Elucidating the mechanism behind the lipid-raising effect of cafestol

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Abstract

Elucidating the mechanism behind the lipid-raising effect of cafestol

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The objective of this thesis was to identify genes that control the response of serum lipid levels to diet. To this end we used cafestol as model substance for a food component that affects serum lipids and therefore health. Cafestol is a cholesterol-raising diterpene present in coffee beans and unfiltered coffee types.

A possible explanation for the cholesterol-raising effect of cafestol is inhibition of bile acid synthesis. This is observed in APOE3Leiden mice upon treatment with cafestol. The nuclear receptors FXR and PXR are key regulators of genes involved in lipid and bile acid metabolism and detoxification. Both these nuclear receptors can mediate inhibition of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis. Therefore, we hypothesized that cafestol is able to activate FXR and/or PXR.

We used promoter-reporter gene assays to show that cafestol interacts with FXR and PXR *in vitro*. This suggests that cafestol can regulate gene expression via these receptors. Indeed cafestol regulated several mRNA levels of target genes of FXR and PXR in livers of APOE3Leiden mice. For a number of target genes these effects were absent in livers of FXR and PXR knockout mice. This confirms that FXR and PXR are involved in the regulation of gene expression by cafestol.

However, we could not confirm suppression of bile acid synthesis in humans. We measured plasma levels of 7α-hydroxy-4-cholesten-3-one, a marker for activity of cholesterol 7α-hydroxylase, in volunteers that consumed coffee oil. Surprisingly, we observed an increase rather than a decrease in the level of 7α-hydroxy-4-cholesten-3-one upon coffee oil treatment.

In conclusion, it is likely that the interaction with FXR and PXR is at least partly responsible for the effect of cafestol on serum lipids in humans. However, the exact mechanism by which cafestol raises serum cholesterol remains to be elucidated. Elucidation of this mechanism will provide insight into how dietary components can affect serum lipid levels.

Abbreviations

Enzymes and transport proteins

ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
BSEP	bile salt export pump
CETP	cholesteryl ester transfer protein
CYP3A4	cytochrome P-450 monooxygenase 3A4
CYP7A1	cholesterol-7α-hydroxylase
IBABP	ileal bile acid binding protein
LCAT	lecithin:cholesterol acyltransferase
NTCP	sodium taurocholate co-transporting polypeptide
PLTP	phospholipid transfer protein

Nuclear receptors

FXR	farnesoid X receptor
LXR	liver X receptor
PXR	pregnane X receptor
RXR	retinoid X receptor
SHP	small heterodimer partner

Various

CA	cholic acid
CDCA	chenodeoxycholic acid
DMSO	dimethylsulfoxide
E3L	APOE3Leiden transgenic mice
HDL	high density lipoproteins
HepG2	human hepatoma cells
LCA	lithocholic acid
LDL	low density lipoproteins
SD	standard deviation
VLDL	very low density lipoproteins

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General introduction

Cafestol is the cholesterol-raising factor in coffee beans

In this thesis a number of studies are described that aim to identify genes that control the serum lipid response to dietary components. By identifying such genes it is possible to elucidate the mechanisms by which diet can affect serum lipids.
The level of serum lipids serves as a marker for the risk of cardiovascular disease (1-3), which is a major course of death worldwide (4). Therefore, it is important to understand how diet affects these risk markers. In this thesis the serum lipid response to cafestol is used as a model system for a food component that affects serum lipid levels. Cafestol is the most potent cholesterol-raising compound identified in the human diet (5). This model makes it feasible to study its effects not only in humans, but also in animal and cell culture studies.

This introduction describes how cafestol was identified as the cholesterol-raising factor in unfiltered coffee and discusses its effect on serum lipids and liver enzyme levels. Furthermore, it summarizes potential mechanisms of the cholesterol-raising effect of cafestol that have been published so far. Finally, the last paragraph gives the objective and outline of this thesis.

Differences in brewing method explain differences in the serum lipid response to coffee

In the 1980's Scandinavian studies showed that consumption of boiled coffee is associated with increased serum cholesterol levels (6). It was demonstrated that withdrawal of boiled coffee consumption lead to a reduction in serum cholesterol of 10% (7, 8). This suggested that boiled coffee causes an increase in serum cholesterol. However, the association between coffee consumption and serum cholesterol levels could not be confirmed by studies performed in the United States or Western Europe (9). Accordingly, it was hypothesized that the brewing method was responsible for the differences in observations. The main difference in the brewing method between Scandinavia versus the United States and Western Europe is the use of paper filters in the latter. In contrast to Scandinavian coffee, in paper-filtered coffee the grounds are not present in the brew.

The importance of the brewing method was confirmed by an intervention study showing that boiled coffee did raise serum cholesterol, whereas filtered coffee had no

effect (10). Subsequently, intervention studies showed that boiled coffee lost its cholesterol-raising effect after passing through a paper filter (11, 12). Together these studies showed that the brewing method used determines the cholesterol-raising potential of coffee.

Identification of cafestol as the cholesterol-raising factor from coffee beans

Zock et al. showed that boiled coffee contains a lipid-rich fraction that raised serum cholesterol in an intervention trial (13). Furthermore, oil pressed from coffee beans caused elevation of serum cholesterol in humans (14, 15). Coffee oil mainly consists of triglycerides, but also contains 15% diterpene esters of fatty acids (16). When coffee oil was stripped of these diterpene esters the cholesterol-raising effect of the oil was lost (17). The most abundant diterpenes in coffee oil are cafestol and kahweol. Several intervention trials showed that for every 10 mg of cafestol per day serum cholesterol increases by 0.13 mmol/l after four weeks of consumption (5).

Cafestol raises serum cholesterol more potently than kahweol does. A mixture of cafestol (60 mg/day) and kahweol (51 mg/day) increased serum cholesterol only slightly more than pure cafestol (64 mg/day) did (18). Results with pure kahweol are not available due to difficulties with purification and stability of this diterpene.

About 80% of the rise in serum cholesterol is due to elevation of low density lipoproteins (LDL) and the remainder by elevation of very low density lipoproteins (VLDL). High density lipoproteins (HDL) are not affected or show a slight decrease (13, 17, 18). Triglycerides are raised by 0.08 mmol/l with every 10 mg of cafestol per day after 2-6 weeks (5). However, this rise in triglycerides is transient. A six-month intervention trial showed that 0.9 liter of unfiltered coffee per day raised serum triglycerides by 26% in the first month, but this effect dropped to 7% after six months of daily consumption (19). The effect of unfiltered coffee on serum cholesterol is more persistent. A 10% raise in serum cholesterol was reduced to 6% after six months of daily intake. This is in agreement with previous epidemiological studies (17, 20, 21).

Together these studies show that cafestol has a permanent effect on LDL and total cholesterol and a transient effect on serum triglycerides.

Consumption of unfiltered coffee affects health

Serum levels of LDL cholesterol are a major risk factor for atherosclerosis and consequently cardiovascular disease (1-3). Early studies already showed that consumption of boiled coffee is associated with an increased risk of cardiovascular disease (22). More recently a case-control study showed that consumption of boiled coffee appears to increase the risk of first non-fatal myocardial infarction (23). In Finland a switch from boiled to filtered coffee was associated with a decrease of 0.3 mmol/l total cholesterol between 1972 and 1992 (24). This decrease in serum cholesterol levels was also associated with a decrease in coronary heart disease.

Therefore, consumption of cafestol in unfiltered coffee increases the risk of cardiovascular disease.

Effect of cafestol on markers of liver function

Cafestol raises serum levels of alanine aminotransferase and aspartate aminotransferase

Coffee oil or unfiltered coffees not only affect serum lipids, but also serum activity of the liver enzymes alanine aminotransferase (ALAT) and to a lesser extent aspartate aminotransferase (ASAT) (15, 17, 19, 25). On average every 10 mg of cafestol or kahweol per day raised serum ALAT by 8-12% (5). Elevation of these liver enzyme activities is indicative of liver damage (26-28). However, the effect of cafestol is not compliant with cholestasis. In cholestasis ASAT activities often exceed ALAT activities and activities of γ-glutamyltranspeptidase and alkaline phosphatase are elevated, whereas cafestol even reduces serum activities of these enzymes (15, 17, 19, 25). ALAT is predominantly located in the cytosol of hepatocytes and ASAT is predominantly present in mitochondria. Elevation of ALAT indicates damage to the membranes of hepatocytes. A larger increase in ASAT indicates more severe damage to liver cells (26-28). Therefore, cafestol seems to compromise membrane integrity of hepatocytes.

Consumption of unfiltered coffee does not cause liver disease in life-long consumers

Although ALAT activity is still elevated in the serum of subjects that consumed unfiltered coffee for six months, life-long consumers do not have elevated ALAT activities (17, 29). Also, mortality rates of liver cirrhosis have been typically low in Scandinavian countries (30). Furthermore, an inverse correlation exists between coffee consumption and risk of alcohol-induced liver cirrhosis (31, 32). This even suggests a protective effect of coffee consumption on development of alcoholic cirrhosis. Together this suggests that the effect of cafestol and kahweol on liver enzyme activities is transient and only causes subclinical damage to hepatocytes.

Possible mechanisms for the effect of cafestol

Cafestol suppresses bile acid synthesis in APOE3Leiden mice

Cafestol and kahweol affect both serum lipids and liver enzymes. Therefore, it seems likely that the liver is the target organ for these diterpenes. Indeed, a number of studies have described effects of cafestol in hepatocytes. The most striking effect of cafestol was the inhibition bile acid synthesis in rat hepatocytes and livers of APOE3-Leiden mice by downregulation of expression and activity of cholesterol 7α-hydroxylase (33, 34). Cholesterol 7α-hydroxylase is the rate-limiting enzyme in the conversion of cholesterol into bile acids in the liver. In addition, the amount of bile acid was reduced by 41% in APOE3-Leiden mice (34). Suppression of bile acid synthesis will lead to an increased pool of hepatic cholesterol, causing downregulation of the LDL-receptor and thereby elevation of serum LDL. Downregulation of the LDL-receptor by cafestol was confirmed *in vitro* (33, 35, 36).

Cafestol affects activity transfer proteins in the liver

Besides raising LDL levels, cafestol also causes a transient rise in serum triglyceride levels. This is probably due to increased production of $VLDL_1$ particles (37).

Furthermore, cafestol increases activity of cholesteryl ester transfer protein (CETP) and phospholipids transfer protein (PLTP) and decreases activity of lecithin:cholesterol acyltransferase (LCAT) (38, 39). However, it remains unclear

whether the elevated activities of CETP and PLTP are a cause or a consequence of the LDL elevation after cafestol consumption (39). The decrease in LCAT activity might account for the slight decrease in HDL levels after cafestol and kahweol consumption observed in some studies (17). However, because LCAT is only synthesized in the liver the decrease in activity could be caused by impairment of liver function (39).

In summary cafestol and kahweol affect bile acid synthesis, production of VLDL, and activities of lipid transfer proteins in the liver. However, it remains unclear how diterpenes regulate these processes and how modulation of these processes contributes to the effects of cafestol and kahweol on serum lipids.

The role of genetic variation and the serum cholesterol response to cafestol

The serum cholesterol response to dietary changes differs considerably between subjects, while the response is to some extent reproducible within subjects (40-44). The between-subjects variation in cholesterol response can possibly be attributed to genetic variation. Accordingly, an alternative strategy to identify genes that are involved in the regulation of the response of serum lipids to cafestol and other dietary compounds is via genetic studies. Indeed, it has been shown that polymorphisms in certain genes may affect the response (45-47). One polymorphism in the apolipoprotein A-I (apoA-I) gene was identified that is associated with the response of serum cholesterol to cafestol. Subjects with the apoA-I 83-CC genotype show a larger increase in serum cholesterol than subjects with the apoA-I 83-CT genotype (48). This shows that genetic variation can at least partly explain differences in the response of serum lipids to cafestol between individuals.

Objective and outline of this thesis

The objective of our studies was to identify genes that regulate the response of serum lipids to diet. In these studies we used cafestol as a model substance.

Cafestol is the most potent cholesterol-raising compound identified in the human diet and can be administered easily in the form of coffee oil. In addition,

cafestol can also be administered to animals and added to the medium of cultured cells.

We first assessed the within-subject reproducibility of the serum lipid response to coffee oil in healthy volunteers (Chapter 2). Before a specific response can be linked to a certain genotype the reproducibly of the response within a person has to be measured. Although the effect of cafestol on serum lipids is highly reproducible on a group level, measurement of the individual response might be hampered by variation in environmental factors that are not stable during the treatment period.

During this reproducibility study we encountered larger effects of coffee oil on the liver enzymes ALAT and ASAT than expected from previous studies. For the reproducibility study we used Arabica coffee oil that contains both cafestol and kahweol. Two previous studies suggested that cafestol is mainly responsible for the effect on serum lipids, whereas kahweol is mainly responsible for the effect on liver enzymes (15, 18). Therefore, we performed a study to assess whether coffee oil from Robusta beans that contain minute amounts of kahweol affects liver enzyme levels in healthy volunteers (Chapter 3). This study showed that coffee oils rich or poor in kahweol have similar effects on liver enzyme levels. Because of the adverse effects in a number of subjects we decided to arrest the trials with coffee oil or cafestol in human volunteers. We continued our investigations into the mechanism by which cafestol raises serum lipids in animal and *in vitro* studies. Chapter 4 shows the combined results of these studies that indicate interaction of cafestol with two nuclear receptors: the farnesoid X receptor and the pregnane X receptor. Upon activation these receptors can downregulate expression of cholesterol 7α-hydroxylase, which is in line with the observation that cafestol downregulates expression and activity of cholesterol 7α-hydroxylase in rat hepatocytes and livers of APOE3-Leiden mice. Chapter 5 describes the results of the measurement of 7α-hydroxy-4-cholesten-3-one in samples from the reproducibility study. This metabolite is present in plasma and reflects activity of cholesterol 7α-hydroxylase in the liver and therefore of bile acid synthesis. Finally, the results of the studies above are discussed in Chapter 6.

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2

Reproducibility of the serum lipid response to coffee oil in healthy volunteers

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Abstract

Background Humans and animals show a certain consistency in the response of their serum lipids to fat-modified diets. This may indicate a genetic basis underlying this response. Coffee oil might be used as a model substance to investigate which genes determine differences in the serum lipid response. Before carrying out such studies our objective was to investigate to what extent the effect of coffee oil on serum lipid concentrations is reproducible within subjects.

Methods The serum lipid response of 32 healthy volunteers was measured twice in separate five-week periods in which coffee oil was administered (69 mg cafestol / day).

Results Total cholesterol levels increased by 24% in period 1 (range:0;52%) and 18% in period 2 (1;48%), LDL cholesterol by 29 % (-9;71%) and 20% (-12;57%), triglycerides by 66% (16;175%) and 58% (-13;202%), and HDL cholesterol did not change significantly: The range of the HDL response was -19;25% in period 1 and -20;33% in period 2.

The correlation between the two responses was 0.20 (95%CI -0.16, 0.51) for total cholesterol, 0.16 (95%CI -0.20, 0.48) for LDL, 0.67 (95%CI 0.42, 0.83) for HDL, and 0.77 (95%CI 0.56, 0.88) for triglycerides.

Conclusions The responses of total and LDL cholesterol to coffee oil were poorly reproducible within subjects. The responses of HDL and triglycerides, however, appeared to be highly reproducible. Therefore, investigating the genetic sources of the variation in the serum-lipid response to coffee oil is more promising for HDL and triglycerides.

Background

The effect of dietary changes on serum lipid levels differs significantly between individuals (1-5). The differences in response may be caused by variation in genes regulating serum lipid levels (6-8). Identification of genes regulating the lipid response may help to clarify the mechanism by which diet raises serum lipid levels. It also might provide leads for dietary and pharmacotherapeutical means of lowering serum cholesterol. Cafestol, a cholesterol-raising substance in coffee oil, can be used to study lipid metabolism and the genes involved because it greatly affects serum lipid levels in humans (9-11). Therefore, coffee oil can be used to affect serum lipid levels without the need to provide a fully controlled diet. The effect of coffee oil on the expression of genes in humans, however, is difficult to study in relevant tissues like liver or intestinal epithelium.

An alternative approach is to study the response of serum lipids to coffee oil in individuals having different genotypes of certain polymorphic candidate genes. For such an approach to succeed, two conditions must be met: First, the response of lipids to coffee oil should be sufficiently different between persons; if this is not the case, the effect of the candidate polymorphisms on the response may be too small to be detected in a study. Second, the individual response of lipids to coffee oil should be sufficiently reproducible; otherwise an efficient study of the effects of genetic factors may not be possible. If these two conditions are not met, the study of individual polymorphisms is not feasible.

The present study was therefore designed to assess the reproducibility and the reliability of the response to coffee oil in subjects from the general population.

Methods

Subjects

Subjects were recruited among the student population of Wageningen, a university town in the Netherlands. Fifty-one men and women were recruited; their health was assessed by means of a questionnaire, and blood and urine testing. We used the following eligibility criteria: serum cholesterol < 8 mmol/l, serum triglycerides < 3.0 mmol/l, no glucosuria, normal liver enzyme activities in serum, no use of medication with effects on serum lipids, and no history of gastrointestinal or liver disease. Because coffee oil can increase the activities of the liver enzymes alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) (9, 12). We measured liver enzyme activities before and during the study. One subject was excluded at baseline because of liver enzyme activity above the upper limit. Consequently, fifty subjects were enrolled in the study.

During the study five subjects withdrew cooperation: three subjects suffered from stomach complaints, one went abroad, and one had a gastrointestinal infection. Another 13 subjects had to be excluded during the study because their serum activities of ALAT and ASAT exceeded previously determined boundaries. These boundaries were 2.7 times the upper limit of normal for ALAT and 1.5 times the upper limit of normal for ASAT.

Thus, thirty-two subjects completed the study. The Medical Ethical Committee of Wageningen University and Research Centre approved the study. Each volunteer gave an informed consent.

Study design

The response of serum lipids to coffee oil consumption was measured twice in each subject. Subjects first entered a run-in period of three weeks in which they received four placebo capsules daily. Placebo capsules contained 0.25 ml sunflower oil and 0.25 ml safflower oil per capsule. After the run-in period subjects took four coffee-oil capsules a day (2 ml oil per day) for a five-week period. The coffee-oil capsules provided 69 mg cafestol and 51 mg of kahweol per day. The run-in and the coffee-oil period together constituted period 1. The change in the level of serum lipids from the end of run-in period 1 to the end of coffee-oil period 1 was defined as response 1.

This first coffee-oil period was followed by a three-week wash-out period in which no capsules were supplied. After the wash-out period subjects repeated the first two periods: a three-week run-in period (run-in 2) and a five-week coffee-oil period (coffee-oil period 2). See figure 1 for a diagram of the study design.

Subjects were asked to maintain their lifestyles and dietary habits for the duration of the study. They reported changes in diet, smoking, physical activity, use of medication, illness, and the number of capsules taken daily in diaries.

We measured body weight at the beginning of the run-in and coffee-oil periods and at the end of the coffee-oil periods.

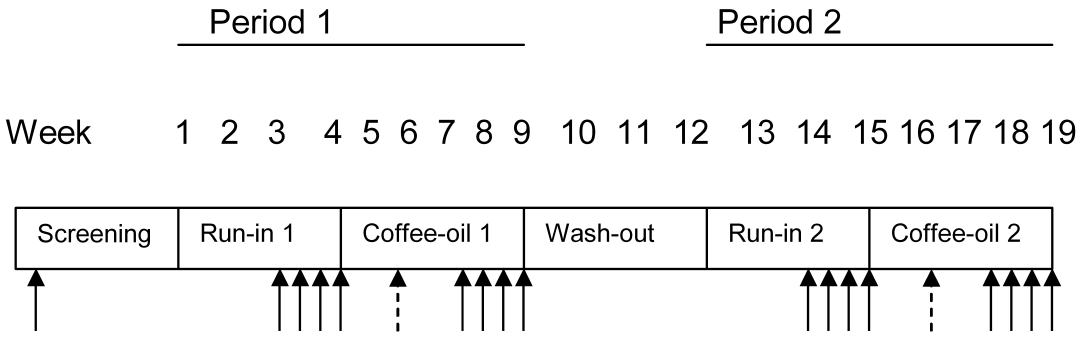

Figure 1 Diagram of the study design
The week number is indicated above. Black arrows indicate blood sampling days. Dashed arrows indicate blood sampling days for determination of liver enzym activities.

Blood lipids

Blood samples were taken from subjects who had fasted overnight on four separate days in the last two weeks of the run-in and coffee-oil periods. In total, each subject had blood drawn 19 times. In 16 samples serum lipid levels were determined. All 16 samples were analysed within one run. Total cholesterol and triglycerides were measured with Cholesterol Flex and Triglycerides Flex reagent cartridge (Dade Behring); HDL was measured with the *Liquid* N-geneous HDL assay (Instruchemie BV.); LDL cholesterol concentrations were calculated (13).

Liver enzymes

To measure liver enzyme activities, we took additional blood samples during the second week of the two coffee-oil periods and within two months after the experiment. In these samples activities of ALAT (Alanine Aminotransferase Flex reagent cartridge, Dade Behring) and ASAT (Aspartate Aminotransferase Flex

reagent cartridge, Dade Behring) were measured at 37° C. Liver enzyme activities are given in multiples of the upper limit of normal because activities vary with the temperature used when determining liver enzyme activities in different laboratories.

The upper limits of normal in this laboratory are 45 IU/l for ALAT and 41 IU/l for ASAT. When ALAT levels exceeded the 2.7 times upper limit of normal or ASAT levels exceeded 1.5 times upper limit of normal, subjects were taken off coffee-oil treatment. These boundaries were based on what is considered a mild increase in liver enzymes activities in plasma (9, 12).

Statistical analysis

A subject's response to coffee-oil in period 1 was defined as the mean serum lipid level at the end of coffee-oil period 1 minus the mean at the end of run-in period 1. The mean level of serum lipids in a period is calculated as the mean of the four repeated measurements in each period. The response in period 2 was calculated in the same way.

We calculated within and between person standard deviations (SD's) of serum lipid levels in the four periods and the two responses. The four periods are: run-in 1, run-in 2, coffee-oil 1 and coffee-oil 2.

We used the following definitions in our calculations:

SD_{total} of run-in 1:	SD of the mean serum lipid level of 32 subjects in run-in 1.
SD_{total} response:	SD of the mean serum lipid response of 32 subjects. The mean response is calculated from the two responses.
SD_{within} level of person1 in run-in 1:	SD of the four measurements in run-in 1 of person 1.
SD_{within} response of person 1:	SD of the two responses for person 1.
$SD_{between}$:	$\sqrt{(SDtotal^2 - SDwithin^2)}$

Results

We analysed data of 32 subjects, 10 men and 22 women. See table 1 for baseline characteristics. During the study 97% of the total amount of capsules was consumed. This was determined by counting returned capsules and checking diaries.

Table 1 Baseline characteristics for all subjects who completed the study

Characteristic	(n=32)
Age (years)	23 ± 4
Height (m)	1.73 ± 0.09
Weight (kg)	67.4 ± 10.4
Body mass index (kg/m^2)	22.4 ± 3.0
Serum total cholesterol (mmol/l)	4.6 ± 0.8
Serum triglycerides (mmol/l)	1.00 ± 0.49
Alanine aminotransferase (IU/l)	25 ± 7
Aspartate aminotransferase (IU/l)	14 ± 4
Current smokers n (%)	7 (22)
Alcohol (glass/week)	3 (1, 7)
median (25th percentile, 75th percentile)	

Variables presented as mean ± sd, current smokers presented as n (%).

Body weight

There was an average weight change of -0.4 kg (range: -3.8 to + 3.8, n=32) from start to end of the experiment. There was no correlation between changes in weight and the response of serum lipids (data not shown).

Blood lipids

Table 2 shows the mean responses of serum lipids to coffee oil in periods 1 and 2. Total cholesterol rose by 24% (range:0;52%) in period 1 and by 18% (1;48%) in period 2, LDL cholesterol by 29% (-9;71%) and 20% (-12;57%), triglycerides by 66% (16;175%) and 58% (-13;202%) and HDL cholesterol rose by 3% in both periods. These changes in HDL levels were not significant. The ranges of the HDL response were -19;25% in period 1 and -20;33% in period 2.

Table 2 Concentrations of blood lipids during the four study periods

	Total cholesterol	HDL	LDL	Triglycerides
Run-in 1 (mmol/l)	4.4±0.7	1.56±0.34	2.39±0.57	1.10±0.38
Coffee oil 1 (mmol/l)	5.5±0.9	1.61±0.44	3.08±0.77	1.82±0.62
Run-in 2 (mmol/l)	4.5±0.8	1.55±0.45	2.41±0.66	1.12±0.47
Coffee oil 2 (mmol/l)	5.3±0.8	1.60±0.45	2.90±0.69	1.77±0.76
Response 1 (mmol/l)	1.1±0.5	0.04±0.18	0.70±0.40	0.72±0.40
Response 2 (mmol/l)	0.8±0.5	0.05±0.20	0.49±0.44	0.65±0.52
Correlation between response 1 and 2	0.20 [-0.16, 0.51]	0.67 [0.42, 0.83]	0.16 [-0.20, 0.48]	0.77 [0.56, 0.88]

Values are mean ± SD. The values for each period are means of four samples. Response1 and response2 are calculated as coffee-oil minus run-in values. Values between brackets are 95% confidence intervals.

Individual responses for total and LDL cholesterol in period 2 hardly correlated with those in period 1. In contrast, individual responses in the two periods for HDL cholesterol (fig. 2) and triglycerides (fig. 3) were highly correlated: the Pearson correlation coefficient was 0.67 for HDL and 0.77 for triglycerides. See table 2 for mean levels and responses.

The total observed standard deviation of the response (SD_{total} response) of serum cholesterol to coffee oil was 0.49 mmol/l. The between subject standard deviation ($SD_{between}$) of the response of serum cholesterol was 0.22 mmol/l and the within subject standard deviation (SD_{within} response) was 0.44 mmol/l. Therefore, a large proportion of the variation in serum cholesterol response to coffee oil was explained by the variation in the response within individuals. The same is true for the LDL response.

The HDL response to coffee oil showed a between subject SD of 0.15 and a within subject SD of 0.11 mmol/l, so the between subject variation was larger than the within subject variation. This explains the high correlation between the two HDL responses.

The triglyceride response showed an SD between individuals of 0.40 mmol/l, which is larger than the within subject SD of 0.22 mmol/l. All standard deviations of serum lipid levels and responses to coffee-oil are presented in Table 3.

Multiple regression analysis showed no association between the response and gender, baseline values, or alcohol use. Only smoking status contributed significantly to the explanation of the variation of the response. However, we did not find a difference in the reproducibility of the serum lipid response to coffee oil between smokers and non-smokers (data not shown).

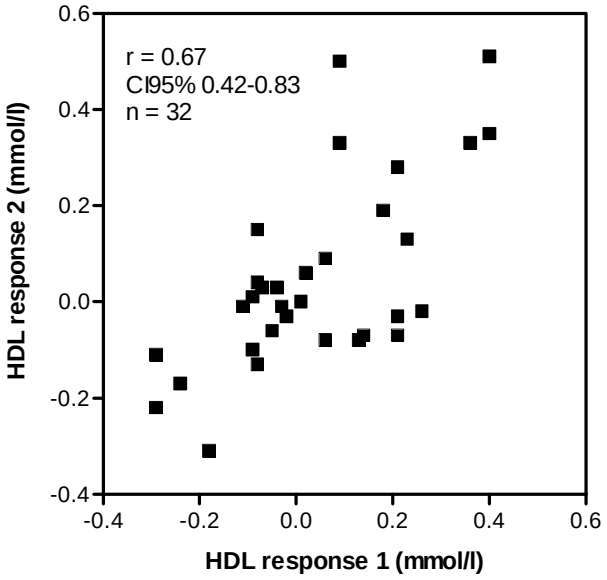

Figure 2 Correlation of HDL response to coffee-oil consumption between period 1 and 2.
On the x-axis the response of coffee-oil in the first period on serum HDL is shown on the y-axis the response to coffee-oil in the second period. Each dot represents one subject.

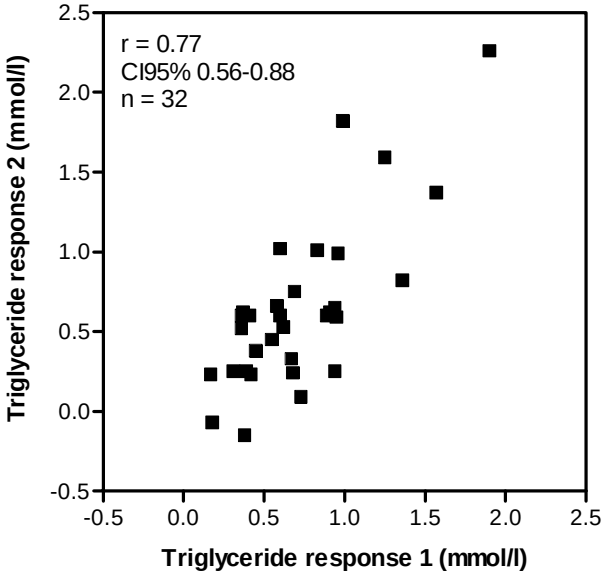

Figure 3 Correlation of triglyceride response to coffee-oil consumption between period 1 and 2. On the x-axis the response of coffee-oil in the first period on serum triglycerides is shown on the y-axis the response to coffee-oil in the second period. Each dot represents one subject.

Table 3 Standard deviations of levels and responses to cafestol of serum lipids

	Mean (mmol/l)	Sdtotal	Sdwithin	Sdbetween
Total cholesterol				
Run-in 1	4.45	0.72	0.26	0.67
Coffee-oil 1	5.51	0.93	0.37	0.85
Run-in 2	4.47	0.79	0.26	0.75
Coffee-oil 2	5.30	0.85	0.33	0.78
Response	0.95	0.49	0.44	0.22
HDL				
Run-in 1	1.56	0.34	0.10	0.32
Coffee-oil 1	1.61	0.44	0.12	0.42
Run-in 2	1.55	0.34	0.10	0.32
Coffee-oil 2	1.60	0.45	0.10	0.44
Response	0.05	0.19	0.11	0.15
LDL				
Run-in 1	2.39	0.57	0.23	0.52
Coffee-oil 1	3.08	0.77	0.34	0.69
Run-in 2	2.41	0.66	0.21	0.63
Coffee-oil 2	2.90	0.69	0.33	0.61
Response	0.60	0.42	0.39	0.16
Triglycerides				
Run-in 1	1.10	0.38	0.23	0.30
Coffee-oil 1	1.82	0.62	0.39	0.48
Run-in 2	1.12	0.47	0.22	0.42
Coffee-oil 2	1.77	0.76	0.42	0.63
Response	0.69	0.46	0.22	0.40

Liver enzymes

Eight subjects dropped out in the first period and five in the second period because of ALAT and ASAT levels exceeding the predetermined boundaries. Eleven of these 13 subjects had returned to normal ALAT (≤ 45 IU/l) and ASAT (≤ 41 IU/l) levels within two months after exclusion. One subject who had elevated liver enzyme activities after two months was referred to a general practitioner. After another two months her liver enzyme levels were within normal limits again. One subject went abroad before the follow-up measurement.

The ALAT levels of the 32 subjects who finished the study rose on average by 0.51 ± 0.47 times the upper limit of normal during the first coffee-oil period and by 0.31 ± 0.29 times the upper limit of normal during the second period. ASAT levels increased by 0.22 ± 0.20 times the upper limit of normal during the first period and by 0.15 ± 0.12 times the upper limit of normal during the second period.

Discussion

In this study we aimed to establish the reproducibility of the serum-lipid response to coffee oil. We found that the response of total serum cholesterol and LDL to coffee oil was poorly reproducible, whereas the responses of HDL and triglycerides to coffee oil proved to be highly reproducible. A high correlation between two intra-individual responses means that the within-subject variability is relatively low. The between-subject variability of HDL and triglycerides is relatively large. The within-subject reproducibility of the responses of HDL and triglycerides to coffee oil in combination with their large between-subject variability indicates that it is more promising to investigate genetic variation that determines the response of HDL and triglycerides to coffee oil than to investigate the responses of total and LDL cholesterol.

The triglyceride response is relatively large compared to the response of total cholesterol. In our study cholesterol increased on average by 21 % and triglycerides by 62%. It has been shown that cafestol increases plasma triglycerides by an increasing production of a fraction of very low density lipoproteins: $VLDL_1$. The

subsequent rise in LDL cholesterol might be caused by enrichment of $VLDL_2$ particles with cholesteryl esters (14).

Group vs. individual response of serum lipids

The average response to the coffee-oil treatment was similar to that in previous studies at our department (11). We expected an increase in total serum cholesterol of 1.0 mmol/l and an increase of serum triglycerides of 0.65 mmol/l, which is close to the observed values (Table 2). The coffee oil did not affect the average HDL concentration. Other studies also found either no effect on HDL or a slight decrease in HDL concentrations (11).

The assessment of the individual response of serum lipids is hampered by day-to-day fluctuations in serum lipid levels (3-5). In this experiment we reduced the effect of these fluctuations by using the mean of four separate measurements (15, 16).

Analyses of the between and within-person standard deviations (SD's) of the responses of serum lipids to coffee oil confirm that total cholesterol and LDL responses are poorly reproducible. The SD's of the responses of total cholesterol and LDL had a within-person component that was clearly larger than the between-person component. For the total cholesterol response to coffee oil we found a total SD of 0.49 mmol/l, an SD_{within} of 0.44 mmol/l, and an $SD_{between}$ of 0.22 mmol/l. This is at variance with the results of Katan et al. who observed an SD of 0.33 mmol/l, an SD_{within} of 0.16 mmol/l, and an $SD_{between}$ of 0.29 mmol/l for total cholesterol (2). This would indicate a better reproducibility than observed in our study. However, Katan et al. selected putative hyperresponders and hyporesponders, which will lead to overestimation of the reproducibility. In our study the response of triglycerides shows a large SD between individuals and a quite small SD within persons. This confirms that the response varies between individuals and that the response is reproducible within persons.

We did not separate laboratory variation in our model for calculation of SD's. We estimated this variation by measuring serum lipids in 32 duplo samples. Coefficients of variation were 1.6% for total cholesterol, 1.8% for HDL, and 2.3% for triglycerides. This means that the laboratory variation is so small that it can be omitted from the model without affecting the calculated values of SD_{within} and $SD_{between}$.

Study limitations

Although cafestol is a potent cholesterol-raising food component, it is not certain that it can be used for the study of variation in genes regulating the serum-lipid response to other foods. It is possible that cafestol regulates different genes than other food components, such as dietary cholesterol or saturated fats do.

Furthermore, we did not use pure cafestol in this study, but coffee oil, which contains many more components, such as triglycerides, free fatty acids, and sterols. However, coffee-oil stripped of diterpenes has no effect on serum lipids (9). Therefore, genes that cause a rise in serum cholesterol are probably affected by diterpenes.

Another limitation of the study is that the subjects were free living and did not receive a controlled diet. Therefore, the response of serum lipids to the coffee oil could be changed by other factors. We instructed the subjects to maintain dietary habits, smoking habits, and physical activity. Changes were recorded in a diary together with use of medication and illness. According to the diaries, subjects maintained their habitual lifestyle. Furthermore, coffee oil has such a large effect on serum-lipid levels that small effects of other factors are not of great concern.

A fourth limitation is the large drop out due to elevations of liver enzymes. There was, however, no correlation between the rise in serum-lipid levels and the rise in liver enzymes (data not shown). Therefore, there is no reason to assume that the reproducibility of the serum lipid response to coffee oil in subjects who showed a considerable increase of liver enzymes differs from the serum lipid response in subjects who did not show a large increase of liver enzymes.

On basis of this study it can not be concluded with certainty that a response to coffee oil that differs between individuals but is reproducible within individuals is determined by genetic variation.

Liver enzymes

Levels of the liver enzymes ALAT and ASAT rose after administrating coffee oil, as was expected from previous studies (9, 11, 12, 17). The rises in ALAT and ASAT indicate that coffee oil can cause acute injury to hepatocytes (18, 19). Alcohol, being hepatotoxic, might be an important cofactor in this effect (20). In this study no association between use of alcohol and drop out due to elevation of liver enzymes

was observed. Levels of ALAT rose more than ASAT levels did. This could mean that the membranes of hepatocytes were damaged (18, 20).

Genetic factors underlying the serum lipid response to cafestol

Given the ratio of between and within person variability of the responses of HDL and triglycerides to coffee oil, research into genetic determinants of the response seems to be feasible.

Other studies have described polymorphisms in genes that have a small effect on the total-cholesterol response to cafestol and dietary fat (6-8). An example is the cholesteryl ester transfer protein (CETP). CETP is a protein that mediates the transfer of cholesteryl esters from HDL to LDL and VLDL. Cafestol, like dietary cholesterol and fat, might increase the transfer of cholesteryl esters by increasing the activity of CETP. Weggemans et al. showed that humans with the CETP Taqlb-1/2 allele have a smaller response of LDL to cafestol, or dietary fat and cholesterol (21). However, it remains to be established whether CETP has a role in the cholesterol-raising effect of cafestol and whether a polymorphism in the gene accounts for some of the variation in response between individuals.

Other candidate genes are genes encoding for proteins in the bile acid metabolism. Cholesterol is converted to bile acids in the liver. There are two pathways involved: the neutral and the acidic pathway. The rate-limiting enzyme in the neutral pathway is 7α-hydroxylase, which is regulated by bile acids through a negative feedback mechanism (22). Chenodeoxycholic acid (CDCA), a primary bile acid, suppresses 7α-hydroxylase activity by binding directly to the farnesoid X receptor (23, 24). It has been shown in mice that cafestol inhibits bile acid synthesis, which could cause the rise in serum cholesterol (25). If functional polymorphisms are present in the genes involved in bile acid metabolism, these could be responsible for the variation in the conversion of cholesterol to bile acids. Therefore, it is interesting to study whether cafestol regulates genes involved in bile-acid metabolism.

Polymorphisms in the genes of the pathways mentioned above could account for the variation in response to cafestol between individuals. There are more possible candidates, such as sterol regulatory element binding proteins, microsomal triglyceride transfer protein, lecithin:cholesterol acyltransferase, lipoprotein lipase, and hepatic lipase.

In this study the responses of HDL and triglycerides to coffee oil showed a sufficient reproducibility. Therefore, our best option is to focus on these serum lipids and characterize an individual's response to coffee oil by the HDL and triglyceride response in future research. The variation between persons in the response of HDL and triglycerides combined with the consistency within persons does not guarantee that a large genetic effect is present. It means that environmental determinants of the response were stable within individuals during the time span of the study, whereas there were differences between individuals in other environmental determinants and/or genetic determinants. The question remains whether possible genetic effects are large enough to be detected and whether variation in these genes is sufficiently prevalent in the population.

Clarification of the mechanism by which cafestol increases serum lipids might provide leads for dietary and pharmacotherapeutical ways to lower serum cholesterol. The 'cafestol model' also could be a trial case for evaluating the possibility of personalized diets. If it is possible to predict people's serum-lipid responses to cafestol on basis of their genetic make-up and to make dietary recommendations based on this genetic information, this could also be applicable in other situations.

Author's contributions

MVB participated in designing and planning the study, headed the investigation during the intervention period, analysed the data and wrote the paper together with MFE, who also contributed to the data analysis. MBK initiated the project and raised the funds. EGS was the senior scientist supervising the project.

Acknowledgements

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3

Coffee bean extracts rich and poor in kahweol both give rise to elevation of liver enzymes in healthy volunteers

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Abstract

Background Coffee oil potently raises serum cholesterol levels in humans. The diterpenes cafestol and kahweol are responsible for this elevation. Coffee oil also causes elevation of liver enzyme levels in serum. It has been suggested that cafestol is mainly responsible for the effect on serum cholesterol levels and that kahweol is mainly responsible for the effect on liver enzyme levels. The objective of this study was to investigate whether coffee oil that only contains a minute amount of kahweol indeed does not cause elevation of liver enzyme levels.

Methods The response of serum alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) to Robusta coffee oil (62 mg/day cafestol, 1.6 mg/day kahweol) was measured in 18 healthy volunteers.

Results After nine days one subject was taken off Robusta oil treatment due to an ALAT level of 3.6 times the upper limit of normal (ULN). Another two subjects stopped treatment due to other reasons. After 16 days another two subjects were taken off Robusta oil treatment. One of those subjects had levels of 5.8 ULN for ALAT and 2.0 ULN for ASAT; the other subject had an ALAT level of 12.4 ULN and an ASAT level of 4.7 ULN. It was then decided to terminate the study. The median response of subjects to Robusta oil after 16 days was 0.27 ULN (n=15, 25^{th},75^{th} percentile: 0.09;0.53) for ALAT and 0.06 ULN (25^{th},75^{th} percentile -0.06;0.22) for ASAT.

Conclusions We conclude that the effect on liver enzyme levels of coffee oil containing hardly any kahweol is similar to that of coffee oil containing high amounts of kahweol. Therefore it is unlikely that kahweol is the component of coffee oil that is responsible for the effect. Furthermore, we conclude that otherwise unexplained elevation of liver enzyme levels observed in patients might be caused by a switch from consumption of filtered coffee to unfiltered coffee.

Background

Consumption of unfiltered coffee types raises serum cholesterol levels in humans (1-4). Unfiltered coffee also causes elevated liver enzyme levels in serum. Cafestol and kahweol are responsible for the effect of unfiltered coffee on serum cholesterol. These diterpenes are present in the oil derived from coffee beans. The only difference between cafestol and kahweol is a double bond present between the C1-C2 atoms in kahweol.

Two types of coffee beans are used for brewing coffee: Arabica and Robusta. Arabica beans contain both cafestol and kahweol, whereas Robusta beans contain half as much cafestol and hardly any kahweol (5). Cafestol raises serum cholesterol more potently than kahweol does. A mixture of cafestol (60 mg/day) and kahweol (51 mg/day) increased serum cholesterol only slightly more than pure cafestol (64 mg/day) did (3). Results with pure kahweol are not available due to difficulties with purification and stability of this diterpene.

Coffee oil also raises serum levels of the liver enzyme alanine aminotransferase (ALAT) and to a lesser extent aspartate aminotransferase (ASAT). Elevation of these liver enzymes may indicate injury of hepatocytes (6-8). For example in acute hepatitis, either viral or drug-induced, both ALAT and ASAT are elevated. The ALAT levels often exceed the ASAT levels. ALAT is predominantly present in the cytosol of hepatocytes and ASAT is predominantly present in the mitochondria. When hepatocytes sustain damage to their membranes ALAT is released from the cytosol, whereas when hepatocytes sustain more severe damage ASAT is released from the

mitochondria (6-8). When ASAT levels are more increased than ALAT levels; this could indicate obstruction of the bile duct or alcohol abuse.

We have earlier suggested that kahweol is mainly responsible for the effect of coffee oil on liver enzyme levels (3, 9). A mixture of cafestol and kahweol raised liver enzyme levels more potently than pure cafestol, whereas the effect on serum cholesterol levels is similar. This would suggest that the structural difference between cafestol and kahweol causes these diterpenes to act on different pathways in the liver. On the basis of the suggestion that kahweol is mainly responsible for the effect on liver enzyme levels, we hypothesized that Robusta oil, which contains a negligible amount of kahweol, would induce no or a smaller response of liver enzymes than Arabica oil, while maintaining its cholesterol-raising effect. In order to test this hypothesis we designed a study in which healthy volunteers consumed Robusta oil.

Methods

Subjects

Subjects were recruited among the student population of Wageningen, a university town in the Netherlands. Twenty-one volunteers were included; their health was assessed by means of a questionnaire, and blood and urine testing. We used the following eligibility criteria: serum cholesterol < 8 mmol/l, serum triglycerides < 3.0 mmol/l, no glucosuria, normal liver enzyme activities in serum, normal bilirubin levels in serum, no use of medication with effects on serum lipids, no consumption of unfiltered coffee, and no history of gastrointestinal or liver disease. The following enzyme activities were measured in serum: ALAT, ASAT, alkaline phosphatase, amylase, γ-glutamyltranspeptidase and lactate dehydrogenase. Three subjects were excluded at baseline due to serum bilirubin concentrations above the upper limit of normal. Thus, eighteen subjects were enrolled in the study. The Medical Ethics Committee of Wageningen University and Research Centre approved the study. It was determined a priori that subjects would be taken off treatment as soon as ALAT levels exceeded 2.5 times upper limit of normal or

ASAT levels exceeded 1.5 times the upper limit of normal. Each volunteer gave an informed consent.

Study design

Green Robusta coffee beans were roasted and ground. Robusta oil was extracted by hexane extraction under food-grade conditions at the Agrotechnology & Food Innovations institute in Wageningen. To make ingestion more convenient the oil was administered as an emulsion containing 50% Robusta oil with 50% water. Subjects consumed 2 ml of Robusta oil twice a day for four weeks resulting in a daily dose of 62 mg cafestol and 1.6 mg kahweol (samples analyzed using DIN 10779, 1999).

After two days of Robusta oil consumption the first blood sample was taken for determination of liver enzyme activities. From then on blood was drawn every seven days and liver enzyme activities were determined in the serum within 24 hours. ALAT (Alanine Aminotransferase Flex reagent cartridge, Dade Behring) and ASAT (Aspartate Aminotransferase Flex reagent cartridge, Dade Behring) were measured at 37° C. Liver enzyme activities are given in multiples of the upper limit of normal because activities vary with the temperature used when determining liver enzyme activities in different laboratories. The upper limits of normal at this laboratory were 45 IU/l for ALAT and 50 IU/l for ASAT.

Subjects were asked to maintain their lifestyles and dietary habits for the duration of the study. They reported the amount of coffee oil taken daily and changes in diet, smoking, physical activity, use of medication, and illness, in diaries.

Statistical analysis

To calculate the effect of Robusta oil on liver enzyme levels we subtracted baseline levels from levels after treatment for each subject. We tested the differences between baseline and treatment levels with the Wilcoxon signed-rank test. We present median differences with the 25^{th} and 75^{th} percentile. The responses of serum cholesterol and triglycerides were also

calculated as the level after treatment minus the baseline level for each subject. Serum lipid responses are presented as means with 95% confidence intervals (CI95%)

We used Bayesian statistics to combine existing evidence of the effect of coffee oil on levels of serum cholesterol with the present data. The Bayes factor was derived from the *P* value of the Student's *t* test: Bayes factor = $exp(-Z^2/2)$, where *Z* is the *Z* score that corresponds to the *P* value obtained with the Student's *t* test. A priori probabilities were converted to a priori odds and multiplied by the Minimum Bayes factor to obtain a posteriori odds. Finally the a posteriori odds were converted to a posteriori probabilities (10).

P values reflect the probability of obtaining the observed results or more extreme ones under the null hypothesis. A priori probabilities reflect our a priori estimation of the probability of the null hypothesis on the basis of the current literature. A posteriori probabilities reflect the probability of the null hypothesis when combining a priori evidence with evidence from the current study. We assigned an a priori probability of 10% to the null hypothesis that the effect of Robusta oil on serum cholesterol levels was equal to zero. The rationale for this postulation is that the effect of coffee oil on serum cholesterol is well established (1-4).

Results

Eighteen subjects were enrolled in the study, three men and 15 women. Figure 1 shows the number of subjects that were screened, enrolled, and excluded. Table 1 provides baseline characteristics of the enrolled subjects. During the study two subjects withdrew: one because of an infectious disease and the other because of the taste of the coffee oil. After nine days one subject was taken off treatment due to an ALAT level of 3.6 times the upper limit of normal (ULN). After 16 days another two subjects had to stop due to elevated ALAT and ASAT levels. One of those subjects had a level of 5.8 ULN for ALAT and 2.0 ULN for ASAT; the other subject had an ALAT level of 12.4 ULN and an ASAT level of 4.7 ULN. It was then decided to terminate the study, as prescribed by the study protocol: Three subjects or more showing a

liver enzyme level above 2.5 times upper limit of normal for ALAT or 1.5 times the upper limit of normal for ASAT.

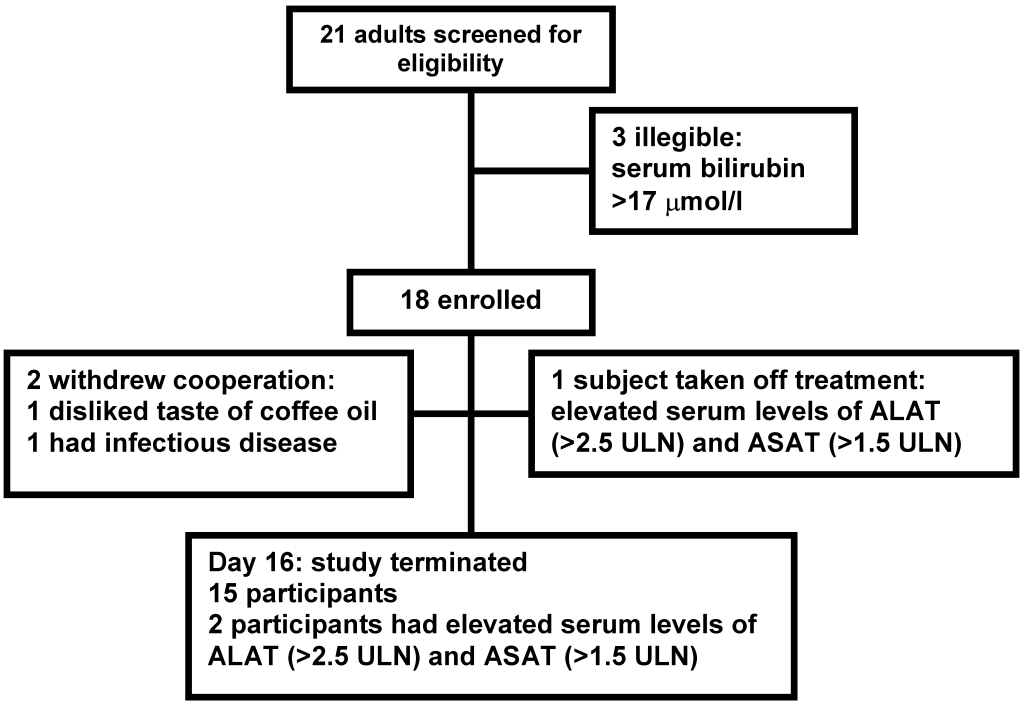

Figure 1 Diagram of the number of subjects that were screened, enrolled, and excluded. ULN = times the upper limit of normal.

Table 1 Baseline characteristics for all subjects who started the study

Characteristic	(n=18)
Age (years)	22 ± 5
Height (m)	1.73 ± 0.09
Weight (kg)	64.3 ± 10.3
Body mass index (kg/m^2)	21.3 ± 1.6
Serum total cholesterol (mmol/l)	4.4 ± 1.0
Serum triglycerides (mmol/l)	0.85 ± 0.38
Alanine aminotransferase (IU/l)	20 ± 8
Aspartate aminotransferase (IU/l)	16 ± 4
Current smokers n (%)	2 (11)
Alcohol (glass/week)	8 (2, 15)
median (25th percentile, 75th percentile)	

Variables presented as mean ± sd, current smokers presented as n (%), and alcohol consumption as median with the 25th and 75th percentile.

Response of liver function parameters to Robusta oil

The median response after 16 days of coffee oil consumption was an increase of 0.27 ULN (25^{th},75^{th} percentile:0.09,0.53) for ALAT and 0.06 ULN (25^{th},75^{th} percentile: -0.06,0.22) for ASAT.

Table 2 shows levels of liver function parameters for all subjects that received Robusta oil treatment for 16 days. Follow-up measurements took place at termination, and after four and eight weeks. After four weeks two subjects had ALAT levels above normal. One of those subjects showed a large response of ALAT after 16 days of treatment, the other subject did not show a response above the upper limit of normal during the treatment period. After eight weeks ALAT and ASAT levels of all subjects were within normal limits again. We observed no significant effect of Robusta oil on alkaline phosphatase, amylase, bilirubin, γ-glutamyltranspeptidase and lactate dehydrogenase levels after 16 days of Robusta oil consumption. However, during the follow-up measurement four weeks after termination we observed increased levels of serum bilirubin and decreased levels of alkaline phophatase. Compared to baseline values bilirubin was increased by 3.0 μmol/l (25th,75th percentile: -2.5,6) and alkaline phosphatase was decreased by 0.05 ULN (25th,75th percentile:-0.16,-0.01).

