Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells

Trine Ranheim, Bente Halvorsen, Anthony C. Huggett,* Rune Blomhoff, and Christian A. Drevon[1]

Section for Dietary Research, Institute for Nutrition Research, University of Oslo, Norway, and Nestec Research Centre,* Lausanne, Switzerland

Abstract The influence of cafestol, a lipid component found in boiled coffee, on low density lipoprotein (LDL) and lipid metabolism was investigated in CaCo-2 cells cultured on filter membranes. The rate of uptake and degradation of ^{125}I-labeled tyramine cellobiose-LDL was increased 50% in CaCo-2 cells incubated with cafestol (20 μg/ml, 63 μM) for 24 h, whereas in cells incubated with 25-hydroxycholesterol (10 μg/ml, 25 μM) the rate of uptake and degradation showed a 30% decrease. A mixture of kahweol and cafestol, both natural components of coffee beans, modestly enhanced the rate of LDL uptake and degradation, as compared to pure cafestol. Incubation of cafestol with CaCo-2 cells induced a 3-fold up-regulation of LDL receptor mRNA, as compared to control cells. In contrast, incubation of the cells with 25-hydroxycholesterol produced a 30% decrease of LDL receptor expression. CaCo-2 cells were transfected with a promoter region containing the sterol regulatory element-1 (SRE-1) coupled to the reporter gene chloramphenicol acetyltransferase (CAT). When cells transfected with SRE-1 promoter were incubated with cafestol, there was a 20% up-regulation of CAT activity, whereas 25-hydroxycholesterol abolished this activity. Cafestol contributed to a significantly lowered secretion of cholesteryl ester and triacylglycerol, regardless of the radiolabeled precursor used ([2-^{14}C]acetic acid, [1,2,3-^{3}H]glycerol, [^{3}H]water, and [1-^{14}C]oleic acid). This reduction in secretion of lipids was accompanied by an increase in trichloroacetic acid-soluble activity when radiolabeled oleic acid was used as a tracer. We conclude that cafestol promotes an enhanced rate of uptake and degradation of LDL, probably due to an increase in transcription of LDL receptor mRNA and a reduced secretion of cholesteryl ester and triacylglycerol in CaCo-2 cells.—**Ranheim, T., B. Halvorsen, A. C. Huggett, R. Blomhoff, and C. A. Drevon.** Effect of a coffee lipid (cafestol) on regulation of lipid metabolism in CaCo-2 cells. *J. Lipid Res.* 1995. **36:** 2079–2089.

Supplementary key words cafestol • 25-hydroxycholesterol • triacylglycerol • cholesteryl ester • low density lipoprotein • intestine • CaCo-2 cells

More than a decade ago, Thelle, Arnesen, and Førde (1) reported a positive correlation between the amount of coffee ingested and serum cholesterol levels in a population-based study in Northern Norway. Although these findings were confirmed in several subsequent studies (2–4), other studies have not shown a cholesterol-raising effect of coffee consumption (5). It was later shown that the method of brewing was an important factor for the hypercholesterolemic effect of coffee. In contrast to boiled coffee, consumption of filtered coffee was found to have little or no association with serum concentration of cholesterol (6–9). Van Dusseldorp et al. (10) demonstrated that the cholesterol-raising effects of boiled coffee could be removed by paper filtration and that this was, at least in part, due to a general adsorption of coffee lipids onto the paper. Ratnayake et al. (11) proposed that the effect was mainly due to removal of suspended coffee particles and not due to a selective adsorption of a particular lipidemic component by the paper filter. