Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts

Bente Halvorsen, Trine Ranheim,[1] Marit S. Nenseter, Anthony C. Huggett,* and Christian A. Drevon

Institute for Nutrition Research, Faculty of Medicine, University of Oslo, 0316 Oslo, Norway, and Nestec Research Center,* Lausanne, Switzerland

Abstract Consumption of boiled coffee promotes an elevation of plasma cholesterol concentration in humans. The active compounds found in the lipid fraction of the coffee have been identified as the diterpenes cafestol and kahweol. We have studied the effects of pure cafestol on cholesterol metabolism in human skin fibroblasts (HSF). The uptake of [^{125}I]-labeled tyramine cellobiose-labeled low density lipoprotein ([^{125}I]TC-LDL) was decreased by about 50% ($P < 0.05$) after 18 h preincubation time with cafestol (20 μg/ml), as compared to the control cells. The specific binding of radiolabeled LDL was reduced by 54% ($P < 0.05$) after preincubation for 18 h with cafestol. A reduced amount of LDL receptors was demonstrated by a protein-normalized Scatchard plot analysis (20% decrease in B_{max}) as well as by immunoblotting (25%) after cafestol incubation. No significant effect was observed on the level of mRNA for the LDL receptor after 11 and 23 h incubation with cafestol. Furthermore, we transfected HSF cells with a promoter region for the LDL receptor gene linked to a reporter gene, chloramphenicol acetyl transferase (CAT). No change was seen in the CAT activity after incubation with cafestol (20 μg/ml). Moreover, cafestol caused a 2.3-fold ($P < 0.05$) higher incorporation of radiolabeled [^{14}C]oleic acid into cholesteryl esters after 24 h incubation, as compared to control cells, suggesting an increased acyl-CoA:cholesterol acyl transferase (ACAT) activity. Incorporation of [^{14}C]acetate into cholesterol was reduced by approximately 40% ($P < 0.05$) with cafestol (20 μg/ml), as compared to control after 24 h preincubation, indicating a decreased 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase activity. Our results suggest that intake of cafestol may cause increased concentration of plasma cholesterol via the down-regulation of low density lipoprotein receptors by post-transcriptional mechanisms.—**Halvorsen, B., T. Ranheim, M. S. Nenseter, A. C. Huggett, and C. A. Drevon.** Effect of a coffee lipid (cafestol) on cholesterol metabolism in human skin fibroblasts. *J. Lipid Res.* 1998. **39:** 901–912.

Supplementary key words cafestol • 25-hydroxycholesterol • kahweol • low density lipoprotein • LDL receptor

Since Thelle, Arnesen, and Førde (1) first observed a significant, positive relationship between the amount of ingested coffee and serum concentration of cholesterol, several intervention trials have supported their findings (2–7). The way the coffee is prepared seems crucial for the cholesterol-raising effect. The typical Scandinavian preparation of boiled coffee provides a drink that markedly raises plasma cholesterol, whereas filtered and instant coffee do not promote a cholesterol-raising effect (2–8). Zock et al. (9) found that after centrifugation of boiled coffee the supernatant contained 1–2 g of lipids per liter, whereas filtered coffee contained hardly any lipids. They also showed that the triacylglycerol- and cholesterol-raising factors were recovered in the floating oily layer after boiling the coffee. Recently it has been reported that an intake of 50–120 mg/day of the purified coffee lipids, cafestol and kahweol (**Fig. 1**), increased plasma cholesterol by 35% in a controlled clinical trial (10). Cafestol and kahweol are diterpenes that so far have been found only in coffee beans.

The mechanisms behind the cholesterol-raising effect of cafestol, and possibly kahweol, are not known. A potential site of action for cafestol may be the low density lipoprotein (LDL) receptor, which is involved in the endocytic process of apoB- and apoE-containing lipoproteins. One important way to regulate the cholesterol content of cells is via feedback repression of the gene for the LDL receptor (11, 12). When the cells are depleted of cholesterol, the LDL receptor gene is transcribed actively, and LDL is cleared from plasma and

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; BSA, bovine serum albumin; CAT, chloramphenicol acetyl transferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HSF, human skin fibroblasts; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; [^{125}I]TC-LDL, ^{125}I-labeled tyramine cellobiose-labeled LDL; PBS, phosphate-buffered saline; SRE-1, sterol regulatory element-1; SDS, sodium dodecyl sulfate; SSC, sodium chloride/sodium citrate; SREBP-1, sterol regulatory element binding protein-1.

[1]To whom correspondence should be addressed.

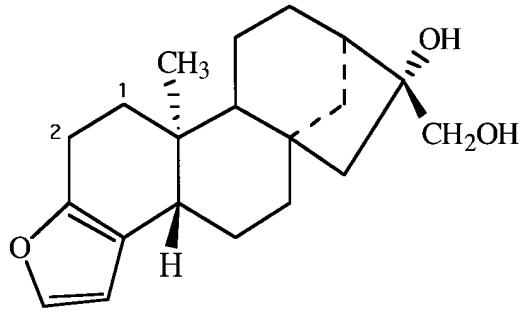


Fig. 1. Chemical structure of cafestol. Kahweol has an additional double-bond between C1 and C2.

taken up by cells expressing this receptor on the cell surface. When cholesterol accumulates within the cells, the number of LDL receptors is down-regulated to obtain a good homeostatic balance.

The aim of this study was to examine the effect of the purified coffee-diterpene cafestol on cholesterol metabolism in human skin fibroblasts (HSF) and to explore the possible mechanism behind the plasma cholesterol-raising effect observed in humans.

MATERIALS AND METHODS

Materials

Na[^{125}I], [2-^{14}C]acetic acid (5 Ci/mol), [^{3}H]leucine (5 Ci/mol), [1-^{14}C]oleic acid (58 Ci/mol), d-threo-dichloroacetyl-[1,2-^{14}C]CAT assay grade (56.8 Ci/mol), and [3-^{32}P]dCTP were obtained from DuPont, NEN Products, Boston, MA. Oleic acid, sodium acetate, bovine serum albumin, acetyl CoA, and heat-inactivated fetal calf serum (FCS) were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin was purchased from Difco Laboratories, Detroit, MI. Nitrocellulose membranes, Hybond N, and Megaprime DNA labeling system 1606 were obtained from Amersham, Buckinghamshire, UK. "Ready to Go" DNA labeling kit was obtained from Pharmacia (Sweden). Autoradiography films were supplied by Fuji Medical X-ray film, Japan, and a phosphor imager and a densitometer from Molecular Dynamics (GmbH, Krefeld, Germany) were used. Thin-layer chromatography (TLC) plates (Silica gel F 1500) were from Schleicher and Schuell, Dassel, Germany; Dulbecco's modified Eagle's medium (DMEM), gentamicin, penicillin, streptomycin, and amphotericin were obtained from Bio Whittaker (Walkersville, MD). Tissue culture dishes were supplied by Costar (Cambridge, MA). BCA Protein Assay was from Pierce Laboratories Inc. (Rockford, IL). Dynabeads Direct kits and oligo-(dT)$_{25}$ Dynabeads were obtained from Dynal (Oslo, Norway). cDNA for human β-actin and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were provided by Clontech (Palo Alto, CA), and the RNA ladder was obtained from Gibco BRL (Sweden). Lovastatin was supplied from Merck, Sharpe & Dohme (Drammen, Norway). Cafestol (purity 99%) and a mixture of cafestol and kahweol (48% cafestol, 47% kahweol, 5% isokahweol, purity 98%) was prepared as free alcohols from coffee oil by R. Bertholet at Nestec Ltd. Research Center, Lausanne, Switzerland (R. Bertholet, Preparation of cafestol, US Patent 4692534, 1987). Isokahweol represents an isomer of kahweol present in oils extracted from spent coffee grounds at less than one tenth the concentration of kahweol (L. Fay, Nestec Ltd. Research Center, Lausanne, Switzerland, personal communication).

Cell culture

HSF have a well defined cholesterol metabolism and were therefore chosen as an in vitro model for studying the effect of cafestol. The fibroblasts were obtained from skin biopsies of two healthy individuals. The HSF cells were maintained in DMEM supplemented with gentamicin (60 μg/ml), l-glutamine (2 mm), penicillin (50 IU/ml), streptomycin (50 μg/ml), amphotericin (2 U/ml), and 20% heat-inactivated FCS at 37°C in a 95% air and 5% CO_2 atmosphere. The culture medium was changed once a week. For subcultures the media were removed, and the cells were detached from culture flasks with 0.25% trypsin. Culture medium with FCS was added to stop trypsination. Cells were then seeded at approximately 1×10^5 per dish (35 mm in diameter), and after 3 days the medium was changed to DMEM supplemented with lipoprotein-deficient human serum (LPDS, 5 mg/ml) for 24 h. All the studies with HSF were performed between the 10th and the 19th passages in culture.

Viability of HSF

The fibroblasts viability after cafestol exposure was examined by incorporation of [^{3}H]leucine into cell-associated proteins to measure protein synthesis. After confluency, the cells were preincubated for 16 h with DMEM supplemented with LPDS (5 mg/ml) and cafestol at concentrations 5–50 μg/ml. The cells were then incubated further for 2 h in the same media supplemented with 5 μCi/ml [^{3}H]leucine. Thereafter, the cells were washed six times with chilled 95% ethanol on ice and then solubilized in 0.2 m NaOH. The total protein-associated radioactivity was measured in a Packard TRI CARB 1900TR Scintillation Counter Spectrophotometer, and an aliquot was taken for protein determination using the BCA Protein Assay with bovine serum albumin (BSA) as standard (13). We also tested the cytotox-

icity after 18 h incubation of HSF with cafestol, cafestol–kahweol mixture, and 25-hydroxycholesterol by measuring the lactate dehydrogenase (LDH) leakage into the media with colorimetric kits provided by Boehringer Mannheim GmbH (Mannheim, Germany). The control cells in the [^{3}H]leucine and LDH experiments were exposed to 0.2% and 0.5% ethanol, respectively.

Isolation and labeling of LDL

LDL was isolated from freshly prepared plasma by sequential ultracentrifugation in a Centrikon T-2060 ultracentrifuge in the density range of 1.019–1.063 g/ml, in a TFT 70.38 rotor at 40,000 rpm for 24 h at 10°C (14). The preparation was dialyzed extensively against 0.15 m NaCl, 20 mm sodium phosphate, and 2 mm EDTA (PBS), pH 7.4. LDL was labeled with ^{125}I-labeled tyramine cellobiose ([^{125}I]TC) (15). The final preparations were dialyzed extensively against PBS. More than 97% of the radioactivity was precipitated by 10% (w/v) trichloroacetic acid (TCA). The final specific activity of ^{125}I-labeled LDL samples was in the range of 200–500 cpm/ng. Before use, the ^{125}I-labeled LDL was diluted with unlabeled LDL to a specific activity of 60 cpm/ng. LDL was stored in the presence of EDTA under nitrogen at 4°C and used within 1–2 weeks. Protein concentration was determined by the BCA Protein Assay (13).

Uptake and degradation of LDL

The cells were preincubated with LPDS medium containing either control (0.1 or 0.2% ethanol), cafestol (0.5–50 μg/ml), 25-hydroxycholesterol (0.5–10 μg/ml), a cafestol–kahweol–isokahweol mixture 48:47:5 (w/w), 1–50 μg/ml) or lovastatin (40 μg/ml) for up to 24 h. Thereafter, the cells were incubated for up to 24 h in fresh LPDS-medium containing test compounds together with radiolabeled LDL (5 μg protein/ml; specific activity 60 cpm/ng) at 37°C. After incubation the cells were chilled on ice and washed 6 times with PBS before the cells were harvested in 1 ml 0.1 m NaOH. Total cell-associated radioactivity was measured in a Packard Gamma Counter. The concentration of cell protein was determined as described previously (13). LDL labeled with radioiodinated TC results in trapping of the LDL-degradation products in the organelles where the degradation takes place. Accordingly, cell-associated radioactivity represents uptake of lipoproteins including degradation products. Degradation products were measured by precipitation with an equal volume of cold TCA (50%, w/v). To obtain a complete precipitation, a 10% BSA solution (2 μl per 1 ml) was used as a carrier. After centrifugation (3,000 rpm) for 10 min, radioactivity was measured in the TCA-soluble (degradation products of LDL) and the TCA-precipitable (undegraded products) fractions.

Binding of LDL

LDL binding assays were carried out at 4°C according to Brown and Goldstein (16). Confluent HSF were pretreated with LPDS-containing medium for 24 h before replacement with fresh LPDS medium containing either control (0.2% ethanol), cafestol (20 μg/ml), or 25-hydroxycholesterol (5 μg/ml) and incubated for 18 h. The cells were then chilled at 4°C and washed with cold, serum-free DMEM. Fresh, cold LPDS medium including 5 μg/ml [^{125}I]TC-LDL (60 cpm/ng) was added, and the dishes were then incubated at 4°C for 4 h. To determine the non-specific binding, identical dishes were incubated with the same concentration of [^{125}I]TC-LDL in the presence of 300 μg/ml of unlabeled LDL. The specific binding is represented by the difference between the total and the non-specific binding. In addition, protein-normalized Scatchard plots were performed and the binding data were analyzed according to the Scatchard methods (17).

Western immunoblotting

After confluency, fibroblasts were exposed to medium containing LPDS (5 mg protein/ml) for 24 h. Then the cells were incubated in the same medium for 24 h in the presence and absence of cafestol (20 μg/ml). Control cells were given ethanol (0.5%). The cells were washed twice in ice-cold PBS (pH 7.2) prior to the addition of lysis buffer (0.1 m NaCl, 20 mm Tris-HCl, pH 7.2, 10 mm EDTA, 1% Triton X-100, 1 mm PMSF (Sigma), 15.2 TIU (trypsin inhibitor units)/L aprotinin (Sigma). The cells were harvested and then shaken vigorously at 4°C for 30 min. The lysate was centrifuged at 12,000 rpm for 5 min at 4°C to remove all cell debris and nuclei. The protein concentration in the supernatant of each sample was determined by the BCA protein assay (Pierce) with BSA as standard. Equal amounts of protein were subjected to 8% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon PVDF filters (Millipore). Membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.2% Tween, followed by incubation at room temperature for 1 h with monoclonal anti-low density lipoprotein receptor (clone C7) RPN 537 (Amersham, Life Science). Proteins were detected by Vectastatin ABC kit (Vector Laboratories) based on the biotin/avidin system using biotinylated anti-rabbit IgG (H + L) for 1 h. The exposed membranes were quantified by laser-scanning densitometry.

Northern blot analysis

Total RNA was extracted by the guanidinium–isothiocyanate method with subsequent phenol/chloroform extractions according to Chomoczynski and Sacchi (18).

Total RNA (20 μg) was electrophoretically separated on 1% agarose/formaldehyde gel, followed by transblotting onto nitrocellulose filters. The filters were prehybridized at 65°C for 2 h under standard hybridization conditions (2 g/L polyvinylpyrrolidone, 2 g/L Ficoll-400, 2 g/L BSA, 0.05 m Tris-HCl, pH 7.5, 1 m NaCl, 2.2 mm sodium pyrophosphate, and 1% SDS, 100 μg/ml of denatured salmon testis DNA) and hybridized with a human cDNA probe for the LDL receptor (pSP 15) (19) after labeling with Megaprime DNA. The filter was washed for 15 min at 40°C with 2 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS, successively. The size of the mRNA was determined with reference to 18S and 28S rRNA, which were visualized by ethidium bromide staining. After autoradiography the signals were determined by scanning the film with a densitometer. To calibrate the mRNA signal levels with an internal standard in addition to total RNA measurements, filters were stripped and rehybridized with a probe for G3PDH (20).

mRNA (poly (A+) RNA) was also isolated from cells using Dynabeads mRNA Direct kit. Six μg poly (A+) RNA was separated on a 1% agarose gel containing 6.7% formaldehyde and transferred to a nylon membrane. cDNA probes for human β-actin and human LDL receptor (see above) were labeled with [^{32}P]dCTP, using "Ready to Go" DNA labeling kit. The prehybridization, hybridization, and washing steps were carried out at 65°C according to Church and Gilbert (21). The size of the mRNA was determined with reference to an RNA standard, which was visualized by methylene blue staining after it was cut off the nylon membrane before hybridization. Finally, the signals were analyzed by a phosphor imager from Molecular Dynamics Inc. and the signals were calibrated against β-actin as an internal standard.

DNA transfection

HSF were plated at 1 × 10^4 cells/cm^2 in 100-mm dishes and transfected 5 days after plating with 15 μg of DNA containing a synthetic active sterol regulatory element-1 (SRE-1) coupled to the gene for CAT. The SRE-1 promoter for the LDL receptor was kindly provided by Goldstein and Brown (22). Briefly, 15 μg of SRE-1-CAT DNA, 2 μg of SRαLac Z, and 3 μg of Bluescript KS+ in 190 μl of 1 mm Tris-HCl/0.05 mm EDTA at pH 7.5 were added to 30 μl 2 m $CaCl_2$ and mixed with 240 μl 2 × HEPES-buffered saline at pH 7.1 (280 mm NaCl, 1.5 mm $Na_2HPO_4 \cdot 2H_2O$, 50 mm HEPES). The precipitate was allowed to form for 30 min at room temperature and thereafter added dropwise to each monolayer. The cells were incubated for 4 h with the DNA, then washed once with 10 ml PBS, and shocked for 3 min with 5 ml 10% glycerol (v/v) in ordinary DMEM, followed by two rinses with 10 ml PBS and refed with 10 ml of DMEM containing LPDS (5 mg protein/ml) in the presence or absence of cafestol (10 μg/ml) or 25-hydroxycholesterol (5 μg/ml). After incubation for 23 h, the cells were harvested for measurement of β-galactosidase (23) and CAT activity (24). Transfected cells were washed twice with PBS, scraped into 180 μl of 0.25 m Tris-HCl/0.05 mm EDTA at pH 7.5, lysed by freezing and thawing six times, and centrifuged at 12,000 rpm for 5 min at 4°C.

Measurement of β-galactosidase

Forty μl of the supernatant obtained from the centrifugation described above was incubated at 37°C for 30–60 min with 140 μl of *o*-nitrophenyl-β-d-galactopyranoside (4 mg/ml) and 700 μl of (60 mm) $NaHPO_4$/(40 mm) NaH_2PO_4/(10 mm) KC1/1 mm) $MgSO_4 \cdot 7H_2O$. Reactions were stopped with 400 μl of 1 m Na_2CO_3, and the amount of *o*-nitrophenol formed was measured spectrophotometrically at 420 nm. The β-galactosidase activity was a measurement for the transfection efficiency.

Measurement of CAT activity

An aliquot of the supernatant was incubated in a final volume of 200 μl containing 4 μl of [^{14}C]chloramphenicol and 25 μl of acetyl CoA (2.6 mg/ml). The aliquot was calculated on the basis of β-galactosidase activity in order to calibrate for unequal transfection efficiency. After incubation for 1 h at 37°C, 1 ml of ice-cold ethyl acetate was added to the tubes and centrifuged at 12,000 rpm for 5 min at 4°C. The organic phase was dried and the residual extract was redissolved in 15 μl ethyl acetate. The ethyl acetylated reaction products were separated by TLC using chloroform–methanol 95:5 (v/v) as the developing solvent. After autoradiography the radioactive spots were cut out and counted in a scintillation counter.

Measurement of cholesterol synthesis

Cholesterol synthesis from [^{14}C]acetate was measured as described by Rustan et al. (25). Briefly, the cells were preincubated with LPDS medium containing either control (ethanol, 0.2%), cafestol (20 μg/ml), 25-hydroxycholesterol (5 μg/ml), or lovastatin (40 μg/ml) for 18 h. The medium was then exchanged with 1 ml medium containing 100 μm acetate including [^{14}C]acetate (1 μCi/ml) and further incubated up to 24 h at 37°C. At the end of the incubation, the culture plates were chilled on ice and the medium was aspirated before the cells were washed once with PBS. The cells were scraped into PBS, homogenized, and an aliquot was taken out for protein determination (13), prior to extraction of lipids (see below).

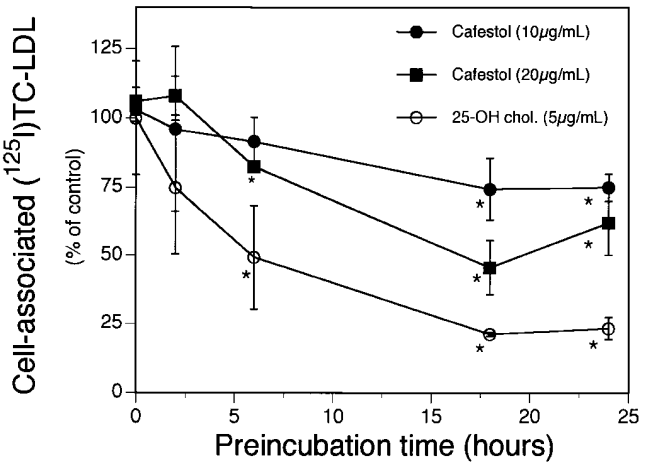


Fig. 2. Cell-association of [^{125}I]TC-LDL in HSF after different preincubation times with cafestol or 25-hydroxycholesterol. Cell-association of [^{125}I]TC-LDL was measured after preincubation with 10 μg/ml cafestol (●), 20 μg/ml cafestol (■), or 5 μg/ml 25-hydroxycholesterol (○). Control cells were given ethanol (0.2%). After confluency, cells were exposed to medium containing LPDS (5 mg protein/ml) for 24 h, then preincubated with cafestol and 25-hydroxycholesterol for indicated time points, and incubated with [^{125}I]TC-LDL (5 μg/ml, 60 cpm/ng) in fresh LPDS medium for 5 h at 37°C. The data are presented as % of control and show means ± SD. Each point is representative of triplicate measurements from three separate experiments. Absolute values for uptake of radiolabeled LDL in control cells at each time point were in the range 578 to 1575 ng LDL/mg cell protein. *Significant difference between cafestol or 25-hydroxycholesterol and control cells at $P < 0.05$.

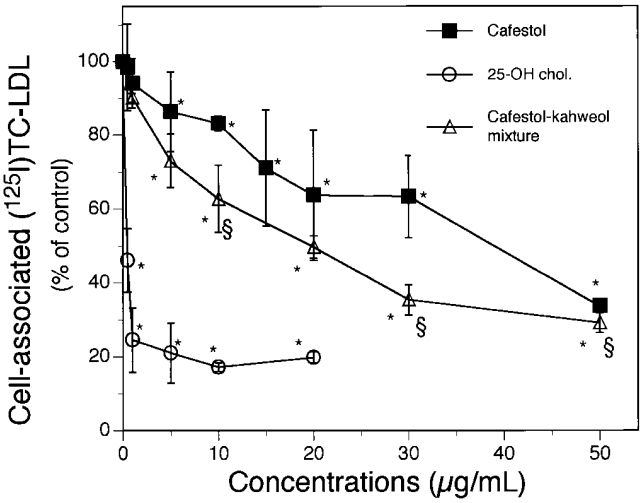


Fig. 3. Effect of cafestol, cafestol–kahweol mixture, and 25-hydroxycholesterol on cell-association of [^{125}I]TC-LDL in HSF. After confluency, fibroblasts were exposed to medium containing LPDS (5 mg protein/ml) for 24 h. Then the cells were incubated in fresh LPDS medium for 18 h with cafestol (■), cafestol–kahweol mixture (△), or 25-hydroxycholesterol (○). Control cells were given ethanol (0.2%). After preincubation, the cells were incubated with [^{125}I]TC-LDL (5 μg/ml, 60 cpm/ng) in fresh LPDS medium for another 5 h at 37°C, together with the test compounds. The data are presented as % of control and show means ± SD. Each point is representative of triplicate measurements from three separate experiments. Absolute values for uptake of radiolabeled LDL in control cells were 1069 ± 377 ng LDL/mg cell protein. *Significant difference between cafestol or 25-hydroxycholesterol and control cells at $P < 0.05$ and §significant difference between cafestol and cafestol–kahweol mixture incubated cells.

Measurement of cholesteryl ester formation

After the cells had reached confluency, they were preincubated with DMEM containing either control (ethanol, 0.2%), cafestol (20 μg/ml), or 25-hydroxycholesterol (5 μg/ml) for 18 h. The medium was then exchanged with 1 ml of medium containing 60 μm oleic acid bound to albumin and [^{14}C]oleic acid (1 μCi/ml) and further incubated up to 24 h at 37°C. The cells were then harvested and homogenized before measurement of protein and lipid extraction (see below).

Lipid extraction and separation

The lipids from the homogenized cell fractions were extracted with chloroform–methanol 2:1 (v/v) (26). The organic phase was dried under nitrogen at 40°C. The residual lipid extract was redissolved in 200 μl hexane and separated on TLC using hexane–diethyl ether–acetic acid 65:35:1 (v/v/v). The various lipids were identified by iodine, and the TLC foils were cut, added to 8 ml liquid scintillation fluid, and counted in Packard TRICARB 1900TR Scintillation Spectrophotometer.

Statistical analysis

The results are presented as means ± SD. Student's two-sample *t*-test (two-tailed) was used for calculation of statistical significance of the difference between the groups. The level of significance was set at $P < 0.05$.

RESULTS

Preincubation of fibroblasts with cafestol

To examine the effect of cafestol on the cell-association of [^{125}I]TC-LDL, we preincubated the HSF with cafestol for up to 24 h (**Fig. 2**). After 6 h preincubation time we observed 9% and 18% reduction in cell-associated LDL with 10 μg/ml and 20 μg/ml cafestol, respectively, and 26% and 55% reduction, respectively, after incubation for 18 h, as compared to control (Fig. 2). After 2 h of preincubation with 25-hydroxycholesterol (5 μg/ml) a 26% reduction in cell-associated LDL was observed, and after 18 h the cell-associated LDL was re-

TABLE 1. Incorporation of [^{3}H]leucine into cell-associated proteins and lactate dehydrogenase activity

Treatment	Cell-Associated [^{3}H]Leucine	Lactate Dehydrogenase Activity
μg/ml	*10^{-3} × dpm/mg cell protein*	*% leakage*[a]
Control	127 ± 7	4.9 ± 4.6
Cafestol		
5	123 ± 15	5.1 ± 6.1
10	150 ± 8	6.8 ± 5.6
20	146 ± 11	6.0 ± 5.9
50	162 ± 10	8.5 ± 9.0
Cafestol–kahweol mixture		
10	ND	2.9 ± 1.3
20	ND	5.7 ± 5.1
25-Hydroxycholesterol		
5	175 ± 14	8.0 ± 6.8

Values for [^{3}H]leucine incorporation into cell-associated protiens are given as means ± SD from six separate cultures, and values for lactate dehydrogenase are given as means ± SD of three separate experiments performed in duplicate; ND, not determined.

[a]One hundred percent leakage is similar to the lactate dehydrogenase activity measured in the cell media after incubation with 2% Triton X-100 for 18 h.

duced by about 80%. The degradation curves for LDL showed a similar pattern as for the cell-associated LDL for cells treated with cafestol or 25-hydroxycholesterol (data not shown). These data suggest that the maximum effect of cafestol was obtained after 18 h preincubation time with cafestol.

Concentration effect of cafestol, kahweol, and 25-hydroxycholesterol

Cafestol, as well as 25-hydroxycholesterol, caused a marked concentration-dependent decrease in cell-associated LDL after 18 h preincubation time (**Fig. 3**). By increasing the concentration of cafestol from 10 to 50 μg/ml we observed a significant reduction in cell-associated LDL from 17% to 66% ($P < 0.05$). No significant cytotoxic effect was observed during the incubation time, as evaluated by microscopy and as measured by both incorporation of [^{3}H]leucine into cell-associated proteins and leakage of lactate dehydrogenase into the medium (**Table 1**).

A similar concentration-dependent rate of uptake of radiolabeled LDL was achieved with cafestol–kahweol mixture similar to what was obtained with the cafestol. However, the cafestol–kahweol mixture at concentrations 10, 30, and 50 μg/ml was significantly more potent than cafestol in inhibiting the LDL uptake by HSF (Fig. 3). No cytotoxic effect was observed after 18 h incubation with the cafestol–kahweol mixture (10 and 20 μg/ml), as measured by the release of LDH (Table 1). Concentrations of 25-hydroxycholesterol from 2 to 20 μg/ml caused an approximately 80% reduction in cell-associated LDL ($P < 0.05$).

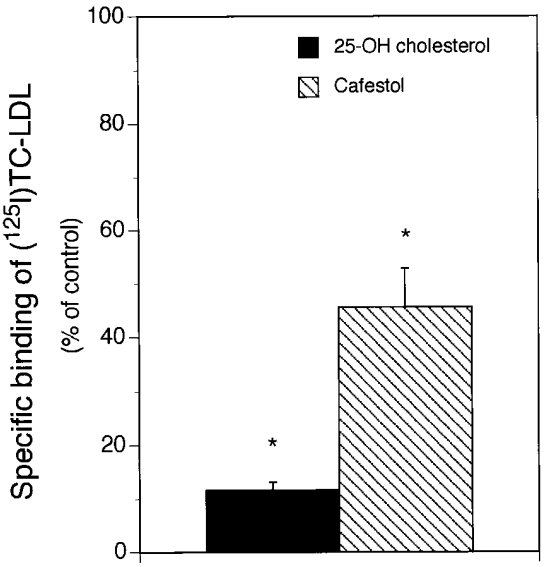


Fig. 4. Specific binding of [^{125}I]TC-LDL to HSF. Confluent cells were incubated with LPDS medium (5 mg/ml) for 24 h. Then the fibroblasts were preincubated for 18 h in LPDS medium with either cafestol (20 μg/ml) or 25-hydroxycholesterol (5 μg/ml) before the binding assay was carried out as described in Methods. Control cells were given ethanol (0.2%). The data are presented as % of control and show means ± SD. Each point is representative of triplicate measurements from three separate experiments. Absolute values for binding of radiolabeled LDL in control cells were 52.6 ± 15.6 ng LDL/mg cell protein. *Significant difference between cafestol or 25-hydroxycholesterol and control cells at $P < 0.05$.

LDL binding by fibroblasts

To examine whether the cafestol-mediated decrease in the cellular uptake and degradation of radiolabeled LDL was due to a reduced number of LDL receptors on the cell surface, binding experiments were performed at 4°C. Cafestol reduced the specific binding of radiolabeled LDL by 54% ($P < 0.05$), whereas 25-hydroxycholesterol reduced the specific binding by 88% ($P < 0.05$) (**Fig. 4**). The nonspecific binding for control, cafestol, and 25-hydroxycholesterol incubated cells was 40 ± 14% (n = 3), 62 ± 11% (n = 3), and 88 ± 4 (n = 3; $P < 0.05$ versus control), respectively. Moreover, no effect on the binding of radiolabeled LDL in the presence of cafestol during the binding experiment was seen (data not shown). In addition, we also performed a protein-normalized Scatchard analysis of LDL receptor binding after incubation of HSF for 18 h in the absence or presence of cafestol (20 μg/ml). Intersection of the best fit-line with the x-axis confirms that cafestol-incubated HSF reduces the binding sites for LDL on the cell surface by nearly 20% compared to control cells (B_{max}, **Table 2**, n = 3; $P < 0.05$). However, the binding affinity was not affected after cafestol exposure (K_d, Table 2).

TABLE 2. Protein-normalized Scatchard analysis of the binding of radiolabeled LDL to the cell surface of the fibroblasts

Treatment	Dose	B_{max}	K_d
	μg/ml	*ng LDL/mg cell protein*	*μg LDL/mg cell protein*
Control		320.2 ± 18.0	101.8 ± 46.1
Cafestol	20	255.4 ± 23.6[a]	115.4 ± 53.3

Binding assays at 4°C were carried out as described in Methods. Values represent means ± SD out of three separate experiments performed in duplicate; B_{max}, total receptor concentration; and K_d, dissociation constant.

[a]Significant difference between cafestol and control cells at $P < 0.05$.

Western blotting of the LDL receptor

To verify the fact that cafestol decreased the binding of radiolabeled LDL (Fig. 4) and the amount of receptors (B_{max}, Table 2), we quantified the LDL receptor by immunoblotting (**Fig. 5**). The figure shows that the amount of LDL receptors was reduced by approximately 25% ($P < 0.01$) after 24 h incubation with cafestol (20 μg/ml).

Expression of the LDL receptor mRNA

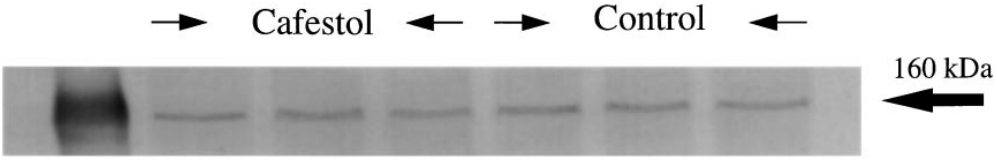
Cells incubated for 23 h with cafestol (20 μg/ml) showed no decrease in the mRNA level, as compared to control cells, whereas the expression of mRNA for the LDL receptor was abolished after treatment with 25-hydroxycholesterol (5 μg/ml) (data not shown). In addition, we also isolated poly (A+) RNA which is a more sensitive method for detection of small variation on the mRNA level. To examine a possible time-dependent effect of cafestol exposure, poly (A+) RNA was isolated after 11 and 23 h incubation with cafestol (20 μg/ml) and 25-hydroxycholesterol (5 μg/ml). No significant differences were observed at the mRNA level for the LDL receptor after incubation with cafestol, as compared to control cells (**Fig. 6**), whereas 25-hydroxycholesterol reduced the LDL receptor mRNA by 80% and 90% (n = 6; $P < 0.05$; Fig. 6).

Interaction of cafestol or 25-hydroxycholesterol with SRE-1

To further study the mechanism for the reduced uptake of radiolabeled LDL, we transfected fibroblasts with an SRE-1 promoter element coupled to the gene for CAT. In this way we could examine the transcriptional effect of cafestol on the LDL receptor gene. The SRE-1 region is a positive element that enhances the LDL receptor gene transcription in the absence of sterol. No difference was observed in CAT activity after incubation of HSF with cafestol (20 μg/ml) (**Fig. 7**), whereas 25-hydroxycholesterol (5 μg/ml) reduced the CAT activity by 52% (n = 4; $P < 0.05$; Fig. 7).

Synthesis of cholesterol and cholesteryl ester

After 18 h preincubation with cafestol (20 μg/ml), the incorporation of radiolabeled acetate into cholesterol was reduced by 34–38% ($P < 0.05$) after 12 to 24 h incubation (**Fig. 8A**). In comparison, 25-hydroxycholesterol



	Experiment 1	Experiment 2 (Relative density)	Experiment 3
Control (ethanol, 0.5 %)	1.00 ± 0.09	1.00 ± 0.04	1.00 ± 0.11
Cafestol (20 μg/ml)	0.72 ± 0.16	0.69 ± 0.13	0.83 ± 0.09

Fig. 5. The effect of cafestol on the amount of protein for the LDL receptor. After confluency, fibroblasts were exposed to medium containing LPDS (5 mg protein/ml) for 24 h. Then the cells were incubated in fresh LPDS medium for 24 h with cafestol (20 μg/ml). Control cells were given ethanol (0.5%). The cells were harvested and lysed as described in Materials and Methods. The figure shows an Immunobilon filter from one typical experiment. Data represent means ± SD of three wells pr experiment. The values from exp. 1, exp. 2, and exp. 3 were added, and data represent a difference between control and cafestol medium at $P < 0.01$ calculated for the overall experiments.

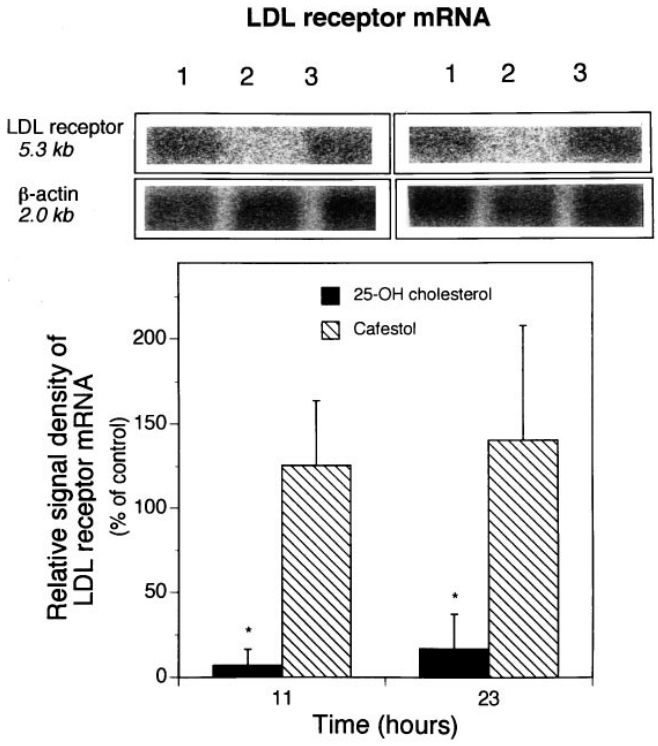


Fig. 6. The effect of cafestol and 25-hydroxycholesterol on the amount of mRNA for the LDL receptor and β-actin. Confluent HSF were incubated with LPDS medium (5 mg/ml) for 24 h, before the cells were incubated for 11 and 23 h in fresh LPDS medium with either control (ethanol, 0.1%, lane 1), 25-hydroxycholesterol (5 μg/ml, lane 2), or cafestol (20 μg/ml, lane 3). Isolation of poly (A+) RNA and Northern blot analysis were performed as described in Methods. The upper panel shows the autoradiogram for one typical experiment out of six separate experiments performed. The lower panel shows means ± SD of LDL receptor mRNA expression. Each value represents the mean ± SD of six separate experiments given as % of control cells. Each experiment was based on cells pooled from two cell cultures. *Significant difference between 25-hydroxycholesterol and control cells at $P < 0.05$.

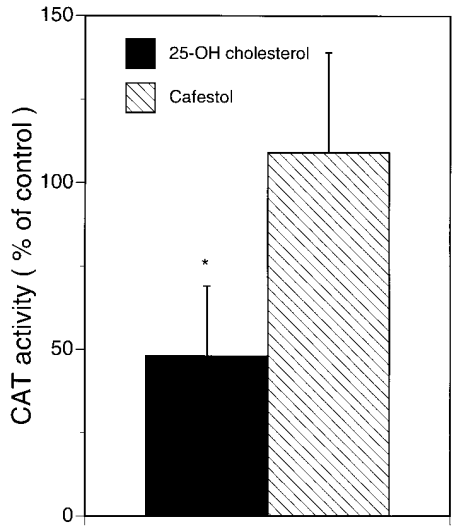


Fig. 7. Effect of cafestol and 25-hydroxycholesterol on chloramphenicol acetyl transferase (CAT) activity in cells transfected with the SRE-1 element. After 90% confluency, the cells were transfected with the SRE-1 coupled to the CAT gene as described in Methods. Thereafter, the cells were incubated for 23 h with LPDS medium containing either ethanol (0.1%), cafestol (20 μg/ml), or 25-hydroxycholesterol (5 μg/ml). Data represent means ± SD of duplicate measurements from four separate experiments. *Significant difference between 25-hydroxycholesterol and control cells at $P < 0.05$.

(5 μg/ml) and lovastatin (50 μg/ml, data not shown) decreased the incorporation of radiolabeled acetate into cholesterol by approximately 90% after 6 h of incubation ($P < 0.05$). Cafestol (20 μg/ml) as well as 25-hydroxycholesterol (5 μg/ml) stimulated the cholesterol esterification. Incorporation of radiolabeled oleic acid into cholesteryl ester increased 2.3-fold ($P < 0.05$) and 3.3-fold ($P < 0.05$) with cafestol and 25-hydroxycholesterol, respectively, after 24 h incubation (Fig. 8B).

DISCUSSION

Our results demonstrate that the coffee lipid, cafestol, significantly reduced the binding, uptake, and degradation of radiolabeled LDL in human fibroblasts. In addition, a reduced amount of LDL receptor protein was confirmed by immunoblotting. Furthermore, a reduction in the synthesis of cholesterol and an increase in cholesterol esterification were observed after cafestol exposure. In contrast, there was no significant effect of cafestol on the transcription of the LDL receptor gene, as assessed by measuring the mRNA level of the LDL receptor and by measuring regulation of gene transcription via the sterol regulatory element. A possible explanation for these observations is that cafestol might act similarly to sterols in the regulation of certain gene products of importance for the metabolism of cholesterol. However, cafestol was not as potent as 25-hydroxycholesterol in reducing LDL binding, uptake, and degradation. Our data suggest that the elevation of plasma cholesterol after intake of boiled coffee may be due to a down-regulation of the activity of LDL receptor.

The unaltered expression of mRNA levels for the LDL receptor after cafestol treatment demonstrates that cafestol does not exert its effect on the LDL receptor gene level and suggests a post-transcriptional-mediated effect of cafestol (Fig. 6). In contrast, incubation with 25-hydroxycholesterol abolished the expression of mRNA for the LDL receptor. Similarly, HSF transfected with a synthetic promoter region for the LDL receptor gene

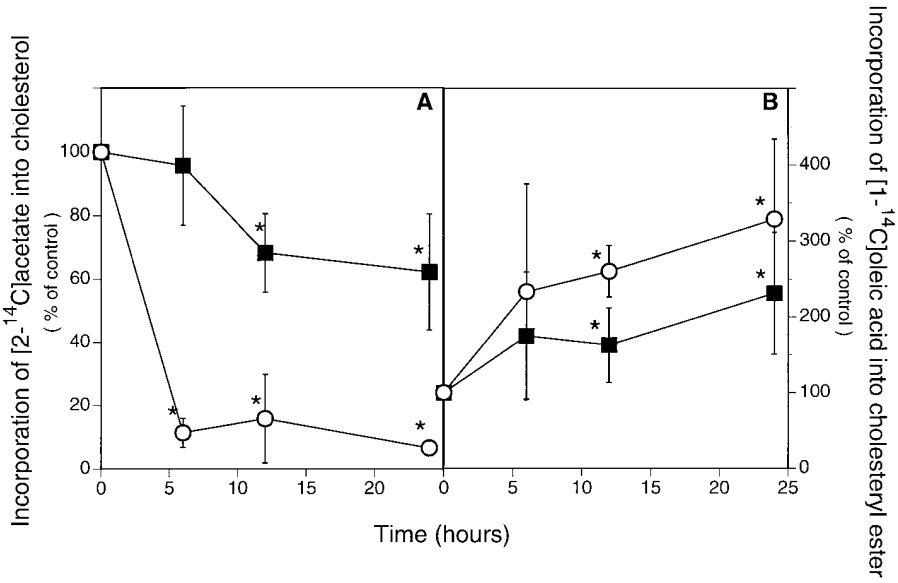


Fig. 8. Time course for incorporation of [2-^{14}C]acetate into cholesterol (panel A) and incorporation of [1-^{14}C]oleic acid into cholesteryl esters (panel B). After confluency the cells were grown in either LPDS medium (panel A) or DME medium containing 20% FCS (panel B) for 24 h. Then the cells were preincubated for 18 h with cafestol (20 μg/ml) (■) or 25-hydroxycholesterol (5 μg/ml) (○). After 18 h of preincubation, fresh medium was provided to the cells together with the test compounds with either radiolabeled acetate (panel A) or radiolabeled oleic acid (panel B), and mixtures were incubated further for the indicated time points. Control cells were given ethanol (0.1%). The data are presented as % of control and show means ± SD of six to twelve cultures. Control values were after 6, 12, and 24 h incubation with [2-^{14}C]acetate (panel A) in the range of 2.1–2.2 nmol/mg cell protein, 1.8–4.2 nmol/mg cell protein, and 1.8–5.2 nmol/mg cell protein, respectively. Control values were after 6, 12, and 24 h incubation with [1-^{14}C]oleic acid (panel B) in the range of 0.2–1.0 nmol/mg cell protein, 0.3–5.5 nmol/mg cell protein, and 2.9–7.6 nmol/mg cell protein, respectively. *Significant difference between cafestol or 25-hydroxycholesterol and control cells at $P < 0.05$.

showed a 52% reduction in CAT activity after exposure of 25-hydroxycholesterol while cafestol had no effect (Fig. 7). These findings are in line with data obtained from studies in HepG2 cells (27) suggesting a post-transcriptional regulation of the LDL receptor. Interestingly, a post-transcriptional regulation of LDL receptor activity in Watanabe heritable hyperlipidemic rabbit fibroblasts that were transduced with a retroviral vector containing the LDL receptor gene has been shown (28).