Serum lipid response to Robusta oil

Total serum cholesterol levels were raised 0.27 mmol/l (n=15, CI95% -0.11;0.64) after 16 days of coffee oil treatment. According to the Student's *t* test this effect was not significantly different from 0 at the $p<0.05$ level. However, Bayesian analysis showed that the evidence from this study actually reduces an a priori probability for no effect on cholesterol of 10% to an a posteriori probability of 4%. See table 3 for the Bayesian analysis including the posteriori probabilities at several a priori probabilities. Serum triglycerides were elevated by 0.46 mmol/l (CI95% 0.26;0.66) in the 15 subjects who were in the study after 16 days.

Table 2 levels of liver parameters at baseline after Robusta oil treatment and during follow-up

Parameter	Normal limits	Baseline	Treatment	Follow-up1	Follow-up2
		n=15	n=15	n=14	n=14
Alanine aminotransferase	< 45 IU/l	0.38 [0.36,0.56]	0.67** [0.58,1.27]	0.49* [0.39,0.72]	0.39 [0.35,0.53]
Aspartate aminotransferase	< 50 IU/l	0.32 [0.26,0.38]	0.44 [0.26,0.56]	0.32 [0.28,0.42]	0.38 [0.28,0.41]
Alkaline phosphatase	40 - 125 U/l	0.45 [0.36,0.58]	0.43 [0.35,0.54]	0.42* [0.34,0.51]	0.49 [0.37,0.53]
Amylase	35 - 130 U/l	0.50 [0.45,0.61]	0.48 [0.38,0.52]	0.49 [0.46,0.58]	0.47 [0.42,0.57]
Bilirubin	< 17 µmol/l	0.47 [0.41,0.65]	0.53 [0.35,0.65]	0.65** [0.57,0.82]	0.53 [0.40,0.74]
γ-glutamyltranspeptidase	< 40 U/l for women, < 75 U/l for men	0.29 [0.24,0.38]	0.25 [0.20,0.45]	0.31 [0.27,0.51]	0.34 [0.25,0.46]
Lactate dehydrogenase	230 - 485 IU/l	0.60 [0.56,0.61]	0.61 [0.49,0.65]	0.60 [0.50,0.64]	0.54 [0.52,0.65]

All values are medians in units of times the upper limit of normal with the 25th and 75th percentile between brackets. Treatment values were obtained after 16 days of Robusta oil treatment. Follow-up 1 took place four weeks after termination of the intervention and Follow-up2 eight weeks after termination.
* value differs significantly from baseline ($p<0.05$) in the Wilcoxon signed-rank test.
** $p<0.01$

Table 3 Change in prior probabilities of cafestol not affecting serum cholesterol to posterior probabilities using data of the present study and Bayesian analysis

Prior probability	Prior odds (Yes/No)	Posterior odds	Posterior probability
0.90 (very strong)	0.9/(1-0.9) = 9	9x Bayes factor = 3.22	3.22/(1+3.22) = 0.76
0.75 (strong)	0.75/(1-0.75) = 3	3x Bayes factor = 1.07	1.07/(1+1.07) = 0.52
0.50 (equivocal)	0.50/(1-0.50)= 1	1x Bayes factor = 0.36	0.36/(1+1.07) = 0.26
0.25 (weak)	0.25/(1-0.25) = 0.33	0.33x Bayes factor = 0.12	0.12/(1+0.12) = 0.11
0.10 (very weak)	0.10/(1-0.10) = 0.11	0.11x Bayes factor = 0.04	0.04(1+0.04) = 0.04

A priori probabilities were converted to a priori odds and multiplied by the minimum Bayes factor*. The obtained a postiori odds were converted to a postiori probabilities.
*Bayes factor = *e* to the power $-Z^2/2$, where *Z* is the *Z*-score corresponding to the *P*-value for obtaining an effect of 0.27 mmol/l under the null hypothesis. *P*-value = 0.15, *Z*-score = 1.43 the minimum Bayes factor = 0.36

Discussion

Robusta oil caused a rise of ALAT levels of more than 2.5 times the upper limit of normal in three out of eighteen subjects (17%). In our previous study with Arabica oil we observed ALAT levels of more than 2.5 times the upper limit of normal in eight out of 50 subjects (16%) in the first period and in five of 40 subjects (13%) not in the first, but only in a second treatment period (11). Therefore we conclude that the effect of Robusta oil on liver enzyme levels is similar to that of Arabica oil. Levels of ASAT were less affected by coffee oil than ALAT levels. ALAT is predominantly present in the cytosol of hepatocytes, whereas ASAT is predominantly present in the mitochondria. This could mean that the outer membranes of hepatocytes have become leaky but that the cells are still largely intact. When hepatocytes sustain more severe damage, the serum levels of ASAT would exceed those of ALAT (6-8). ALAT levels were also elevated in subjects after daily consumption of unfiltered coffee for six months (12), but were not elevated in life-long consumers (1, 13). This suggests that the effect of unfiltered coffee on ALAT levels is transient when consumed over long periods of time and that possibly an adaptation mechanism is present.

Another marker of liver damage, γ-glutamyltranspeptidase (γGT), has been shown to decrease during consumption of boiled coffee. After withdrawal of treatment with coffee lipids or coffee oil an rebound increase above baseline values in serum levels of γGT is observed (1, 3, 14, 15). In the present study we observed a 14% decrease in serum activities of γGT during treatment and an increase of 17% compared to baseline eight weeks after termination. These effects are not statistically significant in the present study. This is due to the limited number of subjects and the short duration of the coffee oil treatment. We also observed a 7% decrease in serum activities of alkaline phosphatase compared to baseline four weeks after termination. A tendency of alkaline phosphatase to be decreased during coffee lipid treatment was observed in previous studies (1, 3). Bilirubin levels were 38% increased during the follow-up measurement after four weeks compared to

baseline. We observed no effect of Robusta oil treatment on amylase, a marker for pancreatitis, or lactate dehydrogenase, which is used for diagnosis of heart, muscle, and liver diseases. Increases of γGT and bilirubin after stopping treatment with coffee oil could indicate cholestasis. Cholestasis is functionally defined as a disruption in secretion of bile acids from the liver. Disrupted secretion causes elevated levels of bile acids in the liver, which causes damage to the hepatocytes. However, in cholestatic disease alkaline phosphatase is strongly increased, which is not the case with coffee oil treatment.

In Scandinavia, where large amounts of unfiltered coffee were commonly consumed, risk of coronary heart disease is high and was associated with consumption of unfiltered coffee but mortality rates of liver cirrhosis have been typically low (16). Therefore, it is unlikely that consumption of unfiltered coffee produces severe damage to the liver. However, it is possible that unfiltered coffee can cause sub-clinical hepatic injury in some individuals. At present we have no evidence that the changes in liver enzyme levels induced by coffee oil or unfiltered coffee are of clinical relevance. Because we have no liver biopsies from subjects we are not able to demonstrate possible liver damage *in vivo*. No results from animal studies showing the effect of coffee oil on the liver have been published.

Interestingly, cafestol and kahweol have been shown to upregulate detoxification pathways in the liver of rat and mice and human cultured cells (17-21). This effect on detoxification is hypothesized to explain the observed inverse association between coffee consumption and certain cancer types (20, 22-24). Possibly, cafestol upregulates these pathways due to its toxicity and as a “side effect” enhances detoxification of carcinogenic compounds.

After 16 days of Robusta oil treatment serum cholesterol levels were elevated but this effect was not statistically significant at the $P<0.05$ level according to the conventional frequentist analysis. This was expected from previous studies: the full effect on serum cholesterol is only observed after 4-6 weeks of coffee oil consumption. After four weeks daily consumption of 62 mg of cafestol results in a rise in serum cholesterol of 0.8 mmol/l. The Bayesian analysis, however, indicates that the present study in fact reinforces the

existing evidence that coffee oil raises serum cholesterol, independent of how strong one judges this prior evidence to be (Table 3). We also found that triglycerides were elevated after 16 days of treatment, which was also expected from previous studies (1-4).

On the basis of our results it is not likely that it is kahweol, which is mainly responsible for the effect of coffee oil on liver enzyme levels, as has been suggested in two previous studies (3, 9). In most subjects coffee oil caused elevation of liver enzyme levels. However, this elevation was more extreme in a small number of subjects. Figure 2 shows ALAT levels and figure 3 shows ASAT levels of subjects at baseline and after 16 days of coffee oil consumption. Although no parallel placebo group was present in this study it is unlikely that such large responses of liver enzymes would be observed with placebo oil. This is supported by previous studies with coffee and placebo oil (1, 9, 25). We found no correlation between the response of liver enzymes to coffee oil and baseline liver enzyme activities, serum lipid response, or alcohol. Moreover, a previous study showed that the liver enzyme response is not consistent within subjects (11). In this study we also found no correlation between alcohol intake and liver enzyme response. Although we cannot rule out the possibility that alcohol affected the liver enzyme response during the study it does not seem likely that alcohol intake could fully explain the observed increases in ALAT and ASAT. Furthermore, γGT is decreased rather than increased during consumption of coffee oil, whereas alcohol causes increases in γGT levels.

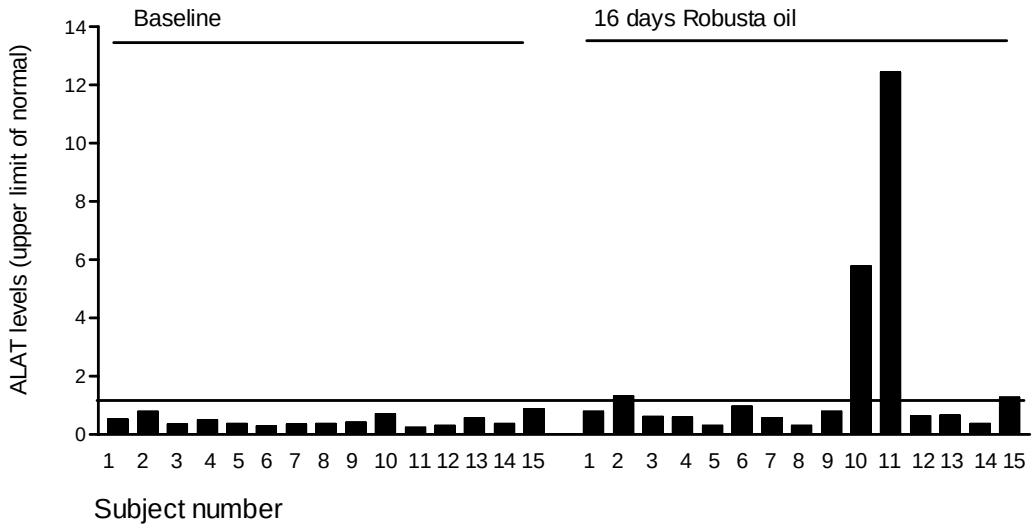

Figure 2 ALAT levels of subjects at baseline and after 16 days of Robusta oil consumption. The horizontal line indicates the upper limit of normal.

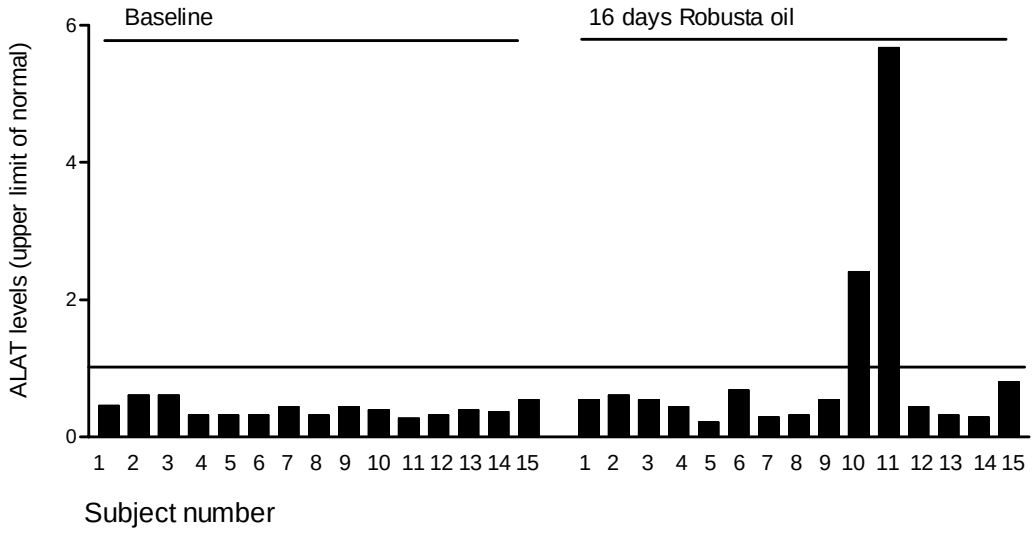

Figure 3 ASAT levels of subjects at baseline and after 16 days of Robusta oil consumption. The horizontal line indicates the upper limit of normal.

Together with the observation that most subjects do not show such a large increase in liver enzyme levels during coffee oil treatment, this suggests that an unknown environmental factor enhances the response of liver enzymes to coffee oil in a number of subjects. We conclude that increased ALAT or ASAT activities in patients may be caused by a switch from filtered to unfiltered coffee. If raised activities of ALAT and ASAT are caused by a change in coffee consumption, the ratio ASAT/ALAT will be smaller then 1 and other markers of liver damage such as γGT and alkaline phophatase will be typically within normal limits. When otherwise unexplained elevation of ALAT and ASAT activities are observed it would be advisable to ask a patient if he/she consumes large amounts of unfiltered coffee such as French press coffee.

Author's contributions

MVB participated in designing and planning the study, headed the investigation during the intervention period, analyzed the data and wrote the paper. EGS participated in designing the study, contributed to analysis of the data and interpretation of the results. MBK was the senior scientist supervising the project.

Acknowledgements

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4

The cholesterol-raising factor from coffee beans, cafestol, activates the farnesoid and pregnane X receptors

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Submitted

Abstract

Unfiltered coffee types, such as Scandinavian boiled coffee and French press coffee, raise serum LDL cholesterol levels in humans. The diterpene lipid cafestol, which is retained by paper filters, was identified as the factor that causes hyperlipidemia. Cafestol is also a candidate anti-carcinogen because it induces detoxifying enzymes. In rodents, cafestol represses bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase (Cyp7A1), the rate-controlling enzyme in the conversion of cholesterol into bile acids. We now show that cafestol is an agonist ligand for the farnesoid X receptor (FXR, NR1H4) which mediates down-regulation of Cyp7A1 expression by bile acids. Cafestol also activated the xenobiotic pregnane X receptor (PXR, NR1I2). Microarray analysis showed that feeding of cafestol to APOE3Leiden mice affected hepatic expression of genes involved in lipid metabolism and detoxification, many of which are regulated by FXR or PXR. Regulation of Cyp7A1 and other FXR target genes by cafestol was abolished in FXR null mice. We conclude that cafestol is an agonist ligand for nuclear receptors that suppress bile acid production, which may explain the lipid-raising effects of cafestol in humans. Activation of the xenobiotic receptor PXR may contribute to the reported anticarcinogenic effects of cafestol.

Introduction

Cafestol is a diterpene from coffee beans that potently raises serum cholesterol and triglycerides in humans (1-4). Paper filters retain cafestol and that explains why paper-filtered coffee does not affect cholesterol levels, while unfiltered coffee brews such as boiled, French press and espresso coffee do. A high intake of boiled coffee has been associated with hypercholesterolemia and coronary heart disease in Norway and Finland (5-7); and a switch from boiled to paper-filtered coffee is thought to be responsible for a 0.3 mmol/l mean fall in serum cholesterol in Finland between 1972 and 1992 (8). Cafestol may also have a beneficial side in that it causes up-regulation of detoxification pathways in liver of rats and mice and in cultured human hepatocytes (9-12). In humans consumption of unfiltered coffee raises the glutathione content in the colorectal mucosa (13). Up-regulation of detoxification pathways by cafestol may explain the inverse association of coffee consumption and risk of certain cancer types (12, 14, 15).

We previously demonstrated that cafestol suppresses synthesis of bile acids from cholesterol in the liver of APOE3Leiden (E3L) mice by down-regulation of expression of the rate-limiting enzyme cholesterol 7α-hydroxylase (Cyp7A1) (16). These E3L mice also show an increase in serum lipids in response to cafestol similar to that observed in humans (16). In humans, a disabling mutation in the Cyp7A1 gene is associated with increased plasma levels of triglycerides and LDL cholesterol (17). This effect on serum lipids is similar to that of cafestol.

The suppression of Cyp7A1 expression and activity suggests a mechanism for the serum lipid response to cafestol, because the rate of conversion of cholesterol to bile acids in the liver affects the production and clearance of lipoproteins. We therefore studied the mechanism through which cafestol affects Cyp7A1 activity. Cyp7A1 activity is under the control of a nuclear receptor cascade. When activated by bile acids, the farnesoid X receptor (FXR) induces expression of the small heterodimer partner (SHP, NR0B2), which potently inhibits the activity of another orphan receptor, liver receptor homologue 1 (LRH-1). LRH-1 is required for Cyp7A1 promoter activity (18, 19). In this negative feedback loop, bile acids can inhibit their own biosynthesis. Another nuclear receptor that inhibits Cyp7A1 expression upon

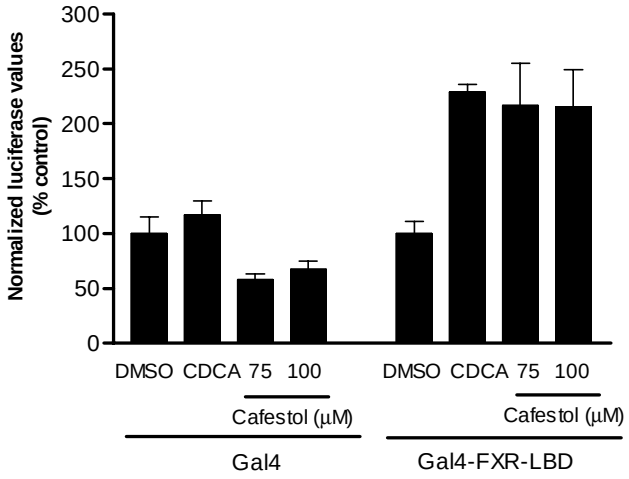
activation is the pregnane X receptor (PXR). PXR is activated by a variety of foreign compounds, or xenobiotics, and also by hydrophobic bile acids and bile acid precursors, and it protects the liver against toxicity of such compounds (20, 21). Although the mechanism by which PXR inhibits Cyp7A1 is poorly understood, it was shown that certain bile acids could inhibit Cyp7A1 expression *in*dependently of SHP and this process is thought to involve PXR (22, 23). Activation of FXR and PXR not only suppresses bile acid synthesis via down-regulation of expression of Cyp7A1, but also increases detoxification of bile acids by up-regulation of 5'-diphosphate-glucuronosyltransferase 2B4 and cytochrome P450 3A enzymes (CYP3A) respectively (24, 25).

Here we report the effects of cafestol on the expression of FXR and PXR controlled genes in pathways of bile acid and lipid metabolism and detoxification processes. Our findings offer an explanation for both the hyperlipidemic and the potential chemopreventive action of this widely consumed dietary component.

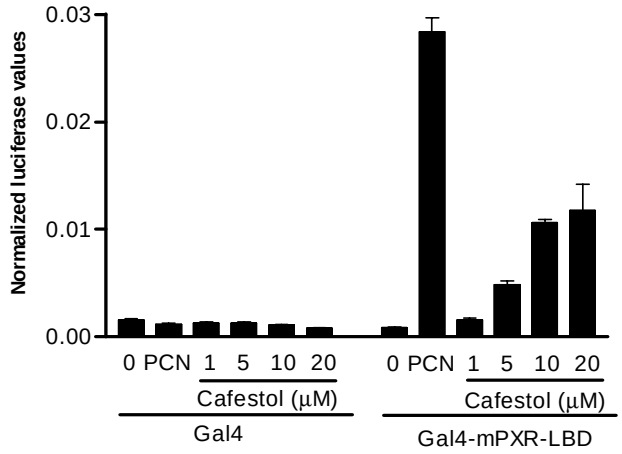
Results

Effects of cafestol on nuclear receptors

A Gal4 based transactivation assay was used to screen for effects of cafestol on a number of nuclear receptors. In this commonly used assay, the ligand binding domains of different nuclear receptors are fused to the Gal4 DNA binding domain and effects on expression directed by a Gal4-dependent reporter plasmid are tested in transient transfections experiments. Out of a large number of receptors tested, cafestol only activated the ligand-binding domains of FXR and PXR (Fig. 1). For example, the nuclear receptors LXRα, RXRα, and PPARα were not activated by cafestol (Fig. 1). Other receptor-LBD chimeras tested, which were not activated by cafestol included mouse and human CAR, mouse PPARγ, human RARα, ERα, GR, TRβ, and VDR, in addition to Gal4 -full length human RORα, RORβ and mouse SHP chimeras (data not shown).


Normalized luciferase values (% control)
300
250
200
150
100
50
0
DMSO CDCA 75 100
DMSO CDCA 75 100
Cafestol (μM)
Cafestol (μM)
Gal4
Gal4-FXR-LBD

A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0.01
0.00
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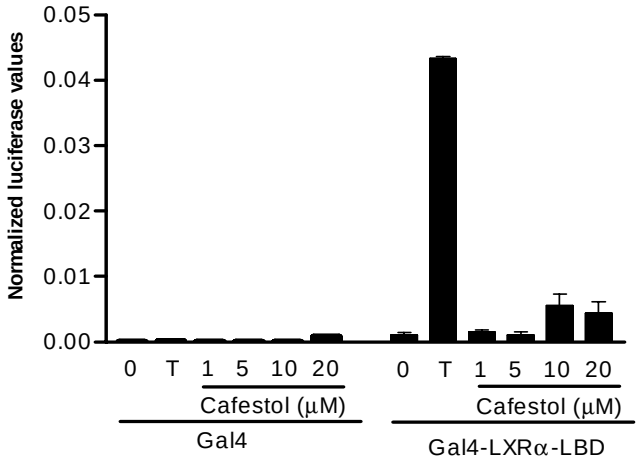
0 PCN 1 5 10 20
Cafestol (μM)
Cafestol (μM)
Gal4
Gal4-mPXR-LBD

B


Normalized luciferase values
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0.03
0.02
0.01
0.00
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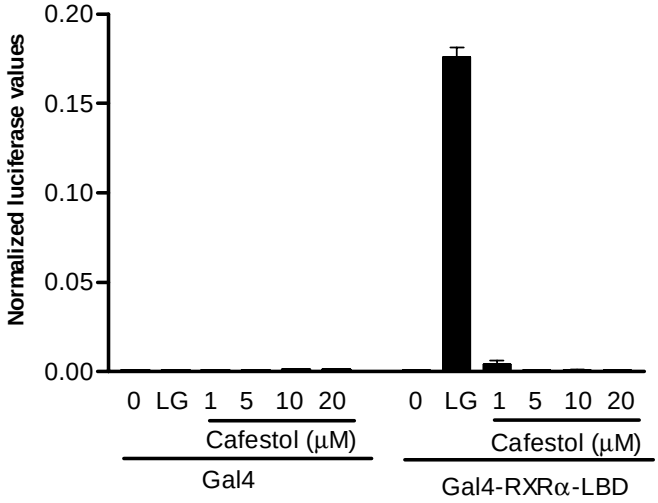
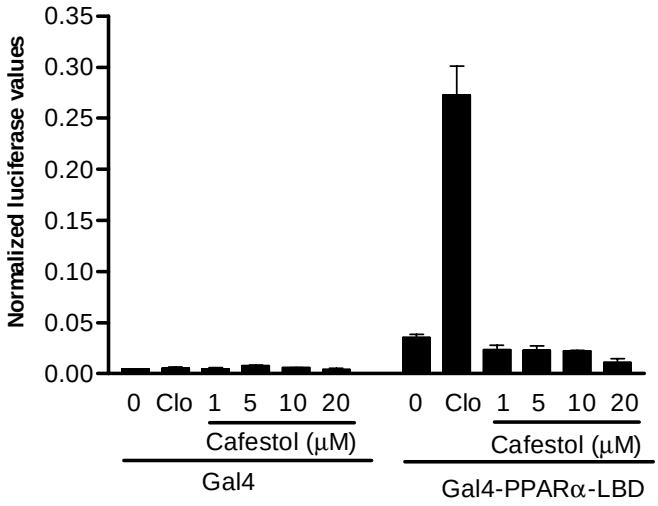
0 T 1 5 10 20
Cafestol (μM)
Cafestol (μM)
Gal4
Gal4-LXRα-LBD

C



D



E

Figure 1 Determination of the ability of cafestol to interact with a range of nuclear receptors *in vitro*. HepG2 cells were co-transfected with the Gal4 luciferase reporter and a series of chimeras in which the Gal4 DNA binding domain is fused to the indicated nuclear hormone receptor ligand-binding domain. The cells were treated with a known receptor-specific agonist or various doses of cafestol as indicated.
Results are expressed as normalized luciferase activity relative to the growth hormone (GH) internal control **(a)** Gal4 alone or Gal4 FXR-LBD in the presence of 100 μM CDCA or 75 or 100 μM cafestol. **(b)** Gal4 alone or Gal4 mouse PXR-LBD in the presence of 1μM PCN or 1, 5, 10 or 20 μM cafestol. **(c)** Gal4 alone or Gal4-LXRα-LBD the presence of 1 μM T0901317 (T) or 1, 5, 10 or 20 μM cafestol. **(d**) Gal4 alone or Gal4-RXR-LBD the presence of 1μM LG101268 (LG) or 1, 5, 10 or 20μM cafestol. **(e)** Gal4 alone or Gal4-PPARα-LBD the presence of 300nM clofibrate (Clo) or 1, 5, 10 or 20 μM cafestol. Results are expressed as normalized luciferase values relative to the GH internal control, mean ± SEM. Similar results were obtained from at least three independent experiments, performed in triplicate.

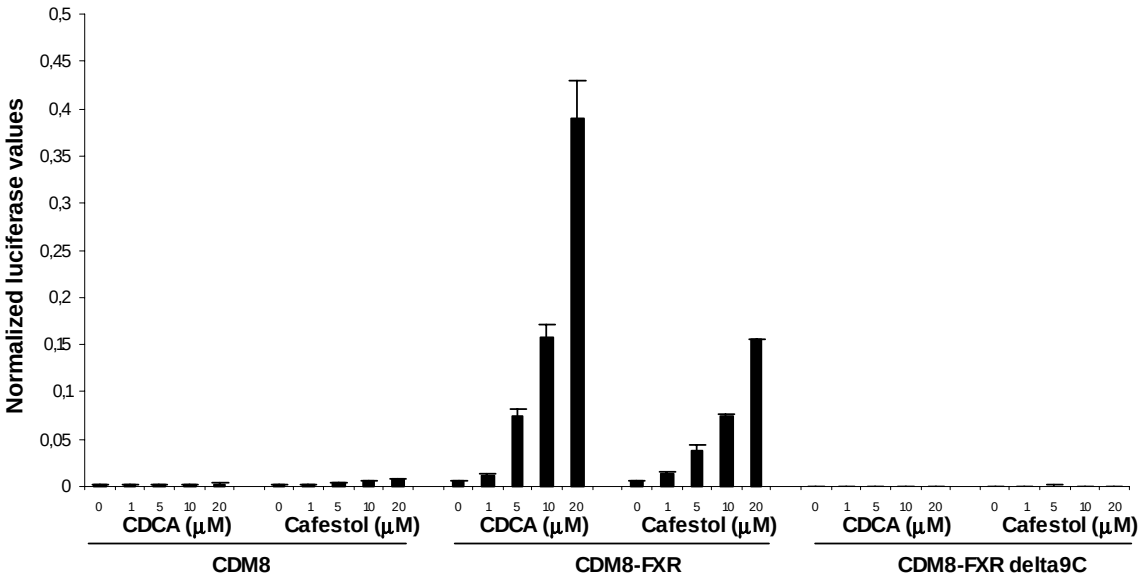
Activation of FXR by cafestol

To confirm the activation of FXR independently, the effect of cafestol on the full-length receptor was compared with that of chenodeoxycholic acid (CDCA), a potent endogenous FXR agonist. Both compounds showed dose dependent activation, with greater response observed with the bile acid (Fig. 2A). These responses were dependent on FXR activation, since they were not observed with the FXR Δ9C mutant, which lacks the terminal amino acids 476-484 corresponding to the helix 12 AF-2 transactivation function (Fig. 2A). In addition, the mammalian two-hybrid assay was used to test the ability of cafestol to induce coactivator recruitment to FXR. In this assay, Gal4 was fused with the coactivator SRC-1 and FXR was fused with the transactivator VP16. The ability of both cafestol and CDCA to induce high levels of luciferase expression from the Gal4 reporter in a dose dependent manner indicates that both compounds induced interaction of FXR with the coactivator SRC-1 (Fig. 2B).

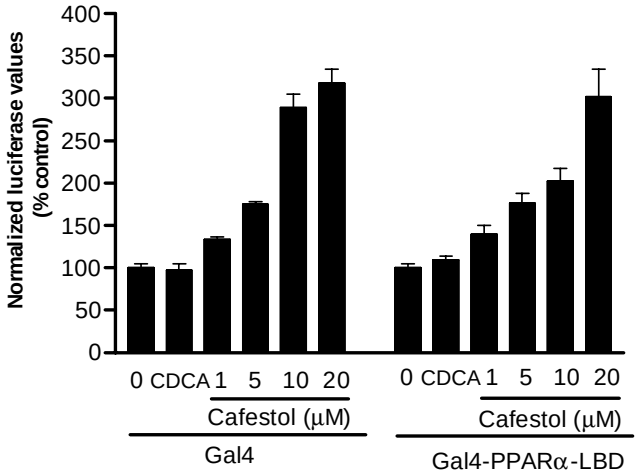
We next analyzed whether a native promoter could be activated *in vitro* to demonstrate the ability of cafestol to activate gene transcription through FXR. For this experiment we used the promoter of the human bile salt export pump (BSEP), which is a well-established FXR target harboring two FXR response elements (26, 27). Both cafestol and CDCA strongly induced BSEP promoter activity in HepG2 cells cotransfected with expression plasmids for FXR and RXR (Fig. 2C). As expected, this response was dependent on both the presence of FXR and RXR expression vectors, and the presence of functional FXR response elements. Similar results were observed when CV-1 and HEK293 cells were transfected (data not shown), although the maximum induction by both cafestol and CDCA was lower in these cells. This may reflect a dependence on liver specific co-activators.

To test the ability of cafestol to specifically activate expression of an endogenous FXR target gene, its effect on BSEP mRNA levels was compared in primary hepatocytes prepared from wild-type and $FXR^{-/-}$ mice. As expected, BSEP expression was increased 3-fold by cafestol in the wild-type cells, but cafestol had no effect on BSEP expression in the $FXR^{-/-}$ hepatocytes (Fig. 2D).

This combination of results demonstrates that cafestol, similar to CDCA, activates FXR and that FXR is required for the induction of BSEP expression *in vitro*.

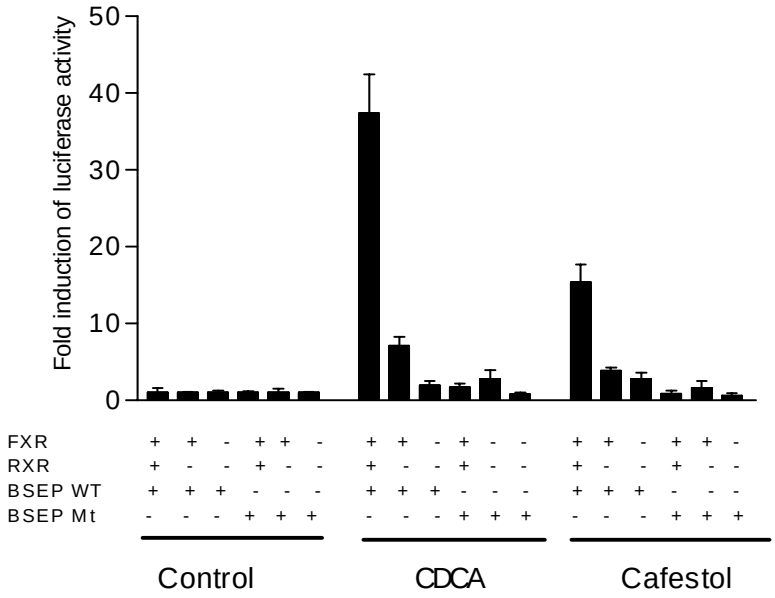


A

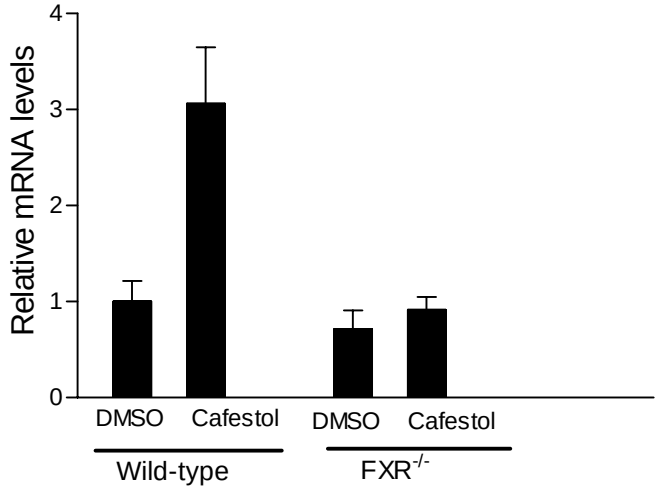


B

Figure 2 (a) Cafestol transactivates FXR. CV-1 cells were co-transfected with a luciferase reporter construct containing an ECRE response element upstream of the TK promoter and expression vectors for either CDM8 vector alone, full-length wild type FXR (wt), or an FXR AF-2 deletion mutant (FXR Δ9C) and RXR along with the internal control (TK-GH). Cells were treated with vehicle alone (DMSO), 1, 5, 10, 20 μM CDCA or 1, 5, 10, 20 μM cafestol as indicated. Results are expressed as normalized luciferase values relative to the GH internal control, mean±SEM. Similar results were obtained from at least three independent experiments, performed in triplicate.
(b) Cafestol enhances FXR interaction with the co-activator SRC-1. CV-1 cells were cotransfected with a luciferase reporter construct containing a Gal4 response element upstream of the TK promoter and expression vectors for Gal4-SRC-1(RID) and VP16 FXR, and RXR, along with the internal control (TK-GH). Cells were treated with vehicle alone (DMSO), 1, 5, 10, 15 or 20 μM CDCA or 1, 5, 10, 15 or 20 μM cafestol, as indicated. Results are expressed as % of the control, normalized to the GH internal control, mean ± SEM. Similar results were obtained from at least three independent experiments, performed in triplicate.



C



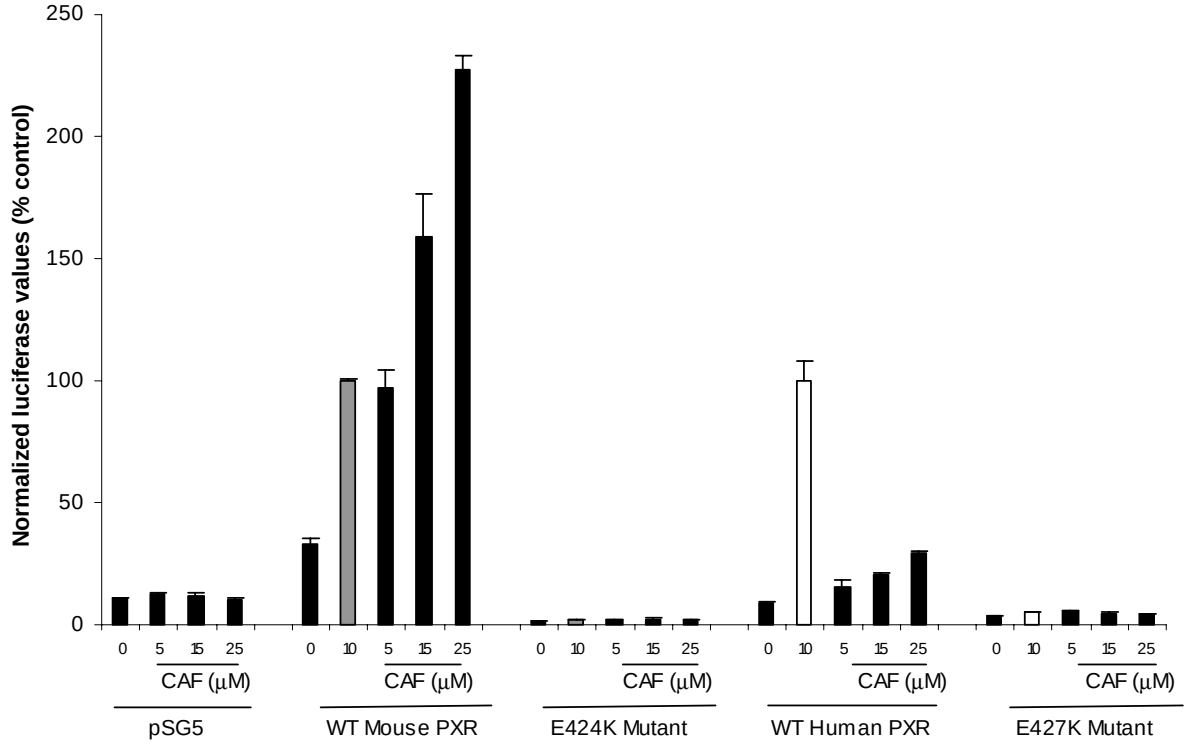
D

Figure 2 (c) Cafestol induces activity of the native BSEP promoter *in vitro*. HepG2 cells were tranfected with a BSEP-promoter-luciferase construct plus expression vectors for rFXR and hRxRα, or rFXR alone, or control vector only. Two different BSEP promoter constructs were used: the wild type (WT) and a mutant (Mt) that contained mutated FXR response elements that do not bind FXR (27). Cells were treated with vehicle alone (DMSO), or 100 μM CDCA, or 56 μM cafestol as indicated. Similar results were obtained from at least three independent experiments performed in quadruplicate. **(d) Cafestol induces BSEP expression in primary hepatocytes.** Quantitative RT-PCR was performed to analyze BSEP expression in hepatocytes from wild-type and $FXR^{-/-}$ mice. Hepatocytes from two wild-type and two knockout mice were isolated and were cultured separately for each mouse in 6-wells plates. Hepatocytes were treated for 24h with DMSO or 56 μM cafestol acetate as indicated. Treatment was performed in nine wells per mouse. This graph shows the combined results in the hepatocytes from both mice. Expression levels were normalized to β-actin. Data are presented as mean ± standard error, n=18.

Activation of PXR by cafestol

Similarly, the capability of cafestol to activate PXR was further examined using the full-length receptor. Since mouse and human PXR differ in their responses to xenobiotics, both the full-length mouse and human PXR were tested. Cafestol was a somewhat more potent and more efficacious activator of mouse PXR than the mouse-specific agonist PCN (pregnenolone-16α-carbonitrile) (Fig. 3A). Cafestol also significantly activated human PXR, but this response was somewhat weaker than that of the human-specific agonist rifampicin (fig 3A). The observed responses of the reporter in this assay were dependent on PXR activation, since they were not observed with PXR mutants lacking the AF-2 transactivation function (Fig. 3A). Finally, as with FXR, cafestol induced coactivator recruitment to PXR as illustrated by the interaction of PXR and the coactivator SRC-1 in the mammalian two-hybrid assay (Fig. 3B).

A reporter construct containing the PXR-response element and the xenobiotic-responsive element (XREM) of the human cytochrome P450 3A4 (CYP3A4) promoter (28) was used to evaluate the ability of cafestol to activate gene expression through PXR. In HepG2 cells cotransfected with expression plasmids for PXR and RXR, both cafestol and rifampicin induced CYP3A4 promoter activity (Fig. 3C). As expected, this response was dependent on both the presence of PXR and RXR expression vectors.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pSG5
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CAF (μM)
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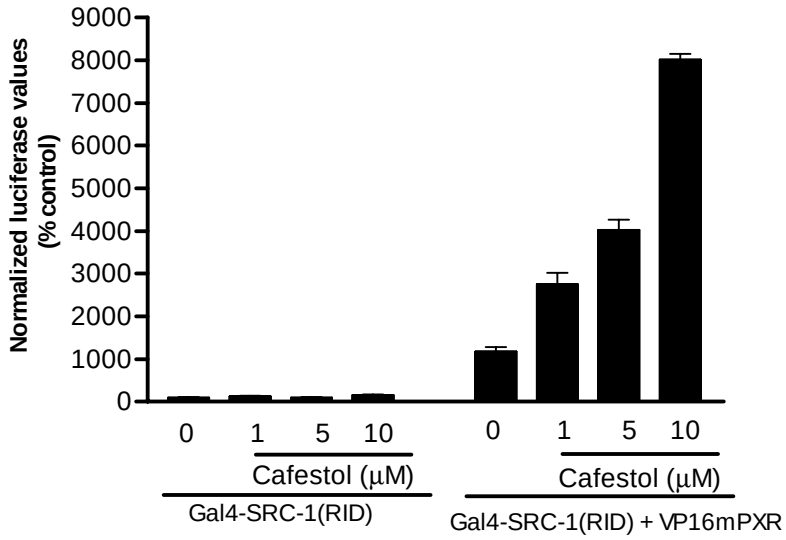
WT Human PXR
0 10 5 15 25
CAF (μM)
E427K Mutant

A


Normalized luciferase values (% control)
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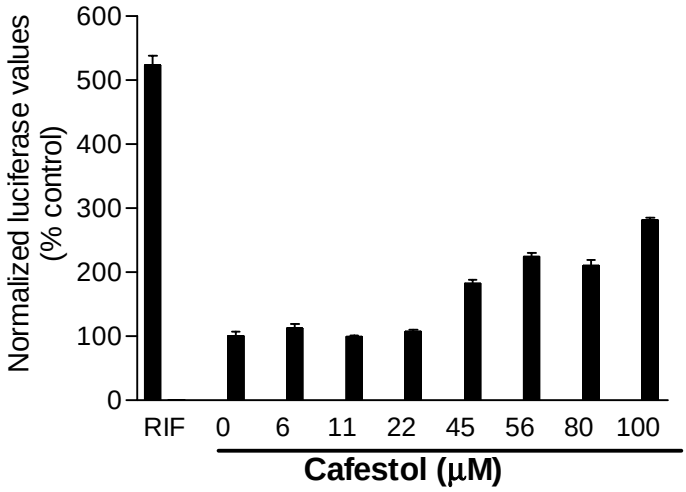
7000
6000
5000
4000
3000
2000
1000
0
0 1 5 10
Cafestol (μM)
Gal4-SRC-1(RID)
0 1 5 10
Cafestol (μM)
Gal4-SRC-1(RID) + VP16mPXR

B



C

Figure 3 (a) Cafestol transactivates both mouse and human PXR. HepG2 cells were co-transfected with a luciferase reporter construct containing a LXRE response element upstream of the TK promoter and expression vectors for wild type mouse PXR or the E424K AF2 mutant or wild type human PXR or the E427K AF2 mutant, and RXR, along with the internal control (TK-GH). Cells were treated with vehicle alone (DMSO), 5, 15, 25μM cafestol (black bars) or 10μM known agonist (open bar), as indicated. Results are expressed as % of the known agonist values (PCN for mouse and RIF for human) normalized to the GH control, mean±SEM. Similar results were obtained from at least three independent experiments, performed in triplicate.
(b) Cafestol enhances PXR interaction with the co-activator SRC-1 *in vivo*. HepG2 cells were co-transfected with a luciferase reporter construct containing a Gal4 response element upstream of the TK promoter and expression vectors for Gal4-SRC-1(RID) alone, Gal4-SRC-1(RID) and VP16 mouse PXR, and RXR, along with the internal control (TK-GH). Cells were treated with vehicle alone (DMSO), 1, 5, or 10μM cafestol, as indicated. Results are expressed as % of the control, normalized to the GH internal control, mean±SEM. Similar results were obtained from at least three independent experiments, performed in triplicate.
(c) Cafestol induces activity of the native CYP3A4 promoter *in vitro*. HepG2 cells were tranfected with a CYP3A4-promoter-luciferase construct plus expression vectors for hPXR and hRxRα, or hPXR alone, or control vector only. Cells were treated with vehicle alone (DMSO), 56μM cafestol, 10μM rifampicin, as indicated. Similar results were obtained from at least three independent experiments performed in triplicate.

Microarray analysis of the cafestol response

The combined *in vitro* data unambiguously show that cafestol has similar effects as the previously known ligands for FXR and PXR. However, the response to nuclear receptor ligands *in vivo* is likely to be complex. To further understand the role of cafestol in metabolism and detoxification, a study was undertaken in which the effect of cafestol on the expression of liver genes was determined at a large scale using microarray analysis.

For this study we used APOE3Leiden (E3L) transgenic mice, a frequently used mouse model to study diet-induced hyperlipidemia (29). When the E3L mice were fed a diet containing cafestol for 30 days serum cholesterol levels were 25% increased as compared to the control group (Table 1). The increase in serum cholesterol is of the same order of magnitude as that observed in humans (30). No effect was seen on serum triglyceride and free fatty acid concentrations (Table 1). Serum levels of bilirubin were increased in the cafestol fed mice in comparison with control mice, but no significant effect was seen on the liver function markers alanine aminotransferase or alkaline phosphatase (Table 1).

Table 1 Weight and serum analysis of control and cafestol fed E3L mice

Groups	Weights	TC	TTG	FFA	ALAT	Bilirubin	Alkaline phosphatase
	g	mmol/l	mmol/l	mmol/l	U/l	µmol/l	U/l
Control group	19.6 ± 1.0	14.8 ± 3.1	1 ± 0.5	0.84± 0.12	74.3 ± 23	< 8.6	289.4 ± 23.9
Cafestol group	19.5 ± 0.9	18.6± 2.6*	1 ± 0.2	0.93± 0.16	126.3± 68.4	20.6 ± 3.7*	268.4 ± 26.7

E3L mice were fed cafestol or control diet. After 30 days serum was prepared and analyzed. TC: total cholesterol; TTG: total triglyceride; FFA: free fatty acids. Values are the mean levels ± SD of 8 mice per group. Means of control and cafestol fed mice were compared using the non-parametric Mann-Whitney test (* $p < 0.05$).

Hepatic mRNA of four individual cafestol-fed mice was subjected to microarray analysis using mRNA of the pooled control group as a reference. Some 648 genes showed a significant ($p<1\times10^{-6}$, Z-test) difference in expression in at least one mouse, as compared to the control group. Hierarchical clustering of these 648 genes revealed 2 clusters of genes showing a consistent direction of up- or down-regulation in all 4 cafestol fed mice. These two clusters harbor 531 genes, i.e. the great majority of the original 648 genes (data not shown). Next, the two gene clusters were categorized using the Gene Ontology (GO) database. The differentially expressed

genes were subdivided into 39 functional classes. Genes that were involved in more than one biological process were categorized in the most relevant category for our study. Notably, glutathione *S*-transferases and monooxygenases were categorized as detoxification genes. The classes containing at least 10 differentially expressed genes per category are shown (Fig.4A). Subsequently, all 9552 genes on the microarray were classified using the same principles, which allowed calculating the fraction of differentially expressed genes in each particular category and compared this number to the 'expected' number of regulated genes. The latter number was determined based on the assumption that all genes in a particular category have a similar chance of being differentially expressed (see the Materials and Methods section for more details). Genes controlling lipid metabolism, detoxification, amino acid metabolism, and the immune response were 2 – 5 times more abundantly present among the 531 genes in the two gene clusters. Hence, these processes are significantly affected by cafestol (Fig. 4A).

The two most relevant categories indicated above, i.e. lipid and detoxification metabolism, consist of 64 genes and were further explored. Using the KEGG database, genes were categorized into the following processes: (1) bile acid metabolism, (2) fatty acid metabolism, (3) sterol biosynthesis, (4) steroid metabolism and (5) detoxification. (Fig. 4B). Some 26 genes show an average fold change greater than 1.4 and are distributed among all 5 categories. Eleven of these genes are known to be under the control of the nuclear receptors FXR and/or PXR: Cyp7A1, PLTP, apoA-I, apoA-V and GST's (31-36). The expression pattern of five genes was tested using Northern blots or quantitative RT-PCR and confirmed the microarray data (see Table 2). In addition we measured expression levels of genes regulated by FXR involved in lipid metabolism that were not present on the cDNA chip and these were: SHP, LRH-1, FXR, NTCP, BSEP, and sterol 12α-hydroxylase (Cyp8B1). (see Table 2).

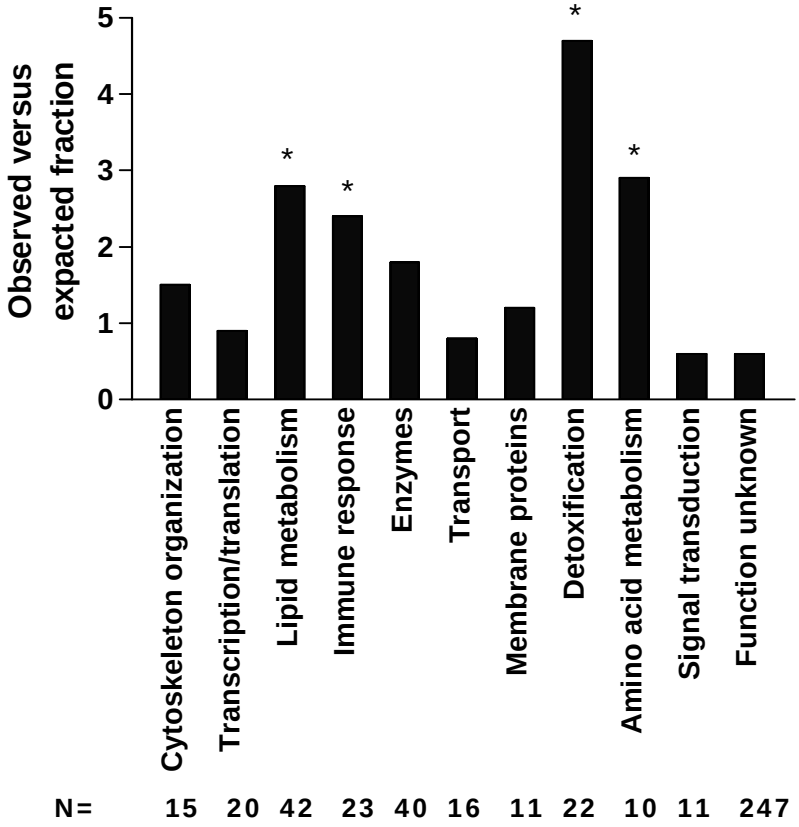
The microarray analysis shows a large impact of cafestol on the regulation of metabolic and detoxifying genes. All data are compatible with an important role of FXR and PXR in the regulation of these genes, except for the expression of SHP. For SHP, additional experiments were performed. Figure 4C shows the acute and chronic effects of cafestol on the expression of SHP and Figure 4D the effects on the expression of Cyp7A1. Four hours after administration of a bolus of cafestol SHP

expression was increased by 33%. In parallel, Cyp7A1 expression was decreased as expected. This initial response of SHP is in agreement with the increase of SHP expression by FXR activators.

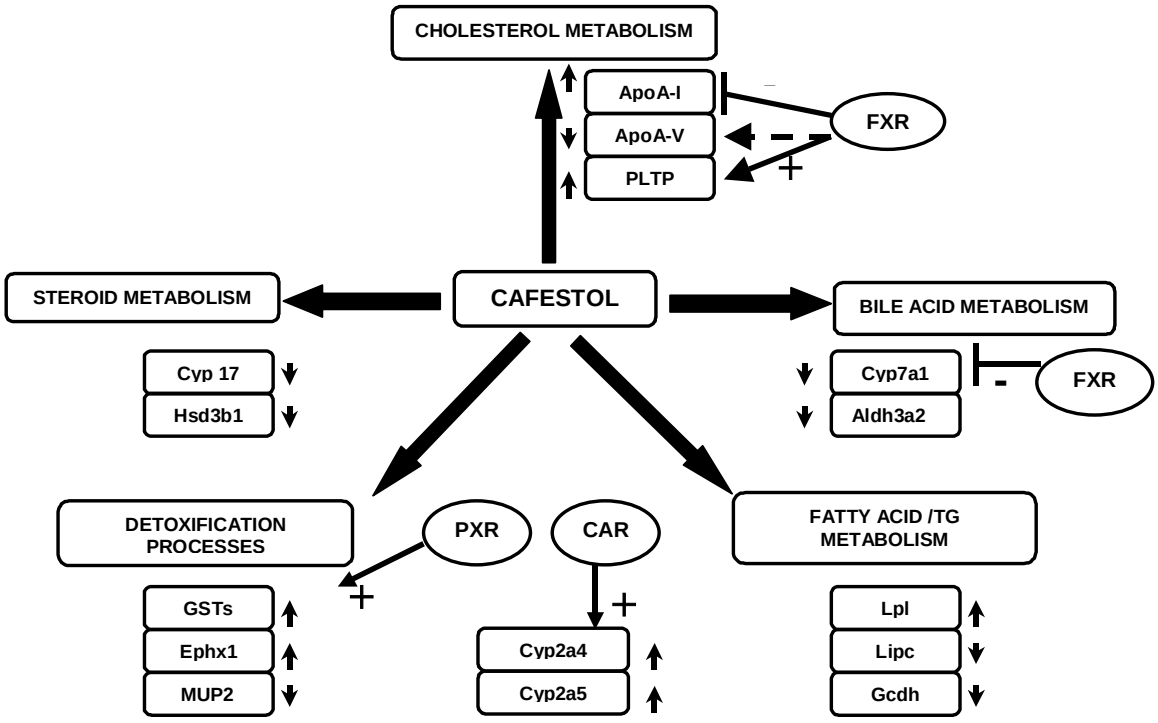
Table 2 Expression of selected FXR and PXR target genes in E3L mice fed cafestol.

Gene	Target gene of	Relative expression	Expected regulation	Remarks
Cyp7A1	FXR and PXR	-85%	decrease	1
GST-p2	PXR	+414%	increase	1
GST-m1	PXR	+375%	increase	1
MUP2	na	-59%	decrease	1
PLTP	FXR	+17%	increase	1
SHP	FXR	-31%	increase	2
BSEP	FXR	+17%	increase	2
Cyp8B1	FXR	-88%	decrease	2
FXR	na	-31%	na	2
LRH-1	na	-48%	decrease	2
NTCP	FXR	-70%	decrease	2

E3L mice were fed cafestol or control diet. After 30 days livers were removed and mRNA was isolated. Gene expression was determined by Northern blot or quantitative RT-PCR. Five genes were analyzed to confirm microarray data and six key genes not present on the array are included as well. Relative expression is expressed as percentage of control, expected regulation based on literature is indicated. Remarks: 1: validation of microarray data 2: genes not present on the microarray. Effects were significant at the $p < 0.05$ level. For figures of the quantitative PCR and Northern blot data see the web (Supplemental Figure I link).


Observed versus expacted fraction
0
1
2
3
4
5
*
*
*
*
Cytoskeleton organization
Transcription/translation
Lipid metabolism
Immune response
Enzymes
Transport
Membrane proteins
Detoxification
Amino acid metabolism
Signal transduction
Function unknown
N= 15 20 42 23 40 16 11 22 10 11 247

A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ApoA-V
PLTP
FXR
STEROID METABOLISM
CAFESTOL
BILE ACID METABOLISM
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Cyp7a1
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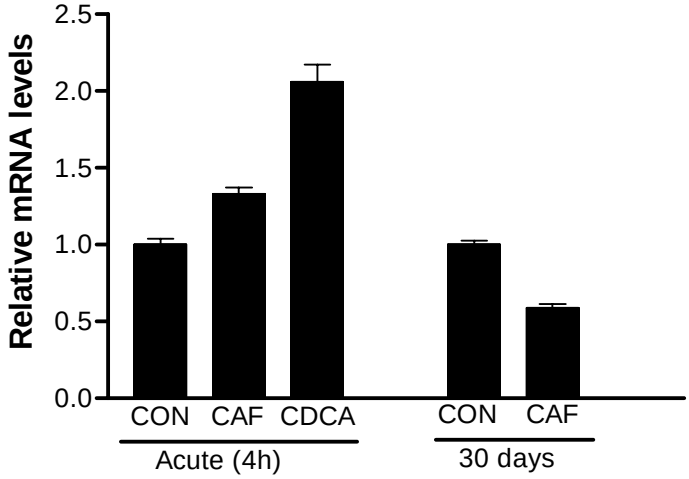
B



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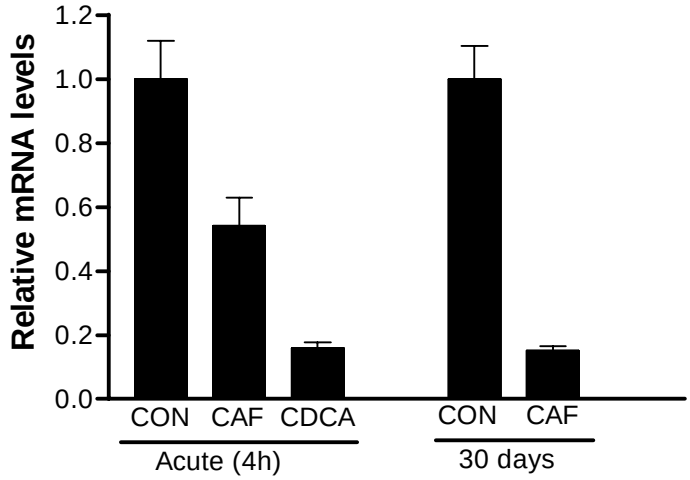


D

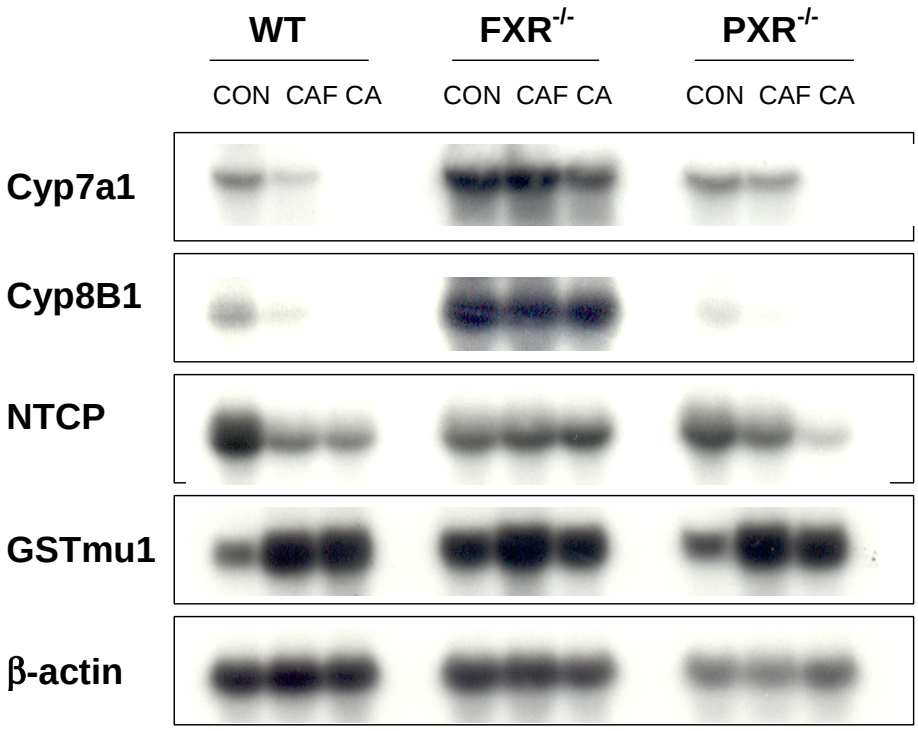
Figure 4 Cafestol differentially regulates FXR and PXR target genes in E3L mice. E3L mice were fed cafestol or control diet. After 30 days hepatic mRNA was isolated and analyzed on Gem 2.03 arrays. Genes were classified using the GO database. **(a)** The number of genes (N) are indicated and the ratio of the observed versus the expected number of regulated genes is shown for the different functional classes. Significantly affected pathways are shown (* $p < 0.001$, Chi-square test).
(b) Overview of genes and pathways affected by cafestol. Arrows indicate the direction of regulation of genes and enzymes. Nuclear receptors through which cafestol may exert its effect (positive: **+**, negative: **-**) are indicated.. **(c)** Expression of SHP Measured by quantitative (real-time) PCR after four hours and 30 days of cafestol treatment. Data are means ± SEM. **(d)** Expression of Cyp7a1 Measured by quantitative (real-time) PCR after four hours and 30 days of cafestol treatment. Data are means ± SEM.

Analysis of cafestol response in FXR and PXR knockout mice

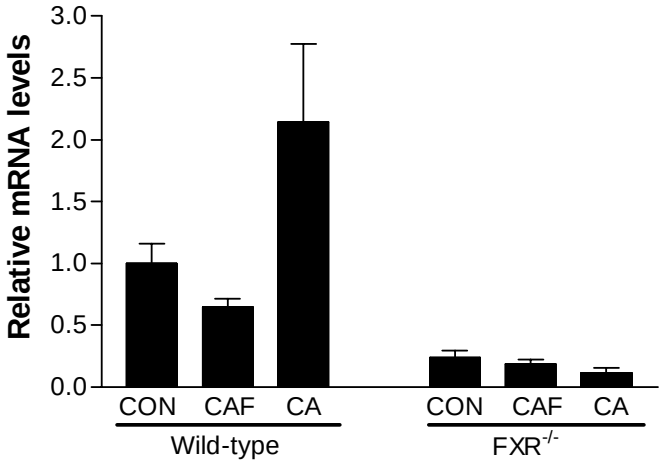
Effects of cafestol on gene expression in wild type, homozygous FXR knockout ($FXR^{-/-}$) and homozygous PXR knockout mice ($PXR^{-/-}$) were compared to analyze the role of these receptors in the *in vivo* response to the coffee compound. When wild type mice were fed the FXR agonist cholic acid (CA) for 7 days, the expression of the three negative FXR target genes Cyp7A1, Cyp8B1 and NTCP was strongly repressed, whereas cafestol feeding resulted in a more modest but reproducible inhibition (Fig. 5A). In the $FXR^{-/-}$ mice, basal expression of all three genes was increased, as expected, and expression was completely unresponsive to either CA or cafestol feeding, demonstrating that FXR is required for the repression of these three genes by both CA and cafestol *in vivo*.

In contrast to these effects on genes down-regulated by FXR, no consistent effects of cafestol feeding were observed *in vivo* on genes known to be up-regulated by FXR. For example, feeding of cafestol did not significantly affect BSEP expression in either wild type or $FXR^{-/-}$ mice (Fig 5B), and induced only a slight increase in BSEP expression in E3L mice (Table 2). In addition, cafestol feeding was able to induce expression of several reported PXR targets, including Gst-m1 (Fig. 5A and Table 2). However, these responses were not lost in the $PXR^{-/-}$ mice.

Together the results of the feeding studies show that cafestol induces expression of several genes in E3L and wild-type mice, and for a number of FXR target genes these effects are abolished in $FXR^{-/-}$ mice. However, the induction of PXR target genes by cafestol observed in E3L and wild type mice was not abolished in $PXR^{-/-}$ mice.



A



B

Regulation of FXR and PXR target gene expression by cafestol in wild type versus knockout mice. (a) Groups of 6 male WT, FXR$^{-/-}$, or PXR$^{-/-}$ null mice were fed with control diet (CO), control diet supplemented with 0.25% (wt/wt) cafestol (CAF), or control diet supplemented with 1% (wt/wt) cholic acid (CA) for 7 days as indicated. Total RNA was prepared from the liver of each individual mouse and equivalent amounts of RNA were pooled together for each treatment group respectively. Twenty micrograms of each RNA sample was used for Northern hybridization with different probes as indicated. **(b)** Quantitative RT-PCR was used to analyze BSEP expression in livers of wild type and FXR$^{-/-}$ mice. Data was normalized to a β-actin as internal control. Data are presented as mean ± SEM, n=6 mice per treatment, per group, in triplicate.

Discussion

Cafestol is the most potent cholesterol-raising compound identified in the human diet, but its mode of action has remained unclear (1-4). We now show that cafestol specifically activates FXR and PXR. Moreover, cafestol induces coactivator recruitment to both receptors indicating that it functions as a direct agonist. One of the strengths of our study is that the regulation of FXR and PXR by cafestol was observed independently in three laboratories (Baylor, Leiden, and Wageningen) using different mouse strains and methods. We observed significant FXR activation at concentrations as low as 20 μM cafestol, which corresponds to 7 μg/ml. Intake of cafestol ranges from 0 to 65 mg or more per day in humans, depending on the amount of unfiltered coffee consumed, of which 70% is absorbed (37). Although there are currently no data available on the concentration of cafestol in serum or liver in humans, it is likely that the amount of cafestol present in hepatocytes after consumption of five or more cups of unfiltered coffee is sufficient to affect FXR activity.

Cafestol does not raise serum cholesterol levels in several animal species but is effective in E3L mice, a well-established model to study diet-induced hyperlipidemia (29). Previous studies showed that in both rat hepatocytes and in livers of E3L mice, cafestol repressed expression and activity of Cyp7A1, the rate-controlling enzyme for the conversion of cholesterol to bile acids (16, 38). In the present study, we observed suppression of Cyp7A1 expression and also of Ntcp and Cyp8B1 in cafestol fed E3L mice and wild-type C57BL/6 mice. The absence of such repression in the $FXR^{-/-}$ mice clearly demonstrates the role of FXR in this response to cafestol. The down-regulation of Cyp7A1 expression by specific FXR activators is well described (18, 19) and is mediated by induction of the negative regulator SHP (22, 23). However, the loss of the effect of high levels of bile acids on their own expression in $FXR^{-/-}$ (48) but not $SHP^{-/-}$ (22, 23) mice strongly suggests that an additional FXR-dependent, but SHP-independent pathway must exist for the negative regulation of Cyp7A1 and other target genes by bile acids. Cafestol may activate this as yet unexplained pathway, since SHP shows only a transient up-regulation cafestol

treated animals that is generally not apparent in those showing decreased expression of the negative FXR target genes.

Experiments with isolated hepatocytes from wild type and $FXR^{-/-}$ mice confirm that cafestol can up-regulate expression of BSEP through FXR. As with SHP, however, the up-regulation of BSEP by cafestol *in vivo* was equivocal and similar effects were observed with PLTP. Lack of responsiveness of some FXR target genes *in vivo* may be due, at least in part, to high levels of endogenous bile acids, which should partially activate FXR and blunt the hepatic response to exogenous cafestol. It is also possible that cafestol is a selective modulator of FXR activity. Similar observations have been made for guggulsterone (39), and the potent synthetic agonists GW4064 and fexaramine have also been reported to regulate quite different, only partially overlapping sets of FXR target genes *in vitro* (40). Thus, the flexibility of FXR responsiveness that is apparent in the structural diversity of these ligands may also be reflected in the ability of this receptor to regulate distinct sets of target genes in response to distinct ligands.

Our *in vitro* analyses show that cafestol can also activate PXR, but the results with the $PXR^{-/-}$ mice did not confirm the requirement of this xenobiotic receptor for the responses of its known target genes to cafestol. Cafestol did not appear to function as a direct ligand for the other xenobiotic receptor, CAR. However, CAR can be activated by an indirect, ligand-independent pathway in response to xenobiotics and CAR target genes overlap with those of PXR (36, 41). Thus, CAR activation could account for the persistent induction of several detoxifying enzymes, such as GST-m1 and Cyp2B10, in the $PXR^{-/-}$ mice.

As outlined above, our studies of the molecular action of cafestol in liver have followed both a hypothesis driven path and a broader, large-scale assessment of the effect of cafestol on the expression of 9552 genes. The gene array approach identified 4 key processes specifically affected by cafestol, i.e. lipid metabolism, detoxification, immune response and amino acid metabolism. The current analysis has focused on lipid metabolism and detoxification, which are governed largely by nuclear receptors. Many nuclear receptor target genes remain to be discovered and it is likely that additional genes in our data set, including key genes in fatty acid metabolism, bile acid metabolism and steroid metabolism (fig. 4b) are being regulated through either FXR or PXR. Such genes are candidates, together with Cyp7A1, to explain the effect of cafestol on plasma cholesterol levels. Of course,

cafestol may also affect the expression of these and other genes by altering the activity of additional non-receptor targets.

In summary, we conclude that cafestol, the cholesterol-raising factor from coffee beans, can directly regulate expression of genes involved in lipid and xenobiotic metabolism by activating the nuclear receptors FXR and PXR. Regulation of such FXR and PXR target genes in the liver may contribute to both the cholesterol-raising effect and the putative anti-carcinogenic effect of cafestol in humans. Further elucidation of the underlying mechanisms will lead to insights into the basis for both the cholesterol-raising and the chemopreventive potential of cafestol and to a more complete understanding of the regulation of serum cholesterol levels.

Materials and Methods

Materials

Cafestol was purchased from LKT laboratories Inc (St. Paul, MN). Cafestol acetate, CDCA, clofibrate, DMSO, PCN and rifampicin were purchased from Sigma. T0901317 and LG101268 were gifts from X-Ceptor Therapeutics (San Diego, CA). All other chemicals were of analytical grade and readily available of commercial suppliers. Molecular biology reagents were obtained from Promega (Madison, WI) or Qiagen (Hilden, Germany). All media and supplements for cell culture were purchased from Invitrogen Corporation (Carlsbad, CA) or Cambrex BioScience (Verviers, Belgium). Cell culture plastics were obtained from Corning (Acton, MA), except otherwise indicated.

Plasmids

The various Gal4 fusions to the ligand-binding domain of various nuclear receptors used in this study have been published (39). The PXR AF2 mutants for both mouse (E424K) and human (E427K) were created by side directed mutagenesis using the Quick-Change site-directed mutagenesis kit from Stratagene (La Jolla, CA), according to the manufacturers protocol. The correct sequence for each mutation was confirmed by sequencing. The Δ9C FXR mutant was reported previously (42). Wild type and mutant human BSEP promoter-reporter constructs were kindly provided by Dr. Plass (University Hospital Groningen, The Netherlands) (27). Dr. Koyano (National Institute of Health Sciences, Tokyo, Japan) generously donated a promoter-reporter construct containing part of the human CYP3A promoter (28). The pCMX-rFXR and pSG5-hRXRα constructs were gifts from Dr. Evans (Salk Institute, La Jolla, CA). Dr Kliewer (University of Texas Southwestern Medical Center, Dallas, TX) kindly provided the pSG5-hPXR construct. pCMV-βGal, used as transfection efficiency control, was purchased from Promega.

Cell cultures

Human hepatoma (HepG2) cells and African green monkey fibroblasts (CV-1) were obtained from the American Type Culture Collection (Manassas, VA) or the European Collection of Animal Cell Cultures (ECAAC) (Salisbury, Wiltshire, UK). HepG2 and CV-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. Primary mouse hepatocytes were isolated and cultured as described (43). Twenty hours after hepatocyte isolation, medium was removed and replaced with William's E medium (Invitrogen) containing 56µM cafestol-acetate, dissolved in DMSO or DMSO only. The concentration of DMSO was always < 0.1% (v/v). After another 24 hours the cells were harvested for RNA isolation (see below). Results are presented in Figure 3D.

***In vitro* interaction assays**

For Gal4 assays (results presented in Fig 1), transfections assessing the effect of cafestol on full length FXR and PXR (Fig 2A and Fig 3A), and co-activator recruitment assays (Fig 2B and Fig 3B), 1.3 x 10^5 (HepG2) or 1 x 10^5 (CV-1) cells were plated in 24-well dishes with DMEM supplemented with 10% charcoal stripped serum. Cells were transfected using the calcium phosphate precipitation method. The next morning, cells were washed with phosphate buffered saline and ligands were added. Typically, transfections included 100 ng receptor plasmids, 200ng of the luciferase reporter plasmid, 10ng CDM-RXRα, 200ng of the growth hormone (GH) internal control plasmid (TK-GH), with 490ng pGEM4 as carrier DNA to make a total of 1µg of plasmid DNA per well. For the Gal4 assays the ligand-binding domains of the following receptors were used: constitutive androstane receptor (CAR) both mouse and human, human estrogen receptor alpha (hERα), farnesoid X receptor (FXR), glucocorticoid receptor, liver X receptor-alpha (LXRα), peroxisome proliferator-activated receptor-alpha (PPARα), peroxisome proliferator-activated receptor-gamma (PPARγ), pregnane X receptor (PXR), retinoid X receptor (RXR), thyroid hormone receptor beta (TRβ) and vitamin D receptor (VDR). The full-length human retinoic acid receptor-related orphan receptor-alpha (RORα), and RORβ, and mouse small heterodimer partner (SHP) were used in this assay.

Cells were assayed for luciferase (Promega) activities 24 hours after addition of ligands, and reporter expression was normalized to GH activity (Quest Diagnostics, Chicago, IL) according to the manufacturers' instructions. Similar results were obtained from at least three independent experiments, each performed in triplicate.
For the studies assessing the effect of cafestol upon the BSEP promoter a construct containing the human BSEP promoter (pGL3-1778) was used to measure activation of FXR by cafestol or CDCA (Fig 2C). A similar construct in which the FXR response element in the BSEP promoter had been knocked out (pGL3-1778Mt) was also used. HepG2 cells were seeded in 12-wells plates at a density of 1.5 x 10^5 cells per well. Cells were transfected using the calcium phosphate method with 250 ng promoter construct, 100 ng pCMX-rFXR, 100 ng pSG5-hRxRα, 100 ng pCMV-βGal, and 450 ng pUC19 as carrier DNA to make a total of 1 µg DNA per well.

In studies assessing the effect of cafestol upon the CYP3A4 promoter (Fig 4C), a reporter-gene construct containing part of the human CYP3A4 promoter was used to measure activation of PXR by cafestol or rifampicin. Transfection conditions were as above for the BSEP promoter study. In studies using the promoter constructs, medium was refreshed 18 hours after transfection. After another 3 hours the medium was replaced with medium containing cafestol-acetate or control ligand (CDCA for FXR and rifampicin for PXR), dissolved in DMSO, or DMSO only. The final concentration of DMSO was 0.1% (v/v) in all media. Twenty-four hours after addition of cafestol or control ligand, cells were harvested for determination of reporter activity. Luciferase activity was measured by the Luciferase Assay System (Promega) on a Fluoroskan ascent FL (Thermo Labsystems, Helsinki, Finland) machine. Luciferase activities were normalized to β-galactosidase activities, which were measured using a Multiskan ascent (Thermo Labsystems).

Animal feeding studies and diets

Mice were housed under standard conditions (12hr light/12 hr dark cycle) in ventilated cages in a temperature-controlled environment. Animals had free access to food and water. Experimental procedures were approved by the local Committees for Care and Use of Laboratory Animals.

Hyperlipidemic E3L mice (line # 2) have been described previously (29). In this study, female E3L mice of 10 weeks of age were used that were of the N21st generation (>99% C57BL/6Jico genetic background). Mice received either the control diet (n=7) or the same diet supplemented with cafestol (n=8). The control diet was a semi-synthetic diet, diet W (18.2 MJ/kg; Hope Farms, Woerden, the Netherlands) enriched with saturated fat (15g/100g) and cholesterol (0.25g/100g) (44, 45). This diet was supplemented with 0.04% (wt/wt) cafestol for the cafestol diet. The cafestol diet also contained 0.02% (wt/wt) of a closely related compound, kahweol. Amounts of diterpenes in the diet were checked by HPLC (46). After 30 days, mice were sacrificed after bleeding and the liver was removed and immediately frozen in liquid nitrogen and stored at –80^{o}C. For an acute feeding study, 10 female E3L mice per treatment group were used. Mice were given by oral gavage either carrier alone (control, methyl-cellulose), cafestol 0.05% (wt/vol) or CDCA (0.5% wt/vol) dissolved in methyl-cellulose, in a volume of 200 µl. Four hours later the mice were sacrificed and their livers harvested.

Hepatocytes were isolated from male wild type ($FXR^{+/+}$) and $FXR^{-/-}$ mice on a mixed C57BL/6J-SV129 background generated by Tularik Inc (San Fransisco, USA) (47). Note that these $FXR^{-/-}$ mice are from a different strain than the $FXR^{-/-}$ mice that were used in the feeding studies.

$FXR^{-/-}$ mice used for feeding experiments were kindly provided by Dr Gonzalez, (NIH, Bethesda, MD) (48), and were maintained with a C57BL/6 background. $PXR^{-/-}$ mice were obtained from Dr Evans (The Salk Institute for Biological studies, La Jolla, CA) and were maintained with a mixed C57BL/6/129 background. C57/BL6 mice were used as the wild type control, backcrossed onto the strain for >4 generations. Routine genotyping was performed on all mice using genomic DNA extracted from tails by PCR using previously reported primer sequences (48, 49). Age-matched groups of 8-10 week-old male wild type (WT), $FXR^{-/-}$ and $PXR^{-/-}$ mice were used in all experiments (n=6 per experimental group). Feeding experiments were performed with a control diet (rodent diet 5001, LabDiet, Brentwood, MO) supplemented with 0.25% cafestol, or 1% cholic acid for 7 days (all wt/wt). On the last day the mice were fasted for 4h, anesthetized and blood was collected from the orbital plexus.

Serum analysis

Blood from E3L mice was collected in a microcuvette tube (Sarstedt, Numbrecht, Germany). Serum was obtained by centrifuging the tubes at 1.200 g for 10 mins, and stored at -20^{o}C. Total serum cholesterol (kit # 236691, Boehringer Mannheim), triglyceride without free glycerol (kit # 337-B, Sigma), alanine aminotransferase (ALAT, kit # 745138 Boehringer Mannheim), free fatty acids (FFA's, NEFA-C kit, WAKO Chemicals) and bilirubin (kit # 905321, Roche) were measured enzymatically after a 4hr fast. Ketone bodies (β-hydroxybutyrate, kit # 310-A, Sigma) were determined after a 24hr fast. Results are presented in Table 1.

For the feeding study of FXR$^{-/-}$ and PXR$^{-/-}$ mice, serum was prepared from whole blood on day 0 and day 7 by centrifugation at 1.200 x g for 10 minutes using Microtainer serum separator tubes (Terumo Medical Corp., Elkton, MD). Serum was frozen in aliquots and stored at –20^{o}C. Enzymatic kits were used for the determination of serum cholesterol and triglycerides (Sigma, St. Louis, MO). Serum ALT levels were measured by routine clinical chemistry testing performed by the chemistry laboratory of the Methodist Hospital, Houston, TX.

Gene expression profiling and functional annotation of genes

Messenger RNA from the caudate liver lobe of E3L mice was isolated using RNA-STAT 60 (Tel-test Inc Friendswood, TX) and labeled with Cy5 and Cy3 fluorescent dyes for hybridization as previously described (50). The labelled RNA was hybridized on Gem 2.03 micro-arrays containing 9552 genes and EST's (Incyte Genomics, Palo Alto, CA). Each sample was hybridized in duplicate. cDNA arrays were scanned on a GenePix 4000A scanner. The data were normalized and analyzed using the GEMTools software version 2.5.1 (Incyte Genomics). To determine if the signal was sufficiently high above background and not the result of non-uniform noise, a spot was included in the analysis when it showed a signal to background ratio of at least 2.5, a signal intensity above 250 arbitrary units for one or both dyes, and a spot size of at least 40% of the spotted area.

For each individual mouse, significant differential expression of the genes was determined by use of the Z-test on the log transformed expression ratios of the duplicate arrays, using all valid (7816) measurements (51, 52). Based on the Z-test,

the overall minimal significant difference in expression was determined at fold change (FC) = 1.4 with a power (1 – β) of 0.8. Hierarchical clustering was performed by average linkage clustering using euclidean distance in Spotfire (Spotfire Inc., Massachusetts). GeneHopper was used to retrieve information from the GeneOntology database (version December 2003) (53, 54). To gain information on metabolic pathways, LocusLink IDs were run through the Kyoto Encyclopedia of Genes and Genomes (KEGG: www.genome.ad.jp/kegg, version December 2003) (55). The expected number of differentially regulated genes per category (E) was calculated as follows: E=C/T x D, where C= total number of genes per category on the array, T= total number of genes on the array and D = total number of differentially expressed genes. Results of the microarray study are presented in Figure 4 A and B. Differential expression of selected target genes was validated by quantitative RT-PCR and Northern blot as previously described (56, 57), and are presented in Table 2.

RNA isolation and mRNA quantitation

Total RNA from primary hepatocytes was isolated using the SV total RNA Isolation System (Promega). A reverse transcription reaction was performed on 3 μg total RNA using the Reverse Transcription System (Promega). cDNA levels of β-actin (endogenous control) and BSEP were measured by quantitative RT-PCR on an iCycler iQ (Biorad, Veenendaal, the Netherlands). Primers and probes used for detection of BSEP and β-actin have been published (58, 59) All reagents for quantitative PCR were from Eurogentec (Seraing, Belgium).

Total RNA was isolated from livers of cafestol-fed wild type, $FXR^{-/-}$ and $PXR^{-/-}$ mice using Tri Reagent (Invitrogen) according to the manufacturer's instructions. Gene expression was analyzed by Northern blot analysis (Fig 5A), performed as described (60), or by quantitative PCR (Fig 5B). Mouse cDNA probes for the genes analyzed in this study were prepared from liver total RNA using the Superscript One-step RT-PCR kit (Invitrogen) using primers designed on published sequences (GeneBank). A single blot was serially hybridized with the various probes, with β-actin serving as the control for equivalent loading. For the quantitation of BSEP mRNA (figure 5b), the Taqman one-step RT-PCR Master mix reagents (Applied

Biosystems, Branhburg, NJ) was used according to the manufacturer's instructions with the same primers and probes as above.

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5

Effect of coffee oil on bile acid synthetic activity in humans

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In preparation

Abstract

Background Unfiltered coffee brews such as espresso contain a lipid from coffee beans named cafestol that raises serum cholesterol in humans. Cafestol decreases expression and activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol into bile acids, in cultured rat hepatocytes and livers of APOE3Leiden mice. Inhibition of bile acid synthesis has been suggested to be responsible for the cholesterol-raising effect of cafestol. Therefore we assessed whether cafestol decreases activity of cholesterol 7α-hydroxylase in humans.

As liver biopsies were not feasible we measured serum levels of 7α-hydroxy-4-cholesten-3-one, which is a marker for the activity of cholesterol 7α-hydroxylase in the liver.

Methods The response of 7α-hydroxy-4-cholesten-3-one in serum was measured in two separate five-week periods in which healthy volunteers consumed coffee oil containing cafestol (69 mg/day).

Results Serum levels of 7α-hydroxy-4-cholesten-3-one increased by 47% (n=38, $CI_{95\%}$ 21;73) in the first period and by 23% (n=31, $CI_{95\%}$ 2;43) in the second treatment period. Serum cholesterol was raised by 23% ($CI_{95\%}$ 20;27) in the first period and by 18% (CI95% 14;23) in the second period. We corrected individual 7α-hydroxy-4-cholesten-3-one levels for serum cholesterol levels, because coffee oil increases serum cholesterol and 7α-hydroxy-4-cholesten-3-one is probably present in the lipoprotein fraction within the serum. After correction the increase in

7α-hydroxy-4-cholesten-3-one was 24% ($CI_{95\%}$ 1;46) in the first period and 5% ($CI_{95\%}$ -13;24) in the second period.

Conclusions Our study showed that coffee oil did not decrease serum levels of 7α-hydroxy-4-cholesten-3-one in humans during two separate treatment periods. Therefore, this study does not support the hypothesis that cafestol decreases bile acid synthesis in humans.

Background

Humans and animals regulate their metabolism in response to dietary compounds. For example, fatty acids can regulate lipid metabolism. *In vitro* studies suggest that these effects are mediated by the peroxisome proliferators-activated receptors, which are transcription factors that belong to the superfamily of nuclear receptors (1). Identification of such key regulators is important in understanding the molecular mechanisms underlying dietary responses. Elucidation of such mechanisms allows us to identify novel dietary components that can affect metabolism and potentially health. However, before one can apply knowledge of molecular mechanisms to identify such compounds in the human diet it has to be established that the potential mechanism indeed is of significance in humans.

In this study we used coffee oil to explore a new potential pathway for the mode of action of food components that affect serum lipids. Oil from coffee beans contains the diterpenes cafestol and kahweol that are responsible for the cholesterol-raising effect of unfiltered coffee types (2-5). Cafestol raises serum cholesterol more potently than the related diterpene kahweol, which is also found in coffee beans (4). Cafestol is the most potent cholesterol-raising substance known. Therefore, elucidation of the mechanism by which cafestol achieves this large effect on serum cholesterol would be helpful in understanding how certain food components can affect lipid metabolism. A number of potential mechanisms have been proposed, such as downregulation of the low density lipoprotein receptor (LDL-R) and regulation of lipid transfer proteins such as cholesteryl ester transfer protein (CETP), phospholipids transfer protein (PLTP), and lecithin:cholesterol acyltransferase (LCAT) (6-8). However**,** it remains unclear by which mechanism cafestol affects these proteins.

Cafestol feeding causes a rise in serum cholesterol in APOE3Leiden mice after three weeks. This rise is accompanied by a decrease in Cholesterol 7α-hydroxylase (Cyp7a1) expression of 58% (9) and faecal bile acid content is reduced by 41% after cafestol feeding (9). Cyp7a1 is the rate-limiting enzyme in the conversion of cholesterol into bile acids in the liver. When rats or mice are fed cholesterol they upregulate Cyp7a1; this enzyme converts the extra cholesterol into bile acids which are excreted with the faeces. In this way rodents avoid the rise in serum cholesterol induced by dietary cholesterol in other species. Cyp7a1 expression is regulated by a number of nuclear receptors including the farnesoid X receptor (FXR), the pregnane X receptor (PXR), and the liver X receptor (LXR). There are differences in regulation of bile acid metabolism between rodents and humans. For example, mice have a response element for LXR in the Cyp7a1 gene that humans lack (10, 11). LXR is an oxysterol receptor that causes upregulation of Cyp7a1 upon activation (12, 13). This could explain why rodents lack the response of serum cholesterol to dietary cholesterol that humans show. Besides oxysterols expression of Cyp7a1 is also regulated by bile acids. Activation of FXR by bile acids causes downregulation of Cyp7a1 through an indirect mechanism (14, 15). PXR also downregulates activity of Cyp7a1, although the mechanism behind this effect is not clear yet (16, 17). See figure 1 for a simplified overview of the regulation of Cyp7a1 by nuclear receptors.

Recently, we showed that cafestol interacts with FXR and PXR (Chapter 4). Interaction of cafestol with these two receptors could explain the observed downregulation of Cyp7a1 in livers of APOE3-Leiden transgenic mice (6, 9, 18). Suppression of bile acid synthesis leads to increased levels of hepatic cholesterol and this could lead to downregulation of the LDL receptor. This results in an increase of serum LDL-cholesterol. In humans, cafestol intake also leads to an increase in serum LDL-cholesterol. Whether this is caused by a decrease in Cyp7a1 activity is not known. However, Cyp7a1 deficiency in humans does lead to an increase in serum lipid levels (19). Due to the differences in regulation of bile acid synthesis between rodents and humans, it is essential to establish whether cafestol also regulates activity of Cyp7a1 in humans.

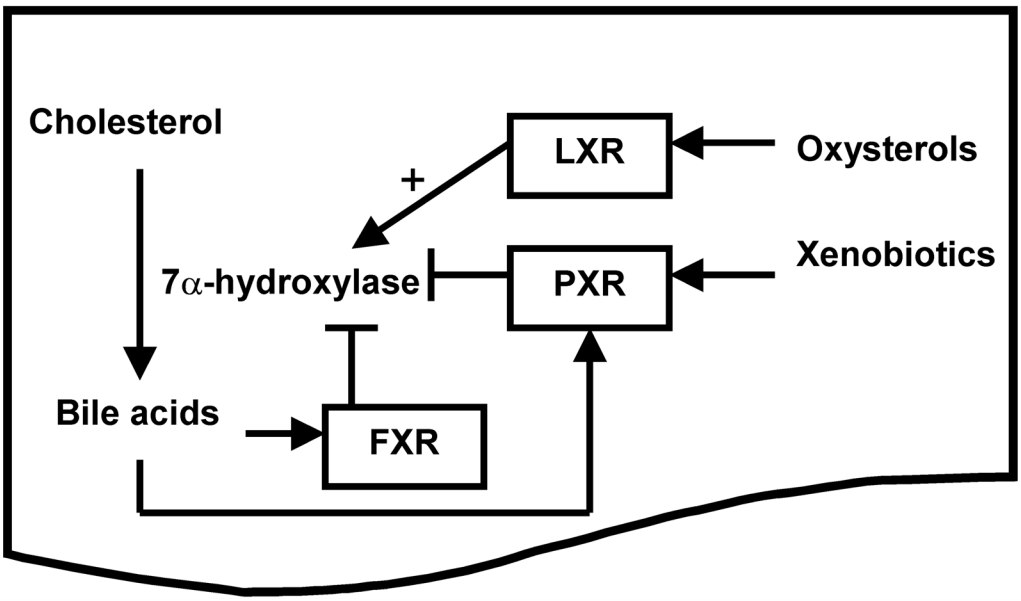

Figure 1 Regulation of cholesterol 7α-hydroxylase by nuclear receptors. The farnesoid X receptor (FXR) is a bile acid receptor that can downregulate 7αhydroxylase via an indirect pathway. The pregnane X receptor (PXR) is a xenobiotic receptor that is also activated by endogenous bile acids. Upon activation PXR can downregulate 7αhydroxylase via an unknown pathway. The liver X receptor (LXR) is an oxysterol receptor that upon activation induces 7αhydroxylase.

Because it is ethically not acceptable to take liver biopsies to study the effect of cafestol on expression and activity of Cyp7a1 in human livers, we used an indirect method. In this method the level of 7α-hydroxy-3-cholesten-4-one, a metabolite of cholesterol, is measured in plasma. 7α-Hydroxy-3-cholesten-4-one is an intermediate in bile acid synthesis and its level in human plasma is considered to reflect Cyp7a1 activity in the liver (20-22).

Methods

Subjects

Subjects were recruited and screened as previously described (23). Fifty subjects were enrolled. During the study five subjects withdrew cooperation: three subjects suffered from stomach complaints, one went abroad, and one had a gastrointestinal infection. Another 13 subjects had to be excluded during the study because their serum activities of ALAT and ASAT exceeded previously determined boundaries. These boundaries were 2.7 times the upper limit of normal for ALAT and 1.5 times the upper limit of normal for ASAT. The Medical Ethical Committee of Wageningen

University and Research Centre approved the study. Each volunteer gave an informed consent in writing.

Study design

Subjects first entered a run-in period of three weeks in which they received four placebo capsules daily. Placebo capsules contained 0.25 ml sunflower oil and 0.25 ml safflower oil per capsule so as to mimic the fatty acid composition of coffee oil. After the run-in period subjects took four coffee-oil capsules a day (2 ml oil per day) for a five-week period. The coffee-oil capsules provided 69 mg cafestol and 51 mg of kahweol per day. The run-in and the coffee-oil period together constituted period 1. The change in the level of serum lipids from the end of run-in period 1 to the end of coffee-oil period 1 was defined as response 1. This first coffee-oil period was followed by a three-week wash-out period in which no capsules were supplied. After the wash-out period subjects repeated the first two periods: a three-week run-in period (run-in 2) and a five-week coffee-oil period (coffee-oil period 2). See figure 2 for a diagram of the study design.

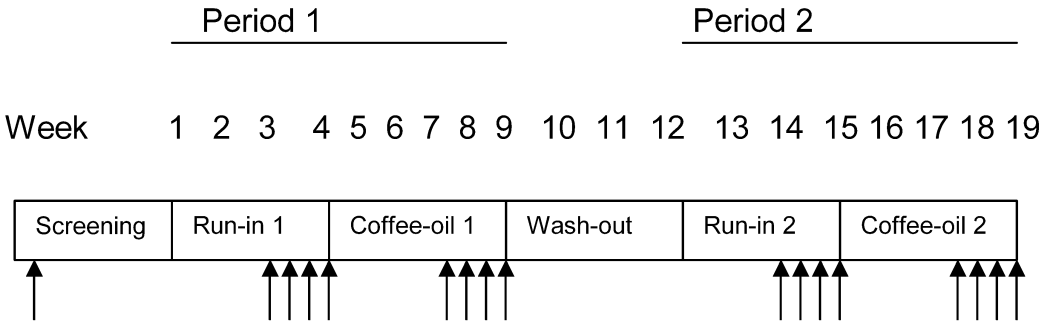

Figure 2 Diagram of the study design
The week number is indicated above. Black arrows indicate blood sampling days.

Laboratory measurements

Blood samples were taken from subjects who had fasted overnight on four separate days in the last two weeks of the run-in and coffee-oil periods. In the 16 serum samples of each person levels of total cholesterol, HDL-cholesterol, triglycerides, alanine aminotransferase, and aspartate aminotransferase were measured as previously described (23). LDL-cholesterol levels were calculated (24).

In addition, a number of liver function markers were measured in the serum samples: alkaline phosphatase, bilirubin, γ-glutamyl transpeptidase, and lactate

dehydrogenase. We also measured levels of amylase, a marker for pancreatitis. All tests were performed using Flex reagents (Dade Behring, Liederback, Germany). Levels of bile acids were also determined in serum using an Enzabile kit (Bio-Stat diagnostic systems, Stockport, UK).

For the analysis of the bile acid precursor 7α-hydroxy-4-cholesten-3-one we pooled plasma samples per subject. This yielded four plasma samples, one for each run-in and one for each coffee-oil period. We determined plasma levels of 7α-hydroxy-4-cholesten-3-one using the HPLC method described by Gälman et al (22). We were not able to measure 7α-hydroxy-4-cholesten-3-one levels in samples of two subjects in period 1 and of one subject in period 2. This was due to the presence of a compound in the serum that interfered with our internal standard. In total we were able to measure 7α-hydroxy-4-cholesten-3-one levels in samples of 38 subjects in the first period and of 31 subjects in the second period (see figure 3 for information on the samples that were analyzed). The four samples were analyzed within one run. In our hands the within-run coefficient of variation was 9.7%.

Statistics

A subject's response to coffee-oil in period 1 was defined as the mean level at the end of coffee-oil period 1 minus the mean at the end of run-in period 1. For the serum samples in which levels of liver function parameters and bile acids were determined the mean level in a period was calculated as the mean of the four repeated measurements in each period. The response in period 2 was calculated in the same way. We compared means using the Student's t test for paired samples.

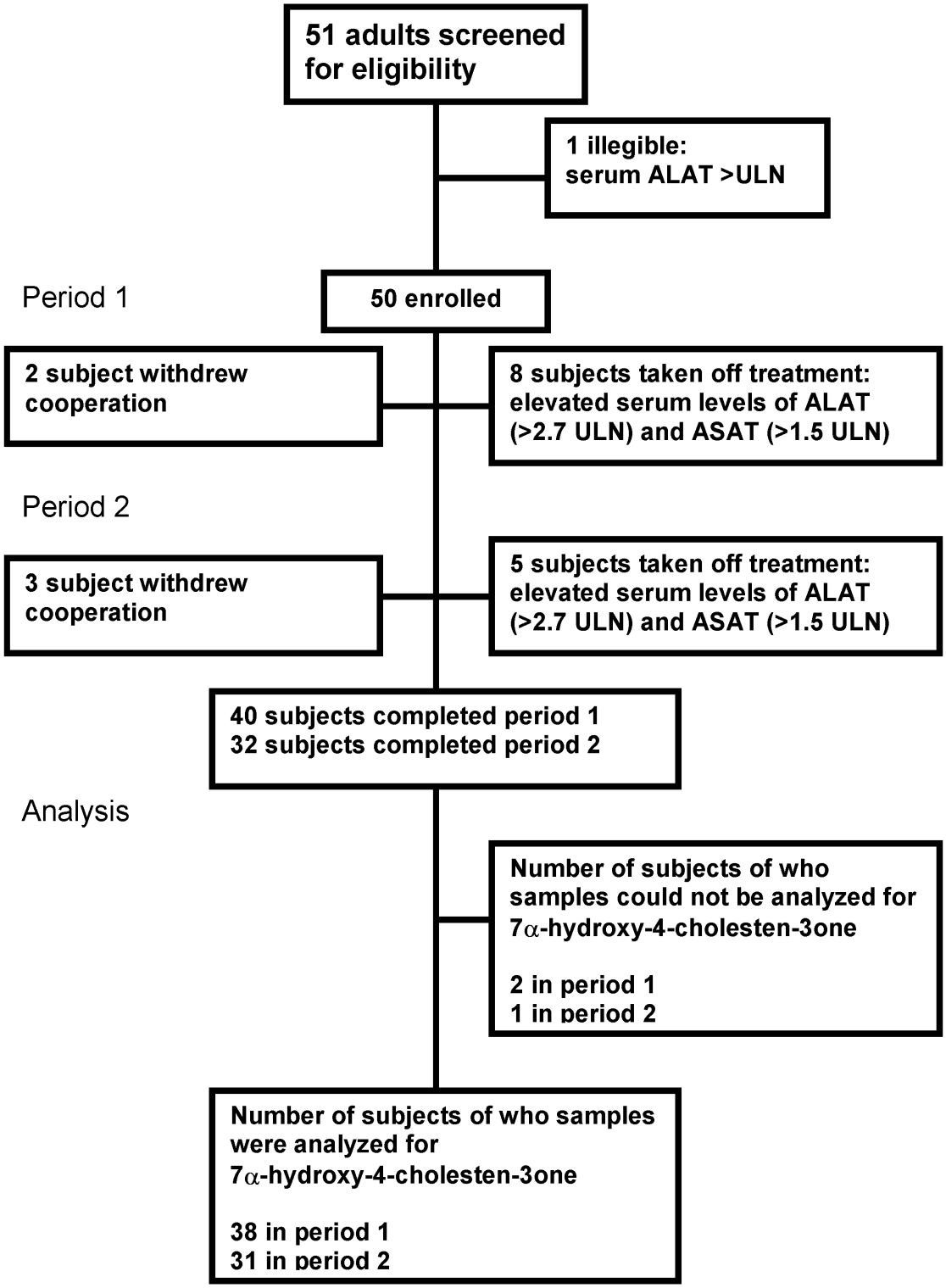

Figure 3 Diagram of the number of subjects that were screened, enrolled, and excluded.
ULN = times the upper limit of normal, ALAT = alanine aminotransferase, and ASAT = aspartate aminotransferase.

Results

Table 1 presents changes in serum variables in units/l or µmol/l for each of the two treatment periods. In the text of this section the results are presented as the mean change over both periods.

Table 1 Response of serum lipids and liver function parameters to coffee oil during two separate periods

Parameter	Baseline	Response period 1		Response period 2	
		n=38		n=31	
7alpha-hydroxy-4-cholesten-3-one (ng/ml)	7.5	3.5**	[1.6;5.5]	1.8*	[0.2;3.5]
Total cholesterol (mmol/l)	4.4	1.0**	[0.9;1.2]	0.8**	[0.6;1.0]
High density lipoproteins (mmol/l)	1.5	0	[-0.07;0.04]	0	[-0.02;0.12]
Low density lipoproteins (mmol/l)	2.4	0.7**	[0.6;0.8]	0.5**	[0.3;0.6]
Triglycerides (mmol/l)	1.1	0.7**	[0.6;0.9]	0.7**	[0.5;0.8]
Alanine aminotransferase (IU/l)	17	27**	[17;37]	14**	[9;18]
Aspartate aminostransferase (IU/l)	17	11**	[6;17]	5**	[3;7]
Alkaline phosphatase (U/l)	64	-5**	[-7;-3]	-9**	[-11;-6]
Amylase (U/l)	75	3	[-0.4;6]	-4*	[-6;-1]
Bile acids (umol/l)	3.3	0.2	[-0.5;0.8]	0.4	[-0.3;1.1]
Bilirubin (umol/l)	12.6	-0.9	[-1.9;0.1]	0.4	[-0.5;1.3]
γ-glutamyltranspeptidase (U/l)	14.9	-0.7	[-1.9;0.4]	-4.5**	[-5.8;-3.2]
Lactate dehydrogenase (U/l)	276	1	[-11;13]	-6	[-14;1]

Values are mean responses with 95% confidence intervals between brackets.
* $p<0.05$ ** $p<0.01$

Serum lipids

Responses of serum lipids to coffee oil in this study were previously published (23). The lipid values presented here are the recalculated values after omission of the serum lipid responses of subjects of whom we could not obtain a response of 7α-hydroxy-4-cholesten-3-one. Serum total cholesterol was raised by 21%, triglycerides were raised by 60%, and LDL-cholesterol was raised by 24%. We did not observe a response of HDL-cholesterol levels to coffee oil treatment.

Liver function parameters and bile acids

Levels of alanine aminotransferase were raised by 112% after coffee-oil treatment and levels of aspartate aminotransferase were raised by 46%. Serum levels of alkaline phosphatase were decreased by 10% and levels of γ-glutamyltranspeptidase were decreased by 15%. The mean response of amylase was -1%. Amylase was non-significantly raised in the first period and significantly decreased in the second period. We observed no significant effects of coffee oil treatment on serum levels of bilirubin and lactate dehydrogenase.

7α-hydroxy-4-cholesten-3-one and bile acid response to coffee oil

We observed a mean increase in plasma 7α-hydroxy-4-cholesten-3-one over both periods of 35%. We found no correlation (n=30, r=-0.02) between the 7α-hydroxy-4-cholesten-3-one responses in the two treatment periods. Plasma oxysterols are mostly found in the lipoprotein fractions (25). Therefore, a rise in the serum level of 7α-hydroxy-4-cholesten-3-one could be explained by an increase in levels lipoprotein fractions in serum. Cafestol treatment causes an increase in cholesterol in serum. In order to correct for this increase in serum cholesterol we divided the individual levels of 7α-hydroxy-4-cholesten-3-one by the corresponding serum levels of total cholesterol. The response of 7α-hydroxy-4-cholesten-3-one after correction was 24% ($CI_{95\%}$ 1;46) in the first period and 5% ($CI_{95\%}$ -13;24) in the second period. The mean increase in 7α-hydroxy-4-cholesten-3-one levels was 14% after correction. Bile acids in serum were increased by 9%. See figure 4 for 7α-hydroxy-4-cholesten-3-one levels.

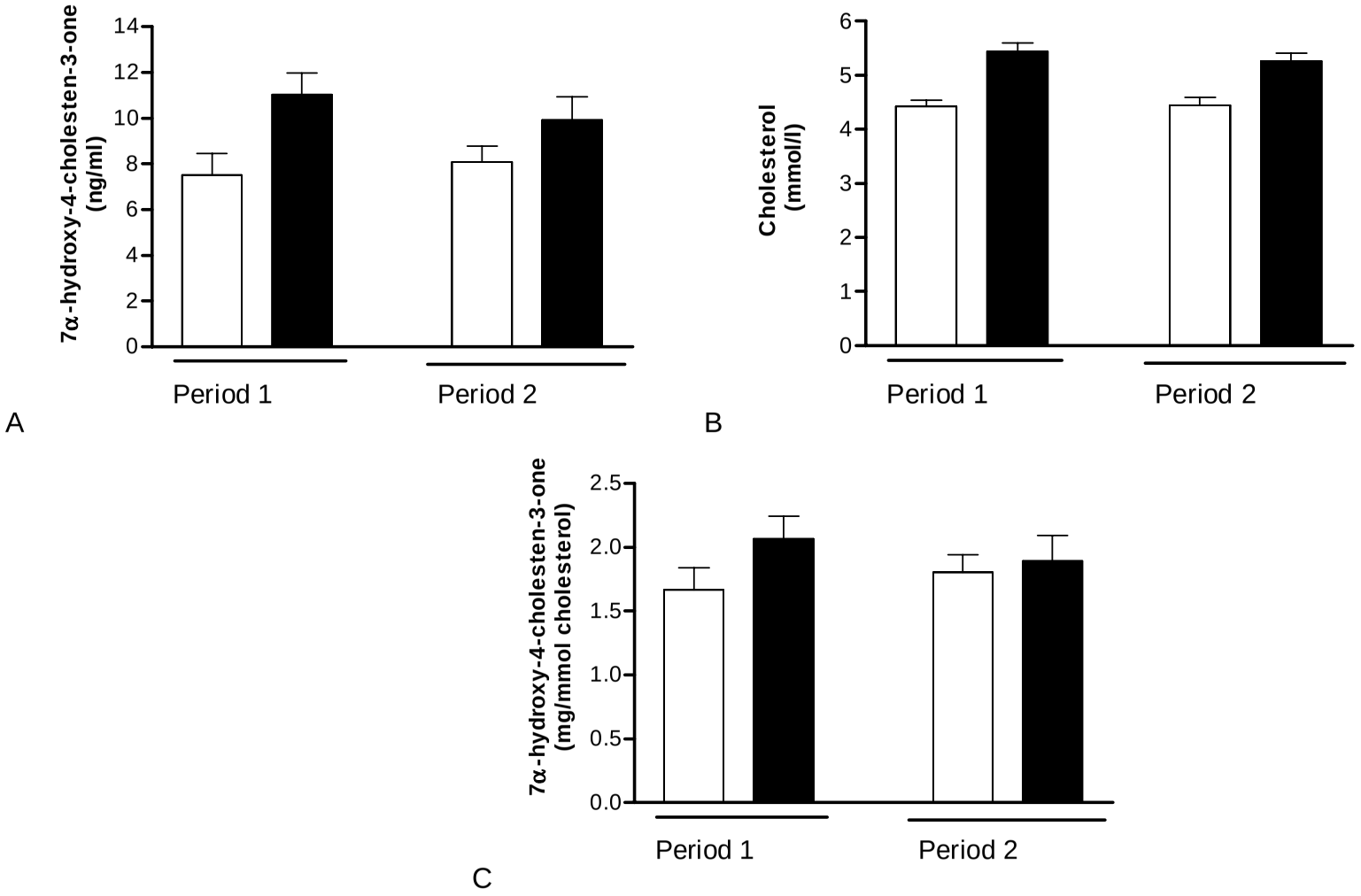

Figure 4 Levels of 7α-hydroxy-3-cholesten-4-one
The white bars indicate the levels in the run-in and the black bars indicate the levels after coffee oil treatment. Levels are given as means with standard error bars. **a)** shows the uncorrected results **b)** shows the serum cholesterol levels **c)** shows the results corrected for cholesterol level.

