the activation of several genes involved in uptake and synthesis of cholesterol (Brown and Goldstein 1997; Sakai, Nohturfft et al. 1998; Brown and Goldstein 1999; Brown, Sun et al. 2002; Espenshade and Hughes 2007). In theory, cafestol could directly or indirectly over stimulate the transcription or activation of an essential enzyme in the cholesterol biosynthesis pathway. It could also inhibit Srebp2, thereby blocking the feedback mechanism that is activated when cholesterol levels are low, resulting in increasing serum cholesterol. Furthermore, the Liver X receptors (LXRs) have been characterized as key transcriptional regulators of lipid metabolism (reviewed in (Baranowski 2008). Their endogenous ligands are oxysterols, which includes oxidized cholesterol. LXR functions as sterol sensors protecting the cells from cholesterol overload by, regulation of cholesterol 7-alpha hydroxylase (Cyp7a1) activity, regulation of cholesterol excretion by regulation of ATP binding cassette transporters Abcg5 and Abcg8 in the liver and regulation of reverse cholesterol transport (Janowski, Willy et al. 1996; Repa, Berge et al. 2002; Yu, York et al. 2003). In short, LXR activation promotes cholesterol transport from the peripheral tissues to the liver for degradation to bile acids and induces Srebp1c that regulates

lipogenesis. Although no data is available, it could be envisioned that interference of cafestol with LXR metabolism could result in altered serum cholesterol levels.

Bile acid biosynthesis from cholesterol

Bile acid synthesis could be another regulatory process modulated by cafestol. Bile acids are synthesized from cholesterol in liver by Cyp7a1, which catalyzes the rate- limiting step in this reaction. Bile acids are secreted by hepatocytes into the bile canaliculi. The bile is subsequently stored in the gallbladder. After consumption of food, bile flows into the duodenum. In the ileum bile acids are absorbed (active transport by transporters such as the apical sodium-dependent bile acid transporter (Asbt) and transported back to the liver by the portal vein (Dawson, Lan et al. 2009). This process is known as the enterohepatic cycle (Thomas, Pellicciari et al. 2008). Previously it was shown that cafestol suppressed Cyp7a1 enzyme activity and mRNA levels (Post, de Roos et al. 2000; Ricketts, Boekschoten et al. 2007). Next to Cyp7a1, also the expression of other enzymes involved in bile acid synthesis such as sterol-27-hydroxylase and oxysterol-7x-hydroxylase were found to be inhibited by cafestol, which may have consequences for the overall process of bile acid production. This is reflected in the excretion of bile acids in faeces which in mice was decreased with 41% after 30 days of cafestol feeding (Post, de Roos et al. 2000).

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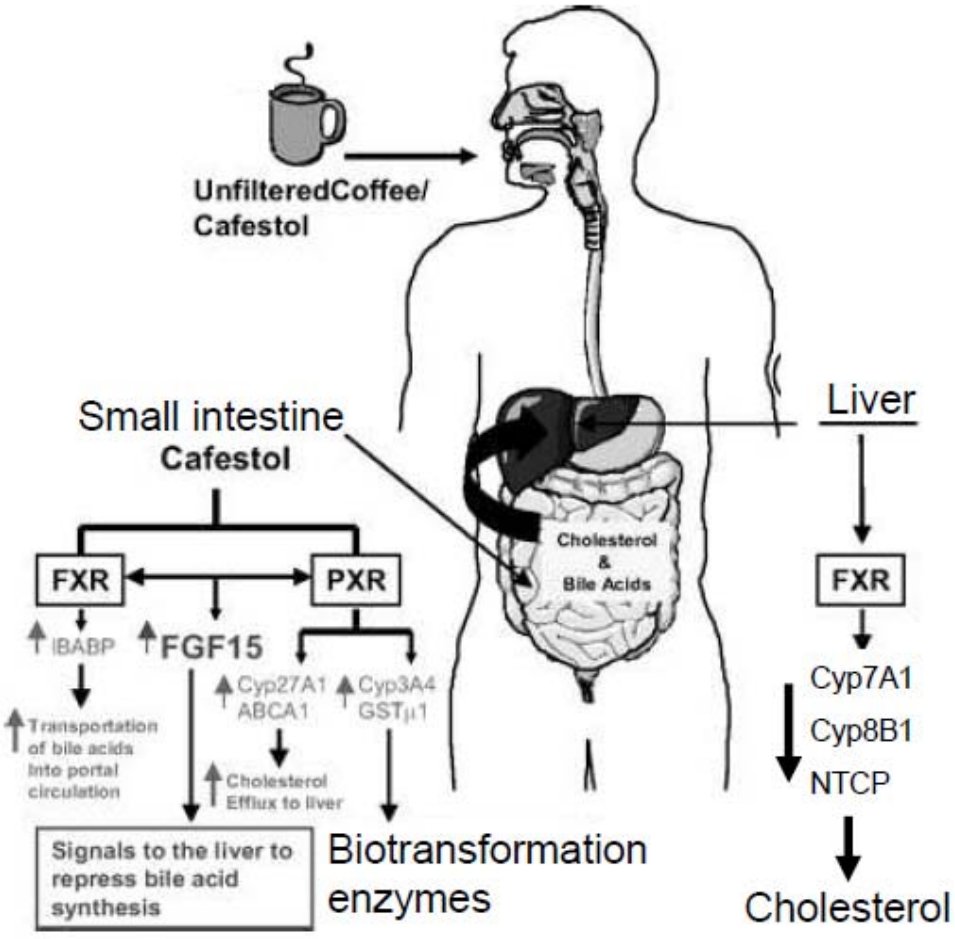

Figure 3: ***Schematic representation of the proposed mechanisms by which cafestol increases cholesterol levels.*** *After cafestol consumption, it enters the small intestine, where it activates Fxr and Pxr. IBABP is induced by cafestol in a Fxr-dependent manner, further increasing the transportation of bile acids into the portal circulation. Upon activation of Pxr, cafestol induces the expression of Cyp27A1 and ABCA1, resulting in an increase in the efflux of cholesterol into the portal circulation. Cafestol also induces Cyp3A11 and GSTμ1 gene expression via Pxr, leading to an increase in biotransformation. Cafestol acts via both Fxr and Pxr to induce FGF15, which signals to the liver to repress bile acid synthesis. In the liver, Cyp7A1, Cyp8B1, and NTCP expression is repressed via FXR, thereby reducing the*

synthesis of bile acids. The direct regulation of such Fxr and Pxr target genes in the intestine combines with indirect effects in the liver to contribute to the cholesterol-raising effect of cafestol in humans (adapted from Ricketts et al. (Ricketts, Boekschoten et al. 2007)).

Recently it became clear that bile acids have more functions than contributing to the solubilisation and digestion of lipid soluble nutrients. Bile acids also function as signaling molecules (Auwerx 2006; Thomas, Auwerx et al. 2008; Thomas, Pellicciari et al. 2008) by activating membrane (TGR5) and intracellular receptors (FXR). Activation of FXR by bile acids results ultimately in inhibition of Cyp7a1 expression and activity, which in turn results in an inhibition of cholesterol conversion to bile acids. FXR also activates the intestinal bile acid binding protein (IBABP) which is a bile acid transporter (Makishima, Okamoto et al. 1999). Although most studies addressing the molecular mechanisms of cafestol have focused on liver, there is evidence that the small intestine has an important regulatory role in this process. Ricketts et al. proposed that cafestol activates intestinal FXR, which activates Fgf15, which similar to activation by bile acids, travels to the liver and activates Fgfr4 thereby inhibiting Cyp7a1 (Ricketts, Boekschoten et al. 2007)(Figure 3). Next to the effects of bile acids on their own synthesis and enterohepatic recirculation, it has recently been shown that bile acids also affect triglyceride, cholesterol, energy and glucose homeostasis, partially through activation of TGR5 (Auwerx 2006; Thomas, Auwerx et al. 2008; Thomas, Pellicciari et al. 2008; Thomas, Gioiello et al. 2009)

Cholesterol transport

The last level of interaction could be modulation of cholesterol transport. Following cafestol intake, approximately 80% of the rise of total cholesterol was caused by LDL cholesterol and the other 20% were caused by a rise in VLDL cholesterol. In some studies HDL slightly decreased with cafestol intake (Zock, Katan et al. 1990; Weusten-Van der Wouw, Katan et al. 1994; Urgert, Schulz et al. 1995). Changes in lipoprotein composition might be mediated by an increased activity of cholesteryl-ester transfer proteins (Cetp) and phospholipid transfer protein (Pltp). In humans the activity of CETP and PLTP is increased by cafestol whereas the activity of lecithin: cholesterol transferase (LCAT) is decreased (van Tol, Urgert et al. 1997). In humans, cafestol has also been shown to increase the pool size of VLDL (most predominantly VLDL1). Cafestol increases the production rate of VLDL1 apolipoprotein B, which results in an increased amount of VLDL particles in the bloodstream (de Roos, Caslake et al. 2001). Because of the hydrophobic nature of cafestol, it is possible that cafestol travels through the body associated with chylomicrons formed in the small intestine. Sofar no studies have been performed which suggest an interaction between cafestol and chylomicrons.

Cafestol induces biotransformation enzymes in rodents and humans

In contrast to its potential harmful effects (increasing plasma cholesterol and liver enzymes), cafestol has also been suggested to have anti-carcinogenic or hepatoprotective effects. This was first reported in 1980 when Gershbein and Baburao showed that feeding the coffee diterpenes to partially hepatectomized rats stimulated liver regeneration (Gershbein and Baburao 1980). After this finding the so-called hepatoprotective properties of cafestol were extensively studied by several research groups that showed that in rodents cafestol decreases

the activity of several CYP450 enzymes and induces glutathione-S-transferase (GSTs) (Di Simplicio, Jensson et al. 1989; Huber, Prustomersky et al. 2002; Huber, Teitel et al. 2004; Huber and Parzefall 2005; Huber, Rossmanith et al. 2008). In rats treated with a 1:1 mixture of cafestol and kahweol (C/K), a dose dependent increase of GSH is seen in the liver and colon. In these rats an increase in glutamylcysteine synthetase (GCS), the first and rate limiting enzyme in the glutathione biosynthesis pathway, is also seen (Scharf, Prustomersky et al. 2001; Huber, Scharf et al. 2002). Furthermore Grubben et al. showed in humans that consumption of unfiltered coffees leads to elevation of glutathione (GSH) content in the colorectal mucosa and in plasma (Grubben, Van Den Braak et al. 2000).

Cafestol metabolism

Remarkably little is known so far regarding the metabolism of cafestol. Lam et al suggested that the furan moiety of cafestol and kahweol is vital to their biological activity as inducers of increased glutathione S-transferase activity (Lam, Sparnins et al. 1987). De Roos et al. studied the metabolism of cafestol in ileostomy patients for 28 days. Participants consumed one, two or three cups of unfiltered coffee every day, and at different time points ileostomy effluent and urine were collected. Approximately 70% of the ingested cafestol and kahweol was absorbed in these patients. The ileostomy effluent contained free cafestol and kahweol. No free cafestol or kahweol was found in urine, but 1% of the ingested amount was recovered in urine as glucuronic or sulphate conjugates. The authors explained their findings by hypothesizing that the major part of the absorbed diterpenes was apparently metabolized extensively to products that were not recovered from urine (De Roos, Meyboom et al. 1998).

Aim and Outline of this thesis

Initially, the general aim of this thesis project was to further elucidate the molecular mechanisms of the effects of cafestol on serum cholesterol levels. As has been described in the previous section, many questions were left open on the kinetics and biotransformation of cafestol itself. Also considering the apparent interaction between cafestol and biotransformation enzymes including CYP450 en GSTs, the possibility was raised that the molecular fate of the cafestol molecule could be key to the interactions with cholesterol. Therefore, the first studies were undertaken to study the uptake, distribution and metabolism of cafestol in mice. These studies are described in **Chapters 2 and 3**. As the results were quite remarkable, indicating a distinctive metabolic pathway and new links with the effects of cafestol on biotransformation pathways, it was decided to shift focus to the role of the liver and intestine and their interaction in cafestol metabolism. In **Chapter 4** it became even more clear that cafestol treatment may result in beneficial metabolic effects. Cafestol is able to prevent diet induced obesity and hepatic steatosis and ameliorates insulin sensitivity in mice. As the results of these studies were pointing towards a possible interference with dietary fat intake this was further investigated in **Chapter 5** in which a nutrigenomics approach was used to gain more insight on the metabolic effects of the interaction between cafestol and diet in liver and intestine. In **Chapter 6**, the overall conclusions are discussed and future recommendations presented.

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Chapter 2

The role of epoxidation and electrophile-responsive element (EpRE)-regulated gene transcription in the potentially beneficial and harmful effects of the coffee components cafestol and kahweol.

Saskia T.J. van Cruchten[1,2*], Laura H.J. de Haan[3], Patrick P.J. Mulder[4], Cindy Kunne[5], Mark V. Boekschoten [1,2,] Martijn B. Katan[6], Jac M.M.J.G. Aarts[3], and Renger F. Witkamp[1].

1 Division of Human Nutrition; Wageningen University, Wageningen, The Netherlands

2 Top Institute of Food and Nutrition, Wageningen, The Netherlands

3 Department of toxicology, Wageningen University, Wageningen, The Netherlands

4 RIKILT, - Institute of Food Safety, Wageningen, The Netherlands

5 *Liver Center, Academic Medical Center, Amsterdam, The Netherlands*

6 Institute of Health Sciences, VU University Amsterdam, The Netherlands

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Abstract

Cafestol and kahweol are diterpene compounds present in unfiltered coffees. Cafestol is known as the most potent cholesterol raising agent that may be present in the human diet. Remarkably, the mechanisms behind this effect have only been partly resolved so far. Even less is known about the metabolic fate of cafestol and kahweol. From the structure of cafestol, carrying a furan moiety, we hypothesized that epoxidation may not only be an important biotransformation route, but that this also plays a role in its effects found. In bile duct cannulated mice, dosed with cafestol, we were able to demonstrate the presence of epoxy-glutathione conjugates, glutathione conjugates and glucuronide conjugates. In addition it was shown that cafestol was able to induce an electrophile-responsive element (EpRE). Using a murine hepatoma cell line with a luciferase reporter gene under control of an EpRE from the human NQO1 regulatory region we also found that metabolic activation by CYP450 enzymes is needed for EpRE induction. Furthermore, raising intracellular GSH resulted in a decrease in EpRE mediated gene induction, whereas lowering intracellular GSH levels increased EpRE mediated gene induction. In conclusion, evidence suggests that cafestol induces EpRE, apparently via a bioactivation process that possibly involves epoxidation of the furan ring. The epoxides themselves appear subject to conjugation with glutathione. The effects on EpRE can also explain the induction of glutathione which seems to be involved in the reported beneficial effects of cafestol, for example when administered with aflatoxin B1 or other toxic or carcinogenic compounds.

Introduction

Cafestol and kahweol (Figure 1) are diterpenes mainly present in unfiltered coffees such as French press, espresso and boiled coffees (Urgert and Katan 1997; Halvorsen, Ranheim et al. 1998; Ranheim and Halvorsen 2005; Ricketts, Boekschoten et al. 2007). Depending on the quality/blend and process of coffee preparation (Speer and Kölling-Speer 2006), concentrations of cafestol and its structural analogue kahweol are each estimated to range between 0.1 and 7 mg/ml coffee (Ranheim and Halvorsen 2005). Cafestol is now regarded as the most potent cholesterol-elevating compound known in the human diet (Weusten-Van der Wouw, Katan et al. 1994; Urgert and Katan 1997; Ricketts, Boekschoten et al. 2007), and most likely responsible for the association between consumption of boiled coffee and an elevated risk for cardiovascular disease (Urgert, Schulz et al. 1995; Urgert, Essed et al. 1997; Urgert and Katan 1997; de Roos and Katan 1999). Although several potential targets involved in cholesterol homeostasis have previously been proposed, many questions regarding its mechanism have remained unsolved (de Roos and Katan 1999; Boekschoten, Hofman et al. 2005; Ranheim and Halvorsen 2005; Ricketts, Boekschoten et al. 2007). In addition, there is still very little information on the metabolic fate of the compound in the body. In contrast to its potential harmful effects, cafestol has also been suggested to have anti-carcinogenic or hepatoprotective effects (Wattenberg 1983; Cavin, Holzhaeuser et al. 2002; Huber, Scharf et al. 2003; Huber, Teitel et al. 2004; Lee, Choi et al. 2007; Higgins, Cavin et al. 2008; Huber, Rossmanith et al. 2008). In rodent studies the compound has been shown to decrease the activity of several CYP450 enzymes and to induce glutathione-S-transferase (GSTs) (Di Simplicio, Jensson et al. 1989; Huber, Prustomersky et al. 2002; Huber, Teitel et al. 2004; Huber and Parzefall 2005; Huber, Rossmanith et al. 2008). In humans, consumption of

unfiltered coffee or cafestol leads to elevation of glutathione (GSH) content in the colorectal mucosa and in plasma (Grubben, Van Den Braak et al. 2000). In rats treated with a 1:1 mixture of cafestol and kahweol (C/K), a dose dependent increase of GSH is seen in the liver and colon. In these rats an increase in glutamylcysteine synthetase (GCS), the first and rate limiting enzyme in the glutathione biosynthesis pathway, is also seen (Scharf, Prustomersky et al. 2001; Huber, Scharf et al. 2002).

Finally, cafestol and/or kahweol and coffee brews that contain these diterpenes cause elevations of serum alanine aminotransferase (ALAT) and to a lesser extent aspartate aminotransferase (ASAT) and a temporary depression of gamma glutamyl transferase (γGT) in plasma (Urgert and Katan 1997; Boekschoten, Schouten et al. 2004; Boekschoten, Hofman et al. 2005).

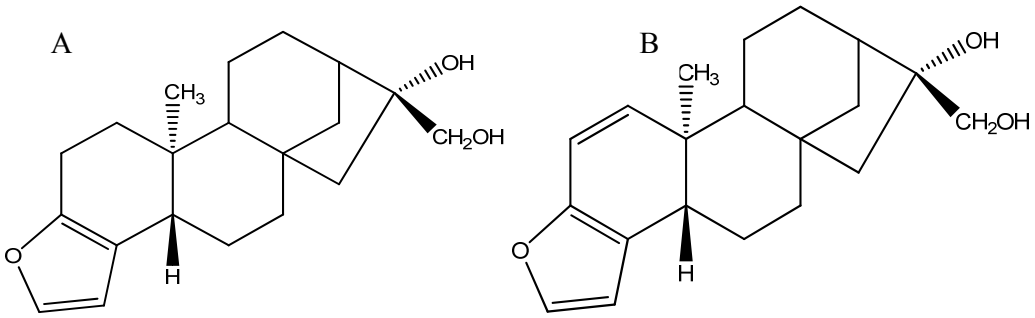

Figure 1: ***Structural formulas of A: cafestol and B: kahweol.***

From the structure of cafestol, which contains a furan moiety, we hypothesized that this apparently two-faced behavior might be related to a bio-activation which includes the formation of one or more furan epoxides. A similar reaction has been described for example for the natural toxin ipomeanol (Alvarez-Diez and Zheng 2004). On the one hand, such furan epoxides could be involved in the hepatotoxic and cholesterol-elevating effects. On the other

hand, epoxidations could explain the induction of glutathione (GSH) biosynthesis, glutathione-S-transferase (GST) and other chemo protective enzyme genes, possibly mediated by an electrophile-responsive element (EpRE) in the regulatory region of the genes involved. The EpRE is a regulatory sequence mediating the coordinated transcriptional activation of genes associated with phase 2 biotransformation, protection against oxidative stress, and other cancer-chemo protective mechanisms (Kobayashi, Kang et al. 2006). The key regulator of EpRE-mediated gene expression is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and, to a lesser extent, Nrf1 (Copple, Goldring et al. 2008). Very recently it was shown that coffee may protect against the adverse effects of aflatoxin B1 via stimulation of the Nrf2-ARE (antioxidant responsive element) pathway (Higgins, Cavin et al. 2008).

The aim of the present study was to further investigate this potential mechanism and the role of bioactivation via epoxide formation for the specific coffee components cafestol and kahweol. First, a study was undertaken to identify glutathione conjugates of cafestol with LC-MS in bile-duct cannulated mice. After this, a series of experiments were carried out using a hepa-1c1c7 cell line stably transfected with a luciferase reporter gene under control of an EpRE from the human NQO1 regulatory region to obtain a reporter cell line responsive to electrophiles. The induction of EpRE-mediated gene expression was studied after exposure to cafestol and kahweol with and without preincubation with S9-mix, a preparation of enzymes of xenobiotic metabolism, particular of phase 1. Therefore, the experiments allowed a determination of the role of metabolic activation in case of cafestol. Furthermore, to investigate whether oxidation was the mechanism leading to Nrf2 release and subsequent gene induction, we modulated the intracellular GSH level using N-acetyl-L-cysteine (NAC) and buthionine-sulfoximine (BSO) to respectively increase and decrease the intracellular GSH concentration.

Methods:

Chemicals

Alpha-Modified Eagle's medium (α-MEM), Hanks balanced salt solution (HBSS), Trypsin, foetal calf serum (FCS), phosphate-buffered saline (PBS), gentamicin and G418 were purchased from Gibco Invitrogen Corporation (Breda, The Netherlands). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (New Yersey, USA). P450 inhibitor, 1-aminobenzotriazole (ABT) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) as were potassium phosphate, NADP+, Glucose-6-phosphate, MgCl2, glucose-6-phosphate dehydrogenase. Clinoleic (20%) was obtained from Baxter (Deerfield, IL, USA). Cafestol was obtained from LKT Laboratories, Inc (St. Paul, MN, USA) and the mixture kahweol/cafestol [K 30.1%/ C 61.9%] was isolated from coffee oil by our department(Urgert, Schulz et al. 1995). Kahweol as single compound could not be obtained in sufficient quantities. For LCMS analysis we used water LC/MS grade and methanol HPLC supra gradient grade were purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid, p.a., was obtained from Merck (Darmstadt, Germany). Ammonium formate 97% was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell line

Hepa-1c1c7 mouse liver hepatoma cells were a kind gift from Dr. M.S. Denison, (University of California, Davis) and were stably transfected with the reporter vector pTI(hNQO1-EpRE)Luc+ carrying the EpRE from the human NQO1 gene regulatory region between basepair -470 and -448 from the transcription initiation site (5'-AGT CAC AGT GAC TCA

GCA GAA TC-3') coupled to a luciferase reporter gene, resulting in the EpRE(hNQO1)-LUX cell line as described elsewhere (Boerboom, Vermeulen et al. 2006), and referred to as EpRE-LUX in this paper. EpRE cells were cultured in α-MEM, supplemented with 10% FCS and 50 μg/mL gentamicin and in addition 0.5 mg/mL G418 to maintain selection pressure on the presence of the reporter gene insertion. The cells were maintained in a humidified atmosphere with 5% CO2 at 37°C.

Animals

Pure-bred wild-type adult, male (C57BL6/j) mice were purchased from Harlan (Horst, The Netherlands). Mice were housed in a light- and temperature-controlled facility and had free access to water and standard laboratory chow (RMH-B, Hope farms, Woerden, The Netherlands). All animal studies were approved by the local Committee for Care and Use of Laboratory Animals.

Animal experiment

Five C57BL6/j mice were fasted overnight. Gallbladder cannulation and bile collection was performed as described previously (Klett, Lu et al. 2004). To maintain a constant bile flow of 300ul/hour/25gram BW, mice were infused in the jugular vein with a bile acid solution containing 30mM tauroursodeoxycholic acid (TUDC) according to standard procedures, also with a flow rate of 300ul/hour/25gram BW) (Klett, Lu et al. 2004). Sixty minutes after infusion, bile flow was considered stable and cafestol was injected through the portal vein. Subsequently, cafestol (total dose 12μg) dissolved in 20% Clinoleic was administrated through the portal vein. Bile was sampled every 15 minutes for the first hour and every 30 min after the

first hour. In total, bile was sampled for 5hours. Bile samples were immediately frozen at −80°C and subsequently analysed by LC-MS. Bile flow was determined gravimetrically assuming a density of 1 g/ml for bile.

LCMS analyses

All LC-MS analyses were carried out using a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Waters LCT Premier time-of-flight (ToF) mass spectrometer. Chromatographic separation was conducted using a Zorbax Eclipse XDB C_8 (Agilent) column (150 x 2.1 mm i.d., 5 µm) thermostatted at 30 ˚C. The injection volume was 5 µl. For analyses carried out in the positive ion electrospray ionization (ESI+) mode, solvent A consisted of 0.02% formic acid in water and solvent B of 0.02% formic acid in methanol. Gradient elution was linearly programmed as follows: 0 min 5% B, 1 min 5% B, 19 min 95% B, and 21 min 5% B at a flow rate of 0.2 ml/min. For negative ion ESI (ESI-) analyses solvent A consisted of 5 mM ammoniumformate in water, and solvent B was methanol. The following linear gradient was used: 0 min 5% B, 1 min 5% B, 16 min 80% B, and 18 min 5% B at a flow rate of 0.2 ml/min.

The ToFMS was equipped with a LockSpray dual ESI source. Source operating conditions were: 120 ˚C ion source temperature, 350 ˚C desolvation temperature, 500 L/h desolvation gas flow rate and 50 L/h cone gas flow rate. The spray voltage was maintained at 3 kV for ESI+ and –2.5 kV for ESI-. Mass spectra were acquired in centroid mode with internal mass correction by scanning from 100 – 1000 m/z. The lock masses used for ToF mass correction in ESI+ and ESI- mode were the $[M+H]^+$ ion (attenuated lock mass) and ^{13}C isotope $[M+H]^+$ ion or the $[M-H]^-$ ion (attenuated lock mass) and ^{13}C isotope $[M-H]^-$ ion of leucine

enkephalin, respectively. Leucine enkephalin (1 ng/µl in water/acetonitrile 80:20 v/v) was delivered to the ion source at 10 µl/min using an HPLC pump. The analyte to reference scan ratio was 9:1. Experiments were performed at spectral acquisition time of 1.0 s. The resolution of the ToFMS was ~10 000 (FWHM). Dynamic range enhancement was switched on.

Metabolic activation of cafestol

To study the role of metabolic activation, we added S9 mix containing 10% S9 enzyme fraction (purchased from Trinova Biochem GmbH, Giessen, Germany), which was prepared from the liver of male Sprague–Dawley rats treated with Aroclor-1254. S9 mix contained an NADPH-generating system consisting of 1.3 M $NADP^+$, 3.3 M glucose-6-phosphate and 0.4 U/ml glucose-6-phosphate dehydrogenase dissolved in 0.5 M phosphate buffer, 0.08 M $MgCl_2$ and 0.330 M KCl prepared with milliQ water. The S9 mix was used at a concentration of 10% in experimental medium. Medium with or without S9 mix and with cafestol or a mixture of cafestol/kahweol was pre-incubated for one hour at 37°C in a rotating bath at 300rpm. After pre incubation, the mixture was added to the cells, which were exposed for 6 hours, where after the cells were harvested and analyzed. Glutathione levels in the cells were modified by treatment with either N-acetyl-L-cysteine (NAC, 40 mM) to generate higher levels of glutathione and buthionine-sulfoximine (BSO, 100 uM) to decrease intracellular GSH levels.

Inhibition of certain P450 enzymes was achieved by exposing the cells for 30min with 1-aminobenzotriazole (ABT, 50µM) (Balani, Zhu et al. 2002; Yang, Choi et al. 2009) and NADPH generating system. ABT inhibits specific P450 enzymes such as CYP3A4, CYP2E1,

CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP1A2 (Balani, Zhu et al. 2002; Yang, Choi et al. 2009). However it predominantly inhibits CYP3A4. Then cells were incubated with α-Mem, S9 mix, cafestol and 50 μM 1-aminobenzotriazole (ABT).

EpRE-LUX

EpRE-mediated induction of gene expression by cafestol and a mixture of cafestol/kahweol was tested using the EpRE-LUX luciferase reporter gene assay as described previously (Boerboom, Vermeulen et al. 2006; Lee-Hilz, Boerboom et al. 2006). Briefly, EpRE-LUX cells were propagated as described above plated in culture medium in 96-wells view-plates (PerkinElmer, 2×10^4 cells/100 μL/well) and incubated for 24 h to allow attachment of the cells to the bottom of the wells and the formation of a confluent monolayer. Next, the culture medium was removed and cells were exposed to 100 μL medium (FCS free) containing different concentrations of cafestol and S9 mix. S9 mix was added to ensure metabolisation of the test compounds with a full spectrum of active P450 enzymes. (S9 mix contained an NADPH-generating system consisting of 1.3 M $NADP^+$, 3.3 M glucose-6-phosphate and 0.4 U/ml glucose-6-phosphate dehydrogenase dissolved in 0.5 M phosphate buffer, 0.08 M $MgCl^2$ and 0.330 M KCl prepared with milliQ water). Medium with different cafestol concentrations and S9 mix were pre-incubated for 1 hour to allow metabolite formation. DMSO concentration in the culture medium was kept constant at 0.4%. After different periods of exposure, cells were washed with 0.5 × concentrated PBS, and lysed by addition of low salt buffer (10 mM Tris, 2 mM dithiothreitol (DTT) and 2 mM trans-1,2-diaminocyclohexane-N,N,N′,N′-tetra-acetic acid monohydrate; pH 7.8) followed by one freezing and thawing cycle. Luciferase reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$,

0.1 mM EDTA, 2 mM DTT, 0.47 mM D-luciferin, 5 mM ATP; pH 7.8) was injected and luciferase activity was immediately measured using a Luminoskan RS (Labsystems) luminometer. The luciferase expression measured was expressed as the induction factor (IF) defined as the potency of cafestol to increase the luciferase expression as compared to cells incubated with medium containing only 0.4% DMSO.

Statistical analysis

Comparisons between a single control and treatment means were made by using post-hoc tests; Bonferroni and a Dunnett's test. Comparisons between metabolically activated and metabolically unactivated cells were made by paired student's t-test. The limit of statistical significance was set at $P < 0.05$. Statistical analysis was performed using SPSS 15.

Results

Identification of cafestol metabolites in bile

Three mice were injected with cafestol and two mice with vehicle as a control. Bile fluid was sampled and analysed for cafestol metabolites by LC-ToF-MS. Only peaks that were detected in the bile fluid of all three treated mice and were absent in the control mice were considered potential metabolites of cafestol. Figure 2 shows the extracted ion chromatogram (EIC) recorded in ESI+ of a candidate glutathione (GSH) metabolite of cafestol. In the chromatogram a protonated molecular ion ($[M+H]^+$) is present of a compound with a retention time of 10.06 min (Figure 2A). This compound was not present in the EIC of the mice that had not received cafestol (Figure 2B).

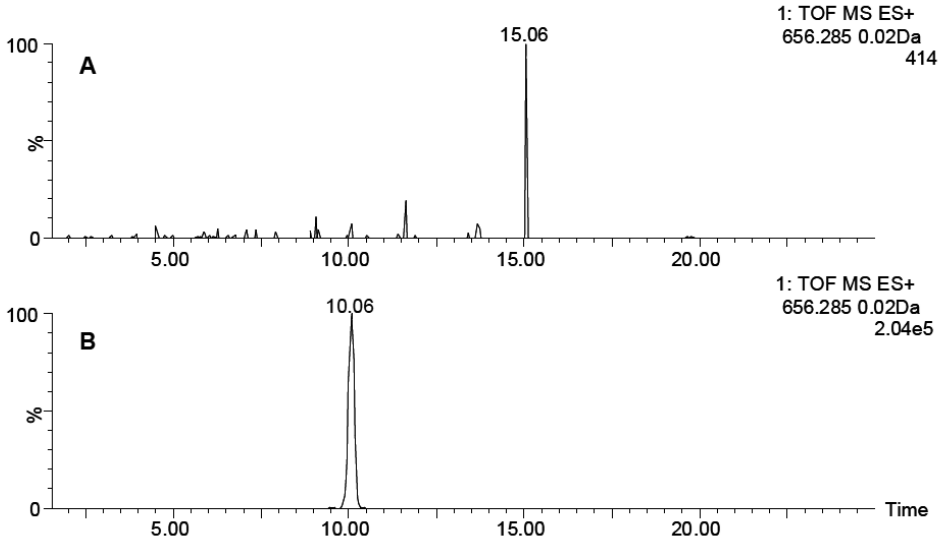

Figure 2: ***Detection of a potential GSH metabolite of cafestol by using LC-ToFMS in ESI+ mode.*** *Extracted ion chromatogram (EIC) for mass 656.285Da using a mass tolerance window of 10 mDa and normalized to the highest peak. A. EIC obtained for a mouse treated*

with cafestol. The peak at 10.06 min in the bile fluid of the cafestol treated mouse is absent in the control mouse. B. EIC obtained for a non-treated (control) mouse.

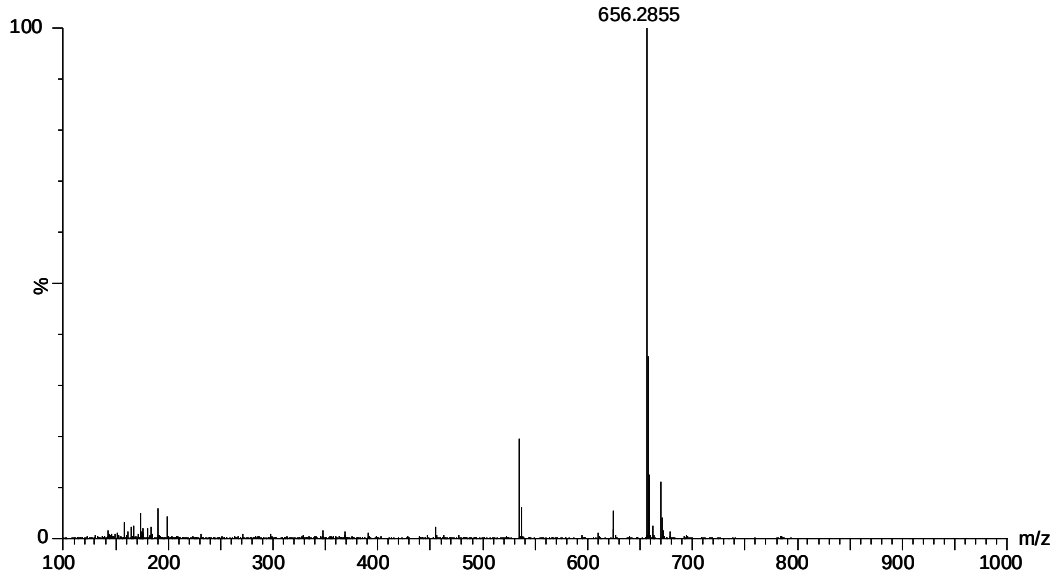

Figure 3: ***ESI+ mass spectrum obtained for the component at a retention time of 10.06 min present in the bile of a mouse treated with cafestol.*** *The $[M+H]^+$ ion with a measured mass of 656.2855 is tentatively identified as a GSH conjugate of 2-hydroxy-cafestol epoxide.*

The mass spectrum acquired of the compound at 10.06 min is shown in Figure 3, for the $[M+H]^+$ ion a mass is observed of 656.2855Da. This matches closely with the elemental composition$C_{30}H_{46}N_3O_{11}S$, for which a theoretical mass of 656.2848Da is calculated. The difference between the measured and theoretical mass of the $[M+H]^+$ ion is 1.1 ppm (0.7 mDa). Similarly, when the same sample was recorded in ESI-, a deprotonated molecular ion ($[M-H]^-$) was found at a retention time of 5.66 min (please note that a different mobile phase composition and gradient was used), with an observed mass of 654.2682Da (Data not shown).

The observed mass matches with an elemental composition $C_{30}H_{44}N_3O_{11}S$ with a deviation of -3.1 ppm (-2.0 mDa).

Taking into account the mass of cafestol epoxide (332.1988Da) and that of glutathione (307.0838Da) the expected adduct, formed by conjugation of glutathione with cafestol epoxide, will have a molecular mass of 640.2898Da and $C_{30}H_{46}N_3O_{10}S$ as elemental composition when measured as the $[M+H]^+$ ion and a mass of 638.2753Da and $C_{30}H_{44}N_3O_{10}S$ as elemental composition when measured as the $[M-H]^-$ ion. The difference between the observed metabolite and the expected cafestol epoxide glutathione adduct equals one oxygen atom. Apparently, the major glutathione adduct present in bile is an oxygenated metabolite.

At this point it remains rather speculative at which position of the molecule oxidation has occurred and at which stage during metabolism. Possible sites of oxidation are the remaining furan double bond (formation of an epoxide), oxidation of the glutathione sulfur (formation of sulfoxide) and hydroxylation at the methylene position next to the furan ring. In the latter case hydroxylation may take place before epoxidation producing 2-hydroxy cafestol. Subsequent epoxidation to 2-hydroxy cafestol epoxide and conjugation with glutathione will produce the putative metabolite (Figure 4). Hydroxylation of steroids is a common metabolism route and may take place to increase the polarity of the lipophilic cafestol. Without the collection of MSMS fragmentation spectra, and preferably, the isolation of the metabolite and subsequent NMR analysis, the exact structure of this metabolite can not be determined with certainty. The collected amounts of bile fluid were too small to allow such a detailed analysis, however.

At least two other glutathione containing metabolites were detected in the bile fluids of cafestol treated mice (Data not shown). Considering the mass of these metabolites, they contain an extra oxygen compared to the 655Da metabolite.

Next to glutathione conjugates of cafestol, also a glucuronide conjugate was detected in the bile.

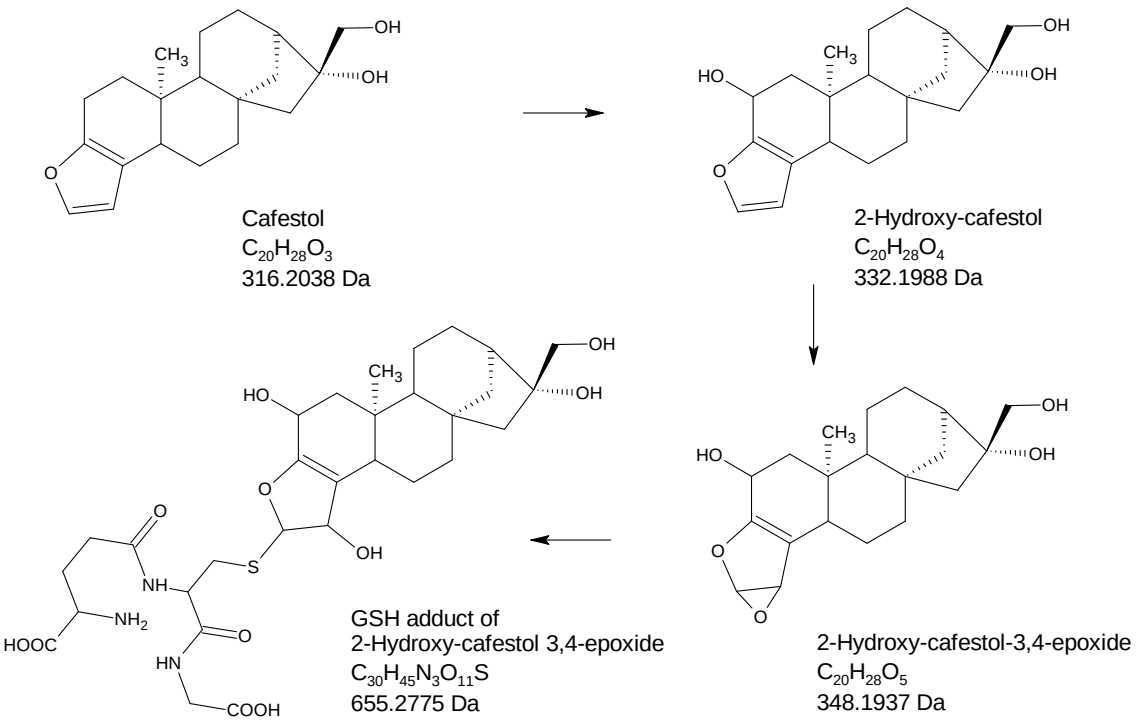

Figure 4: ***Proposed schematic representation of the formation of a glutathione conjugate of cafestol.*** *In this scheme cafestol is hydroxylated prior to epoxidation of the furan moiety of cafestol by P450 enzymes.*

Electrophile-responsive element (EpRE)-mediated effects of cafestol and kahweol.

In the EpRE-Lux cells exposed to cafestol an obvious luciferase induction response (IF) is only seen after metabolic stimulation (Figure 5, $p<0.001$).

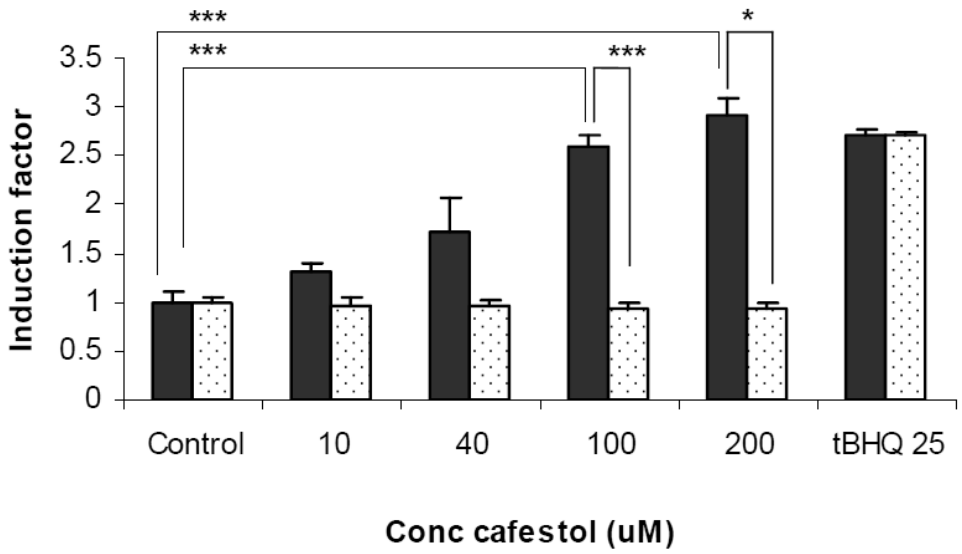

Figure 5: ***Induction of EpRe-mediated gene transcription by cafestol*** *. (■ cafestol with metabolic activation ▒ cafestol without metabolic activation). TBHQ (25µM) is used as a positive control for EpRE induction. Data are presented as means with standard deviation based on six independent measurements. Treatments groups 100µM and 200µM cafestol differ significantly compared to control (p≤0.001). Metabolic activated cells (■) differ significantly from control cells (▒).* ***P≤0.001, **P≤0.002.*

A mixture of cafestol/kahweol (68.2% cafestol; 31.8% kahweol) induced EpRE mediated gene induction at a concentration of 50 µM (34.1µM cafestol + 15.9 µM kahweol) after 6 hours of exposure (Figure 6). However, at high concentrations of the mixture C/K (200 and 300uM, total amount diterpenes are similar in treatments) cells died, whereas similar cafestol concentrations showed no effect on cell viability (morphologically checked).

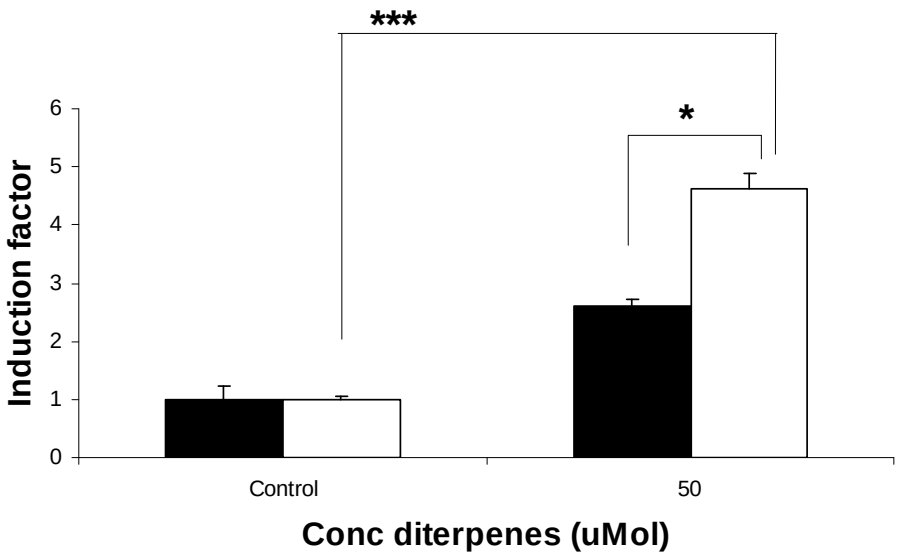

Figure 6: A mixture of cafestol and kahweol induces more EPRE mediated gene induction compared to cafestol alone. Cells treated with a mixture of cafestol/kahweol (□) induce more EpRE mediated activation compared to cafestol treated cells (■).Data are presented as means with standard deviation based on six independent measurements. ******P≤0.001, *P≤0.05.***

Increase of intracellular glutathione levels by NAC resulted in a 4.5- to 5-fold decrease of EpRE-mediated gene induction as compared to control without NAC treatment (Figure 7). When cells were glutathione depleted by means of pre-treatment with BSO, metabolically activated cafestol caused a 2.5-fold increase of EpRE mediated gene induction compared to exposed cells with unchanged glutathione levels (Figure 7). Inhibition of specifically P450 enzymes, by adding ABT (Balani, Zhu et al. 2002), resulted in a lower induction of the EpRE-controlled reporter gene expression compared to control (Data not shown). ABT inhibits specific P450 enzymes such as CYP3A4, CYP2E1, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP1A2 (Balani, Zhu et al. 2002; Yang, Choi et al. 2009). Therefore we conclude that P450 enzymes such as CYP3A4 are essential for metabolisation and epoxidation of cafestol.

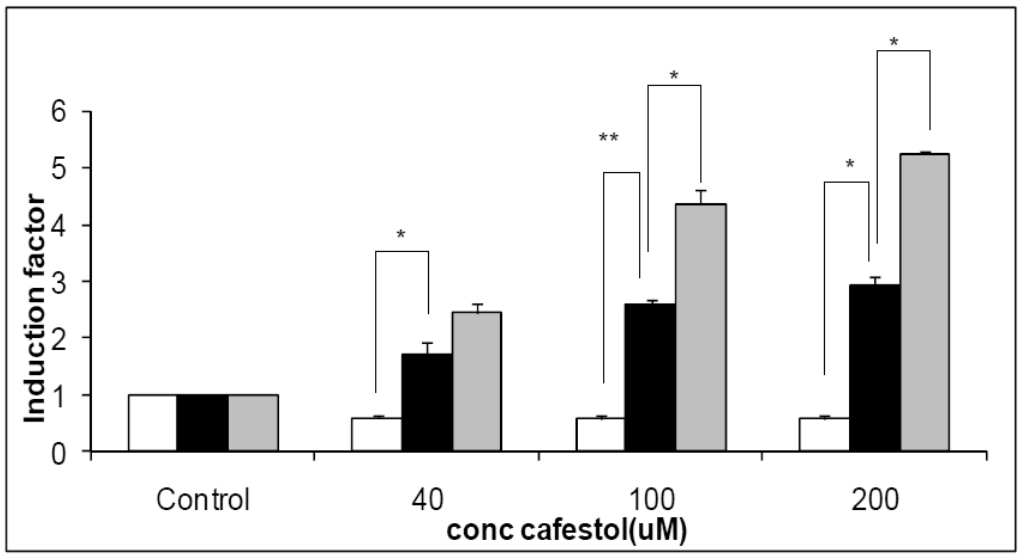

*Figure 7: Effects of changes in intracellular glutathione levels on cafestol induced EpRE activity. □ Glutathione increase (NAC), ■ Cafestol with metabolic activation. In Grey; Glutathione depletion and cafestol (BSO). *P<0.001, **P=0.076. An increase in total glutathione concentration (□ NAC) results in a significant decrease in cafestol induced EpRE induction. In glutathione depleted cells (grey), cafestol induced EpRE induction increases 2.5 fold. Data are presented as means with standard deviation based on six independent measurements.*

Discussion

It has become clear from several studies that the effects of cafestol on human health can be positive and negative. The beneficial effects include a possible reduction of toxicant activity after consumption of cafestol, whereas the increase in plasma cholesterol concentration can be considered as potentially harmful. Both outcomes are considered as relevant for a normal coffee consumption pattern (Urgert, Schulz et al. 1995; Post, de Wit et al. 1997; Urgert and Katan 1997; Urgert, Weusten-van der Wouw et al. 1997; de Roos and Katan 1999; De Roos, Van Tol et al. 2000; de Roos, Caslake et al. 2001; Ranheim and Halvorsen 2005; Ricketts, Boekschoten et al. 2007). The present study contributes to a further mechanistic understanding of this balance between beneficial and potentially detrimental effects of cafestol.

We provide evidence that the metabolism of cafestol plays a key role in the induction of glutathione-S-transferases (GSTs) and other phase II enzymes via the electrophile-responsive element (EpRE). Epoxidation of the furan moiety is probably involved in this activation. EpRE-, initially referred to as antioxidant-responsive element (ARE) is an important gene-regulatory enhancer mediating induction of the expression of a battery of genes involved in the defense against electrophilic and other reactive species. From the structure of cafestol we predicted that epoxidation in the furan moiety is a possible biotransformation route. A number of metabolites were found in bile of the bile-duct cannulated mice. Further analysis revealed the identity of other cafestol metabolites including epoxy-glutathione conjugates and glutathione conjugates. Epoxidation has been described for several other furan containing molecules, including furan, menthofuran, ipomeanine, 4-ipomeanol, furosemide and teucrin A (Khojasteh-Bakht, Chen et al. 1999; Alvarez-Diez and Zheng 2004; Baer, Rettie et al. 2005;

Peterson, Cummings et al. 2005; Chen, DeRose et al. 2006). For some of these compounds, including the natural toxin ipomeanol, the role of specific CYP450s has been established and furan-epoxides have been associated with cellular toxicity (Alvarez-Diez and Zheng 2004). To the best of our knowledge the formation of epoxides has not been described before for cafestol.

An important role in the potential anti-carcinogenic properties of cafestol has been attributed to both a decreased activity of bio-activating CYP450 enzymes or to an induction of glutathione-S-transferases (GSTs). Evidence for a decrease of CYP450 expression or activity by cafestol is coming from a number of studies. For example Huber et al. (Huber, Rossmanith et al. 2008) recently demonstrated inhibition of a fairly wide pattern of P450 enzymes in rats that had received a cafestol/kahweol mixture. The effect was most pronounced with CYP1A2 for which inhibition was detectable at the level of activity and mRNA. Interestingly, this P450 enzyme is generally associated with bio-activation of several pro-carcinogens, which often includes the formation of an epoxide. Similar conclusions were obtained by Cavin et al. (Cavin, Holzhaeuser et al. 2002) who studied the effect of coffee in rats and human hepatocytes. Also in this study, CYP1A1/2 was inhibited in rats and hepatocytes, as was the rat-specific CYP2C11. By contrast, CYP4503A enzymes were not affected by coffee in this study. However, inhibition of several P450 enzymes including CYP3A4 by ABT in our EpRE experiments showed a significant decrease in EpRE mediated gene induction.

Evidence for a GST-inducing effect by cafestol and/or kahweol is obtained from a number of studies, including (Ricketts, Boekschoten et al. 2007; Higgins, Cavin et al. 2008). This induction can lead to increased glutathione conjugation of potentially toxic or carcinogenic compounds, as has been described for aflatoxin B1 (Cavin, Mace et al. 2001), azoxymethane(Huber, Haslinger et al. 2007) and carbon tetrachloride (Lee, Choi et al. 2007).

In contrast to these potentially beneficial effects, cafestol is currently regarded as the most potent cholesterol-elevating bioactive compound that may be present in the human diet (Ricketts, Boekschoten et al. 2007). Remarkably, the precise mechanisms behind this effect are still not completely known. It appears that a number of key enzymes and transporters that are involved in the metabolism of cholesterol, including CYP7A1, sterol 12α- hydroxylase, and Na^+-taurocholate cotransporting polypeptide can be down-regulated by cafestol. Many of these enzymes are under control of the farnesoid X- (FXR) and the pregnane X (PXR) receptors (Ricketts, Boekschoten et al. 2007). At the same time, cafestol may also have direct toxic effects on the liver. It was shown that coffee oil, cafestol (and also kahweol) can cause an increase of serum alanine aminotransferase (ALAT) and to a lesser extent aspartate aminotransferase (ASAT) in a majority of the subjects (Urgert, Meyboom et al. 1996).

There are some studies that indicate that PXR and FXR also regulate GSTs (Falkner, Pinaire et al. 2001; Maglich, Stoltz et al. 2002; Ricketts, Boekschoten et al. 2007). However, recent evidence suggests that the induction of GSTs by cafestol is due to an induction of the Nrf2-mediated gene expression pathway (Higgins, Cavin et al. 2008) (Tao, Wang et al. 2008). Nrf2 is the key regulator of EpRE-mediated gene expression. It belongs to the nuclear basic leucine zipper transcription factors. The major regulator of Nrf2 is supposed to be Keap1, which represses Nrf2 transcription activation by cytoplasmic sequestration and mediation of the degradation of Nrf2 (Copple, Goldring et al. 2008; Surh 2008; Surh and Na 2008). When the disulfide bridges of keap 1 are oxidised, Nrf2 is released from Keap1(Copple, Goldring et al. 2008).

In the present study we used a stably transfected Hepa1c1c7 cell line with a luciferase reporter gene under the control of the EpRE derived from the human NQO1 gene as previously described (Boerboom, Vermeulen et al. 2006). Cafestol caused a dose-dependent luciferase

induction. However, this induction was only seen in the presence of S9 mix, suggesting the need for a metabolic activation process. When combined with P450 inhibitors such as ABT (Balani, Zhu et al. 2002; Yang, Choi et al. 2009) specifically inhibiting CYP3A4, CYP2B6, CYP2E1, CYP2C9, CYP2C19, 2D6 and CYP1A2, the EpRE induction was significantly decreased. This finding supports the possible role of different P450 enzymes such as CYP3A4 and CYP2E1 in cafestol metabolism. Furthermore, these findings also suggest a role of CYP450 in the bio-activation which possibly occurs via epoxidation. Depletion of glutathione (by BSO) was found to cause an increase of EpRE mediated gene induction whereas elevation of intracellular glutathione (by NAC) inhibited EpRE mediated gene induction. This is in line with our analytical data showing that glutathione conjugation is probably an important elimination route for cafestol, and/or prevents the formation of epoxide metabolites capable of oxidation reactions with Keap1 thiol groups critical for Nrf2 release and activation of EpRE-controlled gene transcription. Data from the present study suggest that epoxide formation in the furan ring is probably a key step in this process(Chen, DeRose et al. 2006). So far it is not clear whether epoxide formation also plays a role in the apparently transient liver toxicity of cafestol, as shown by elevated ASAT and ALAT levels. From its structure it could be predicted that the other diterpene in coffee, kahweol would be an even more potent inducer of the EpRE at least in vitro, since the double bond between position 1-2 makes it more prone to epoxidation. This is in line with our experimental results. Interestingly, there are some indications that certain coffee blends and mixtures with higher kahweol contents produce a higher ALAT / ASAT response in humans. However, further investigation is needed to verify this. It is not clear to what extent epoxidation or glutathione conjugation may contribute to the cholesterol-elevating effect of cafestol. One possibility might be that the glutathione conjugate after removal of the glutamyl (by gamma-glutamyl transferases) and glycine

moieties is metabolized to a (toxic) compound that affects bile production. Interestingly, while the effects of ASAT and ALAT are acute, cholesterol levels slowly increase. This would be consistent with a gradual increase in glutathione conjugation due to induction of gene expression as found in the present study.

In conclusion, we provide evidence that cafestol induces EpRE, apparently via a bioactivation process that possibly involves epoxidation of the furan ring. The epoxides themselves appear subject to conjugation with glutathione. Further studies should further reveal the role of the epoxide(s) and the glutathione conjugates in the elevation of plasma cholesterol caused by the coffee components cafestol and kahweol.

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Chapter 3

Absorption, distribution and biliary excretion of cafestol, a potent cholesterol elevating compound in unfiltered coffees in mice.

Saskia.T.J. van Cruchten, D.R. de Waart, C. Kunne, G.J.E.J Hooiveld, M.V. Boekschoten, M.B. Katan, R. P. J. Oude Elferink and R.F. Witkamp.

Division of Human Nutrition; Wageningen University, Wageningen, The Netherlands (SvC, GH, MB, RW)

Nutrigenomics consortium, Top Institute of Food and Nutrition; Wageningen, The Netherlands (SvC, GH, MB)

AMC Liver Center, Academic Medical Center, Amsterdam, The Netherlands (DRdW, CK,ROE)

Institute of Health Sciences, VU University Amsterdam, The Netherlands (MK)

TNO Quality of Life, Zeist, The Netherlands (RW)

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Non-standard abbreviations

ALT	alanine amino transferase
AST	aspartate aminotransferase
CMC	Carboxy methyl cellulose
EpRE	electrophile-response element
HPLC-RA	high-performance liquid chromatography with radioactive detection
LCMS	Liquid Chromatography mass spectrometry
Nrf2	NF-E2-related factor 2
SI	Small intestine
TUDC	tauroursodeoxycholic
QWBA	Quantitative whole body autoradiography

Abstract

Cafestol is a diterpene present in unfiltered coffees. It is the most potent cholesterol-elevating compound present in the human diet. However, the precise mechanisms underlying this effect are still unclear. In contrast, cafestol is also known as a hepatoprotective compound which is likely to be related to the induction of glutathione biosynthesis and conjugation. In the present study we investigated whole body distribution, biliary excretion and portal bioavailability of cafestol in mice. First, dissection was used to study distribution. Five hours after an oral dose with ^{3}H labeled cafestol, most activity was found in small intestine, liver and bile. These results were confirmed by quantitative whole body autoradiography in a time course study which also showed elimination of all radioactivity within 48 hours after administration. Next, radiolabeled cafestol was dosed i.v. to bile duct cannulated mice. Five hours post dose 20% of the radioactivity was found in bile. Bile contained several metabolites but no parent compound. After intestinal administration of radioactive cafestol to portal vein cannulated mice, cafestol was shown to be rapidly absorbed into the portal vein as parent compound, a glucuronide and an unidentified metabolite. From the presence of a glucuronide in bile that can be deconjugated by a bacterial enzyme and the prolonged absorption of parent compound from the GI tract we hypothesize that cafestol undergoes enterohepatic cycling. Together with our earlier observation that epoxidation of the furan ring occurs in liver these findings merit further research on the process of accumulation of this coffee ingredient in liver and intestinal tract.

Introduction

Cafestol (Figure 1) is a diterpene with a characteristic furan group which is present in unfiltered coffees such as French press coffee, Scandinavian boiled coffee, cafetiere coffee, and to a lesser extent espresso (Urgert and Katan 1997; Ranheim and Halvorsen 2005; Ricketts, Boekschoten et al. 2007). The compound is regarded as the major compound responsible for the increase in serum cholesterol observed after consumption of these coffee preparations (Weusten-Van der Wouw, Katan et al. 1994; Urgert and Katan 1997; Ricketts, Boekschoten et al. 2007). For example, three cups of French press (plunger pot) coffee, providing the equivalent of 10mg cafestol, consumed for 4 weeks are estimated to increase serum cholesterol by 0.13 mmol/l (Urgert and Katan 1996). In addition, cafestol transiently increases serum triglycerides and levels of liver alanine amino transferase (ALT), and aspartate aminotransferase (AST) (Urgert, Meyboom et al. 1996; Urgert, Essed et al. 1997; Boekschoten, Schouten et al. 2004). However, from a toxicological perspective cafestol shows a remarkable two-faced behavior. In addition to its deleterious effects on cholesterol levels and liver enzymes, cafestol has also been identified as an anti-mutagenic compound (Cavin, Holzhaeuser et al. 2002; Huber, Prustomersky et al. 2002; Huber, Scharf et al. 2002; Huber, Scharf et al. 2003; Huber, Teitel et al. 2004; Huber and Parzefall 2005). This potential beneficial effect was shown to be related to an induction of glutathione biosynthesis and conjugation, and a decreased activity of CYP450 enzymes involved in the bio-activation of some pro-carcinogens (Cavin, Bezencon et al. 2003; Huber, Teitel et al. 2004; Lee, Choi et al. 2007; Huber, Rossmanith et al. 2008). Recently we demonstrated that cafestol is able to induce an electrophile-response element (EpRE) *in vitro* after metabolic activation with S9 mix obtained from liver of male Sprague–Dawley rats treated with Aroclor-1254 (van

Cruchten, de Haan et al. 2009). The EpRE is a regulatory sequence mediating the coordinated transcriptional activation of genes associated with phase 2 biotransformation, protection against oxidative stress, and other cancer-chemo protective mechanisms (Kobayashi, Kang et al. 2006). The key regulator of EpRE-mediated gene expression is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and, to a lesser extent, Nrf1 (Copple, Goldring et al. 2008). This Nrf2 activation may be responsible for the increase in glutathione biosynthesis and conjugation. We hypothesized that metabolites formed via epoxidation of the furan moiety are involved in this induction process. Indeed we were able to demonstrate the presence of cafestol epoxides and their epoxy-glutathione conjugates in bile of mice dosed with cafestol. In addition, we also found a glucuronide conjugate of cafestol in mouse bile (van Cruchten, de Haan et al., 2009).

The present studies were undertaken to study body distribution, portal bioavailability and biliary excretion of cafestol in mice in more detail. The selection of the mouse as a model was based on previous studies showing that among various animal models, the ApoE*3-Leiden transgenic mouse is the only model that responds to cafestol as humans do (Post, de Roos, et al. 2000).

Distribution after oral administration of radiolabeled cafestol was studied by liquid scintillation counting of dissected tissues, and by quantitative whole body autoradiography. Following the observation that the distribution of the radiolabel remained largely restricted to the liver and intestinal tract, the question was addressed whether enterohepatic cycling plays a role. This was first investigated by studying biliary excretion of cafestol and metabolites after i.v. administration to gallbladder cannulated mice. Finally, portal delivery of cafestol and potential metabolites was assessed after intestinal administration.

Material and methods

Chemicals

Chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated. Solvable™ (tissue solubilizing fluid) was purchased from Perkin and Elmer (Groningen, The Netherlands). Methanol was purchased from Baker (Mallinckrodt Baker, Deventer, The Netherlands).

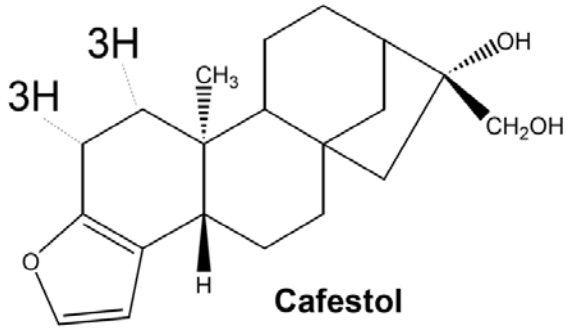

Figure 1: ***Structure of cafestol****. Labeling was on the C1 and C2 positions. Before each use, radiochemical purity of* ^{3}*H Cafestol was verified (99%).*

Radio labelling

^{3}H Cafestol (1mCi/mmol) was synthesized by RC Tritec (Teufen, Switzerland). The compound was made by hydrogenation of kahweol (Alexis Biochemicals, U.S.A) to cafestol under tritium gas atmosphere using $Pd/SrCO_3$ in EtOH. Labeling was on the C1 and C2 positions. Before each use, radiochemical purity (> 99 %) of ^{3}H Cafestol was verified by HPLC-RA. Exchange of the 3H label with water was checked by collecting and counting

water evaporating at 105 °C from a treated liver sample using a Carbolite furnace (Keison, Essex, UK).

Animals

Pure bred wild-type adult (8-10 weeks), male (C57BL6/j) mice were purchased from Harlan (Horst, Netherlands). Mice were housed in a light- and temperature controlled facility and had free access to water and standard laboratory chow (CRME, SDS diets, The Netherlands). All animal studies were approved by the Local Committee for Care and Use of Laboratory Animals of Wageningen University, or the Amsterdam Medical Centre (for the studies with gallbladder and portal vein cannulated mice), Amsterdam, The Netherlands.

^{3}H radiolabeled preparations for administration

Preparations for administration were made by mixing ethanol stock solutions of unlabeled cafestol with appropriate amounts of ^{3}H cafestol. These solutions were administered after mixing with either 0.1% of carboxymethyl cellulose in water (experiment I), olive oil (experiment II) or Clinoleic 20% emulsion (Baxter, IL, U.S.A, experiment III).

Experimental setup

Four experiments were performed. The aim of experiment I was to study distribution of the radiolabel after oral administration, using liquid scintillation counting of organs and tissues. Mice were fasted for 4 hours. Then 1.5mg of cafestol enriched with ^{3}H cafestol to an equivalent of 10μCi was given to each animal by oral gavage (mixed with a 0.1% solution of

carboxymethyl cellulose in water). Five hours after dosing, mice were killed and organs and blood samples collected. In experiment II, quantitative whole body autoradiography (QWBA) was used to study tissue distribution after oral administration in more detail and at more points in time. Mice (eight in total) were dosed 1.5mg unlabeled cafestol in olive oil via oral gavage mixed with a trace amount of ^{3}H cafestol (1mCi/kg BW). At different time points (0.5, 1, 2, 4, 8, 12, 24 and 48 hours) mice were anesthetized with a mixture of isoflurane and oxygen and blood was sampled via orbital punction and puncture of the tail vein. Then mice were euthanized with a mixture of isoflurane and oxygen, fixated and subsequently frozen in a hexane-dry ice bath. Whole body autoradiography was performed according to standard operation procedures (Solon, Balani et al. 2002) as described below. Experiment III was performed to study biliary excretion of cafestol and its metabolites after i.v. administration of labeled cafestol. For this experiment, five mice were fasted overnight. Mice were anaesthetized, subsequently their gallbladder was cannulated and bile collected (Kuipers, van Ree et al. 1996; Klett, Lu et al. 2004). To maintain a constant bile flow, mice were infused with a bile acid solution containing tauroursodeoxycholic (TUDC) acid (conc. 600nmol/100gramBW/min) according to standard procedures (Klett, Lu et al. 2004). After 60 minutes of infusion, bile flow was considered constant based on our previous experience and cafestol was injected through the tail vein. Every mouse was injected with 200µl of a mixture containing 12µg unlabeled compound and 10µCi ^{3}H cafestol dissolved in Clinoleic (20%) in the tail vein. Bile was sampled every 15minutes during the first hour and then every half hour. After 5 hours, blood samples were drawn from the systemic circulation by orbital puncture. Then mice were killed and liver, intestine and kidneys were isolated. Bile and plasma samples were immediately frozen at −80°C. Bile flow was determined gravimetrically assuming a density of 1g/ml for bile. Total radioactivity in the samples was determined by scintillation

counting. These values were averaged for the number of mice. Organ samples were processed as described in the organ sample preparation section.

In the fourth experiment (experiment IV), the portal vein of C57Bl6 mice that had fasted for 4hours was cannulated. A solution containing cafestol mixed with 60µCi ^{3}Hcafestol was administered at a dose of 1.5 mg directly into the duodenum. Portal blood was sampled at different time points: 2, 5, 10, 20, 30, 40 and 50minutes. At the end (50 minutes post dose) a blood sample from the systemic circulation was collected by orbital puncture. Blood samples were centrifuged at 4000g for 10minutes and plasma was stored at -80ºC until further HPLC analyses.

Organ sample preparation

Organ samples were cut in pieces of approximately 25mg and incubated overnight at room temperature with 1ml Solvable. After incubation, samples were decoloured with 300µl hydrogen peroxide (30%) (1000µl in case of spleen), and counted. Blood samples (30µl) were centrifuged and the radioactivity in plasma was determined. Five microliters of bile were diluted with 5ml scintillation fluid (Ultima Gold^{R}, Perkin and Elmer, Groningen, The Netherlands) and incubated at room temperature overnight in a dark environment. The radioactivity in all samples was measured using a liquid scintillation counter (model 3255, Packard Instrument Co., Downer's Grove, IL, U.S.A.), with a quench curve used for correction.

Quantative whole body autoradiography

The animals were rapidly frozen by total immersion in a hexane/solid carbon dioxide mixture (ca. -80°C) and retained at -80°C for quantitative whole body autoradiography. Following removal of the whiskers, legs and tail, each frozen carcass was placed in a block of carboxymethyl cellulose (1% aqueous solution, w/v) and mounted onto the stage of a Leica CM3600 cryomicrotome maintained at ca. -20°C. Sagittal sections (nominally 30µm) of each animal were subjected to whole-body autoradiography using procedures based on the work of Ullberg (Ullberg 1977), at five different levels of the animal body (to include as many tissues as possible). Three sections were taken at each level and freeze-dried. One section from each level was selected and, along with a ^{3}H-whole blood standard curve, placed in contact with FUJI imaging plates. The imaging plates were placed in light-tight cassettes and allowed to expose for 7 days. After exposure, and under subdued lighting, the sections were removed from the plates and the plates processed using a FUJI BAS 1500 Bio-image analyser (Fuji). These analyses were performed by Quotient Bioresearch (Rushden, UK).

HPLC analysis with radiochemical detection of cafestol and metabolites

Five microliters of bile sample were mixed with 45µl milliQ water. This sample was analyzed by a HPLC system equipped with a ChromSpher column (Varian, Middelburg, The Netherlands), 100*4.6 mm i.d, packed with 3µm omnisphere C18 material (Varian). The mobile phase was delivered using a system with Gynkotech pumps (Germering, Germany) at a flow rate of 0.4ml/min. Solvent A was 5mM phosphate buffer pH 6.3 and solvent B was 100% methanol. A linear gradient of 0minutes 10%B to 20minutes, 100%B was used to separate the components. Detection was performed using a flow scintillation analyzer (500TR

series, Packard Instrument Co., Downer's Grove, IL, U.S.A., run in parallel with a absorbance detector (spectroflow 757, Kratos Analytical Instruments, Ramsey, USA).

Deconjugation of cafestol metabolites

Bile samples (5µl) were deconjugated by adding 10µl of E.coli derived β-glucuronidase solution. This mixture was incubated at 37°C for 1hour. HPLC analysis was performed with these samples as described.

Results

Tissue distribution of ^{3}H cafestol (metabolites)

Five hours after oral administration of ^{3}H labeled cafestol, the major amount of the radioactivity was found in the intestinal tract (Figure 2). Within the gastro intestinal tract, radioactivity was found both in the lumen of the small intestine (SI) (14%) and in the SI itself (10%), 34% was in the cecum (combined contents and mucosa), 6% in the colon lumen and 1% in the colon mucosa. After the intestinal tract, the largest fractions of radioactivity were found in liver (5%) and bile (2%). Approximately 1% of the radioactivity was detected in kidneys. No radioactivity was detected in esophagus, pancreas, spleen, adrenals, brain, heart, lung, white adipose tissue and muscle. No radioactivity was also detected in systemic blood and only 1 % of the dose was excreted per 100µl of urine. Total recovery of radioactivity was 98% (±31%). In this experiment it was not further investigated whether the radioactivity in these tissues was due to cafestol or to cafestol metabolites.

Quantitative whole body autoradiography

Every mouse was cut at 5 different levels of its body. From every level, 3 sections were made. Selected whole-body auto-radiograms are given in figure 3. QWBA confirmed the distribution pattern as obtained by dissection. Eight different time points (0-48 hours) were investigated by QWBA. Thirty minutes post dose, almost all radioactivity was found in liver and small intestines. Trace amounts were found in kidneys. Apart from liver and intestinal tract and the traces in kidney, no radioactivity was seen in any other organs or tissues that were analyzed. Blood samples were collected separately and consistent with the data from the

dissection study, no radioactivity was found. This relative distribution pattern remained essentially the same during the first 24hours post dose. At 48hours post dose, no radioactivity was detected anymore.

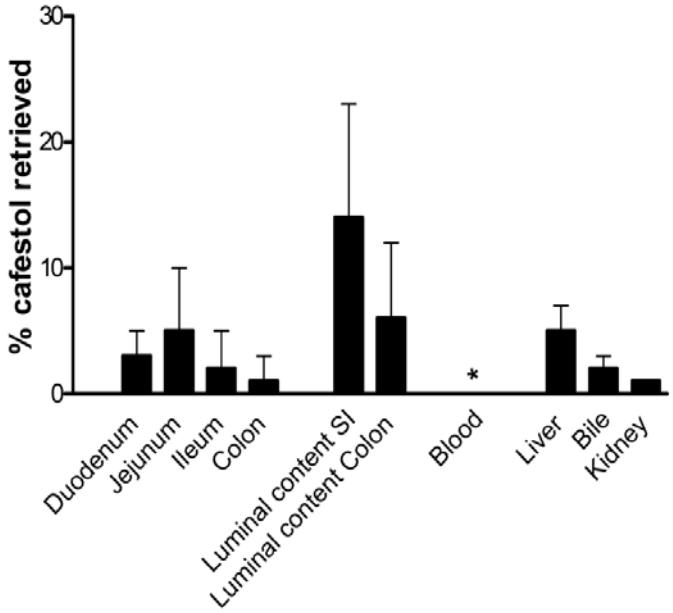

Figure 2: ***Organ distribution of cafestol in mice****. Data show the levels of 3H cafestol(metabolites) 5 hours after oral administration to mice, expressed as percentage of administrated dose corrected for organ weight. Trace amounts of cafestol were detected in total blood volume (0.001%) (*). No cafestol could be detected in; esophagus, pancreas, spleen, adrenals, brain, heart, lung, white adipose tissue and muscle. Values are expressed in averages ± SEM.*

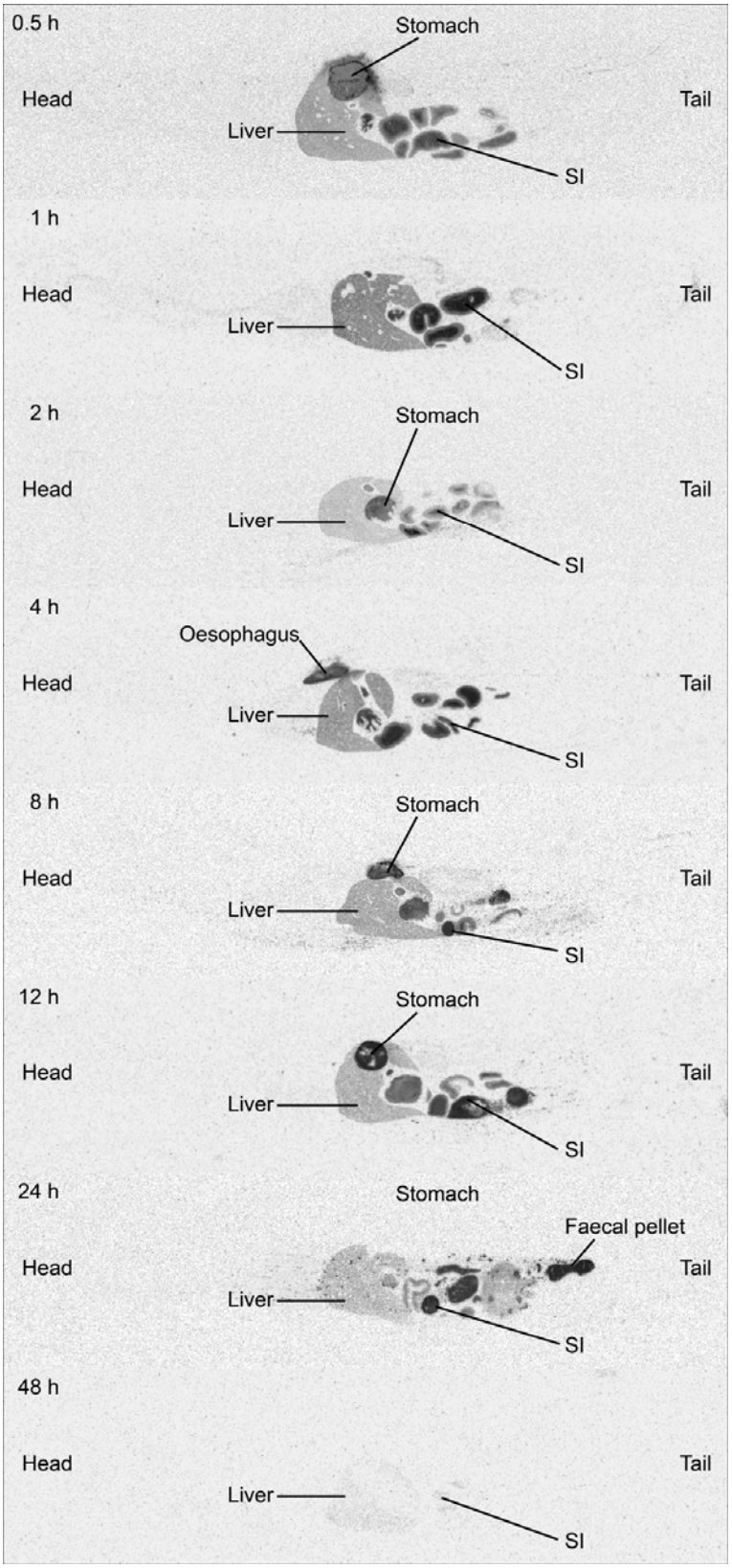

Figure 3: ***Quantitative whole body autoradiography (QWBA) of cafestol fed C56BL6/j mice.*** *Thirty minutes post dose, almost all radioactivity is found in liver and small intestine. Trace amounts were found in systemic blood and kidneys. Apart from liver and intestinal tract, no radioactivity was detected in all other sections that were analyzed. At 48hours post dose, no radioactivity was detected anymore.*

Biliary excretion of cafestol metabolites

The biliary excretion of radioactive cafestol after i.v. administration is depicted in figure 4. Five hours after administration a cumulative amount of 20% of the administered dose of radioactivity was secreted in bile. In the blood samples taken at that moment by orbital puncture, no radioactivity was detected.

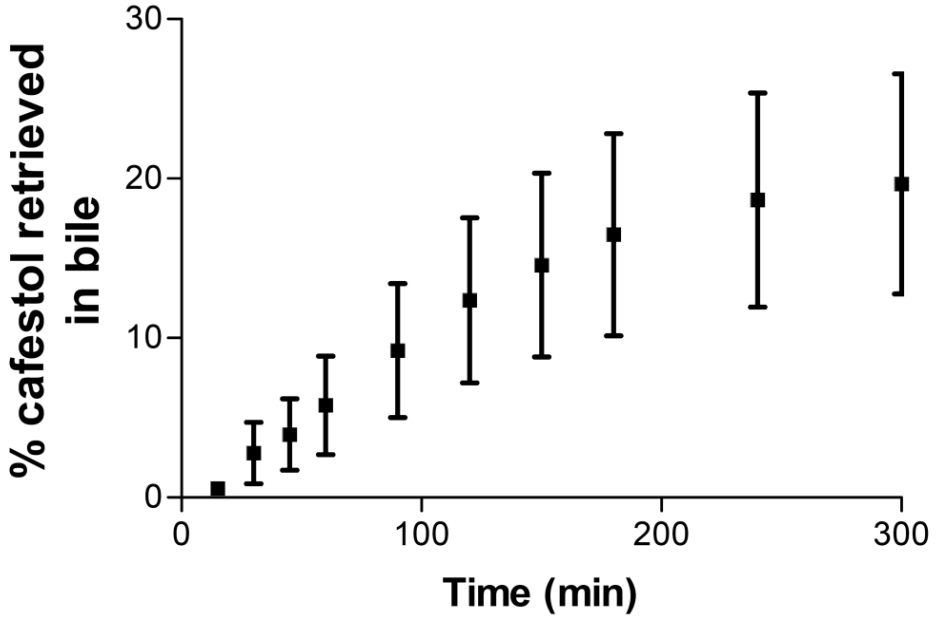

Figure 4: ***Cafestol metabolites are transported via bile in mice****. This graph shows the ^{3}H cafestol (metabolites) present in bile at the different time points. Bile samples were taken at different time points and relative amounts ^{3}H cafestol were determined. Each value is the mean of 5 mice and variation is the SEM. Cafestol percentages are plotted cumulative.*

HPLC analysis of bile showed that there was no parent compound detectable. Instead, several apparent cafestol metabolites were found. As can be seen in Figure 5a, bile samples contained multiple ^{3}H peaks. These peaks are designated as peak I, II, III, and IV, eluting at retention

time 3.5, 10, 13 and 18 minutes, respectively. To further characterize these peaks, conjugation and deconjugation reactions were performed. Deconjugation with β-glucuronidase resulted in a decrease of peak IV. A new peak (V) eluted at retention time (RT) 22 minutes, which corresponds to the parent compound cafestol (Figure 5B). This was confirmed by conjugation reactions (data not shown) in which uridine 5- diphosphoglucoronic acid (UDPGA) and microsomes were added to radioactive cafestol. From the present study no direct structural information regarding the identity of the compounds can be derived. It is clear that the most abundant cafestol metabolite in bile is cafestol glucuronide conjugate (41%). The identity of the other three metabolites, representing the majority of the radioactive cafestol metabolites in bile (RT 3.5 minutes, 7%; RT 10 minutes, 32%, RT 13 minutes, 20%), was not further elucidated.

Analyses of cafestol and metabolites in portal blood

Very rapidly after duodenal administration three radio-active compounds appeared in portal blood. Figure 6 shows their relative abundance in time. At all time points, the most abundant radioactive compound in portal blood was found to be the parent cafestol (50% of total radioactivity). In addition, intestinal biotransformation led to two major metabolites. One of the two has the same retention time as the glucuronide found in bile suggesting that it is the glucuronide. At 10 min post dose 70% of parent compound is present in portal blood. After this the concentration of parent compound decreases (to 41%) whereas at 50 min post dose, the concentration parent compound in portal blood is increased up to 70% again. Taken together, absorption of parent compound and metabolites to the portal vein is prolonged, and apparently follows a cyclic pattern (figure 6). In the sample taken from the systemic circulation at t=50 min no radioactivity was detected.

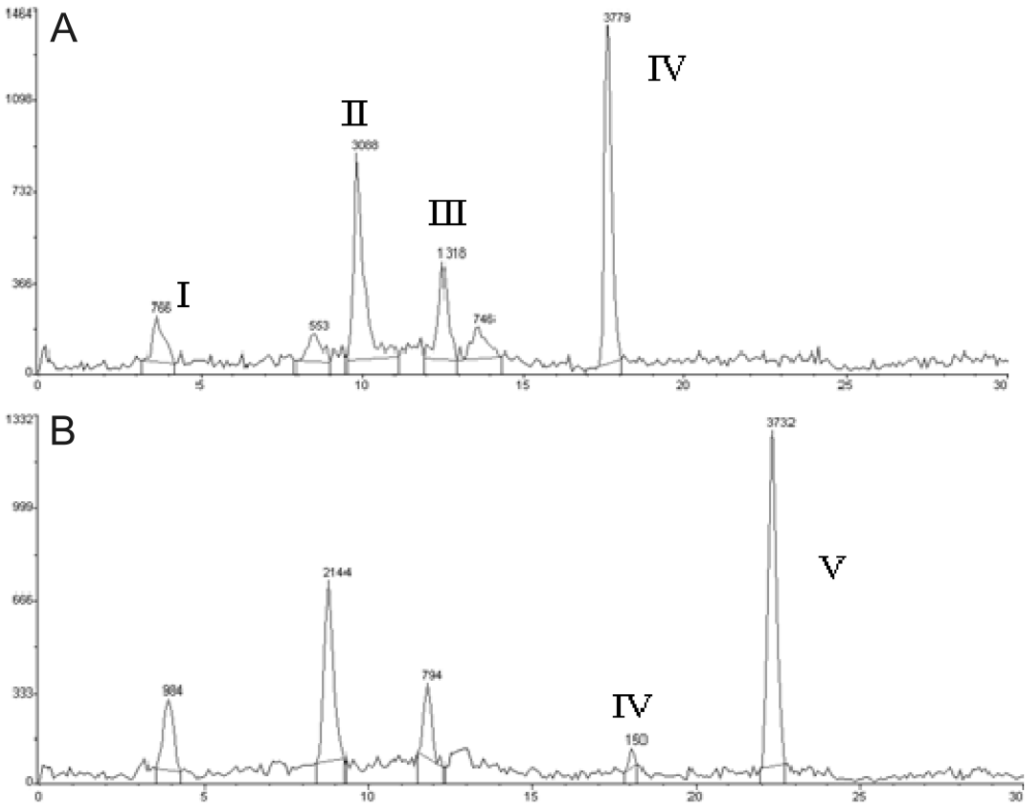

Figure 5: ***Metabolite at retention time 18minutes is a cafestol glucuronide conjugate****. In figure A is a bile sample of a mouse treated with cafestol at time point 5minutes. (y-as; DPM present in bile fraction); x-as; retention time (minutes). Figure 5b is the same bile sample but then deconjugated with β-glucuronidase.*

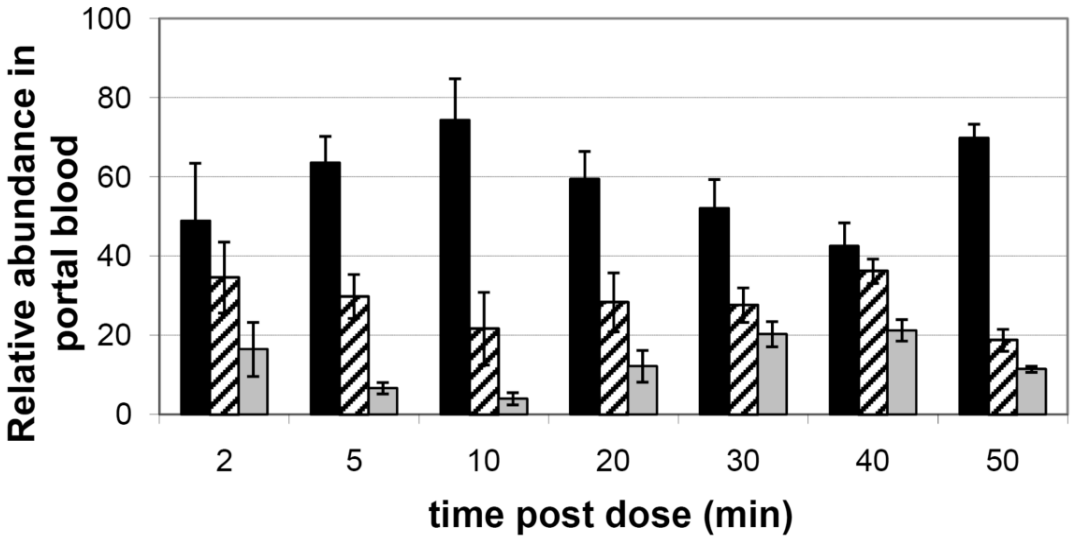

Figure 6: ***Cafestol and metabolites in portal blood****. Data shows that after duodenal administration cafestol is absorbed and transported by portal vein. No cafestol is present in systemic blood. Most abundant in portal blood is the parent compound (±70%), however cafestol is also partly metabolized by intestine, resulting in two metabolites of which one is a glucuronide. (Black box, parent compound; Striped box peak 1, Grey box, cafestol glucuronide). Values are expressed in averages ± SEM.*

Discussion

Results from both distribution studies indicate that cafestol and/or its metabolites strongly accumulate in the liver and gastro-intestinal tract. After oral administration, hardly any distribution to other parts of the body was found. This distribution pattern remains fairly constant for at least 24hours after administration. Both after i.v. and oral administration no radioactive fraction corresponding to the parent compound was found in bile. This confirms our earlier results using LC-MS, where we showed that cafestol is extensively metabolized by the liver to epoxy-glutathione conjugates, glutathione conjugates and glucuronide conjugates (van Cruchten, de Haan et al. 2009). These metabolites are subsequently excreted into the bile. Although the radioactive HLPC analyses in the present study do not provide direct structural confirmation, the conjugation-deconjugation experiment confirms our previous observations that one of the major metabolites in bile is a glucuronide. The same glucuronide is also found in portal blood (already 2min post dose), which indicates that glucuronidation also takes place in the intestinal epithelium. However, parent cafestol is also rapidly absorbed into the portal vein. Two minutes after dosing, the parent compound represented 50% of the total radioactivity present in portal blood. Remarkably, cafestol absorption continued during the next 50 minutes, still representing 70 % of the activity present in portal blood at 50min post administration. The presence of a glucuronide in bile found to be easily deconjugated by a bacterial enzyme, together with the prolonged absorption of parent compound from the GI tract suggests that cafestol undergoes enterohepatic cycling. It should be mentioned that the cafestol dose used in the oral studies, 1.5 mg per mouse, is rather high compared to the amount present in coffee. Depending on the brewing, coffees may contain up to 3.5 mg per cup of 100 ml (Ranheim and Halvorsen, 2005). If cafestol kinetics would be non-linear in

mice, a relatively high concentration in the intestinal lumen might partly explain the prolonged absorption and accumulation in intestinal tissue. Cafestol is the most potent cholesterol-elevating compound by weight present in the human diet (Urgert and Katan 1997). Several studies have shown that cafestol produces a clinical significant rise of serum cholesterol, which is a relevant risk for the development of cardio vascular diseases. Epidemiological studies in Scandinavians have shown that drinking large amounts of cafestol-rich coffee is indeed associated with an increased risk for coronary heart disease (Stensvold and Tverdal 1995; Urgert, Schulz et al. 1995; Stensvold, Tverdal et al. 1996; Urgert and Katan 1996). High intakes have also shown to increase serum levels of ALT and AST indicating liver damage (Urgert, Essed et al. 1997; Urgert and Katan 1997). In a previous study we proposed that epoxidation of the furan ring plays a role in these hepatotoxic effects of cafestol (van Cruchten, de Haan et al. 2009). We also showed *in vitro* induction by cafestol of an electrophile-responsive element (EpRE) derived from the human NQO1 regulatory region (van Cruchten, de Haan et al. 2009). Further studies are needed to elucidate in which form accumulation in the liver occurs. In combination with our earlier observations, it could be speculated that the accumulation of radioactivity is related to binding of reactive intermediates to cellular macromolecules. Data from the present study show that the liver is exposed to significant amounts of cafestol which can lead to the formation epoxides. This has been described for several other furan containing molecules, including furan, menthofuran, ipomeanine, 4-ipomeanol, furosemide and teucrin A (Khojasteh-Bakht, Chen et al. 1999; Alvarez-Diez and Zheng 2004; Baer, Rettie et al. 2005; Peterson, Cummings et al. 2005; Chen, DeRose et al. 2006). For some of these compounds, including the natural toxin ipomeanol, formation of furan-epoxides has indeed been associated with cellular toxicity. To the best of our knowledge, no evidence for the presence of either cafestol or its metabolites in

human plasma is available. In the present study, we also found no cafestol in the peripheral circulation of mice that orally received cafestol. The only human data on cafestol pharmacokinetics and metabolite formation in more detail have been published by De Roos et al. (De Roos, Meyboom et al. 1998). These colleagues investigated cafestol disposition in healthy ileostomy volunteers. From the recovery of cafestol metabolites in the ileostomy effluent it was estimated that approximately 70% was absorbed from the GI tract. As only about 1% of the dose was recovered in urine, it was concluded from that study that cafestol is subject to extensive metabolism in the human body. These observations in humans are in line with those of the present study but our new data put the original conclusion in a slightly different perspective. Indeed, cafestol seems to be absorbed very rapidly and its passage to the systemic circulation is near to zero. However, in addition to direct biotransformation, accumulation in the liver and enterohepatic cycling play important roles in the kinetics of the compound. The causal relations between epoxide formation, liver accumulation and cholesterol elevation remain to be elucidated. The effect of cafestol on blood lipids in humans is unusually slow. It takes at least 4 weeks to reach new steady-state levels of blood lipids. Our data do not suggest that this is due to a slow accumulation of cafestol in some body pool, because cafestol does not seem to penetrate beyond the enterohepatic axis and is cleared fairly rapidly. Secondary changes in liver metabolism induced by cafestol may explain its unusually protracted effect on blood lipids.

In conclusion, data from the present study provide evidence that cafestol is efficiently absorbed and partially metabolized by the gut, cleared and further metabolized by the liver and excreted into bile. Because the main metabolite is a glucuronide and because the parent

compound appears to be present in portal blood, we suggest that cafestol is likely to undergo enterohepatic circulation.

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Chapter 4

Cafestol prevents diet-induced obesity in mice.

S.T.J. van Cruchten[1, 2], A. Knol[1], I. Felicidade[1], H. Schipper[3], M.V Boekschoten[1, 2] , M.B. Katan[4], G.J.E.J. Hooiveld[1, 2] and M. Müller[1, 2].

[1]*Division of Human Nutrition, Wageningen University, the Netherlands*

[2]*Nutrigenomics Consortium, Top Institute Food & Nutrition, Wageningen, the Netherlands*

[3]*Experimental Zoology Group, Wageningen University, the Netherlands*

[4]*Department of Health Sciences, Free University, Amsterdam, the Netherlands*

Submitted for publication.