Two essential genes in the cholesterol metabolism, HMG-CoA synthase and LDL receptor, are known to possess the sterol regulatory element (SRE-1) in their promoter regions (29). These elements are able to bind sterol regulatory element binding protein-1 (SREBP-1) and thereby increase the transcription of these genes. SREBP-1 is anchored to the cholesterol-poor endoplasmic reticulum (ER). When the cells are depleted of sterols, this membrane-bound precursor is cleaved by a protease and translocated to the nucleus where it binds to SRE-1 and thereby activates the genes. It is speculated that the cholesterol-mediated changes in the ER membrane may activate the cleavage of the active SREBP-1 (29–31). The SRE-1-transfected cells incubated with 25-hydroxycholesterol in the present study showed a reduced CAT activity, which is in agreement with former studies (27, 32), whereas SRE-1-transfected cells incubated with cafestol showed no reduction in CAT activity, as compared to control cells. These data, in combination with the unchanged mRNA level for the LDL receptor in the presence of cafestol, indicate that cafestol is not involved in the transcription of the LDL receptor gene, whereas 25-hydroxycholesterol probably regulates the transcription of the LDL receptor gene by an inhibition of the cleavage of the SREBP-1.

Our results clearly show that the decrease in binding, uptake, and degradation of radiolabeled LDL (Figs. 2–4 and Table 2), as well as the reduced amount of LDL receptor protein (Fig. 5) is caused by cafestol. A possible explanation for this decrease induced by cafestol may be a reduced transport of newly synthesized LDL receptors to the cell surface or an intracellular accumulation of newly synthesized LDL receptors (33, 34). Alternatively, the phosphorylation/dephosphorylation of key regulatory proteins in cholesterol metabolism may

be affected by cafestol. A phosphorylation/dephosphorylation mechanism for regulation of the scavenger receptor has recently been described (35, 36).

In order to reveal a potentially increased degradation of newly synthesized LDL receptor after cafestol (20 μg/ml) incubation, protein synthesis was inhibited by cycloheximide. Then, binding of [^{125}I]TC-LDL up to 24 h was measured. No effect of cafestol was seen on the binding of radiolabeled LDL, indicating that cafestol probably does not cause increased degradation of the receptor (data not shown). It has previously been reported that HMG-CoA reductase is regulated through a sterol-dependent degradation of its protein (37, 38). In addition, ubiquitin-dependent protein degradation has been reported for several receptors as an important regulatory mechanism (39).

Our data also demonstrate that cafestol, like 25-hydroxycholesterol, influences the synthesis of cholesterol and cholesteryl ester promoted by HMG-CoA reductase and ACAT, respectively, as assessed by indirect measurements (Fig. 8). Both enzymes are controlled by the cellular concentration of sterols (40–42). ACAT functions as a cholesterol buffer in the cells; an elevated pool of intracellular cholesterol will cause increased esterification of cholesterol (43). Conflicting data have been reported concerning the regulation of ACAT by protein phosphorylation (44–46).

The mechanism responsible for the observed changes in HMG-CoA reductase activity after exposure to cafestol is not clear. In some cell lines, it has been shown that the rate of synthesis of the enzyme is controlled by oxysterols acting both on transcriptional and translational stages. HMG-CoA reductase is a highly regulated enzyme, with its catalytic activity influenced by the amount (synthesis and degradation), as well as the activation state (phosphorylation and dephosphorylation) of the enzyme (47–49).

In contrast to HSF, incubation of CaCo-2 cells, a human cancer colon cell line, with cafestol enhanced the rate of uptake and degradation of radiolabeled LDL by 50%, whereas 25-hydroxycholesterol caused a 30% decrease in both the uptake and degradation of radiolabeled LDL (32). A 20% increase in uptake of radiolabeled LDL was also seen in P19 cells, a murine embryonic carcinoma cell line, and in J774 cells, a murine macrophage-like cell line, while 25-hydroxycholesterol decreased the rate of uptake of radiolabeled LDL in both these cell lines (unpublished data). These data demonstrated that cafestol and 25-hydroxycholesterol may influence the cholesterol metabolism differently in different cell lines. These observed differences in the regulation of LDL receptor may be caused by the fact that mucosal cells (CaCo-2 cells), liver cells (HepG2 cells), and fibroblasts have different metabolic functions. Interestingly, cafestol is the only dietary lipid known to have this ability to regulate cholesterol metabolism differently in different cell types. The effects of cafestol and kahweol seem to be unique to humans. The same batch of coffee that raised cholesterol in humans produced no effect in monkeys, hamsters, or rabbits. One may speculate that differences in absorption or metabolism of coffee diterpenes account for this marked species specificity (8).

In conclusion, our studies showed that cafestol decreased the binding, uptake, and degradation of LDL in HSF. In addition, cafestol decreased the LDL receptor protein level. Cafestol also reduced the cholesterol synthesis and increased the cholesterol esterification in these cells. The mRNA level for the LDL receptor was unaffected after incubation of HSF with cafestol, and cells transfected with the synthetic promoter SRE-1 for the LDL receptor and HMG-CoA synthase were insensitive to cafestol. These findings suggest that the mechanisms responsible for the cholesterol-raising effects of cafestol involve a post-transcriptional down-regulation of the LDL receptor.

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REFERENCES

1. Thelle, D. S., E. Arnesen, and O. H. Førde. 1983. The Tromsø heart study. Does coffee raise serum cholesterol? *N. Engl. J. Med.* **308:** 1454–1457.
2. Bønaa, K., E. Arnesen, D. S. Thelle, and O. H. Førde. 1988. Coffee and cholesterol: is it all in the brewing? The Tromsø Study. *Br. Med. J.* **297:** 1103–1104.
3. Aro, A., J. Teirila, and C. G. Gref. 1990. Dose-dependent effect on serum cholesterol and apoprotein B concentrations by consumption of boiled, non-filtered coffee. *Atherosclerosis* **83:** 257–261.
4. Aro, A., J. Tuomilehto, E. Kostiainen, U. Uusitalo, and P. Pietinen. 1987. Boiled coffee increases serum low density lipoprotein concentration. *Metabolism* **36:** 1027–1030.
5. Bak, A. A., and D. E. Grobbee. 1989. The effect on serum cholesterol levels of coffee brewed by filtering or boiling. *N. Engl. J. Med.* **321:** 1432–1437.

