Stellingen

1. Een goed proefdiermodel voor het effect van cholesterol en vetzuren op het lipoproteinenmetabolisme hoeft niet noodzakelijk ook een goed proefdiermodel te zijn om het mechanisme van cafestol te bestuderen. (*dit proefschrift*).

2. In apolipoproteine E*3-Leiden transgene muizen verhoogt cafestol het LDL cholesterol primair door remming van de galzuursynthese (*dit proefschrift*).

3. Cafestol verhoogt het serum triglyceriden en het LDL cholesterol gehalte onafhankelijk van elkaar (*dit proefschrift*).

4. In mechanistisch onderzoek naar effecten van stoffen op het lipoproteinen metabolisme is informatie over veranderingen in lipoproteinen (-subfracties) minstens zo belangrijk als informatie over veranderingen in lipiden.

5. Het is vooralsnog niet bewezen dat voedingsvezel beschermt tegen het optreden van colonkanker (*o.a. Fuchs et al., NEJM 1999;340:169-176*).

6. De ongenuanceerde uitspraken over voeding en gezondheid in populaire magazines impliceren dat een voedingskundige zich naast de rol van 'wetenschapper' beter zou moeten richten op de rol van 'voorlichter'.

7. De publieke opinie rond de introductie van genetisch gemodificeerde produkten in Groot-Brittannie wordt gemanipuleerd door de media.

8. Gebrek aan kennis over het gebruik van proefdieren in medisch onderzoek is voor een groot deel verantwoordelijk voor een negatieve houding ten opzichte van dierexperimenteel onderzoek (*New Scientist, 22 mei 1999*).

9. De tijd die nodig is voor het afronden van een manuscript is positief gecorreleerd met het aantal co-auteurs.

10. Personen van dezelfde sexe gaan zich doorgaans sexe-stereotieper gedragen als ze onder elkaar zijn (*Psychologie, maart 1999*).

Stellingen behorende bij het proefschrift "Mechanistic studies on the lipid-raising coffee diterpenes cafestol and kahweol in monkeys, mice and man", te verdedigen door Baukje de Roos op 14 januari 2000.

Mechanistic studies on the lipid-raising coffee diterpenes cafestol and kahweol in monkeys, mice and man

Baukje de Roos

Promotor:

dr. M.B. Katan
Persoonlijk hoogleraar bij de afdeling Humane Voeding & Epidemiologie

Mechanistic studies on the lipid-raising coffee diterpenes cafestol and kahweol in monkeys, mice and man

Baukje de Roos

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr. C. M. Karssen,
in het openbaar te verdedigen
op vrijdag 14 januari 2000
des namiddags te vier uur in de Aula.

The studies described in this thesis were supported by a grant from the Netherlands Heart Foundation (grant no. 900-562-091) through the Netherlands Organization of Scientific Research and from the Foundation for Nutrition and Health Research.

The PhD-project of Baukje de Roos was part of the research program of the Graduate School VLAG.

Financial support by the Netherlands Heart Foundation and the Dr. L.A. Buma Stichting for the publication of this thesis is gratefully acknowledged.

Mechanistic studies on the lipid-raising coffee diterpenes cafestol and kahweol in monkeys, mice and man
Baukje de Roos

Thesis Wageningen University. - With ref. - With summary in Dutch.
ISBN 90-5808-172-9

Printing: Grafisch Bedrijf Ponsen & Looijen BV, Wageningen, The Netherlands

Abstract

Mechanistic studies on the lipid-raising coffee diterpenes cafestol and kahweol in monkeys, mice and man

PhD thesis by Baukje de Roos, Division of Human Nutrition & Epidemiology, Wageningen University, Wageningen, The Netherlands.

Cafestol and kahweol are lipid-raising diterpenes present in unfiltered coffee. The objective of this thesis was to study their lipid-raising action in man. Unravelling this action might lead to new insights into the regulation of serum cholesterol levels.

We first studied the absorption and urinary excretion of cafestol and kahweol in eight ileostomy volunteers. About 70% of the consumed cafestol and kahweol was absorbed and thus available for raising serum lipids in humans. Only 1.2% of the diterpenes was subsequently excreted as a conjugate of glucuronic acid or sulphate in urine, suggestive for an extensive metabolism of coffee diterpenes.

We then searched for an animal model to study the mechanism of action of cafestol and kahweol. In African green monkeys, both diterpenes raised total cholesterol less pronounced than in man. Unlike humans, the rise in cholesterol was predominantly due to a rise in HDL cholesterol rather than LDL cholesterol. In apolipoprotein E*3-Leiden mice, cafestol and kahweol increased total cholesterol with 61% after eight weeks of treatment. The increase in total cholesterol was mainly due to a rise in VLDL and IDL cholesterol. After three weeks of treatment, total cholesterol was increased due to suppression of bile acid synthesis, which caused a decreased expression of the LDL receptor. In addition, VLDL became enriched in cholesteryl esters.

Two mechanistic studies were performed in healthy human subjects. Consumption of cafetiere (French-press) coffee increased CETP activity by 15% after 12 weeks of intervention. The increase in CETP activity clearly preceded the increase in LDL cholesterol. Cafestol increased serum triglycerides by an 80% increase in the production rate of $VLDL_1$ apolipoprotein B after two weeks of intervention. This resulted in an increased amount of $VLDL_1$ particles in the circulation. Cafestol did not change the composition of $VLDL_1$. $VLDL_2$ became enriched with cholesteryl esters.

In conclusion, cafestol first increases plasma triglycerides by increasing the production rate of $VLDL_1$ apolipoprotein B. The subsequent rise in LDL cholesterol might be due to suppression of bile acid synthesis, which probably leads to down-regulation of the LDL receptor and/or to an enrichment of $VLDL_2$ with cholesteryl esters. The mechanisms of action that raise plasma cholesterol and plasma triglycerides in humans might be regulated independently in the liver.

Abbreviations

ACAT	acyl-coenzyme A:cholesterol-acyltransferase
ALAT	alanine aminotransferase
ApoB	apolipoprotein B
ApoE	apolipoprotein E
C	cholic acid
CDC	chenodeoxycholic acid
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
D_3-leucine	L-[5,5,5-$^2H_3$]leucine
DC	deoxycholic acid
DMSO	dimethylsulfoxide
GAPDH	glyceraldehyde-3-phosphate
HDC	hyodeoxycholic acid
HDL	high density lipoprotein
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
LC	litocholic acid
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	LDL receptor
LpL	lipoprotein lipase
LRP	LDL receptor-related protein
MC	muricholic acid
MTP	microsomal triglyceride transfer protein
PABA	para-amino benzoic acid
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator-activated receptor
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
Sf	Svedbergs flotation rate
SREBP	sterol regulatory element binding protein
UDC	ursodeoxycholic acid
VLDL	very low density lipoprotein
βVLDL	β-migrating very low density lipoprotein

Contents

1

General introduction

Possible mechanisms underlying the cholesterol-raising effect of the coffee diterpene cafestol

Baukje de Roos and Martijn B. Katan. *Current Opinion in Lipidology 1999; 10: 41-45.*

Abstract

Cafestol, a coffee diterpene present in unfiltered coffee brews, potently raises serum lipids in humans. The mechanism through which this dietary compound influences lipoprotein metabolism is largely unknown. Unravelling the mechanism of action might lead to new insights into the regulation of serum cholesterol levels in humans. This review summarises ways in which cafestol may act on serum lipids.

Introduction: coffee diterpenes raise serum lipids in humans

Cafestol and kahweol are fat-soluble diterpenes in coffee beans. They are present in commercially important blends of Arabica and Robusta *Coffea* species, as well as in wild African *Coffea* species (1). Unfiltered coffee brews such as Scandinavian boiled coffee, cafetiere coffee, and Turkish coffee contain 3-6 mg of each diterpene per cup (2;3). Cafestol and kahweol potently raise serum cholesterol and triglycerides in humans (4;5). Of the two diterpenes, cafestol is responsible for more than 80% of the effect on serum lipids (6). This review therefore focuses on cafestol and its possible mode of action.

Cafestol is the most potent cholesterol-elevating compound known. Each 10 mg of cafestol increases serum cholesterol by 0.13 mmol/L and serum triglycerides by 0.08 mmol/L after consumption for four weeks (7). When 22 volunteers consumed 0.9 L of cafetiere coffee per day (8), serum cholesterol reached a maximum increase of 17% after eight to twelve weeks. About 80% of the rise in total cholesterol was due to LDL cholesterol, and the rest was due to a rise in VLDL cholesterol. In this study cafetiere coffee did not affect serum HDL cholesterol. However, in other studies HDL decreased slightly with cafestol intake (5;6;9). Serum triglycerides increased by 26% after two to four weeks of cafetiere coffee, but they were back to baseline after 24 weeks (8). Thus the effect on triglycerides is transient while the effect on LDL cholesterol is permanent. This is in good agreement with epidemiological observations (5;10;11). A number of mechanisms can be involved to explain the effect of cafestol on lipoproteins. The most important candidates are discussed below.

Proposed mechanism: the SREBP pathway

Any proposed mechanism should explain both a transient rise in VLDL and a permanent rise in LDL in subjects consuming cafestol. Both effects could hypothetically be explained by a primary effect of cafestol on the sterol regulatory element binding proteins (SREPB)-pathway. SREBP are membrane bound transcription factors, which are important regulators of cholesterol biosynthesis in humans. Sterols induce a proteolytic cleavage of the NH_2-terminal domain of the SREBP. This domain binds subsequently to specific DNA sequences, by which it can modulate the transcription of certain genes involved in lipid metabolism, such as genes for HMG-CoA reductase, lipoprotein lipase and the LDL receptor (12).

The rise in VLDL and LDL could now be explained as follows (figure 1A).

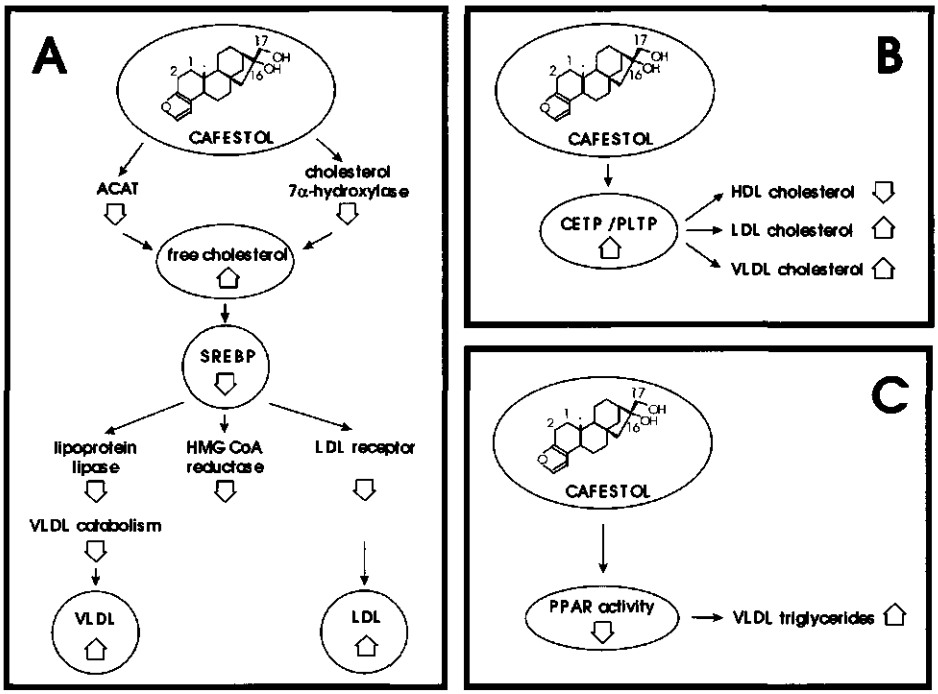

Figure 1. Three proposed mechanisms for the action of cafestol on lipoprotein metabolism. ACAT: acyl-CoA:cholesterol acyltransferase; HMG-CoA: 3-hydroxy-3-methyl-glutaryl CoA; VLDL: very low density lipoproteins; LDL: low density lipoproteins; HDL: high density lipoproteins; CETP: cholesterol ester transfer protein; PLTP: phospholipid transfer protein; PPARs: peroxisome proliferator-activated receptors.

An effect of cafestol on SREBP may be generated by modulating the pool of free cholesterol in the liver cell. For example, cafestol might inhibit cholesterol acyl-CoA:cholesterol acyltransferase (ACAT) activity, and thereby the conversion of unesterified cholesterol into its cholesterol ester. Cafestol indeed decreased cholesterol esterification in CaCo-2 cells (13). However, it increased cholesterol esterification in human skin fibroblasts (14). Cafestol did not affect cholesterol esterification in HepG2-cells, suggesting that ACAT activity was not modified (15) (figure 2). Thus it is unclear whether cafestol affects the pool of free cholesterol through modulation of ACAT. Alternatively, cafestol might inhibit the activity of cholesterol 7α-hydroxylase, a rate-limiting enzyme in the conversion of cholesterol into bile acids. Cafestol did not affect bile acid formation in HepG2-cells (15) (figure 2). However, cafestol decreased bile acid mass production in rat hepatocytes in a dose-dependent matter. In addition, cafestol reduced the activity of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase. These reductions paralleled a reduction in

cholesterol 7α-hydroxylase and in sterol 27-hydroxylase mRNA (16). Thus suppression of bile acid synthesis may explain the cholesterol-raising effects of cafestol. Therefore, it would be interesting to study changes in bile acid mass production *in vivo*.

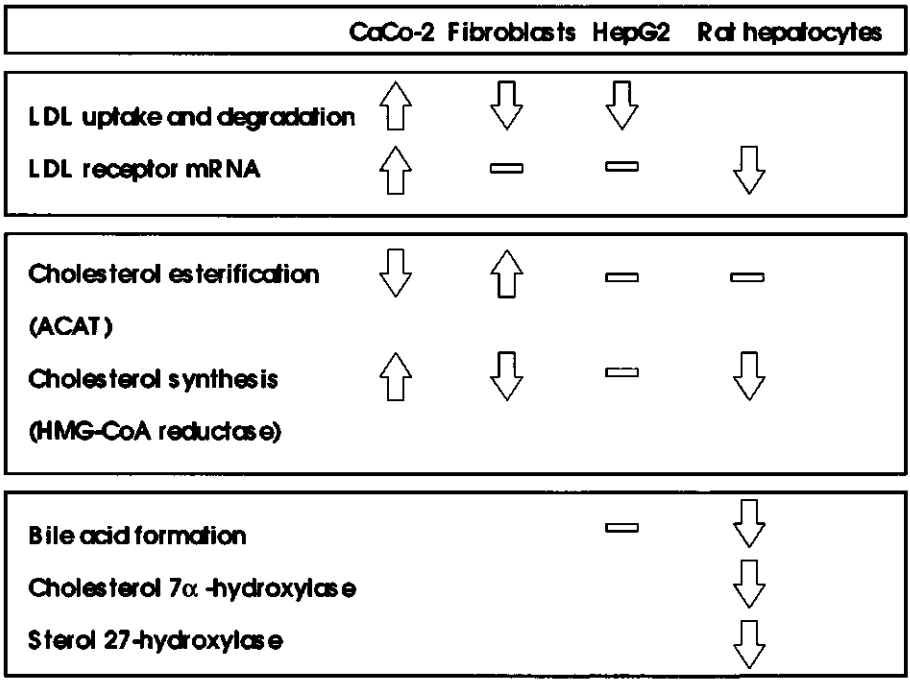

Figure 2. Summary of the effects of cafestol on cholesterol metabolism in CaCo-2 cells (a human colon adenoma cell line), in fibroblasts (human skin fibroblast cells), in HepG2 cells (a human hepatoma cell line), and in rat hepatocytes. LDL: low density lipoproteins; ACAT: acyl-CoA:cholesterol acyltransferase; HMG-CoA: 3-hydroxy-3-methyl-glutaryl CoA.

When the pool of free cholesterol in the liver cell increases, the activity of SREBP will be reduced (12). This may result in a decreased transcription of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA reductase) gene. HMG-CoA reductase is a key enzyme involved in the synthesis of cholesterol. However, both *in vivo* and *in vitro* studies do not provide clear evidence for a cafestol-induced decreased transcription of this enzyme. Boiled coffee did not affect the ratio of serum lathosterol:total cholesterol - a measure of whole-body cholesterol synthesis - in humans (17). Cafestol reduced HMG-CoA reductase mRNA in rat hepatocytes (16) and in human skin fibroblasts (14). However, cafestol did not affect cholesterol synthesis in HepG2 cells (15), while it increased cholesterol synthesis in CaCo-2 cells (13) (figure 2). Alternatively, down-regulation of SREBP activity may result in a

decreased transcription of the lipoprotein lipase gene. Subsequently, triglyceride-rich VLDL can not efficiently be cleared from the plasma by lipoprotein lipase, and plasma VLDL will increase. An alternative explanation for increased VLDL concentrations might be an elevated VLDL production rate. A subsequent recovery in lipoprotein lipase activity might explain why triglycerides eventually return to baseline. However, the role of lipoprotein lipase in the mechanism of action of cafestol has not been established yet. Down-regulation of SREBP activity may also result in a decreased transcription of the LDL receptor gene. As a consequence, LDL receptor activity is suppressed, uptake of LDL from the plasma is impaired and plasma cholesterol will increase (18). Results on the ability of cafestol to modify LDL receptor activity *in vitro* are however contradictory. Cafestol decreased LDL receptor mRNA in rat hepatocytes (16), whereas LDL receptor mRNA was not affected in human skin fibroblasts (14). However, cafestol reduced the uptake of radio-labelled LDL and the number of LDL receptors in human skin fibroblasts (14) and in HepG2 cells (15). The authors concluded that down-regulation of the LDL receptor by post-transcriptional mechanisms might explain the effects of cafestol on plasma cholesterol. Unlike the previously mentioned cell lines, cafestol increased the uptake of radio-labelled LDL and the amount of LDL receptor mRNA in CaCo-2 cells (13) (figure 2). Since *in vitro* studies do not provide consistent evidence for involvement of the LDL receptor in the mechanism of cafestol, an animal model to study LDL receptor expression *in vivo* would be very useful.

Proposed mechanism: cholesterol ester transfer proteins

Another mechanism that might explain the effects of cafestol on lipoprotein metabolism is via cholesterol ester transfer proteins. Cafestol might elevate plasma LDL and VLDL cholesterol and decrease HDL cholesterol by changing their composition. Cafestol could lead to an enrichment of LDL with cholesteryl esters, which results in elevated LDL cholesterol levels. This change in lipoprotein composition might be mediated by an increased activity of cholesteryl-ester transfer proteins (CETP) and phospholipid transfer protein (PLTP) (figure 1B).

In humans, consumption of diterpenes increased the activity of CETP and PLTP and decreased the activity of lecithin:cholesterol acyltransferase (LCAT) (19). However, consumption of unfiltered coffee did not affect lipoprotein composition, although apolipoprotein B100 was slightly increased (de Roos, van Tol, unpublished results). Two other studies also showed a similar rise in apolipoprotein B100 (17;20). The increase in VLDL and LDL cholesterol appears thus more likely due to changes in the number of lipoprotein particles than to a change in lipoprotein composition. Therefore, the role of CETP and PLTP in the mechanism of action of cafestol is doubtful.

Proposed mechanism via PPARs

A third mechanism by which cafestol might affect serum triglycerides and LDL cholesterol is via peroxisome proliferator-activated receptors (PPARs) (figure 1C). PPARs can be considered as key messengers which are responsible for the translation of nutritional, pharmacological and metabolic stimuli into changes in the expression of genes. PPARs compose a subfamily of the nuclear hormone receptors. To date, three different types of PPARs - α, β, and γ - have been identified. PPARα plays a key role in the β-oxidation of fatty acids, while PPARγ is involved in the storage of fatty acids. They are activated by various fatty acid metabolites as well as by several drugs. Activated PPARs heterodimerise with another nuclear receptor: the retinoid X receptor. This complex will bind to specific peroxisome proliferator response elements, by which they alter the transcription of target genes (21). We investigated whether cafestol binds to PPARs and whether cafestol changes transcription mediated by PPARα, β, and γ.

We established that cafestol in concentrations up to 10 μM did not cause a detectable displacement of specific radioligands for PPARα, β, and γ from any of its three receptors (K. MacNaul, D. Moller, unpublished results). High concentrations of cafestol, i.e. up to 20 μM, inhibited transcription of a Gal4-responsive reporter gene in transfected cells expressing chimaeric Gal4-PPAR constructs for each of the three receptor isoforms (D. Moller, J. Berger, unpublished results). However, given the lack of binding and the fact that all 3 PPARs were affected in a similar fashion, we interpret this to be a non-specific cell toxicity effect. Therefore, it appears unlikely that PPARs play a role in the mechanism of action of cafestol.

Conclusion

At this moment, the most plausible mechanism by which cafestol elevates cholesterol in humans is via the SREBP-pathway. However, data from *in vitro* studies are inconsistent and indicate that cafestol or its metabolites may act differently in various cell types. A different regulation of cholesterol levels and the LDL receptor may be caused by the fact that mucosal cells (CaCo-2 cells), fibroblasts and liver cells (HepG2 cells and rat hepatocytes) have different metabolic functions. Because of these conflicting results from *in vitro* studies, and especially because of the sparse knowledge we have on the mechanism of cafestol in man, we can not deduce which finding might be applicable *in vivo*. We thus need to know how cafestol affects lipoprotein composition, synthesis, and degradation in humans. In addition, it would be interesting to know how cafestol is transported in the plasma, as well as how it is metabolised in the liver. Then, we might be able to define a model - either *in vitro* or *in vivo* – which can answer questions on the action of cafestol.

Objective and outline of this thesis

The objective of our research was to study the mechanism of action of cafestol (and kahweol) in man.

We first assessed which part of the consumed coffee diterpenes is absorbed in healthy ileostomy volunteers and thus available for raising serum lipoproteins in humans (*Chapter 2*). Considering the effects of cafestol and kahweol on lipoprotein metabolism and serum liver enzymes, it appears likely that the liver is the target organ for their action. In this case, the possibilities for mechanistic studies in humans are limited, and an animal model would therefore be useful. *Chapter 3* reviews the validity of various animal models for the cholesterol-raising effects of coffee diterpenes in humans. This chapter also describes the effects of cafestol and kahweol on serum lipoproteins in African green monkeys, an animal model which shows many similarities with humans in the effects of dietary cholesterol and fatty acids on plasma lipoproteins. *Chapter 4* continues with the effects of cafestol and kahweol on serum lipoproteins in two hyperlipidaemic mouse strains: heterozygous LDL receptor knock-out mice and apolipoprotein E*3-Leiden transgenic mice. In the latter mouse strain we subsequently performed studies on the mechanism of the cholesterol-raising effect. The following two chapters describe two mechanistic studies in humans. We first studied the possible involvement of cholesteryl ester transfer proteins (CETP) in the cholesterol-raising action of cafetiere coffee (*Chapter 5*). We then determined the production and fractional catabolic rate of large very low density lipoprotein ($VLDL_1$) apolipoprotein B in order to explain the triglyceride-raising effect of cafestol (*Chapter 6*). Finally, the main outcomes of the mechanistic studies with cafestol are discussed in *Chapter 7*.

Acknowledgements

We thank the Foundation for Nutrition and Health Research for funding, co-workers and colleagues for their contributions in the research described above, and Dr. K. MacNaul, Dr. D. Moller, Dr. J. Berger and Dr. S. Wright, Merck Research Laboratories, Rahway, USA, for performing the PPAR-binding and PPAR-function studies with cafestol.

References

1. de Roos B, van der Weg G, Urgert R, van de Bovenkamp P, Charrier A, Katan MB. Levels of cafestol, kahweol, and related diterpenoids in wild species of the coffee plant Coffea. J Agric. Food Chem. 1997;45:3065-3069.
2. Urgert R, van der Weg G, Kosmeijer-Schuil TG, van de Bovenkamp P, Hovenier R, Katan MB. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. J Agric. Food Chem. 1995;43:2167-2172.
3. Gross G, Jaccaud E, Huggett AC. Analysis of the content of the diterpenes cafestol and kahweol in coffee brews. Food Chem. Toxicol. 1997;35:547-554.
4. Heckers H, Gobel U, Kleppel U. End of the coffee mystery: diterpene alcohols raise serum low-density lipoprotein cholesterol and triglyceride levels [letter]. J Intern. Med 1994;235:192-193.

5. Weusten-van der Wouw MPME, Katan MB, Viani R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res. 1994;35:721-733.
6. Urgert R, Essed N, van der Weg G, Kosmeijer-Schuil TG, Katan MB. Separate effects of the coffee diterpenes cafestol and kahweol on serum lipids and liver aminotransferases. Am. J Clin. Nutr. 1997;65:519-524.
7. Urgert R, Katan MB. The cholesterol-raising factor from coffee beans. Annu. Rev. Nutr. 1997;17:305-324.
8. Urgert R, Meyboom S, Kuilman M, Rexwinkel H, Vissers MN, Klerk M, Katan MB. Comparison of effect of cafetiere and filtered coffee on serum concentrations of liver aminotransferases and lipids: six month randomised controlled trial. BMJ. 1996;313:1362-1366.
9. Zock PL, Katan MB, Merkus MP, van Dusseldorp M, Harryvan JL. Effect of a lipid-rich fraction from boiled coffee on serum cholesterol. Lancet 1990;335:1235-1237.
10. Stensvold I, Tverdal A, Foss OP. The effect of coffee on blood lipids and blood pressure. Results from a Norwegian cross-sectional study, men and women, 40-42 years. J Clin. Epidemiol. 1989;42:877-884.
11. Pietinen P, Aro A, Tuomilehto J, Uusitalo U, Korhonen H. Consumption of boiled coffee is correlated with serum cholesterol in Finland. Int. J Epidemiol. 1990;19:586-590.
12. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331-340.
13. Ranheim T, Halvorsen B, Huggett AC, Blomhoff R, Drevon CA. Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells. J Lipid Res. 1995;36:2079-2089.
14. Halvorsen B, Ranheim T, Nenseter MS, Huggett AC, Drevon CA. Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts. J Lip. Res. 1998;39:901-912.
15. Rustan AC, Halvorsen B, Huggett AC, Ranheim T, Drevon CA. Effect of coffee lipids (cafestol and kahweol) on regulation of cholesterol metabolism in HepG2 cells. Arterioscl. Thromb. Vasc. Biol. 1997;17:2140-2149.
16. Post SM, de Wit EC, Princen HMG. Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase in rat hepatocytes. Arterioscler. Thromb. Vasc. Biol. 1997;17:3064-3070.
17. van Dusseldorp M, Katan MB, van Vliet T, Demacker PN, Stalenhoef AF. Cholesterol-raising factor from boiled coffee does not pass a paper filter. Arterioscler. Thromb. 1991;11:586-593.
18. Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem. 1977;46:897-930.
19. van Tol A, Urgert R, de Jong-Caesar R, et al. The cholesterol-raising diterpenes from coffee beans increase serum lipid transfer protein activity levels in humans. Atherosclerosis 1997;132:251-254.
20. Aro A, Teirila J, Gref CG. Dose-dependent effect on serum cholesterol and apoprotein B concentrations by consumption of boiled, non-filtered coffee. Atherosclerosis 1990;83:257-261.
21. Schoonjans K, Staels B, Auwerx J. The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochim. Bioph. Acta 1996;1302:93-109.

2

Absorption and urinary excretion of the coffee diterpenes cafestol and kahweol in healthy ileostomy volunteers

Baukje de Roos, Saskia Meyboom, Truus G. Kosmeijer-Schuil and Martijn B. Katan.
Journal of Internal Medicine 1998; 244: 451-460.

Abstract

The objective of this study was to determine the absorption and urinary excretion of the cholesterol-raising coffee diterpenes cafestol and kahweol in man. Nine healthy ileostomists consumed a dose of 1, 2, or 3 cups of French-press coffee together with a standardised breakfast on three separate days in random order. Subsequently, ileostomy effluent was collected for 14 h and urine for 24 h. We measured absorption and urinary excretion of diterpenes and the stability of cafestol and kahweol was also assessed under simulated gastro-intestinal tract conditions. Corrected mean absorption expressed as percentage of the amount consumed and as percentage of the amount entering the duodenum was 67% and 88%, respectively, for cafestol, and 72% and 93%, respectively, for kahweol. We found losses of diterpenes during incubation *in vitro* with gastric juice (cafestol 24%, kahweol 32%), during storage with ileostomy effluent (cafestol 18%, kahweol 12%), and during freeze-drying (cafestol 26%, kahweol 32%). Mean excretion of glucuronidated plus sulphated conjugates in urine was 1.2% of the ingested amount for cafestol and 0.4% of the ingested amount for kahweol. In conclusion, about 70% of the ingested cafestol and kahweol is absorbed in ileostomy volunteers. Possibly, undetected metabolites are present in ileostomy effluent, resulting in lower absorption percentages. Only a small part of the diterpenes is excreted as a conjugate of glucuronic acid or sulphate in urine. Therefore these compounds are extensively metabolised in the human body.

Introduction

Cafestol and kahweol (figure 1) are naturally-occurring diterpenes in coffee beans (1), where they are present as fatty esters, mainly of palmitic and linoleic acids (2). They are responsible for the low density lipoproteins (LDL) and very low density lipoproteins (VLDL)-raising effect of Scandinavian-type boiled coffee (3;4), which contains 3-4 mg of each per cup (1). Levels of diterpenes are also high in other unfiltered coffee brews such as Turkish/Greek and French-press or cafetiere coffee (1). The LDL- and VLDL raising effect of cafestol and kahweol seems to be unique for humans: serum lipids in various animal models did not respond to the two diterpenes (5).

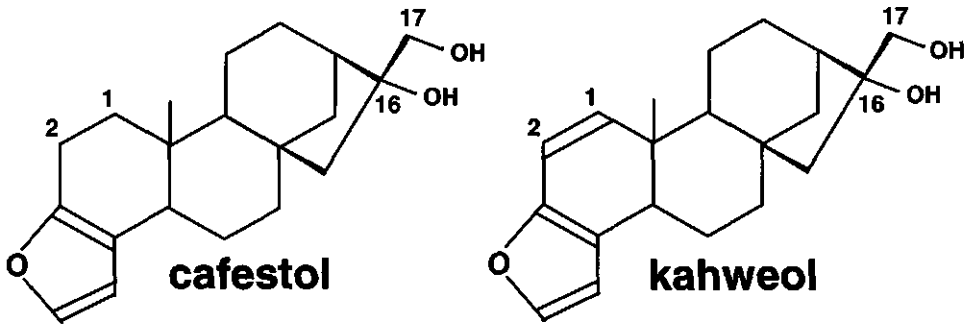

Figure 1. Structure of the coffee diterpenes cafestol and kahweol. Diterpenes occur in coffee beans mainly esterified to fatty acids at the C16 or C17 position.

In humans, cafestol appears to be mainly responsible for the effects on serum lipids (5;6). A meta-analysis on eleven trials showed that a daily dose of 10 mg of cafestol for four weeks raised serum cholesterol by 0.13 mmol/L, while kahweol raised serum cholesterol by 0.02 mmol/L. About 80% of the rise in serum cholesterol was accounted for by LDL cholesterol. A daily dose of 10 mg of cafestol or kahweol for four to six weeks raised serum triglycerides by 0.08 mmol/L and 0.01 mmol/L, respectively. Most of the rise in serum triglycerides subsides with chronic intake of coffee diterpenes (5). Both cafestol and kahweol also appear to affect liver cell functioning in humans: each 10 mg of cafestol or kahweol per day increased alanine aminotransferase by 2-3 U/L after four weeks (5). The different capacities of cafestol and kahweol to raise serum lipids or serum liver enzymes indicate that possibly two distinct mechanisms of action are involved.

Knowledge about the absorption and the mechanisms of action of these coffee diterpenes in humans is limited. *In vitro* studies suggest that the coffee diterpenes decrease receptor-mediated uptake of LDL cholesterol (7;8). A lower hepatic uptake of LDL cholesterol may contribute to the cholesterol-raising effect of cafestol observed in humans. Absorption values are now required to assess which part of the consumed diterpenes is actually responsible for the rise in serum lipids. Factors such as dose might influence absorption, and thus the effects observed. In addition, absorption values indicate which part of the consumed diterpenes are unabsorbed and thus enter the colon, where they might have beneficial effects: intake of coffee diterpenes reduced the frequency of adenocarcinoma of the colon in rats (9), and in some human epidemiological studies a lower incidence of colon cancer with coffee intake was found (10). More insight into the metabolism of cafestol and kahweol in the human body might lead us to the compound that actually raises serum lipids. This active compound could be cafestol or kahweol, but it could also be a metabolite. Such insights would facilitate further studies of the mechanism of action.

We assessed the absorption of cafestol and kahweol from increasing doses of French-press coffee by measuring coffee diterpenes in ileostomy effluent of healthy ileostomy volunteers. We also provide evidence for the excretion of conjugated cafestol and kahweol metabolites in urine.

Subjects and methods

We obtained prior approval for the experiment from the Medical Ethical Committee of the Department of Human Nutrition, Wageningen University.

Subjects

We recruited subjects by writing to members of the Dutch association of ileostomists and by approaching volunteers from a previous study (11). Thirteen subjects filled out a general and a medical questionnaire and blood samples were taken for standard laboratory assays. Exclusion criteria were: signs of Crohn's disease or malabsorption, resection of more than 50 cm of the terminal ileum, an ileostomy that did not function properly, a history of hepatobiliary disease, renal disease or diabetes, use of drugs influencing gastro-intestinal transit, present illness, pregnancy or lactation, a serum haemoglobin concentration of less than 8.6 mmol/L for men or less than 7.4 mmol/L for women, serum cholesterol levels of more than 8.0 mmol/L, triglyceride levels of more than 2.37 mmol/L, or ALT levels exceeding the upper limit of normal. We checked urine samples for protein and glucose. The medical questionnaires and the results of the blood tests were judged by an independent gastro-enterologist. Four subjects were excluded from this study

because of Crohn's disease (n=1), liver and gall bladder disease (n=1), small intestine disease (n=1) or removal of more than 50 cm of the terminal ileum (n=1).

Nine subjects (4 men and 5 women) with a mean age of 60 years (range 43-71 years) and mean (±SD) body mass index of 26.8±3.0 kg/m^2 were admitted to the study. All subjects had undergone proctocolectomy 3-27 years earlier because of ulcerative colitis (n=8) or polyposis coli (n=1), and all had an intact ileum. The subjects gave their informed consent before the start of the study.

Supplements

We prepared French-press coffee by pouring 1 L of boiling water onto 65 g of coarsely ground coffee (Roodmerk, Douwe Egberts, The Netherlands) in a glass jug (Kaffee Primo, BMF, Germany). The brew was thoroughly stirred and allowed to stand for 3 min. The plastic strainer was pushed down and the liquid was decanted. For each subject we weighed 150, 300 or 450 mL of the coffee brew into a thermos flask so as to provide 1, 2 or 3 cups of coffee, respectively. We stored another 100 mL of each individual brew at -20 °C for diterpene analysis.