Abstract

Cafestol is the most potent cholesterol-raising compound in human diet and is present in unfiltered coffees. Cafestol inhibits bile acid synthesis and reduces hepatic triglyceride content. In the current studies we investigated whether cafestol could prevent the development of hepatic steatosis in mice fed a high fat diet, and had a protective effect on the development of HFD-induced metabolic syndrome. Mice were fed a low or high fat diet supplemented or not with cafestol (0.5%) up to 8 weeks. Bodyweight and food intake was measured weekly, and body composition was measured using dual energy X-ray absorptiometry (DEXA). Food intake between all diet groups was similar at all times. Compared to LFD, HFD feeding significantly increased body weight, fat content and percentage during the whole experiment. Cafestol reduced body weight gain and adiposity in HFD animals, which after 8 weeks resulted in 20,8 % less weight gain. Feeding a HFD resulted in clear signs of fatty livers but this was prevented by cafestol. Cafestol increased fecal fat content but decreased the white and brown adipose tissue depots. Plasma leptin levels paralleled the decrease in WAT weight. Supplementation of cafestol to a HFD normalized fasting plasma glucose and insulin levels, suggesting improved insulin sensitivity cafestol supplemented mice compared to HFD. In conclusion, our results provide novel findings on the effects of cafestol on obesity and associated metabolic dysfunction. We demonstrated that cafestol prevented diet induced obesity, adiposity and liver steatosis under high-fat feeding, all of which are related to the observed improved insulin sensitivity.

Introduction

Cafestol and kahweol are diterpenes present in unfiltered coffees, such as French press, Scandinavian and espresso. Cafestol is the most potent cholesterol-raising agent known in human diet. Of the two diterpenes, cafestol accounts for 80% of the cholesterol-raising effect (Urgert and Katan 1997). Therefore, in the current studies we focused on the effects of cafestol. The exact molecular mechanism underlying the cholesterol-raising effect is unknown. However, it has been demonstrated that cafestol inhibits hepatic bile acid metabolism and triglyceride (TG) accumulation (Post, de Roos et al. 2000). In liver, cafestol suppresses both the mRNA expression and enzyme activity of cholesterol 7α-hydroxylase (Cyp7a1), by which it inhibits the conversion of cholesterol to bile acids (Post, de Roos et al. 2000). Cafestol also activates the nuclear receptors Fxr and Pxr (Ricketts, Boekschoten et al. 2007). Via Fxr cafestol activates transcription of the fibroblast growth factor (Fgf) 15 in small intestine (Ricketts, Boekschoten et al. 2007). Subsequently, Fgf15 travels to the liver and binds to fibroblast growth factor receptor (Fgfr) 4, thereby inhibiting bile acid synthesis (Inagaki, Choi et al. 2005). Activation of the nuclear receptor Pxr by cafestol may lead to increased plasma cholesterol levels (Zhou, King et al. 2009). In addition, it has been reported that in mice very low density lipoproteins (VLDL) triglyceride (TG) production rate was reduced two fold by cafestol, which coincided with reduced hepatic TG levels (Post, de Roos et al. 2000). These data suggested that cafestol impairs hepatic TG synthesis. Both findings are thought to be involved in the cholesterol-raising mechanism of cafestol.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver disorders (Clark, Brancati et al. 2002; Bedogni, Miglioli et al. 2005; Jump, Botolin et al. 2008). NAFLD is considered as the hepatic component of the metabolic syndrome, being closely

associated with insulin resistance and hypertriglyceridemia (Chitturi, Weltman et al. 2002; Marchesini, Marzocchi et al. 2005), and in non-diabetic subjects there is a correlation between body mass index and liver fat (Gastaldelli, Cusi et al. 2007). Fatty liver disease is a major contributor to cardiovascular and overall obesity-related morbidity and mortality (Angulo 2007; Loria, Lonardo et al. 2007). It has been suggested that dietary factors may contribute to the development of liver steatosis in humans (Zivkovic, German et al. 2007). We and others have shown that in mice overnutrition induced by feeding a high-fat diet (HFD) results in hepatic steatosis and NAFLD (Patsouris, Reddy et al. 2006; Stienstra, Mandard et al. 2007; Zivkovic, German et al. 2007; Radonjic, de Haan et al. 2009). Therefore, in the current studies we set out to investigate whether cafestol could prevent the development of liver steatosis in mice fed a HFD, and had a positive effect on the development of HFD-induced metabolic syndrome. We indeed observed protective effects of cafestol on hepatic steatosis, but unexpectedly also on whole body adiposity, adipose tissue mass and insulin sensitivity. We provide evidence that these effects may be mediated through Nrf2-mediated reduction of hepatic ER stress and increased energy expenditure due to mitochondrial uncoupling.

Material and methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). Cafestol was obtained from LKT Laboratories, Inc (St. Paul, MN).

Animals

Pure-bred 11-12-weeks old, male C57BL6/J mice were purchased from Harlan (Horst, the Netherlands). Mice were housed in a light- and temperature-controlled facility and had free access to water and diets. All animal studies were approved by the local Committee for Care and Use of Laboratory Animals.

Diets

Semi-synthetic diets were used in this study. The LFD and HFD were based on OpenSource diets D12450B and D12451, respectively, in which lard was replaced by palm oil. The fat fraction provided either 10% (LFD) or 45% (HFD) of the caloric content of the diet. In addition, the sucrose content of the LFD was made equal to that of the HFD. The only other variable besides fat content was the amount of corn starch, which was the main source of carbohydrates. Cafestol was mixed in the diets at a concentration of 0.05% (w/w). All diets were made by Research Diet Services (Wijk bij Duurstede, the Netherlands) (for exact composition see supplemental data (de Wit, Bosch-Vermeulen et al. 2008).

Experimental setup

Two experiments were performed. Experiment A: mice were fed the LFD or HFD for 4 weeks (n=90 mice per group). After this run-in period, designated as t=0, 10 mice per group were killed for collection of tissues. The remaining 160 mice were stratified on body weight into four groups. The LFD group was split in either a group that continued to receive the LFD, and a group that received the LFD supplemented with cafestol (LFD-C). Similarly, the HFD group was split into a HFD and a HFD-C group. At t=1, 4 and 8 weeks, 10 mice from every group were killed for tissue sampling. To study the effects of acute exposure to cafestol, 10 mice of the LFD and HFD groups were dosed 300 µl olive oil with or without 0.05% cafestol (1.5mg) by oral gavage at t=0. Six hours post dose, mice were killed and tissues removed. Experiment B: This experiment was an exact copy of experiment A with 4 mice per group, except that only body composition was analyzed at t=0 and 8 weeks.

Tissue sampling

Mice were fasted at 8.00 am for 4 hours and subsequently sacrificed. Mice were anaesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%), and oxygen (30%). Blood was sampled in heparin coated microtainers (BD, NJ) and plasma isolation was performed as described by the manufacturer. After blood sampling the liver, small intestine, epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) were excised and weighed. Pieces of tissue were snap-frozen in liquid nitrogen for RNA isolation, whereas other pieces were fixed by immersion in 4% PBS-buffered formaldehyde for histological analyses.

Expression analysis

RNA isolation and quality control.

Total RNA was isolated from tissue samples using TRIzol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. RNA was treated with DNase and purified using the Qiagen RNeasy kit (Qiagen, Venlo, the Netherlands). Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits and displayed no chromosomal peaks or RNA degradation products. Single-stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the reverse-transcription system from iScript cDNA synthesis kit (Biorad, Veenendaal, the Netherlands) according to the supplier's protocol. qRT-PCR was performed on a MyIQ thermal cycler (Bio-Rad) using Platinum Taq DNA polymerase (Invitrogen) and SYBR green (Molecular Probes, Leiden, the Netherlands). The majority of primer sequences were obtained from the PrimerBank at Harvard University (Spandidos, Wang et al. 2008). Primer sequences are available upon request. Samples were analyzed in multiple pools each comprising RNA of 3-4 mice, and standardized to 36B4 expression.

Histology/Immunohistochemistry

After fixing, tissues were processed in an automatic tissue processor, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin using standard protocols. For

detection of macrophages/monocytes, an F4/80+ antibody (Serotec, Oxford, UK) was used. Sections were preincubated with 20% normal goat serum followed by overnight incubation at 4 °C with the primary antibody diluted 1:50 in phosphate-buffered saline, 1% bovine serum albumin. After incubation with the primary antibody, a goat anti-rat IgG conjugated to horseradish peroxidase (Serotec) was used as a secondary antibody. Visualization of the complex was done using 3,3′-diaminobenzidene for 5 min. Negative controls were used by omitting the primary antibody. Sections were examined on a CKX41 microscope (Olympus, Zoeterwoude, the Netherlands) equipped with calibrated DP software, version 3.2 (Olympus). This software was used to measure the number and cell surface area of adipocytes in WAT and BAT.

Plasma analysis

Fasting plasma glucose was measured by means of an Accu check Compact meter. Fasting insulin was measured using mouse ultrasensitive EIA (Alpco diagnostics, NH). Insulin sensitivity was indicated through calculation of the homeostasis model assessment (HOMA) index as follows: HOMA = insulin (mU/m) x [glucose (mmol/L)/22.5] (Matthews, Hosker et al. 1985).

Hepatic triglyceride analysis

Hepatic triglycerides were determined in 10% liver homogenates prepared in buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5) using a commercially available kit from Instruchemie (Delfzijl, the Netherlands).

Body composition

Dual energy x-ray absorptiometry (DEXA) was used to measure body composition, fat and lean body mass. Scans were performed on anesthetized mice with a PIXImus imager (GE Lunar, Madison, WI) and Lunar PIXImus 2 software. The head region was excluded from the analysis. Values were obtained for the total tissue mass (calculated by the software, in g), total area (cm2), fat content (g), lean content (total tissue mass minus fat content, g) and percent fat (fat content divided by total tissue mass). Body weight was measured on a scale just prior to scanning.

Fecel fat analysis

Feces were sampled and frozen at -20°C. After freeze-drying, the feces were grinded. To quantify fat in feces an acidified water – ethanol – petroleum ether (PEE) extract was obtained from 60-80 mg feces. H_2O/12 N HCl/Ethanol/PEE (40-60°C: 60-80°C = 1:1) (0.55:0.05:1:4,v/v/v/v).The extraction procedure was repeated twice with PEE. The PEE phase was obtained after centrifugation (10 min, 4000 *g*) and evaporated under nitrogen. The difference in weight of the tube (collecting the supernatants) before and after adding the supernatant was the fat content corresponding to the weight of the feces.

Statistical analyses

Comparisons between a single control (LFD) and treatments were made by using Univariate ANOVA. Comparisons between cafestol and control were made by student's t-test. A p-value

of less then 0.05 was considered to be significant. Statistical analysis was performed using SPSS version 15 (SPSS Inc, Chicago, Il).

Results

Cafestol prevents body weight gain of mice fed a HFD

First, we assessed the overall change in body weight during the diet intervention. At t=0, after the 4 week run-in period, HFD feeding caused significantly more body weight gain compared with LFD feeding, as expected (Figure 1A). However, supplementation of the HFD with 0.05% cafestol reduced body weight gain already after 1 week of intervention, and body weight gain remained reduced throughout the whole experiment (Figure 1A). When supplemented to the LFD, cafestol had no effect on body weight gain. Food intake was similar in all diet groups (Figure 1B). We next evaluated whether the cafestol-mediated reduction in weight gain could be attributed to an increased fecal fat loss. Indeed, after 8 weeks of cafestol supplementation the amount of dietary fat absorbed was significantly reduced (Figure 1C). However, it should be noted that this increase in fecal fat excretion cannot fully explain the reduction in body weight gain caused by cafestol supplementation.

Cafestol reduces adiposity

Triggered by these findings, we performed a new experiment in which body composition of the animals was measured more detailed by means of a DEXA scan. Total fat and lean body mass was determined both at the beginning and at the end of the experiment. As expected at t= 0, mice fed the HFD showed a significant higher bodyweight, absolute fat content and a higher fat percentage compared to LFD animals (Table 1). These differences were even more pronounced at t=8 weeks (Table 1). HFD-C animals had significant lower body weight,

absolute fat and lower fat percentages in comparison to HFD. On a LFD, cafestol had no significant effect on these parameters.

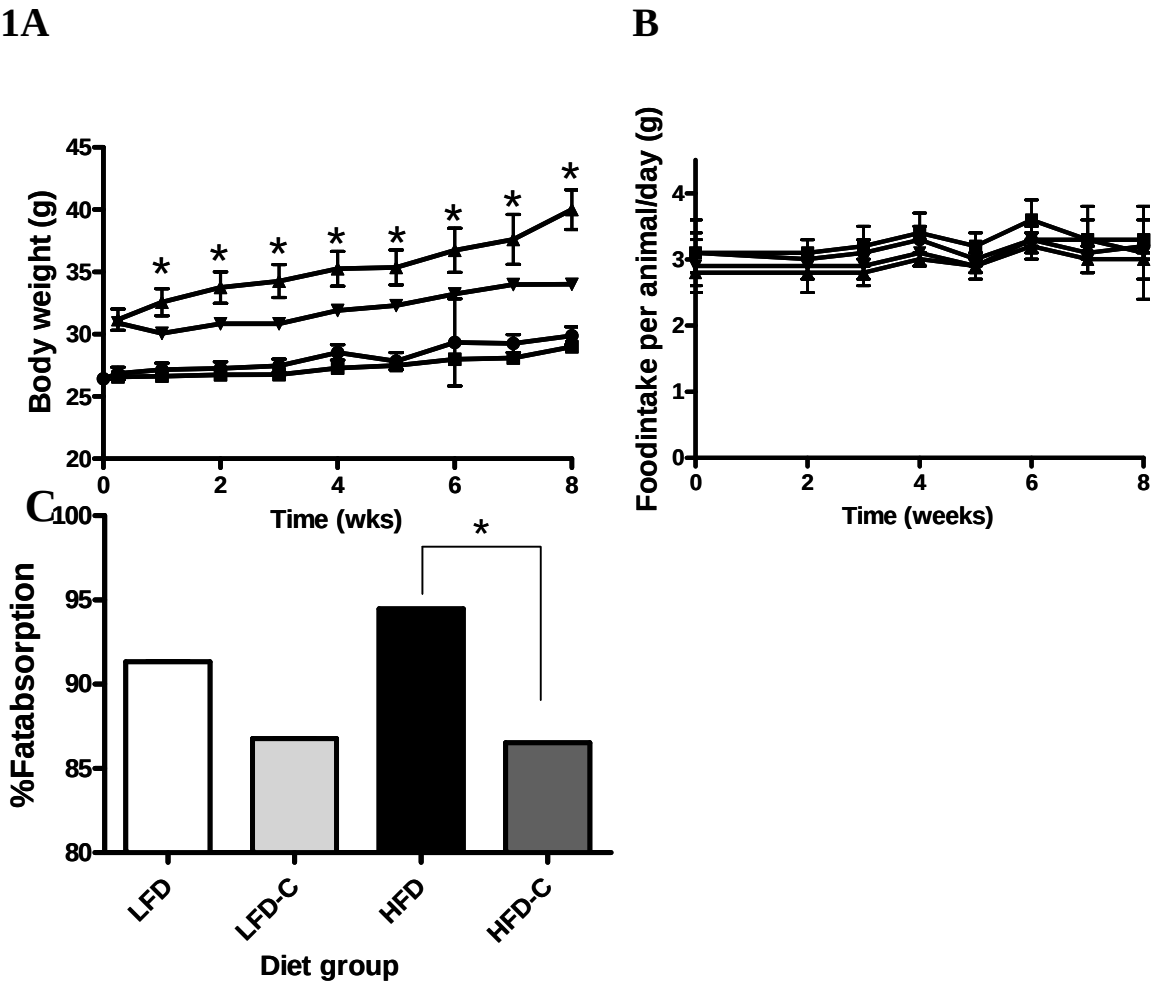


Figure 1A: ***Bodyweight in time****. Mice fed a high-fat diet and 0.05% cafestol (▼) have significant lower body weights compared to control (▲, high fat animals) already after 1 week cafestol diet (n=10 per diet group per time point). This difference in body weight becomes more pronounced in time. After 9 weeks experimental diets, cafestol fed mice weight 20% less then their high fat control mice (*p<0.05). (■) represents body weight of mice fed a low fat and 0.05% cafestol diet, whereas (●) represents LFD.* ***1B;*** *Food intake during experiment expressed in gram food per mouse per day. All mice consumed equal amounts of diet.* ***1C****: Fat absorption in mice fed different diets. After 8 weeks of experimental diets, cafestol reduces fat absorption in HFD animals (p<0.001). On a low fat background a trend is seen in which cafestol reduces fat absorption. Values expressed in mean ±SEM.*

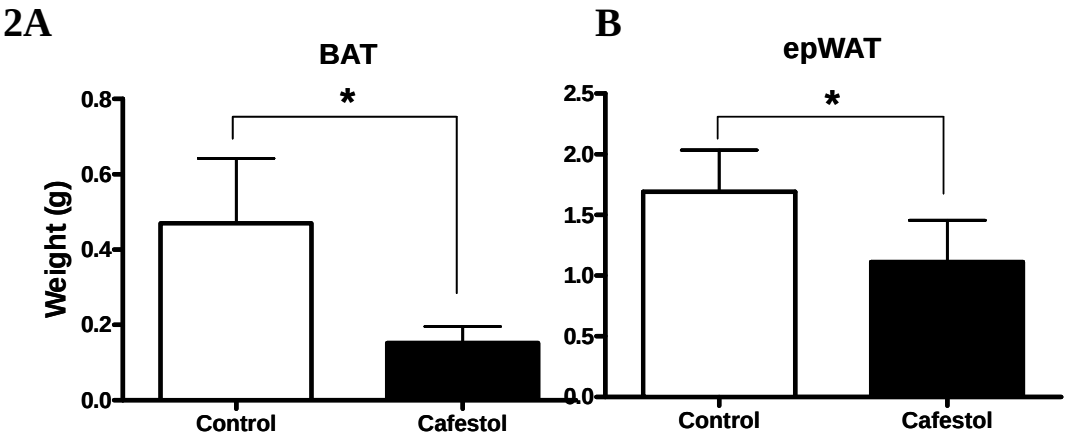


*Figure 2**: Cafestol induces a decrease in brown adipose tissue (A) and epididymal white adipose tissue (B).** Cafestol fed animals have only 1/3 the amount of BAT ($P<0.05$) and total epidydimal WAT content is decreased by 1/3 compared to control. Control animals are LFD and HFD, and cafestol are mice fed LFD and cafestol and HFD and cafestol. Values expressed in mean ±SEM.*

Table 1: **Body composition of mice fed the various experimental diets.** ***DEXA scan was used to measure body composition, body fat mass, and lean body mass.*** *At the beginning (t=0) and at the end (t=8wks) of the experiment, body composition was analyzed by a DEXA scan. After a run-in period of 4 weeks on either a low fat or high fat diet, there is a clear significant difference between the body weight and fat content of the animals. After 12 weeks these effects are more pronounced. HFD-C animals have significant lower body weight, lean mass, absolute fat and lower fat percentages. On a LFD these parameters are not significantly different. Ψ Significant difference between LFD and HFD. * Significant difference between HFD and HFD-C.*

T=0

Diet group	LFD	LFD-C	HFD	HFD-C
Tot. body weight(g)	24.85 ± 1.27	-	31.78 ± 2.57Ψ	-
Lean mass (g)	20.20 ± 1.27	-	22.70 ± 1.19Ψ	-
Fat (g)	4.58 ± 0.63	-	9.075 ± 2.44Ψ	-
Total weight (g)	24.81 ± 1.68	-	31.8 ± 3.56Ψ	-
% Fat	18.4 ± 1.91	-	28.1 ± 4.38Ψ	-

T=8wks

Diet group	LFD	LFD-C	HFD	HFD-C
Tot. body weight(g)	27.77± 2.04	27.83±1.42	42.53±3.59 Ψ	34.63±7.4*
Lean mass (g)	18.83±1.69	20.10±1.45	22.40±1.13 Ψ	21.87±0.71*
Fat (g)	5.30±1.82	4.23±0.40	16.88±2.27 Ψ	10.17± 6.93*
Total weight (g)	24.27±3.34	24.30±1.48	39.23±2.76Ψ	32.03±6.65*
% Fat	21.33± 4.21	17.45± 1.65	42.83± 3.14 Ψ	30.47±10.61*

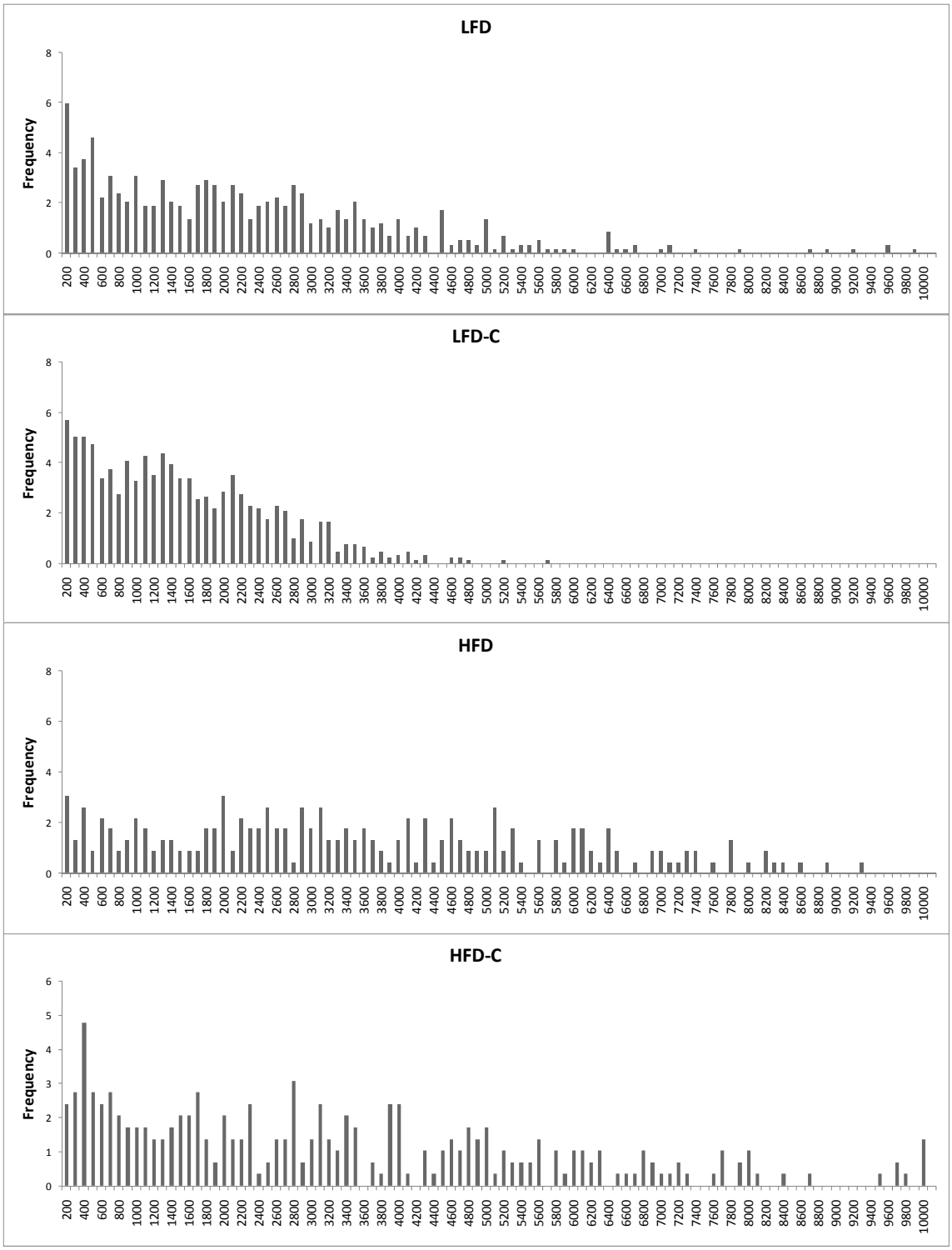


Figure 3: ***Effect of high fat diet and cafestol on adipocyte size****. Feeding high fat diet resulted in hypertrophy of adipocytes. On both diets, cafestol reduced adipocyte size.*

Cafestol reduces adipose tissue stores

Changes in weights of BAT and WAT in response to the diets were also evaluated. Compared to LFD, HFD significantly increased the adipose tissue to body weight ratio, similar to the DEXA outcomes (Table 1). However, cafestol supplementation resulted in reduced adipose tissue weights, both for BAT and WAT (Figure 2A/B). In line with previous data, morphological analyses of WAT showed that HFD feeding resulted in adipocyte hypertrophy (Stienstra, Duval et al. 2008). Addition of cafestol to the HFD reversed this hypertrophy, but had no effect on the LFD (Figure 3). These results were corroborated by the reduction of plasma leptin concentrations, a sensitive marker for adipose tissue stores (Galic, Oakhill et al. 2009), upon cafestol treatment of HFD-fed animals (Figure 4).

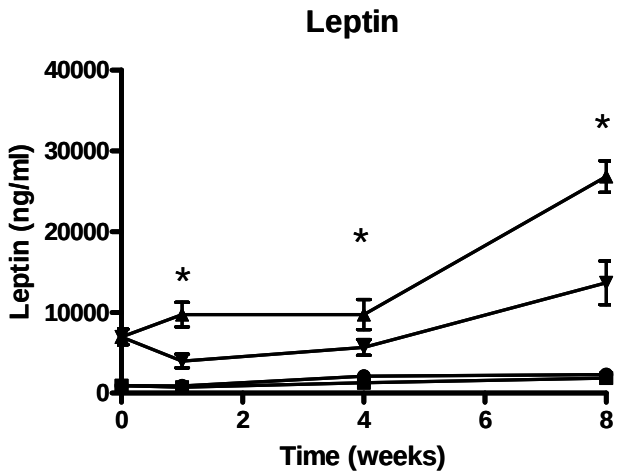


Figure 4: ***Leptin levels are 50% lower in HFD cafestol animals compared to control****. There is no difference between LFD (●) and LFD C (■) in plasma leptin. However, as expected there is a big difference between ▲ HFD and ▼ HFD-C fed animals. This is in accordance with the decrease in fat percentage as demonstrated by DEXA scan analysis. Values are expressed as averages ±SEM. * Significant difference between HFD and HFD-C.*

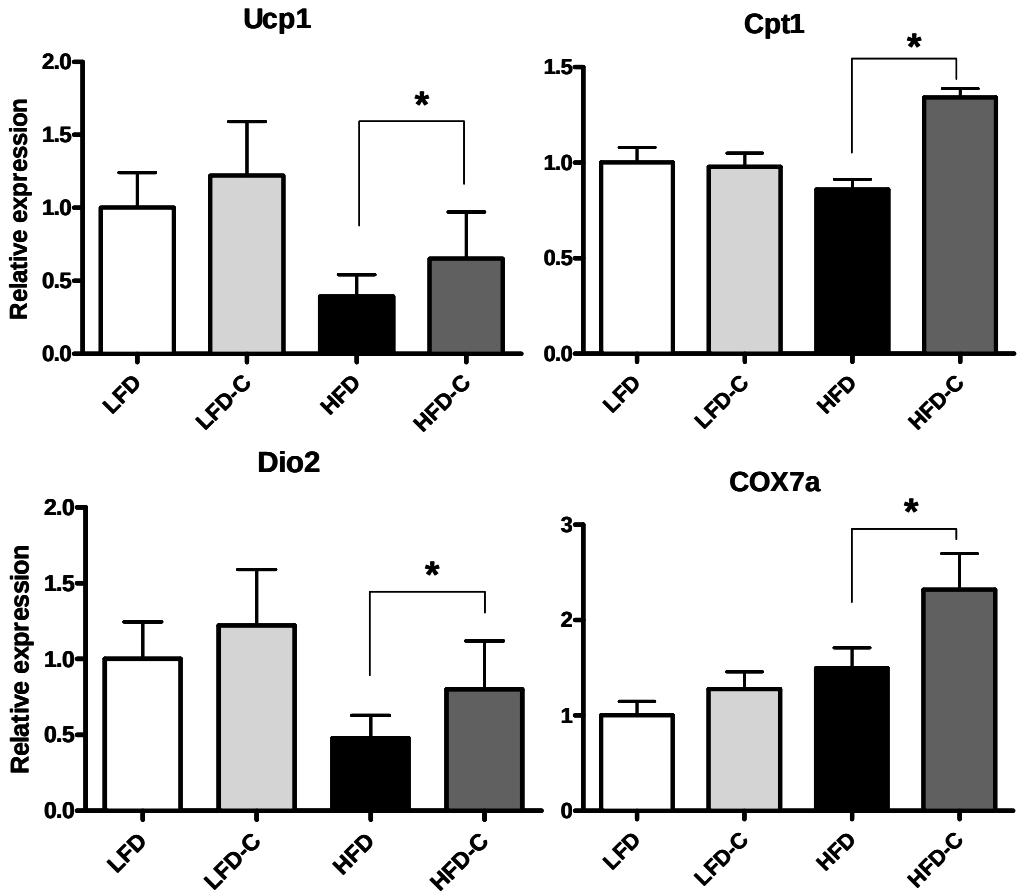


Figure 5: ***Cafestol induces energy expenditure in BAT.*** *On a high-fat background diet cafestol induces DIO2, CPT 1, UCP-1 and COX7a expression in BAT. Thermogenic uncoupling has been proven only for UCP1 in brown adipose tissue. Values expressed in mean ±SEM. * Significant difference between HFD and HFD-C.*

Cafestol increases expression of genes involved in energy expenditure in BAT

Gene expression profiling showed that cafestol induced expression of thyroid hormone deiodinase (Dio2), carnitine palmitoyltransferase (Cpt)-1, uncoupling protein (Ucp)-1 and cytochrome c oxidase (Cox)-7a expression in BAT (Figure 5A-D). Since these are all key

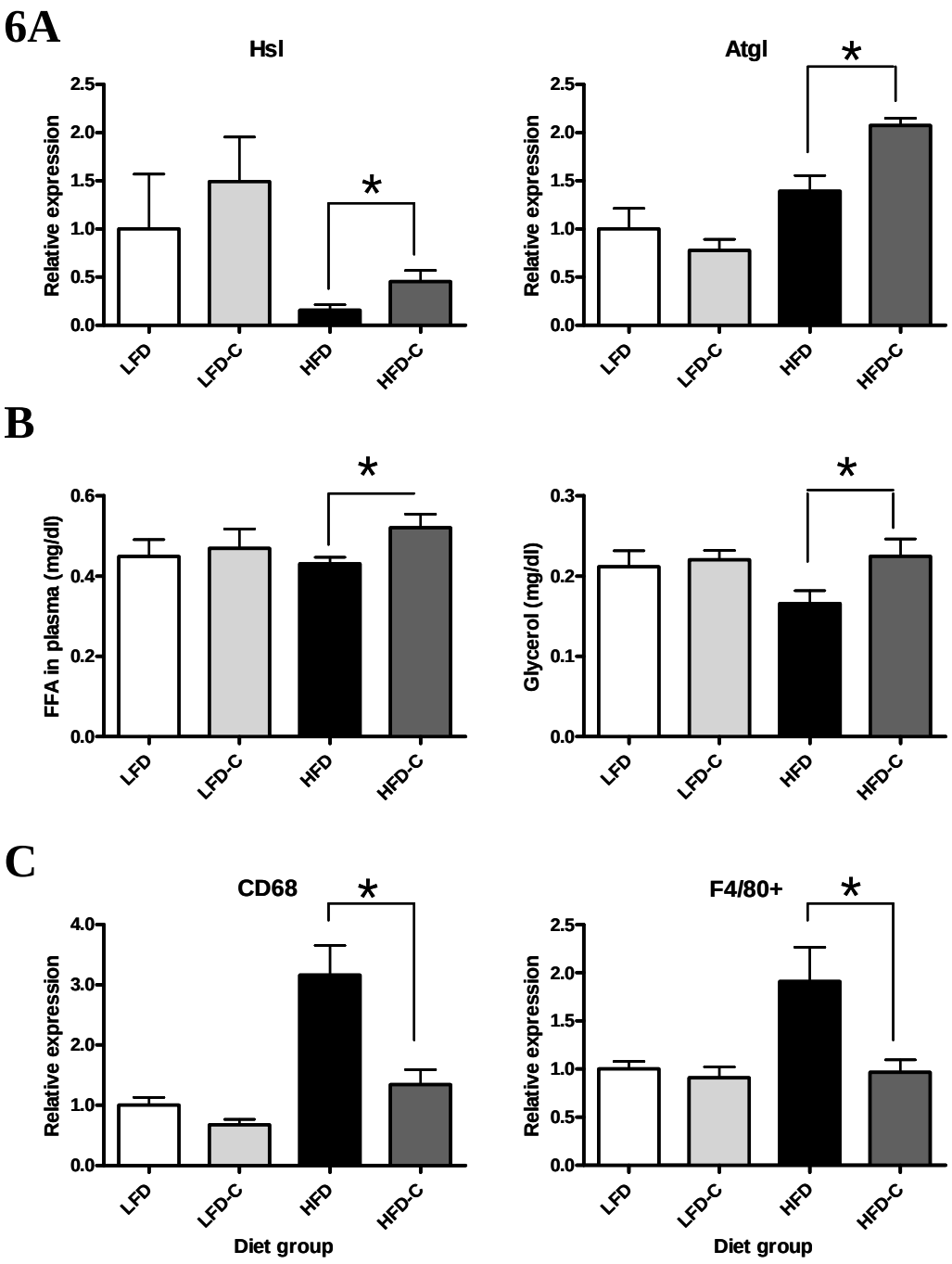
markers for energy expenditure (Auwerx 2006), these data suggest that cafestol increases energy expenditure, which in turn may be partially responsible for the observed reduction in weight loss of mice fed the HFD-C diet compared to HFD.