Discussion

In this study we showed that cafestol did not decrease serum 7α-hydroxy-4-cholesten-3-one levels during two separate treatment periods in humans. This would suggest that Cyp7a1 activity in the liver was not decreased by the coffee oil treatment. Previous studies observed a decrease in expression and activity of Cyp7a1 after treatment with cafestol in cultured rat hepatocytes and livers of APOE3-Leiden mice (18). Based on these studies, we expected a decrease in Cyp7a1 activity and therefore in serum 7α-hydroxy-4-cholesten-3-one in humans upon cafestol treatment upon cafestol intake. We designed the study to detect a decrease or increase in 7α-hydroxy-4-cholesten-3-one levels of at least 20%. The other variables measured in the serum of subjects in this study showed the typical response to cafestol: total cholesterol and LDL-cholesterol were increased and the liver enzymes ALAT and ASAT were also increased (2, 5). Furthermore, alkaline phosphatase was decreased and γ-glutamyltranspeptidase was deceased during the second treatment period as has been observed in previous studies (2, 5, 26).

The increases in 7α-hydroxy-4-cholesten-3-one levels can be partly explained by the increase in serum cholesterol. Due to its lipohilic nature 7α-hydroxy-4-cholesten-3-one is, like other oxysterols, probably present in the lipoprotein fractions. However this does not explain why we did not observe a decrease in 7α-hydroxy-4-cholesten-3-one levels as we expected. Due to the consecutive design of the study we cannot exclude the possibility of a seasonal effect on serum levels of 7α-hydroxy-4-holesten-3-one. However, after the wash-out period the serum level of 7α-hydroxy-4-cholesten-3-one was comparable to the level at the beginning of period 1 and therefore, it is unlikely that the observed increase is due to seasonal variation. We measured 7α-hydroxy-4-cholesten-3-one levels in the last two weeks of both coffee-oil treatment periods. It is possible that Cyp7a1 activity and therefore 7α-hydroxy-4-cholesten-3-one levels were decreased in the first three weeks of the treatment periods. However, it would then be unlikely that the effect of cafestol on Cyp7a1 contributes to the serum lipid response, because the effect on LDL-cholesterol is still present after six months of daily consumption of French press

coffee (26). Furthermore, influence of diurnal variation on the outcome of this study is also unlikely, because blood was taken at the same time each blood-sampling day.

It is possible that the response of 7α-hydroxy-4-cholesten-3-one to cafestol in serum of humans does not reflect the change in Cyp7a1 activity in liver. However, in patients serum levels of 7α-hydroxy-4-cholesten-3-one reflect Cyp7a1 activity in the liver (20, 21). Furthermore, serum levels of 7α-hydroxy-4-cholesten-3-one are closely correlated with the synthesis rate of chenodeoxycholic acid and cholic acid in humans (27). To our knowledge it is not known if the level of 7α-hydroxy-4-cholesten-3-one reflects the response of Cyp7a1 activity to dietary changes in humans. However, treatment of patients with chenodeoxycholic acid reduced serum levels of 7α-hydroxy-4-cholesten-3-one by more than 80% in two patients (28). This suggests that this marker responds to treatment. In addition, serum levels of 7α-hydroxy-4-cholesten-3-one follow diurnal variation in Cyp7a1 activity in rat livers (22). In rabbits cholesterol feeding causes an increase in Cyp7a1 activity, while bile drainage causes a decrease in Cyp7a1 activity. The response of 7α-hydroxy-4-cholesten-3-one in serum is correlated with Cyp7a1 activity in rabbit livers after correction for the response of serum cholesterol (29). Together, these studies indicate that serum levels of 7α-hydroxy-4-cholesten-3-one reflect activity of Cyp7a1 in the liver.

Another possibility is that 7α-hydroxy-4-cholesten-3-one in the serum does not reflect a change in Cyp7a1 activity upon treatment with cafestol specifically. Interestingly, a study in which subjects were treated with the antibiotic rifampicin shows a 70% increase in serum levels of 7α-hydroxy-4-cholesten-3-one (30). This study also shows that levels of deoxycholic acid are decreased upon treatment with rifampicin. This suggests that rifampicin upregulates clearance of these secondary bile acids, which can result in upregulation of Cyp7a1 activity via a FXR mediated negative feedback mechanism. However, upon coffee oil treatment we did not observe a decrease in serum levels of bile acids. Therefore, it is unlikely that cafestol increases clearance of secondary bile acids and subsequently upregulates Cyp7a1 activity. Like rifampicin, cafestol regulates activity of several enzymes involved in detoxification (31). It is possible that cafestol disturbs clearance of 7α-hydroxy-4-cholesten-3-one itself. If this is indeed the case 7α-hydroxy-4-cholesten-3-one is not a useful marker for change in activity of Cyp7a1

upon treatment with such compounds. However, it remains to be established whether cafestol indeed disturbs clearance of 7α-hydroxy-4-cholesten-3-one.

Recently, we showed that cafestol is a ligand for both the farnesoid X receptor (FXR) and the pregnane X receptor (PXR) (Chapter 4). Activation of these receptors causes a decrease in Cyp7a1 activity (14, 15, 32). Interaction of cafestol with either of these receptors could explain the decrease in Cyp7a1 expression and activity in livers of APOE3Leiden mice and rat heptocytes (9, 18). However, the present study did not provide evidence that supports this hypothesis in humans. It is possible that the mechanism behind the serum lipid response to cafestol is unique for humans. Previous studies showed that several animals including monkeys, hamsters, rabbits, wild-type rats and gerbils do not show an increase in LDL-cholesterol upon cafestol feeding (33). There is a precedent for differences in regulation of bile acid metabolism between rodents and humans, and that is the regulation of the Cyp7a1 gene by the liver X receptor (LXR). In rodents oxysterols activate LXR and this causes upregulation of Cyp7a1 expression and bile acid synthesis (10, 12, 13). In humans activation of LXR has no effect on Cyp7a1 expression and bile acid synthesis. This is explained by the absence of a LXR response element in the human Cyp7a1 gene (10, 11). Another example is the fact that the human and mouse orthologue of PXR are activated by different ligands (34). Moreover, the genes that are regulated by PXR differ between species, due to differences between the receptor orthologues (35).

We conclude that coffee oil containing cafestol did not decrease serum levels of 7α-hydroxy-4-cholesten-3-one in humans. This suggests that Cyp7a1 activity and bile acid synthesis are not decreased. Therefore, this study does not support the hypothesis that cafestol raises serum cholesterol by decreasing synthesis of bile acids. Furthermore, elucidation of the mechanism behind the cholesterol-raising effect of cafestol is hampered by differences in the regulation of bile acid and cholesterol metabolism between species. Animal models are widely used for studying lipid metabolism, but caution is necessary when translating results from animal studies to the human situation. Mechanisms elucidated in animals often remain to be established in humans.

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6

General discussion

Our objective was to identify genes that control serum lipid levels. We intended to use the response of serum lipids to cafestol as a model system. The first section describes the approach we followed to identify dietary response genes. The second section describes the large response of liver enzymes to coffee oil we observed in some individuals, which is the reason that we abandoned coffee oil as a model system in humans. Mice studies indicated a role for the farnesoid X receptor (FXR) and the pregnane X receptor (PXR) in the response to cafestol. Therefore, the third and fourth section of this discussion will focus on these two receptors and their role in the response to cafestol respectively.

Identification of dietary response genes

We designed a study in which we would measure the serum lipid response to cafestol in 400 volunteers. We planned to link this response to variation in candidate genes. These candidate genes were derived from mice studies in which genes were identified that are differentially expressed after cafestol feeding. Those genes were identified using the serial analysis of gene expression and micro array techniques (1).

Identification of genes by haplotype analysis

Because it is not feasible to measure the effect of cafestol on gene expression in relevant tissues such as liver in humans we planned to use haplotype analysis (2). This type of analysis allows the identification of genes that are involved in the regulation of the serum lipid response to cafestol. Genes that are involved in the response to cafestol could also be involved in the response to other food components such as dietary cholesterol or fatty acids.

Reproducibility of the serum lipid response to cafestol

In order to link a haplotype to the serum lipid response to cafestol it is important to know whether this lipid response can be reproducibly measured within a subject. The effect of genetic factors on variation in the serum lipid response between individuals can only be studied when it can be excluded that environmental factors affect a person's response during the study period. Within-subject reproducibility does not

mean that the response is not determined by environmental factors, but rather that these factors are stable during the study period. The first study described in this thesis shows that the responses to coffee oil containing cafestol of total cholesterol and LDL cholesterol are poorly reproducible (Chapter 2). However, the triglyceride response to cafestol was highly reproducible within subjects. The HDL response was also highly reproducible within subjects. This might seem anomalous because on a group level there was no response to cafestol of HDL. However, HDL was increased as well as decreased in a similar number of subjects. The high degree of reproducibility between the two responses shows that we are not dealing with random variation, but that the direction of the effect is consistent within subjects.

Therefore, we concluded that investigation of the contribution of genetic factors to the serum lipid response to cafestol would be feasible for triglycerides and HDL, but not for total and LDL cholesterol.

The use of coffee oil as a model system

Coffee oil causes large responses of liver enzymes in a number of subjects

During the study investigating the reproducibility of the serum lipid response we encountered a problem with the use of coffee oil containing cafestol as a model system. A number of subjects showed more extreme responses of liver enzymes alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) to cafestol then was expected on the basis of previous studies (3-5). During this study we used Arabica coffee oil that contained both cafestol and kahweol. Two previous studies suggested that kahweol was the component of coffee oil that is responsible for the effect on liver enzymes, whereas cafestol is mainly responsible for the effect on serum cholesterol (4, 6). Therefore, we performed a study in which we tested if Robusta coffee oil, which contains negligible amounts of kahweol, would have little effect on liver enzyme levels while maintaining its cholesterol-raising effect.

We observed no differences in the response of liver enzyme levels to Robusta oil compared to Arabica oil. Indeed, a similar percentage of subjects showed a large response of liver enzymes ALAT and ASAT to both oils (Chapter 3).

Coffee oil could cause injury to hepatocytes

Elevation of these enzymes could indicate injury to hepatocytes caused by the coffee oil treatment. Other markers of liver damage such as γ-glutamyltranspeptidase and alkaline phosphatase were not increased, which excludes the possibility that coffee oil induces cholestasis. Furthermore, we were not able to identify a parameter that could predict a subject's response to coffee oil, such as alcohol intake or baseline levels of liver functions tests. Although we have no clinical evidence that injury to hepatocytes indeed occurs, we decided not to perform further studies with coffee oil or cafestol in human volunteers.

Candidate genes were acquired from mice studies

Not being able to perform further studies with cafestol in humans posed a problem to our original design of the study of the cholesterol response to cafestol combined with certain haplotypes. Interestingly, results from the studies in mice showed that cafestol regulated expression of a number of genes involved in lipid metabolism and detoxification. Instead of testing these candidate genes by haplotype analysis a number of *in vitro* experiments were designed to elucidate the mechanism by which cafestol raises serum lipids. The effect of cafestol on the expression of several genes implicated a role for two nuclear receptors: the farnesoid X receptor and the pregnane X receptor. By means of detailed *in vitro* experiments the interaction of cafestol with these receptors could be studied thereby providing insight in the possible mechanism by which cafestol affects serum lipids.

Nuclear receptors play a central role in regulation of lipid and bile acid metabolism

This section will start with some background concerning nuclear receptors in general and the farnesoid X receptor and the pregnane X receptor in particular. The following sections will discuss the interaction of natural compounds with these receptors in general and specifically addresses the interaction of cafestol with FXR and PXR.

Orphan nuclear receptors

The existence of nuclear receptors was first hypothesized based on the mode of action of steroids. Steroids were shown to translocate from the cytoplasm to the nucleus. This translocation is accompanied by binding to specific high affinity receptor proteins. Subsequently, target genes were identified that are responsive to steroid hormones. Together this constituted the classic model of the action of steroid hormones. On the basis of this model it was hypothesized that binding of the hormone to the receptor caused a change in the hormone-receptor complex that allowed it to modulate transcription. Cloning of their cDNA's identified a number of receptors: the glucocorticoid receptor, the estrogen receptor, the thyroid hormone receptor, and the retinoic acid receptor. This suggested that a superfamily of nuclear receptors might exist. In support of this hypothesis receptors were identified for all known steroid hormones as well as a number of receptors that had no known ligands at the time. The receptors without known ligands were called orphan nuclear receptors. See Mangelsdorf et al for a review on the nuclear receptor superfamily (7).

Nuclear receptor share structural and functional domains

Nuclear receptors have two highly conserved domains that determine their function as transcription factors. The first is the DNA binding domain located toward the amino-terminus and consists of two "zinc fingers". Zinc fingers are cysteine rich regions that complexes zinc and form protruding structures resembling fingers, hence "zinc finger". This DNA-binding structure allows the receptor to bind specific DNA sequences, also known as response elements, in the promoters of various genes.

The second domain that almost all nuclear receptors share is the ligand-binding domain located at the C-terminus. This domain allows the receptor to act as a molecular switch. According to the general model of nuclear receptor action, ligand binding brings the nuclear receptor into its transcriptionally active state. This active state is reached by a conformational change that is caused by dissociation of co-repressors and recruitment of co-activators (8). While some nuclear receptors can bind to DNA as monomers, the majority forms obligatory homodimers or heterodimers. See figure 1 for the general structure of nuclear receptors and how they regulate gene expression.

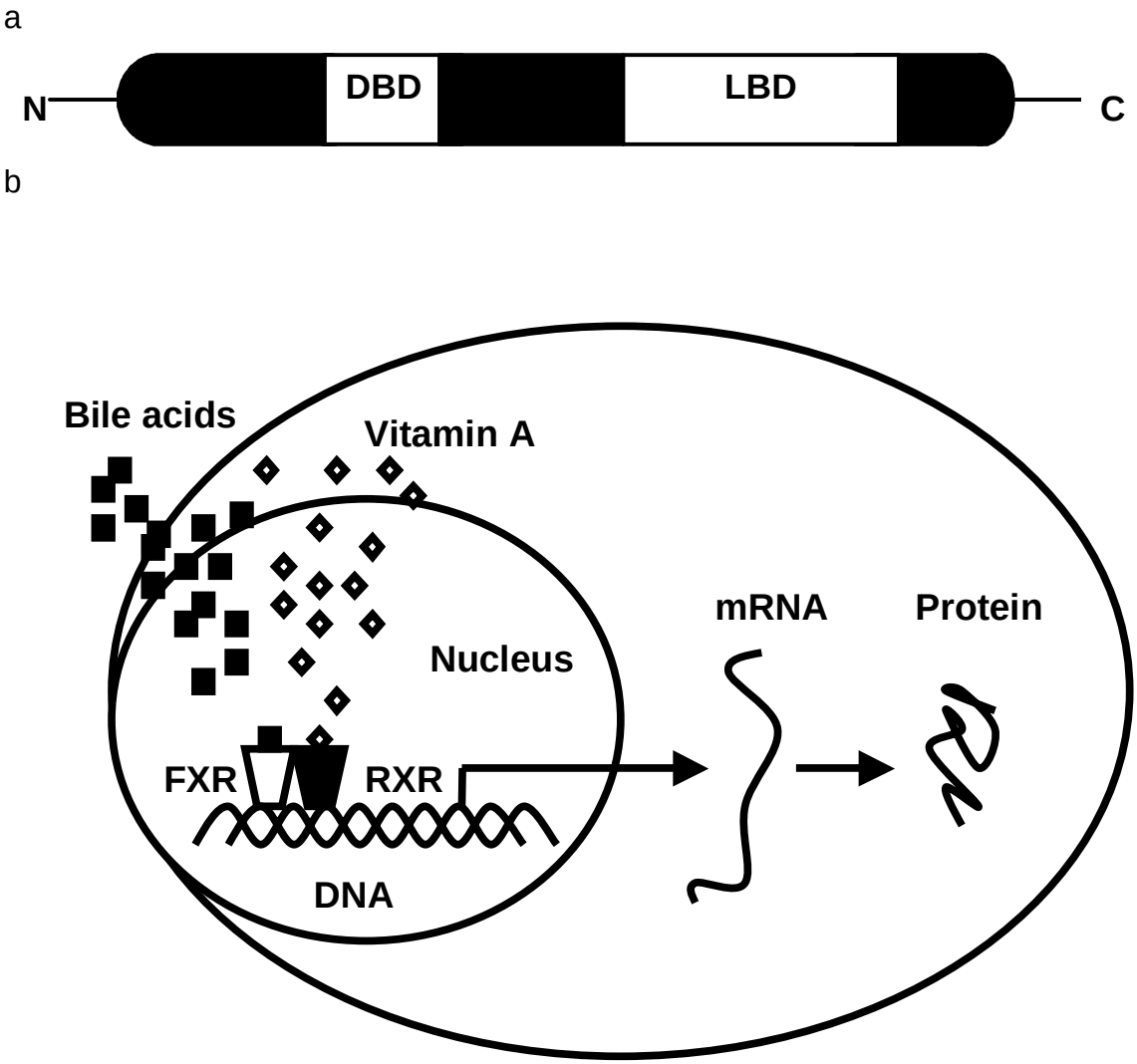

Figure 1 a) Diagram of the domains nuclear receptors share. A DNA-bindining domain (DBD) is located at the amino-terminal (N), a ligand-binding domain (LBD) is located at the COOH-terminal (C). **b) The farnesoid X receptor (FXR) as an example of how nuclear receptors regulate gene expression.** FXR is activated by bile acids it forms an obligatory heterodimer with the retinoid X receptor (RXR), which is activated by vitamin A metabolites. FXR then binds to a response element within the promoter of a gene. Binding of the response elements induces transcription.

The farnesoid X receptor is a central regulator in bile acid and lipid metabolism

The farnesoid X receptor (FXR) is a nuclear receptor that was named after farnesol, the first FXR-ligand identified (9). FXR is expressed in liver and intestine and forms an obligatory heterodimer with the retinoid X receptor (RXR). RXR is a nuclear receptor that is activated by a vitamin A derivative (10). Although FXR binds a variety of endogenous compounds, it is now well established that the physiological ligands for the receptor are bile acids (11, 12). The importance of FXR as a bile acid receptor was first put into context by the demonstration that FXR mediates the negative feedback of bile acids on their own synthesis. Upon activation by bile acids, FXR induces expression of the small heterodimer partner (SHP). SHP in turn inhibits the activity of the liver receptor homologue 1 (LRH-1), which is a nuclear receptor that upregulates cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol into bile acids in the liver (13-15). As a consequence expression of cholesterol 7α-hydroxlase is repressed. Besides mediating this negative feedback mechanism, FXR also regulates several other genes involved in bile acid metabolism, such as the bile salt export pump (BSEP) (16, 17), the sodium taurocholate co-transporting polypeptide (NTCP) (18), and the ileal bile acid binding protein (IBABP) (19, 20). BSEP transports bile acids across the canalicular membrane into the bile and is upregulated by FXR activation. At the same time, FXR downregulates NTCP, which transports bile acids from the serum into the hepatocyte. In combination with the suppression of bile acid synthesis by FXR these effects on NTCP and BSEP are thought to protect the hepatocyte from toxic concentrations of bile acids (21, 22). In contrast to liver, very little is known about the role of FXR in the intestine. In the intestine FXR increases expression of IBABP, which is thought to facilitate uptake of bile acids and intracellular bile acid transport in enterocytes (11). However, In FXR null mice IBABP is lost from the intestine, yet the turnover rate of bile acids is not affected (23), casting doubt on the proposed role of IBABP. The role FXR in bile acid metabolism is depicted schematically in figure 2. FXR also regulates genes involved in lipoprotein metabolism, such as apolipoprotein A-I (24), apolipoprotein C-II (25), apolipoprotein CIII (26), apolipoprotein E (27), the phospholipids pump MDR3 (28), and phospholipid transfer protein (PLTP) (29).

Although much of the function of FXR remains to be elucidated it is clear that this nuclear receptors plays an important role in bile acid and lipid homeostasis.

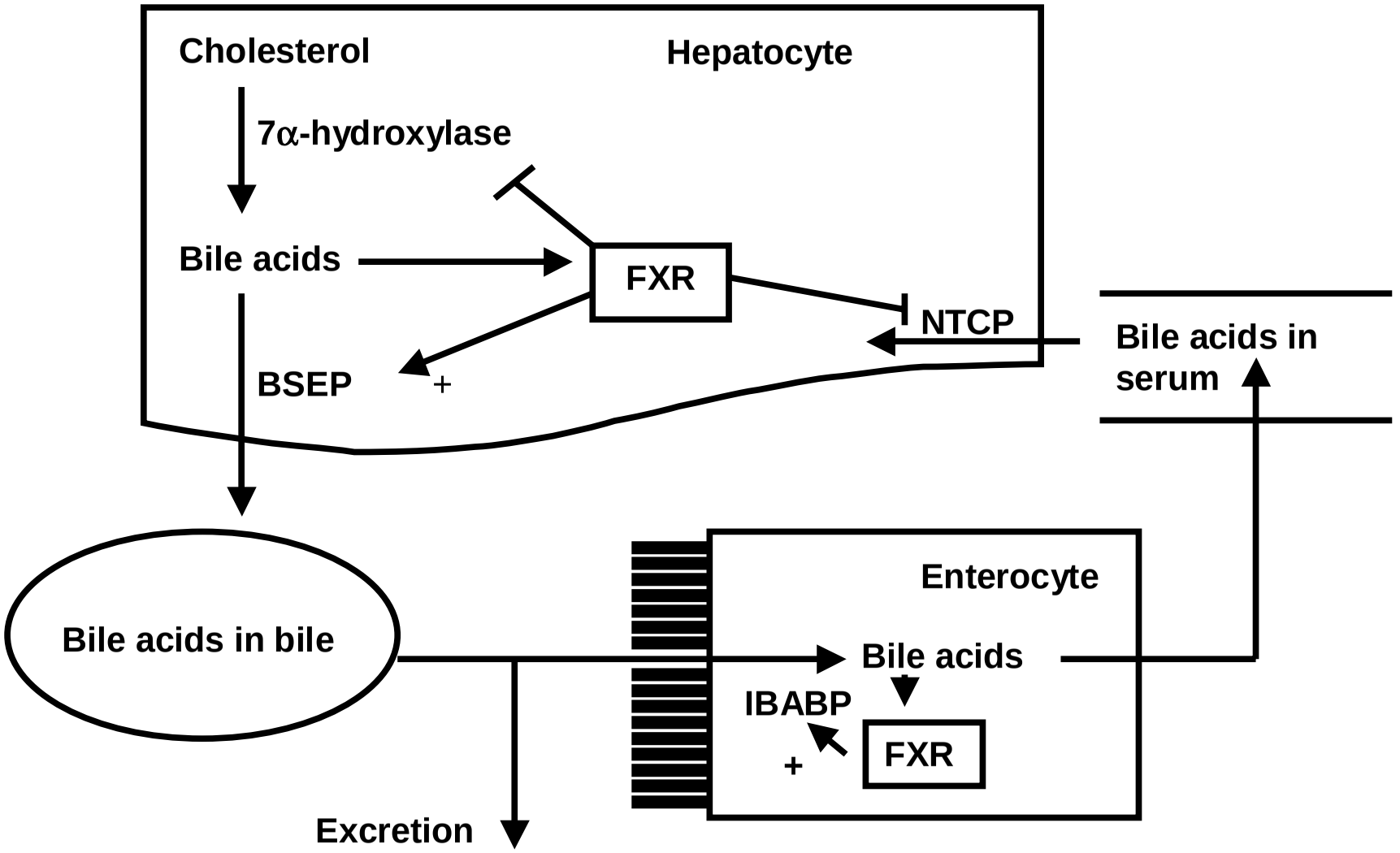

Figure 2 FXR plays a central role in the regulation of levels of bile acids in the hepatocyte. Upon activation FXR inhibits cholesterol 7α-hydroxylase, upregulates the bile salt export pump (BSEP), and downregulates the sodium taurocholate co-transporting polypeptide (NTCP). In enterocytes FXR upregulates the ileal bile acid binding protein (IBABP).