Study design

We used a multiple cross-over design (figure 2) in which all volunteers first followed a run-in period of seven days. On day seven, subjects received a standardised breakfast without coffee. Subsequently, they were randomly assigned to three treatment sequences, each consisting of three treatment days separated by one wash-out week. Treatment on day 14, 21 and 28 consisted of consumption of the same standardised breakfast as on day seven, plus either one, two or three cups of French-press coffee. Subjects also swallowed 20 radio-opaque barium-salt-impregnated plastic ringlets with an outer diameter of 3 mm (TD Medical, Eindhoven, The Netherlands). These markers provide a validated method for the assessment of solid food recovery in ileostomists (12). Subjects were restricted from eating and drinking for 2 h after breakfast, except for having tea without milk or sugar, or water. On all treatment days, subjects swallowed with their meals three capsules containing 80 mg of *para*-aminobenzoic acid each per day to check for complete urine collection. *Para*-aminobenzoic acid is completely absorbed and excreted with urine in humans (13). The participants were allowed to drink only filtered or instant coffee throughout the study, as these coffee types contain negligible amounts of cafestol and kahweol (1). Participants recorded any deviation from their usual diet and physical activity pattern in a diary, as well as any signs of illness, and medication used.

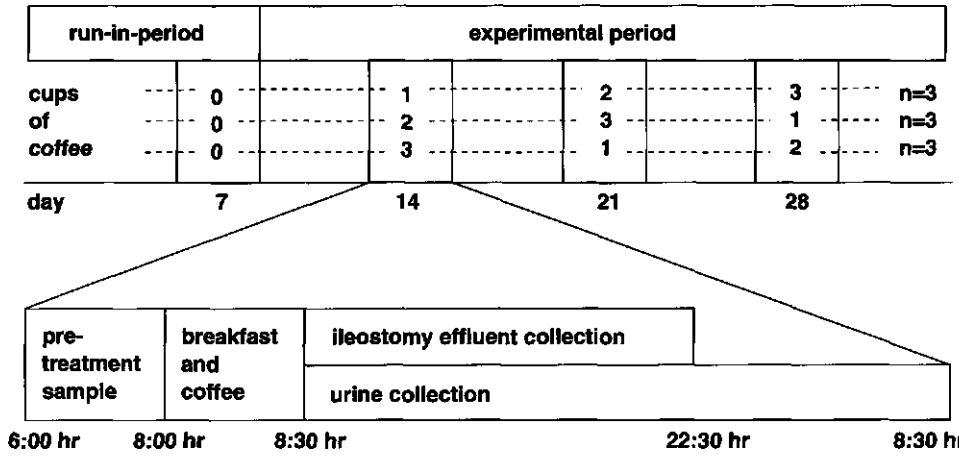

Figure 2. Design of the study. Nine healthy ileostomy volunteers followed a run-in period of seven days and were randomly assigned to three treatment sequences, each consisting of three treatment days. On day seven, a standardised breakfast was provided and subsequently ileostomy effluent was collected every 2 h for about 14 h. On day 14, 21 and 28, a standardised breakfast with a single dose of either one, two or three cups of French-press coffee was provided, ileostomy effluent was collected every 2 h for about 14 h, and urine was collected for 24 h. On the day when three cups of French-press coffee had to be consumed, subjects collected urine every 2 h. In the night preceding every experimental day, a pre-treatment sample of ileostomy effluent and urine was obtained.

Collection of samples

On days 7, 14, 21 and 28, subjects collected ileostomy effluent throughout the day for 14.1±0.7 (mean±SD) h after coffee consumption. They also collected a pre-treatment sample during the night preceding each experimental day. Subjects changed ileostomy bags every 2.1±0.2 h and immediately stored them on dry ice. We counted the number of radio-opaque ringlets in the ileostomy samples by X-raying within the next two days. We separated the plastic ileostomy bags from their contents using liquid nitrogen, and freeze-dried the contents. We checked sample weights once or twice a day during the process of freeze-drying, and immediately removed them from the freeze-dryer when stable weight was reached. Samples were ground to pass a 0.5 mm sieve and stored at -20°C until analysis.

Subjects collected urine for 24 h after each coffee dose. Control urine samples were collected during the night preceding each treatment day. On the day on which 3 cups of French-press coffee were provided, subjects collected 24-h urine in timed portions every 2.0±0.1 h during daytime and in 1 portion during the night. Urine was voided into 0.5 L nalgene bottles containing 5 mL toluene (Janssen Chimica) each, and immediately put on dry ice. The following morning we warmed the urines to

37°C, mixed, and took aliquots for cafestol, kahweol and *para*-aminobenzoic acid analysis. Aliquots were stored at -20°C.

In vitro incubation of diterpenes with gastro-intestinal fluids

We evaluated the stability of cafestol and kahweol palmitate in gastric and duodenal juice *in vitro* in order to assess possible losses of diterpenes during gastro-intestinal transit. Gastric and duodenal juice were obtained from 2 healthy fasting volunteers at the department of Gastro-enterology, Nijmegen University Hospital, The Netherlands, and stored at -20°C. We incubated 6, 12 and 18 mL of French-press coffee with 4 mL of gastric juice and 1 mL of water each for 0, 20 and 60 min at 37°C. This mimicked stomach contents after the administration of 1, 2 and 3 cups of coffee (14;15). To assess if a low pH affects the recovery of diterpenes during stomach passage, we made five solutions of HCl in demineralised water with pH values of 0.72, 1.43, 2.19, 3.56, and 4.05. One volume of each solution was added to 3 volumes of French-press coffee. This yielded incubation samples with pH values of 1.56, 3.89, 4.63, 4.81 and 4.92, respectively. Distilled water was used for the control incubation. Samples were incubated at 37°C for 20 and 60 min, frozen immediately, and stored at -20°C. In addition, we incubated 6, 12 and 18 mL of French-press coffee with 2 mL of duodenal juice (Department of Gastro-enterology, Nijmegen University Hospital, The Netherlands) plus 1 mL of water each for 0, 1 and 4 h: 1 and 4 h reflect the estimated mean and maximum transit time in the human small intestine, respectively (16;17). All samples were frozen with liquid nitrogen immediately after the incubation, and stored at -20°C.

We also assessed stability of cafestol and kahweol palmitate during collection of the effluent in the ileostomy bag and during freeze-drying. To that end, we incubated 70 mg of a mixture of cafestol plus kahweol palmitate dissolved in 0.5 mL ethanol with 50 g of fresh ileostomy effluent obtained from 8 subjects for 2 and 0 h, respectively, at 30°C. The samples were shaken every 15 min during incubation. Samples were immediately frozen in liquid nitrogen, freeze-dried and stored at -20°C. To asses the stability of cafestol and kahweol during freeze-drying in detail, we dissolved 40.7 mg aliquots of cafestol plus kahweol palmitate in 0.4 mL ethanol plus 9.6 mL distilled water, and freeze-dried such samples for various time periods.

Analytical methods

Cafestol and kahweol in ileostomy effluent. We analysed samples with and without pre-treatment with an enzyme preparation having ß-glucuronidase activity (100,000 units/mL) and sulphatase activity (5,000 units/mL) (Sigma, G7017). The pre-treatment consisted of incubating 300 mg of thawed ileostomy effluent with 200 µL of ß-glucuronidase and 2 mL of 0.4 mol/L phosphate buffer (pH 5) in a water bath of 37°C for 1 h. Subsequently, we prepared samples for diterpene analysis as

described by Urgert et al (1). Briefly, we added 2 mL of 5 mol/L methanolic KOH to both pre-treated and not pre-treated samples and saponified the samples for 1 h at 80°C. After cooling the samples, lipids were extracted by adding 5 mL of di-isopropyl ether (Merck, Darmstadt, Germany, no. 867), shaking for 10 min at 250 oscillations/min and centrifuging for 10 min at 3000 rpm. We collected the ether phase, and re-extracted the water phase twice with diisopropyl ether. The combined ether phases were washed twice with 3 mL of distilled water. The ether phase was evaporated with nitrogen, and the remnant was dissolved in 1 mL of methanol (Lab Scan, no. C2517). Of each sample, 10 µL was injected into an HPLC system (Shodex degas, SP8875 autosample, SP8800 ternary HPLC pump, Spectra Focus detector, Thermo Separation Products) equipped with two serially connected reverse phase Spherisorb ODS columns, 100 x 3 mm each (Chrompack, Middelburg, The Netherlands). The elution solvent, which consisted of 62.5% (v/v) methanol and 37.5% filtered distilled water, was administered at a flow rate of 0.4 mL/min. Detection was at both 220 and 290 nm, which reflects the optimal absorption of cafestol and kahweol, respectively. The coefficients of variation for a control sample of faeces containing a known amount of cafestol and kahweol were 10.6% within and 7.0% between runs over a 6 month's period for cafestol, and 8.2% and 7.1%, respectively, for kahweol.

Cafestol and kahweol in urine. The urine samples were thawed and centrifuged. Two mL of the supernatant was incubated with 1 mL of 0.4 mol/L phosphate buffer (pH 5) and 200 µL of ß-glucuronidase at 37°C for 1 h, adsorbed to a 500 mg reverse phase C18 column (Varian products, USA), washed twice with 1 mL of distilled water, and extracted with 3 mL of methanol. The extract was evaporated and the remnant dissolved in 250 µL methanol/H_2O, 62.5/37.5 v/v. Twenty µL was injected into the HPLC system, equipped with two serially connected reverse phase ChromSpher C8 columns, 100 x 3 mm each (Chrompack). Other conditions were as described for ileostomy effluent. Two urine samples from a previous experiment (18), one with a low and one with a high cafestol and kahweol content, showed a coefficient of variation for cafestol of 14.4% and 4.6%, respectively, within, and 15.8% and 9.8%, respectively, between runs over a 6 month's period. For kahweol, coefficients of variation were 25.8% and 11.1%, respectively, within, and 12.8% and 16.1%, respectively, between runs.

Cafestol and kahweol in coffee and gastro-intestinal fluids. We analysed coffee and fluid samples as described by Urgert et al. (1). The coefficients of variation for a control pool of boiled coffee were 2.5% within and 9.5% between runs over a 6 month's period for cafestol and 2.3% and 7.2% for kahweol, respectively.

Para-aminobenzoic acid in urine. We determined *para*-aminobenzoic acid spectrophotometrically with fluorescamine (Roche, 071088) as described previously

(19). Addition of 200 µg of para-aminobenzoic acid to 1 mL of urine yielded a recovery of 96% (n=2).

Results

Collection of ileostomy effluent and urine

Of the twenty radio-opaque ringlets swallowed, 93±13% (mean±SD) were recovered in the ileostomy bags. One subject refrained from swallowing the ringlets on the third treatment day after consultation with the researchers, because during the first two experimental days she had excreted less than 50% of the 20 ingested radio-opaque ringlets. Data analyses were performed with and without this subject's results on diterpene excretion in ileostomy effluent.

Recovery of *para*-aminobenzoic acid in 24-h urine was 85±16% (mean±SD). Four persons showed on ten separate occasions a urinary recovery of *para*-aminobenzoic acid of less than 85%. Data analyses were performed with and without the results of these ten occasions.

Stability of cafestol and kahweol during incubation with gastro-intestinal fluids

During *in vitro* incubation in gastric juice, recoveries of cafestol decreased by 25±10% (mean±SD, n=6 incubations) after 20 min and 23±9% after 60 min. Recovery of kahweol decreased by 22±9% (n=6 incubations) after 20 min and 25±6% after 60 min. Outcomes were irrespective of the dose of French-press coffee, and therefore data were pooled per incubation time period. Recoveries of cafestol and kahweol after incubation with HCl dilutions with pH values of 0.7, 1.4, 2.2, 3.6, and 4.1, were 89±9% (mean±SD, n=8 incubations), 112±3%, 113±6%, 116±5% and 113±6%, respectively. We pooled data per pH because outcomes were irrespective of the incubation time. The diterpenes were stable during incubation in duodenal juice for 1 and 4 h. Incubation of cafestol and kahweol with ileostomy effluent for 2 h plus subsequent freeze-drying yielded a recovery of 56±5% (n=6 incubations) for both cafestol and kahweol.

Stability of cafestol and kahweol during freeze-drying

Recovery of cafestol and kahweol palmitate in ileostomy effluent during freeze-drying was 74±5% (n=4 incubations) for cafestol and 68±4% (n=4 incubations) for kahweol. Cafestol plus kahweol palmitate dissolved in ethanol and distilled water were stable during freeze-drying until all water had disappeared, i.e. after 8 h. Then recovery decreased to 60% for cafestol and 37% for kahweol during an additional 22 h of freeze-drying. Therefore, loss of cafestol and kahweol during freeze-drying appears to occur mainly after samples lost their water content.

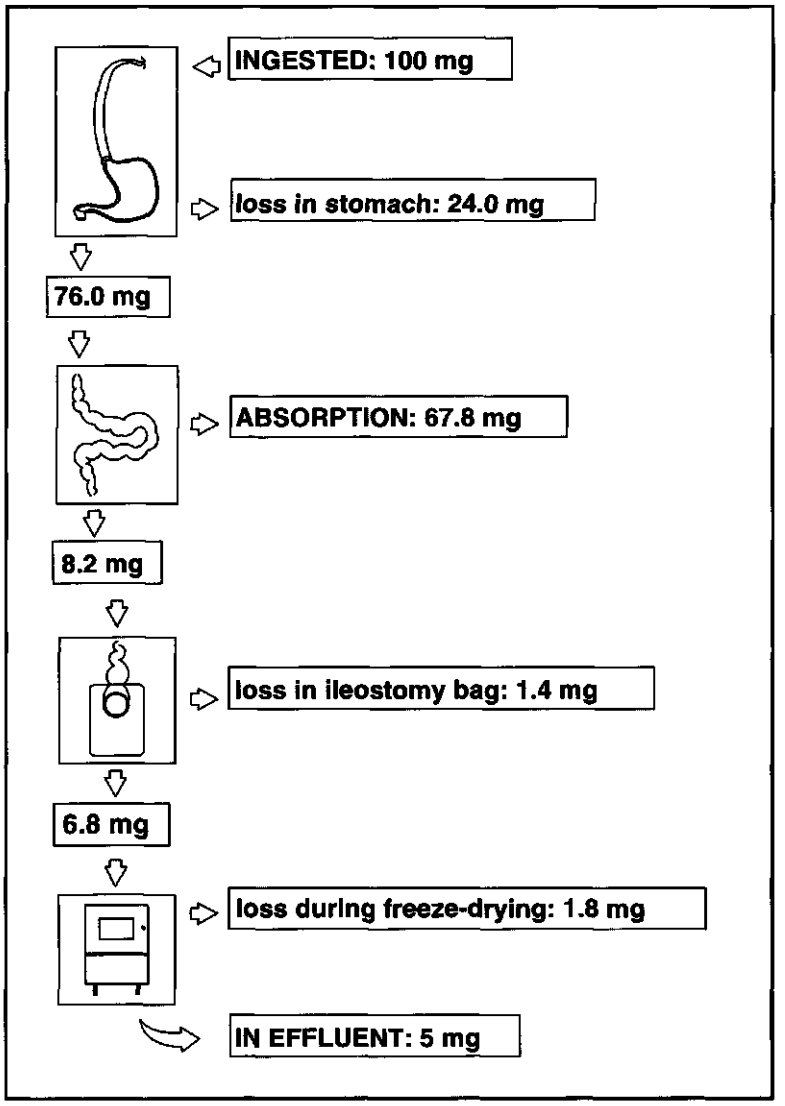

Figure 3. Algorithm to calculate absorption of cafestol and kahweol. Data were calculated as described in the Results section. Losses during incubation with gastric juice, during storage in ileostomy effluent and during freeze-drying were estimated with data obtained from in vitro experiments.

Calculations

We used an algorithm to correct the absorption of cafestol and kahweol in the small intestine for losses in gastric juice, in ileostomy effluent and during freeze-drying (figure 3). We calculated the individual absorption of cafestol and kahweol expressed as the percentage of the amount consumed as follows:

Cafestol absorption as percentage of the amount consumed (%) =
*(((X-0.24X)-((Y/0.74)/0.83))/X)*100*
Kahweol absorption as percentage of amount consumed (%) =
*(((X-0.23X)-((Y/0.68)/0.88))/X)*100*
where X was the amount consumed with the French-press coffee, and Y the amount detected in freeze-dried ileostomy effluent. Absorption of cafestol and kahweol expressed as percentage of the amount entering the duodenum was calculated as follows:
Cafestol absorption as percentage of the amount entering the duodenum (%) =
*(((X-0.24X)-((Y/0.74)/0.83))/(X-0.24X))*100*
Kahweol absorption as percentage of the amount entering the duodenum (%) =
*(((X-0.23X)-((Y/0.68)/0.88))/(X-0.23X))*100*
Cafestol and kahweol excretions in urine were corrected for estimated losses in the stomach (cafestol 24% and kahweol 23%).

Absorption and excretion of coffee diterpenes

No cafestol or kahweol was found in ileostomy effluent collected on the pre-experimental control day (figure 2, day 7), when no French-press coffee was provided. However, significant amounts of the coffee diterpenes were found in pre-treatment samples of two subjects collected in the night preceding an experimental day. Data analyses were therefore performed with and without the results obtained from the subsequent two experimental days. The reason for the presence of the coffee diterpenes in the pre-treatment samples is not clear. Both subjects declared not to have consumed any coffee other than filtered coffee during the experiment. It might be that cafestol and kahweol are not unique for coffee beans. So far, however, these coffee diterpenes have not been found in other foods.

Only free cafestol and kahweol was detected in ileostomy effluent; addition of β-glucuronidase with sulphatase activity did not result in a higher diterpene content (data not shown). Mean (±SD) absorption of cafestol and kahweol expressed as percentage of the ingested amount and as percentage of the amount entering the duodenum, after correction for the estimated losses, is illustrated in table 1.

No free cafestol or kahweol was found in urine. Urinary excretion of glucuronidated or sulphated cafestol and kahweol occurred mostly within 8 h after consumption of the French-press coffee, indicating that collection was complete. There was a considerable variation in the subjects' excretion rates, possibly due to interindividual differences in phase II enzyme activities (figure 4). Mean (±SD) excretion of cafestol and kahweol in 24-h urine is illustrated in table 1.

Table 1. Intake, excretion, and estimated absorption of cafestol and kahweol from French-press coffee expressed as percentage of the ingested amount and expressed as percentage of the amount entering the duodenum in nine healthy ileostomy volunteers*. Subjects consumed on three separate days a standardised breakfast with one, two or three cups of French-press coffee. They subsequently collected ileostomy effluent for 14 h and urine for 24 h.

Diterpene and cups of coffee	n	Intake	Excretion in ileostomy effluent	Absorption of ingested amount†	Absorption of amount entering the duodenum†	Excretion in 24-h urine‡	
		(mg)	*(mg)*	*(%)*	*(%)*	*(mg)*	*(%)*
Cafestol							
1 cup	9	6.1 ± 1.3 (4.6-7.6)	0.4 ± 0.4 (0.1-1.0)	64.2 ± 10.2	84.5 ± 13.5	0.1 ± 0.1 (0.0-0.4)	1.8 ± 4.1
2 cups	9	12.9 ± 2.6 (9.3-16.1)	0.8 ± 0.6 (0.2-2.0)	65.8 ± 7.7	86.6 ± 10.1	0.1 ± 0.1 (0.0-0.1)	0.4 ± 0.4
3 cups	9	20.9 ± 4.8 (12.7-24.3)	0.7 ± 0.6 (0.1-1.6)	70.6 ± 3.9	92.9 ± 5.1	0.2 ± 0.2 (0.0-0.5)	1.4 ± 0.7
Kahweol							
1 cup	9	6.8 ± 1.4 (5.2-8.2)	0.3 ± 0.3 (0.0-0.8)	70.2 ± 7.6	91.1 ± 9.8	0.0 ± 0.0 (0.0-0.1)	0.5 ± 1.1
2 cups	9	14.4 ± 2.7 (10.8-17.5)	0.4 ± 0.4 (0.1-1.4)	7.2 ± 4.5	93.9 ± 5.8	0.0 ± 0.0 (0.0-0.1)	0.2 ± 0.2
3 cups	9	23.0 ± 4.7 (14.7-26.3)	0.5 ± 0.5 (0.1-1.7)	73.2 ± 3.0	95.0 ± 3.9	0.1 ± 0.1 (0.0-0.2)	0.5 ± 0.4

*Mean ± SD (range). Analyses were performed on all subjects. Mean absorption of cafestol expressed as percentage of the amount consumed - after rejection of the results of two occasions where cafestol and kahweol were found in pre-treatment samples and of the results of one subject who refrained from swallowing the radio-opaque ringlets - were 66.7% (n=7), 65.4% (n=8) and 70.8% (n=7) after consumption of 1, 2, or 3 cups of French-press coffee, respectively. Mean absorption of kahweol was 72.0%, 72.1% and 72.7%, respectively. Cafestol absorption expressed as percentage of the amount entering the duodenum was 87.8%, 86.1% and 93.1% after consumption of one, two, or three cups of French-press coffee, respectively. Mean absorption of kahweol was 93.5%, 93.6% and 94.4%, respectively. Mean excretion of cafestol in 24-h urine after exclusion of the results of the two occasions where diterpenes were found in pre-treatment samples and of the results of the occasions where less than 85% of *para*-aminobenzoic acid was recovered in urine was 0.0% (n=5), 0.2% (n=5) and 0.9% (n=6) after consumption of 1, 2, or 3 cups of French-press coffee, respectively. Mean excretion of kahweol was 0.1%, 0.1% and 0.3%, respectively.

†Corrected for estimated loss in gastric juice (cafestol 24% and kahweol 23%), estimated loss in the ileostomy bag (cafestol 18% and kahweol 12%) and estimated loss during freeze-drying (cafestol 26% and kahweol 32%) and calculated as described in the Results section.

‡Corrected for estimated loss in gastric juice (cafestol 24% and kahweol 23%).

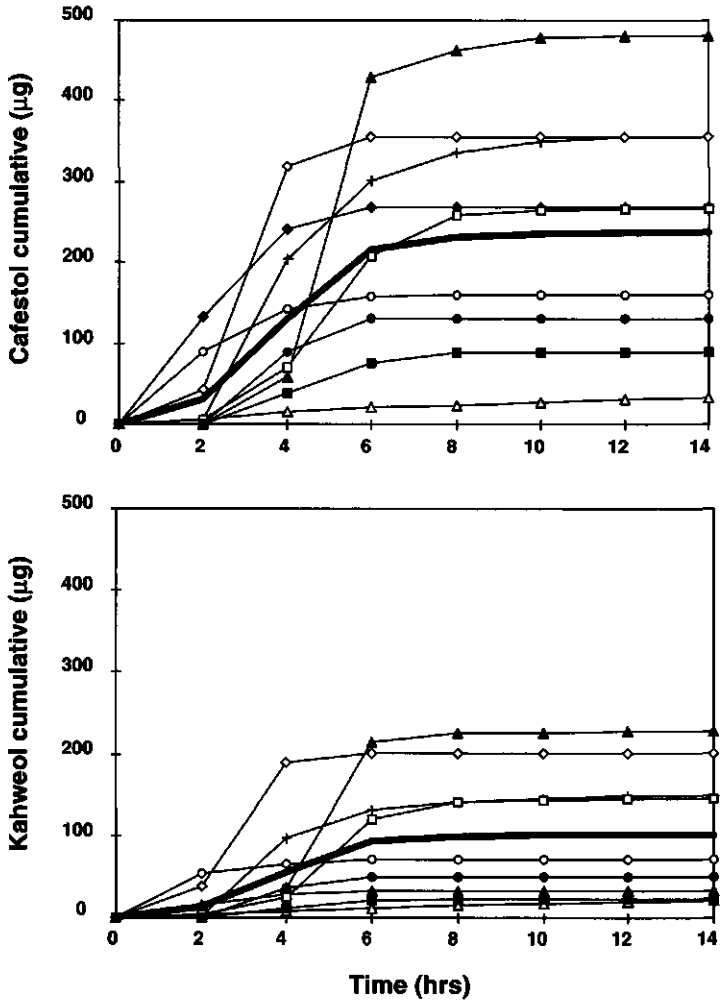

Figure 4. Cumulative excretion of cafestol and kahweol in urine of 9 subjects after consumption of 3 cups of French-press coffee. The heavy line represents the mean.

Discussion

We found that about 70% of the ingested cafestol and kahweol from French-press coffee was absorbed in ileostomy volunteers. Most of the other 30% was degraded in gastric fluid before it could reach the duodenum. Absorption of the cafestol and kahweol that reached the duodenum was about 90%. Only a small part was subsequently excreted as a conjugate of glucuronic acid or sulphate in urine.

Validity of the ileostomy model

We studied the excretion of cafestol and kahweol in subjects whose colon had been resected. The advantage of the ileostomy model is the absence of microbial degradation in the colon. When 2-h portions of ileostomy effluent during daytime and one portion during the night were subjected to immediate freezing on dry ice, virtually no microbial degradation on protein, fat, and dietary fibre in the ileostomy bags was found (20;21), and degradation of bile acids and neutral sterols was absent or minimal (22;23). In our samples, however, we did find a mean degradation of 18% of cafestol and of 12% of kahweol 2 h after addition to freshly collected ileostomy effluent. A part of the cafestol and kahweol reaching the ileostomy bag might be degraded by microflora excreted from the terminal ileum. Substantial bacterial colonisation of the terminal ileum in ileostomists has been reported (24-26). If indeed bacteria are responsible for the degradation of cafestol and kahweol in ileostomy effluent, use of the ileostomy model does not appear to exclude effects of microflora on all substances present in the ileostomy bag.

We could not avoid freeze-drying, since this is the only way to obtain homogeneous samples for the analysis. Losses of cafestol and kahweol during freeze-drying might be due to the formation and sublimation of cafestolene and kahweolene: these contain an additional double bond between C15 and C16 compared to the original diterpenes (27). Loss of cafestol and kahweol became high after the water had disappeared. However, we immediately removed samples from the freeze-dryer upon reaching stable weight. Therefore, losses of cafestol and kahweol have been minimised.

Gastro-intestinal transit of coffee diterpenes

We found a considerable loss of cafestol and kahweol present in French-press coffee during *in vitro* incubation with representative amounts of gastric juice. Although the number of *in vitro* incubations was few, the results were consistent. Therefore, these *in vitro* data strongly indicate that at least a part of the consumed diterpenes is transformed into other compounds during gastric passage. As the recovery of cafestol and kahweol with gastric juice were equally affected, the double bond between C1 and C2 in the kahweol molecule does not appear to play an important role. Recovery of cafestol and kahweol was slightly smaller after incubation with a solution with a pH value of 0.72, compared to solutions with pH values ranging from 1.43-4.05. Therefore, high concentrations of HCl might in part be responsible for the transformation of diterpenes during incubation with gastric juice. However, the 24% decrease in recovery of coffee diterpenes during incubation with gastric juice cannot be fully explained by low pH.

Absorption of coffee diterpenes

An algorithm based on losses determined *in vitro* enabled us to estimate the absorption of cafestol and kahweol expressed as percentage of the amount consumed and expressed as percentage of the amount entering the duodenum. Both calculated mean absorptions might show variation between the 9 subjects because of interindividual differences in the proportion of diterpenes in ileostomy effluent compared to the amount consumed. Our algorithm, however, does not consider the variation of the mean losses determined *in vitro* in the calculation of individual absorption values. Therefore, the real variation in mean absorption will be underestimated. We calculated that the standard deviation of the amount of cafestol and kahweol leaving the stomach (in the formula presented as X-0.24X for cafestol and X-0.23X for kahweol) has 10 times more influence on the total variation of an individual absorption value than the standard deviation of the amount entering the colon (in the formula presented as (Y/0.74)/0.83 for cafestol and (Y/0.68)/0.88 for kahweol). These standard deviations taken together lead to a coefficient of variation of 16% for the absorption of cafestol expressed as percentage of the ingested amount and of 13% for the absorption of kahweol as percentage of the ingested amount. This indicates that two thirds of the absorption values expressed as percentage of the ingested amount will lie in a range of 52-84% for cafestol and 56-81% for kahweol.

As estimated losses were about equal for both cafestol and kahweol, excretion in ileostomy effluent is expected to be similar. However, kahweol excretion was somewhat lower than that of cafestol. This is in agreement with earlier observations in faeces (28). Kahweol might be absorbed more efficiently than cafestol. Also, the double bond of the kahweol molecule might be hydrogenated by bacteria present in the terminal ileum.

Collection of ileostomy effluent during the daytime appeared to cover all cafestol and kahweol excreted. Pilot experiments revealed that no cafestol and kahweol was present in ileostomy effluent sampled 12 h after the consumption of French-press coffee (data not shown).

Urinary excretion of coffee diterpenes

We measured excretion of diterpenes as conjugate of glucuronic acid and sulphate in urine, because glucuronidation and sulphation are the major pathways of xenobiotic biotransformation in mammalian species (29). We hypothesised that at least a part of the ingested cafestol and kahweol will not undergo phase I metabolisation, as they already possess hydroxyl groups. Conjugation of this part with a hydrophilic moiety appears to be necessary for excretion of the fat-soluble coffee diterpenes by the kidney. Indeed, no free cafestol and kahweol was present in urine from subjects receiving diterpene-rich supplements in previous experiments

(28). However, since only about 1% of the ingested amount was excreted as conjugate of glucuronic acid or sulphate in urine, the major part of the absorbed diterpenes must be metabolised more extensively than just glucuronidation or sulphation of the cafestol and kahweol molecule.

There are some indications that phase I metabolites might undergo conjugation with glutathione; kahweol palmitate and to a lesser extent cafestol palmitate induced glutathione S-transferases activity in the mucosa of the small intestine and in the liver of mice (30). The furan moiety of cafestol and kahweol appears vital for this effect (31).

We did not find glucuronidated or sulphated conjugates of cafestol and kahweol in ileostomy effluent. This can implicate that cafestol and kahweol conjugates of glucuronic acid or sulphate are small enough to be excreted into urine instead of into bile, or that the ileostomy effluent matrix inhibits ß-glucuronidase. Also, the fact that we found only a small percentage of the absorbed cafestol and kahweol as conjugates of glucuronic acid or sulphate in urine suggests that glucuronidation or sulphation of cafestol and kahweol are minor processes. Since we did not measure other metabolites of cafestol and kahweol than conjugates of glucuronic acid and sulphate, the presence of other metabolites might overestimate the absorption percentages as calculated with the algorithm.

In conclusion, about 90% of the cafestol and kahweol that enters the small intestine is absorbed there. Absorption of these coffee diterpenes expressed as percentage of the amount consumed is about 70%. This indicates that of each 10 mg of cafestol in French-press coffee consumed, 7 mg of cafestol is absorbed in the small intestine and available for raising serum cholesterol by about 0.13 mmol/L in man. The question remains which part of the ingested diterpenes eventually raises serum lipids, since we did not study the fate of cafestol and kahweol in the pre-systemic circulation. Only 0.8 mg will enter the colon; the amounts available for the presumed anticarcinogenic effects of coffee diterpenes (10) are thus very small. Moreover, only a very small amount of the cafestol and kahweol that enters the circulation is subsequently excreted as conjugate of glucuronic acid or sulphate in urine. Therefore, the major part of the ingested cafestol and kahweol must be metabolised different from just glucuronidation or sulphation of the cafestol and kahweol molecule.

Acknowledgements

This study was supported by the Netherlands Heart Foundation (grant No. 900-562-091 of the Netherlands Organisation of Scientific Research) and the Foundation for Nutrition and Health Research. We thank all volunteers for participating; J. De Vries and 'Harry Bacon' for their contribution to the recruitment of subjects; G. van der Weg, J. Barendse, P. van der Bovenkamp, H. Boer, A. Terlouw, T. van der Doorn, M. Sins en J. Lenting for technical assistance; J. Burema for statistical

assistance; M. Maas for providing us with gastric and duodenal juice and J. Kruimel for medical supervision.

References

1. Urgert R, van der Weg G, Kosmeijer-Schuil TG, van de Bovenkamp P, Hovenier R, Katan MB. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. J Agric. Food Chem. 1995;43:2167-2172.
2. Folstar P. Chemistry. In: Clarke RJ, Macrae R, eds. Coffee. London/New York: Elsevier Applied Science Publishers, 1985:210-213.
3. Weusten-van der Wouw MPME, Katan MB, Viani R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res. 1994;35:721-733.
4. Heckers H, Gobel U, Kleppel U. End of the coffee mystery: diterpene alcohols raise serum low-density lipoprotein cholesterol and triglyceride levels [letter]. J Intern. Med. 1994;235:192-193.
5. Urgert R, Katan MB. The cholesterol-raising factor from coffee beans. Annu. Rev. Nutr. 1997;17:305-324.
6. Urgert R, Essed N, van der Weg G, Kosmeijer-Schuil TG, Katan MB. Separate effects of the coffee diterpenes cafestol and kahweol on serum lipids and liver aminotransferases. Am. J Clin. Nutr. 1997;65:519-524.
7. Ranheim T, Halvorsen B, Huggett AC, Blomhoff R, Drevon CA. Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells. J Lipid Res. 1995;36:2079-2089.
8. Rustan AC, Halvorsen B, Huggett AC, Ranheim T, Drevon CA. Effect of coffee lipids (cafestol and kahweol) on regulation of cholesterol metabolism in HepG2 cells. Arterioscler. Thromb. Vasc. Biol. 1997;17:2140-2149.
9. Gershbein LL. Action of dietary trypsin, pressed coffee oil, silymarin and iron salt on 1,2-dimethylhydrazine tumorgenesis by gavage. Anticancer Res. 1994;14:1113-1116.
10. IARC Working Group. Coffee, tea, mate, methylxanthines and methylglyoxal. Vol 51. 1991. Lyon, France, International Agency for Research on Cancer. Monographs on the evaluation of carcinogenic risks to humans.
11. Hollman PCH, de Vries JHM, van Leeuwen SD, Mengelers MJB, Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am. J Clin. Nutr. 1995;62:1276-1282.
12. Silvester KR, Cummings JH. Can radio-opaque markers be used to measure mouth-to-stoma transit time in ileostomates? Eur. J Clin. Nutr. 1996;50:343-345.
13. Bingham S, Cummings JH. The use of 4-aminobenzoic acid as marker to validate the completeness of 24-h urine collections in man. Clinical Science 1983;64:629-635.
14. Jebbink MCW, Lamers CBH, Mooy DM, Rovati LC, Jansen JBMJ. Effect of loxiglumide on basal and gastrin and bombesin-stimulated gastric acid and serum gastrin levels. Gastroenterology 1992;103:1215-1220.
15. Roxburgh JC, Whitfield PF, Hobsley M. Effect of acute cigarette smoking on gastric secretion. Gut 1992;33:1170-1173.
16. Malagelada JR, Robertson JS, Brown ML, et al. Intestinal transit of solid and liquid components of a meal in health. Gastroenterology 1984;87:1255-1263.
17. Layer P, Jansen JBMJ, herian L, amers CBHW, oebbel H. Feedback regulation of human pancreatic secretion. Effects of protease inhibition on duodenal delivery and small intestine transit of pancreatic enzymes. Gastroenterology 1990;98:1311-1319.
18. Urgert R, Schulz AG, Katan MB. Effects of cafestol and kahweol from coffee grounds on serum lipids and serum liver enzymes in humans. Am. J Clin. Nutr. 1995;61:149-154.
19. Eisenwiener HG, Morger F, Lergier W, Gillesen D. Die Bestimmung der *p*-Aminobenzoesäure mit Fluram im Urin nach Durchführing des Pankreasfunktiontests mit Bentiromid. J. Clin. Chem. Clin. Biochem. 1982;20:557-565.
20. Sandberg A-S, Andersson H, Hallgren B, Hasselblad K, Isaksson B. Experimental model for *in vivo* determination of dietary fibre and its effect on the absorption of nutrients in the small intestine. Br. J Nutr. 1981;45:283-294.
21. Tornqvist H, Rissanen A, Andersson H. Balance studies in patients with intestinal resection: how long is enough? Br. J Nutr. 1986;56:11-16.
22. Bosaeus IG, Carlsson N-G, Sandberg A-S, Andersson H. Effect of wheat bran and pectin on bile acid and cholesterol excretion in ileostomy patients. Human Nutrtion: Clinical Nutrition 1986;40C:429-440.

23. Bosaeus IG, Andersson HBO. Short-term effect of two cholesterol-lowering diets on sterol excretion in ileostomy patients. Am. J Clin. Nutr. 1987;45:54-59.
24. Gorbach SC, Nahas L, Weinstein L, Levitan R, Patterson JF. Studies of intestinal microflora. IV. The microflora of ileostomy effluent: a unique microbial ecology. Gastroenterology 1967;53:874-880.
25. Dowsett J, Gibney MJ, Kennedy NP. Bacterial fermentation occurs in the terminal ileum of ileostomates. Proc. Nutr. Soc. 1990;49:110A
26. Fuller MF, Milne A, Harris CI, Rein TMS, Keenan R. Amino acid losses in ileostomy fluid on a protein-free diet. Am. J Clin. Nutr. 1994;59:70-73.
27. Viani R. Physiologically active substances in coffee. In: Clarke RJ, Macrae R, eds. Physiology. London/New York: Elsevier Applied Science Publishers, 1988:1-30.
28. Urgert R, Kosmeijer-Schuil TG, Katan MB. Intake levels, sites of action and excretion routes of the cholesterol-elevating diterpenes from coffee beans in humans. Biochem. Soc. Trans. 1996;24:800-806.
29. Klaassen CD, Amdur MO, Doull J. Casarett and Doull's Toxicology, the basic science of poisons. 5 ed. McGraw-Hill Companies, Inc., 1996.
30. Lam LK, Sparnins VL, Wattenberg LW. Isolation and identification of kahweol palmitate and cafestol palmitate as active constituents of green coffee beans that enhance glutathione S-transferase activity in the mouse. Cancer Res. 1982;42:1193-1198.
31. Lam LK, Sparnins VL, Wattenberg LW. Effects of derivatives of kahweol and cafestol on the activity of glutathione S-transferase in mice. J Med. Chem. 1987;30:1399-1403.

3

Validity of animal models for the cholesterol-raising effects of coffee diterpenes in humans

Baukje de Roos, Janet K. Sawyer, Martijn B. Katan and Lawrence L. Rudel.
Proceedings of the Nutrition Society, in press.

Abstract

Cafestol and kahweol - coffee lipids present in unfiltered coffee brews - potently increase low density lipoprotein (LDL) cholesterol concentration in humans. We searched for an animal species in which cafestol similarly increases LDL cholesterol. Such an animal model could subsequently be used as a model to study the mechanism of action of cafestol and kahweol. Cafestol and kahweol increased serum lipids in African green monkeys, Cebus and Rhesus monkeys, hamsters, rats, and gerbils differently than in humans. In African green monkeys, the rise in total cholesterol was less significant than in man. In addition, the increase in total cholesterol was predominantly due to a rise in HDL cholesterol rather than LDL cholesterol. Therefore, the rise in plasma lipids might illustrate the mechanism in these monkeys rather than the mechanism in humans. In other animal species, cafestol and kahweol did not raise cholesterol consistently. The variability in effects on serum lipids could not be explained by the mode of administration or dose of diterpenes, nor by the amount of cholesterol in the diet. In conclusion, we did not find an animal model in which cafestol and kahweol elevate plasma lipoproteins to the same extent as in man. For the time being, therefore, studies on the mechanism of action should preferably be done in humans.

Introduction

Unfiltered coffee brews markedly increase serum lipids in humans. The compounds responsible for this effect are cafestol and kahweol, which are present in coffee beans (1;2). The mechanism by which coffee diterpenes influence lipoprotein metabolism is largely unknown. An animal model with a response to cafestol and kahweol similar to that in man would allow mechanistic studies that otherwise could not be done in man. We here review the use of animal models for understanding the action of cafestol and kahweol in humans.

Effect of the coffee diterpenes on serum lipids in humans

The cholesterol raising potential of unfiltered coffee depends mainly on its content of cafestol (3). Unfiltered coffee brews such as Scandinavian boiled coffee, cafetiere (French-press) coffee, and Turkish coffee contain about 3-6 mg of cafestol per cup (4;5). Filtered coffee does not contain cafestol, because coffee diterpenes are insoluble in water and do not pass through a paper filter (6). In short term studies each 10 mg of cafestol ingested raised serum cholesterol an average of 0.13 mmol/L and serum triglycerides an average of 0.08 mmol/L after four weeks. Effects are linear up to 100 mg of cafestol ingested per day (7). In a long term study the cafestol content of five cups of cafetiere coffee ingested per day caused a persistent rise in serum total cholesterol of 11-17% and a rise in LDL cholesterol of 9-14% after 24 weeks (8). Hence, about 80% of the rise in total cholesterol was accounted for by LDL cholesterol, and the rest was due to a rise in VLDL cholesterol. Cafetiere coffee did not affect HDL cholesterol levels, although coffee diterpenes did slightly decrease HDL cholesterol concentration in some previous studies (2;3;7;9). Serum triglycerides rose markedly (26%) after two to four weeks of intake of cafetiere coffee. However, the rise in serum triglycerides subsided with chronic intake of coffee diterpenes (8).

To study how cafestol affects serum lipoproteins in humans, we would like to use an animal model with a lipoprotein metabolism similar to humans. One criterion would be that the animal's response to dietary cholesterol is similar to that in man. Rats, mice, dogs and squirrel monkeys respond to dietary cholesterol by down-regulation of cholesterol synthesis and by up-regulation of bile acid production (i.e. cholesterol 7α-hydroxylase) in the liver. As a consequence, plasma lipoprotein concentrations do not increase (10). In contrast, bile acid production is not affected when humans increase their cholesterol intake, while serum LDL and HDL cholesterol concentrations will increase. Hamsters, guinea pigs, cynomolgus monkeys, Cebus monkeys, Rhesus monkeys, African green monkeys, baboons, and pigs have, like humans, a low rate of cholesterol synthesis in the liver (11). In addition, these species do not increase bile acid synthesis when fed cholesterol. Instead, liver

cholesterol content increases, LDL receptor activity is down-regulated and LDL cholesterol concentration in the plasma increases (10). Therefore, the response to dietary cholesterol in these animal species is similar to man.

Male African green monkeys show many similarities with humans in the effects of dietary cholesterol and fatty acids on plasma lipoproteins and cholesterol metabolism (12-14). Therefore, we studied whether the African green monkey is also a good model for the effect of cafestol and kahweol on lipoprotein metabolism in man. In addition, we here review whether we can use Rhesus or Cebus monkeys, hamsters, rats, gerbils, or mice as a model to study the mechanism of action of the coffee diterpenes.

Effect of coffee diterpenes in African green monkeys and man

We fed eight male African green monkeys with a mean age of 15.9 years and a mean weight of 4.4 kg a "Western type" diet with a caloric distribution of 17% protein, 35% fat (15% saturated fat) and 48% carbohydrate. 96 mg Crystalline cholesterol per MJ of diet was added in order to produce total plasma cholesterol concentrations of 5-8 mmol/L, i.e. the range seen in humans. This diet was either supplemented with placebo oil consisting of sunflower oil and palm oil (3:2 wt/wt) or with coffee oil. Both oils had a similar content of fatty acids (15). The diets provided 0.26 g of these oils per kg body weight per day; as a result the coffee oil diet provided 8 mg cafestol and 7 mg kahweol per kg body weight per day.

During a run-in period of six weeks, all monkeys consumed the placebo oil diet. During the first treatment periods of seven weeks, four animals were given the placebo oil diet, while the other four received the coffee oil diet. The two groups had similar initial cholesterol concentrations (mean 6.41 (SD 2.76) vs. mean 6.37 (SD 2.07)). This period was followed by a wash-out period of five weeks, where only the placebo diet was provided. Then followed a second treatment period of seven weeks, in which the placebo oil diet and the coffee oil diet were switched. Blood samples were taken in week 5 of the run-in period, in week 5, 6 and 7 of both treatment periods, and in week 5 of the wash-out period. In plasma we determined total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides as described by Carr *et al* (16).

Food intake and body weight did not change during the experimental period. Coffee oil raised total plasma cholesterol by 14%, LDL cholesterol by 8%, and HDL cholesterol levels by 23% (table 1). About 32% of the rise in cholesterol was accounted for by LDL cholesterol and 57% by HDL cholesterol. We assume that the remaining 11% was accounted for by VLDL+IDL cholesterol. Coffee oil increased plasma triglycerides by 35% compared to placebo oil.

Plasma levels of alanine aminotransferase did not differ between the placebo and the coffee oil periods (table 1), which indicates that liver cell integrity was not disturbed. In two previous animal studies, coffee diterpenes also did not affect plasma alanine aminotransferase (15;17).

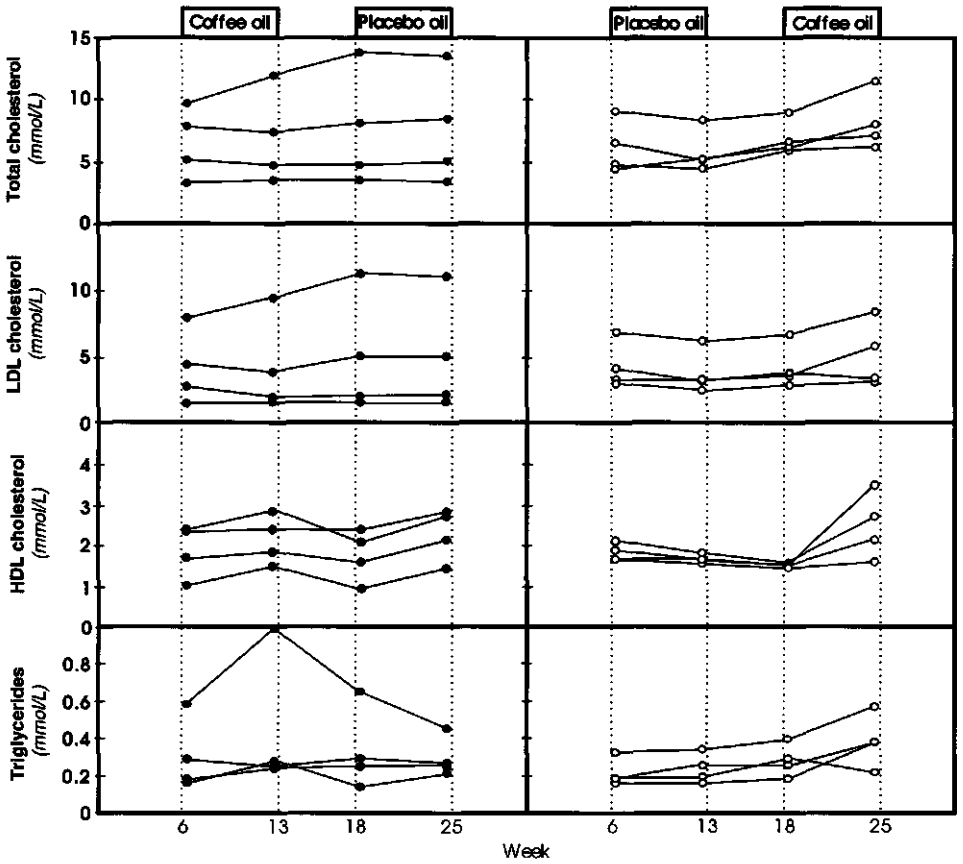

Figure 1. Individual values of plasma total cholesterol, LDL- and HDL cholesterol, and triglycerides in eight African green monkeys after a high-fat high-cholesterol diet supplemented with either placebo oil or coffee oil in a cross-over design. Treatment with the placebo diet or the coffee-oil diet lasted for seven weeks. Treatments were separated by a wash-out period of five weeks. ●: Monkeys that first received the coffee oil diet; ○: monkeys that first received the placebo oil diet.

Table 1. Plasma concentrations of total-, LDL-, and HDL-cholesterol, triglycerides and alanine aminotransferase in eight male African green monkeys after consumption of placebo- or coffee oil for seven weeks in a cross over design*†.

		Placebo oil		Coffee oil		Coffee versus placebo oil	
	Group	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Difference (95% CI)	P‡
Total cholesterol	all	7.01 (SD 3.4)	6.82 (SD 3.16)	6.62 (SD 2.12)	7.57 (SD 2.96)	1.14 (0.21 to 2.06)	0.03
(mmol/L)	COF-PL					0.40	
	PL-COF					1.87	
LDL cholesterol	all	4.58 (SD 3.28)	4.34 (SD 3.04)	4.30 (SD 2.20)	4.70 (SD 3.02)	0.65 (-0.13 to 1.42)	0.09
(mmol/L)	COF-PL					0.17	
	PL-COF					1.29	
HDL cholesterol	all	1.80 (SD 0.39)	2.01 (SD 0.52)	1.75 (SD 0.44)	2.33 (SD 0.69)	0.37 (-0.18 to 0.91)	0.14
(mmol/L)	COF-PL					-0.23	
	PL-COF					0.97	
Triglycerides	all	0.28 (SD 0.16)	0.27 (SD 0.09)	0.30 (SD 0.14)	0.42 (SD 0.27)	0.13 (0.00 to 0.26)	0.06
(mmol/L)	COF-PL					0.19	
	PL-COF					0.07	
Alanine aminotransferase	all	46 (SD 15)	47 (SD 17)	47 (SD 29)	50 (SD 31)	2(-5 to 8)	0.34
(U/L)	COF-PL					1	
	PL-COF					3	

* Pre-treatment samples were obtained in week 5 of the run-in and wash-out period. Treatment samples were obtained in week 5, 6 and 7 of the placebo- or coffee oil treatment period.

† Mean (SD) indicated for all 8 monkeys, for the four monkeys who first consumed the coffee oil diet followed by the placebo diet (COF-PL) and for the four monkeys who first consumed the placebo diet followed by the coffee oil diet (PL-COF).

‡ Plasma lipids were different at the start of the treatment period with coffee- or placebo oil due to carry-over effects. Therefore, we calculated the response to coffee oil or placebo oil treatment by subtracting pre-treatment values from the treatment values. Differences in responses between the coffee or placebo oil treatment were tested using the one-tailed paired Student's T-test.

The response in plasma cholesterol was higher in the four animals that consumed the coffee oil diet in the second treatment period than in the four animals that consumed the coffee oil in the first treatment period: 26% versus 2% for total cholesterol; 21% versus -5% for LDL cholesterol; and 59% versus -12% for HDL cholesterol, respectively (figure 1). The reason for this could be the relatively short run-in period of 6 weeks on the high fat, high cholesterol placebo oil diet. In monkeys, a steady state in proportions of fatty acids in the liver pools, in the distribution of unesterified and esterified cholesterol in liver, in LDL receptor activity in the liver, in LDL cholesterol production, and in plasma LDL cholesterol are reached in approximately three months of feeding a high cholesterol, high fat diet (18). The four monkeys that consumed the coffee oil diet in the second treatment period had consumed the placebo oil diet for 18 weeks before they received the coffee oil diet. Therefore, the data of these latter four monkeys may give a better indication of the lipid-elevating effect of the coffee oil.

The monkeys received per day on average 35 mg of cafestol and 31 mg of kahweol at an energy intake of 2.21 MJ. The daily intake would have been 160 mg cafestol and 140 mg kahweol if the monkeys were fed the same amount of energy as humans, i.e. 10 MJ per day. This amount of diterpenes is present in 25 cups of unfiltered coffee (7), and it would raise serum cholesterol by on average 2.05 mmol/L or 40% and serum triglycerides by on average 1.26 mmol/L or 120% in humans (7). The response of plasma cholesterol to coffee oil in African green monkeys was in the same direction, but significantly weaker than in man. Also, unlike humans, in the monkeys the rise in cholesterol was primarily due to increases in HDL cholesterol rather than in LDL cholesterol. Therefore, the action of cafestol and kahweol on lipoprotein metabolism may illustrate that the mechanism in this monkey species is different than the mechanism in humans. These data show that an animal species which is a good model to study the mechanisms of dietary cholesterol and fatty acids on lipoprotein metabolism may not necessarily be a good model to study the mechanism of cafestol and kahweol.

Coffee diterpenes fail to show consistent results in other animal species

In contrast to the present findings in African green monkeys, coffee oil did not affect plasma cholesterol and triglycerides in Cebus and Rhesus monkeys (15). These monkeys consumed a daily amount of cafestol corresponding to that present in 12-13 cups of boiled coffee per 10 MJ of diet (figure 2). This dose is half that given to the African green monkeys, which might explain why the Cebus and Rhesus monkeys did not respond. In addition, the Cebus and Rhesus monkeys received a lower dose of dietary cholesterol (figure 2), which also might help to explain the lack of effect on serum lipids, because in various animal experiments the influence of fed

components on serum cholesterol appeared greater against a dietary background rich in cholesterol (19).

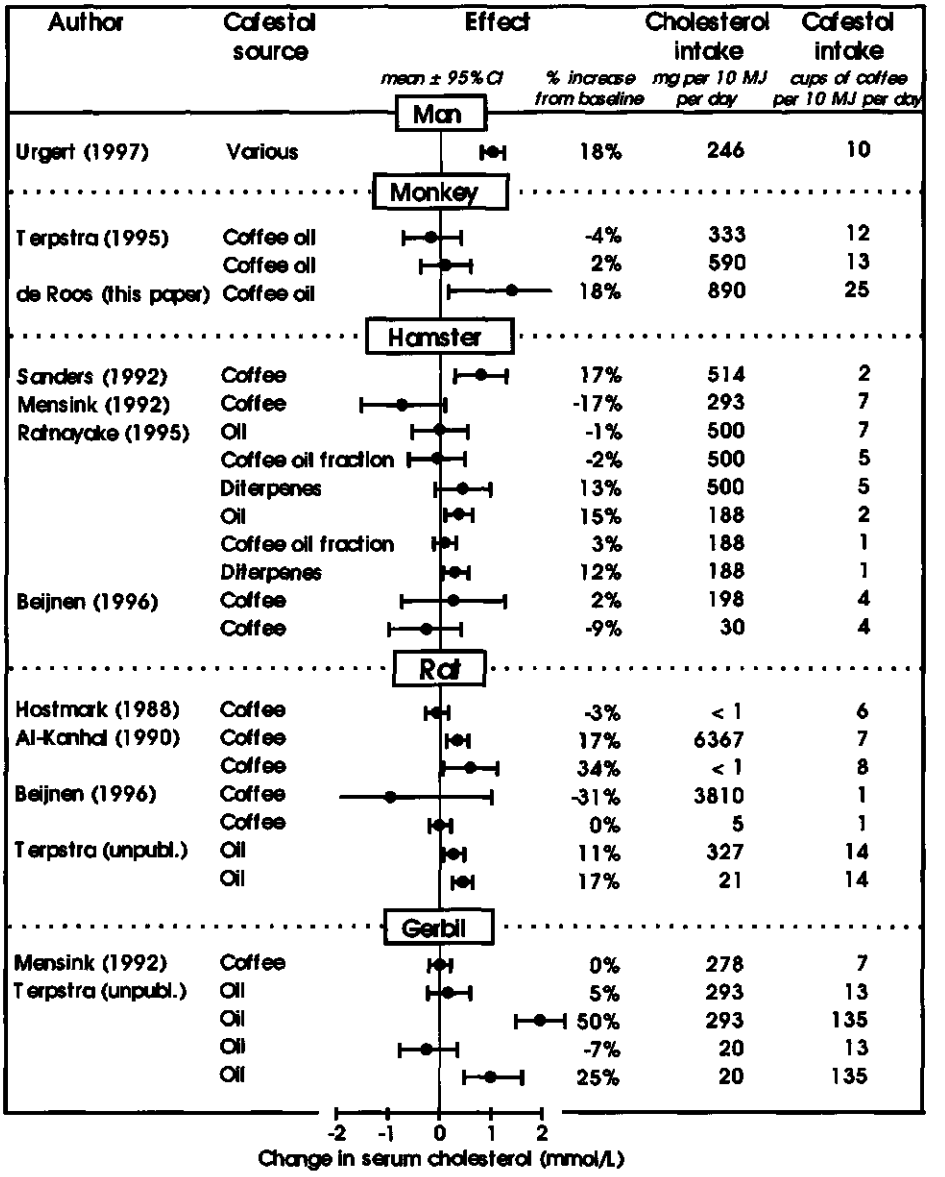

Figure 2. Comparison of the effect of cafestol in man with that in various animal species. Bars indicate the mean ± 95% confidence intervals. Treatment periods varied from two to twenty weeks. Cafestol intake was recalculated to the amount of cups of coffee that would need to be consumed per 10 MJ per day, assuming that each 150 mL-cup of unfiltered coffee contains 6.2 mg of cafestol. The mean preparations of known diterpene content were given.

In male Syrian hamsters, coffee diterpenes had no consistent effect on serum lipids (figure 2). Boiled coffee elevated plasma cholesterol and triglycerides in one study with Syrian hamsters by Sanders & Sandaradura (20), but our attempt to replicate this result was unsuccessful (17). In another study by Ratnayake *et al.* (1995) coffee oil, a diterpene rich fraction from coffee beans and purified diterpenes also did not affect serum lipids in hamsters (21), even though the animals received the same amount of dietary cholesterol and a much higher dose of cafestol than the hamsters in the study of Sanders & Sandaradura (20). Paradoxically, serum cholesterol was significantly increased when Ratnayake *et al* (1995) repeated this experiment with a much lower dose of cafestol, dietary cholesterol, and saturated fatty acids (21).

Rats also failed to show a consistent response of serum lipids upon cafestol treatment (figure 2). Boiled coffee (22) and coffee oil (Terpstra, unpublished observations) significantly increased serum cholesterol both on a low and high cholesterol diet. However, in two other studies using Wistar rats, serum lipids were not affected by unfiltered coffee (17;23) although one of these studies used a background diet which was relatively high in cholesterol (17).

Two studies presented data on the effects of coffee in male gerbils (figure 2). In our hands, freeze-dried boiled coffee did not affect serum cholesterol in gerbils (24). However, the process of freeze-drying may remove or modify the cafestol in the diets. In a second study we fed gerbils coffee oil from the same source and lot as we used in our human studies. Here, coffee oil significantly elevated plasma cholesterol compared to placebo oil (Terpstra, unpublished observations).

We have also studied the effects of cafestol and kahweol on serum lipoproteins in hyperlipidaemic LDL receptor knock-out mice and apolipoprotein E*3-Leiden mice (chapter 4). A dose equivalent to 8 or 40 cups of unfiltered coffee per 10 MJ per day raised serum cholesterol to the same extent in these mouse species as in man. The coffee diterpenes also raised serum cholesterol in the wild type (C57Bl/6) mice (chapter 4). However, the rise in total cholesterol was predominantly due to a rise in VLDL and IDL cholesterol and not to a rise in LDL cholesterol.

The form in which diterpenes were given - as pure compounds, as coffee oil or as unfiltered coffee - does not appear to explain the inconsistent effects on serum lipids in animals. Also the dose of coffee diterpenes does not explain the variability of the effects. Both high and low doses of diterpenes caused a significant rise in serum lipids in some animals (figure 3). In addition, a dietary background rich in cholesterol is neither necessary nor sufficient to produce a rise in serum cholesterol in animals (figure 3). As yet, there is no animal species in which cafestol and kahweol uniformly raised serum cholesterol. Therefore, criteria such as study design and chance fluctuations might explain why cafestol raised serum cholesterol in some animal species, whereas other species did not respond to coffee diterpenes.

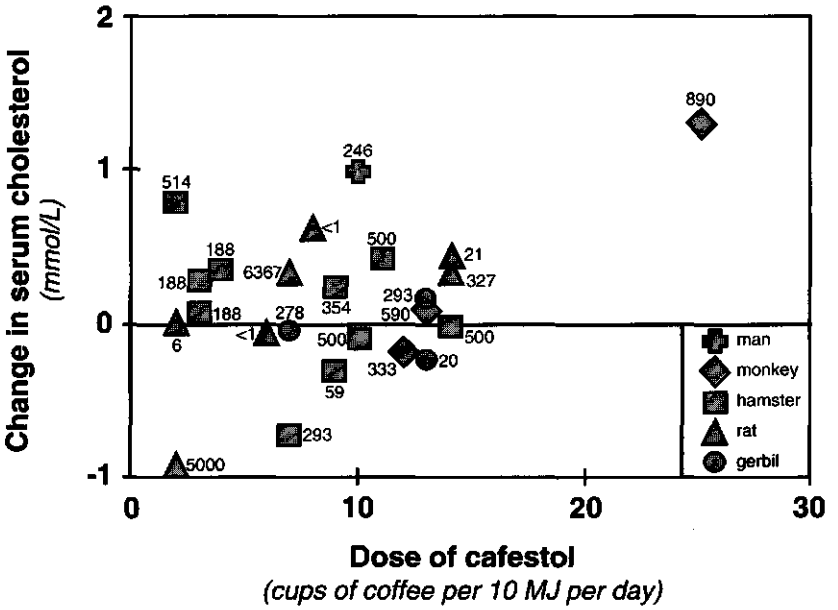

Figure 3. Effect of cafestol intake (expressed as cups of coffee per 10 MJ per day) on the response in serum cholesterol in various animal studies. Responses were adjusted for the mean changes in the control group, if present. The values next to the grey marks indicate the amount of background dietary cholesterol (expressed in mg cholesterol per 10 MJ per day) used in these studies.

Conclusion

The effect of cafestol and kahweol on lipoprotein metabolism in monkeys, hamsters, rats and gerbils differs from that in humans. In African green monkeys, coffee diterpenes appeared to raise HDL as much as LDL, although no statistical significance for either lipoprotein was found. The small group size and time on diet appeared to limit the significance of the outcome. In the other animal species, cafestol and kahweol did not raise serum cholesterol consistently and to the same extent as in man. We will gain little knowledge by extrapolating these effects of cafestol and kahweol on plasma lipoproteins in animals to humans unless the animal model has first shown to respond to the intervention as humans do. For the time being, studies on the mechanism of action should preferably be done in humans.

Acknowledgements

We thank the Netherlands Heart Foundation (grant no. 900-562-091), the Netherlands Organisation of Scientific Research and the Foundation for Nutrition and Health Research for funding, Ms. Kathryn Kelley for technical assistance, and Dr. A.H.M. Terpstra for advice.

References

1. Heckers H, Gobel U, Kleppel U. End of the coffee mystery: diterpene alcohols raise serum low-density lipoprotein cholesterol and triglyceride levels [letter]. J Intern. Med. 1994;235:192-193.
2. Weusten-van der Wouw MPME, Katan MB, Viani R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res. 1994;35:721-733.
3. Urgert R, Essed N, van der Weg G, Kosmeijer-Schuil TG, Katan MB. Separate effects of the coffee diterpenes cafestol and kahweol on serum lipids and liver aminotransferases. Am. J Clin. Nutr. 1997;65:519-524.
4. Urgert R, van der Weg G, Kosmeijer-Schuil TG, van de Bovenkamp P, Hovenier R, Katan MB. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. J Agric. Food Chem. 1995;43:2167-2172.
5. Gross G, Jaccaud E, Huggett AC. Analysis of the content of the diterpenes cafestol and kahweol in coffee brews. Food Chem. Toxicol. 1997;35:547-554.
6. van Dusseldorp M, Katan MB, van Vliet T, Demacker PN, Stalenhoef AF. Cholesterol-raising factor from boiled coffee does not pass a paper filter. Arterioscler. Thromb. 1991;11:586-593.
7. Urgert R, Katan MB. The cholesterol-raising factor from coffee beans. Annu. Rev. Nutr. 1997;17:305-324.
8. Urgert R, Meyboom S, Kuilman M, et al. Comparison of effect of cafetiere and filtered coffee on serum concentrations of liver aminotransferases and lipids: six month randomised controlled trial. BMJ. 1996;313:1362-1366.
9. Zock PL, Katan MB, Merkus MP, van Dusseldorp M, Harryvan JL. Effect of a lipid-rich fraction from boiled coffee on serum cholesterol. Lancet 1990;335:1235-1237.
10. Spady DK, Turley SD, Dietschy JM. Rates of low density lipoprotein uptake and cholesterol synthesis are regulated independently in the liver. J Lipid Res. 1985;26:465-472.
11. Spady DK, Dietschy JM. Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. J Lipid Res. 1983;24:303-315.
12. Rudel L, Parks JS, Bond MG. Dietary polyunsaturated fat effects on atherosclerosis and plasma lipoproteins in African green monkeys. In: Scarpelli DG, Migaki G, eds. Current topics in nutrition and disease in comparative pathobiology. New York: Alan R Liss, Inc, 1986:501-523.
13. Rudel LL, Haines JL, Sawyer JK. Effects on plasma lipoproteins of monounsaturated, saturated, and polyunsaturated fatty acids in the diet of African green monkeys. J Lipid Res. 1990;31:1873-1882.
14. Rudel L, Sawyer JK, Parks JS. Dietary fat, lipoprotein structure, and atherosclerosis in primates. Athero. Rev. 1991;23:41-50.
15. Terpstra AH, Katan MB, Weusten-van der Wouw MPME, Nicolosi RJ, Beynen AC. Coffee oil consumption does not affect serum cholesterol in rhesus and cebus monkeys. J Nutr. 1995;125:2301-2306.
16. Law MR, Wald NJ, Thompson SG. By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? BMJ. 1994;308:367-372.
17. Beynen AC, Weusten-van der Wouw MPME, de Roos B, Katan MB. Boiled coffee fails to raise serum cholesterol in hamsters and rats. Br. J Nutr. 1996;76:755-764.
18. Kris EP, Dietschy J. Design criteria for studies examining individual fatty acid effects on cardiovascular disease risk factors: human and animal studies. Am. J Clin. Nutr. 1997;65:1590S-1596S.
19. Beynen AC, West CE. Mechanisms underlying nutritional effects on serum cholesterol concentrations. In: Cliff WJ, Schoefl GI, eds. Coronaries and Cholesterol. London: Chapman and Hall Medical, 1989:89-114.
20. Sanders TA, Sandaradura S. The cholesterol-raising effect of coffee in the Syrian hamster. Br. J Nutr. 1992;68:431-434.
21. Ratnayake WM, Pelletier G, Hollywood R, Malcolm S, Stavric B. Investigation of the effect of coffee lipids on serum cholesterol in hamsters. Food Chem.Toxicol. 1995;33:195-201.

22. Al-Kanhal MA, Tariq M, Iqbal SS. Effect of gahwa (arabian coffee) on serum lipid and lipoprotein levels in rats. Research Communications in Substances of Abuse 1990;11:185-194.
23. Hostmark AT, Lystad E, Haug A, Bjerkedal T, Eilertsen E. Effect of boiled and instant coffee on plasma lipids and fecal excretion of neutral sterols and bile acids in the rat. Nutrition Reports International 1988;38:859-864.
24. Mensink RP, Zock PL, Katan MB, Beynen AC. Boiled coffee does not increase serum cholesterol in gerbils and hamsters. Z. Ernahrungswiss. 1992;31:82-85.

4

Cafestol, the cholesterol-raising factor in boiled coffee, increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis

Baukje de Roos and Sabine M. Post, Martijn Vermeulen, Lydia Afman, Miek C. Jong, Vivian E.H. Dahlmans, Louis M. Havekes, Frans Stellaard, Martijn B. Katan, Hans M.G. Princen. *Conditionally accepted.*

Abstract

Cafestol, a diterpene present in unfiltered coffee brews, potently increases serum cholesterol levels in humans. So far, no suitable animal model has been found to study the biochemical background of this effect. We determined the effect of cafestol on serum cholesterol and triglycerides in different strains of mice and studied subsequently the mechanism of action in apolipoprotein E*3-Leiden transgenic mice.

Apolipoprotein (apo) E*3-Leiden, heterozygous LDL receptor knock-out (LDLR+/-) or wild type C57Bl/6 (WT) mice were fed a high (0.05% w/w) or low (0.01% w/w) cafestol diet or a placebo diet for eight weeks. Standardised to energy intake, these amounts equal 40, 8 or 0 cups of unfiltered coffee per 10 MJ per day in humans. In apoE*3-Leiden mice, serum cholesterol was increased by 33% (3.46 mmol/L; 95%CI [1.62;5.30]) on the low and by 61% (6.35 mmol/L; 95%CI [4.47;8.22]) on the high cafestol diet. In LDLR+/- and WT mice, the increases were 20% (0.85 mmol/L; 95%CI [-0.25;1.94]) and 24% (0.62 mmol/L; 95%CI [0.34;0.90]), respectively, on the low cafestol diet, and 55% (2.37 mmol/L; 95%CI [0.73;4.01]) and 46% (1.21 mmol/L; 95%CI [0.92;1.50]), respectively, on the high cafestol diet. The increase in total cholesterol was mainly due to a rise in VLDL and IDL cholesterol in all three mouse strains.

To investigate the mechanism of the cholesterol-raising effect, apoE*3-Leiden mice were fed a high cafestol or a placebo diet for three weeks. Cafestol suppressed enzyme activity and mRNA levels of cholesterol 7α-hydroxylase by 57% and 58% (both $p<0.05$), respectively. mRNA levels of enzymes involved in the alternative pathway of bile acid synthesis, i.e. sterol 27-hydroxylase and oxysterol 7α-

hydroxylase, were reduced by 32% ($p<0.05$) and 48% ($p<0.005$), respectively. The total amount of bile acids secreted in faeces was decreased by 41%. Cafestol did not affect hepatic free and esterified cholesterol, but it decreased LDLR mRNA levels by 37% ($p<0.05$). VLDL particles contained a three times higher amount of cholesteryl esters, indicative for the secretion of a ßVLDL-like particle. This was confirmed by a decreased VLDL triglyceride production, as measured by the increase in triglycerides after Triton injection, in mice treated with cafestol (35.1±13.8 µmol/h/kg) compared to placebo treatment (63.1±17.5 µmol/h/kg). This was a result of a reduction in hepatic triglyceride content by 52% ($p<0.05$).