Cafestol induces lipolysis of WAT on a HFD

To identify a potential mechanism for the reduced WAT mass, we analyzed the expression of several genes involved in adipose tissue lipolysis. Expression of hormone sensitive lipase (Hsl) was decreased in HFD compared to LFD animals, as reported before (Alvarez, Lasuncion et al. ; Winzell, Holm et al. 2003). Supplementation of cafestol to a high-fat diet attenuated the HFD-mediated inhibition of Hsl expression 3-fold. (Figure 6A). Cafestol induced the expression of adipose triglyceride lipase (Atgl) on a HFD (Figure 6A). In agreement with these data we found that levels of free fatty acids and glycerol in plasma were increased in HFD-C compared to HFD littermates (Figure 6B). Combined these data suggest that upon HFD feeding cafestol induces lipolysis of WAT.

Cafestol mediated reduction of WAT mass is associated with reduced level of infiltration of macrophages

It has been reported that obesity is associated with a state of chronic low grade inflammation, which in addition to adipocyte hypertrophy is caused by infiltration of macrophages into adipose tissue (Hotamisligil 2006). We therefore evaluated the expression and presence of markers of macrophages in WAT. Supplementation of HFD fed animals with cafestol resulted in a significant decrease in gene expression of macrophage markers CD68 and F4/80+ (Figure 6C). This was confirmed with antibody staining for F4/80+ (Figure 6D).


6A
Hsl
Atgl
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
LFD
LFD-C
HFD
HFD-C
B
FFA in plasma (mg/dl)
0.6
0.4
0.2
0.0
Glycerol (mg/dl)
0.3
0.2
0.1
0.0
C
CD68
F4/80+
4.0
3.0
2.0
1.0
Diet group

D

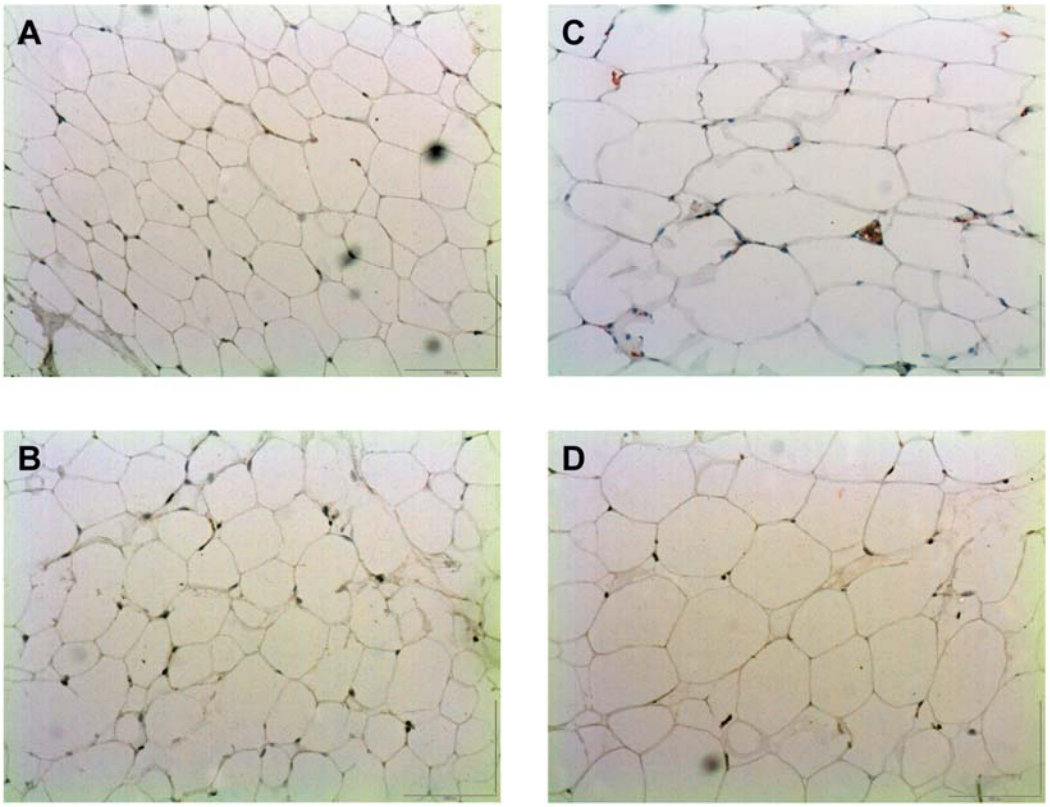


Figure 6: ***Cafestol induces gene expression of key players of lipolysis in WAT on a high-fat background diet.*** *A: Cafestol induces hormone sensitive lipase and desnutrin (ATGL) on a high fat background diet. B**:** Supplementation of HFD animals with cafestol increases FFA and glycerol in plasma. This suggests an increase in lipolysis**. C:** Supplementation with cafestol reduces CD68 and F4/80+ expression in WAT. Values expressed in mean ±SEM. D: F4/80 staining in liver slides. Red dots are the F4/80+ staining, nuclei are stained blue. Magnification 200x. In correspondence with the gene expression data, HFD increases macrophage infiltration (red dots) in WAT, whereas supplementation with cafestol reduces this.*

Cafestol reduces hepatic steatosis

Compared to LFD, HFD significantly increased the liver weight after 8 weeks of dietary intervention (Table 2), indicative for the development of steatosis (Stienstra, Mandard et al. 2007). However, cafestol supplementation resulted in reduced liver weight (Table 2). Detailed histological analysis showed no lipid droplets in livers of mice fed the LFD (Figure 7A) or LFD-C (Figure 7B) diets. However, as expected, a large number of lipid droplets were observed in HFD-fed animals (Figure 7C), demonstrating the well-known development of HFD-induced steatosis (Patsouris, Reddy et al. 2006; Stienstra, Mandard et al. 2007; Zivkovic, German et al. 2007; Radonjic, de Haan et al. 2009).The number and size of lipid droplets were dramatically reduced in livers of mice fed the HFD-C diet (Figure 7D). These data were confirmed by quantitative measurements of hepatic TG levels (Table 3). Combined, these data show that cafestol prevents HFD-induced steatosis.

Table 2: ***Liver weights during time course experiment.*** *Cafestol supplementation significantly reduces liver weight after 8 weeks. Values expressed in mean ±SEM.*

** Significant difference between HFD and HFD-C.*

Time / Diet group	LFD	LFD-C	HFD	HFD-C
0	1.1±0.043	-	1.12±0.043	-
6 hrs	1.07±0.046	0.98±0.033	1.11±0.048	1.04±0.030
1	1.05±0.045	1.13±0.049	1.24±0.077	1.18±0.044
4	1.12±0.038	1.08±0.039	1.31±0.065	1.20±0.034
8	1.10±0.048	1.04±0.047	1.55±0.078	1.28±0.064*

Table 3: ***Hepatic TG levels. Cafestol reduces hepatic TG with 50% regardless of background diet.*** *Values expressed in mean ±SEM (mg TG/gram liver). Ψ Significant difference between LFD and LFD-C. * Significant difference between HFD and HFD-C.*

Time(weeks) / Diet	LFD	LFD-C	HFD	HFD-C
0	6.59 ±1.0		8.35±0.68	
6 hrs	11.66±0.9	11.63±1.87	10.98±0.90	10.15±0.50
1	9.04±1.30	6.3±0.58Ψ	12.132±1.22	6.16±0.50*
4	13.10±1.76	7.2±0.83Ψ	12.38±0.83	7.16±0.58*
8	15.48±*0.9*	8.964±0.68Ψ	20.48±2.74	10.69±1.04*

We next evaluated the expression of several genes involved in hepatic lipid metabolism. Cafestol reduced the expression of the master regulator sterol regulatory element-binding protein (Srebp)-1c, fatty acid synthase (Fasn), ATP citrate lyase (Acly), acetyl-Coenzyme A carboxylase alpha (Acaca), and diacylglycerol O-acyltransferase (Dgat)-2, all key players in hepatic lipogenesis and fat storage, for most genes in both cafestol-treatment groups compared to their respective controls (Figure 8A-E). Furthermore, the expression of microsomal triglyceride transfer protein (Mttp) was increased in HFD, but was reduced in HFD-C livers (Figure 8F). These expression data indicate that cafestol prevented steatosis by reduction of hepatic *de novo* lipogenesis as well as decreasing lipid storage.

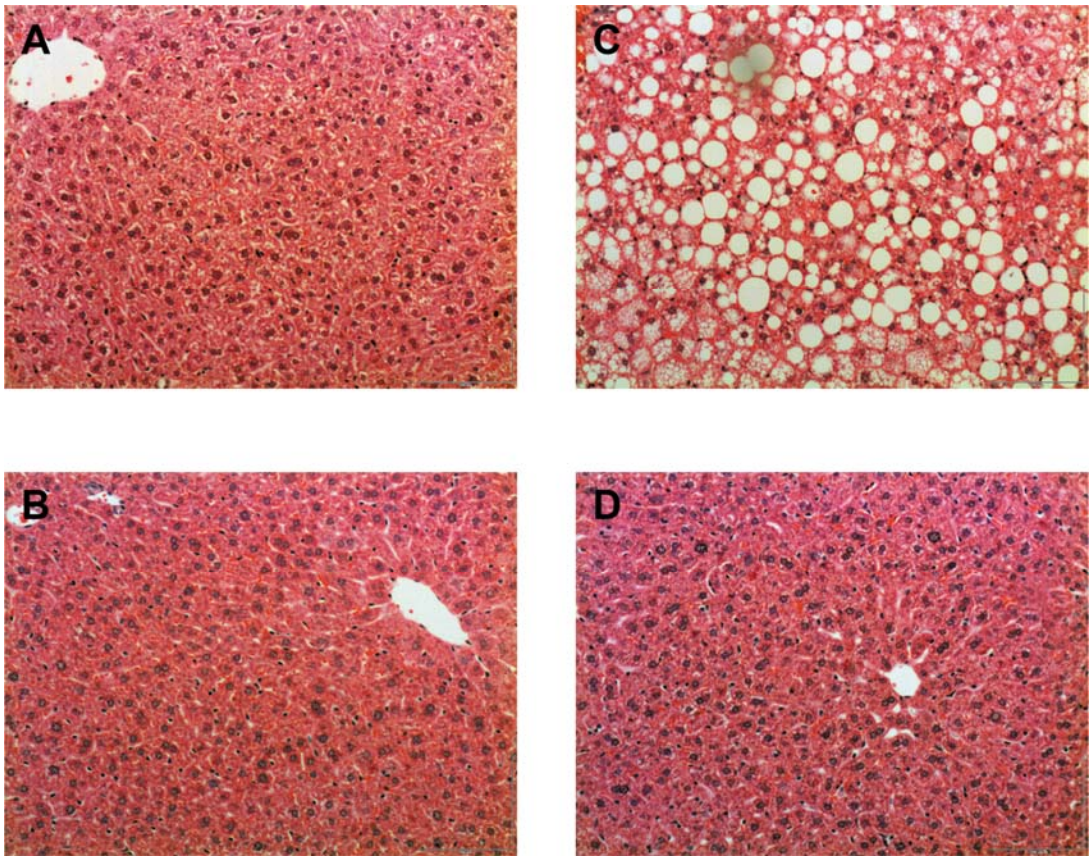


Figure 7: ***Liver histology.*** *Livers from mice fed either a low fat (A: 10 energy % fat) or high fat (B; 45energy % fat) with or without cafestol were stained with H&E (magnification 200x). No morphologic differences are seen between LFD (Figure A) and LFD and cafestol (Figure B). Many fat droplets were observed in the livers of high fat control mice (HFD, Figure C), suggesting that fatty liver (steatosis) was induced by intake of the high-fat diet. However, the amounts of fat droplets were scarcely observed in HFD cafestol mice (Figure D). These results indicate that consumption of cafestol prevents hepatic steatosis induced by intake of a high-fat diet.*

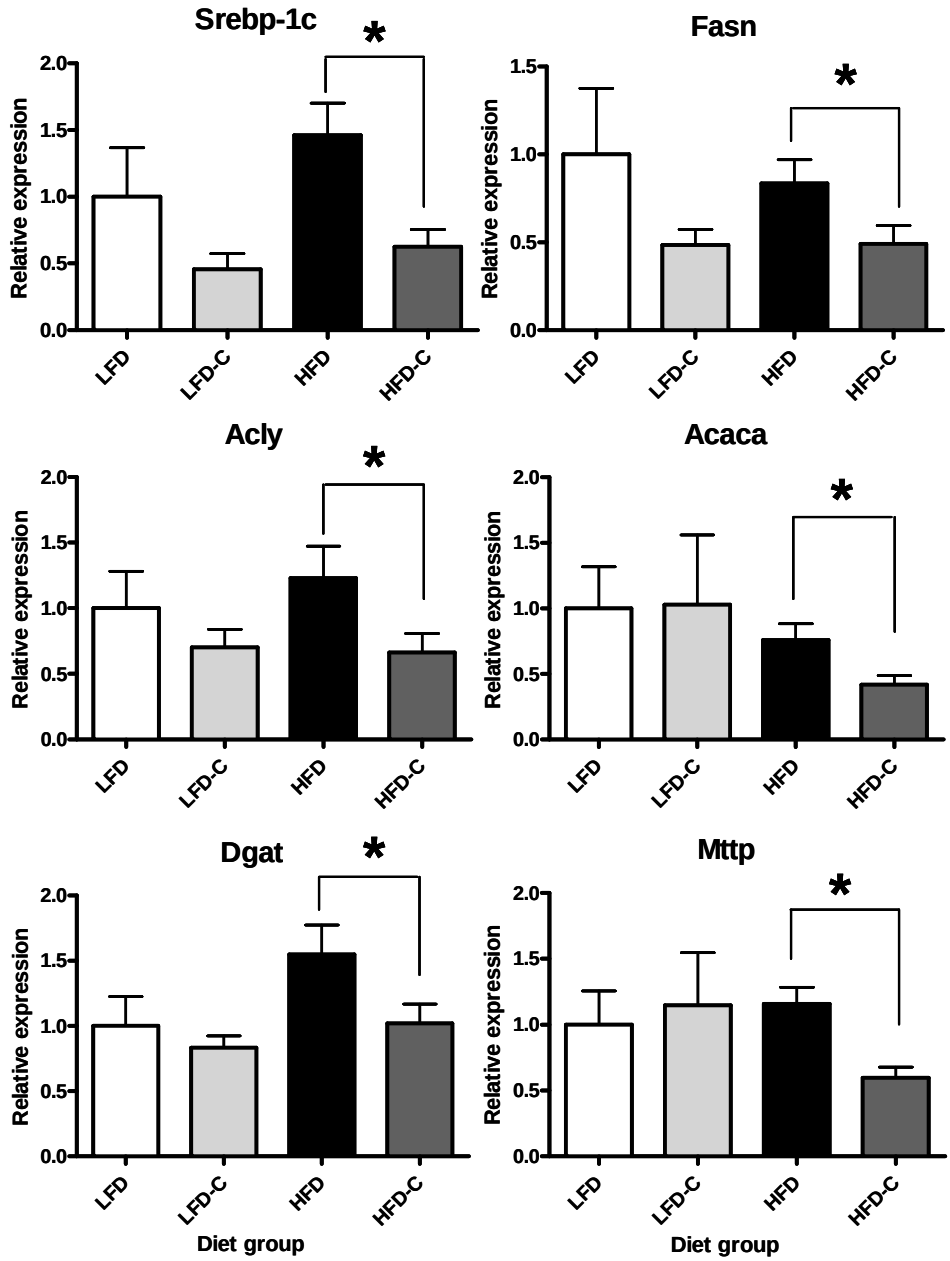


Figure 8: ***Cafestol (partly) inhibits lipogenesis in liver****. Cafestol reduces Srebp-1c expression (A). Srebp-1c inhibition results in down regulation of key players of lipogenesis such as FasN (B), ATP citrate lyase (C), Acaca (D) and Dgat2 (E). Cafestol supplementation to a high fat diet also decreased transcription of Mttp in liver (F). Values are expressed in mean ±SEM.*

Cafestol activates Nrf2 mediated gene expression and reduces ER stress in liver

Nrf2 is a transcription factor that protects cells and tissues from oxidative stress by activating protective antioxidant and biotransformation enzymes (Nguyen, Nioi et al. 2009). Moreover, it is known that an increase in Nrf2 mediated biotransformation and oxidative stress defense may result in a decrease of endoplasmic reticulum (ER) stress (Nair, Xu et al. 2007), which in turn affects the expression and activity of Srebp-1c (Kammoun, Chabanon et al. 2009; Kammoun, Hainault et al. 2009). To evaluate whether cafestol reduced ER stress through activation of Nrf2, we measured its effect on expression in liver of Nrf2 target genes as well as on markers for ER stress. On both LFD and HFD diets, cafestol upregulated expression of typical Nrf2 target genes, such as epoxide hydrolase, glutathione s-transferase (Gst)-m4, Gst-m3 and Gst-m5 (Chapter 5). Cafestol reduced expression of Chop10 and Herpud1 (Figure 9A/B), two marker genes of ER stress (Lawrence, McGlynn et al. 2007). In addition, cafestol induced expression of glucose-regulated protein 78 (Grp78) (Figure 9C), a protein recently shown to inhibit ER stress-mediated Srebp-1c cleavage and the expression of Srebp-1c and Srebp2 target genes (Kammoun, Chabanon et al. 2009). Combined, these results show that the supplementation of diets with cafestol activated Nrf2 and reduced ER stress, which in turn may contribute to decreased hepatic lipogenesis.

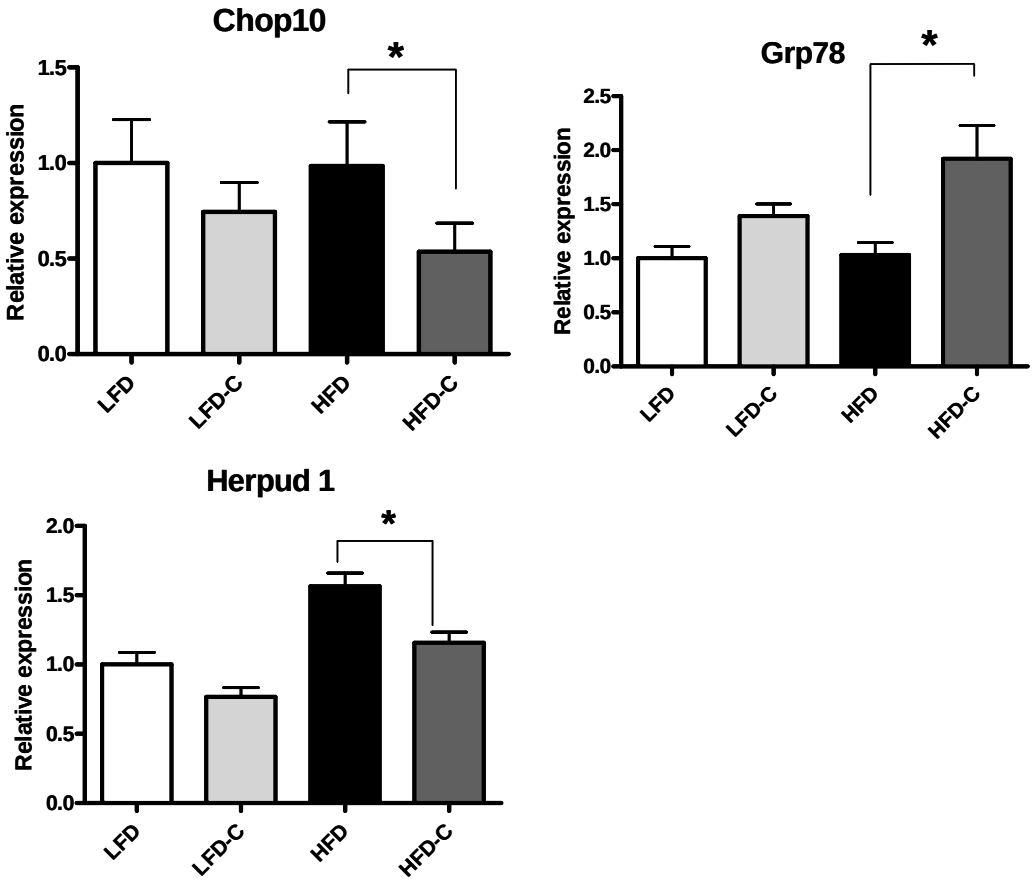


Figure 9: ***Cafestol reduces ER stress as can be seen on transcriptional level of Chop10, Grp78 and Herpud1.*** *Values expressed in mean ±SEM. * Significant difference between HFD and HFD-C.*

Cafestol improves insulin sensitivity

It is well established that obesity, chronic inflammation of adipose tissue and hepatic steatosis are closely associated with insulin insensitivity (Hotamisligil 2006). Since all these parameters were reduced by cafestol treatment, we determined fasting plasma glucose and insulin levels (Table 4) and used these to determine the HOMA index, which we used as measurement of insulin sensitivity. On a low fat background diet cafestol did not change glucose levels in plasma, however on a high-fat background diet cafestol reduced fasting plasma glucose after 8wks consumption (Table 4, P=0.007). Plasma insulin was significantly decreased in cafestol treated animals (Table 4). Feeding mice run-in diets of either LFD (HOMA index 6.21) or HFD (HOMA index 13.73) results in significantly different HOMA indexes, which suggest decreased insulin sensitivity in HFD fed animals. Supplementation of cafestol to HFD reduced HOMA indexes with 40% already after 1 week consumption of experimental diets. Over time this effect becomes even more pronounced. After 8 weeks of cafestol supplementation to the HFD diet (3.24), HOMA indexes are reduced 3-fold compared to HFD control animals (10.84). We conclude that cafestol improves insulin sensitivity in mice fed a high-fat diet.

Table 4: ***Cafestol ameliorates insulin sensitivity.*** *Fasting plasma glucose, fasting plasma insulin and HOMA index in mice fed 4 experimental diets. Already after one week, cafestol decreases plasma glucose and insulin and subsequently decreases the HOMA index with 150%. These differences on the high fat diet suggest better insulin sensitivity in mice fed cafestol. These effects became more pronounced during time. Values expressed in mean ±SEM. Ψ Significant difference between LFD and HFD. * Significant difference between HFD and HFD-C (P<0.05).*

Time (weeks) Diet group	LFD	LFD-C	HFD	HFD-C
T=0				
Glucose (mmol/l)	*15.11± 0.*68		*15.10±.1.15*	
Insulin (mU/l)	*9.01± 0.87*		*19.77± 0.64 Ψ*	
HOMA index	*6.21±0.8*		*13.73± 1.24Ψ*	
6 hrs				
Glucose (mmol/l)	*14.63±1.12*	*14.71±0.78*	*18.83±1.34*	*16.89±0.66*
Insulin (mU/l)	*5.37±1.47*	*4.31±0.56*	*9.24±1.63*	*11.68±1.73*
HOMA index	*3.73±1.23*	*2.36±0.43*	*8.22±2.01*	*10.60±1.41*
T=1				
Glucose (mmol/l)	*15.95±1.06*	*16.79±0.84*	*21.34±0.94*	*18.26±0.93*
Insulin (mU/l)	*3.92±0.43*	*11.09±1.31*	*16.83±2.16*	*13.11±1.00*
HOMA index	*3.00±0.46*	*7.77±0.93*	*17.35±2.20*	*7.87±1.28**
T=4				
Glucose (mmol/l)	*17.18±0.82*	*16.97±0.68*	*19.37±0.98*	*19.53±0.75*
Insulin (mU/l)	*5.67±0.93*	*5.40±2.00*	*16.49±1.33*	*9.00±1.47**
HOMA index	*4.30±0.86*	*4.50±1.24*	*15.09±1.31*	*7.94±1.4**
T=8				
Glucose (mmol/l)	*13.80*±0.94	*14.19*±0.55	*17.78±0.43*	*15.39±0.55*
Insulin (mU/l)	*9.50*±3.77	*7.88*±1.47	*27.84±4.61*	*10.16±1.90**
HOMA index	*2.88*±1.18	*2.35*±0.45	*10.84±1.85*	*3.24±0.63**

Discussion

The major findings of this study are that in mice cafestol prevents high-fat diet induced obesity, concordantly with reduced adiposity and hepatic steatosis, all of which are associated with improved insulin sensitivity, as indicated by the HOMA index.

Our data suggest that the lower body weight of HFD-C mice compared to HFD is mainly due to the greatly reduced fat depots and lower liver weight (Figure 4A and table 2). Since food intake was identical between these groups (Figure 2B), we exclude that cafestol-mediated reduction in body weight gain is caused by differences in food intake. Moreover, our data also indicate that cafestol does not act through inhibition of lipid absorption, since this was only slightly diminished in cafestol-treated mice. It should be noted that we are the first to report the detailed effects of cafestol on adiposity and bodyweight gain. Previously it has been shown that cafestol reduces TG in liver in Apoe3 mice (Post, de Roos et al. 2000).

Several mechanisms could potentially explain the effects of cafestol on weight gain and adiposity. It is know that cafestol is an agonist for two bile acid receptors, Fxr and Pxr (Ricketts, Boekschoten et al. 2007) and is likely to undergo extensive enterohepatic circulation (van Cruchten, de Waart et al. 2010). These data indicate that cafestol may interfere with bile acid metabolism. In this respect it is important to note that it has been recently demonstrated that bile acids are able to activate the G-protein-coupled receptor (GPCR) TGR5. This results in an increase in cyclic AMP content and induces the expression and activity of Dio2. This induction enhances local conversion of inactive thyroid hormone to its active form, ultimately resulting in increased energy expenditure in BAT and other tissues expressing TGR5 such as skeletal myocytes (Auwerx 2006; Wondisford 2006). We show that key markers for energy expenditure were increased in BAT in HFD-C fed animals. We

therefore hypothesize that in addition to its interaction with Fxr and Pxr cafestol (metabolites) also interact with TGR5, either directly or indirectly (through changes in bile acid metabolism), resulting in increased energy expenditure which may contribute to the reduced body weight gain. However, this hypothesis requires further investigations.

Cafestol reduced WAT mass (Figure 4A and Table 1). We believe this is partly due to the increased expression of the enzyme Atgl together with the cafestol-mediated diminished suppression of Hsl expression in WAT normally observed after HFD intervention (Winzell, Holm et al. 2003). Atgl activity accounts for 60-70% of triglyceride lipase activity in adipose and appears to be essential for the control of normal weight (Huijsman, van de Par et al. 2009). Expression of this enzyme was increased in HFD-C mice, which contributes to an increase in lipolysis. Hsl catalyzes the rate-limiting step in the mobilization of free fatty acids from WAT by hydrolyzing TGs (Kraemer and Shen 2002). Hsl is regulated by the several hormones, including insulin (Watt 2009). Since insulin levels are increased in HFD compared to LFD, as also shown previously, we speculate this explains the suppressed expression of Hsl (Winzell, Holm et al. 2003). However, cafestol treatment of HFD-fed mice normalizes insulin levels, thereby partially lifting its inhibition on Hsl. The cafestol-mediated reduction in WAT mass was accompanied with a significant decrease of macrophage infiltration. Taken together, supplementation of cafestol to a HFD overall results in the appearance of ‘healthier’ adipose tissue.

It is known that cafestol activates Nrf2, which in turn triggers transcription and activation of several biotransformation enzymes such as Gsts and epoxide hydrolase (van Cruchten, de Haan et al. 2009). This increase in Nrf2 mediated biotransformation may result from a

decrease of ER stress (Cullinan and Diehl 2004; Nair, Xu et al. 2007). The ER is a vast network of membranes in which all the secretory and membrane proteins are assembled into their secondary and tertiary structures. The proper folding, maturation, storage and transport of these proteins takes place within this organelle, and unfolded or misfolded proteins are detected, removed from the ER, and degraded by the proteasome system. An accumulation of unfolded or misfolded proteins could result from e.g. an increased demand on the synthetic machinery, inhibition of protein glycosylation or overnutrition. Such an accumulation would give rise to perturbations in the ER lumen, and create stress (Ozcan, Cao et al. 2004; Nakatani, Kaneto et al. 2005; Song, Scheuner et al. 2008; van der Kallen, van Greevenbroek et al. 2009). In livers and adipose tissue of obese animals, activation of the ER stress pathway has been associated with development of hepatic steatosis (Ozcan, Cao et al. 2004; Nakatani, Kaneto et al. 2005; Song, Scheuner et al. 2008; van der Kallen, van Greevenbroek et al. 2009). Although ER stress is largely regulated by means of phosphorylation, there are some markers regulated at the level of transcription (Hayashi, Kasahara et al. 2007). Expression of Chop10 and Herpud1, indicators of ER stress, was reduced by cafestol, whereas levels of the protective chaperone protein Grp78 were increased. Recently it has been reported that reduced ER stress suppressed the expression and activity of Srebp-1c (Kammoun, Chabanon et al. 2009; Kammoun, Hainault et al. 2009). In our studies cafestol suppressed Srebp-1c expression in liver, which coincided with a reduced expression of key enzymes of lipogenesis, Fasn, Acaca, Acly and Dgat2. It is tempting to speculate that cafestol mediated activation of Nrf2 results in a reduction of HFD-induced ER stress in liver, which subsequently prevents and protects against development of hepatic steatosis. Alternatively, a role of TGR5 could also be envisioned. In addition to muscle and brown adipose tissue, TGR5 is also expressed in

liver, an organ exposed to high levels of cafestol (metabolites) that might serve as agonists for TGR5 receptor. The physiological function of TGR5 in liver is still unknown, but a very recent study reported that a synthetic TGR5 agonist reduced liver steatosis (Thomas, Gioiello et al. 2009), similarly to cafestol.