6. Ratnayake, W. M., R. Hollywood, E. O'Grady, and B. Stavric. 1993. Lipid content and composition of coffee brews prepared by different methods. *Food Chem. Toxicol.* **31:** 263–269.
7. van Dusseldorp, M., M. B. Katan, T. van Vliet, P. N. Demacker, and A. F. Stalenhoef. 1991. Cholesterol-raising factor from boiled coffee does not pass a paper filter. *Arterioscler. Thromb.* **11:** 586–593.
8. Urgert, R., and M. B. Katan. 1996. The cholesterol-raising factor from coffee beans. *J. R. Soc. Med.* **89:** 618–623.
9. Zock, P. L., M. B. Katan, M. P. Merkus, M. van Dusseldorp, and J. L. Harryvan. 1990. Effect of a lipid-rich fraction from boiled coffee on serum cholesterol. *Lancet.* **335:** 1235–1237.
10. Weusten-Van der Wouw, M. P., M. B. Katan, R. Viani, A. C. Huggett, R. Liardon, R. Liardon, P. G. Lund-Larsen, D. S. Thelle, I. Ahola, A. Aro, S. Meyboom, and A. C. Beynen. 1994. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. *J. Lipid. Res.* **35:** 721–733.
11. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
12. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature* **343:** 425–430.
13. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76–85.
14. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
15. Pittman, R. C., T. E. Carew, C. K. Glass, S. R. Green, C. Taylor, Jr., and A. D. Attie. 1983. A radioiodiated, intracellularly trapped ligand for determining the sites of plasma protein degradation in vivo. *Biochem. J.* **212:** 791–800.
16. Brown, M. S., and J. L. Goldstein. 1975. Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. *Cell.* **6:** 307–316.
17. Hulme, E. C., and N. J. M. Birdsall. 1992. Strategy and tactics in receptor-binding studies. *In* Receptor–Ligand Interactions: a Practical Approach. E. C. Hulme, editor. Oxford University Press, New York, NY. 63–174.
18. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
19. Peacock, S. L., M. P. Bates, D. W. Russell, M. S. Brown, and J. L. Goldstein. 1988. Human low density lipoprotein receptor expressed in *Xenopus* oocytes. Conserved signals for O-linked glycosylation and receptor-mediated endocytosis. *J. Biol. Chem.* **263:** 7838–7845.
20. Arcari, P., R. Martinelli, and F. Salvatore. 1984. The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple mRNA species. *Nucleic Acids Res.* **12:** 9179–9189.
21. Church, G. M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* **81:** 1991–1995.
22. Yokoyama, C., X. Wang, M. R. Briggs, A. Admon, J. Wu, X. Hua, J. L. Goldstein, and M. S. Brown. 1993. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell.* **75:** 187–197.
23. Lee, F., C. V. Hall, G. M. Ringold, D. E. Dobson, J. Luh, and P. E. Jacob. 1984. Functional analysis of the steroid hormone control region of mouse mammary tumor virus. *Nucleic Acids Res.* **12:** 4191–4206.
24. Gorman, C. M., L. F. Moffatt, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2:** 1044–1051.
25. Rustan, A. C., J. O. Nossen, H. Osmundsen, and C. A. Drevon. 1988. Eicosapentaenoic acid inhibits cholesterol esterification in cultured parenchymal cells and isolated microsomes from rat liver. *J. Biol. Chem.* **263:** 8126–8132.
26. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
27. Edwards, P. A., A. M. Fogelman, and G. Popjak. 1976. A direct relationship between the amount of sterol lost from rat hepatocytes and the increase in activity of HMG-CoA reductase. *Biochem. Biophys. Res. Commun.* **68:** 64–69.
28. Sharkey, M. F., A. Miyanohara, R. L. Elam, T. Friedmann, and J. L. Witztum. 1990. Post-transcriptional regulation of retroviral vector-transduced low density lipoprotein receptor activity. *J. Lipid. Res.* **31:** 2167–2178.
29. Wang, X., M. R. Briggs, X. Hua, C. Yokoyama, J. L. Goldstein, and M. S. Brown. 1993. Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. *J. Biol. Chem.* **268:** 14497–14504.
30. Wang, X., R. Sato, M. S. Brown, X. Hua, and J. L. Goldstein. 1994. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell.* **77:** 53–62.
31. Gasic, G. P. 1994. Basic-helix-loop-helix transcription factor and sterol sensor in a single membrane-bound molecule. *Cell.* **77:** 17–19.
32. Ranheim, T., B. Halvorsen, A. C. Huggett, R. Blomhoff, and C. A. Drevon. 1995. Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells. *J. Lipid. Res.* **36:** 2079–2089.
33. Edwards, E. H., E. A. Sprague, J. L. Kelley, J. J. Kerbacher, C. J. Schwartz, and A. D. Elbein. 1989. Castanospermine inhibits the function of the low-density lipoprotein receptor. *Biochemistry.* **28:** 7679–7687.
34. Lodish, H. F., and N. Kong. 1984. Glucose removal from N-linked oligosaccharides is required for efficient maturation of certain secretory glycoproteins from the rough endoplasmic reticulum to the Golgi complex. *J. Cell Biol.* **98:** 1720–1729.
35. Fong, L. G. 1994. Posttransitional regulation of macrophage scavenger receptor by protein phosphorylation. *Circulation.* **90(suppl I):** I–189. Abstract.
36. Fong, L. G. 1996. Modulation of macrophage scavenger receptor transport by protein phosphorylation. *J. Lipid. Res.* **37:** 574–587.
37. Tanaka, R. D., A. C. Li, A. M. Fogelman, and P. A. Edwards. 1986. Inhibition of lysosomal protein degradation inhibits the basal degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid. Res.* **27:** 261–273.
38. Tanaka, R. D., P. A. Edwards, S. F. Lan, and A. M. Fogelman. 1983. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in avian myeloblasts. Mode of action of 25-hydroxycholesterol. *J. Biol. Chem.* **258:** 13331–13339.
39. Hochstrasser, M. 1996. Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* **30:** 405–439.
40. Drevon, C. A., D. B. Weinstein, and D. Steinberg. 1980. Regulation of cholesterol esterification and biosynthesis

in monolayer cultures of normal adult rat hepatocytes. *J. Biol. Chem.* **255:** 9128–9137.
41. Drevon, C. A., A. D. Attie, S. H. Pangburn, and D. Steinberg. 1981. Metabolism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *J. Lipid. Res.* **22:** 37–46.
42. Hashimoto, S., C. A. Drevon, D. B. Weinstein, J. S. Bernett, S. Dayton, and D. Steinberg. 1983. Activity of acyl-CoA: cholesterol acyltransferase and 3-hydroxy-3-methylglutaryl-CoA reductase in subfractions of hepatic microsomes enriched with cholesterol. *Biochim. Biophys. Acta.* **754:** 126–133.
43. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
44. Gavey, K. L., D. L. Trujillo, and T. J. Scallen. 1983. Evidence for phosphorylation/dephosphorylation of rat liver acyl-CoA:cholesterol acyltransferase. *Proc. Natl. Acad. Sci. USA.* **80:** 2171–2174.
45. Suckling, K. E., E. F. Stange, and J. M. Dietschy. 1983. In vivo modulation of rat liver acyl-coenzyme A:cholesterol acyltransferase by phosphorylation and substrate supply. *FEBS Lett.* **159:** 29–32.
46. Kwok, C. T., J. L. Smith, S. P. Pillay, and I. R. Hardie. 1984. Inactivation of acyl-CoA:cholesterol acyltransferase by ATP+ Mg2+ in rat liver microsomes. *Biochem. Int.* **8:** 271–282.
47. Faust, J. R., K. L. Luskey, D. J. Chin, J. L. Goldstein, and M. S. Brown. 1982. Regulation of synthesis and degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by low density lipoprotein and 25-hydroxycholesterol in UT-1 cells. *Proc. Natl. Acad. Sci. USA.* **79:** 5205–5209.
48. Luskey, K. L., and B. Stevens. 1985. Human 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Conserved domains responsible for catalytic activity and sterol-regulated degradation. *J. Biol. Chem.* **260:** 10271–10277.
49. Omkumar, R. V., and V. W. Rodwell. 1994. Phosphorylation of Ser^{871} impairs the function of His^{865} of Syrian hamster 3-hydroxy-3-methylglutaryl-CoA reductase. *J. Biol. Chem.* **269:** 16862–16866.