In conclusion, cafestol increases serum cholesterol levels in apoE*3-Leiden transgenic mice by suppression of the major regulatory enzymes in the bile acid synthesis pathways, leading to decreased LDLR mRNA levels and increased secretion of cholesteryl esters by the liver. In analogy, we suggest that suppression of bile acid synthesis may provide an explanation for the cholesterol-raising effect of cafestol in humans.

Introduction

Unfiltered coffee brews markedly increase serum cholesterol levels in humans. The responsible compounds for this effect are cafestol and kahweol, two diterpenes that are present in coffee beans (1). From our experiments, cafestol appeared to be by far the most potent compound (2). We estimated that each 10 mg of cafestol ingested per day raises serum cholesterol levels by 0.13 mmol/L (3). In humans, about 80% of the rise in total cholesterol is accounted for by low density lipoprotein (LDL) cholesterol, and the rest is due to a rise in very low density lipoprotein (VLDL) cholesterol (4).

The mechanism by which coffee diterpenes influence lipid metabolism is largely unknown. Recently, we reported that cafestol suppressed bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase (5). Suppression of bile acid synthesis will lead to an increased pool of regulatory cholesterol, resulting in a decreased expression of the hepatic LDL-receptor. This may provide an explanation for the cholesterol-raising effect of cafestol in humans (5). The availability of an animal model to study this hypothesis *in vivo* would be of great value, since it may help us to validate our *in vitro* experiments and eventually to discover the metabolic control points of cafestol. However, in previous studies, various animal models like hamsters (6-8), rats (6;9), gerbils (8), Cebus, Rhesus and African green monkeys (10) did not respond to cafestol and kahweol as humans do, regardless of the dosage, the mode of administration or the duration of the treatment. Differences in the absorption and/or metabolism of coffee diterpenes,

or in their effects on lipoprotein metabolism, might underlie the negative results in a range of animal species.

We here studied the effects of cafestol and kahweol on serum lipoproteins in apolipoprotein E*3-Leiden (apoE*3-Leiden) transgenic mice, in heterozygous LDL receptor deficient (LDLR+/-) mice, and in wild type (WT) C57Bl/6 mice. We chose to use transgenic mice over-expressing human apoE*3-Leiden, since these mice are highly susceptible to diet-induced hyperlipoproteinemia, primarily due to a partial defect in hepatic uptake of remnant lipoproteins (11;12). Since it was anticipated from our studies in cultured rat hepatocytes that cafestol would have an indirect effect on the expression of the LDL-receptor (5), experiments were also performed with LDLR+/- mice. Lipoprotein profiles from LDLR+/- mice are more similar to humans than those from WT mice, which transport most of their cholesterol in the HDL lipoprotein fraction (13). The cholesterol-raising effect of cafestol appeared to be most pronounced in apoE*3-Leiden transgenic mice, allowing us to investigate the mechanism of the cholesterol-raising effect of cafestol in this mouse strain.

Animals and methods

Animals, housing and diet

Twenty-four female apoE*3-Leiden mice (mean age 19 ± 5 weeks), 24 female LDLR+/- mice (mean age 32 ± 2 weeks) and 24 female WT C57Bl/6 mice (mean age 19 ± 5 weeks) were held under standard conditions in Macrolon type III cages housing 4 animals. All cages were placed under filtertops to prevent infection. They were maintained on 12-h dark and 12-h light cycles and were allowed free access to food and water. Body weight of the mice and the consumption of diet and water were recorded weekly during the whole experimental period. Mice were fed a common challenge diet enriched with saturated fat and cholesterol (18.2 MJ/kg) containing per 100 g: cacao butter 15 g, corn oil 1 g, cholesterol 0.25 g, sucrose 40.5 g, corn starch 10 g, cellulose 5.9 g, minerals 2.6 g, and vitamins 8.2 g (Hope Farms, Woerden, The Netherlands). This diet was supplemented with either 0.05% (w/w) cafestol and 0.025% (w/w) kahweol (high cafestol diet), 0.01% (w/w) cafestol and 0.005% (w/w) kahweol (low cafestol diet), or no cafestol and kahweol (placebo diet). Standardised to daily energy intake, these amounts are comparable with a daily amount of 40, 8 or 0 cups of unfiltered coffee, respectively, per 10 MJ (the average daily energy intake in humans). Diets were stored at -20°C until use and food was renewed twice a week. Institutional guidelines for animal care were observed in all experiments.

Experimental design

Per mice strain, animals were randomly divided into three experimental groups of eight mice each, matched by age and serum cholesterol. During a run-in period of four weeks, all mice received the placebo diet. During the treatment period of eight weeks, the groups consumed either the high or the low cafestol diet, or the placebo diet. About 100 µL of blood was taken at week 0, 2, 4 and 8 of the treatment period by orbital puncture after an overnight fasting period. Faeces were sampled during three days in week 3 and in week 6 of the experimental period. They were stored at -20°C until analysis of bile acids. After eight weeks of treatment, mice were anaesthetised with ether, bled and cervically dislocated.

Measurement of serum lipids and lipoproteins

In serum, total cholesterol and triglycerides (without free glycerol) were measured enzymatically (CHOD-PAP method, Boehringer Mannheim, #236691, and GPO-trinder, Sigma, #337-B, respectively). Alanine aminotransferase was measured enzymatically (GPT, Boehringer Mannheim, #745138) after pooling the serum of four mice in one cage. Subsequently, serum lipoproteins were separated by ultracentrifugation. For this, 25 µL of serum per mouse was pooled per treatment group. 200 µL of pooled serum was layered with 1 mL of potassium bromide (ρ=1.21), 2.58 mL of sodium chloride (ρ=1.063), and 8 mL of distilled water in a poly-allomer tube (Beckman instruments, Mijdrecht, The Netherlands). The tubes were centrifuged for 18 h at 40.000 rpm at 4°C in a Beckman SW41 rotor. Then, the volume was fractionated in 47 fractions using a peristaltic pump (LKB Micro Perspex) and a fractionating apparatus (LKB Redifrac). In each fraction, cholesterol was measured enzymatically as described above, and the density was checked with a densitometer (Mettler DMA 45, Graz, Austria).

Enzyme activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

In livers from mice which had been on a high cafestol or placebo diet for three weeks, enzyme activities of cholesterol 7α-hydroxylase in microsomes and sterol 27-hydroxylase in mitochondria were determined essentially according to Chiang (14). This method measures the mass conversion of cholesterol into 7α- and 27-hydroxycholesterol. Briefly, 1 mg of either microsomal or mitochondrial protein was incubated in 1 mL of buffer containing 0.1 mol/L potassium phosphate pH 7.2, 50 mmol/L NaF, 5 mmol/L DTT, 1 mmol/L EDTA, 20% (w/v) glycerol and 0.015 % (w/w) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (CHAPS). Twenty µL of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated under agitation for 10 min at 37 °C. Then 200 µL of a regenerating system containing 10 mmol/L sodium isocitrate, 10 mmol/L $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase was added at 37°C. After 20 min

of incubation, 60 µL of a stop solution containing 20% (w/v) sodium cholate and 1 µg 20α-hydroxycholesterol, which served as a recovery standard, were added. After addition of 100 µL buffer containing 0.1% (w/v) cholesteroloxidase (Calbiochem, USA, #228250), 10 mmol/L potassium phosphate pH 7.4, 1 mM DTT and 20% glycerol (w/v), steroid products were oxidised at 37 °C for 45 min. The reaction was stopped by the addition of 2 mL ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues were re-suspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v). This mixture was analysed using a C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 50% acetonitril and 50% (v/v) methanol at a flow rate of 0.8 mL/min. The amount of the products formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

RNA isolation, blotting and hybridisation procedures

Isolation of total RNA, and subsequent electrophoresis, northern-blotting and hybridisation techniques were performed as described previously (5;15). The following DNA fragments were used as probes in hybridisation experiments: a 1.6 kb PCR-synthesised fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region; a 1.6 kb *Hin*dIII/*Xba*I fragment of rat sterol 27-hydroxylase cDNA, a 1.2 kb *Hin*dIII fragment of murine oxysterol 7α-hydroxylase cDNA (16), and a 2.2 kb *Eco*RI fragment of rat LDL-receptor cDNA. As controls, a 1.2 kb *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and a 3.8 mDa *Eco*RI fragment of the human 18S ribosomal DNA were used (17). The GAPDH mRNA or 18S rRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels were quantitated using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Determination of total bile acid concentration and bile acid composition in faeces

Dried faeces (25 mg) were treated with 1 mL alkaline methanol (methanol : 1 mol/L NaOH 3:1 (v/v)) for 2 h at 80°C in screw capped tubes. Then 9 mL of distilled water was added and the tubes were mixed and centrifuged. Total bile acid concentration was determined enzymatically/fluorimetrically on 100 µL of the supernatant applying 3α-hydroxysteroid dehydrogenase (18;19). The total residual supernatant was subsequently applied to a prepared Sep-Pak C18 solid phase extraction cartridge for determination of individual bile acid concentrations (19). After a clean up by wash procedures, bile acids were eluted with 75% methanol (20). Coprostanol was used as an internal standard. The eluate was evaporated to

dryness and the bile acids were methylated with acetyl chloride/methanol 1:20 (v/v) for 30 min at 60°C. The samples were then evaporated to dryness and silylated with 100 µl bis-trimethylsilyl-trifluoroacetamide (BSTFA)/pyridine/trimethylchlorosilane (TMCS) 5:4:1 (v/v) at room temperature for at least 1 h. The methyl-TMS derivatives were separated on a 25 m x 0.25 mm capillary OV-1701 GC column (CP Sil 19CB, Chrompack International, Middelburg, The Netherlands) (20) in a HP6890 gas chromatograph (Hewlett Packard, Palo Alto, Ca, USA) equipped with a flame ionisation detector (FID). The injector was kept at 280°C, the FID detector at 300°C. Helium was used as carrier gas at a flow rate of 0.8 mL/min. The column temperature was programmed from 240 to 280°C at a rate of 10°C/min. Bile acid derivatives were introduced by split-injection (split ratio 20:1). Quantitation was based on the area ratio of the individual bile acid to the internal standard.

Determination of neutral sterol composition and concentration in faeces

Dried faeces (25 mg) were treated with 1 mL alkaline methanol as described for bile acid measurement to liberate neutral sterols from faeces material. Prior to this treatment, 5α-cholestane was added as internal standard. After treatment, the tubes were cooled to room temperature and the neutral sterols were extracted three times with 3 mL petroleum ether. The combined petroleum ether layers were evaporated to dryness and the neutral sterols silylated to TMS derivatives using the same protocol as described for bile acids. Analysis of the TMS derivatives was performed by GC applying the same column and analytical conditions as described for the methyl TMS derivatives of bile acids. Quantitation was based on the area ratio of the individual neutral sterol to the internal standard 5α-cholestane.

Measurement of liver lipids

Liver samples from mice which had been on a high cafestol or placebo diet for three weeks were homogenised and samples were taken for measurement of protein content. Two µg of cholesterol acetate was added per sample as an internal standard. Then, lipids were extracted from the homogenate according to Bligh & Dyer (21). The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 pre-coated plates as described previously (5;22). Quantitation of the lipid amounts was performed by scanning the plates with a Hewlett Packard Scanjet 4c and by integration of the density areas with the computer program Tina version 2.09.

***In vivo* hepatic VLDL production in apoE*3-Leiden mice**

Mice which had been on a high cafestol or placebo diet for three weeks were fasted for 4 h (from 8.00-12.00 a.m.) and then injected with Triton WR 1339 (500 mg/kg body weight). Triton virtually completely inhibits serum VLDL clearance (23).

Subsequently, serum triglycerides were determined prior to injection (t=0 min) and 30, 60, 90, 120 and 180 min after Triton injection. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as µmol/h/kg body weight. Serum collected 180 min after Triton injection was pooled per treatment group and VLDL was subsequently isolated by ultracentrifugation in triplicate. Triglycerides, total and free cholesterol, and phospholipids were measured enzymatically as described previously (24). Cholesteryl esters were subsequently calculated as the difference between total and free cholesterol.

Statistical analyses

We calculated the change in serum lipids per mouse by subtracting values at the start of the experimental period from values obtained during the experimental period. After checking for normality, differences in changes between treatment groups and the control group were tested using the one-tailed unpaired Student's t-test. Other data were analysed statistically using a two-tailed Student's unpaired t-test with the level of significance selected to be $p<0.05$. Values are expressed as means ± SD.

Results

Food, body weight and alanine aminotransferase

In all mouse strains, the average change in body weight was significantly higher in mice fed the placebo diet compared to mice fed the high cafestol diet ($p<0.05$); average daily food intake was significantly higher in mice fed the placebo diet compared to mice fed the low and high cafestol diet ($p<0.05$). Concentrations of the liver enzyme alanine aminotransferase (ALT) did not significantly increase during consumption of cafestol in all mouse strains (table 1).

Table 1. Food intake, body weight and serum ALT in female apolipoproteinE*3-Leiden (apoE*3-Leiden), heterozygous LDL receptor knock-out (LDLR+/-) and wild type (WT) C57Bl/6 mice fed on moderate cholesterol diets enriched with either 0.05% w/w (high cafestol diet), 0.01% w/w (low cafestol diet) or no cafestol (placebo diet) for eight weeks.

	Placebo diet	Low cafestol diet	High cafestol diet
ApoE*3-Leiden mice			
Food intake *(g/d)*	2.8 ± 0.2	2.6 ± 0.2†	2.3 ± 0.2†
Body wt *(g)*			
Initial	20.4 ± 2.9	20.1 ± 2.4	19.8 ± 0.9
Final	23.3 ± 2.7	21.9 ± 2.0	20.9 ± 1.1†
ALT *(U/L)*			
Initial	100.3 ± 16.5	95.8 ± 5.9	92.3 ± 3.2
Final	107.0 ± 4.2	57.2 ± 7.8	68.4 ± 8.5
LDLR+/- mice			
Food intake *(g/d)*	2.6 ± 0.2	2.4 ± 0.1†	2.2 ± 0.2†
Body wt *(g)*			
Initial	20.4 ± 2.6	21.1 ± 2.5	23.0 ± 2.4
Final	22.1 ± 2.8	22.2 ± 2.8	21.3 ± 1.6†
ALT *(U/L)*			
Initial	54.1 ± 24.4	71.9 ± 6.6	51.0 ± 22.3
Final	30.9 ± 9.5	28.3 ± 2.8	33.8
WT mice			
Food intake *(g/d)*	2.7 ± 0.1	2.5 ± 0.1†	2.4 ± 0.4†
Body wt *(g)*			
Initial	21.3 ± 1.3	21.0 ± 0.8	21.2 ± 1.3
Final	23.4 ± 0.8	22.2 ± 1.0	20.9 ± 1.1†
ALT *(U/L)*			
Initial	33.1 ± 5.1	41.7 ± 10.9	54.6 ± 32.4
Final	26.3 ± 6.0	26.5 ± 8.6	23.0 ± 3.3

* Mean (± SD) of eight mice per strain per diet (ALT analyses were determined in duplicate per mice strain per diet, except for the final value of ALT in LDLR+/- mice on the high cafestol diet, which was determined in one pool sample).

† ($p<0.05$) compared to the placebo diet (Student's T-test on changes during the experimental period).

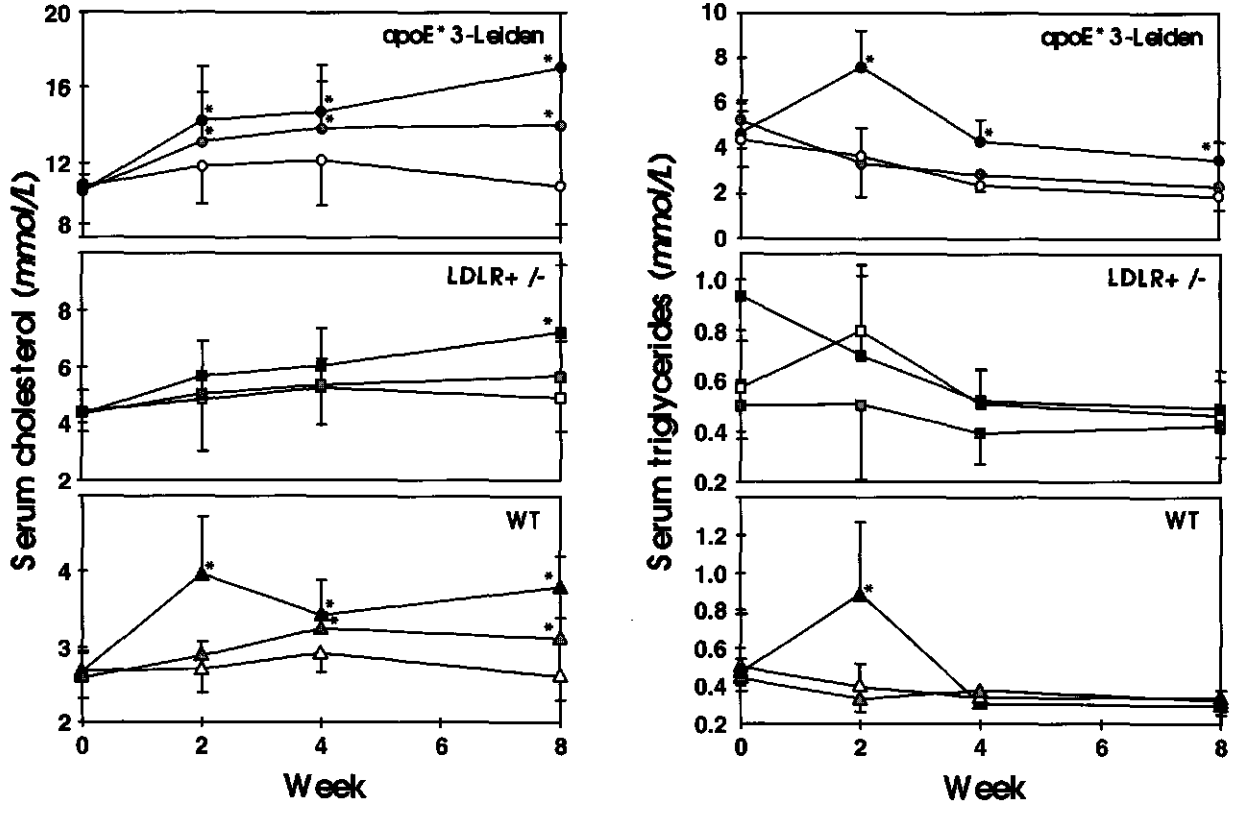

*Figure 1. Effect of a high cafestol (0.05% w/w) diet (black marks), a low cafestol (0.01% w/w) diet (grey marks) and a placebo diet (white marks) on serum cholesterol and triglycerides in apoE*3-Leiden mice (circles), LDLR+/- mice (squares) and WT (C57Bl/6) mice (triangles). Significant differences between the cafestol and the placebo treatment are indicated by an asterisk.*

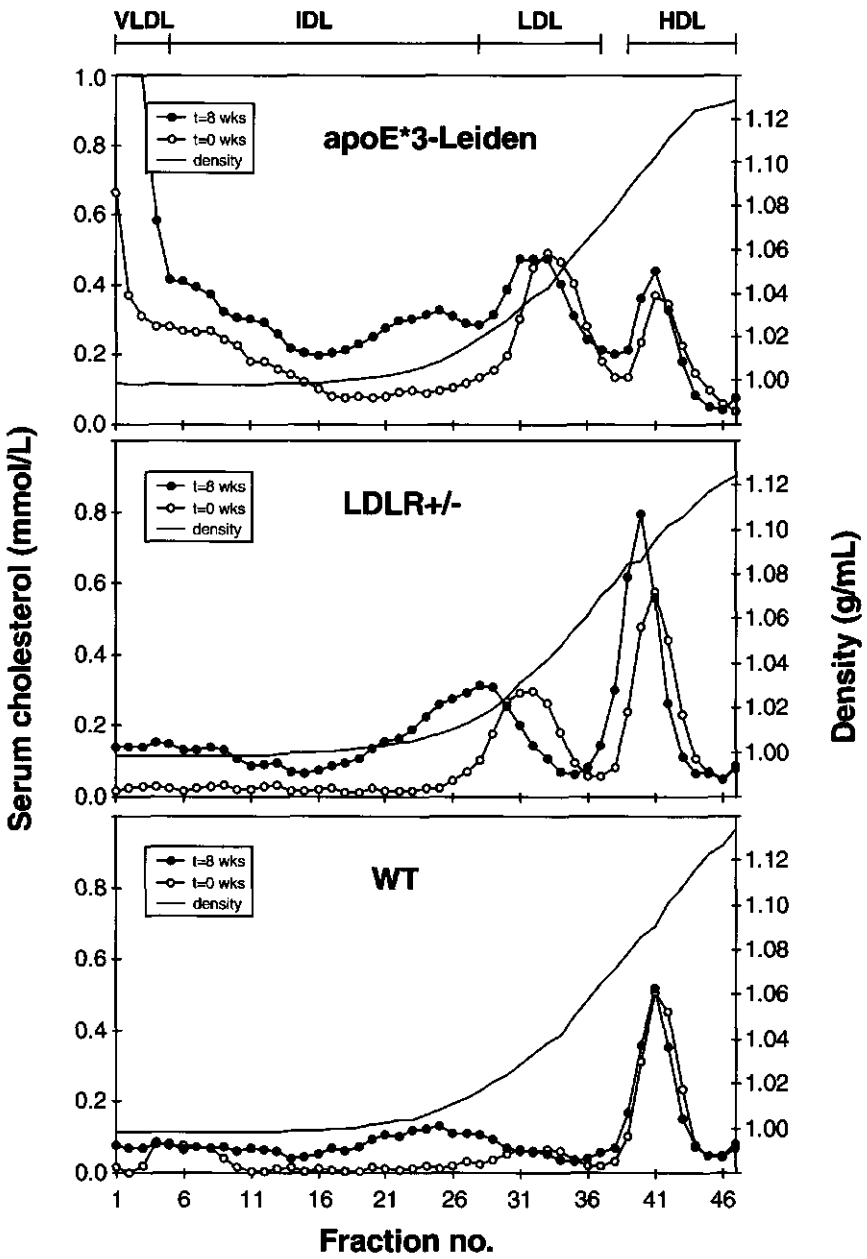

*Figure 2. Effect of a high cafestol (0.05% w/w) diet on cholesterol profiles in apolipoprotein E*3-Leiden mice (upper part), LDLR+/- mice (middle part) and WT (C57Bl/6) mice (lower part). The white marks indicate the cholesterol profile from pooled serum of 8 mice at the start of the experimental period (t=0). The black marks indicate the cholesterol profile from pooled serum of 8 mice after 8 weeks of treatment with the high cafestol diet. Serum was fractionated as described in Animals and Methods.*

Cafestol increases serum lipid and lipoprotein levels

Cafestol raised serum cholesterol in all three mouse strains after eight weeks of dietary intervention (figure 1). In apoE*3-Leiden transgenic mice, serum cholesterol was raised by 33% (3.46 mmol/L; 95%CI [1.62;5.30]) in the low cafestol diet group and by 61% (6.35 mmol/L; 95%CI [4.47;8.22]) in the high cafestol diet group. In LDLR+/- mice, serum cholesterol was raised by 20% (0.85 mmol/L; 95%CI [-0.25; 1.94]) in the low cafestol diet group and by 55% (2.37 mmol/L; 95%CI [0.73;4.01]) in the high cafestol diet group. In WT mice, serum cholesterol was raised by 24% (0.62 mmol/L; 95%CI [0.34;0.90]) in the low cafestol diet group and by 46% (1.21 mmol/L; 95%CI [0.92;1.50]) in the high cafestol diet group. In all mouse strains, the rise in serum cholesterol was predominantly due to a rise in VLDL and IDL cholesterol (figure 2). Serum triglycerides were increased after two weeks in apoE*3-Leiden transgenic and WT mice and remained significantly higher in the apoE*3-Leiden transgenic mice during cafestol treatment compared to placebo treatment (figure 1). Since the effects on serum cholesterol were most pronounced in the apoE*3-Leiden transgenic mice, we proceeded with this animal model to study the mechanism of the cholesterol-raising effects of cafestol.

Cafestol decreases hepatic enzymes in bile acid synthesis and faecal excretion of bile acids

To validate the effects of cafestol on bile acid synthesis obtained in cultured rat hepatocytes (5), we determined the effect of a high cafestol diet during three weeks on enzymes involved in bile acid synthesis and on faecal bile acid excretion in apoE*3-Leiden transgenic mice. Cafestol decreased the cholesterol 7α-hydroxylase activity and mRNA levels by 57% and 58%, respectively (both $p<0.05$) (table 2). Cafestol also decreased sterol 27-hydroxylase mRNA levels by 32% ($p<0.05$), while the enzyme activity was paradoxically increased by 40% ($p<0.05$) (table 2). It is well-known, however, that mitochondria may contain oxysterol 7α-hydroxylase (25), as found in pigs (26) and in humans (27), converting 27-hydroxycholesterol into 7α,27-dihydroxycholesterol (25-27). Thus, it is possible that the apparent increase in sterol 27-hydroxylase activity can be attributed to accumulation of its product, 27-hydroxycholesterol, caused by a blockade in the subsequent metabolic conversion. Therefore, we measured mRNA levels of oxysterol 7α-hydroxylase. The expression of this enzyme was decreased by 58% ($p<0.005$), giving an explanation for the apparent increase in sterol 27-hydroxylase activity (table 2).

Table 2. Effect of cafestol on hepatic mRNA and activity levels in apo E*3-Leiden mice

	Placebo diet		High cafestol diet	
	activity	mRNA	activity	mRNA
	(% of placebo)			
Cholesterol 7α-hydroxylase	100 ± 5	100 ± 41	43 ± 1*	42 ± 7*
Sterol 27-hydroxylase	100 ± 10	100 ± 12	140 ± 5*	68 ± 17*
Oxysterol 7α-hydroxylase	N.D.	100 ± 21	N.D.	41 ± 16**
LDL receptor	N.D.	100 ± 26	N.D.	63 ± 14*

ApoE*3-Leiden mice were treated with a high cafestol diet or a placebo diet for 3 weeks. Hepatic enzyme activities and mRNA levels of mice treated with a high cafestol (0.05% w/w) or placebo diet were determined after a 4 hour fasting period from 8 till 12 a.m. The amount of mRNA was corrected for differences in total RNA applied to the gel, using 18 S ribosomal RNA as an internal standard. Absolute activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase from apoE*3-Leiden mice treated the placebo diet were 1.83 nmol/h/mg protein and 1.76 nmol/h/mg protein, respectively. Data shown are means ± SD (n=4 per group). A significant difference between placebo and cafestol diet is indicated by an asterisk* ($p<0.05$), ** ($p<0.005$). N.D.: not determined.

Since down-regulation of enzymes involved in bile acid synthesis has consequences for the overall process of bile acid production, the amount of total and individual bile acids was measured in faeces. Cafestol decreased the total amount of bile acids excreted in the faeces with 41% as compared to the placebo diet (table 3). In addition, cafestol changed the relative amount of the various bile acids in the faeces showing an increase in the ratio between bile acids formed only via the neutral or 7α-hydroxylated pathway (deoxycholate and cholate) and those formed via the neutral as well as the acidic or 27-hydroxylated pathway (remaining bile acids) (28;29) (table 3). Furthermore, the amount of faecal neutral sterols excreted in the cafestol treated group was slightly lower as compared to the placebo group indicating that inhibition of bile acid synthesis does not lead to an increased secretion of free cholesterol into bile (table 3). The effect on the excretion and composition of faecal bile acids and neutral sterols was similar after three and six weeks on the high cafestol or placebo diet.

Table 3. Effects of cafestol on faecal bile acid excretion and composition and on excretion of neutral sterols in apo E*3-Leiden mice

Treatment	Bile acid excretion	DC	C	LC	CDC	HDC	UDC	α/βMC	ωMC	ratio (7α/7α+27)	Neutral sterol excretion
	(μmol/day per 100g BW)					*(%)*					*(μmol/day per 100g BW)*
Placebo diet	6.8 ± 0.7 (100%)	21	15	4	1	2	3	28	26	36/64	34.3 ± 1.3 (100%)
High cafestol diet	4.0 ± 0.1 (59%)	30	13	3	1	2	3	16	32	43/57	27.9 ± 2.8 (81%)

Mice were treated with a high cafestol diet or a placebo diet for three weeks. In faeces, total bile acids, neutral sterols and bile acid composition was determined as described in Animals and methods. Data are means ± range of two individual faeces samples of each group. DC: deoxycholic acid; C: cholic acid; LC: lithocholic acid; CDC: chenodeoxycholic acid; HDC: hyodeoxycholic acid; UDC: ursodeoxycholic acid; MC: muricholic acid. Ratio 7α/7α+27 indicates Σ(DC+C)/Σ(LC+CDC+HDC+UDC+all MC's). Values between parenthesis represent the percentage of the value obtained in animals treated with the placebo diet.

Effect of cafestol on VLDL production and hepatic lipid metabolism in apoE*3-Leiden transgenic mice

In order to investigate the effects of a decreased bile acid synthesis on hepatic lipid metabolism, we determined the amount of hepatic lipids of apoE*3-Leiden transgenic mice treated with a high cafestol or placebo diet for three weeks. Cafestol decreased the hepatic triglyceride content by 52% ($p<0.05$), but it did not alter the liver free and esterified cholesterol levels (table 4). Although the hepatic content of free cholesterol in apoE*3-Leiden mice was apparently not affected by cafestol, the regulatory pool of cholesterol was increased as indicated by a decreased expression of the LDL receptor (-37%, $p<0.05$) (table 2).

Table 4. Effect of cafestol on hepatic cholesterol and triglyceride content in apo E*3-Leiden mice

Treatment	Free cholesterol	Cholesteryl ester	Triglycerides
		(µg/mg protein)	
Placebo	15.5 ± 1.5	38.3 ± 7.7	86.3 ± 10.6
Cafestol	14.4 ± 1.3	32.8 ± 11.9	41.1 ± 8.7*
	(93%)	(85%)	(48%)

Mice were treated with a high cafestol diet or a placebo diet for three weeks. In liver homogenates, free cholesterol, cholesteryl esters and triglycerides were determined using a h.p.t.l.c. method (see Animals and methods) after a 4 hour fasting period. Data are means ± SD (n=4). Values between parenthesis represent the percentage of the value obtained in animals treated with the placebo diet. A significant difference between control and treated mice is indicated by an asterisk * ($p<0.05$).

Table 5. Effect of cafestol on VLDL composition in apo E*3-Leiden mice.

Treatment	Triglycerides	Free cholesterol	Cholesteryl ester	Phospholipids
		% of total lipid (by weight)		
Placebo	52.0 ± 3.0	7.8 ± 0.8	17.5 ± 2.9	22.7 ± 5.0
Cafestol	28.9 ± 10.1*	6.6 ± 1.1	49.2 ± 12.7*	15.3 ± 1.7

VLDL was isolated by ultracentrifugation from pooled serum of eight fasted apo E*3-Leiden transgenic mice treated with a high cafestol or a placebo diet for three weeks. The serum was collected 180 min after Triton injection. Triglycerides, total cholesterol, free cholesterol and phospholipids were measured enzymatically and the amount of cholesteryl ester was calculated (see Animals and methods). Data are means ± SD of three individual VLDL samples of each group. A significant difference between control and cafestol treated mice is indicated by an asterisk * ($p<0.05$).

Since the excess hepatic cholesterol was not excreted into the bile, it might have been secreted by the liver in VLDL particles. Therefore, we measured nascent VLDL production in apolipoprotein E*3-Leiden transgenic mice after three weeks on a high cafestol or placebo diet. The relative amount of cholesteryl esters in the VLDL particles upon cafestol treatment was almost three times higher compared to placebo treatment ($p<0.05$), while the relative amount of triglycerides in the particles was decreased by about 50% (table 5). This was reflected in a two times lower VLDL triglyceride production rate (35.1±13.8 µmol/h/kg after the high cafestol diet versus 63.1±17.5 µmol/h/kg after the placebo diet) (figure 3).

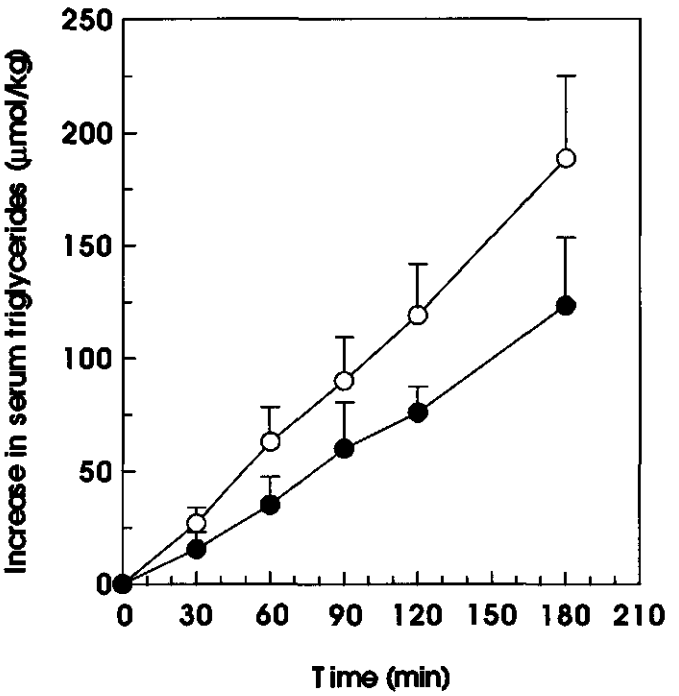

*Figure 3. Effect of cafestol on the VLDL triglyceride production rate in apoE*3-Leiden mice. Triton WR 1339 (500 mg/kg body weight) was injected into placebo (white marks) and cafestol (0.05% w/w) (black marks) treated apoE*3-Leiden mice (n=8 fasted mice per group). Serum triglyceride levels were determined at 30, 60, 90, 120 and 180 min and corrected for the triglyceride level at the time of injection (t=0 min). The values shown are means ± SD.*

Discussion

In this study, cafestol increased serum cholesterol levels in apoE*3-Leiden, heterozygous LDL receptor deficient, and in wild type (C57Bl/6) mice, mainly in the VLDL and IDL fraction. In apoE*3-Leiden transgenic mice, cafestol decreased bile acid synthesis reflected by a reduction in the total amount of faecal bile acids by down-regulation of expression of enzymes involved in the neutral as well as in the alternative bile acid synthetic pathway. The decrease in bile acid synthesis resulted in a decline in LDL receptor mRNA levels and an increased secretion of VLDL cholesteryl ester.

So far, no suitable animal model has been found to study the mechanism of action of cafestol, since various animal models did not respond to this coffee diterpene as humans do (6-10). ApoE*3-Leiden transgenic mice are the first animals showing a similar increase in serum cholesterol due to cafestol as observed in humans, making this species a good model to investigate the biochemical background of the cholesterol-raising effect of cafestol in humans. It should be noted that the increase in serum cholesterol in humans is mainly present in LDL, whereas in the mice the rise is predominantly found in the VLDL-IDL range.