Whereas mice fed a HFD for 12 weeks show several features of metabolic syndrome, among others obesity, hepatic steatosis, insulin insensitivity (high levels of insulin, increased HOMA index) and low-grade inflammation, supplementation of cafestol for 8 weeks to the same diet results in an improvement of all of these parameters. The improvement in insulin sensitivity is likely due to a combination of protective effects of cafestol in WAT, liver and skeletal muscle, as discussed above.

In conclusion, our results provide novel findings on the effects of cafestol on obesity and associated metabolic dysfunction. We demonstrated that cafestol prevented diet induced obesity, adiposity and liver steatosis under high-fat feeding. However, whether cafestol supplementation also prevents diet induced obesity in humans remains to be established.

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Chapter 5

High saturated fat diet modulates the effects of cafestol on lipid metabolism and biotransformation pathways

Saskia T.J. van Cruchten[1, 2], E. Dillon[1], M.V Boekschoten[1, 2], M.B. Katan[3], G.J.E.J Hooiveld[1, 2] and M. Müller[1, 2].

[1]*Division of Human Nutrition, Wageningen University, the Netherlands*

[2]*Nutrigenomics Consortium, Top Institute Food & Nutrition, Wageningen, the Netherlands*

[3]*Department of Health Sciences, Free University, Amsterdam, the Netherlands.*

In preparation.

Supplemental data is available at http://nutrigene.4t.com/data/SKphd

Abstract

Cafestol is a compound present in unfiltered coffees and is the most potent cholesterol-raising agent known in human diet. It is known that the metabolism of cafestol, bile acids and lipids are closely interrelated. Diet composition has a significant impact on intermediary metabolism and biotransformation. In the current study we investigated the interaction between the fat content of the diet and the effects of cafestol in liver and small intestine. Mice were fed cafestol supplemented to either a low fat (LFD) or high fat diet (HFD) for 8 weeks. We performed genome-wide transcriptome analyses of liver and small intestine. The effect of cafestol on composition of bile was also investigated. We found that fat content modulated the effects of cafestol on genes involved in bile acid metabolism, which could be linked to changes in bile composition. On both LFD and HFD cafestol profoundly induced biotransformation pathways under the transcriptional control of Nrf2. Nrf2 activation in small intestine was more pronounced than in liver. Transcriptome analysis demonstrated that cafestol regulated two distinct sets of genes on LFD and HFD, again indicating that dietary lipid content is an important determinant for the effects of cafestol. Moreover, several new processes were identified that were affected by cafestol treatment. However, a series of future studies is required to evaluate the relevance of these findings.

Introduction

Cafestol is a compound present in unfiltered brewed coffees, such as French press, espresso and Turkish coffees. It is the most cholesterol raising compound known in human diet (Urgert and Katan 1997). Previous studies have shown that cafestol also increases plasma cholesterol in animals (de Roos, Sawyer et al. 1999). The precise underlying molecular mechanisms are not clear, but it has been suggested that the nuclear receptors Fxr and Pxr, and the G-coupled protein receptor Tgr5 are involved (Ricketts, Boekschoten et al. 2007), Chapter 4). These transcription factors are critical for metabolic regulation, especially for bile acid and lipid homeostasis (Lefebvre, Cariou et al. 2009; Tiwari and Maiti 2009; Wada, Gao et al. 2009). Moreover, Post et al. showed that on a high fat diet cafestol suppressed Cyp7a1 activity and expression in mice (Post, de Roos et al. 2000). Finally, cafestol inhibits diet-induced obesity, resulting in improved metabolic flexibility (Chapter 4). Combined, these data imply that cafestol and metabolism of bile acids and lipids are closely related.

Recently we demonstrated that by the consecutive action of several phase I and II biotransformation enzyme systems in liver cafestol is metabolized into several metabolites, such as epoxide-, glutathione- and glucuronide conjugates (van Cruchten, de Haan et al. 2009). Yet it is important to realize that diet composition is also well known to influence the efficiency of these reactions (Pantuck, Hsiao et al. 1975; Bidlack and Smith 1984; Anderson and Kappas 1991; Jodynis-Liebert and Murias 2002; Adithan 2005).

Based on these findings, we hypothesized that changes in diet composition may influence the effects of cafestol on intermediary metabolism and biotransformation, and *vice versa*. In this respect it is relevant to note that up until now in all animal studies the effects of cafestol are investigated on a background of high fat diet containing animal fat and often also additional cholesterol (Post, de Roos et al. 2000; Ricketts, Boekschoten et al. 2007). Therefore, to

investigate a possible interaction between diet and cafestol, we performed genome-wide transcriptome analyses of liver and small intestine of mice that were fed cafestol supplemented to either a low fat or high fat diet. We primarily evaluated changes in expression of genes involved in bile acid and lipid metabolism, and biotransformation, because these processes are most likely primarily affected by cafestol as suggested by previous studies. Since cafestol is likely to undergo enterohepatic circulation and accumulates mainly in liver and small intestine (van Cruchten, de Waart et al. 2010), we focused on these two organs.

Material and methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). Cafestol was obtained from LKT Laboratories (St. Paul, MN).

Animals

Pure-bred 11-12-weeks old, male C57BL6/J mice were purchased from Harlan (Horst, the Netherlands). Mice were housed in a light- and temperature-controlled facility and had free access to water and diets. All animal studies were approved by the local Committee for Care and Use of Laboratory Animals.

Diets

Semi-synthetic diets were used in this study. The LFD and HFD were based on OpenSource diets D12450B and D12451, respectively, in which lard was replaced by palm oil. The fat fraction provided either 10% (LFD) or 45% (HFD) of the caloric content of the diet. It should be noted that these diets contain mainly saturated fats and are devoid of any cholesterol. Cafestol was mixed in the diets at a concentration of 0.05% (w/w). All diets were made by Research Diet Services (Wijk bij Duurstede, The Netherlands).

Experimental setup

Mice were fed the LFD or HFD for 4 weeks. After this run-in period, designated as t=0, mice were stratified on body weight (BW) into four groups. The LFD group was split in either a group that continued to receive the LFD, and a group that received the LFD supplemented with cafestol (LFD-C). Similarly, the HFD group was split into a HFD and a HFD-C group. At t = 8 weeks, mice from every group were killed for tissue sampling. At t=9 weeks, mice were fasted for 4 hours, and gal bladder cannulation was performed as described earlier (Klett, Lu et al. 2004). Bile was sampled every 15 minutes for the first hour and every 30 min after the first hour. Bile samples were immediately frozen at −80°C. Bile flow was determined gravimetrically assuming a density of 1 g/ml for bile. Bile composition was analysed as described before (Kok, Bloks et al. 2003; van Cruchten, de Haan et al. 2009)..

Organ sampling

After a 4hr fast period, starting at 8:00 AM, mice were anaesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%), and oxygen (30%). The liver and small intestine were excised, weighed, snap-frozen and stored at - 80ºC until analyses.

Expression analysis

RNA isolation and quality control.

Total RNA was isolated from liver and small intestine using TRIzol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. RNA was treated with DNase and purified using the Qiagen RNeasy kit (Qiagen, Venlo, the Netherlands).

Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits and displayed no chromosomal peaks or RNA degradation products.

Affymetrix GeneChip oligoarray hybridization, scanning and quality control

Total RNA (100 ng) was labelled using the Affymetrix GeneChip WT Sense Target Labeling and Control Reagents kit (P/N 900652) (Affymetrix, Santa Clara, CA). The correspondingly labeled RNA samples were hybridised on GeneChip Mouse Gene 1.0 ST arrays (Affymetrix), washed, stained and scanned on an Affymetrix GeneChip 3000 7G scanner. The mouse Gene 1.0 ST array contains 906,259 probes that detect expression levels of 20,985 unique genes. For each experimental group four biological replicates were hybridized. Detailed protocols for the handling of the arrays can be found in the Genechip Expression Analysis Technical Manual, section 2, chapter 2 (Affymetrix; P/N 701028, revision 5). Libraries from the Bioconductor project were used for analyzing the scanned Affymetrix arrays (Gentleman, Carey et al. 2004). Various advanced quality metrics, diagnostic plots, pseudo-images and classification methods were applied to ascertain only excellent quality arrays were used in the statistical and functional analyses (Heber and Sick 2006).

Statistical and functional analyses of microarray data

Probesets were redefined according to Dai et al. (Dai, Wang et al. 2005)) because the genome information utilized by Affymetrix at the time of designing the arrays is not current anymore, resulting in unreliable reconstruction of expression levels. In this study probes were

reorganized based on the Entrez Gene database, build 37, version 1 (remapped CDF v12). Expression estimates were obtained by robust multi-array (RMA) analysis (Irizarry, Hobbs et al. 2003). Differentially expressed probe sets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularization of standard errors (Smyth 2004). The moderated t-test statistic has the same interpretation as an ordinary t-test statistic, except that the standard errors have been moderated across genes, i.e. shrunk to a common value, using a Bayesian model. To adjust for both the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity, the moderated t-statistic was extended by a Bayesian hierarchical model to define a intensity-based moderated T-statistic (IBMT) (Sartor, Tomlinson et al. 2006). Probe sets that satisfied the criterion of a fold change >1.2 combined with an IBMT p-value < 0.05 were considered to be significantly regulated.

Changes in gene expression were related to functional changes using gene set enrichment analysis and Ingenuity Pathways Analysis (IPA v 8.0; Ingenuity Systems, Mountain View, CA). GSEA takes into account the broader context in which gene products function, namely in physically interacting networks, such as biochemical, metabolic or signal transduction routes (Subramanian, Tamayo et al. 2005). In this study, GSEA was performed using pathway information derived from the Kyoto Encyclopedia of Gene and Genomes. IPA is based on a large expert-curated repository of molecular interactions, regulatory events, gene-to-phenotype associations, and chemical knowledge, mainly obtained from peer-reviewed scientific publications. GSEA was used to identify significantly regulated processes (FDR q-value <0.25), and the corresponding canonical pathways from Ingenuity were then used for visualizing processes.

Quantitative PCR analysis

Quantitative PCR analyses were performed exactly as described before (Chapter 4). Samples were analyzed in multiple pools each comprising RNA of 3-4 mice, and standardized to 36B4 expression.

Statistical analyses

Comparisons between a single control (LFD) and treatments were made by using Univariate ANOVA. Comparisons between cafestol and control were made by student's t-test. A p-value of less then 0.05 was considered to be significant. Statistical analysis was performed using SPSS version 15 (SPSS Inc, Chicago, Il).

Results and discussion

Previous studies have suggested that changes in diet composition may influence the effects of cafestol on intermediary metabolism and Nrf2-mediated biotransformation, and *vice versa* (Chapter 4). Therefore in the current study we modulated the fat content of the diet and investigated its effects on the hepatic and intestinal adaptive response to cafestol.

Effects of cafestol on HFD or LFD in liver.

We first determined by quantitative PCR the effect of cafestol on either a LFD or HFD on the expression of Cyp7a1 and Cyp8b1, two key enzymes in bile acid synthesis, because cafestol is known to suppress Cyp7a1 (Post, de Roos et al. 2000; Ricketts, Boekschoten et al. 2007). We also analyzed the regulation of various Nrf2-target genes, since previous work showed cafestol induces these genes (van Cruchten, de Haan et al. 2009). As expected, cafestol reduced expression levels of Cyp7a1 and Cyp8b1 on both diets (Figure 1).

However, the extent of suppression on a LFD was much smaller than observed on a HFD. Moreover, when comparing the levels of expression between the four experimental groups, it is clear that the HFD-mediated induction of Cyp7a1 and Cyp8b1 was completely prevented when cafestol was present in the diet. Comparable results were found for the induction of Nrf2-target genes because the regulation by cafestol was for almost all genes more pronounced on a HFD than on a LFD (Figure 2).

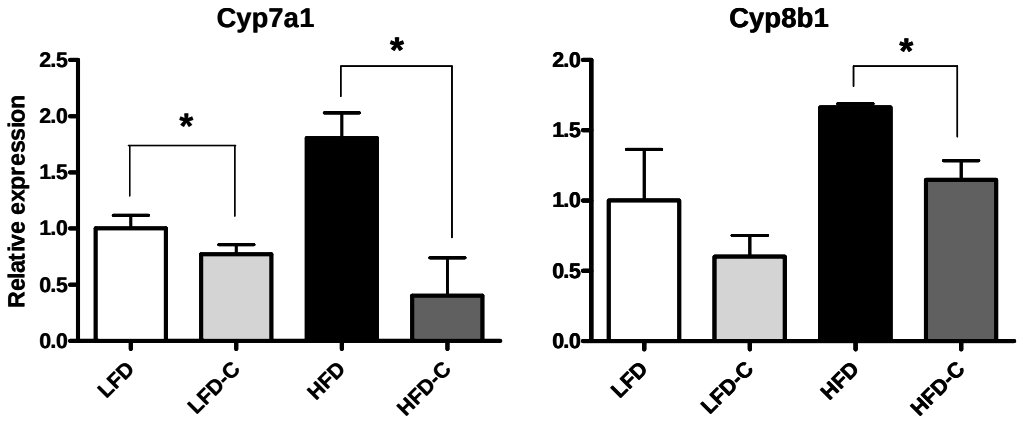

Figure 1: ***Cafestol suppresses bile acid synthesis in liver****. For two key enzymes in bile acid metabolism (Cyp7a1, Cyp8b1) the effect of cafestol on hepatic gene expression was studied by qPCR analyses in mice that were fed cafestol for 8weeks (pools of in total 10 animals). Cafestol suppresses Cyp7a1 on LFD by 23%. On a HFD Cyp7a1 expression is induced (181%) compared to LFD. Cafestol supplementation to HFD suppresses Cyp7a1 expression to 40%. Cafestol suppressed Cyp8b1 expression on HFD. Messenger levels were standardized to 36B4; LFD was set to 1. Data are presented as mean ±SEM. * indicates significant difference between groups (p<0.05).*

However, the extent of suppression on a LFD was much smaller than observed on a HFD. Moreover, when comparing the levels of expression between the four experimental groups, it is clear that the HFD-mediated induction of Cyp7a1 and Cyp8b1 was completely prevented when cafestol was present in the diet. Comparable results were found for the induction of Nrf2-target genes because the regulation by cafestol was for almost all genes more pronounced on a HFD than on a LFD (Figure 2).

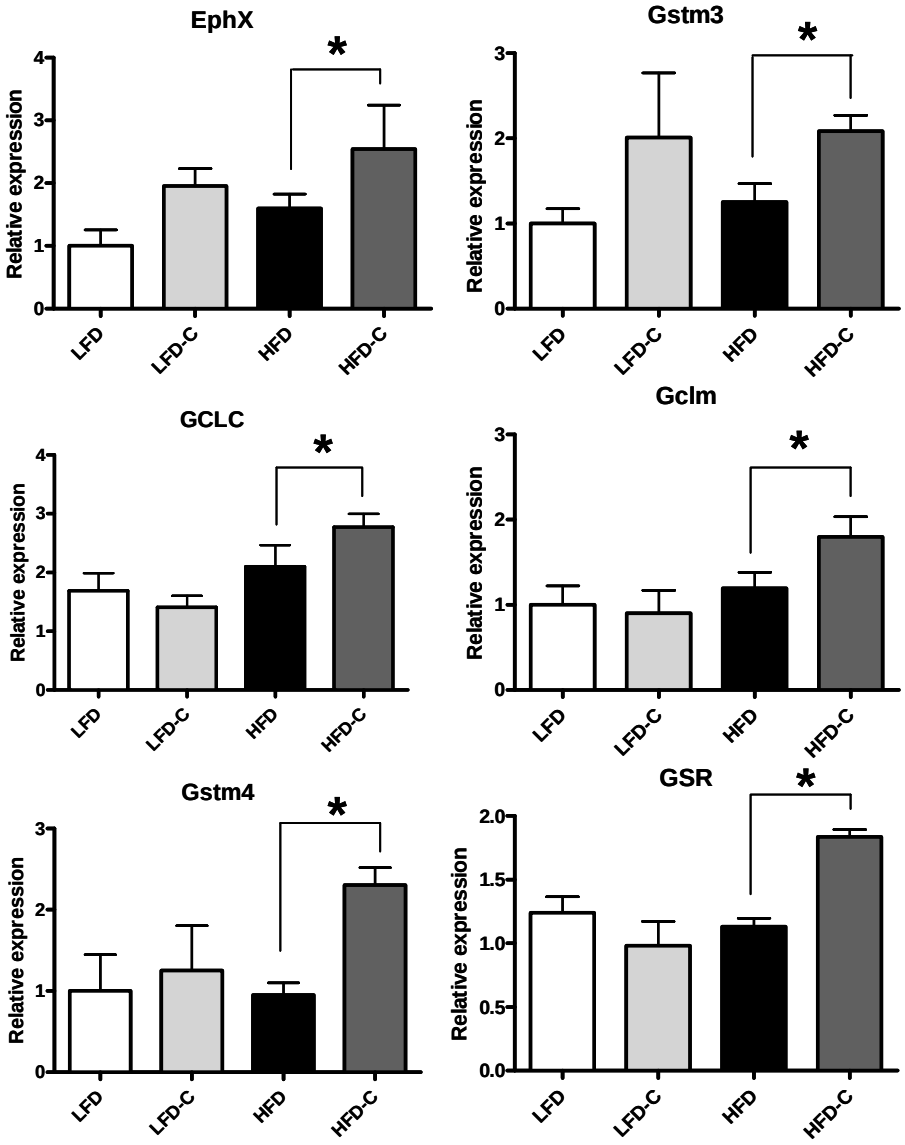

Figure 2: ***Cafestol induces Nrf2 mediated biotransformation enzymes in liver****. For selected Nrf2 target genes such as, Ephx,Gstm3, GCLC, GCLM, Gstm4 and Gsr, the effects of cafestol on hepatic gene expression was studied by qPCR analyses in mice that were fed cafestol for 8weeks (n=10). Messenger levels were standardized to 36B4; LFD was set to 1. Data are presented as mean ±SEM. * indicates significant difference between groups (p<0.05).*

Since conversion into bile acids and their subsequent secretion into bile is the only route for the removal of excess cholesterol, we next evaluated for additional genes involved in bile acid metabolism whether cafestol likewise obstructed the adaptive response to a HFD. To this end expression data was visualized on a canonical pathway representing the role of Fxr in bile acid and lipoprotein homeostasis (Figure 3A and 3B). Results presented in Figure 3A show that for many genes the regulation by cafestol was similar on both LFD and HFD, although the effect size was often most pronounced on a HFD. However, in contrast to Cyp7a1 and Cyp8b1, for most genes cafestol did not attenuate the adaptive response to a HFD (Figure 3B). Again, comparable results were found when genes were overlaid on a canonical pathway representing Nrf2-mediated stress response (Figure 4A + 4B).

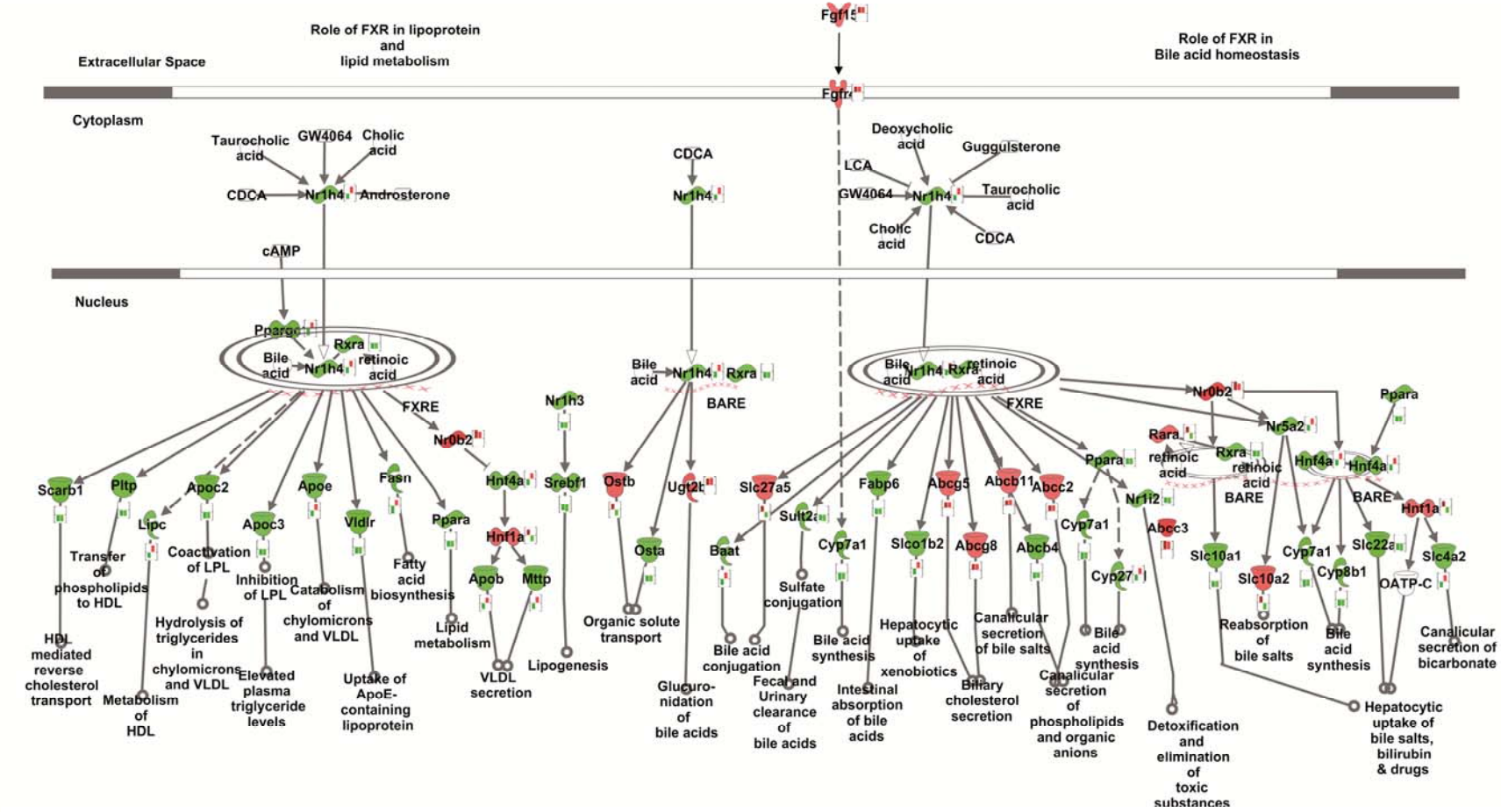

Figure 3A: ***Effects of cafestol supplemented to either a LFD or HFD on genes involved in bile acid and lipoprotein homeostasis in liver.*** *Regulation by cafestol on a LFD (left bar) and HFD (right bar) of a canonical pathway representing the role of Fxr in bile acid and lipoprotein homeostasis. Color coding: red means induced and green means suppressed gene expression in cafestol group versus baseline. Size of the bars corresponds to magnitude of regulation.*

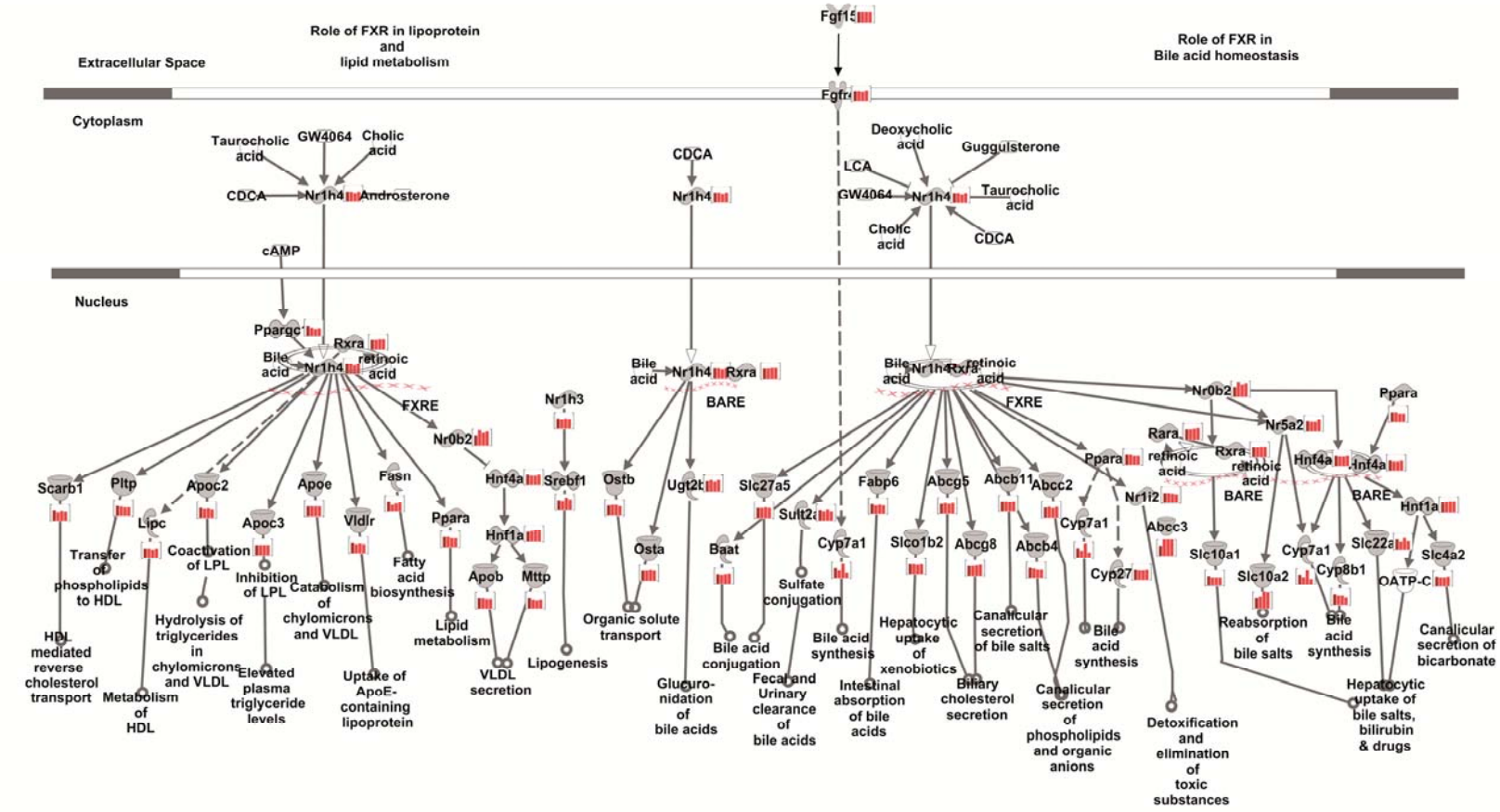

Figure 3B: ***Effects of cafestol supplemented to either a LFD or HFD on genes involved in bile acid and lipoprotein homeostasis in liver.*** *Relative expression levels of genes in (from left to right) 1) LFD, 2) LFD + cafestol (LFD-C), 3) HFD, and 4) HFD + cafestol (HFD-C). The size of the bars represent the relative expression level compared to LFD.*

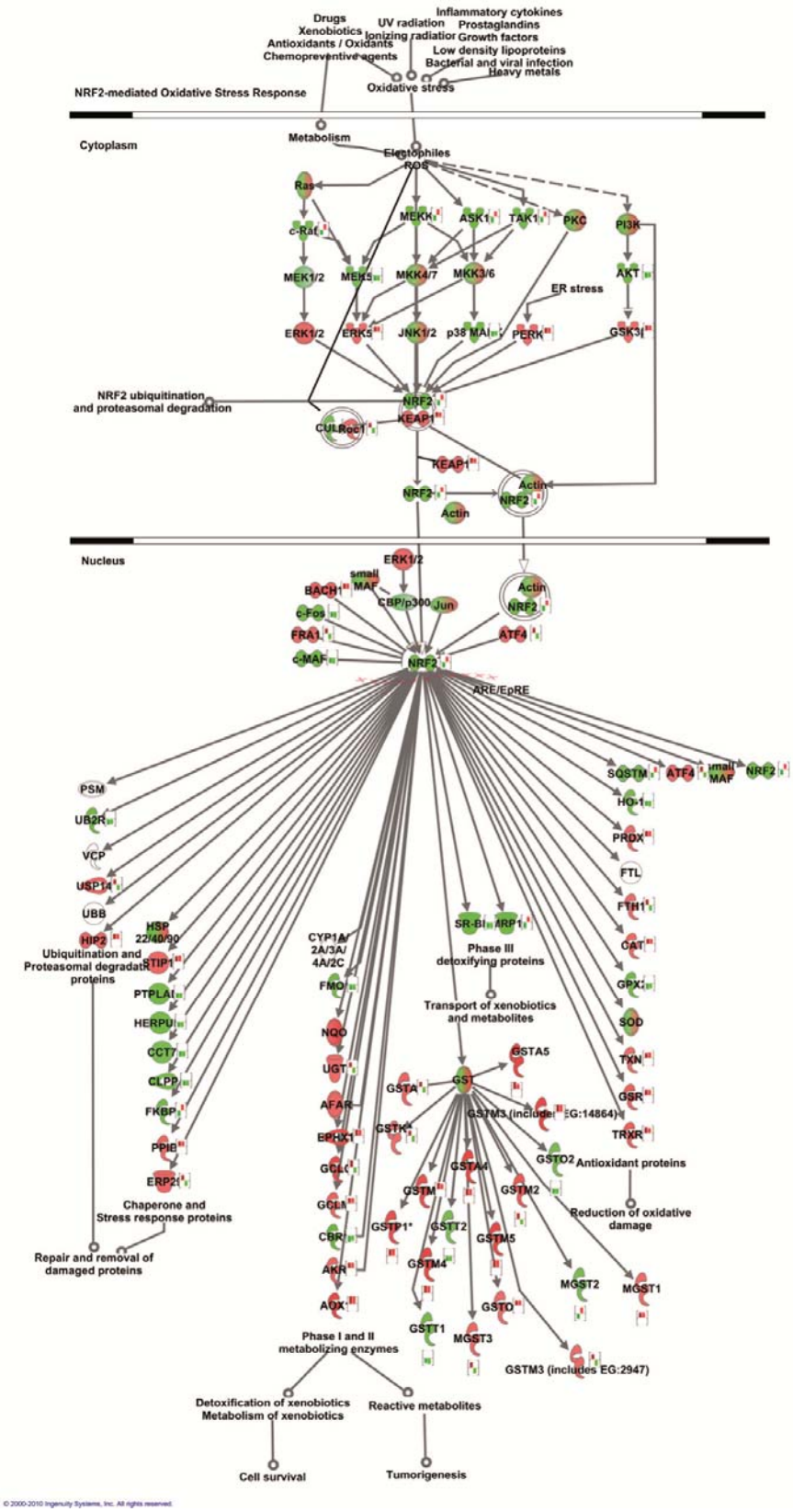

Figure 4A: ***Effects of cafestol supplemented to either a LFD or HFD on genes involved in the Nrf2-mediated stress response in liver. Panel A****. Regulation by cafestol on a LFD (left bar) and HFD (right bar) of a canonical pathway representing Nrf2-mediated stress response. Color coding: red means induced and green means suppressed gene expression in cafestol group versus baseline. Size of the bar corresponds to magnitude of regulation.*

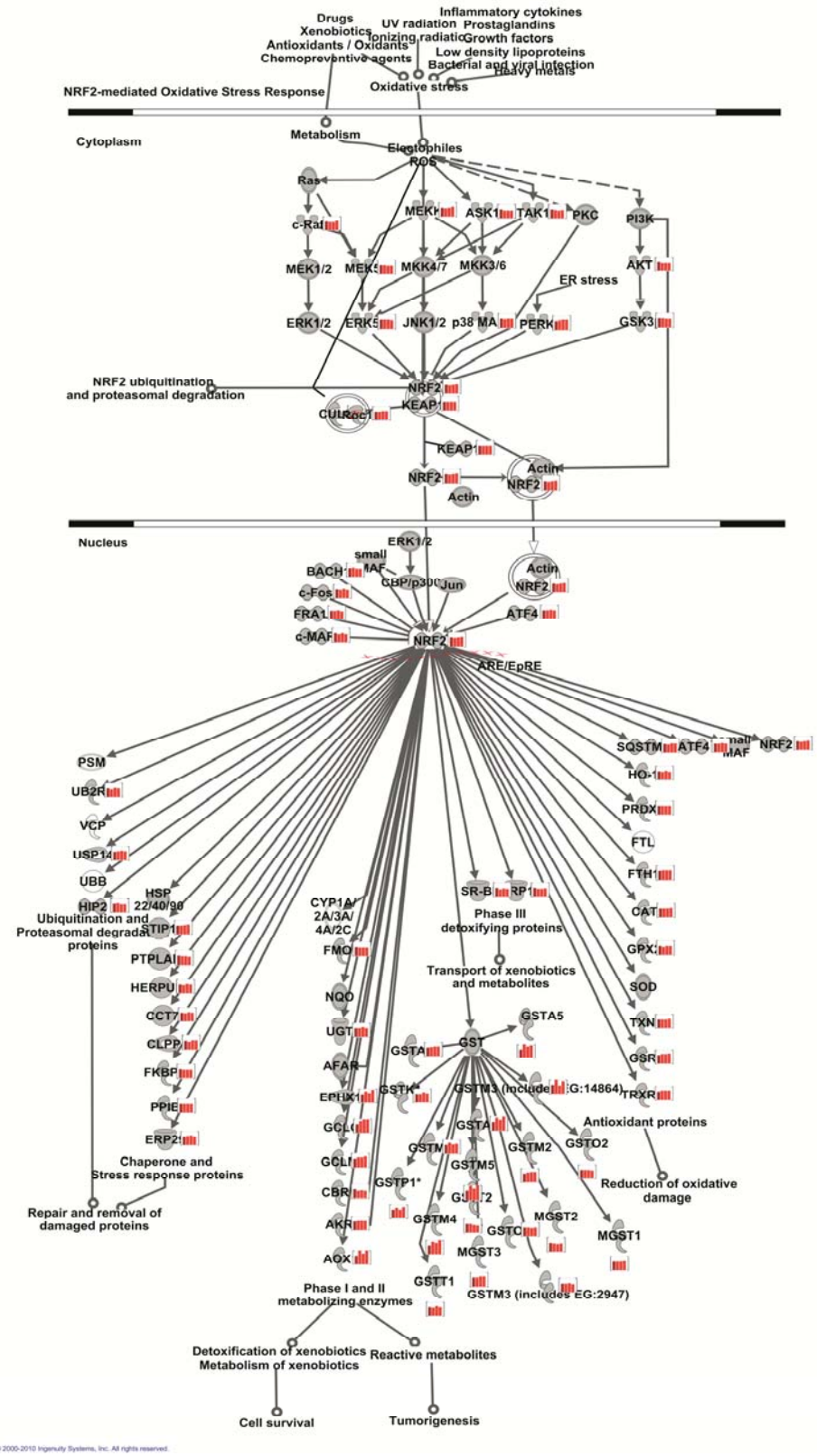

Figure 4B: ***Effects of cafestol supplemented to either a LFD or HFD on genes involved in the Nrf2-mediated stress response in liver. B****: relative expression levels of genes in (from left to right) 1) LFD, 2) LFD + cafestol (LFD-C), 3) HFD, and 4) HFD + cafestol (HFD-C). The size of the bars represents the relative expression level compared to LFD.*

Our data clearly show that cafestol modulated bile acid metabolism. To evaluate the functional implications of this regulation, we analyzed bile composition of mice that were fed cafestol for 9 weeks. On a HFD, cafestol reduced the concentration of biliary bile acids as well as the output of cholate and deoxycholate, but no significant effects were observed on a LFD (Table 1). This functional inhibition of bile acid synthesis coincided with decreased hepatic levels of cholesterol esters (Chapter 4 and (Post, de Roos et al. 2000)), but resulted in increased serum cholesterol levels (Figure 5) as well as enhanced biliary output of cholesterol (Table 1). The latter is in line with the increased expression of the canalicular transporters Abcg5 and Abcg8 (Figure 3A).

With respect to genes involved in lipoprotein metabolism, it is evident that cafestol regulated many of these, but independent of diet (Figure 3A + 3B). Interestingly, cafestol suppressed expression of Apoc2 and Apoc3, two apolipoproteins involved in the catabolism of VLDL (Kawakami, Aikawa et al. 2006; Pollin, Damcott et al. 2008). This regulation may be linked to (transient) rise of plasma TG observed after cafestol treatment (Urgert, Schulz et al. 1995; Urgert, Meyboom et al. 1996; Urgert and Katan 1997; Post, de Roos et al. 2000).

Table 1: ***Bile flow and bile acid concentrations in C57BL6/j mice which are fed LFD of HFD with and without 0.05% cafestol for 9 weeks****. Bile was sampled at 0-3 hours at different intervals (n=5). Subsequently bile flow was measured. In the HFD fed animals cafestol induced average bile flow with 25% compared to HFD. In addition, biliary bile output is decreased in HFD-C animals compared to HFD. Cholesterol output in bile is higher in cafestol fed animals regardless of background diet. Values are expressed in averages ± SEM. Ψ Significant difference between LF and LF and cafestol. * Significant difference between HFD and HFD and cafestol. ** p=0.06*

	LFD	LFD-C	HFD	HFD-C
Bile				
Total Bile acids conc (mM)	10.80±1.39	11.91±1.17	10.29±1.4	7.79±0.53*
Mean BA flow (µl [min−1 [100 g−1)	2.94±0.44	2.73±0.19	3.1±0.23	3.99±0.33*
Cholesterol (mM)	0.06±0.01	0.23±0.024 Ψ	0.06±0.015	0.16±0.028**
Phospholipids (mM)	4.63±0.89	5.13±0.63 Ψ	3.46±0.53	3.03±0.40
Biliary output				
Total Bile salts (nmol [min−1 [100 g−1)	31.96±8.47	33.36±5.28	36.4±6.08	26.44±3.81*
CA	11.64±3.7	13.37±2.63	16.88±2.59	11.04±1.76*
CDCA	0.79±0.27	0.62±0.09	1.03±0.08	0.52±0.06*
Cholesterol(nmol [min−1 [100 g−1)	0.176±0.051	0.628±0.11Ψ	0.186±0.093	0.638±0.266*
Phospholipids (nmol [min−1 [100 g−1)	13.61±2.21	14.0±1.71	10.72±1.86	12.09±1.45

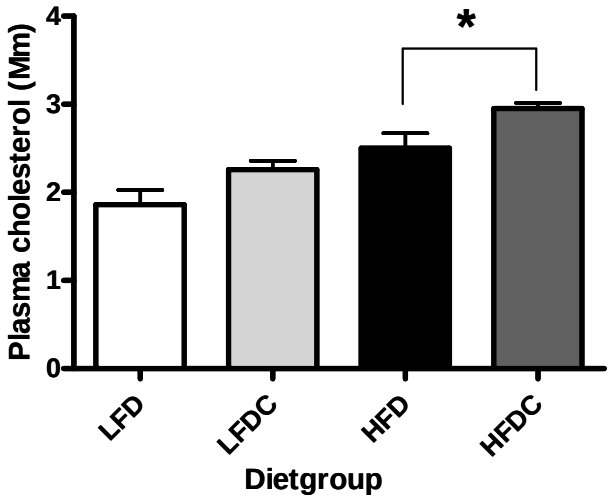

Figure 5: ***Cafestol increased plasma cholesterol in mice fed a high fat diet for 8weeks.*** *Plasma cholesterol was determined in mice fed cafestol for 8 weeks (pools of in total 10 animals). Data are presented as mean ±SEM. * indicates significant difference between groups ($p<0.05$).*

Thus, this data show that cafestol inhibits many genes involved in bile acid and lipoprotein homeostasis, including Cyp7a1 and Cyp8b1. For most genes this inhibition is most pronounced at a HFD. Moreover, the deregulation of the adaptive response to HFD of Cyp7a1 and Cyp8b1 by cafestol shows that cafestol somehow interferes with the signaling of bile acids, which ultimately contributes to increased serum and biliary cholesterol levels.

In addition to this targeted view on processes known to be affected, the array analysis provided the opportunity to also have more global look at the effects of cafestol. Supplementation of a LFD or HFD with cafestol for 8 weeks resulted in the significant regulation of 652 and 1111 genes, respectively, compared to control (Supplemental table 1). This translated into the significant regulation of 39 and 18 pathways, respectively (Table 2 in

appendix). The overlap was low, both at the level of individual genes and pathways (Figure 6).

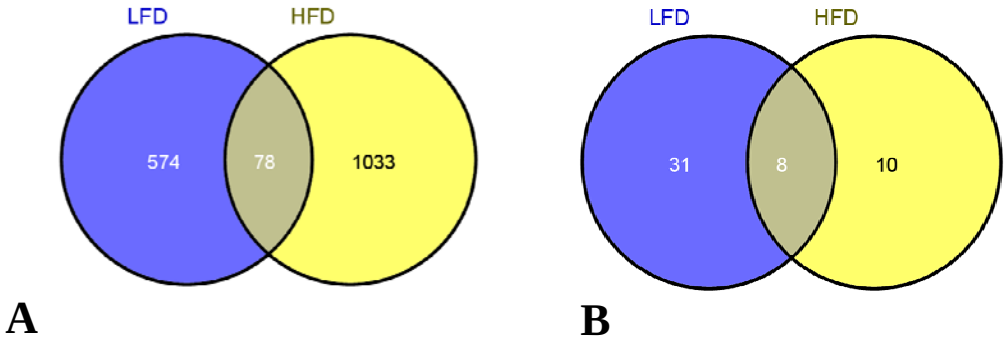

Figure 6: ***Number of differential expressed genes (A) and KEGG pathways (B) in liver after 8 weeks supplementation with cafestol on a LFD or HFD.*** *Fat content of the diet has a major influence on the response of the liver to cafestol because two distinct sets of genes are regulated on LFD or HFD, as reflected on the level of individual genes (panel A) and pathways (panel B)*

Processes that were induced by cafestol on both diets mainly reflected cytochrome P450- and Nrf2-mediated biotransformation, whereas lipogenesis, lipoprotein metabolism and energy metabolism were suppressed. Specific for HFD was the induction of heme-containing enzymes, referring to increased levels of CypP450 isoforms, and the suppression of cholesterol esterification and steroid synthesis. More processes were regulated on a LFD, and overall these corresponded to increased metabolism of glutathione and reduced energy and fatty acid metabolism. Taken together, the array data identified several processes already known to be functionally regulated by cafestol, such as CypP450- and Nrf2-mediated

biotransformation (van Cruchten, de Haan et al. 2009), lipogenesis and energy metabolism (Chapter 4) and bile acid metabolism (Post, de Roos et al. 2000; Ricketts, Boekschoten et al. 2007), but also provided clues for additional processes that are affected by cafestol. Moreover, these data do imply that diet is an important determinant of the hepatic effects of cafestol, since distinct sets of genes are regulated by cafestol on a LFD or HFD as well as because most genes responded more pronounced on a HFD.

Effects of cafestol on HFD or LFD in small intestine

We also had an initial look at the interaction between cafestol and diet in small intestine. Since previous data demonstrated that cafestol is extensively metabolized in intestine (van Cruchten, de Waart et al. 2010), we first analyzed regulation of a set of Nrf2 target genes by quantitative PCR (Figure 7). Cafestol induced expression of all genes, and this regulation was independent of diet. Similar results were found when genes were overlaid on a canonical pathway representing Nrf2-mediated stress response (Figure 8A + 8B). Moreover, on both diets the extent of regulation was larger in intestine compared to liver (compare Figure 2 and Figure 7). We believe this is due to the direct contact of the intestine with cafestol in the diet. These results may also be linked to the presumed anti carcinogenic effects of cafestol, specifically to the increase of glutathione levels in colon in human volunteers when consuming unfiltered coffees (Grubben, Van Den Braak et al. 2000) and data on glutathione levels obtained in rats (Huber, Teitel et al. 2004; Huber and Parzefall 2005; Huber, Rossmanith et al. 2008).

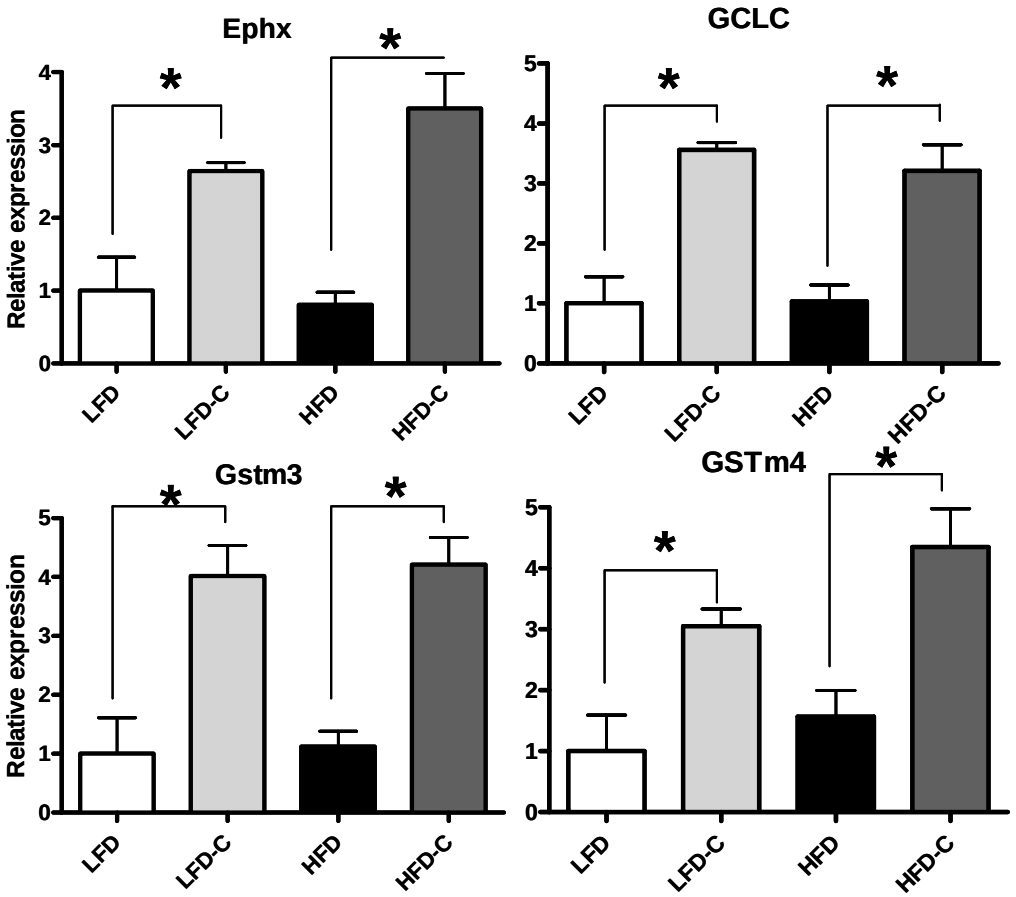

Figure 7: ***Cafestol activates Nrf2 mediated biotransformation enzymes in the small intestine after 8weeks consumption on both LFD and HFD.*** *For selected Nrf2 target genes the effects of cafestol on gene expression was studied by qPCR analyses in mice that were fed cafestol for 8 weeks (n=10). Messenger levels were standardized to 36B4; LFD was set to 1. Data are presented as mean ±SEM. * indicates significant difference between groups (p<0.05).*

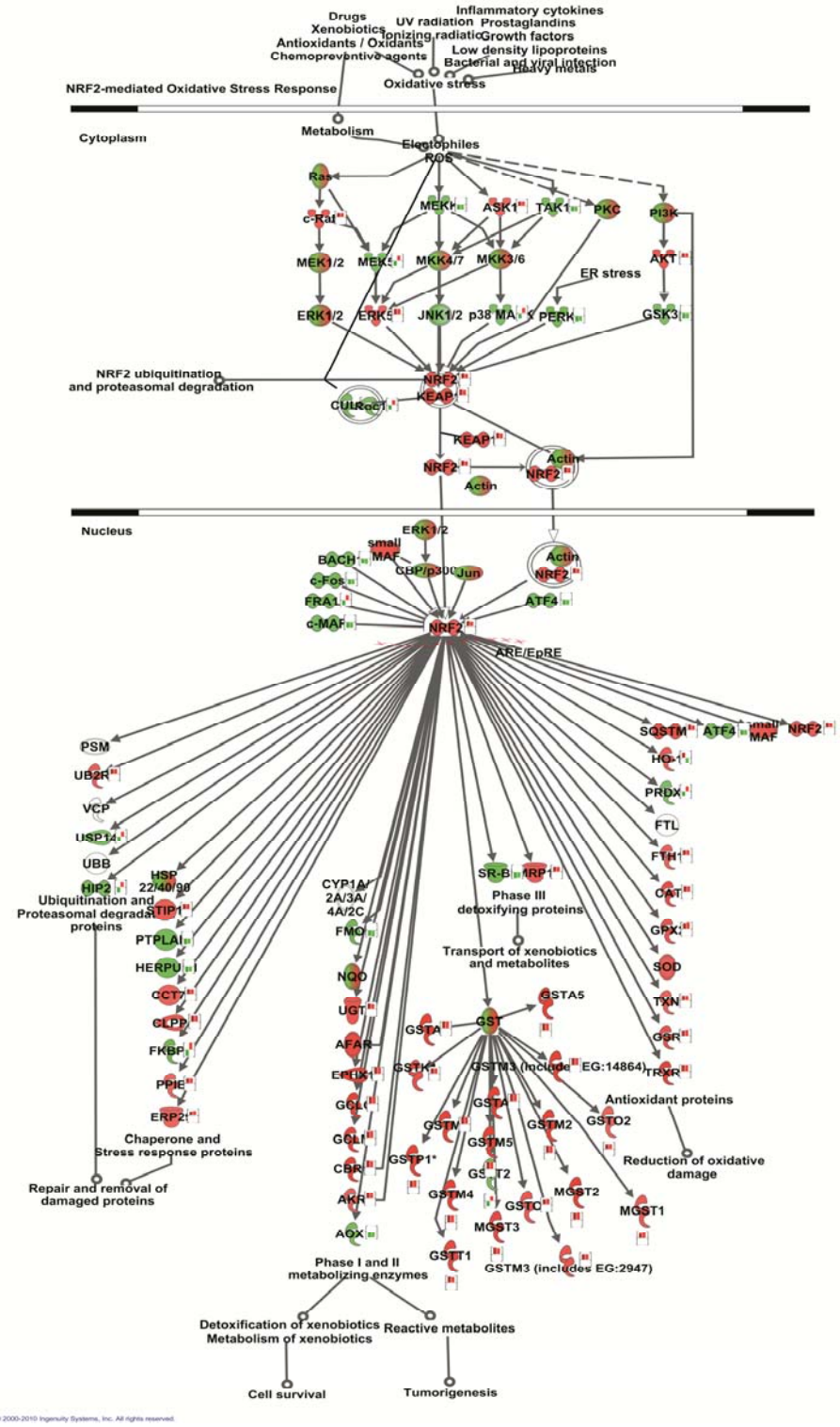

Figure 8A: ***Effects of cafestol supplemented to either a LFD or HFD on genes involved in the Nrf2-mediated stress response in small intestine. Panel A****. Regulation by cafestol on a LFD (left bar) and HFD (right bar) of a canonical pathway representing Nrf2-mediated stress response. Color coding: red means induced and green means suppressed gene expression in cafestol group versus*

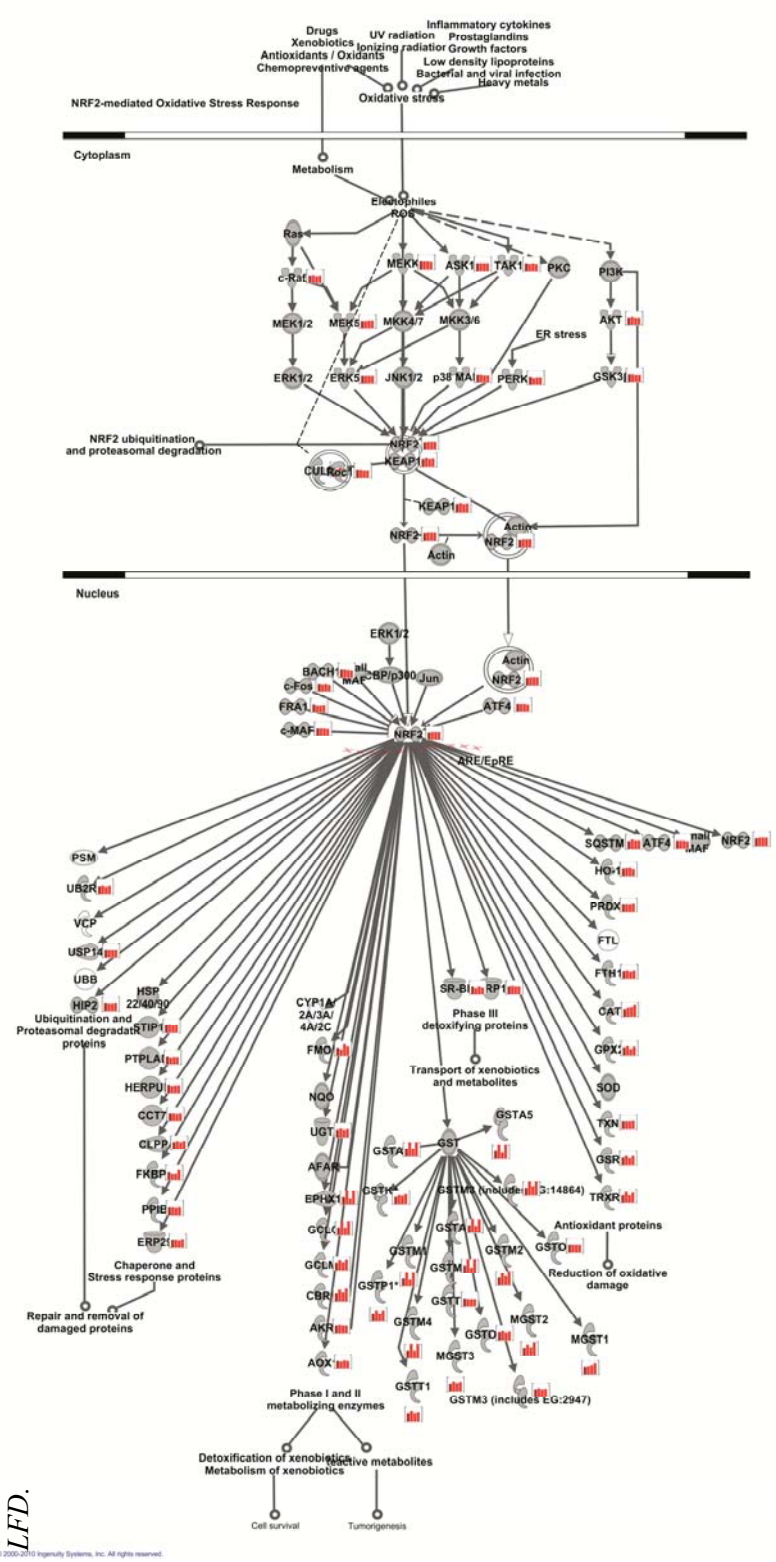

Figure 8B: ***Effects of cafestol supplemented to either a LFD or HFD on genes involved in the Nrf2-mediated stress response in small intestine. Panel B****: relative expression levels of genes in (from left to right) 1) LFD, 2) LFD + cafestol (LFD-C), 3) HFD, and 4) HFD + cafestol (HFD-C). The size of the bars represents the relative expression level compared to LFD.*

Array analyses revealed that on a LFD cafestol significantly regulated the expression of 1104 genes, whereas this number was 570 on a HFD (Supplemental table 2). This corresponded to significant regulation of 47 and 56 pathways, respectively (Table 3). On both diets cafestol regulated distinct set of genes and pathways (Figure 9), but compared to liver the overlap of regulated genes and pathways was much higher.

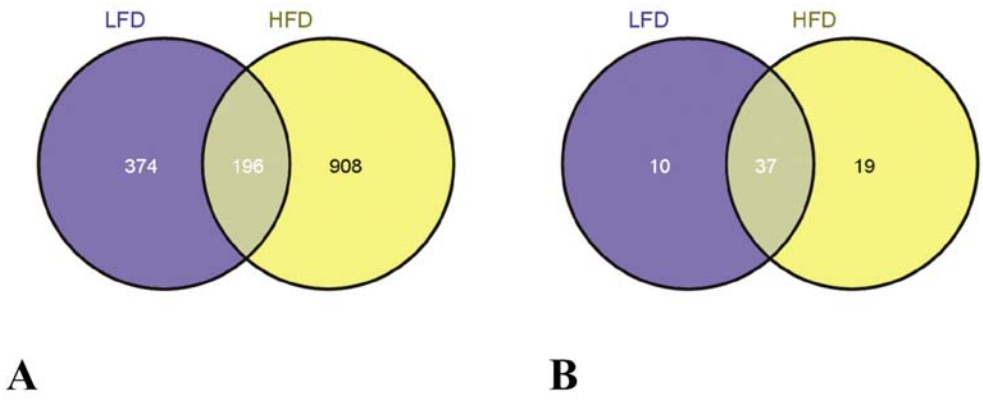

Figure 9: ***Number of differential expressed genes (A) and KEGG pathways (B) in small intestine after 8 weeks supplementation with cafestol on a LFD or HFD.*** *Fat content of the diet does influence the response of the intestine to cafestol because two distinct sets of genes are regulated on LFD or HFD, as reflected on the level of individual genes (panel A) and pathways (panel B).*

Processes that were regulated on both diets corresponded to CypP450- and Nrf2-mediated biotransformation, and to a lesser extent to bile acid and energy metabolism, barrier function, and cell growth and differentiation. The processes that were specific for either LFD or HFD

could be related to the pathways that were regulated on both diets, suggesting that diet had a smaller modulating effect on the effects of cafestol in intestine compared to liver.

Conclusion

The major finding of this study is that fat content of the diet is an important determinant of the effects of cafestol. This has been evaluated for several processes already known to be influenced by cafestol, such as bile acid metabolism and Nrf2-mediated biotransformation. The latter was more pronouncedly regulated in intestine than liver. Moreover, in addition to these, array analysis identified several new processes that are affected by cafestol treatment. However, a series of future studies is required to evaluate the relevance of these findings.

Acknowledgements

We would like to thank Jenny Jansen en Mechteld Grootte Bromhaar for their skillful processing of the samples for microarray analysis.

Appendix

Table 2: ***Supplementation of a LFD or HFD with cafestol for 8 weeks resulted in liver in the significant regulation of 39 and 18 KEGG pathways, respectively, compared to control (p<0.05).***

LFD-C to LFD

METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450
NRF2-REGULATED_GENES_COMBINED
NRF2-REGULATED_XENOBIOTIC_DETOXIFICATION_GENES
CELL_COMMUNICATION
OXIDATIVE_PHOSPHORYLATION
LIPOGENESIS
CITRATE_CYCLE_(TCA_CYCLE)
LIPOPROTEIN_METABOLISM
ASCORBATE_AND_ALDARATE_METABOLISM
NUCLEOTIDE_SUGARS_METABOLISM
GLUTATHIONE_METABOLISM
COMPLEMENT_AND_COAGULATION_CASCADES
BILE_ACID_BIOSYNTHESIS
PPAR_SIGNALING_PATHWAY
INSULIN_SIGNALING_PATHWAY_KEGG
MISCELLANEOUS_LIPID_METABOLISM_(LIST_IS_BIASED_TOWARDS_PUTATIVE_PPARA_TARGETS)
ADIPOCYTOKINE_SIGNALING_PATHWAY
MAPK_SIGNALING_PATHWAY
LONG-TERM_DEPRESSION
GLYCOLYSIS___GLUCONEOGENESIS
GAP_JUNCTION
CARBON_FIXATION
FRUCTOSE_AND_MANNOSE_METABOLISM
PENTOSE_PHOSPHATE_PATHWAY
CELL_CYCLE
GLYCAN_STRUCTURES_-_BIOSYNTHESIS_2
PHENYLALANINE_METABOLISM
TYROSINE_METABOLISM
CHRONIC_MYELOID_LEUKEMIA
TYPE_I_DIABETES_MELLITUS
GLYCEROLIPID_METABOLISM
BENZOATE_DEGRADATION_VIA_COA_LIGATION
MITOCHONDRIAL_FATTY_ACID_OXIDATION
GNRH_SIGNALING_PATHWAY
GLYCEROPHOSPHOLIPID_METABOLISM
ETHER_LIPID_METABOLISM
PYRUVATE_METABOLISM
REGULATION_OF_ACTIN_CYTOSKELETON
JAK-STAT_SIGNALING_PATHWAY

HFD-C to HFD

METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450
NRF2-REGULATED_GENES_COMBINED
NRF2-REGULATED_XENOBIOTIC_DETOXIFICATION_GENES
CELL_COMMUNICATION
OXIDATIVE_PHOSPHORYLATION
LIPOGENESIS
CITRATE_CYCLE_(TCA_CYCLE)
LIPOPROTEIN_METABOLISM
BASAL_TRANSCRIPTION_FACTORS
HEME_CONTAINING_ENZYMES_AND_PROTEINS
ALKALOID_BIOSYNTHESIS_II
AMINOACYL-TRNA_BIOSYNTHESIS
ANDROGEN_AND_ESTROGEN_METABOLISM
CHOLESTEROL_SYNTHESIS_ESTERIFICATION
UREA_CYCLE_AND_METABOLISM_OF_AMINO_GROUPS
ALANINE_AND_ASPARTATE_METABOLISM
PROPANOATE_METABOLISM
GLYCAN_STRUCTURES_-_DEGRADATION

Table 3: ***Supplementation of a LFD or HFD with cafestol for 8 weeks resulted in the small intestine in the significant regulation of 47 and 56 KEGG pathways, respectively, compared to control (p<0.05).***

LFD-C to LFD

GLUTATHIONE_METABOLISM
METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450
NRF2-REGULATED_GENES_COMBINED
NRF2-REGULATED_XENOBIOTIC_DETOXIFICATION_GENES
OXIDATIVE_PHOSPHORYLATION
PENTOSE_PHOSPHATE_PATHWAY
GALACTOSE_METABOLISM
FRUCTOSE_AND_MANNOSE_METABOLISM
NRF2_REGULATED_ANTIOXIDANT_GENES
ARACHIDONIC_ACID_METABOLISM
CARBON_FIXATION
GLYCOLYSIS___GLUCONEOGENESIS
HEME_CONTAINING_ENZYMES_AND_PROTEINS
BUTANOATE_METABOLISM
NUCLEOTIDE_SUGARS_METABOLISM
GLUTAMATE_METABOLISM
LINOLEIC_ACID_METABOLISM
LYSINE_DEGRADATION
BILE_ACID_BIOSYNTHESIS
ASCORBATE_AND_ALDARATE_METABOLISM
PROTEASOME
CITRATE_CYCLE_(TCA_CYCLE)
SELENOAMINO_ACID_METABOLISM
GAMMA-HEXACHLOROCYCLOHEXANE_DEGRADATION
FATTY_ACID_METABOLISM
GLYCAN_STRUCTURES_-_DEGRADATION
PYRUVATE_METABOLISM
NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY
TGF-BETA_SIGNALING_PATHWAY
T_CELL_RECEPTOR_SIGNALING_PATHWAY_KEGG
CYTOKINE-CYTOKINE_RECEPTOR_INTERACTION
OLFACTORY_TRANSDUCTION
NEUROACTIVE_LIGAND-RECEPTOR_INTERACTION
FOCAL_ADHESION
CALCIUM_SIGNALING_PATHWAY
ANTIGEN_PROCESSING_AND_PRESENTATION
COMPLEMENT_AND_COAGULATION_CASCADES
GAP_JUNCTION
HEMATOPOIETIC_CELL_LINEAGE
WNT_SIGNALING_PATHWAY_KEGG
B_CELL_RECEPTOR_SIGNALING_PATHWAY
CELL_ADHESION_MOLECULES_(CAMS)
CELL_CYCLE
LONG-TERM_POTENTIATION
TYPE_I_DIABETES_MELLITUS
MAPK_SIGNALING_PATHWAY
CHOLESTEROL_SYNTHESIS_ESTERIFICATION

HFD-C to HFD

NRF2-REGULATED_GENES_COMBINED
METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450
GLUTATHIONE_METABOLISM
NRF2-REGULATED_XENOBIOTIC_DETOXIFICATION_GENES
NRF2_REGULATED_ANTIOXIDANT_GENES
PENTOSE_PHOSPHATE_PATHWAY
PROTEASOME
AMINOACYL-TRNA_BIOSYNTHESIS
OXIDATIVE_PHOSPHORYLATION
LINOLEIC_ACID_METABOLISM
GALACTOSE_METABOLISM
ARACHIDONIC_ACID_METABOLISM
FRUCTOSE_AND_MANNOSE_METABOLISM
BILE_ACID_BIOSYNTHESIS
GLUTAMATE_METABOLISM
HEME_CONTAINING_ENZYMES_AND_PROTEINS
GLYCINE,_SERINE_AND_THREONINE_METABOLISM
LYSINE_DEGRADATION
DNA_POLYMERASE
ONE_CARBON_POOL_BY_FOLATE
NUCLEOTIDE_SUGARS_METABOLISM
GLYCOLYSIS___GLUCONEOGENESIS
BUTANOATE_METABOLISM
SELENOAMINO_ACID_METABOLISM
ASCORBATE_AND_ALDARATE_METABOLISM
PYRIMIDINE_METABOLISM
PORPHYRIN_AND_CHLOROPHYLL_METABOLISM
PYRUVATE_METABOLISM
GLYCEROLIPID_METABOLISM
CARBON_FIXATION
HEMATOPOIETIC_CELL_LINEAGE
ECM-RECEPTOR_INTERACTION
FOCAL_ADHESION
GAP_JUNCTION
CELL_ADHESION_MOLECULES_(CAMS)
TGF-BETA_SIGNALING_PATHWAY
AXON_GUIDANCE
MAPK_SIGNALING_PATHWAY
ADHERENS_JUNCTION
OLFACTORY_TRANSDUCTION
LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION
CYTOKINE-CYTOKINE_RECEPTOR_INTERACTION
WNT_SIGNALING_PATHWAY_KEGG
NEUROACTIVE_LIGAND-RECEPTOR_INTERACTION
LIPOPROTEIN_METABOLISM
B_CELL_RECEPTOR_SIGNALING_PATHWAY
REGULATION_OF_ACTIN_CYTOSKELETON
LONG-TERM_POTENTIATION
COMPLEMENT_AND_COAGULATION_CASCADES
STEROL_TRANSPORT_TRAFICKING
CALCIUM_SIGNALING_PATHWAY
PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM
PANCREATIC_CANCER

HUNTINGTON'S_DISEASE
CELL_COMMUNICATION
GNRH_SIGNALING_PATHWAY

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Chapter 6

General discussion

Since its discovery in the early 90s of last century as a compound responsible for an increase of plasma cholesterol levels, cafestol has been subject of several studies. Nevertheless, many questions are left open regarding the mechanisms of this effect. As there were indications that cafestol absorption, distribution, metabolism and excretion are playing crucial roles in this respect, the current project was initiated. The first studies were undertaken to study uptake, distribution and metabolism of cafestol in mice. In **Chapter 2,** it is shown that cafestol can be metabolized by the liver into several metabolites, and that these metabolites can activate Nrf2. Results of **Chapter 3** show that cafestol is mainly distributed to liver and small intestine, and that hardly any cafestol becomes available in the systemic circulation. Furthermore, the compound is extensively metabolized by the liver and possibly the intestinal wall. From its kinetic behaviour, we hypothesized that cafestol is likely to undergo enterohepatic cycling. Results described in **Chapter 5** show that cafestol not only activates Nrf2 in liver, but also in the small intestine. As a result, cafestol consumption leads to induction of Nrf2 regulated biotransformation enzymes in these tissues. As these results were quite remarkable, indicating a distinctive metabolic pathway and new links with the effects of cafestol on biotransformation pathways, it was decided to shift focus more specifically towards the role of the liver and intestine in cafestol metabolism. Surprisingly, results described in **Chapter 4**, showed that cafestol was able to prevent diet induced obesity and the development of hepatic steatosis, and that it ameliorated insulin sensitivity in high fat-fed mice. As the results of these studies were pointing towards a possible interference with dietary fat intake, this was further investigated as described in **Chapter 5.** To address this**,** a nutrigenomics approach has been

followed in order to gain more insight in the metabolic interactions of cafestol with different background diets.

Absorption, distribution, metabolism and excretion

During the course of this project, the metabolism and distribution of cafestol has been investigated extensively in mice and rats (not reported). In both species the compound was found to undergo rapid metabolism, with feces being the major route of excretion. Following oral administration, cafestol was mainly found in intestine and liver. Hardly any cafestol was present in peripheral blood. Remarkably, a similar pattern was seen after intravenous administration. Cafestol was rapidly cleared from the circulation, thereby accumulating in the gastrointestinal tract and liver. This typical distribution pattern and the lack of urinary elimination suggested that cafestol is subject to extensive hepatic elimination and probably undergoes enterohepatic circulation. To study this hypothesis, portal vein cannulated mice were dosed with radioactive cafestol. In portal blood the parent compound was found to be the most abundant. However, 2 other metabolites were present from which one was identified as a glucuronide conjugate of cafestol. In bile duct-cannulated animals the presence of several metabolites could be demonstrated in bile, whereas no parent compound was present here. These metabolites were identified as epoxy-glutathione conjugates, glutathione conjugates and glucuronide conjugates. In portal blood, parent compound and cafestol metabolites were detected. Together these findings support the hypothesis that cafestol undergoes enterohepatic circulation.

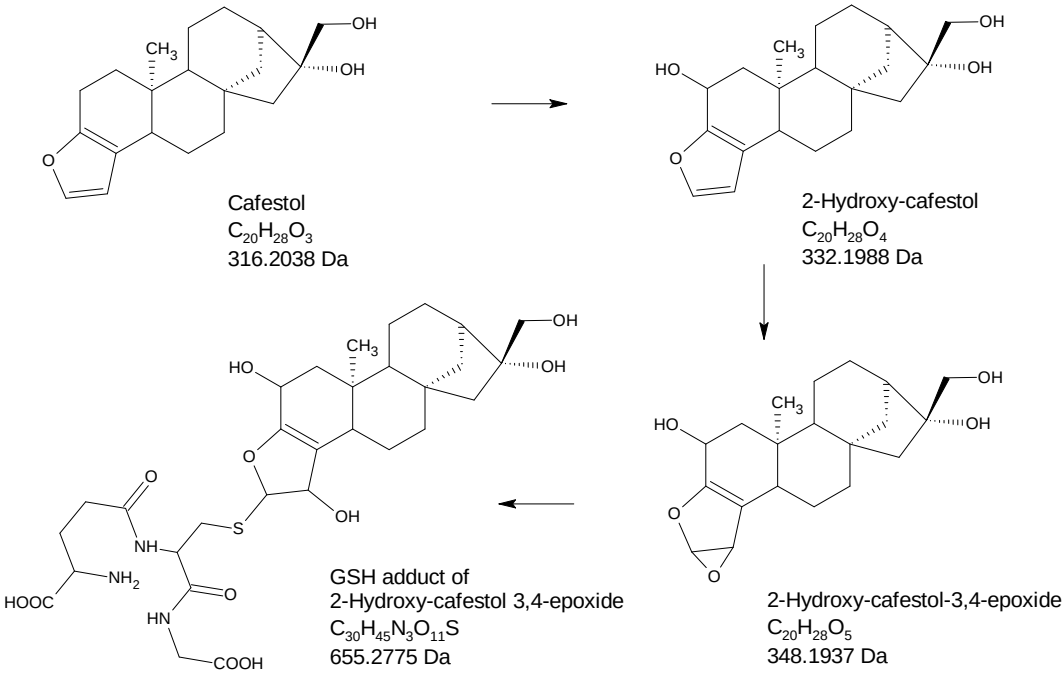

Figure 1: ***Proposed schematic representation of the formation of a glutathione conjugate of cafestol.*** *In this scheme cafestol is hydroxylated prior to epoxidation of the furan moiety of cafestol by CYP450 enzymes (adapted from van Cruchten et al. Journal of Nutritional Biochemistry; 2009 in press.)*

As mentioned before, the presence of epoxy-glutathione conjugates, glutathione conjugates and glucuronide conjugates was demonstrated in bile, while no parent cafestol was present. In **Chapter 2** evidence is provided that the metabolism of cafestol plays a key role in the induction of glutathione-S-transferases (GSTs) and other phase II enzymes via the electrophile-responsive element (EpRE), after binding of the transcription factor Nrf2. Epoxidation of the furan moiety is probably involved in this activation (Figure 1) (van Cruchten, de Haan et al. 2009). EpRE-, initially referred to as antioxidant-responsive element (ARE) is an important gene-regulatory enhancer mediating induction of the expression of a

battery of genes involved in the defense against electrophilic and other reactive species. Based on structurally similar compounds like ipomeanol and teucrin A, it was hypothesized that CYP450 enzymes are probably responsible for the epoxidation of cafestol. Among the different metabolites formed, we hypothesize that cafestol epoxide is the main metabolite responsible for Nrf2 activation thereby triggering activation of phase 2 enzymes such as epoxide hydrolase and glutathione biosynthesis enzymes. The *in vivo* experiments confirmed induction of Nrf2 mediated biotransformation enzymes including epoxide hydrolase, both in liver and small intestine (**Chapter 5**). The finding that cafestol-metabolites induce Nrf2 mediated biotransformation enzymes both in liver and small intestine is highly relevant and may explain the protection against potentially carcinogenic compounds (Cavin, Holzhauser et al. 1998; Cavin, Mace et al. 2001; Cavin, Holzhaeuser et al. 2002; Huber, Prustomersky et al. 2002; Cavin, Bezencon et al. 2003; Huber, Scharf et al. 2003; Huber, Teitel et al. 2004; Huber and Parzefall 2005; Huber, Rossmanith et al. 2008).

These remarkable results indicate a distinctive metabolic pathway and new links with the effects of cafestol on biotransformation pathways. Therefore it was decided to shift focus to the role of the liver and intestine in cafestol metabolism. In **chapter 4** the metabolic effects of cafestol were studied in mice. Surprisingly, it was found that cafestol reduced body weight gain by over 20% and ameliorated the metabolic effects of high fat feeding such as hepatic steatosis and insulin resistance. These apparently beneficial effects are in contrast to the negative effects associated with elevation of serum cholesterol and liver enzymes.

Potential mechanisms of body weight gain reduction by cafestol

The chemical structure of cafestol resembles that of bile acid metabolites, and it was proposed that cafestol might interfere with bile acid synthesis and turnover (Post, de Roos et al. 2000; Ricketts, Boekschoten et al. 2007). Moreover, it was found that cafestol is likely to undergo enterohepatic circulation (**Chapter 3**), and is able to activate FXR both in liver and small intestine (Ricketts, Boekschoten et al. 2007). Interestingly recently bile acids have been found to activate the G-protein coupled receptor TGR5 (Auwerx 2006; Watanabe, Houten et al. 2006). TGR5 is highly expressed in human intestine and gall bladder (Vallim and Edwards 2009), which are normally exposed to high levels of bile acids circulating in the enterohepatic circulation, whereas low levels of TGR5 are present in muscle and brown adipose tissue, tissues that are exposed to lower levels of bile acids present in peripheral blood. Activation of this receptor in muscle and brown adipose tissue by bile acids results in an increase in cyclic AMP content and induces thyroid hormone deiodinase-2 expression, thereby enhancing local conversion of inactive T4 to the active thyroid hormone T3 which leads to induced energy expenditure due to mitochondrial uncoupling. This ultimately results in lower body weight gain and a decrease in fat depots, thereby preventing obesity and ameliorating insulin sensitivity (Watanabe, Houten et al. 2006). Similar observations were made in mice fed a high fat diet supplemented with cafestol (Chapter 4). Since cafestol and bile acid metabolism seem to be tightly intertwined, it is reasonable to hypothesize that cafestol-mediated body weight gain reduction is either the result of activation of TGR5 due to changes in bile acid profiles, or alternatively, cafestol or its metabolites. However, obviously, further research into this topic is required.

Cafestol prevents hepatic steatosis

In addition to the effect of cafestol on body weight gain, cafestol also prevents the development of hepatic steatosis on a high fat diet. It seems likely that this is linked to the overall reduction in body weight gain of the animals. However, evidence is provided that a decrease in HFD-induced endoplasmic reticulum (ER) stress may also contribute to reduction in hepatic steatosis. In livers and adipose tissue of obese animals, activation of the ER stress pathway has been associated with development of hepatic steatosis (Ozcan, Cao et al. 2004; Song, Scheuner et al. 2008; van der Kallen, van Greevenbroek et al. 2009). Although ER stress is mainly regulated on the short term by post-translational events such as phosphorylation of key regulator proteins, there is also transcriptional control more relevant for long term regulatory control of ER stress. In **Chapter 4** cafestol was shown to reduce expression of *Chop10*, and *Herpud 1*, indicating a reduction in ER stress (Lawrence, McGlynn et al. 2007; Song, Scheuner et al. 2008). In addition, cafestol induced the expression of the ER chaperone glucose-regulated protein (GRP)-78/BiP, which serves as a master controller of this stress response (Quinones, de Ridder et al. 2008; Kammoun, Chabanon et al. 2009). Nrf2 activation and ER stress are linked (Cullinan and Diehl 2006) and consequently we speculated that activation of Nrf2 mediated biotransformation enzymes, reduces ER stress. Therefore, activation of Nrf2 by cafestol metabolites may result in an increased activity of Nrf2 regulated biotransformation enzymes which in turn might ultimately reduce ER stress in liver (Cullinan and Diehl 2006; Nair, Xu et al. 2007). Kammoun et al. recently reviewed that a reduction in ER stress reduces the transcription of Srebp1c (Kammoun, Hainault et al. 2009). Cafestol suppressed Srebp1c expression in liver, and through this subsequently also suppressed the transcription of key players of lipogenesis including fatty acid synthase

(FasN), acetyl carboxylase A (Acaca), ATP citrate lyase (Acly) and diacylglycerol O-acyltransferase 2 (Dgat2).

Finally, a role of TGR5 could also be envisioned. In addition to muscle and brown adipose tissue, TGR5 is also expressed in small intestine and liver, two organs exposed to high levels of cafestol and its metabolites that might serve as an agonists for TGR5 receptor. The physiological function of TGR5 in these organs is still unknown, but a very recent study reported that a synthetic TGR5 agonist induces the release of glucagon-like peptide (GLP)-1 in small intestine, reduced liver steatosis, which ultimately resulted in normalized glucose tolerance in obese and diet-induced insulin resistant mice (Thomas, Gioiello et al. 2009), similarly to cafestol.

In **Chapter 5** the molecular mechanism behind the different effects of cafestol were further studied using a nutrigenomics approach. Whole genome transcriptomics data generated in this study confirmed earlier data presented in this thesis and offered valuable clues for future research. In addition, fat content of the diet was modulated and the effects of this modulation to hepatic and intestinal response to cafestol were investigated. We showed that dietary fat content is an important determinant of the effects of cafestol. This has been evaluated for several processes already known to be influenced by cafestol, such as bile acid metabolism and Nrf2-mediated biotransformation. The microarray analyses presented in **Chapter 5** confirmed that cafestol feeding leads to activation of Nrf2, which causes the increased expression of genes encoding several biotransformation enzymes. Next to activation of Nrf2 in liver, cafestol also triggered activation of Nrf2 in the small intestine. Furthermore, but not surprisingly, it was found that cafestol modulates lipid and bile acid metabolism in liver. Previously, Post *et al* showed that cafestol inhibits Cyp7a1 which resulted in a decrease in

total bile acids in faeces (Post, de Roos et al. 2000). In **Chapter 5** we show that chronic consumption of cafestol reduced biliary bile acid concentration but increased biliary cholesterol output, which suggests inhibition of bile acid synthesis.

Based on the data presented in this thesis it is concluded that cafestol behaves as an hormetic compound. Cafestol elicits a combination of mechanisms which together determine the balance between positive and negative health outcomes. Although for some mechanisms, *i.e.* the induction of biotransformation enzymes and acute liver toxicity, a connection seems plausible, several questions remain regarding their interrelations. The research described in this thesis has generated new mechanistic insights in the multi-faced behavior of cafestol. More studies, including in humans, are needed to study its dose-effect relations and interactions with dietary compounds. For the time being it is advisable to keep cafestol under scrutiny.

Recommendations for future research.

Since still many people consume unfiltered coffees every day, there is clear need for further studying the potentially toxic effects of cafestol and kahweol on liver and gut. The medicinal plant Germander was recalled from the market after its use as an adjuvant to slimming diets resulted in an epidemic of hepatitis in France. Later it was discovered that one of the constituents of Germander, the diterpene teucrin A was responsible for the hepatitis (Kouzi, McMurtry et al. 1994; Lekehal, Pessayre et al. 1996; Tanira, Wasfi et al. 1996; Druckova, Mernaugh et al. 2007). Both teucrin A and cafestol contain a similar furan ring (Figure 2). They also show similar effects in human and laboratory animals with regards to the weight loss and prevention of steatosis (Tanira, Wasfi et al. 1996).

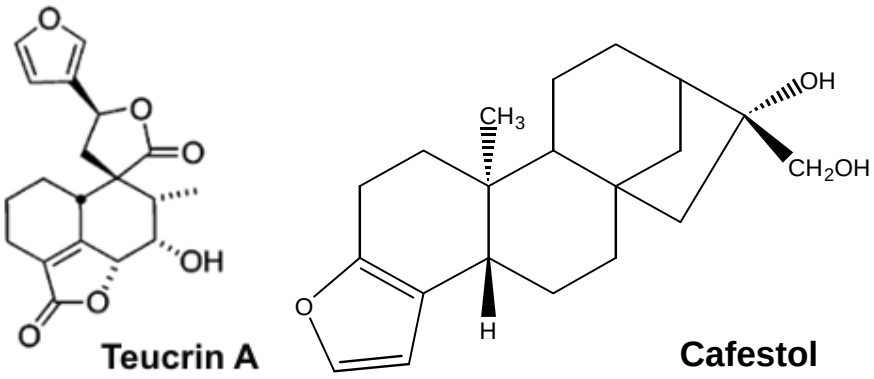

Figure 2: ***Structure formulas of the diterpenes teucrin A and cafestol****. They both contain a furan ring, which can be epoxidized by CYP3A4.*

Cafestol and the cholesterol raising mechanism.

This thesis provides a broader view of the effects of cafestol. In addition to the cholesterol raising effect, cafestol interacts with several other mechanisms which might contribute to the increase in serum cholesterol. With regards to the increase in serum cholesterol we hypothesize that metabolisation of cafestol by P450 enzymes result in an epoxide-conjugate of cafestol which might have a toxic effect on hepatocytes (as described for teucrin A (Kouzi, McMurtry et al. 1994; Laliberte and Villeneuve 1996; Lekehal, Pessayre et al. 1996; Tanira, Wasfi et al. 1996)). Previous observations show that after a prolonged period of cafestol consumption, cafestol might induce bile duct proliferation in mice (unpublished data). The reported increase in liver enzymes ALT and AST (Urgert, Schulz et al. 1995; Urgert, Meyboom et al. 1996; Urgert, Essed et al. 1997; Urgert and Katan 1997)indicates changes in membrane permeability of hepatocytes. Damage to hepatocytes might lead to a reduced capacity for storing additional cholesterol. Furthermore, the function of hepatocytes in conversion of cholesterol into bile acids is compromised, since cafestol inhibits the enzyme that converts cholesterol to bile acids, Cyp7a1 ((Post, de Roos et al. 2000) and **Chapter 4**). However further research is needed to study the hepatotoxic effects of cafestol (as explained before) and study the effects of this toxicity on cholesterol homeostasis in the liver.

In chapter 4 we show that cafestol might influence ER stress. ER stress is regulated through 3 receptors; ATF6, IRE1 and Perk (Kammoun, Hainault et al. 2009). Nrf2 activation is regulated by Perk activation (Cullinan, Zhang et al. 2003; Cullinan and Diehl 2004; Cullinan and Diehl 2006) which indicates that cafestol may, directly or indirect via Nrf2, act on this receptor. Furthermore, it has been shown that changes in ATF6 activation influence plasma

cholesterol (Meex, Weissglas-Volkov et al. 2009). This hypothesis merits further investigations.

Cafestol prevents diet induced obesity and its metabolic effects

Previously we hypothesized that activation of the TGR5 receptor can explain the effects of cafestol on body weight, adiposity and hepatic steatosis. To further unravel the molecular mechanism behind these effects, studies with TGR5 KO animals would provide us with more answers regarding the molecular pathways involved. A second hypothesis has been postulated in which cafestol might reduce ER stress in liver, thereby preventing hepatic steatosis. As indicated there is a possibility that the effects of cafestol on ER stress are (partly) responsible for the increase in plasma cholesterol.

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Samenvatting

Cafestol en kahweol zijn twee stoffen die in de koffieboon voorkomen. Wanneer koffie wordt gezet zonder het gebruik van een papieren filter, zoals bijvoorbeeld bij espresso en cafetière koffie, kunnen deze terecht komen in het product zoals het wordt gedronken. Van cafestol en kahweol is aangetoond dat zij bij langdurige consumptie kunnen bijdragen aan een stijging van het cholesterolgehalte in het bloed, wat weer kan leiden tot een verhoogd risico op hart en vaatziekten. In dit proefschrift wordt duidelijk dat cafestol naast 'slechte' effecten op het cholesterolgehalte in het bloed en op de lever ook 'gunstige' effecten kan hebben. In **hoofdstuk 1** wordt de literatuur samengevat die laat zien dat cafestol niet alleen het cholesterol verhoogt, maar ook zorgt voor een (tijdelijke) stijging van de concentratie in het bloed van de twee leverenzymen ALAT en ASAT. Dit wordt in het algemeen gezien als een aanwijzing voor leverschade. Aan de andere kant is gevonden dat cafestol de effecten van sommige stoffen op het ontstaan van kanker zou kunnen remmen. In hetzelfde hoofdstuk wordt ook een aantal mechanismen besproken waarop cafestol het cholesterol metabolisme zou kunnen beïnvloeden. In **hoofdstuk 2** laten we zien dat in de muis cafestol na opname vanuit het maag-darmkanaal geheel wordt omgezet tot metabolieten (omzettingsproducten). Deze omzettingsproducten kunnen in de cel een Electrophilic Response Element (EpRE) activeren. Dit EpRE element is aanwezig in de promoter regio van Nrf2 en dit suggereert dat cafestol metabolieten het Nrf2 activeren. Dit zou vervolgens weer kunnen leiden tot een verhoogde activiteit van bepaalde biotransformatie enzymen, hetgeen de beschermende werking van cafestol zou kunnen verklaren. In **hoofdstuk 3** laten we zien dat al binnen 30 minuten na consumptie het cafestol wordt verdeeld over de lever en de darm en dat er bijna geen cafestol verschijnt in de algemene bloedsomloop. Ook laten onze resultaten zien dat na

48uur bijna alle cafestol is uitgescheiden. Bij elkaar suggeren de bevindingen dat cafestol net als bijvoorbeeld galzouten een entero-hepatische cyclus ondergaat. Naast het metabolisme van cafestol zijn verder ook de verschillende effecten van cafestol bestudeerd. In **hoofdstuk 4** wordt duidelijk dat cafestol de gewichtstoename van muizen die een hoog vet dieet krijgen kan remmen. Ook leidt het toevoegen van cafestol aan een hoog vet dieet er toe dat de verwachte vervetting van de lever niet meer optreedt. Tenslotte ontwikkelen met cafestol behandelde muizen op een hoog vet dieet geen diabetes. In **hoofdstuk 5** wordt duidelijk dat de effecten van cafestol op de verschillende processen die door Nrf2 en de galzout receptor FXR worden gecontroleerd, zoals de biotransformatie en het metabolisme van galzouten en lipiden, worden beïnvloed door het vetgehalte van het dieet dat de muizen krijgen.

Samenvattend kan worden geconcludeerd dat cafestol zich gedraagt als een molecuul met zowel goede als slechte kanten. Het is duidelijk dat een aantal mechanismen met elkaar samenhangt zoals bijvoorbeeld de toename van de door Nrf2 gecontroleerde biotransformatie enzymen en de mogelijke effecten van cafestol op levertoxiciteit. Toch blijven er verschillende vragen over betreffende de interacties tussen de verschillende mechanismen. Om deze te kunnen beantwoorden blijven er nieuwe studies noodzakelijk waarin de effecten van cafestol consumptie in de mens onder de loep worden genomen. Vooral het effect van de dosis en de invloed van verschillen in samenstelling van het voedsel zijn daarbij van belang. Voorlopig is het aan te raden om de blootstelling aan cafestol, dus het gebruik van ongefilterde koffie, zo beperkt mogelijk te houden.

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Saskia

Curriculum vitae

Saskia van Cruchten was born the 13th of august 1981, in Sittard, the Netherlands. During her bachelor and master in Health sciences at the University of Maastricht (UM), she specialized in molecular biology. In the Academic Hospital Maastricht (AzM), she performed her MSc-thesis on the receptor identification of Anginex, an angiogenesis inhibitor, in the departement of pathology of Prof. Dr. Arjan Griffioen under the supervision of Dr. Victor Thijssen. After finishing her master, she decided to leave the South of the Netherlands, and came to Wageningen where she started as a PhD-candidate (promotors: Prof. Dr. Renger Witkamp and Prof. Dr. Michael Müller). In this project she studied the metabolism and molecular mechanism of cafestol, the most potent cholesterol raising agent known in human diet. During this project we collaborated with several parties such as; the department of Toxicology (WUR) , Human Nutrition (WUR), Nutrition, Metabolism and Genomics groups, and AMC liver centre (AMC). Saskia is experienced in animal studies, bioavailability studies, pharmacokinetics, metabolism, transport, and expression profiling and various analytical and cellular techniques such as isolation of HUVECs or hepatocytes, and she published several research-papers.

List of publications

Boekschoten, M. V., S. T. van Cruchten, et al. (2006). "[Negligible amounts of cholesterol-raising diterpenes in coffee made with coffee pads in comparison with unfiltered coffee]." Ned Tijdschr Geneeskd **150**(52): 2873-5. PMID: 17319220

van Cruchten, S. T., L. H. de Haan, et al. (2009). "The role of epoxidation and electrophile-responsive element-regulated gene transcription in the potentially beneficial and harmful effects of the coffee components cafestol and kahweol." J Nutr Biochem. **doi:10.1016/j.jnutbio.2009.05.001**. PMID; 19616929

van Cruchten, S. T., D. R. de Waart, et al. (2010). "Absorption, distribution and biliary excretion of cafestol, a potent cholesterol elevating compound in unfiltered coffees in mice." Drug Metab Dispos. **38**(4): *635-640*. PMID; 20047988

van Cruchten, S.T.J., Knol, A., Felicidade, I. Schipper, H., Boekschoten, M.V., Katan, M.B., Hooiveld, G.J.E.J. and Müller, M. "Cafestol prevents diet induced obesity."

van Cruchten, S.T.J., Dillon, E, Boekschoten, M.V., Katan M.B., Hooiveld, G.J.E.J. and M. Müller. "High saturated fat diet modulates the effects of cafestol on lipid metabolism and biotransformation pathways."

Overview of completed training activities

Discipline specific activities

Courses

Radiation expert 5B, Larenstein, Velp, 2005

Introduction course NUGO, 2006, Munich

Micro array course 2005, NUGO, Maastricht

Micro array course 2006, NUGO, Maastricht

Master class nutrigenomics Wageningen, 2007

Plant Metabolomics: Sander van der Kroll, Wageningen; 2006.

Meetings

Wageningen Nutritional Sciences Forum, 2009 Too much-Too little

FIGON geneesmiddelen dagen 2009

FIGON geneesmiddelen dagen 2008

FASEB summer conference, Molecular biology of Intestinal lipid transport and Metabolism;

July 2006, Tucson, Arizona.

General courses

Career perspectives, Wageningen Graduate Schools, 2009

Communication of Science; how to write a press release? 2009; TIFN

Scientific writing, Wageningen University, 2008

Nederlands tijdschrift van geneeskunde schrijfcursus 2006

VLAG PhD week, 2005

Optionals

Preparation PhD research proposal

PhD tour UK and Scotland 2005

Literature study program NMG group, 2005-2009, Wageningen

Overview of completed training activ

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