The pregnane X receptor is a xenobiotic receptor that regulates detoxification

The pregnane X receptor (PXR) is an orphan nuclear receptor that was named after pregnenolone, which together with progesterone was identified as a ligand (30, 31). The human homologue of PXR is also known as the steroid and xenobiotic receptor (SXR) (32). Like FXR, PXR forms a functional unit with the common heterodimeric partner RXR. The first indication of the function of PXR was the observation that ligands for this receptor are know to induce expression of cytochrome P-450 monooxygenase 3A4 (CYP3A4) (33). CYP3A4 is a member of the CYP3 subfamily of cytochrome P-450 monooxygenase, which are involved in the detoxification of a wide variety of xenobiotics and natural compounds including steroids and bile acids.

It has been established that nearly all compounds known to induce CYP3A4 are ligands for PXR (34). Induction of CYP3A4 is species specific. In mice CYP3A4 is potently induced by pregnenolone-16alpha-carbonitrile or dexamethasone, whereas in humans CYP3A4 is potently induced by rifampicin. Studies in transgenic mouse that express the human form of PXR showed that the differences in response to these ligands is due to differences between the human and the mice orthologues of PXR, rather than differences in the response element within the promoter of the target-gene CYP3A4 (35). The interspecies difference in PXR explains the variation in CYP3A4 response to several compounds between species (36). Besides CYP3A4, PXR also induces other enzymes involved in detoxification, such as phase II glutathione-S-transferases (37).

PXR binds a large variety of compounds including endogenous bile acids

Besides several xenobiotics including prescription drugs, several endogenous compounds were identified as ligands for PXR (34). The most potent endogenous ligand is lithocholic acid (38, 39). This implies that PXR is not only involved in the response to xenobiotics, but also in detoxification of endogenous compounds that are potentially toxic. In addition to governing detoxification, PXR also inhibits bile acids synthesis by suppressing cholesterol 7α-hydroxylase (38). In contrast to the mechanism by which FXR downregulates bile acid synthesis, PXR acts independently from SHP (40, 41).

It is remarkable that PXR binds such a wide array of compounds, since most other nuclear receptors bind their ligands in a highly selective manner. The structure of this receptor may explain this promiscuity. Structural studies have shown that in

contrast to other nuclear receptors except the PPAR's, PXR has a relatively large and smooth binding pocket (42, 43). The ability to bind such a wide variety in compounds together with the ability to induce detoxification pathways renders PXR a key regulator in the response to xenobiotic and endobiotic toxins (34).

The role of FXR and PXR in the response to cafestol

Interaction of natural compounds with the nuclear receptors FXR and PXR

As has been discussed in the previous paragraph, nuclear receptors can bind a variety of ligands of both natural and synthetic origin. The former group includes many compounds in our diet (44). For example, the estrogen receptor can bind both phytoesterogens and synthetic xenoestrogens (45). Another example is the interaction of hyperforin with PXR (46, 47). Hyperforin is the active compound in St. John's Wort, which is used as an herbal remedy for depression. In this paragraph we will focus on the best-described example of a natural compound that interacts with FXR and PXR: guggulsterone. Guggulsterone is present in the gum resin of the *Commiphora mukul* tree. In traditional Indian Ayurveda medicine, this extract is used to treat obesity and disorders of lipid metabolism. For a review on guggulsterone the reader is referred to Urizar et al (48). Guggulsterone has been shown to serve as an antagonist for the FXR receptor *in vitro* (49, 50). Animal experiments showed that guggulsterone lowered hepatic cholesterol in wildtype, but not in FXR null mice (49). These data confirm that FXR plays a role in the putative lipid-lowering effect of guggulsterone.

It was hypothesized that guggulsterone lowers cholesterol levels by upregulating cholesterol 7α-hydroxylase activity via FXR and thereby increases bile acid synthesis. However, guggulsterone was also shown to be an agonist of PXR (49, 50). In cell culture guggulsterone was shown to strongly inhibit cholesterol 7α-hydroxylase probably via PXR (51). This would suggest that guggulsterone actually inhibits bile acid synthesis. However, PXR agonists do not lower lipid levels in mice (49). Therefore, the interaction of guggulsterone with PXR probably does not contribute to the effect of this compound on lipid levels. Recently, it has been shown that guggulsterone not only interacts with FXR and PXR, but also with the alpha isoform of the estrogen receptor and the progesterone receptor (52). In addition to

the difficulties in identifying the mechanism by which guggulsterone lowers serum lipids, the lipid lowering effect itself has also been under debate. In contrast to several Indian studies, a recently conducted intervention trial in the USA with hypercholesterolemic adults did not show lowering of serum lipid levels after eight weeks (53). This study even suggested an increase in LDL cholesterol caused by guggulsterone. Another study suggested that the beneficial effects of guggulsterone towards atherosclerosis could be explained by inhibition of LDL oxidation by guggulsterone (54). In summary, guggulsterone interacts with several nuclear receptors, which could explain the beneficial effects of guggulsterone on serum lipid levels observed in some studies. However, due to the complexity of the potential mechanisms involved the explanation is not straightforward and the beneficial effect of guggulsterone has yet to be convincingly shown.

Does interaction of cafestol with FXR and PXR explain the cholesterol-raising effect of cafestol?

In Chapter 4 we show that cafestol interacts with FXR and PXR *in vitro*. This could explain the decrease in cholesterol 7α-hydroxylase activity observed in APOE3Leiden mice (55) as both receptors are able to inhibit this enzyme. Inhibition of bile acid synthesis leads to an increase in hepatic cholesterol and subsequent downregulation of the LDL receptor would result in an increase in serum LDL levels. However, a study in mice showed that bile acids lower serum triglyceride levels via FXR. This is in contrast with the effect of cafestol and unfiltered coffee, which potently increase serum triglyceride levels. As we showed in Chapter 2 this effect of cafestol on serum triglycerides is highly reproducible within subjects. Furthermore, the results from the *in vivo* studies in mice are inconclusive. Several FXR and PXR target genes were regulated by cafestol according to micro array (Chapter 4). However, while the effect of cafestol on a number of target genes was abolished in FXR and PXR null mice, other target genes were still regulated by cafestol. Also, some of the effects were opposite to what was expected. This suggests that cafestol also affects gene expression via other pathways than those mediated by FXR and PXR, possibly through other nuclear receptors. Furthermore, it suggests that cafestol can act as an antagonist rather than an agonist on some target-genes. As the example of guggulsterone in the previous paragraph showed, it is difficult to explain

the effect of a compound on serum lipids by simple interaction of that compound with one or even two receptors.

Interspecial differences hamper elucidation of the cholesterol-raising mechanism

Another obstacle in elucidating the mechanism involved in the cholesterol-raising effect of cafestol is the existence of interspecies differences. Indeed, while in rodents the liver X receptor (LXR) upregulates bile acid synthesis in response to oxysterols (56-58), humans do not respond to oxysterols in such a fashion. This is explained by the absence of a LXR response element in the promoter of the cholesterol 7α-hydroxylase gene in humans (58, 59).

Furthermore, often differences exist in the affinity of ligands for the human and rodent isoforms of nuclear receptors. For example, as has been mentioned above human and mice PXR are bound by different ligands. In summary, differences between species can be reflected in the properties of the nuclear receptors themselves, but also between the target genes they induce. This makes it more difficult to translate the effects of ligands for nuclear receptors observed in animal models to humans.

Cafestol did not decrease a marker of cholesterol 7α-hydroxylase activity in humans

Our studies failed to confirm that cafestol decreases the activity of cholesterol 7α-hydroxylase in humans. We even found an increase in serum levels of 7α-hydroxy-4cholesten-3-one, which is a marker of cholesterol 7α-hydroxylase in humans treated with coffee oil (Chapter 5). This increase could partly be explained by an increase in serum lipids upon cafestol treatment. Due to its lipophilic nature 7α-hydroxy-4cholesten-3-one is probably present in the lipoprotein fractions. One possible explanation for the observed decrease in cholesterol 7α-hydroxylase activity in mice is that this is connected with an acute phase response rather than be the consequence of an interaction of cafestol with FXR or PXR. During an acute phase response expression of cholesterol 7α-hydroxylase and several nuclear receptors including FXR, PXR, and SHP is decreased (60). Another explanation would be that cafestol could act as an agonist or antagonist depending on the conditions in the

hepatocyte. For example, cafestol could compete with bile acids for binding to FXR and PXR. The net effect on gene expression depends on the concentration of other ligands in the cell and the maximal level of activation achieved by cafestol. At this moment it is not clear under which conditions cafestol could act as an antagonist rather than an agonist.

Although the level of 7α-hydroxy-4cholesten-3-one in serum reflects activity of cholesterol 7α-hydroxylase in the liver, it is not entirely clear if this marker also reflects change in cholesterol 7α-hydroxylase activity in the liver upon cafestol treatment. Cafestol regulates activity of a number of detoxifying enzymes and this could affect clearance of the marker itself. If this is indeed the case the level of 7α-hydroxy-4cholesten-3-one in serum is not useful as a marker of cholesterol 7α-hydroxylase activity upon treatment with such compounds. However, it remains to be established whether cafestol treatment indeed affects clearance of 7α-hydroxy-4cholesten-3-one.

Recommendations for further research

The interaction of cafestol with the nuclear receptors FXR and PXR provides a clue as to how cafestol raises serum lipids. The presence of response elements for these receptors in several target genes indicates which genes are involved in the serum lipid response to cafestol. However, the example of guggulsterone shows us that it is difficult to fully explain the effect of such a compound on serum lipid levels by interaction with a nuclear receptor *in vitro*. This is illustrated by the fact that we, contrary to expectations, did not find a decrease in bile acid synthesis in humans. However, one major advantage of cafestol as a model system is that the cholesterol-raising effect of cafestol in humans is firmly established (5). Therefore, any model of the mechanism of the response to cafestol should explain the observed effects on serum lipids. The clear effect on serum lipids makes cafestol a substance that is useful in revealing how dietary components can effects serum lipids.

In order to further elucidate the mechanism by which cafestol raises serum lipids a number of experiments need to be performed. First the role of the nuclear receptors FXR and PXR needs to be confirmed in knockout models. Such experiments should not only show effects on gene expression, but also on bile acid

synthesis. Thereafter the results need to be confirmed in ‘humanized’ mice models such as APOE3Leiden mice or transgenic mice carrying a human gene for cholesterol 7α-hydroxylase. Crossbreeds of relevant knockout models with these transgenic mice can be created. This would allow us to make a better model of the effect of cafestol on serum lipids in humans.

Besides the experiments in animals, it would be informative to further investigate the interaction of cafestol with FXR and PXR. Our experiments did not show direct binding of cafestol to these receptors. Furthermore, cafestol could act as a partial agonist. A partial agonist is a ligand that activates a receptor, but to a lesser extent than other ligands. Therefore, cafestol could compete for the receptor with other ligands that activate a receptor more potently and thereby acts as an antagonist rather than an agonist. Reporter promoter-gene assays with cells cultured in the presence of cafestol and other ligands such as bile acids could show if cafestol indeed is a partial agonist for FXR and/or PXR.

Another interesting experiment would be to test compounds related to cafestol such as kahweol or 16-*O* -methylcafestol for interaction with FXR and PXR. The only structural difference between cafestol and kahweol is the presence of a double bond between the C1 and C2 atom in kahweol. However, an intervention study comparing a mixture of cafestol and kahweol was only slightly more potent in raising cholesterol than pure cafestol (4). This suggested that cafestol is the compound mainly responsible for the cholesterol-raising effect of unfiltered coffee. Results from intervention studies with pure kahweol are not available due to difficulties in purification of sufficient amounts. Nowadays small quantities of kahweol are commercially available that can be used for *in vitro* studies.

Concluding remarks

Our studies showed that cafestol interacts with two nuclear receptors: FXR and PXR. This provides us with an important lead into which genes are involved in the regulation of the serum lipid response to cafestol. Although the mechanism still remains to be elucidated this lead allows us to further explore the role of nuclear receptors in the regulation of the response to cafestol and possibly other dietary compounds. However, it remains to be established whether interaction of cafestol

with FXR and PXR can explain the raise in serum lipids in response to unfiltered coffee observed in humans.

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Summary

Cafestol is a diterpene from coffee beans that is present in unfiltered coffee types such as Scandinavian boiled coffee, French press coffee, and espresso. This diterpene is responsible for the cholesterol-raising effect of unfiltered coffee. Consumption of unfiltered coffee raises serum levels of LDL and triglycerides. Elevated levels of LDL in serum are a major cause of cardiovascular disease. We aimed to identify genes that control serum lipid levels in response to diet. In the studies described in this thesis we used cafestol as a model for a dietary component that affects serum lipids in humans.

We first assessed whether the serum lipid response to coffee oil that contains cafestol was reproducible within subjects. This is necessary to be able to link the response to a genotype. If the response cannot be measured reproducibly within persons this means that environmental factors affect the response during de study period. Therefore the identification of genetic factors that possibly are involved is hampered. We designed a study in which subjects consumed coffee oil during two separate five-week treatment periods. The correlation between the two responses in each period indicates the reproducibility within subjects. We found that the responses of total and LDL cholesterol were poorly reproducible within subjects, whereas the responses of HDL and triglycerides were highly reproducible. Therefore, linking a genotype with the serum lipid response to coffee oil is more promising for HDL and triglycerides.

During the study of the reproducibility of the serum lipid response we also observed larger responses of liver enzyme levels to coffee oil in serum of a number of subjects than was expected on the basis of previous studies. In the first treatment period we had to exclude eight subjects that had levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) above predetermined boundaries of 2.7 times the upper limit of normal for ALAT and 1.5 times the upper limit of normal for ASAT. In the second period we had to exclude another 5 subjects due elevation of liver enzymes.

In two previous studies it was suggested that kahweol is the component of coffee oil that is mainly responsible for the effect on liver enzymes. Kahweol is a diterpene that closely resembles cafestol. The only structural difference is the

presence of a double bond between the C1 and C2 atom of kahweol. Kahweol is present in the oil of Arabica coffee beans, which we used during the reproducibility study. Oil from Robusta coffee beans contains negligible amounts of kahweol. Therefore, we designed a study to assess whether Robusta coffee oil affects liver enzyme levels to a lesser extent than Arabica coffee oil does. After two weeks of Robusta oil consumption we already observed elevations of ALAT and ASAT above predetermined boundaries of 2.5 times the upper limit of normal for ALAT and 1.5 times the upper limit of normal for ASAT in three of eighteen subjects (17%). This is comparable to the number of subjects that were excluded due to elevations of liver enzymes during the reproducibility study in which we used Arabica oil: eight out of 50 subjects (16%) in the first treatment period and five out of forty subjects (13%) in the second period. We concluded that Robusta oil has a similar effect on liver enzyme levels as Arabica oil has. Furthermore, we were not able to identify factors that could predict a person's response such as alcohol intake or baseline levels of liver function parameters. Therefore we decided not to perform further intervention studies with coffee oil or cafestol in human volunteers.

We continued our research using *in vitro* and animal studies. It was previously demonstrated that cafestol suppresses bile acid synthesis in cultured rat hepatocytes and APOE3Leiden mice by downregulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol into bile acids in the liver. Expression of this enzyme is under the control of several nuclear receptors including the farnesoid X receptor (FXR), the pregnane X receptor (PXR), and the liver X receptor (LXR). Both FXR and PXR mediate a negative feedback loop inhibiting bile acid synthesis upon activation by bile acids. LXR upregulates bile synthesis when activated by oxysterols. Our studies showed that cafestol interact with FXR and PXR *in vitro*. This could explain the suppression of bile acid synthesis observed in mice.

Comparison of gene expression in livers from APOE3Leiden mice that were fed a cafestol containing diet or a control diet showed that several target-genes of FXR and PXR are regulated by cafestol. The involvement of FXR en PXR was confirmed by the absence of these effects on a number of target-genes in FXR and PXR knockout mice. However, not all the effects of cafestol on gene expression could be explained by interaction with FXR or PXR. Possibly, other nuclear receptors are involved in this response.

In order to confirm whether cafestol raises serum lipids by suppression of bile acid synthesis in humans we measured levels of 7α-hydroxy-4-cholesten-3-one in serum of the reproducibility study. The level of 7α-hydroxy-4-cholesten-3-one in serum is a marker for the activity of 7α-hydroxylase in the liver. We found that 7α-hydroxy-4-cholesten-3-one was raised by 47% in the first treatment period with coffee oil and by 23% in the second period. After correction of the individual levels of 7α-hydroxy-4-cholesten-3-one for the total cholesterol levels these effects were reduced to +24% in the first period and +5% in the second period. This suggests that bile acid synthesis is not suppressed by cafestol in humans.

We conclude that cafestol interacts with the nuclear receptors FXR and PXR and via these receptors regulates expression of several genes involved in lipid metabolism and detoxification. These receptors possibly mediate the suppression of bile acid synthesis by cafestol observed in mice. However, the mechanism by which cafestol raises serum lipids in humans still remains to be elucidated.

Samenvatting

Cafestol, een diterpeen uit koffiebonen, is aanwezig in ongefilterde koffie types zoals Scandinavische kookkoffie, Cafetière koffie en espresso. Dit diterpeen is verantwoordelijk voor het cholesterol verhogende effect van ongefilterde koffie. Consumptie van ongefilterde koffie veroorzaakt een verhoging van LDL cholesterol (ook wel bekend als het “slechte cholesterol”) en triglyceriden in het serum. Een verhoogd niveau van LDL cholesterol in het serum is een belangrijke oorzaak van hart- en vaatziekten. Het doel van het onderzoek was het identificeren van genen die de respons van serum lipiden op voeding reguleren. In de studies die beschreven staan in dit proefschrift is cafestol gebruikt als model voor een voedingscomponent dat invloed heeft op serum lipiden in mensen.

Als eerste hebben we onderzocht of de respons van serum lipiden op cafestol houdende koffieolie reproduceerbaar is binnen personen. Dit is nodig om de respons te kunnen koppelen aan een genotype. Wanneer een respons niet reproduceerbaar is binnen personen, dan betekent dit dat de respons wordt beïnvloed door omgevingsfactoren gedurende de studie periode. Dit verstoort de identificatie van genetische factoren die de respons eventueel beïnvloeden. Om dit te onderzoeken hebben we een studie uitgevoerd waarin mensen koffieolie consumeerden gedurende twee afzonderlijke periodes van ieder vijf weken. De correlatie tussen de respons in de eerste periode en de respons in de tweede periode laat zien hoe reproduceerbaar de respons is binnen personen. We vonden dat de respons van totaal en LDL cholesterol slecht reproduceerbaar was binnen personen, terwijl de respons van triglyceriden en HDL (ook wel bekend als het “goede cholesterol”) cholesterol zeer goed reproduceerbaar was. Dit betekent dat het uitvoerbaar is om de respons van HDL cholesterol en triglyceriden te koppelen aan een genotype.

Tijdens de studie naar de reproduceerbaarheid van de respons van serum lipiden op koffieolie vonden we ook een groter effect van koffieolie op leverenzymen in het serum van een aantal deelnemers dan we op grond van eerdere studies hadden verwacht. In de eerste behandelperiode werden we genoodzaakt acht mensen van het onderzoek uit te sluiten op grond van niveaus van alanine aminotransferase (ALAT) en aspartaat aminotransferase (ASAT) boven de van tevoren vastgestelde grenzen van 2,7 maal de bovengrens van normaalwaarden

voor ALAT en 1,5 maal de bovengrens van normaalwaarden voor ASAT. In de tweede behandelperiode moesten we nog eens vijf mensen uitsluiten wegens verhoging van de leverenzymen.

Twee eerdere studies suggereerden dat het effect van koffieolie op de leverenzymen voornamelijk wordt veroorzaakt door kahweol. Kahweol is een diterpeen dat zeer veel lijkt op cafestol. Het enige structurele verschil tussen deze stoffen is de aanwezigheid van een dubbele binding tussen de C1 en C2 atomen in kahweol. Kahweol is aanwezig in de olie van Arabica koffiebonen die we gebruikten tijdens de studie naar de reproduceerbaarheid. Olie van Robusta koffiebonen bevat verwaarloosbare hoeveelheden kahweol. Om deze reden ontwierpen we een studie waarin we testten of Robusta koffieolie minder effect heeft op de leverenzymen dan Arabica koffieolie. Na twee weken consumptie van Robusta olie zagen we verhoging van de leverenzymen boven de van tevoren vastgestelde grenzen van 2,5 maal de bovengrens van normaal voor ALAT en 1,5 maal de bovengrens van normaal voor ASAT in drie van de achttien deelnemers (17%). Dit is vergelijkbaar met het aantal deelnemers dat werd uitgesloten in de studie naar de reproduceerbaarheid waarin we Arabica olie gebruikten: acht van de vijftig deelnemers (16%) in de eerste behandelperiode en vijf van de veertig deelnemers (13%) in de tweede periode. We concludeerden dat Robusta olie een vergelijkbaar effect heeft op de leverenzymen als Arabica olie. Verder konden we geen factoren onderscheiden die de respons van een persoon konden voorspellen, zoals bijvoorbeeld alcohol inneming of beginwaardes van leverfunctie tests. Daarom besloten we om geen interventie studies meer uit te voeren met koffieolie of cafestol in mensen.

We hebben het onderzoek voortgezet met *in vitro* experimenten en dierstudies. Eerdere studies lieten zien dat cafestol de galzuursynthese onderdrukt in gekweekte hepatocyten van ratten en in APOE3Leiden muizen door remming van cholesterol 7α-hydroxylase. Dit enzym is het sleutelenzym in de omzetting van cholesterol in galzuren in de lever. Expressie cholesterol 7α-hydroxylase staat onder controle van verschillende nucleaire receptoren zoals de farnesoid X receptor (FXR), de pregnane X receptor en de liver X receptor (LXR). Zowel FXR als PXR zijn onderdeel van een negatief feedback mechanisme en remmen de galzuursynthese wanneer ze geactiveerd zijn door galzuren. LXR kan de galzuursynthese opreguleren na activatie door galzuren. Onze studies lieten zien dat cafestol *in vitro* interactie

heeft met FXR en PXR. Dit zou een verklaring kunnen zijn voor de remming van galzuur synthese door cafestol in muizen. Het verschil in genexpressie in levers van APOE3Leiden muizen die cafestol-houdend voedsel verstrekt kregen of die controle voedsel kregen liet zien dat verschillende genen die onder controle staan van FXR en PXR door cafestol worden gereguleerd. De rol van FXR en PXR hierin werd bevestigd doordat deze effecten op een aantal genen onder controle van FXR en PXR niet aanwezig waren in muizen die geen functioneel FXR of PXR hebben. Niet alle effecten op genexpessie van cafestol konden verklaard worden door interactie met FXR of PXR. Mogelijk zijn andere nucleaire receptoren bij de respons betrokken.

Om te onderzoeken of in mensen de verhoging van serum lipiden door cafestol kan worden verklaard door remming van de galzuursynthese maten we de respons van 7α-hydroxy-4-cholesten-3-one in serum van onze reproduceerbaarheidstudie. Het niveau van 7α-hydroxy-4-cholesten-3-one in serum is een maat voor de activiteit van 7α-hydroxylase in de lever. We vonden dat het niveau van 7α-hydroxy-4-cholesten-3-one was verhoogd met 47% gedurende de eerste behandelperiode met koffieolie en met 23% gedurende de tweede periode. Na correctie van individuele 7α-hydroxy-4-cholesten-3-one waarden voor de waarden van totaal serum cholesterol daalden deze effecten naar +24% in de eerste periode en +5% in de tweede periode. Dit suggereert dat cafestol de galzuursynthese niet remt in mensen.

De conclusie van dit proefschrift is dat cafestol aangrijpt op de nucleaire receptoren FXR en PXR en zo de expressie reguleert van verschillende genen die betrokken zijn bij het lipiden-metabolisme. Dit zou een verklaring kunnen zijn voor de remming van de galzuursynthese door cafestol in muizen. Het is echter nog niet duidelijk hoe de interactie van cafestol met deze receptoren in mensen leidt tot een verhoging van de serum lipiden.

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Bij het project zijn ook twee afstudeerstudenten betrokken geweest: Mariëlle Engberink en Esther van Straten. Bedankt voor jullie bijdrage aan het onderzoek en veel succes met jullie eigen AIO-projecten.

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Iedereen bedankt!

Curriculum vitae

Mark Vincent Boekschoten was born March 16, 1977, in Hilversum, The Netherlands. In 1995 he passed secondary school, gymnasium, at the Comenius College in Hilversum. The same year he started the study ‘Medical Biology’ at the Vrije Universiteit Amsterdam. During his study he worked for six months at the division of Neuropathology of the Academic Hospital Vrije Universiteit Amsterdam on the expression of β-chemokines and their receptors in Multiple Sclerosis lesions. For eight months he worked at the Department of Cell Biology of the Vrije Universiteit on regulation of gene expression by glucocorticoids in human monoctyes. He wrote a thesis entitled ‘Diversity of T-cell populations and immune reconstitution during treatment of HIV-infection’. In 2000 he received his MSc degree in Medical Biology with specializations in cell biology, immunology, and pathology.

In January 2000 he was appointed as a PhD student on the project ‘Identification of dietary response genes controlling plasma lipid levels in mice and humans; the analysis of the response to cafestol as a model system’. He joined the education program of the graduate school VLAG that offers advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences. In 2003 he attended the summer research conference ‘Molecular biology of intestinal lipid transport’ of the Federation of American Societies for Experimental Biology, in Snowmass Village, Colorado, USA. At this conference he received the ‘Molecular biology presentation award’. He was a member of the information technology committee and the laboratory committee within the Division of Human Nutrition. He was also member of the editorial board of the newsletter of the Wageningen Postgraduate Programme in Human Nutrition & Epidemiology and member and chair of the committee of temporary scientific staff within the Division of Human Nutrition.

Training and Supervision plan

Genetic variation and consequences for health	Nutrim	2000
Molecular approaches in genetics	NIHES	2000
Genetic epidemiology	NIHES	2000
Organising and supervising student projects	WUR	2000
PhD student week	VLAG	2000
Bio-informationtechnology	WUR	2000
Journal club	Human Nutrition, WUR	2000-2002
Meeting of Nutritional science community		2000-2003
Programme meeting "Nutrition and chronic disease"	ZonMW	2000-2004
Nutritional & lifestyle epidemiology	VLAG	2001
From Nutrigenomics to healthy food	VLAG	2001
Food summit "Biomarkers for assessing effects of diet on cardiovascular health"	WCFS	2001
PhD study tour	VLAG	2001
Molecular biology of intestinal lipid transport	FASEB	2003
English Scientific writing	CENTA	2003
Functioning successfully within organisations	VLAG	2003

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