Previously, we reported that cafestol suppressed bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression in rat hepatocytes (5). This is now confirmed *in vivo* in apolipoprotein E*3-Leiden transgenic mice. In this study we also showed that the total bile acid mass in faeces of the cafestol-treated group was decreased. Cafestol predominantly affected cholesterol 7α-hydroxylase and oxysterol 7α-hydroxylase, but the effects on sterol 27-hydroxylase also contributed to the decreased faecal bile acid mass since cafestol changed the faecal bile acid composition. The ratio between bile acids formed only via the neutral or 7α-hydroxylated pathway (deoxycholate and cholate) and those formed via the neutral as well as the acidic or 27-hydroxylated pathway (remaining bile acids) (28;29) increased. This indicates inhibition of the acidic pathway in bile acid synthesis next to an inhibitory effect on the neutral pathway.

Theoretically, a suppressed bile acid synthesis would increase the pool of free cholesterol in the liver cell. We did not find an effect on hepatic free cholesterol levels, but different metabolic pathways might have converted the free cholesterol into cholesteryl esters or removed it from the liver as such and/or via VLDL particles to maintain the hepatic cholesterol homeostasis. Since we did not find an increase in faecal excretion of neutral sterols or a hepatic accumulation of cholesteryl esters, it appears plausible that the cholesterol which becomes available due to inhibition of bile acid synthesis is directly removed from the liver via VLDL particles (see below). In addition, high amounts of free cholesterol in the cell membranes (30) may overshadow subtle changes in free cholesterol caused by inhibition of bile acid

synthesis. We found a substantial decrease in LDLR mRNA, which is a sensitive measure to detect changes in the regulatory pool of free cholesterol. Subtle increases in intracellular cholesterol prevent processing of sterol regulatory element binding protein (SREBP), resulting in a down-regulation of LDL-receptor gene transcription (31). A similar decrease in LDLR mRNA levels has been shown *in vitro* in cultured rat hepatocytes (5) and in HepG2 cells (32). In contrast, divergent data were reported in other cell types (33;34), possibly because of the different metabolic functions of these cells. Our results plead in favour of the hypothesis that the cholesterol-raising effect of cafestol can be explained at least in part by a reduced expression of the LDL receptor.

The rise in serum cholesterol upon cafestol treatment may also partly be explained by an increased secretion of cholesteryl esters in VLDL. The relative amount of cholesteryl esters in the VLDL particles upon cafestol treatment was significantly higher compared to placebo treatment. Concomitantly, the relative amount of triglycerides in the particles decreased. This reduction was to the same extent as the decline in the VLDL-triglyceride production rate. This suggests that not the number of particles but the composition is changed, resulting in the production of a ßVLDL-like particle. The decrease in the VLDL-triglyceride production rate and the reduced triglyceride content of the liver suggests an impaired triglyceride synthesis. Whether this is due to a direct or indirect effect of cafestol on activity or expression of enzymes involved in triglyceride synthesis awaits further investigation.

In conclusion, we found that cafestol inhibits bile acid synthesis by down-regulation of both the neutral and the acidic pathway leading to a decrease in the expression of the LDL receptor and an elevated secretion of cholesteryl esters in VLDL. Suppression of bile acid synthesis may provide an explanation for the cholesterol-raising effects of unfiltered coffee in humans.

Acknowledgements

We thank the Netherlands Heart Foundation (grant no. 94.049), the Netherlands Organization for Scientific Research (grant no. 900-562-091) and the Foundation for Nutrition and Health Research for funding, and Gerrit van Tintelen, Bert Weijers, Rene Bakker, and Renze Boverhof for technical assistance.

References

1. Weusten-van der Wouw MPME, Katan MB, Viani R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res. 1994;35:721-733.
2. Urgert R, Essed N, van der Weg G, Kosmeijer-Schuil TG, Katan MB. Separate effects of the coffee diterpenes cafestol and kahweol on serum lipids and liver aminotransferases. Am. J Clin. Nutr. 1997;65:519-524.
3. Urgert R, Katan MB. The cholesterol-raising factor from coffee beans. Annu. Rev. Nutr. 1997;17:305-324.

4. Urgert R, Meyboom S, Kuilman M, et al. Comparison of effect of cafetiere and filtered coffee on serum concentrations of liver aminotransferases and lipids: six month randomised controlled trial. BMJ. 1996;313:1362-1366.
5. Post SM, de Wit EC, Princen HMG. Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase in rat hepatocytes. Arterioscler. Thromb. Vasc. Biol. 1997;17:3064-3070.
6. Beynen AC, Weusten-van der Wouw MPME, de Roos B, Katan MB. Boiled coffee fails to raise serum cholesterol in hamsters and rats. Br. J Nutr. 1996;76:755-764.
7. Ratnayake WM, Pelletier G, Hollywood R, Malcolm S, Stavric B. Investigation of the effect of coffee lipids on serum cholesterol in hamsters. Food Chem. Toxicol. 1995;33:195-201.
8. Mensink RP, Zock PL, Katan MB, Beynen AC. Boiled coffee does not increase serum cholesterol in gerbils and hamsters. Z. Ernahrungswiss. 1992;31:82-85.
9. Hostmark AT, Lystad E, Haug A, Bjerkedal T, Eilertsen E. Effect of boiled and instant coffee on plasma lipids and fecal excretion of neutral sterols and bile acids in the rat. Nutrition Reports International 1988;38:859-864.
10. Terpstra AH, Katan MB, Weusten-van der Wouw MPME, Nicolosi RJ, Beynen AC. Coffee oil consumption does not affect serum cholesterol in rhesus and cebus monkeys. J Nutr. 1995;125:2301-2306.
11. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, et al. Diet -induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin. Invest. 1994;93:1403-1410.
12. Jong MC, Dahlmans VE, Van Gorp PJ, et al. Both lipolysis and hepatic uptake of VLDL are impaired in transgenic mice coexpressing human apolipoprotein E*3Leiden and human apolipoprotein C1. Arterioscler. Thromb. Vasc. Biol. 1996;16:934-940.
13. Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J Clin. Invest. 1994;93:1885-1893.
14. Chiang JY. Reversed-phase high-performance liquid chromatography assay of cholesterol 7 alpha-hydroxylase. Methods Enzymol. 1991;206:483-491.
15. Twisk J, Lehmann EM, Princen HMG. Differential feedback regulation of cholesterol 7 alpha-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem. J 1993;290:685-691.
16. Stapleton G, Steel M, Richardson M, et al. A novel cytochrome P450 expressed primarily in brain. J Biol. Chem. 1995;270:29739-29745.
17. Wilson GM, Hollar BA, Waterson JR, Schmickel RD. Molecular analysis of cloned human 18S ribosomal DNA segments. Proc. Natl. Acad. Sci. USA 1978;75:5367-5371.
18. Murphy GM, Billing BH, Baron DN. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J. Clin. Pathol. 1970;23:594-598.
19. Setchell KD, Worthington J. A rapid method for the quantitative extraction of bile acids and their conjugates from serum using commercially available reverse-phase octadecylsilane bonded silica cartridges. Clin. Chim. Acta 1982;125:135-144.
20. Stellaard F, Sackmann M, Sauerbruch T, Paumgartner G. Simultaneous determination of cholic acid and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using 13C-labeled bile acids. J Lipid Res. 1984;25:1313-1319.
21. Bligh EG, Dyer WJ. A rapid method for total lipid extraction and purification. Can. J Biochem. 1959;193:911-917.
22. Havekes LM, de Wit EC, Princen HMG. Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity. Biochem. J 1987;247:739-746.
23. Aalto-Setala K, Fisher EA, Chen X, et al. Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles. J Clin. Invest. 1992;90:1889-1900.
24. Jong MC, Dahlmans VE, Van Gorp PJ, et al. In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in trangenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor-associated protein-sensitive pathway. J Clin. Invest. 1996;98:2259-2267.
25. Schwarz M, Lund EG, Lathe R, Bjorkhem I, Russell DW. Identification and characterization of a mouse oxysterol 7alpha-hydroxylase cDNA. J Biol. Chem. 1997;272:23995-24001.

26. Toll A, Shoda J, Axelson M, Sjovall J, Wikvall K. 7 alpha-hydroxylation of 26-hydroxycholesterol, 3-beta-hydroxy-5-cholestenoic acid and 3-beta-hydroxy-5-cholenoic acid by cytochrome P-450 in pig liver microsomes. FEBS Lett. 1992;296:73-76.
27. Shoda J, Toll A, Axelson M, Pieper F, Wikvall K, Sjovall J. Formation of 7 alpha- and 7 beta-hydroxylated bile acid precursors from 27-hydroxycholesterol in human liver microsomes and mitochondria. Hepatology 1993;17:395-403.
28. Russell DW, Setchell KD. Bile acid biosynthesis. Biochemistry 1992;31:4737-4749.
29. Princen HMG, Post SM, Twisk J. Regulation of bile acid biosynthesis. Curr. Pharmaceutical Design 1997;3:59-84.
30. Lange Y, Ramos BV. Analysis of the distribution of cholesterol in the intact cell. J Biol. Chem. 1983;258:15130-15134.
31. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425-430.
32. Rustan AC, Halvorsen B, Huggett AC, Ranheim T, Drevon CA. Effect of coffee lipids (cafestol and kahweol) on regulation of cholesterol metabolism in HepG2 cells. Arterioscler. Thromb. Vasc. Biol. 1997;17:2140-2149.
33. Halvorsen B, Ranheim T, Nenseter MS, Huggett AC, Drevon CA. Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts. J Lipid Res. 1998;39:901-912.
34. Ranheim T, Halvorsen B, Huggett AC, Blomhoff R, Drevon CA. Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells. J Lipid Res. 1995;36:2079-2089.

5

Consumption of cafetiere coffee raises cholesteryl ester transfer protein activity levels before LDL cholesterol in normolipidaemic subjects

Baukje de Roos and Arie van Tol, Rob Urgert, Leo M. Scheek, Teus van Gent, Rien Buytenhek, Hans M.G. Princen, and Martijn B. Katan. *Submitted.*

Abstract

Cafestol and kahweol, diterpene lipids present in unfiltered coffee, raise serum LDL cholesterol concentrations in humans. We investigated whether the serum lipid transfer proteins cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) might be involved in the mechanism of action of unfiltered coffee. In addition, we determined the long-term effects of cafetiere coffee consumption on the activity level of serum lipid transfer proteins and lecithin: cholesterol acyltransferase (LCAT). Forty-six healthy normolipidaemic subjects consumed 0.9 L of either cafetiere or filtered coffee for 24 weeks. Relative to baseline, cafetiere coffee significantly increased average CETP activity by 12% after 2 weeks, by 18% after 12 weeks, and by 9% after 24 weeks. LDL rose by 2% after 2 weeks, by 12% after 12 weeks, and by 7% at 24 weeks. The increase in CETP clearly preceded the increase in LDL cholesterol. PLTP activity was significantly increased by 10% after 12 and 24 weeks. LCAT activity was significantly decreased by 6% after 12 weeks and by 7% after 24 weeks. We conclude that the increased CETP activity during consumption of cafetiere coffee may contribute to the rise in LDL cholesterol.

Introduction

Long-term consumption of unfiltered coffee potently raises serum low density lipoprotein (LDL) cholesterol in humans (1). The lipid soluble diterpenes cafestol and kahweol - present in coffee beans (2) - are responsible for this effect (3). These diterpenes are the most potent cholesterol-raising substances from the diet that are known. Therefore, the action of cafestol and kahweol offers an interesting model to study the effect of dietary substances on cholesterol metabolism in humans.

The mechanism of action of cafestol and kahweol is poorly understood. *In vitro* studies show contradictory results in various cell lines. Cafestol decreased LDL receptor activity in human fibroblasts (4), in HepG2-cells (5) and in primary rat hepatocytes (6). However, in CaCo-2 cells, cafestol enhanced LDL cholesterol uptake and degradation (7). We earlier found that in humans, consumption of diterpenes is associated with increased serum activity levels of the lipid transfer proteins cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) and with decreased activity levels of lecithin: cholesterol acyltransferase (LCAT) (8). CETP catalyses the transfer of cholesteryl esters - synthesised by LCAT - from HDL to the apolipoprotein B-containing lipoproteins LDL and VLDL (9). PLTP can affect the net mass transfer of phospholipids between lipoproteins (10) and it also converts small-sized HDL (HDL_3) both into larger (HDL_2-sized) and smaller (preß-migrating) HDL particles (11-13). Both CETP and PLTP appear to play a major role in determining the concentration and size of HDL particles in plasma (10;14). In our earlier study (8) it remained unclear whether the elevated serum CETP and PLTP activity levels in subjects consuming diterpenes were a cause or a consequence of the raised LDL cholesterol concentrations.

In the present experiment we studied whether serum lipid transfer proteins might play a role in the mechanism of action of cafestol and kahweol in humans. For this purpose, we compared the initial changes occurring in lipid transfer protein activity levels with the initial changes in LDL and HDL cholesterol levels during cafetiere coffee consumption. Filtered coffee was used as a control. We reasoned that an increase in a lipid transfer protein activity prior to an increase in LDL cholesterol could indicate a role of this protein in the mechanism of action. In addition, we determined the long-term effects of consumption of cafetiere coffee on lipid transfer protein activity levels and LCAT.

Subjects and Methods

Subjects

Approval for the experiments was obtained from the Human Ethics Committee of the Division of Human Nutrition & Epidemiology, Wageningen Agricultural University. Forty-six subjects gave their written informed consent and participated in the study. These subjects had a mean age of 30 years (range 19-69) and a BMI of 23±3 (mean±SD) kg/m^2. Sixteen of these subjects were smokers. Alcohol consumption was limited to less than three beverages per day per subject.

Design

During a run-in period of four weeks subjects consumed 0.9 L of filtered coffee per day. Subsequently, they were stratified by age and serum alanine aminotransferase and then randomly allocated to consume 0.9 L of either filtered coffee (n=24 subjects; 12 male and 12 female) or cafetiere coffee (n=22 subjects; 11 male and 11 female) per day for 24 weeks. Subjects brewed their coffee at home. Filtered coffee was prepared by scooping 33 g of finely ground coffee into a paper filter and pouring 1.1 L of boiling water onto the grounds. Boiled coffee was prepared by scooping 39 g of coarse grounds into a cafetiere (Kaffee Primo, BMF, Germany) and pouring 1.2 L of boiling water onto the grounds. Subjects stirred the brew for 10 seconds, allowed it to stand for 2-5 minutes, pushed down the plunger to separate the grounds from the brew, and then decanted the brew. This amount of cafetiere coffee provided about 38 mg cafestol and about 33 mg kahweol per day. Filtered coffee provided less than 1 mg of either diterpene a day. Both coffee brews contained similar amounts of caffeine.

Blood sampling and assays

Fasting venous blood samples were taken after 0, 2, 12 and 24 weeks of intervention and after 12 weeks of follow up. Serum was obtained by centrifugation and stored at –80°C. Total serum triglycerides, LDL cholesterol and HDL cholesterol were assayed as described (1). Serum CETP activity levels were assayed in duplicate after removal of endogenous VLDL + LDL (15) using excess exogenous substrates. The isotope assay measures the transfer of [1-^{14}C-oleate]cholesteryl ester from labelled LDL to an excess of unlabelled pooled normal HDL, while LCAT is inhibited with dithiobis-2-nitrobenzoic acid (16). CETP activity was calculated as the bi-directional transfer between labelled LDL and HDL. The CETP activity levels obtained by this method correlate well with CETP mass (17). Serum PLTP activity levels were assayed in duplicate in a phospholipid vesicles-HDL system, as described (15). In short, small serum samples of 0.5-1.0 µl were incubated with [^{3}H]phosphatidylcholine labelled liposomes and an excess of pooled normal HDL,

followed by precipitation of the liposomes with a mixture of NaCl, $MgCl_2$ and heparin (final concentrations of 230 mmol/L, 32 mmol/L and 200 U/mL, respectively). The measured PLTP activity is not influenced by the phospholipid transfer promoting properties of CETP (15). Serum LCAT activity levels were measured in duplicate, using excess exogenous substrate containing [^{3}H]cholesterol as described (18). The measured LCAT activity levels vary linearly with the amount of serum added to the incubation mixture. The measured activity is indicative for the serum LCAT concentration (19). Serum CETP, PLTP and LCAT activity levels were related to human pool serum and expressed in arbitrary units (AU, which corresponds to the percentages of the activities present in the serum pool). All subjects were analysed using one batch of substrates. The within assay coefficients of variation of CETP, PLTP and LCAT were 2.7%, 3.5% and 4.5%, respectively.

Statistics

Responses were calculated by subtracting baseline values from values obtained during treatment. All variables were normally distributed. Differences in responses between the cafetiere and filtered-coffee group were tested against zero with unpaired t-tests.

Results

CETP activity was significantly elevated already after two weeks of cafetiere coffee consumption compared to filtered coffee consumption (table 1). At this point, CETP had already reached 68% of its maximal increase that was reached after 12 weeks of intervention. The increase in LDL cholesterol only became significant after 12 weeks (figure 1). At two weeks, LDL cholesterol had only reached 18% of its maximal increase that was reached after 12 weeks of intervention. A maximum increase of 18% over baseline value in CETP activity and 10% over baseline value in PLTP activity was reached after 12 weeks (figure 1). Both CETP and PLTP activity dropped just below baseline after consumption of cafetiere coffee had ceased. LCAT activity was significantly decreased by about 6% after 12 and 24 weeks of cafetiere coffee consumption and returned to baseline after cessation of treatment (table 1). As reported previously (1), triglycerides and thus presumably VLDL peaked already at the first measuring point. No significant changes were detected in HDL cholesterol (figure 1).

Table 1. Mean serum activity levels of cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP) and lecithin:cholesterol acyltransferase (LCAT) in 46 healthy normolipidaemic subjects before, during, and after consumption of filtered coffee (n=24) or cafetiere coffee (n=22) for six months.

Variable	Run-in	Treatment period			Treatment effect at 24 weeks	Follow up
	Week 0	Week 2	Week 12	Week 24	(95% CI) †	Week 36‡
			(*percentage of activity in a human pool serum*)			
CETP						
Filtered coffee	105.0	102.9	102.5	104.8		107.4
Cafetiere coffee	107.7	118.4¶	124.1¶	116.9¶	9.4 (1.5 to 17.3)	108.7
PLTP						
Filtered coffee	95.5	95.6	98.6	95.7		88.6
Cafetiere coffee	91.6	96.5	104.0¶	100.9¶	9.0 (1.9 to 16.2)	83.0
LCAT						
Filtered coffee	95.5	94.9	96.2	96.1		96.6
Cafetiere coffee	95.7	90.7	90.9¶	90.0¶	-6.3 (-11.8 to -0.8)	93.5

† Calculated by subtracting the mean change from baseline at 24 weeks in the group drinking filtered coffee from that in the group drinking cafetiere coffee.
‡ Value of one subject drinking filtered coffee missing.
¶ Significantly different from zero (*t-test, $p<0.05$).*

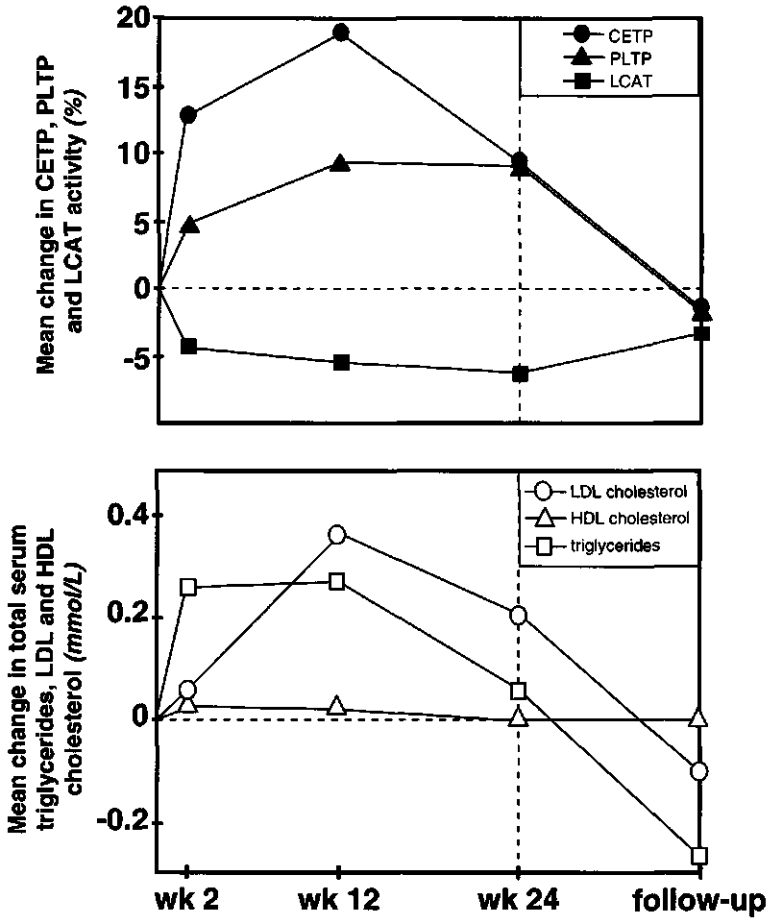

Figure 1. Mean changes from baseline values in serum activity levels of CETP, PLTP and LCAT and of serum concentrations of LDL and HDL cholesterol and triglycerides in 22 subjects drinking 0.9 L of cafetiere coffee per day for 24 weeks. For each time point, mean changes from baseline in 24 subjects drinking filtered coffee were subtracted from those in the subjects drinking cafetiere coffee.

Discussion

We found that consumption of cafetiere coffee increased CETP activity before it increased LDL cholesterol concentrations in a group of healthy normolipidaemic subjects. During consumption of cafetiere coffee, CETP activity levels had already reached 68% of their maximum levels at the first measurement point, two weeks after subjects had started to drink unfiltered coffee rich in cafestol. At that point in time, LDL levels had reached only 18% of their maximum level (figure 1). PLTP

activity levels were significant increased and LCAT activity levels were significantly decreased only after 12 and 24 weeks of intervention.

The present findings extend our earlier data obtained with purified coffee diterpenes (8). In our previous experiments, it remained unclear whether elevated activity levels of CETP and PLTP due to cafestol or kahweol consumption were a cause or a consequence of elevated serum LDL cholesterol levels (8). Earlier human studies on the effects of a high fat, high cholesterol diet on serum CETP also do not provide clear evidence for a causal relationship, e.g. in favour of a mechanism with increased serum CETP activity levels causing increased serum LDL cholesterol levels (20-22). Data from animal models are not conclusive either. Some studies in transgenic mice suggest that increased CETP levels may be secondary to increased LDL cholesterol levels. When mice carrying the human CETP gene were cross-bred with hyperlipidaemic mice which had a deficiency in either apolipoprotein E or the LDL receptor, plasma CETP levels were increased to values higher than those found in normolipidaemic mice carrying the human CETP gene (23). However, other studies showed that expression of the monkey CETP gene in mice raised plasma levels of LDL, a class of lipoproteins which is virtually absent in wild-type mice (24). Rats injected intravenously with purified human CETP also showed increased LDL and apolipoprotein B concentrations (25). In the present experiment the increase in CETP activity levels preceded the increase in LDL cholesterol, as illustrated in figure 1. This supports a role for CETP in the effect of cafestol on LDL levels in man. However, a caveat is in place because triglycerides and presumably VLDL rose rapidly on cafestol; thus the rise in CETP may itself have been caused by the rise in VLDL.

PLTP activity also increased upon long term cafetiere coffee consumption. This is in agreement with data from our previous study in which subjects consumed coffee diterpenes (8). PLTP and CETP belong to the same lipid transfer protein/lipopolysaccharide binding protein (LTP/LBP) gene family (26). Both proteins are up-regulated by cholesterol feeding in animal studies (27;28), suggesting that their genes contain cholesterol-responsive elements (9). A parallel increase in both lipid transfer proteins might therefore be expected. Serum LCAT activity levels decreased slightly after consumption of cafetiere coffee. A comparable decrease was observed earlier after consumption of purified coffee diterpenes (8). Since LCAT is solely synthesised in the liver (19), an impairment of liver function, as evident from increased activities of serum transaminases after cafetiere coffee consumption (1) may explain the decrease in serum LCAT levels. Because LCAT primarily functions in the formation of HDL cholesteryl esters, a lower LCAT activity may result in decreased HDL cholesterol levels, especially when CETP activity is increased. Indeed, cafestol and kahweol were found to lower serum HDL cholesterol in previous studies (3;29;30), though not in the present study.

If CETP is involved in the mechanism of action of coffee diterpenes then it might increase the cholesteryl ester content of VLDL and/or LDL (31). The effect of coffee diterpenes on the composition of the various lipoproteins is not known. Two earlier studies showed a significant increase in apolipoprotein B100 levels of 0.09 g/L (32) and 0.13 g/L (33) after consumption of boiled coffee. This suggests that the increase in LDL cholesterol is at least partly explained by changes in the number of LDL particles. An additional change in lipoprotein composition can however not be excluded. Since the rise in CETP activity preceded the rise in LDL cholesterol upon consumption of cafetiere coffee, CETP might contribute to the LDL cholesterol–raising effect of coffee diterpenes.

Acknowledgements

This study was supported by the Netherlands Heart Foundation through grant No. 900-562-091 of the Netherlands Organisation of Scientific Research (NWO) and by the Foundation for Nutrition and Health Research (SOVG). We thank the volunteers for participation in this study on effects of coffee consumption. Marga van der Steen, Geke Groenwold, Joke Barendse, Jan Harryvan, Robert Hovenier and Guido van der Weg are thanked for performing various analyses. Saskia Meyboom, Marjan Kuilman, Henny Rexwinkel, Maud Vissers and Mariska Klerk are acknowledged for their assistance and Joanna Kruimel, MD for medical supervision.

References

1. Urgert R, Meyboom S, Kuilman M, et al. Comparison of effect of cafetiere and filtered coffee on serum concentrations of liver aminotransferases and lipids: six month randomised controlled trial. BMJ. 1996;313:1362-1366.
2. Urgert R, van der Weg G, Kosmeijer-Schuil TG, van de Bovenkamp P, Hovenier R, Katan MB. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. J Agric. Food Chem. 1995;43:2167-2172.
3. Weusten-van der Wouw MPME, Katan MB, Viani R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res. 1994;35:721-733.
4. Halvorsen B, Ranheim T, Nenseter MS, Huggett AC, Drevon CA. Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts. J Lipid Res. 1998;39:901-912.
5. Rustan AC, Halvorsen B, Huggett AC, Ranheim T, Drevon CA. Effect of coffee lipids (cafestol and kahweol) on regulation of cholesterol metabolism in HepG2 cells. Arterioscler. Thromb. Vasc. Biol. 1997;17:2140-2149.
6. Post SM, de Wit EC, Princen HMG. Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase in rat hepatocytes. Arterioscler. Thromb. Vasc. Biol. 1997;17:3064-3070.
7. Ranheim T, Halvorsen B, Huggett AC, Blomhoff R, Drevon CA. Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells. J Lipid Res. 1995;36:2079-2089.
8. van Tol A, Urgert R, de Jong-Caesar R, et al. The cholesterol-raising diterpenes from coffee beans increase serum lipid transfer protein activity levels in humans. Atherosclerosis 1997;132:251-254.
9. Tall A. Plasma lipid transfer proteins. Annu. Rev. Biochem. 1995;64:235-257.
10. Bruce C, Chouinard R, Tall AR. Plasma lipid transfer proteins, high density lipoproteins, and reverse cholesterol transport. Annu. Rev. Nutr. 1998;18:297-330.
11. Jauhiainen M, Metso J, Pahlman R, Blomqvist S, van TA, Ehnholm C. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. J Biol.Chem 1993;268:4032-4036.
12. von Eckardstein A, Jauhiainen M, Huang Y, et al. Phospholipid transfer protein mediated conversion of high density lipoproteins generates pre beta 1-HDL. Biochim. Biophys. Acta 1996;1301:255-262.

13. Lusa S, Jauhiainen M, Metso J, Somerharju P, Ehnholm C. The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion. Biochem. J 1996;313:275-282.
14. Tollefson JH, Ravnik S, Albers JJ. Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. J Lipid Res. 1988;29:1593-1602.
15. Speijer H, Groener JE, van Ramshorst E, van Tol A. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. Atherosclerosis 1991;90:159-168.
16. Groener JE, Pelton RW, Kostner GM. Improved estimation of cholesteryl ester transfer/exchange activity in serum or plasma. Clin. Chem. 1986;32:283-286.
17. Hannuksela M, Kesaniemi YA, Savolainen MJ. Evaluation of plasma cholesteryl ester transfer protein (CETP) activity as a marker of alcoholism. Alcohol 1992;27:557-562.
18. Dullaart RP, Sluiter WJ, Dikkeschei LD, Hoogenberg K, van Tol A. Effect of adiposity on plasma lipid transfer protein activities: a possible link between insulin resistance and high density lipoprotein metabolism. Eur. J Clin. Invest. 1994;24:188-194.
19. Floren CH, Chen CH, Franzen J, Albers JJ. Lecithin: cholesterol acyltransferase in liver disease. Scand. J Clin. Lab.I nvest 1987;47:613-617.
20. van Tol A, Zock PL, van Gent T, Scheek LM, Katan MB. Dietary trans fatty acids increase serum cholesterylester transfer protein activity in man [see comments]. Atherosclerosis 1995;115:129-134.
21. Quig DW, Zilversmit DB. Plasma lipid transfer activities. Annu. Rev. Nutr. 1990;10:169-193.
22. Martin LJ, Connelly PW, Nancoo D, et al. Cholesteryl ester transfer protein and high density lipoprotein responses to cholesterol feeding in men: relationship to apolipoprotein E genotype. J Lipid Res. 1993;34:437-446.
23. Masucci ML, Plump A, Jiang XC, Walsh A, Breslow JL, Tall AR. Profound induction of hepatic cholesteryl ester transfer protein transgene expression in apolipoprotein E and low density lipoprotein receptor gene knockout mice. A novel mechanism signals changes in plasma cholesterol levels. J Clin. Invest. 1996;97:154-161.
24. Marotti KR, Castle CK, Murray RW, Rehberg EF, Polites HG, Melchior GW. The role of cholesteryl ester transfer protein in primate apolipoprotein A-I metabolism. Insights from studies with transgenic mice. Arterioscler. Thromb. 1992;12:736-744.
25. Groener JE, van Gent T, van Tol A. Effect of lipid transfer protein on plasma lipids, apolipoproteins and metabolism of high-density lipoprotein cholesteryl ester in the rat. Biochim. Biophys. Acta 1989;1002:93-100.
26. Beamer LJ, Carroll SF, Eisenberg D. Crystal structure of human BPI and two bound phospholipids at 2.4 angstrom resolution. Science 1997;276:1861-1864.
27. Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. J Clin. Invest. 1992;90:1290-1295.
28. Jiang XC, Brudal S. Regulation of murine plasma phospholipid transfer protein activity and mRNA levels by lipopolysaccharide and high cholesterol diet. J Biol. Chem. 1995;270:17133-17138.
29. Urgert R, Essed N, van der Weg G, Kosmeijer-Schuil TG, Katan MB. Separate effects of the coffee diterpenes cafestol and kahweol on serum lipids and liver aminotransferases. Am. J Clin. Nutr. 1997;65:519-524.
30. Zock PL, Katan MB, Merkus MP, van Dusseldorp M, Harryvan JL. Effect of a lipid-rich fraction from boiled coffee on serum cholesterol. Lancet 1990;335:1235-1237.
31. Dinchuk J, Hart J, Gonzalez G, Karmann G, Schmidt D, Wirak DO. Remodelling of lipoproteins in transgenic mice expressing human cholesteryl ester transfer protein. Biochim. Biophys. Acta 1995;1255:301-310.
32. van Dusseldorp M, Katan MB, van Vliet T, Demacker PN, Stalenhoef AF. Cholesterol-raising factor from boiled coffee does not pass a paper filter. Arterioscler. Thromb. 1991;11:586-593.
33. Aro A, Teirila J, Gref CG. Dose-dependent effect on serum cholesterol and apoprotein B concentrations by consumption of boiled, non-filtered coffee. Atherosclerosis 1990;83:257-261.

6

The coffee diterpene cafestol increases plasma triglycerides by increasing the production rate of large very low density lipoprotein apolipoprotein B in normolipidaemic subjects

Baukje de Roos, Muriel J. Caslake, Anton F.H. Stalenhoef, Dorothy Bedford, Pierre N.M. Demacker, Martijn B. Katan, Chris J. Packard. *Submitted.*

Abstract

Cafestol is a diterpene present in unfiltered coffee that raises plasma triglycerides in humans. We studied whether cafestol increases plasma triglycerides by increasing the production rate or decreasing the fractional catabolic rate of $VLDL_1$ (Svedbergs flotation rate 60-400) apolipoprotein B. In addition, we studied the effect of cafestol on the composition of $VLDL_1$ and $VLDL_2$ (Svedbergs flotation rate 20-60). Eight healthy normolipidaemic men were administered a daily dose of 75 mg of cafestol for two weeks. A bolus injection of 7 mg/kg of body weight of d_3-leucine (L-[5,5,5-$^2H_3$]leucine) was given after a baseline period with no cafestol and again after treatment with cafestol. We derived kinetic constants to describe the metabolism of $VLDL_1$ apolipoprotein B using a multicompartmental model. Cafestol increased plasma triglycerides by 31% or 0.32 mmol/L (95%CI [0.03-0.61]). The rise in triglycerides was mainly due to a rise in $VLDL_1$ triglycerides of 57% or 0.23 mmol/L (95%CI [-0.02;0.48]). The pool size of $VLDL_1$ apolipoprotein B was increased by 59% or 34 mg (95%CI [-1;102]). Cafestol increased the mean rate of $VLDL_1$ apolipoprotein B production by 80% or 755 mg/day (95%CI [0.2;5353]), whereas it did not significantly change the mean fractional catabolic rate of $VLDL_1$ apolipoprotein B (mean increase of 3 pools/day; 95%CI [-4;10]). Cafestol did not change the composition of $VLDL_1$. $VLDL_2$ became enriched with cholesteryl esters at the cost of triglycerides, as indicated by a significant increase in the $VLDL_2$ cholesteryl ester/triglyceride ratio. We conclude that cafestol increases plasma triglycerides by an increased production rate of $VLDL_1$ apolipoprotein B, probably via an increased assembly of $VLDL_1$ in the liver. The enrichment of $VLDL_2$ particles with cholesteryl

esters – rather than the increased amount of triglyceride-rich $VLDL_1$ particles - might play a role in the subsequent rise of LDL cholesterol levels.

Introduction

Unfiltered coffee brews such as Scandinavian-type boiled coffee, cafetiere (or French-press) coffee, and Turkish coffee raise plasma triglycerides and low density lipoprotein (LDL) cholesterol concentrations in humans (1). The responsible compound is cafestol, a lipid-soluble diterpene in coffee beans (2). Unfiltered coffee brews contain 3-6 mg of this diterpene per cup (3). Filtered coffee does not contain cafestol, because the diterpene is retained by a paper filter (4).

In human subjects, the effects of cafestol on plasma triglycerides and on LDL cholesterol differ in their kinetics. When 22 volunteers consumed 0.9 L of cafetiere coffee per day, plasma triglycerides reached a maximum increase of about 30% after 2-4 weeks. The effect on triglycerides was transient since levels were back at baseline after 24 weeks (5). The onset of the rise in LDL cholesterol was much slower: a maximum increase of about 12% was reached only after 8-12 weeks of cafetiere coffee consumption. The rise in LDL cholesterol is not transient; epidemiological studies show that chronic consumers of boiled coffee have permanently elevated levels of plasma cholesterol (6-8). Since the rise in triglycerides is the first step in a cascade of changes in lipoprotein metabolism upon consumption of cafestol (1;5), this rise might play a role in the subsequent increase in LDL cholesterol levels.

In this study we examined the mechanism by which cafestol increases plasma triglycerides in normolipidaemic men. In humans, the majority of triglycerides is transported in very low density lipoproteins (VLDL). However, VLDL can be fractionated into two structurally and metabolically discrete components: $VLDL_1$ (S_f 60-400) and $VLDL_2$ (S_f 20-60). Compared to $VLDL_1$, $VLDL_2$ are smaller, enriched in cholesteryl ester, depleted in triglycerides and have a lower ratio of apolipoprotein E and apolipoprotein C to apolipoprotein B (9). There is evidence for an independent regulation of the secretion of $VLDL_1$ and $VLDL_2$ by the liver (10-13). High levels of triglyceride rich $VLDL_1$ have been suggested to give rise to a preponderance of small dense LDL, which appears – rather than large more buoyant LDL – a risk factor for coronary heart disease (9). Since $VLDL_1$ carries almost half of the total amount of triglycerides in the circulation, whereas $VLDL_2$ carries only about one fifth, we used a modelling approach to quantify the kinetic properties of $VLDL_1$ apolipoprotein B metabolism (14). In addition, we determined the effects of cafestol on $VLDL_1$ and $VLDL_2$ composition. Our hypothesis was that an increased secretion of $VLDL_1$ apolipoprotein B into the circulation - i.e. an increased production rate - or a

decreased clearance of $VLDL_1$ apolipoprotein B from the circulation - i.e. a decreased fractional catabolic rate - might cause an increased amount of $VLDL_1$ particles in the circulation, either or not enriched with triglycerides, explaining the rise in plasma triglycerides upon consumption of cafestol.

Subjects & methods

Prior approval for this study was obtained from the Research Ethics Committee of the Glasgow Royal Infirmary, University N.H.S. Trust, and the Committee on Experimental Research in Humans of the Nijmegen University Hospital.

Subjects

Fifty-two men entered a medical screening. All had normal hepatic and renal function, and none had haematological abnormalities. No subject was taking any medication known to affect lipid metabolism. Eight healthy normolipidaemic men with plasma triglyceride levels between 0.9 and 2.0 mmol/L were selected to participate in the study. They had a mean (±SEM) age of 21±1 years and a mean (±SEM) body mass index of 22.30±0.65 kg/m^2. Three volunteers had the apolipoprotein E phenotype 4/3, three the apolipoprotein E phenotype 3/3, and two the apolipoprotein E phenotype 3/2. The first two subjects were studied at the Department of Pathological Biochemistry, Glasgow Royal Infirmary, Scotland. The remaining 6 subjects were studied at the Department of Internal Medicine, Nijmegen Academic Hospital, The Netherlands. The subjects were well informed about the purpose and protocol of this experiment. They gave their written informed consent before the start of the study.

Preparation of the cafestol supplements and the tracer

Cafestol esters were hydrolysed and extracted from coffee oil and then re-esterified with palmitate in the laboratory of the Department of Human Nutrition and Epidemiology, Wageningen Agricultural University, The Netherlands. It had a purity of 85%; the impurities consisted of free cafestol, cafestol dipalmitate and palmitic acid. Cafestol palmitate was dissolved in a mixture of sunflower oil and palm oil (3:2 by mass). Earlier studies have shown that this mixture of sunflower oil and palm oil has no effect on plasma cholesterol levels. The daily amount of cafestol provided in this study, i.e. 75 mg cafestol (= 131.5 mg cafestol palmitate) was divided over two capsules in order to spread the cafestol uptake over a day.

D_3-leucine (L-[5,5,5-$^2H_3$]leucine) with a purity higher than 99% was purchased from Cambridge Isotope Laboratory (Woburn, MA). It was dissolved in 0.9% saline to a concentration of 10 mg/mL, sterilised and checked for pyrogens.

Study design

Plasma lipids, $VLDL_1$ and $VLDL_2$ apolipoprotein B, the production and fractional catabolic rates of $VLDL_1$ apolipoprotein B, and the composition of $VLDL_1$ and $VLDL_2$ were measured after a cafestol-free baseline period and directly after two weeks of treatment with cafestol. We first performed a VLDL turnover study to obtain baseline values. For this, subjects were fasted for 12 h overnight. At approximately 8 a.m. the tracer d_3-leucine was administered as an intravenous bolus injection (7.0 mg/kg of body weight). We took plasma samples immediately before tracer injection and at multiple time points throughout the following 48 h: at 2, 5, 10, 15, 20, 30, 40, 60, 80, 120 min; at 3, 4, 6, 8, 10, 11, 14, 24, 36 and 48 h. A total amount of 240 mL of blood was sampled per VLDL turnover study. To keep the chylomicron production minimal during the initial 12-h phase of the turnover, participants were fasted until 6 p.m. and were then permitted a light fat-free meal. Blood samples taken after 24 and 48 h were also taken in the fasting state. At least two weeks after the first turnover, subjects swallowed two capsules with 37.5 mg of cafestol each per day for a period of two weeks. On the last two days of this 2-week treatment-period, a second VLDL turnover study was performed as described above. The 16 VLDL turnover studies were done over a period of nine months. We always performed two turnovers on the same occasion; a VLDL turnover after a cafestol-free baseline period in one subject was combined with a VLDL turnover after treatment with cafestol in a second subject. This will reduce the impact of drifts in the variables of interest with time.

The subjects did not drink any other coffee than filtered coffee for at least two months prior to the start of the experiment. In addition, subjects were not allowed to drink any other coffee brews than drip filter coffee, percolated coffee or instant coffee throughout the study. During the whole experimental period, subjects were asked to maintain their usual pattern of diet and physical activity. Alcohol consumption was restricted to a maximum of two units per day. The subjects were asked to record any deviation from the usual diet and physical activity in a special diary. In addition, subjects were asked to record the time of consuming the capsules with cafestol, the daily amount of alcoholic intake, signs of illness, and medications used.

Plasma lipids and apolipoprotein E phenotype

We determined plasma cholesterol and triglycerides by enzymatic methods (CHOD-PAP and GPO-PAP, respectively, Boehringer-Mannheim, Mannheim, Germany, cat no 237574 and no 701912). HDL cholesterol was determined by a precipitation method using phosphotungstic/Mg^{2+} (15). LDL cholesterol was calculated as described by Friedewald (16). We isolated total VLDL by ultracentrifugation of plasma for 16 hours at 36,000 rpm (168,000 g) using the TFT 45.6 fixed angle rotor (Kontron, Zürich, Switzerland), in a Beckman L7-55 ultracentrifuge (Beckman, Palo Alto, California) (17). In this VLDL fraction we

analysed total cholesterol and triglycerides as described above. Apolipoprotein E phenotypes were determined after iso-electric focusing of VLDL apolipoproteins, as described previously (18). Results were periodically checked with apolipoprotein E genotyping according to Hixson et al (19).

Lipoprotein composition and apolipoprotein B pool size

We isolated $VLDL_1$ and $VLDL_2$ from 2 mL of plasma by cumulative flotation gradient ultracentrifugation using a six-step discontinuous salt gradient (20;21). The lipid composition of the two VLDL subfractions was determined using the methods mentioned above for total cholesterol and triglyceride determination. We used enzymatic reagents for the determination of free cholesterol and phospholipids (Boehringer cat. no. 310328 and 691844, respectively). The amount of cholesteryl esters was subsequently calculated as the difference between total and free cholesterol. To correct for losses of lipids and protein during ultracentrifugation, we multiplied the measured content of lipids and protein in both VLDL subfractions with the ratio VLDL triglycerides/($VLDL_1$+$VLDL_2$) triglycerides. The mean recovery (±SEM) of $VLDL_1$+$VLDL_2$ triglycerides compared to VLDL triglycerides was 70±6% after the cafestol-free baseline period and 64±9% after treatment with cafestol.

We precipitated apolipoprotein B from both VLDL subfractions by addition of an equal volume of isopropanol (22). We determined the apolipoprotein B content of $VLDL_1$ and $VLDL_2$ as the difference between total and isopropanol soluble protein (20;22;23). To correct for losses of apolipoprotein B during ultracentrifugation, we multiplied the measured apolipoprotein B content with the ratio VLDL triglycerides/($VLDL_1$+$VLDL_2$) triglycerides as described above. $VLDL_1$ and $VLDL_2$ apolipoprotein B pool sizes were calculated as the product of plasma volume (assumed to be 4% of the body weight) and the plasma concentration of apolipoprotein B in $VLDL_1$ and $VLDL_2$. The leucine content of the apolipoprotein B pools was assumed to be 12.12% of apolipoprotein B (24).

D_3-leucine enrichment in apolipoprotein B

The precipitated apolipoprotein B from the $VLDL_1$ fraction was delipidated with ethanol-ether (3:1) and dried with ether. We subsequently hydrolysed apolipoprotein B with 1.0 mL 6 N HCl at 110°C for 24 h (20).

From plasma, we precipitated proteins with trichloroacetic acid as described (14). We determined the enrichment of leucine with d_3 -leucine (i.e. the tracer/tracee ratio's) in apolipoprotein B hydrolysates and in plasma free amino acids by gas chromatography-mass spectrometry (GC-MS) (14). The maximal level of enrichment of leucine with d_3 leucine is approximately similar in all turnovers. The mean tracer/tracee curves are shown in figure 1 (upper panel). Tracer mass was calculated by multiplying the tracer/tracee ratio's with the tracee mass (figure 1, lower panel).

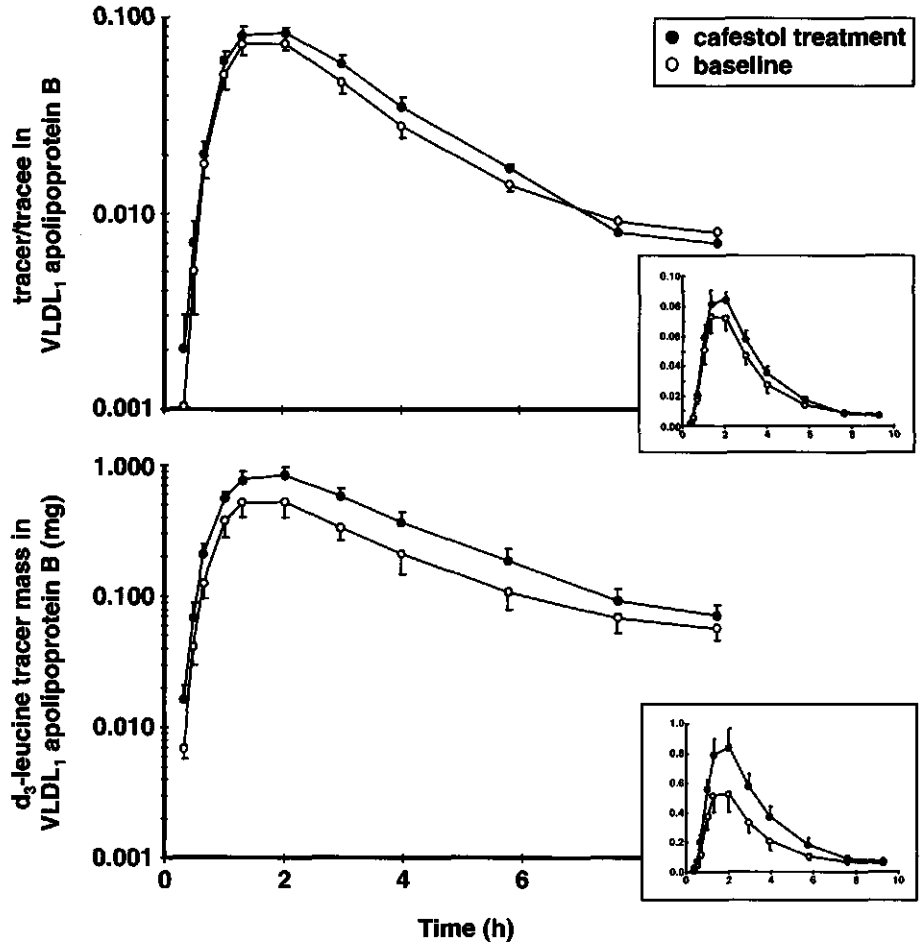

Figure 1. Mean tracer/tracee in $VLDL_1$ apolipoprotein B (upper panel) and incorporation of d_3-leucine mass into $VLDL_1$ apolipoprotein B (lower panel) after a baseline period with no cafestol treatment (○) or after a 2-week treatment period with cafestol (●) in eight normolipidaemic men. D_3-leucine was given as a bolus infusion at time zero. Tracer mass was calculated by multiplying the tracer/tracee ratio (as determined by a GC-MS method) with the tracee mass. Bars indicate SEM. In the inserts, the tracer/tracee and tracer mass are depicted on a linear scale.

Kinetic analysis and multicompartmental modelling

We adapted a model (figure 2) which was developed and described previously (14). The first four compartments explain the kinetics of plasma leucine. D_3-leucine is injected into the plasma compartment (compartment 1). An intracellular pool in the liver (compartment 2) is the immediate precursor of the d_3-leucine. Compartments 3 and 4 represent body protein with a slow turnover. Input of leucine into $VLDL_1$ apolipoprotein B occurs from compartment 2 via a delay compartment (compartment

5). The delay indicates the time between the injection of d_3-leucine in the plasma compartment and its appearance in $VLDL_1$ apolipoprotein B. The delay was set at 0.5 h initially but was adjusted between 0.4 and 0.6 h when required to obtain a better fit of the curves. Input of apolipoprotein B into $VLDL_1$ occurred at compartment 6. Compartments 6 and 7 represent a delipidation chain. Compartment 8 represents a remnant particle that does not undergo further delipidation but is eventually eliminated from the plasma.

Before fitting this model to the data, we introduced parameter dependencies to prevent that the number of unknown parameters (transfer rate constants) exceeded the number of equations. For the plasma kinetics, the transfer rate constants $k_{1,2}$ and $k_{2,1}$ were both fixed at 2.5, and the value for $k_{3,4}$ was set at $0.1*k_{4,3}$. These values have been determined in previous studies on long term data in a large group of subjects (14). We assumed that the delipidation rate from compartments in $VLDL_1$ was similar. Therefore, the delipidation rate constants $k_{7,6}$ and $k_{0,7}$ were made equal. These parameter dependencies were applied in all 16 turnover studies.

The measured data, i.e. the tracer/tracee ratios and the $VLDL_1$ apolipoprotein B leucine masses, were analysed with the SAAMII program (SAAM institute, University of Washington, Seattle, WA). This program derives the value of the kinetic rate constants that produce the best fit between the calculated and the measured tracer/tracee ratios. Initially, the tracer/tracee ratio's were assigned a standard deviation that was related to the precision of their measurement by GC-MS. Previous studies have shown that GC-MS analysis of the tracer/tracee values show a coefficient of variation (or relative SD) of about 1% (25). Thus, we assumed that the SD at the peak value of the tracer/tracee curve, which in our study was typically between 0.05 and 0.1, equalled 1% of that peak height. SAAMII subsequently calculated a weight for each tracer/tracee ratio, which is the reciprocal of the square of the standard deviation assigned to these data. During the fitting process, peak values of tracer/tracee were assigned a lower SD, thus more weight, when this would lead to a better fit of the model to the data. The weights were adjusted until there was no further improvement in the sum of squares. Eventually, we applied a mean standard deviation of approximately $3x10^{-5}$ to the peak ratio's of $VLDL_1$ apolipoprotein B leucine, which represented a coefficient of variance of about 0.1%. The derived kinetic constants and the transport rates were considered acceptable when the calculated curves fitted the observed data without systematic error and when the calculated masses for $VLDL_1$ apolipoprotein B were within 10% of the measured apolipoprotein B pool sizes.

The $VLDL_1$ apolipoprotein B production rate (in mg $VLDL_1$ apolipoprotein B/day) was calculated from the output from compartment 2 (i.e. $k_{5,2}$ * the mass of leucine in compartment 2). The $VLDL_1$ apolipoprotein B fractional catabolic rate (in pools/day) was calculated by dividing the sum of outputs from compartments 7 and 8 (i.e. $k_{0,7}$ *

mass of leucine in compartment 7 and $k_{0,8}$ * mass of leucine in compartment 8; both expressed in mg leucine/day) by the $VLDL_1$ apolipoprotein B mass (i.e. the combined masses of leucine in compartments 6, 7 and 8).

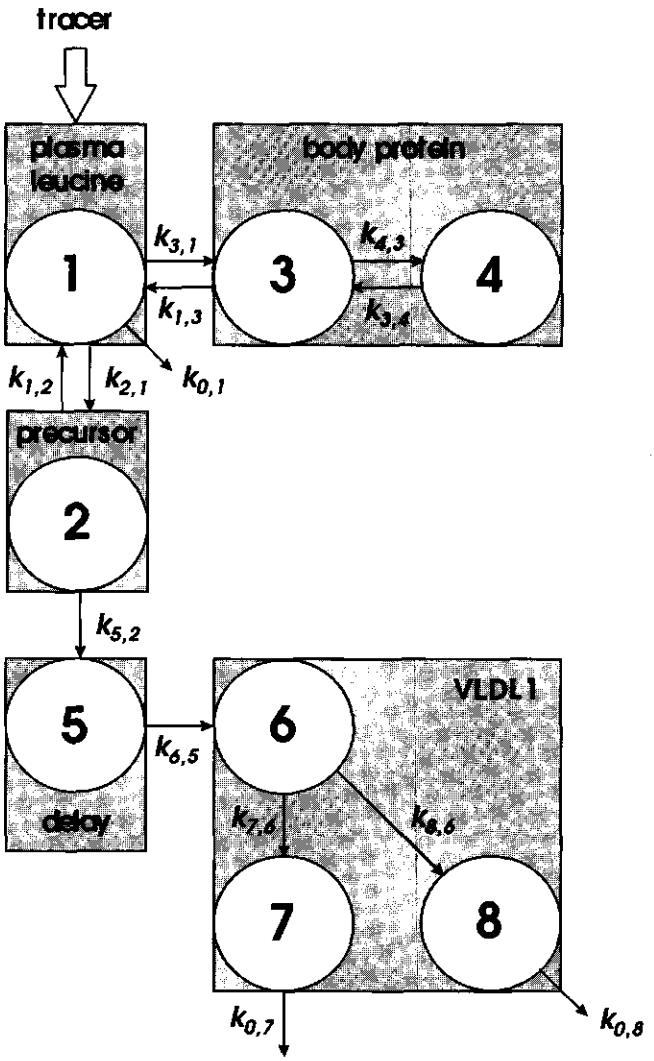

*Figure 2. Multicompartmental model for apolipoprotein B metabolism. Bolus infusion data were analysed with the same model in all 8 subjects. Plasma leucine (compartment 1) receives the d_3-leucine tracer and distributes it to the body protein pools (compartment 3 and 4) and to the intracellular precursor pool for apolipoprotein B synthesis (compartment 2). After the delay (compartment 5), the tracer appears in $VLDL_1$ via compartment 6, and then throughout delipidation in compartment 7, or throughout remnant formation in compartment 8. Parameter dependencies were k(2,1)=k(1,2)=2.5; k(3,4)=0.1*k(4,3); k(7,6)=k(0,7).*

Statistical analysis

All data are expressed as mean±SEM. We tested whether cafestol increases the $VLDL_1$ apolipoprotein B production rate or decreases the $VLDL_1$ apolipoprotein B fractional catabolic rate with a paired two-tailed Student's t-test. In addition, we tested whether cafestol affects the cholesteryl ester/triglyceride ratio of $VLDL_1$ and $VLDL_2$ with a paired two-tailed Student's t-test. The variables $VLDL_1$ apolipoprotein B mass and $VLDL_1$ apolipoprotein B production had a skewed distribution and were therefore logarithmically transformed before statistical comparisons.

Results

Plasma triglycerides and composition of $VLDL_1$ and $VLDL_2$

Cafestol treatment for two weeks increased average total triglycerides by 31% (table 1). $VLDL_1$ triglycerides were increased by 57%, whereas $VLDL_2$ triglycerides were increased by only 11%. About 72% of the rise in plasma triglycerides was due to a rise in $VLDL_1$ triglycerides, and an additional 7% could be explained by a rise in $VLDL_2$ triglycerides.

Table 1. Mean plasma lipids before and after a daily dose of 75 mg of cafestol for two weeks in eight healthy normolipidaemic men.

	Baseline	Cafestol treatment	Treatment effect (95% CI)
Plasma lipids (mmol/L)			
Triglycerides	1.05	1.37	0.32 (0.03 to 0.61)
VLDL triglycerides	0.61	0.86	0.25 (-0.15 to 0.65)
$VLDL_1$ triglycerides	0.40	0.63	0.23 (-0.02 to 0.48)
$VLDL_2$ triglycerides	0.21	0.24	0.02 (-0.04 to 0.08)
Total cholesterol	4.08	4.23	0.15 (-0.06 to 0.36)
VLDL cholesterol	0.40	0.50	0.10 (-0.07 to 0.27)
LDL cholesterol	2.53	2.62	0.09 (-0.16 to 0.34)
HDL cholesterol	1.16	1.14	-0.02 (-0.11 to 0.07)

Cafestol increased the $VLDL_1$ apolipoprotein B pool size by 59% (figure 3). This increase was of the same magnitude as the increase in $VLDL_1$ triglycerides, suggesting that the composition of $VLDL_1$ was not altered (table 2). Indeed, the $VLDL_1$ cholesteryl ester/triglyceride ratio - an index of particle composition - was not significantly changed after cafestol treatment (mean increase of 0.02; 95% CI [-0.09; 0.05]).

Cafestol increased the $VLDL_2$ apolipoprotein B pool size by 31% or 32 mg (95%CI [-27;90]). This increase was larger than the increase in $VLDL_2$ triglycerides, suggesting that $VLDL_2$ was depleted in triglycerides. Indeed, the percentage cholesteryl ester in $VLDL_2$ was increased (table 2), which was reflected by an

increased $VLDL_2$ cholesteryl esters/triglycerides ratio (mean increase of 0.12; 95% CI [0.07;0.16]).

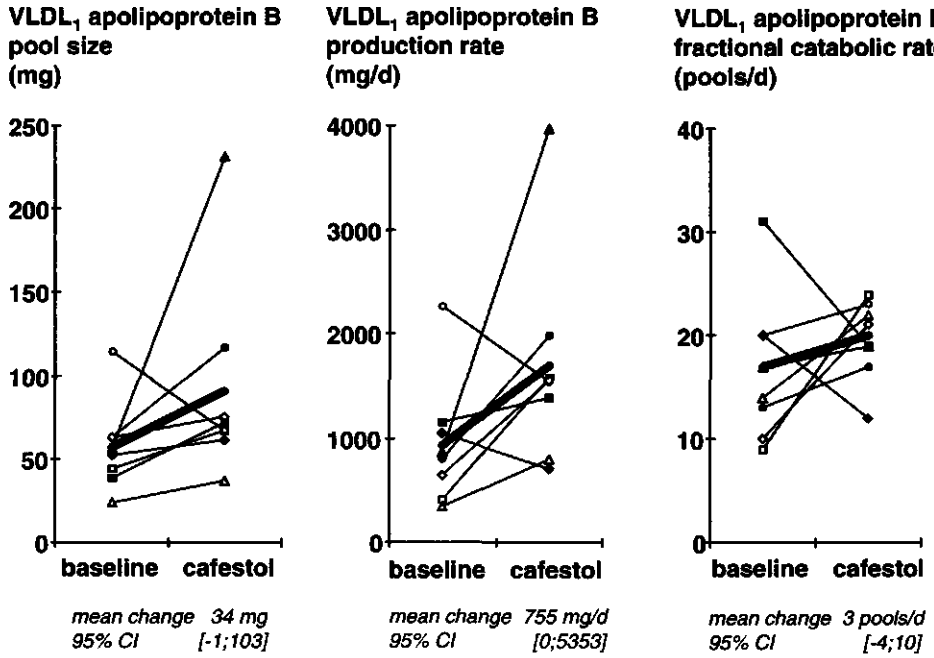

Figure 3. Individual changes in the pool size, the production rate and the fractional catabolic rate of $VLDL_1$ apolipoprotein B after cafestol treatment for two weeks. The heavy line indicates the mean change.

Production and fractional catabolic rate of $VLDL_1$ apolipoprotein B

Inspection of the tracer mass curves by eye revealed that cafestol treatment induced more tracer mass in $VLDL_1$ apolipoprotein B due to a more rapid appearance (i.e. a higher production rate), whereas this treatment induced only a minor increase in the clearance of the tracer from the circulation (figure 1, lower panel). This suggested that cafestol increased the amount of $VLDL_1$ apolipoprotein B mass, and thus the amount of $VLDL_1$ particles, mainly because of an increased production rate of $VLDL_1$ apolipoprotein B.

Multicompartmental modelling of the tracer/tracee data revealed that cafestol treatment elevated $VLDL_1$ apolipoprotein B (figure 1, lower panel) by significantly increasing the mean production rate of $VLDL_1$ apolipoprotein B by 80% (figure 3). Cafestol non-significantly increased the mean fractional catabolic rate of $VLDL_1$ apolipoprotein B by 17% (figure 3).

Table 2. Effect of a daily dose of 75 mg of cafestol for two weeks on the composition of $VLDL_1$ and $VLDL_2$ in eight healthy men[†].

	Baseline		Cafestol treatment		Treatment effect	
	(mg/dL plasma)	*(% of total mass)*	*(mg/dL plasma)*	*(% of total mass)*	*(mg/dL plasma)*	*(mg/mg apolipoprotein B)*
$VLDL_1$						
triglycerides	35.8 ± 12.9	67	56.3 ± 28.5	66	20.5 ± 11.2	-1.2 ± 1.8
free cholesterol	2.4 ± 2.1	5	4.2 ± 2.0	5	1.8 ± 0.9	0.5 ± 0.5
cholesteryl esters	4.1 ± 3.0	8	6.4 ± 4.2	8	2.4 ± 1.5	-0.7 ± 0.8
phospholipids	6.2 ± 3.5	12	10.2 ± 4.4	12	4.0 ± 1.6	0.2 ± 0.5
protein	5.0 ± 2.2	9	8.1 ± 4.5	10	3.2 ± 1.9	
$VLDL_2$						
triglycerides	19.0 ± 6.1	47	21.0 ± 7.3	42	2.0 ± 2.9	-1.1 ± 0.6
free cholesterol	2.9 ± 1.3	7	3.9 ± 1.6	8	0.9 ± 0.5	0.0 ± 0.1
cholesteryl esters	6.2 ± 4.1	15	9.5 ± 5.1	19	3.3 ± 1.1	0.3 ± 0.2
phospholipids	7.0 ± 2.2	17	9.4 ± 4.6	19	2.5 ± 1.4	-0.0 ± 0.3
protein	5.4 ± 2.5	13	6.6 ± 2.4	13	1.2 ± 1.3	

[†] Data are expressed as mean±SEM (n=8 subjects).

Discussion

In this study we showed that in healthy normolipidaemic subjects, cafestol increased plasma triglycerides mainly by increasing the production rate of $VLDL_1$ apolipoprotein B. This resulted in an increased amount of $VLDL_1$ particles in the circulation. Cafestol did not change the composition of $VLDL_1$. $VLDL_2$ became enriched with cholesteryl esters at the cost of triglycerides.

The secretion of apolipoprotein B-containing lipoproteins appears to be regulated primarily at a post-translational level, since mRNA levels of apolipoprotein B did not change in many situations in which the secretion of apolipoprotein B from cultured liver cells was altered over a wide range. The amount of VLDL apolipoprotein B reaching the circulation is therefore largely determined by the proportion that escapes degradation after the synthesis of the protein (26). Substances like oleate or insulin, which are known to affect the secretion of VLDL apolipoprotein B without altering its levels of mRNA (27-29), might do this via modulation of $VLDL_1$ assembly in the liver, which in turn affects the rate at which $VLDL_1$ apolipoprotein B is secreted into the circulation. Therefore, cafestol might increase the rate at which $VLDL_1$ apolipoprotein B is secreted into the circulation by increasing the assembly of apolipoprotein B with lipids inside liver cells.

The assembly of apolipoprotein B-containing lipoproteins by the liver is suggested to be a 2-step process (figure 4). To initiate lipoprotein assembly, the N-terminal portion of apolipoprotein B is thought to be translocated into the lumen of the rough endoplasmic reticulum, where it receives a small amount of lipid (whether this is cholesteryl ester or triglyceride is still a matter of debate) via the action of microsomal triglyceride transfer protein (MTP) (30;31). If this addition of lipid does not occur, or if MTP is absent (32), apolipoprotein B may be degraded. Thus MTP appears to control the number of apolipoprotein B lipoprotein particles secreted by the liver and thereby the apparent production rate of $VLDL_1$ apolipoprotein B. Therefore, up-regulation of MTP activity is one possible explanation for the increased secretion of $VLDL_1$ apolipoprotein B upon consumption of cafestol (figure 4).

In the second step of VLDL assembly, the primordial lipoprotein coalesces with a large apolipoprotein B-free droplet of triglycerides at the junction of rough and smooth endoplasmic reticulum to form large triglyceride-rich VLDL (figure 4) (30;31;33). This second step depends on the availability of hepatic triglycerides (34). An alternative explanation for the action of cafestol is therefore that it increases the availability of triglycerides in the liver by increasing the synthesis of triglycerides or by decreasing the rate of β-oxidation of fatty acids. The rate of β-oxidation of fatty acids is modulated by peroxisome proliferator activated receptor α (PPARα) - activity, which is however not affected by cafestol (35). Therefore, the two most likely mechanisms for the effect of cafestol on $VLDL_1$ apolipoprotein B production are that

cafestol increases MTP-action or that it increases the synthesis of triglycerides in the liver. Both might lead to apolipoprotein B being used for the production of VLDL$_1$ instead of being broken down.

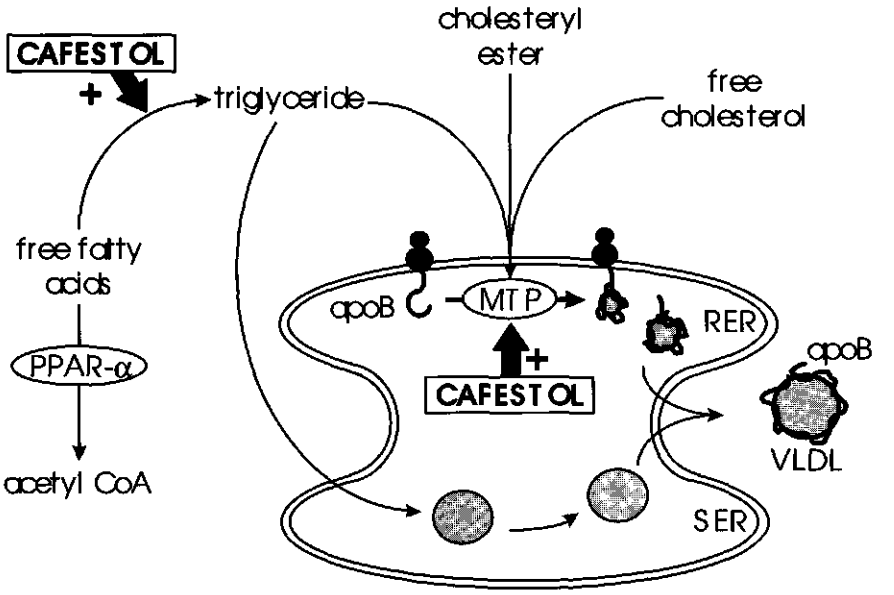

Figure 4. Possible action of cafestol on the assembly of VLDL$_1$ in the liver. Cafestol might increase the secretion of VLDL apolipoprotein B by increasing the assembly of VLDL$_1$. This effect is possibly established by an increased action of MTP and/or by an increased synthesis of triglycerides in the liver cell. ApoB: apolipoprotein B; PPARα: peroxisome proliferator activated receptor α; acetyl CoA: acetyl coenzyme A; MTP: microsomal triglyceride transfer protein; RER: rough endoplasmic reticulum; SER: smooth endoplasmic reticulum.

Cafestol did not change the composition of VLDL$_1$, but VLDL$_2$ became enriched with cholesteryl esters at the cost of triglycerides. This was reflected in a significant increase in the VLDL$_2$ cholesteryl ester/triglyceride ratio. Recently, a changed VLDL composition upon cafestol treatment has also been found in apolipoprotein E*3-Leiden transgenic mice (36). VLDL particles became enriched with cholesteryl esters, suggestive of the secretion of a βVLDL-like particle. In this transgenic mouse strain, cafestol significantly suppressed bile acid synthesis (36). We hypothesised that the cholesterol which comes available in the liver due to inhibition of bile acid synthesis might be directly removed via secretion into VLDL particles. Availability of intrahepatic cholesterol might therefore be an important regulator of the secretion of VLDL$_2$ apolipoprotein B.

In conclusion, our data indicate that cafestol raises plasma triglycerides predominantly by an increased production rate of VLDL$_1$ apolipoprotein B after two

weeks of intervention. This results in a higher amount of $VLDL_1$ particles in the circulation. It appears unlikely that this higher amount of $VLDL_1$ particles is mainly responsible for the subsequent rise in LDL cholesterol. Evidence from kinetic studies indicates that only a small part of $VLDL_1$ is delipidated to LDL (37;38), whereas $VLDL_2$ particles are rapidly and efficiently converted to LDL (37). Therefore, cholesteryl ester-enriched $VLDL_2$ particles appear a more likely candidate to play a role in the subsequent rise of LDL cholesterol levels. In addition, *in vitro* studies suggest that cafestol elevates plasma LDL cholesterol levels at least partly by down-regulation of the LDL receptor (39-41). This finding was recently confirmed *in vivo* in apolipoprotein E*3-Leiden transgenic mice (36). Down-regulation of the LDL receptor appears to be initiated by a suppression of bile acid synthesis (36;41). The action of cafestol on plasma triglycerides and plasma cholesterol might thus be regulated independently in the liver.

Acknowledgements

We would like to thank the Netherlands Heart Foundation (grant no. 900.562.091 through the Netherlands Organization of Scientific Research) and the Foundation for Nutrition and Health Research for funding, the volunteers for participating in this study, Til Terburg, Jacqueline de Graaf, Helga Toenhake, Heidi Hak, Magda P. Hectors, Pamela Doherty and Grace Stewart for skillful technical assistance, and John Millar for his advice on multicompartmental modelling.

References

1. Urgert R, Katan MB. The cholesterol-raising factor from coffee beans. Annu. Rev. Nutr 1997;17:305-324.
2. Urgert R, Essed N, van der Weg G, Kosmeijer-Schuil TG, Katan MB. Separate effects of the coffee diterpenes cafestol and kahweol on serum lipids and liver aminotransferases. Am. J Clin. Nutr. 1997;65:519-524.
3. Urgert R, van der Weg G, Kosmeijer-Schuil TG, van de Bovenkamp P, Hovenier R, Katan MB. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. J Agric. Food Chem. 1995;43:2167-2172.
4. van Dusseldorp M, Katan MB, van Vliet T, Demacker PN, Stalenhoef AF. Cholesterol-raising factor from boiled coffee does not pass a paper filter. Arterioscler. Thromb. 1991;11:586-593.
5. Urgert R, Meyboom S, Kuilman M, et al. Comparison of effect of cafetiere and filtered coffee on serum concentrations of liver aminotransferases and lipids: six month randomised controlled trial. BMJ. 1996;313:1362-1366.
6. Weusten-van der Wouw MPME, Katan MB, Viani R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res. 1994;35:721-733.
7. Stensvold I, Tverdal A, Foss OP. The effect of coffee on blood lipids and blood pressure. Results from a Norwegian cross-sectional study, men and women, 40-42 years. J Clin. Epidemiol. 1989;42:877-884.
8. Pietinen P, Aro A, Tuomilehto J, Uusitalo U, Korhonen H. Consumption of boiled coffee is correlated with serum cholesterol in Finland. Int. J Epidemiol. 1990;19:586-590.
9. Packard CJ, Shepherd J. Lipoprotein heterogeneity and apolipoprotein B metabolism. Arterioscler. Thromb. Vasc. Biol. 1997;17:3542-3556.
10. Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnikar V, Sacks FM. Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. N. Engl. J Med. 1991;325:1196-1204.

11. Gaw A, Packard CJ, Lindsay GM, et al. Overproduction of small very low density lipoproteins (Sf 20-60) in moderate hypercholesterolemia: relationships between apolipoprotein B kinetics and plasma lipoproteins. J Lipid Res. 1995;36:158-171.
12. Malmstrom R, Packard CJ, Caslake M, et al. Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM. Diabetologia 1997;40:454-462.
13. Malmstrom R, Packard CJ, Watson TD, et al. Metabolic basis of hypotriglyceridemic effects of insulin in normal men. Arterioscler. Thromb. Vasc. Biol. 1997;17:1454-1464.
14. Demant T, Packard CJ, Demmelmair H, et al. Sensitive methods to study human apolipoprotein B metabolism using stable isotope-labeled amino acids. Am. J. Physiol. 1996;270:E1022-E1036
15. Demacker PN, Hessels M, Toenhake DH, Baadenhuijsen H. Precipitation methods for high-density lipoprotein cholesterol measurement compared, and final evaluation under routine operating conditions of a method with a low sample-to-reagent ratio. Clin. Chem. 1997;43:663-668.
16. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin. Chem. 1972;18:499-502.
17. Demacker PN, Vos JH, Jansen AP, van-'t Laar A. Evaluation of the dual-precipitation method by comparison with the ultracentrifugation method for measurement of lipoproteins in serum. Clin. Chem. 1977;23:1238-1244.
18. Weidman SW, Suarez B, Falko JM, et al. Type III hyperlipoproteinemia: development of a VLDL ApoE gel isoelectric focusing technique and application in family studies. J Lab. Clin. Med. 1979;93:549-569.
19. Hixson JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with Hhal. J Lipid Res. 1990;31:545-548.
20. Demant T, Bedford D, Packard CJ, Shepherd J. Influence of apolipoprotein E polymorphism on apolipoprotein B-100 metabolism in normolipidemic subjects. J Clin. Invest. 1991;88:1490-1501.
21. Lindgren FT, Jensen LC, Wills RD, Freeman NK. Flotation rates, molecular weights and hydrated densities of the low density lipoproteins. Lipids 1969;4:337-344.
22. Egusa G, Brady DW, Grundy SM, Howard BV. Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein B. J Lipid Res. 1983;24:1261-1267.
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol. Chem. 1951;193:265-275.
24. Hardman DA, Kane JP. Isolation and characterisation of apolipoprotein B-48. In: Segrest JP, Albers JJ, eds. Methods and Enzymology. Plasma Lipoproteins (Part A): Preparation, Structure, and Molecular Biology. Orlando, FL: Academic Press, 1986:262-272.
25. Demant T, Packard CJ, Stewart P, et al. A sensitive mass spectrometry technique for the measurement of human apolipoprotein B metabolism in-vivo. Clin. Chem. 1994;40:1825-1827.
26. Ginsberg HN. Synthesis and secretion of apolipoprotein B from cultured liver cells. Curr. Opin. Lipidol. 1995;6:275-280.
27. Pullinger CR, North JD, Teng BB, Rifici VA, Ronhild de Brito AE, Scott J. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of mRNA halflife. J Lipid Res. 1989;30:1065-1076.
28. Moberly JB, Cole TG, Alpers DH, Schonfeld G. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco2 cells occurs post-transcriptionally. Biochim. Biophys. Acta 1990;1042:70-80.
29. Dashti N, Williams DL, Alaupovic P. Effects of oleate and insulin on the production rates and cellular mRNA concentrations of apolipoproteins in HepG2 cells. J Lipid Res. 1989;30:1365-1373.
30. Pease RJ, Leiper JM. Regulation of hepatic apolipoprotein B-containing lipoprotein secretion. Curr. Opin. Lipidol. 1996;7:132-138.
31. Gordon DA. Recent advances in elucidating the role of the microsomal triglyceride transfer protein in apolipoprotein B lipoprotein assembly. Curr. Opin. Lipidol. 1997;8:131-137.
32. Wetterau JR, Aggerbeck LP, Bouma ME, et al. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 1992;258:999-1001.
33. Boren J, Rustaeus S, Wettesten M, Andersson M, Wiklund A, Olofsson SO. Influence of triacylglycerol biosynthesis rate on the assembly of apoB-100-containing lipoproteins in Hep G2 cells. Arterioscler. Thromb. 1993;13:1743-1754.

34. Gordon DA, Jamil H, Gregg RE, Olofsson SO, Boren J. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. J Biol. Chem. 1996;271:33047-33053.
35. de Roos B, Katan MB. Possible mechanisms underlying the cholesterol-raising effet of the coffee diterpene cafestol. Curr. Opin. Lipidol. 1999;10:41-45.
36. de Roos B, Post SM, Vermeulen M, et al. The coffee diterpene cafestol decreases bile acid synthesis by down-regulation of cholesterol 7a-hydroxylase in apolipoprotein E*3-Leiden mice. Atherosclerosis 1999;144:95
37. Packard CJ, Munro A, Lorimer AR, Gotto AM, Shepherd J. Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects. J Clin. Invest. 1984;74:2178-2192.
38. Stalenhoef AF, Malloy MJ, Kane JP, Havel RJ. Metabolism of apolipoproteins B48 and B100 of triglyceride rich lipoporteins in normal an lipoprotein lipase deficient humans. Proc. Natl. Acad. Sci. USA 1984;81:1839-1843.
39. Halvorsen B, Ranheim T, Nenseter MS, Huggett AC, Drevon CA. Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts. J Lipid Res. 1998;39:901-912.
40. Rustan AC, Halvorsen B, Huggett AC, Ranheim T, Drevon CA. Effect of coffee lipids (cafestol and kahweol) on regulation of cholesterol metabolism in HepG2 cells. Arterioscl. Thromb. Vasc. Biol. 1997;17:2140-2149.
41. Post SM, de Wit EC, Princen HMG. Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase in rat hepatocytes. Arterioscler. Thromb. Vasc. Biol. 1997;17:3064-3070.

7

General discussion

The main objective of this research was to study the mechanism of action of coffee diterpenes in humans. The general introduction discussed a number of mechanisms that could explain the effect of cafestol on lipoproteins. We hypothesised that the most plausible mechanism by which cafestol elevates cholesterol in humans appears to be via the sterol regulatory element binding proteins (SREBP-) pathway (1). In this chapter we will discuss that the SREBP-pathway is probably not the only pathway involved in the mechanism of action of coffee diterpenes.

Main outcome from mechanistic studies

Modulation of bile acid metabolism

In apolipoprotein E*3-Leiden transgenic mice, cafestol increased serum cholesterol concentrations primarily by suppressing the synthesis of bile acids. Cafestol decreased the expression and activity of cholesterol 7α-hydroxylase, and the amounts of primary and secondary bile acids in faeces were reduced after 3 weeks of intervention (chapter 4).

Is cafestol able to alter bile acid metabolism in humans similarly as in apolipoprotein E*3-Leiden mice? Bile acid metabolism in mice is much more easily affected by dietary components than that in humans. Mice, who have a high rate of hepatic cholesterol synthesis (2), primarily respond to dietary cholesterol by down-regulation of hepatic cholesterol synthesis and up-regulation of hepatic bile acid production. As a consequence, plasma lipoprotein concentrations do not increase as easily (3) as in humans, who have a limited ability to activate bile acid synthesis in response to dietary cholesterol intake. There are however dietary compounds known to affect bile acid metabolism in man. Psyllium, a mucilage from the seeds of *Plantago ovata*, increased faecal excretion of bile acids in man (4). Consumption of oat bran increased faecal secretion of bile acids in ileostomy subjects, normo- and hyperlipidaemic men, which may explain the concomitant decrease in LDL cholesterol (5-8). In analogy, suppression of bile acid synthesis may thus provide an explanation for the cholesterol-raising effects of unfiltered coffee in humans.

In healthy volunteers, consumption of cafetiere (French-press) coffee for 2 weeks did not significantly affect the secretion of primary (cholic acid and chenodeoxycholic acid) and secondary (lithocholic acid, iso-lithocholic acid, deoxycholic acid and iso-deoxycholic acid) bile acids in faeces (M. Grubben, unpublished results). However, two weeks of treatment with cafestol may be too short to detect a significant decrease in faecal output of bile acids.

Modulation of the synthesis and composition of VLDL lipoproteins

In healthy male volunteers, intervention with cafestol for two weeks resulted in a significant rise in serum triglycerides. This effect was due to an increased production of $VLDL_1$ apolipoprotein B, whereas the fractional catabolic rate of this apolipoprotein remained unchanged. This resulted in a higher amount of $VLDL_1$ particles in the circulation. The increased production rate of $VLDL_1$ apolipoprotein B might be due to an increased activity of microsomal triglyceride transfer protein (MTP) or an increased synthesis of hepatic triglycerides. Cafestol did not change the composition of $VLDL_1$, but $VLDL_2$ became enriched with cholesteryl esters at the cost of triglycerides (chapter 6).

One determinant of the $VLDL_1$ concentration is the $VLDL_1$ production rate, which has been proposed to be under the influence of hormones. In normoglycaemic subjects, insulin suppressed the production of $VLDL_1$ apolipoprotein B, without any change in the production of $VLDL_2$ (9;10). In post-menopausal women, oestrogen treatment resulted in an increased secretion of both $VLDL_1$ and $VLDL_2$. In addition, oestrogen also increased the fractional catabolic rate of $VLDL_2$ (11). In patients with growth hormone deficiency, VLDL apolipoprotein B secretion was increased as compared to control subjects (12). Cafestol might be directly responsible for the increased production rate of $VLDL_1$ apolipoprotein B, but it might as well exert its action via modulation of hormones.

Across a range of plasma lipids, however, the rate of delipidation (e.g. the mass transfer from $VLDL_1$ into $VLDL_2$) appeared to be the main predictor of plasma triglycerides in a population of healthy subjects (C.J. Packard, unpublished observations). The conversion of $VLDL_1$ to $VLDL_2$ is dependent on lipoprotein lipase (LpL), since the fractional transfer rate from $VLDL_1$ to $VLDL_2$ is reduced by 90% in patients with LpL deficiency (13;14). LpL activity can be modulated by both apolipoprotein C-III as well as apolipoprotein C-II (15-17). However, $VLDL_1$ has two distinct metabolic fates. In addition to being converted to $VLDL_2$, $VLDL_1$ lipoproteins can also be directly catabolised. It has been proposed that remnant receptors, i.e. the LDL receptor-related protein (LRP) or VLDL receptor (18;19), mediate the direct removal of large triglyceride-rich VLDL. It appears unlikely that the LDL receptor is involved in the direct catabolism of $VLDL_1$, since the rate of $VLDL_1$ direct catabolism was similar to normal in patients with homozygous familial hypercholesterolaemia (20). In our experiment, the plasma- and $VLDL_1$ model did not allow us to distinct between direct catabolism (via receptor-mediated processes) and transfer to $VLDL_2$ (via lipolysis). The sum of the two routes, the fractional catabolic rate of $VLDL_1$ apolipoprotein B, did not appear to be altered by cafestol after two weeks of intervention (chapter 6).

As proposed in chapter 6, it appears unlikely that an increase in the concentration of $VLDL_1$ particles upon treatment with cafestol is responsible for the subsequent rise in LDL cholesterol. Evidence from kinetic studies in human subjects indicates that only a small fraction of $VLDL_1$ is delipidated to LDL (13;21), whereas $VLDL_2$ particles are rapidly and efficiently converted to LDL (21). Therefore, cholesteryl ester-enriched $VLDL_2$ particles appear to be a more likely candidate responsible for the subsequent rise of LDL cholesterol levels.

Modulation of cholesterol ester transfer protein (CETP) activity

Previous studies have shown that dietary compounds that lead to hypercholesterolaemia also increase CETP activity in humans (22-26). Cafestol, dietary cholesterol, trans- and saturated fatty acids might increase the transfer of cholesteryl esters by increasing CETP mass as well as by changing the structure of plasma lipoproteins, resulting in better substrates for CETP action. From these experiments, it remained unclear whether elevated levels of CETP and phospholipid transfer protein (PLTP) are either a cause or a consequence of elevated LDL cholesterol levels. We found that cafestol increased CETP activity levels before it increased LDL cholesterol levels (chapter 5). This indicates that an increased CETP activity during consumption of cafetiere coffee might contribute to the rise in LDL cholesterol. However, we also noticed that triglycerides and thus presumably VLDL rose rapidly in response to cafestol. Thus the rise in CETP may itself have been caused by the rise in VLDL cholesterol or triglycerides.

CETP mediates the transfer of cholesteryl esters - synthesised by lecithin:cholesterol acyltransferase (LCAT) - from HDL to the apolipoprotein B-containing lipoproteins LDL and VLDL (27). The role of CETP in neutral lipid exchange and lipoprotein composition is illustrated in humans with a CETP deficiency. CETP deficiency is associated with marked hyper-alphalipoproteinaemia and a two- to sixfold increase in HDL_2. In these patients, HDL lipoproteins are increased in size and enriched in cholesteryl esters (28). In addition, the ratio cholesteryl ester/triglycerides is decreased in VLDL and IDL lipoproteins (29). In human CETP transgenic mice, HDL cholesterol levels and HDL particle size were uniformly reduced in three separate studies (30-32). However, it appears that a change in CETP activity does not necessarily always have to occur with a change in VLDL and IDL/LDL lipoprotein composition. Only in one of these transgenic mice studies, VLDL and IDL lipoproteins were enriched with cholesteryl esters (31). In the other two studies, CETP activity levels did not or only very modestly changed the composition of VLDL and LDL lipoproteins in human CETP transgenic mice (30;32).

In mechanistic studies on drugs or food substances, the role of CETP in a change in lipoprotein composition is difficult to assess. The component of interest might change the composition also via the action of for example LCAT, lipoprotein lipase or

hepatic lipase. Therefore, changes in the chemical composition of lipoproteins can only provide support for a potential mechanism via CETP when more is known on the alternative pathways in the mechanism of action of cafestol. At this moment, however, we can not exclude a role of CETP in the LDL cholesterol-raising action of cafestol.

Action of cafestol on LDL cholesterol and VLDL triglycerides

Most *in vitro* studies suggest that cafestol elevates LDL cholesterol levels by down-regulation of the LDL receptor (33-35). This finding was confirmed *in vivo* in apolipoprotein E*3-Leiden transgenic mice (chapter 4). Therefore, increasing evidence suggests that a down-regulated LDL receptor activity might, at least in part, be responsible for the increased levels of LDL cholesterol. In apolipoprotein E*3-Leiden mice, down-regulation of the LDL receptor was primarily due to a decreased bile acid secretion (figure 1). The SREBP pathway (36) is likely to play an important role in this hypothesised mechanism (figure 1). The cholesterol which becomes available in the liver due to inhibition of bile acid synthesis might be directly removed from the liver as a component of VLDL particles, since we found that VLDL lipoproteins in apolipoprotein E*3-Leiden mice and $VLDL_2$ particles in human subjects became enriched with cholesteryl esters. As proposed in chapter 6, the enrichment of VLDL lipoproteins with cholesteryl esters might also play a role in the rise of LDL cholesterol. However, whether cafestol reduces bile acid synthesis in humans remains to be established first.

The effect of cafestol on plasma triglycerides can be explained by an increased production rate of $VLDL_1$ apolipoprotein B after two weeks of cafestol treatment (chapter 6), at which time the increase in plasma triglycerides is maximal (37). The initial rise in the production rate of $VLDL_1$ apolipoprotein B might be a result of an increased activity of MTP or an increased synthesis of hepatic triglycerides, as discussed in chapter 6. However, the rise in plasma triglycerides upon cafestol treatment is transient, and the liver appears to adapt in a mechanism by which triglycerides eventually return to baseline levels. This could be a result of either an increased activity of LpL or by a decreased secretion of VLDL triglycerides, which might be caused by a depleted store of hepatic triglycerides. We previously suggested that once cafestol increased plasma triglycerides, this effect might be compensated for by an increased LpL activity. This could explain why triglycerides eventually return to baseline after six months of intervention with cafestol (1). However, results from studies in apolipoprotein E*3-Leiden mice give evidence for the alternative hypothesis. Cafestol caused a maximal increase in serum triglycerides after two weeks, after which serum triglycerides rapidly decreased back to baseline. After three weeks of intervention with cafestol, when serum triglycerides were on their way back to baseline levels, the VLDL triglyceride production rate had

decreased by 50%. In addition, we found that hepatic triglycerides had also decreased by 50% (chapter 4). A depleted store of hepatic triglycerides might therefore explain the return of plasma triglycerides to baseline levels.

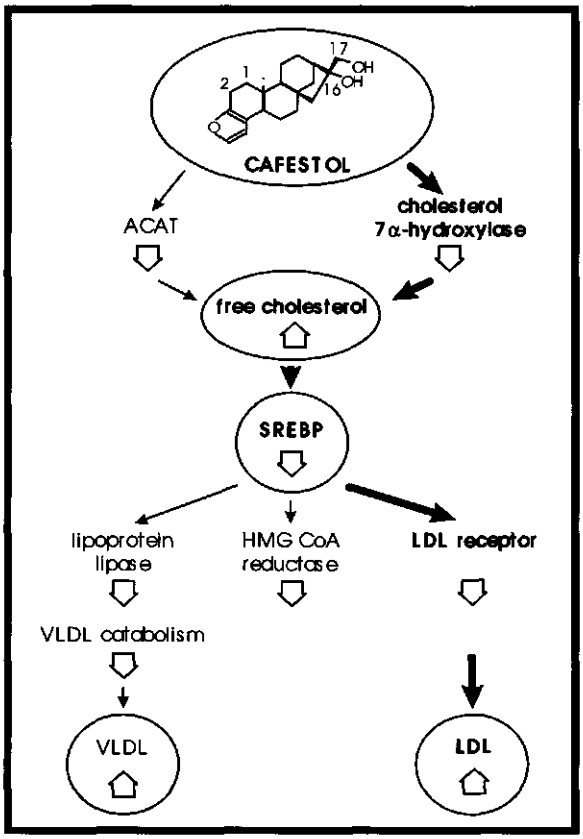

Figure 1. Proposed mechanism for the action of cafestol on LDL cholesterol (thick arrows). ACAT: acyl-coenzyme A:cholesterol acyltransferase; SREBP: sterol regulatory element binding protein; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A.

Validity of animal models

Data from mechanistic studies on cafestol and kahweol in an animal model may provide additional data to support and extend that obtained from human studies. Two animal models were tested on their ability to serve as a good model for the cholesterol-raising properties of coffee diterpenes in humans. A major criterion of a good animal model is that cafestol induces a similar change in lipoprotein profile as compared to humans. However, even if the changes in lipoprotein profile of the animal model and humans are similar, this alone does not offer proof that the mechanisms by which these changes occur are the same.

The African green monkey – an animal species that was previously shown to be a good model to study the mechanisms of dietary cholesterol and fatty acids on lipoprotein metabolism (38-40) - did not appear to be a good model to study the mechanism of action of cafestol and kahweol. The response of plasma cholesterol to coffee oil was much weaker than in humans. Also, unlike humans, the rise in total cholesterol in the monkeys was primarily due to an increase in HDL cholesterol rather than LDL cholesterol.

We also studied the effects of cafestol and kahweol in mice expressing the human apolipoprotein E*3-Leiden gene. Due to the presence of this receptor-defective apolipoprotein E variant, the clearance of remnant lipoproteins is impaired in these mice. Remnant lipoproteins accumulate in the plasma, and these mice easily develop diet-induced hyperlipidaemia and atherosclerosis (41-43). We observed that cafestol significantly raised total cholesterol in these mice species. However, the rise in total cholesterol was predominantly due to a rise in VLDL and IDL lipoproteins, and not in LDL lipoproteins as in humans. Previously, cholesterol feeding also induced an accumulation of predominantly cholesterol- and apolipoprotein E-rich VLDL remnant lipoproteins in apolipoprotein E*3-Leiden mice (42). The distribution of cholesterol among lipoproteins is a major difference between mouse and man. A second difference in lipoprotein metabolism is that the mouse edits apolipoprotein B100 to the truncated apolipoprotein B48 in both the intestine and the liver, whereas in humans apolipoprotein B editing only occurs in the intestine (44;45). Furthermore, a mouse lacks CETP as well as lipoprotein (a) (45), two parameters that are both affected by cafestol treatment (25;46). These differences should be taken into account when results from mice studies are extrapolated to the human situation.

Human subjects remain preferable for studying the action of cafestol and kahweol, but considering the methodological and ethical constraints of mechanistic research in humans, the apolipoprotein E*3-Leiden mouse model provides a satisfactory animal model.

Why are we interested in the action of an atherogenic compound?

Cardiovascular diseases are the leading causes of death in humans in industrialised countries. Elevated levels of plasma total or LDL cholesterol (47-49) and triglycerides (50-52) are important risk factors for the development of atherosclerosis. In the past years, a wide array of cholesterol-lowering drugs has been developed by industry, and many studies have been executed to determine their mechanisms of action. Then, why would one be interested in determining the mechanism of a potentially pro-atherogenic compound?

First, research on the mechanism of action of both a cholesterol-lowering drug and a cholesterol-elevating compound as cafestol can provide insight into the regulation

of lipoprotein metabolism in humans. All of the lipid-regulating drugs or food compounds that are currently available have a defined role on different aspects of controlling serum lipoprotein metabolism (53). Statins lower LDL cholesterol by inhibition of 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA), the enzyme for the rate limiting step in the synthesis of cholesterol in the liver (53). Fibrates lower triglycerides by decreasing the secretion of VLDL, increasing the lipoprotein lipase activity and decreasing the release of free fatty acids from peripheral adipose tissue via modulation of peroxisome proliferator-activated receptor (PPAR) α (54). Resins lower LDL cholesterol by increasing the activity of cholesterol 7α-hydroxylase resulting in a higher demand for cholesterol in the liver (55). Dietary plant stanol esters reduce LDL cholesterol by inhibition of cholesterol absorption, despite a concomitant increase in the synthesis of cholesterol (56). Thus several cholesterol-lowering drugs as well as cholesterol-elevating or -lowering dietary compounds have specific and unique actions on lipoprotein metabolism in humans. Elucidation of the mechanism of action of cafestol might be of help in getting insight in the regulation of hepatic cholesterol metabolism, and with that in the development of new tools for the treatment of abnormal serum lipoprotein levels in humans.

Second, cafestol is the most potent cholesterol-raising food component known. It is almost as potent in increasing LDL cholesterol as the most powerful cholesterol-lowering drugs are able to lower it. A daily dose of 40 mg of a statin will decrease LDL cholesterol by the same percentage as a daily dose of 200 mg of cafestol will increase LDL cholesterol (53;57). Relative high amounts of this powerful cholesterol-raising food component are present in unfiltered coffee brews such as Scandinavian boiled coffee, cafetiere (French-press) coffee, Turkish and Greek coffee (58). Boiled coffee was once a popular coffee brew in Scandinavia, but nowadays most Scandinavians use filtered coffee. This switch in brewing practices is thought to explain one third (59) to one half (60) of the 10% fall in serum cholesterol in Scandinavia since 1970, and to have contributed significantly to the concurrent fall in coronary mortality (61;62). On the other hand, cafetiere coffee is gaining popularity in many European countries and in the USA. High intakes of cafetiere coffee are likely to be associated with an increased risk of coronary heart disease, similar to what has been observed with boiled coffee (37;63). Mocha and espresso coffee appear harmless with consumption of a few cups per day (57). We do not exactly know how much cafestol is present in coffee brews from coffee vending machines. Coffee brews from these machines are regularly consumed in public places. Preliminary data suggest that the amounts of cafestol in brews from machines that use freeze-dried coffee are negligible. Amounts of cafestol in brews from machines that use suction or pressure ranged from 10 to 100% of the amounts detected in boiled coffee (unpublished observations). However, we need to analyse a larger amount of coffee brew samples obtained from different types of machines in order to provide a better

estimation of the amount of cafestol in brews from coffee vending machines. From a public health perspective, patients with high serum cholesterol concentrations or patients with an increased risk for ischaemic heart disease should limit their intake of cafestol-rich coffee brews.

Recommendations for further research

The first mechanistic studies on cafestol have been undertaken, but the major part of the action of cafestol in humans is still unknown. This hampers a proper extrapolation of more fundamental data from *in vitro* or *in vivo* animal studies to the human situation.

Cafestol clearly inhibited bile acid synthesis in apolipoprotein E*3-Leiden mice. As was mentioned above, it is of great interest to know how cafestol affects bile acid metabolism in humans. A decreased secretion of bile acids by the liver might explain the cholesterol-raising properties of coffee diterpenes. It is worthwhile to perform a study in which faecal bile acids from subjects treated with cafestol for longer than 2 weeks are determined with the conventional analysis method, e.g. determination of primary and secondary bile acids in freeze-dried faeces after extraction. In addition to the measurement of bile acid in faeces, we might be able to detect changes in the serum concentration of cholesterol 7α-hydroxylase (64) upon cafestol treatment.

We found that cafestol increases the production of $VLDL_1$ lipoproteins in humans. In order to assess if MTP is involved in this action, we might determine the expression and activity of MTP in hepatocytes of apolipoprotein E*3-Leiden mice after short- and long-term treatment with cafestol. We also found in both humans and in apolipoprotein E*3-Leiden mice that cafestol enriches $VLDL_2$ lipoproteins with cholesteryl esters at the cost of triglycerides. However, we still do not know how cafestol and kahweol affect the composition, the synthesis and catabolism of IDL, LDL and HDL lipoproteins. A detailed overview of changes in the lipoprotein profile and in apolipoprotein concentrations might help to estimate the atherogenicity of the LDL cholesterol-raising effect of unfiltered coffee.

To elucidate the role of LpL and hepatic lipase activity in lipoprotein metabolism during treatment with cafestol, it might be interesting to measure post heparin lipolytic activity. This measurement, however, does not provide information on the amount of LpL present or the affinity of LpL for lipoproteins. An alternative method to measure LpL activity *in vivo* is to study the triglyceride profiles after an oral fat load during several stages of cafestol treatment. Information on levels of VLDL apolipoprotein CII - which is required for activation of LpL - and CIII - which inhibits LpL activity - might also prove valuable in the assessment of LpL action.

About 70% of the ingested cafestol and kahweol is absorbed in humans. In order to give a more precise estimation of the absorption of coffee diterpenes, we need to

know how cafestol and kahweol are metabolised in the human body, and which metabolites are subsequently excreted into urine. It is possible that not cafestol itself, but a metabolite, is responsible for the cholesterol-raising effect. Attempts to measure cafestol in serum might be undertaken in order to estimate the bio-availability of cafestol. In addition, serum analyses will provide us with an opportunity to determine the kinetics and the transport mechanism of cafestol in the circulation.

Concluding remarks

Our mechanistic studies suggest that cafestol raises plasma triglycerides and LDL cholesterol by separate mechanisms. The mechanism to raise plasma triglycerides is relatively rapid, cafestol causing an increase in the production rate of $VLDL_1$ apolipoprotein B. This effect might be a result of an increased MTP activity or by an increased synthesis of hepatic triglycerides. The mechanism by which plasma triglycerides eventually return to baseline levels in humans is unknown, but a depleted store of hepatic triglycerides might play a role in this mechanism. The mechanism to raise LDL cholesterol appears to be much slower. Results from both *in vitro* and transgenic mice studies suggest that cafestol increases LDL cholesterol by suppression of bile acid synthesis. Subsequently, this might lead to down-regulation of the LDL receptor via a decreased activity of SREBP or via an enrichment of $VLDL_2$ particles with cholesteryl esters. However, whether cafestol or unfiltered coffee brews suppress the secretion of bile acids in humans remains to be established.

References

1. de Roos B, Katan MB. Possible mechanisms underlying the cholesterol-raising effect of the coffee diterpene cafestol. Curr. Opin. Lipidol. 1999;10:41-45.
2. Spady DK, Dietschy JM. Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. J Lipid Res. 1983;24:303-315.
3. Spady DK, Turley SD, Dietschy JM. Rates of low density lipoprotein uptake and cholesterol synthesis are regulated independently in the liver. J Lipid Res. 1985;26:465-472.
4. Bell LP, Hectorn KJ, Reynolds H, Hunninghake DB. Cholesterol-lowering effects of soluble-fiber cereals as part of a prudent diet for patients with mild to moderate hypercholesterolemia. Am. J Clin. Nutr. 1990;52:1020-1026.
5. Lia A, Hallmans G, Sandberg AS, Sundberg B, Aman P, Andersson H. Oat beta-glucan increases bile acid excretion and a fiber-rich barley fraction increases cholesterol excretion in ileostomy subjects. Am. J Clin. Nutr. 1995;62:1245-1251.
6. Marlett JA, Hosig KB, Vollendorf NW, Shinnick FL, Haack VS, Story JA. Mechanism of serum cholesterol reduction by oat bran. Hepatology 1994;20:1450-1457.
7. Anderson JW, Story L, Sieling B, Chen WJ, Petro MS, Story J. Hypocholesterolemic effects of oat-bran or bean intake for hypercholesterolemic men. Am. J Clin. Nutr. 1984;40:1146-1155.
8. Kirby RW, Anderson JW, Sieling B, et al. Oat-bran intake selectively lowers serum low-density lipoprotein cholesterol concentrations of hypercholesterolemic men. Am. J Clin. Nutr. 1981;34:824-829.
9. Malmstrom R, Packard CJ, Caslake M, et al. Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM. Diabetologia 1997;40:454-462.
10. Malmstrom R, Packard CJ, Watson TD, et al. Metabolic basis of hypotriglyceridemic effects of insulin in normal men. Arterioscler. Thromb. Vasc. Biol. 1997;17:1454-1464.

11. Campos H, Walsh BW, Judge H, Sacks FM. Effect of estrogen on very low density lipoprotein and low density lipoprotein subclass metabolism in postmenopausal women. J Clin. Endocrinol. Metab. 1997;82:3955-3963.
12. Cummings MH, Christ E, Umpleby AM, et al. Abnormalities of very low density lipoprotein B-100 metabolism contribute to the dyslipidaemia of adult growth hormone deficiency. J Clin. Endocrinol. Metab. 1997;82:2010-2013.
13. Stalenhoef AF, Malloy MJ, Kane JP, Havel RJ. Metabolism of apolipoproteins B48 and B100 of triglyceride rich lipoporteins in normal an lipoprotein lipase deficient humans. Proc. Natl. Acad. Sci. USA 1984;81:1839-1843.
14. Demant T, Carlson LA, Holmquist L, et al. Lipoprotein metabolism in hepatic lipase deficiency: studies on the turnover of apolipoprotein B and on the effect of hepatic lipase on high density lipoprotein. J Lipid Res. 1988;29:1603-1611.
15. Le NA, Gibson JC, Ginsberg HN. Independent regulation of plasma apolipoprotein CII and CIII concentrations in very low density and high density lipoproteins: implications for the regulation of the catabolism of these lipoproteins. J Lipid Res. 1988;29:669-677.
16. Ginsberg HN, Le NA, Goldberg IJ, et al. Apolipoprotein B metabolism in subjects with deficiency of apolipoproteins CIII and AI: evidence that apolipoprotein CIII inhibits catabolism of triglyceride-rich lipoproteins by lipoprotein lipase in vivo. J Clin. Invest. 1986;78:1287-1295.
17. Fojo SS, de Gennes JL, Beisiegel U, et al. Molecular genetics of apoC-II and lipoprotein lipase deficiency. Adv. Exp. Med. Biol. 1991;285:329-333.
18. Beisiegel U. Receptors for triglyceride-rich lipoproteins and their role in lipoprotein metabolism. Curr. Opin. Lipidol. 1995;6:117-122.
19. Takahashi S, Suzuki J, Kohno M, et al. Enhancement of binding of triglyceride-rich lipoproteins to the very low density lipoprotein receptor by apolipoprotein E and lipoprotein lipase. J Biol. Chem. 1995;270:15747-15754.
20. James RW, Martin B, Pometta D, et al. Apolipoprotein B metabolism in homozygous familial hypercholesterolemia. J Lipid Res. 1989;30:159-169.
21. Packard CJ, Munro A, Lorimer AR, Gotto AM, Shepherd J. Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects. J Clin. Invest. 1984;74:2178-2192.
22. Groener JE, van Ramshorst E, Katan MB, Mensink RP, van Tol A. Diet-induced alteration in the activity of plasma lipid transfer protein in normolipidemic human subjects. Atherosclerosis 1991;87:221-226.
23. Martin LJ, Connelly PW, Nancoo D, et al. Cholesteryl ester transfer protein and high density lipoprotein responses to cholesterol feeding in men: relationship to apolipoprotein E genotype. J Lipid Res. 1993;34:437-446.
24. van Tol A, Zock PL, van Gent T, Scheek LM, Katan MB. Dietary trans fatty acids increase serum cholesterylester transfer protein activity in man [see comments]. Atherosclerosis 1995;115:129-134.
25. van Tol A, Urgert R, de Jong-Caesar R, et al. The cholesterol-raising diterpenes from coffee beans increase serum lipid transfer protein activity levels in humans. Atherosclerosis 1997;132:251-254.
26. Abbey M, Nestel PJ. Plasma cholesteryl ester transfer protein activity is increased when trans-elaidic acid is substituted for cis-oleic acid in the diet. Atherosclerosis 1994;106:99-107.
27. Tall A. Plasma lipid transfer proteins. Annu. Rev. Biochem. 1995;64:235-257.
28. Yamashita S, Sprecher DL, Sakai N, Matsuzawa Y, Tarui S, Hui DY. Accumulation of apolipoprotein E-rich high density lipoproteins in hyperalphalipoproteinemic human subjects with plasma cholesteryl ester transfer protein deficiency. J Clin. Invest. 1990;86:688-695.
29. Koizumi J, Inazu A, Yagi K, et al. Serum lipoprotein lipid concentration and composition in homozygous and heterozygous patients with cholesteryl ester transfer protein deficiency. Atherosclerosis 1991;90:189-196.
30. Agellon LB, Walsh A, Hayek T, et al. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. J Biol. Chem. 1991;266:10796-10801.
31. Jiang XC, Masucci ML, Mar J, et al. Down-regulation of mRNA for the low density lipoprotein receptor in transgenic mice containing the gene for human cholesteryl ester transfer protein. Mechanism to explain accumulation of lipoprotein B particles. J Biol. Chem. 1993;268:27406-27412.
32. Dinchuk J, Hart J, Gonzalez G, Karmann G, Schmidt D, Wirak DO. Remodelling of lipoproteins in transgenic mice expressing human cholesteryl ester transfer protein. Biochim. Biophys. Acta 1995;1255:301-310.
33. Halvorsen B, Ranheim T, Nenseter MS, Huggett AC, Drevon CA. Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts. J Lipid Res. 1998;39:901-912.

34. Rustan AC, Halvorsen B, Huggett AC, Ranheim T, Drevon CA. Effect of coffee lipids (cafestol and kahweol) on regulation of cholesterol metabolism in HepG2 cells. Arterioscler. Thromb. Vasc. Biol. 1997;17:2140-2149.
35. Post SM, de Wit EC, Princen HMG. Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase in rat hepatocytes. Arterioscler. Thromb. Vasc. Biol. 1997;17:3064-3070.
36. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331-340.
37. Urgert R, Meyboom S, Kuilman M, et al. Comparison of effect of cafetiere and filtered coffee on serum concentrations of liver aminotransferases and lipids: six month randomised controlled trial. BMJ. 1996;313:1362-1366.
38. Rudel L, Parks JS, Bond MG. Dietary polyunsaturated fat effects on atherosclerosis and plasma lipoproteins in African green monkeys. In: Scarpelli DG, Migaki G, eds. Current topics in nutrition and disease in comparative pathobiology. New York: Alan R Liss, Inc, 1986:501-523.
39. Rudel L, Sawyer JK, Parks JS. Dietary fat, lipoprotein structure, and atherosclerosis in primates. Athero. Rev. 1991;23:41-50.
40. Rudel LL, Haines JL, Sawyer JK. Effects on plasma lipoproteins of monounsaturated, saturated, and polyunsaturated fatty acids in the diet of African green monkeys. J Lipid Res. 1990;31:1873-1882.
41. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, et al. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol. Chem. 1993;268:10540-10545.
42. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, et al. Diet -induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin. Invest. 1994;93:1403-1410.
43. van Vlijmen BJ, van 't Hof HB, Mol MJ, et al. Modulation of very low density lipoprotein production and clearance contributes to age- and gender- dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. J Clin. Invest. 1996;97:1184-1192.
44. Higuchi K, Kitagawa K, Kogishi K, Takeda T. Developmental and age-related changes in apolipoprotein B mRNA editing in mice. J Lipid Res. 1992;33:1753-1764.
45. Paigen B, Plump AS, Rubin EM. The mouse as a model for human cardiovascular disease and hyperlipidemia. Curr. Opin. Lipidol. 1994;5:264
46. Urgert R, Weusten-van der Wouw MPME, Hovenier R, Meyboom S, Beynen AC, Katan MB. Diterpenes from coffee beans decrease serum levels of lipoprotein(a) in humans: results from four randomised controlled trials. Eur. J Clin. Nutr. 1997;51:431-436.
47. Stamler J, Wentworth D, Neaton JD. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). JAMA 1986;256:2823-2828.
48. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. JAMA 1986;256:2835-2838.
49. Kromhout D, Bosschieter EB, Drijver M, de-Lezenne CC. Serum cholesterol and 25-year incidence of and mortality from myocardial infarction and cancer. The Zutphen Study. Arch. Intern. Med. 1988;148:1051-1055.
50. Austin MA. Plasma triglyceride and coronary heart disease. Arterioscler Thromb. 1991;11:2-14.
51. Anonymous. National Cholesterol Education Program. Second Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II). Circulation 1994;89:1333-1445.
52. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J Cardiovasc. Risk 1996;3:213-219.
53. Dujovne CA. New lipid lowering drugs and new effects of old drugs. Curr. Opin. Lipidol. 1997;8:362-368.
54. Staels B, Dallongeville J, Auwerx J, choonjans K, eitersdorf E, ruchart JC. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 1998;98:2088-2093.
55. Einarsson K, Ericsson S, Ewerth S, et al. Bile acid sequestrants: mechanisms of action on bile acid and cholesterol metabolism. Eur. J Clin. Pharmacol. 1991;40 [Suppl 1]:S53-S58
56. Miettinen TA, Gylling H. Regulation of cholesterol metabolism by dietary plant sterols. Curr. Opin. Lipidol. 1999;10:9-14.

57. Urgert R, Katan MB. The cholesterol-raising factor from coffee beans. Annu.Rev.Nutr. 1997;17:305-324.
58. Urgert R, van der Weg G, Kosmeijer-Schuil TG, van de Bovenkamp P, Hovenier R, Katan MB. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. J Agric. Food Chem 1995;43:2167-2172.
59. Aro A. The effect of coffee on serum lipids and its clinical considerations. Cardiovascular risk factors 1993;3:238-243.
60. Tuomilehto J, Pietinen P. Coffee and cardiovascular disease. Cardiovascular risk factors 1991;1:165-173.
61. Johansson L, Drevon CA, Aa BG. The Norwegian diet during the last hundred years in relation to coronary heart disease. Eur. J Clin. Nutr. 1996;50:277-283.
62. Pietinen P, Vartiainen E, Seppanen R, Aro A, Puska P. Changes in diet in Finland from 1972 to 1992: impact on coronary heart disease risk. Prev. Med. 1996;25:243-250.
63. Tverdal A, Stensvold I, Solvoll K, Foss OP, Lund LP, Bjartveit K. Coffee consumption and death from coronary heart disease in middle aged Norwegian men and women. BMJ. 1990;300:566-569.
64. Bjorkhem I, Reihner E, Angelin B, Ewerth S, Akerlund JE, Einarsson K. On the possible use of the serum level of 7 alpha-hydroxycholesterol as a marker for increased activity of the cholesterol 7 alpha-hydroxylase in humans. J Lipid Res. 1987;28:889-894.

Summary

Cafestol and kahweol are diterpenes present in unfiltered coffee brews such as boiled or French-press coffee. Cafestol is the most powerful cholesterol-raising food substance known. It also causes a transient rise in triglycerides. The mechanism through which cafestol influences lipoprotein metabolism is largely unknown. Unravelling the mechanism of action might lead to new insights into the regulation of serum cholesterol levels in humans. The objective of this thesis was to study the mechanism of action of cafestol (and kahweol) in man.

We first studied the absorption and urinary excretion of cafestol and kahweol in man. Absorption values are required to assess which part of the consumed diterpenes is actually responsible for the rise in serum lipids. Urinary excretion values might provide more insight into the metabolism of cafestol and kahweol in the human body. This might lead us to the compound that actually raises serum lipids: the diterpenes themselves or a metabolite. Such insights would facilitate further studies of the mechanism of action. In this study, nine healthy ileostomists consumed a known dose of French-press coffee, after which they collected ileostomy effluent for 14 hrs and urine for 24 hrs. We measured absorption and urinary excretion of diterpenes; their stability was also assessed under simulated gastro-intestinal tract conditions. About 90% of the cafestol and kahweol that entered the small intestine was absorbed there. Absorption of these coffee diterpenes expressed as percentage of the amount consumed was about 70%. Possibly, undetected metabolites were present in ileostomy effluent, resulting in lower absorption percentages. We found losses of diterpenes during incubation *in vitro* with gastric juice (about 28%), during storage with ileostomy effluent (about 15%), and during freeze-drying (about 29%). In conclusion, about 70% of the consumed diterpenes was absorbed in ileostomy volunteers, whereas only 1.2% of the diterpenes was excreted as a conjugate of glucuronic acid or sulphate in urine. Therefore, these compounds are extensively metabolised in the human body (chapter 2).

We have searched for an animal species in which cafestol and kahweol similarly increase plasma cholesterol as in humans. Such an animal model might be a good model to study the mechanism of action of these diterpenes. Previous studies showed that cafestol and kahweol affected serum lipids in Cebus and Rhesus monkeys, hamsters, rats, and gerbils inconsistently and differently than in humans. The variability in effects on serum lipids could not be explained by the mode of administration or dose of diterpenes, nor by the amount of cholesterol in the diet. Therefore, these animal species do not appear to be suitable animal models to study the action of coffee diterpenes. Since male African green monkeys show many similarities with humans in the effects of dietary cholesterol and fatty acids on plasma lipoproteins and cholesterol metabolism, they might be a good model to study the effects of cafestol and kahweol on lipoprotein metabolism in man. However, in African green monkeys the rise in total cholesterol was less pronounced than in man.

Also, unlike humans, the rise in cholesterol was predominantly due to a rise in HDL cholesterol rather than LDL cholesterol. Thus, the rise in plasma lipids might illustrate the mechanism in these monkeys rather than the mechanism in humans (chapter 3). Therefore, the African green monkey does also not appear to be a good model to study the mechanism of action of cafestol and kahweol.

We also determined whether cafestol and kahweol raise serum cholesterol in apolipoprotein E*3-Leiden, heterozygous LDL receptor knockout or wild type (C57Bl/6) mice similarly as in humans. Heterozygous LDL receptor knockout mice cannot clear VLDL and LDL particles with the same efficiency as wild type mice - and possibly other animal models -, and hence they might be more responsive to cafestol. Transgenic mice over-expressing human apolipoprotein E*3-Leiden are highly susceptible to diet-induced hyperlipoproteinemia due to a defect in hepatic uptake of remnant lipoproteins. A diet with cafestol (0.05% w/w) and kahweol (0.03% w/w) increased serum cholesterol by 61% in apolipoprotein E*3-Leiden mice, by 55% in LDL receptor knock-out mice and by 46% in wild type mice after eight weeks of treatment. The increase in total cholesterol was mainly due to a rise in VLDL and IDL cholesterol in all three mouse strains. Since the effects of cafestol and kahweol on total cholesterol were most pronounced in apolipoprotein E*3-Leiden mice, we subsequently studied the mechanism of action of the diterpenes in this mouse strain. After three weeks of treatment, cafestol and kahweol suppressed the major regulatory enzymes in the bile acid synthesis pathways: activity of cholesterol 7α-hydroxylase was reduced by 57% and mRNA levels of cholesterol 7α-hydroxylase were reduced by 58%; mRNA levels of sterol 27-hydroxylase were reduced by 32%. The total amount of faecal bile acids was decreased by 41%. Cafestol and kahweol did not affect hepatic free and esterified cholesterol, but they decreased LDL receptor mRNA levels by 37%. VLDL particles contained a three times higher amount of cholesteryl esters, indicative for the secretion of a ßVLDL-like particle. This was confirmed by a decreased rate of VLDL triglyceride production in mice treated with cafestol and kahweol (35.1±13.8 µmol/h/kg) compared to placebo treatment (63.1±17.5 µmol/h/kg) as a result of a reduction in hepatic triglyceride content by 52%. Therefore, cafestol and kahweol might increase serum cholesterol levels by suppression of bile acid synthesis (chapter 4).

A second study on the mechanism of action of coffee diterpenes was performed in humans. We investigated whether the serum lipid transfer proteins CETP and PLTP might be involved in the mechanism of action of cafestol and kahweol. In our earlier study it remained unclear whether the elevated serum CETP and PLTP activity levels were a cause or a consequence of the raised LDL cholesterol concentrations. The activity level of the serum enzyme LCAT was studied as well. Forty-six healthy normolipidaemic subjects consumed either 0.9 L of French-press coffee or filtered coffee for 24 weeks. French-press coffee increased CETP activity by 15% and PLTP

activity by 12% after 12 weeks in both male and female subjects. French-press coffee decreased LCAT activity by 5% after 12 weeks. Changes in CETP clearly preceded the changes in LDL cholesterol. Therefore, we concluded that the increased CETP activity during consumption of French-press coffee may contribute to the rise in LDL cholesterol (chapter 5).

We studied whether cafestol increases plasma triglycerides by increasing the production rate or decreasing the fractional catabolic rate of $VLDL_1$ apolipoprotein B. Eight healthy normolipidaemic men consumed a daily dose of 75 mg of cafestol for 2 weeks. A bolus injection of 7 mg/kg of body weight of d_3-leucine (L-[5,5,5-$^2H_3$]leucine) was given as a tracer before and directly after treatment with cafestol. We derived kinetic constants to describe the metabolism of $VLDL_1$ apolipoprotein B using a multicompartmental model. Cafestol increased plasma triglycerides by 31% after two weeks of intervention. The rise in plasma triglycerides was mainly due to a rise in $VLDL_1$ triglycerides. The pool size of $VLDL_1$ apolipoprotein B was increased by 59%. The rise in $VLDL_1$ apolipoprotein B was accompanied by an increased $VLDL_1$ apolipoprotein B production rate of 80%, while the fractional catabolic rate of $VLDL_1$ apolipoprotein B was not significantly changed. Cafestol did not change the composition $VLDL_1$. $VLDL_2$ became enriched with cholesteryl esters at the cost of triglycerides. Therefore, cafestol increases serum triglycerides by an increased production rate of $VLDL_1$ apolipoprotein B. The enrichment of $VLDL_2$ particles with cholesteryl esters - rather than the increased amount of triglyceride-rich $VLDL_1$ particles - might play a role in the subsequent rise of LDL cholesterol levels.

We conclude that cafestol raises plasma triglycerides and LDL cholesterol by separate mechanisms. The initial mechanism to raise plasma triglycerides is a fast action, where cafestol causes an increase in the production rate of $VLDL_1$ apolipoprotein B. The second mechanism by which cafestol increases LDL cholesterol appears to be much slower. Results from the studies with apolipoprotein E*3-Leiden mice suggest that cafestol increases LDL cholesterol by suppression of bile acid synthesis. This might lead to down-regulation of the LDL receptor via a decreased activity of sterol regulatory element binding protein (SREBP) and to an enrichment of $VLDL_2$ particles with cholesteryl esters. However, whether cafestol or unfiltered coffee brews suppress bile acids secretion in humans remains to be established. In addition, CETP might as well play a role in the rise of LDL cholesterol upon consumption of coffee diterpenes.

Samenvatting

Cafestol en kahweol zijn stoffen die voorkomen in ongefilterde koffie zoals kookkoffie en cafetierekoffie. Van deze twee is cafestol de meest cholesterol- en triglyceriden verhogende voedingsstof die we op dit moment kennen. Hoe cafestol het cholesterol en triglyceriden gehalte in het bloed van mensen verhoogt is echter niet bekend. Het ontrafelen van het werkingsmechanisme zou ons misschien nieuwe inzichten kunnen geven in de regulatie van deeltjes die cholesterol en triglyceriden vervoeren in het bloed: de lipoproteinen. Er zijn vier verschillende soorten lipoproteinen die worden onderscheiden op hun dichtheid: HDL (vervoert het "goede cholesterol"), LDL (vervoert het "slechte" cholesterol), IDL (vervoert zowel cholesterol als triglyceriden) en VLDL (vervoert voornamelijk triglyceriden). Het doel van dit proefschrift is dan ook het bestuderen van het werkingsmechanisme van cafestol.

Allereest hebben we de opname, omzetting en uitscheiding van cafestol en kahweol in het menselijk lichaam onderzocht. Absorptie-waarden zeggen iets over het deel van de geconsumeerde cafestol en kahweol dat verantwoordelijk is voor de stijging van cholesterol en triglyceriden in het bloed. Informatie over de omzetting van cafestol en kahweol in de lever zegt iets over welke stof nu eigenlijk het bloed cholesterol en triglyceriden verhoogt: cafestol en kahweol zelf, of misschien een afgeleide stof. Deze kennis kan vervolgens helpen bij het oplossen van het werkingsmechanisme. Om de aborptie en uitscheiding van cafestol en kahweol te bepalen, dronken negen mensen bij het ontbijt een vastgestelde hoeveelheid cafetierekoffie, en gedurende de 24 uren hierna hebben we de uitscheiding van cafestol en kahweol in stoma-vloeistof en in urine gemeten. Ongeveer 70% van de cafestol en kahweol uit de geconsumeerde cafetierekoffie werd geabsorbeerd. Cafestol en kahweol werden echter ook afgebroken tijdens hun tocht door het maagdarmkanaal en tijdens hun korte verblijf in het stomazakje. We vonden slechts 1,2% van de geconsumeerde cafestol en kahweol terug in de urine. De omzetting van deze stoffen in het lichaam is waarschijnlijk dermate ingewikkeld dat onze meetmethoden deze niet kunnen detecteren (hoofdstuk 2).

We hebben gezocht naar een diersoort waarbij cafestol eenzelfde soort cholesterol-verhogend effect veroorzaakt als in mensen. Zo'n diersoort kan vervolgens worden gebruikt om het werkingsmechanisme van cafestol en kahweol nader te onderzoeken. Voorgaande studies hebben al laten zien dat cafestol en kahweol het bloed cholesterol in Cebus en Rhesus apen, hamsters, ratten en gerbils niet zo consistent verhogen als in mensen. De verschillen konden hierbij niet worden verklaard door de toedieningsvorm, de dosis, of de hoeveelheid cholesterol in het voer. In de groene meerkat verhogen cholesterol en vetzuren uit het voer het bloed cholesterol op eenzelfde manier als in mensen. Daarom hebben we in deze apensoort gekeken of ook cafestol en kahweol op eenzelfde manier het bloed cholesterol verhogen als in mensen. We vonden dat beide stoffen het bloed cholesterol in deze apen wel verhoogden, maar deze verhoging was aanzienlijk

zwakker dan in mensen. Verder veroorzaakten cafestol en kahweol in deze apen een ander soort cholesterolstijging: terwijl ze in mensen voornamelijk LDL cholesterol verhogen, ging in deze apen juist het HDL cholesterol omhoog. Het cholesterol verhogende effect zegt daarom waarschijnlijk meer iets over het werkingsmechanisme in deze apen dan over een werkingsmechanisme in mensen (hoofdstuk 3).

We hebben ook het effect van cafestol en kahweol op bloed cholesterol in apolipoproteine E*3-Leiden, heterozygote LDL receptor knock-out en wild type muizen onderzocht. Cafestol en kahweol verhoogden het bloed cholesterol met 61% in apolipoproteine E*3-Leiden muizen, met 55% in LDL receptor knock-out muizen en met 46% in wild type muizen na een behandelingsperiode van acht weken. Deze verhoging was net als in de apen anders dan in mensen; in deze muizen ging voornamelijk het VLDL en IDL cholesterol omhoog. Omdat de respons van het bloed cholesterol op cafestol en kahweol het sterkst was in de apolipoproteine E*3-Leiden muizen, hebben we vervolgens bij deze muizenstam het werkingsmechanisme van cafestol en kahweol nader onderzocht. Na drie weken bleken cafestol en kahweol twee enzymen die in de lever zorgen voor de omzetting van cholesterol naar galzouten met ongeveer 60% te remmen. De uitscheiding van galzouten in de faeces was geremd met 41%. Cafestol en kahweol hadden geen invloed op de hoeveelheid cholesterol in de lever, al was de hoeveelheid LDL receptor (een constructie die er voor zorgt dat LDL deeltjes uit het bloed in de cel worden opgenomen) verlaagd met 37%. Cafestol en kahweol verhoogden de cholesterol concentratie in de VLDL deeltjes, terwijl de VLDL triglyceriden productie was verlaagd met ongeveer 50% als gevolg van een 50% lagere concentratie aan triglyceriden in de lever. Mogelijk is dus een remming van de galzuursynthese (mede) verantwoordelijk voor het cholesterol-verhogende effect van cafestol (hoofdstuk 4).

In mensen hebben we onderzocht of CETP en PLTP (twee eiwitten die cholesterol vanuit HDL deeltjes naar LDL en VLDL deeltjes vervoeren in ruil voor triglyceriden) zijn betrokken bij het werkingsmechanisme van cafestol. Uit eerder onderzoek bleek namelijk niet duidelijk of CETP nu het LDL cholesterol verhoogt of andersom. We hebben tevens gekeken naar het effect van cafestol op de aktiviteit van het enzym LCAT wat betrokken is bij de aanmaak van HDL deeltjes. Zesenveertig gezonde proefpersonen dronken hiervoor gedurende een half jaar lang per dag een thermosfles vol cafetiere ofwel filterkoffie. De consumptie van cafetierekoffie verhoogde de aktiviteit van CETP in het bloed met 15% en die van PLTP in het bloed met 12% na 12 weken in zowel mannen als vrouwen. Na 12 weken was de aktiviteit van LCAT in het bloed met 5% gedaald. De stijging in CETP liep duidelijk vooruit op de stijging in LDL cholesterol. De stijging in CETP is dus mogelijk betrokken bij het LDL cholesterol-verhogende effect van cafetiere koffie (hoofdstuk 5).

Veranderingen in de aanmaak- of afbraaksnelheid van grote, lichte VLDL deeltjes (deze deeltjes vervoeren relatief de meeste triglyceriden in het bloed) zouden kunnen verklaren waarom na het drinken van ongefilterde koffie het triglyceriden gehalte in het bloed gaat stijgen. Daarom hebben we gekeken naar het effect van cafestol op de kinetiek van grote, lichte VLDL deeltjes in 8 gezonde mannen. Cafestol had na 2 weken het triglyceridengehalte in het bloed met gemiddeld 31% verhoogd. Cafestol verhoogde daarnaast de hoeveelheid apolipoproteine B eiwit in de lichtere VLDL fractie met 59% en de hoeveelheid apolipoproteine B eiwit in de zwaardere VLDL fractie met 31%. Omdat elk VLDL deeltje slechts één apolipoproteine B eiwit bevat, wijst dit op meer VLDL deeltjes in het bloed. Cafestol had geen invloed op de samenstelling van de lichte VLDL deeltjes, terwijl de zware VLDL deeltjes relatief meer cholesterol en minder triglyceriden bevatten na behandeling met cafestol. Om de kinetiek van de VLDL deeltjes te kunnen beschrijven, kregen de proefpersonen voor en na behandeling met cafestol een injectie met een gemerkt aminozuur (d_3-leucine). Dit gemerkte aminozuur kwam terecht in het apolipoproteine B eiwit van de grote, lichte VLDL deeltjes. De snelheid waarmee dit gebeurt zegt iets over de aanmaak- en afbraaksnelheid van deze VLDL deeltjes. Wij vonden dat cafestol de aanmaak van grote, lichte VLDL deeltjes verhoogde, terwijl de afbraaksnelheid niet veranderde. De stijging in de aanmaaksnelheid van lichte VLDL deeltjes is dus de oorzaak van de stijging van triglyceriden in het bloed na consumptie van cafestol (hoofdstuk 6).

De conclusie van dit proefschrift is dat een remming van het galzuurmetabolisme mogelijk de stijging in LDL cholesterol na consumptie van ongefilterde koffie veroorzaakt. We weten echter nog steeds niet goed of mensen na het drinken van ongefilterde koffie minder galzuren in de faeces gaan uitscheiden. Dit zou als eerste uitgezocht moeten worden. De tijdelijke stijging in triglyceriden wordt veroorzaakt door een verhoogde produktie van grote, lichte VLDL deeltjes. Na 4-6 weken zet het lichaam waarschijnlijk een compensatie-mechanisme in werking waarmee de triglyceriden weer teruggaan naar hun oorspronkelijke niveau. De verhoging in LDL cholesterol en triglyceriden wordt mogelijk door twee verschillende mechanismen van cafestol veroorzaakt.

Dankwoord

Het proefschrift is af! De afgelopen vier jaar heb ik zo'n 1000 keer de Wageningse berg op en af gefietst om te kijken hoe het bloed cholesterol van apen, muizen en mensen reageert op twee stoffen uit ongefilterde koffie. Binnenkort moet ik de inhoud van dit boekje in mijn eentje verdedigen, maar het onderzoek had ik zeker niet alleen kunnen doen! Graag wil ik iedereen bedanken die heeft bijgedragen aan het tot stand komen van dit boekje. Een aantal mensen wil ik met name noemen.

Martijn, ik heb ontzettend veel geleerd van je kritische instelling en je vermogen om alle resultaten te deduceren tot een 'bottom line'. Daarnaast waardeer ik het erg dat je me op pad hebt gestuurd om met verschillende prominente onderzoekers in binnen- en buitenland een aantal experimenten op te zetten en uit te voeren. Professor Hautvast, ondanks het feit dat ik uw naam fout had gespeld op mijn afstudeerverslag ('begeleider: prof. Houtvast') heeft u toch ingestemd met mijn aanstelling als AIO. Bedankt voor uw interesse in zowel mijn AIO onderzoek als mijn Schotse toekomstplannen, maar ook voor uw bijdrage in mijn begeleidings-commissie.

Mijn AIO-periode was zonder de samenwerking met onderzoekers buiten de vakgroep zeker niet zo leerzaam geweest. Dear Dr. Rudel and Janet Sawyer, I very much enjoyed my visit to Winston Salem in 1996. Thanks for the realisation of the monkey-trial. Despite being on two different continents, the frequent e-mails pushed us through the preparation of the manuscript. Dear Chris, Muriel, Dorothy, and all the people from the Department of Pathological Biochemistry, thanks for all the instructive discussions on the kinetics of lipoproteins, your help with the organisation and realisation of the turnover studies and GC-MS analyses in Glasgow, and the ever lasting hospitality. John, I'd like to thank you for proof-reading the discussion of this thesis. Professor Stalenhoef, beste Anton, ik weet nog steeds niet goed wat je nu van een 'Wageningen-AIO' vindt, maar ik denk in ieder geval met veel plezier terug aan onze samenwerking. Bedankt voor je professionele begeleiding van de turnover experimenten in Nijmegen en je bijdrage in mijn begeleidingscommissie. Til, zonder de inzet van 'die jonge verpleegkundige met die moderne bril' zouden de turnover studies zeker een stuk minder gezellig zijn verlopen! Pierre, Magda, Heidi, Helga, bedankt voor jullie hulp bij het opzetten en uitvoeren van de analyses op het lab in

Nijmegen, en Jacqueline, bedankt voor je hulp bij de bloedafname's. Beste Aad, bedankt voor al je input bij de CETP/PLTP analyses en je grote belangstelling voor het koffie-onderzoek; ik heb veel geleerd van de discussies die we over ons onderzoek hebben gevoerd. Hans en Sabine, na wat ongewenste zwangerschappen hebben we toch nog hele leuke resultaten behaald met de transgene muizen. Bedankt voor jullie uitgebreide inzet en bijdrage in het 'moleculaire' deel van mijn AIO-onderzoek. Tom Vree en Roelof v.d. Meer, jullie wil ik graag bedanken voor je bijdrage in mijn begeleidingscommissie.

In mijn studies heb ik het de proefpersonen niet gemakkelijk gemaakt, en ik wil hun dan ook graag bedanken voor hun deelname. Martijn, Lydia, Hanja en Yvonne, jullie hebben als studenten een wezenlijke bijdrage geleverd aan de muizen- en turnover studies. Bedankt voor jullie hulp en inzet, ik vond het erg leuk om met jullie samen te werken. Truus, Peter, Guido, Robert, Frans, Joke, Marga, Jan, Pieter, Paul, ik heb heel wat uurtjes bij jullie op lab doorgebracht (en ik was er echt niet alleen als er taart was..). Bedankt voor al jullie hulp bij de diterpeen analyses, de bloedafnames, en het opzetten van meetapparatuur. Jo, Gerrit, Bert, Rene, Maria en alle andere mensen van het Centrum voor Kleine Proefdieren, ik had jullie al leren kennen in mijn studententijd, maar ook tijdens mijn AIO-tijd heb ik mogen profiteren van jullie professionele begeleiding van de dierstudies, bedankt! Saskia, bedankt voor je geweldige inzet bij het stoma-experiment, hierdoor kon ik snel van start met mijn AIO-onderzoek. Ben en Dirk, bedankt voor de IT ondersteuning en al de Bonzo-dropjes.

De grote groep van AIO en post-doc collega's heeft absoluut bijgedragen aan een zeer geslaagde tijd op de vakgroep. Rob, ik heb hele goede herinneringen aan onze discussies die gelukkig lang niet altijd over het koffie-onderzoek gingen! Bedankt voor het kritisch doorlezen van mijn artikelen en discussie. Liesbeth, de laatste 2½ jaar waren zeer gezellig met jou als kamergenote! Ik vind het heel leuk dat jij mij bij de promotie gaat ondersteunen als paranimf. Nicole, Ingeborg, Nynke, Frouwkje, Louise, Peter, Margreet, Maud, Rianne, Machteld, en alle andere collega's op de vakgroep, bedankt voor al die uitstapjes buiten kantooruren, WK-voetbal gatherings met oranje kaarsen, squash-uitdagingen en soesjes tijdens de koffie-pauze. Ik zal dit alles zeker gaan missen in Schotland!

Tot slot wil ik graag mijn familie en schoonfamilie bedanken voor hun interesse in mijn onderzoek. Alle vrienden die voor de nodige afleiding hebben gezorgd tijdens filmpjes, feesten, borrels en gezellige weekenden: bedankt! Marieke, leuk dat je mijn paranimf wilt zijn, je hoeft echt geen roze jurkje aan hoor! Pieter, lieve knul, jij hebt me de afgelopen 4 jaar ruimschoots voorzien van steun, goede adviezen en glazen lekkere whisky. Veertien januari 2000 is voor ons beiden het 'officiele' einde van onze AIO-loopbaan, en hopelijk het begin van heel veel nieuwe spannende uitdagingen!

Curriculum Vitae

Baukje de Roos was born on October 11, 1970, in Tijnje, The Netherlands. In 1989, she passed secondary school, Atheneum, at the Nassau College, Heerenveen and started the study 'Human Nutrition' at the Wageningen Agricultural University. During this study she worked for 6 months at the Food and Nutrition Research Institute, Manila (The Philippines). She received her license as laboratory animal researcher in February 1994. In August 1995, she obtained her MSc degree in Human Nutrition with main topics in biochemistry, toxicology and molecular biology. She was then appointed as a PhD student on the project: 'Mechanism of action of the cholesterol-raising diterpenes from coffee beans". As part of this project, she collaborated with research groups from the Department of Comparative Medicine, Wake Forest University, Winston-Salem (USA), Department of Biochemistry, COEUR, Erasmus University Rotterdam (The Netherlands), TNO-PG, Gaubius Laboratory, Leiden (the Netherlands), the Department of Pathological Biochemistry, Glasgow Royal Infirmary (Scotland, UK), and the Department of Internal Medicine, Academic Hospital Nijmegen (the Netherlands). In 1996, she attended the 'Annual New England Epidemiology Summer Program' at Tufts University, Boston (USA), and the course Pharmacokinetics', Oss (The Netherlands), organised by the Leiden/Amsterdam Centre for Drug Research. She was a member of the PhD-excursion committee that organised a study-tour to Scandinavia in 1997. Currently, she is employed as a research assistant at the Department of Pathological Biochemistry, Glasgow Royal Infirmary, Scotland.

List of publications

Full papers:

Beynen AC, Weusten-van der Wouw MPME, **De Roos B**, Katan MB. Boiled coffee fails to raise serum cholesterol in hamsters and rats. Br. J. Nutr. 1996; 76: 755-764.

De Stoppelaar JM, **De Roos B**, Mohn GR, Hoebee B. Analysis of DES induced micronuclei in binucleated rat fibroblasts: comparison between FISH with satellite I probe and immunocytochemical staining with CREST serum. Mutation Res. 1997; 392: 139-149.

Van Tol A, Urgert R, de-Jong Ceasar R, Van Gent T, Scheek LM, **De Roos B**, Katan MB. The cholesterol-raising diterpenes from coffee beans increase serum lipid transfer protein activity levels in humans. Atherosclerosis 1997; 132: 251-254.

De Roos B, Van der Weg, G, Urgert, R, Van de Bovenkamp P, Charrier A, Katan MB. Levels of cafestol, kahweol, and related diterpenoids in wild species of the coffee plant *Coffea*. J. Agric. Food Chem. 1997; 45: 3065-3069.

De Roos B, Meyboom S, Kosmeijer-Schuil TG, Katan MB. Absorption and urinary excretion of the coffee diterpenes cafestol and kahweol in healthy ileostomy volunteers. J. Int. Med. 1998; 244: 451-460.

Terpstra AHM, Katan MB, Weusten-van der Wouw MPME, **De Roos B**, Beynen AC. The feeding of coffee oil raises plasma cholesterol concentrations in gerbils and rats. (submitted).

De Roos B, Katan MB. Possible mechanisms underlying the cholesterol-raising effect of cafestol. Curr. Opin. Lipidology 1999; 10: 41-45.

De Roos B, Sawyer JK, Katan MB, Rudel LL. Validity of animal models for the cholesterol-raising effects of coffee diterpenes in humans. Proc. Nutr. Soc. (in press).

De Roos B, Post SM, Vermeulen M, Afman L, Jong MC, Dahlmans VEH, Havekes LM, Stellaard F, Katan MB, Princen HMG. Cafestol, the cholesterol-raising factor in boiled coffee, increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis. (conditionally accepted).

De Roos B, Van Tol A, Urgert R, Scheek LM, Van Gent T, Buytenhek R, Princen HMG, Katan MB. Consumption of cafetiere coffee raises cholesteryl ester transfer protein activity levels before LDL cholesterol in normolipidemic subjects. (submitted).

De Roos B, Caslake MJ, Stalenhoef AFH, Bedford D, Demacker PNM, Katan MB, Packard CJ. The coffee diterpene cafestol increases plasma triglycerides by increasing the production rate of large very low density lipoprotein apolipoprotein B in normolipidemic subjects. (submitted).

Other publications:

De Roos B, Meyboom S, Kosmeijer-Schuil TG, Katan MB. Absorption and metabolism of the cholesterol-raising coffee diterpenes cafestol and kahweol in healthy ileostomy volunteers. Atherosclerosis 1997; 134 (supplement):29 (abstract).

De Roos B, Post SM, Vermeulen M, Afman L, Jong MC, Dahlmans VEH, Havekes LM, Stellaard F, Katan MB, Princen HMG. The coffee diterpene cafestol decreases bile acid synthesis by down-regulation of cholesterol 7a-hydroxylase in apolipoprotein E*3-Leiden mice. Atherosclerosis 1999; 144 (suppl. 1): 95 (abstract).

De Roos B, Meyboom S, Kosmeijer-Schuil TG, Katan MB. Absorptie en metabolisme van de cholesterolverhogende koffie-diterpenen cafestol en kahweol. Voeding 1997; 58: 27-28 (in Dutch).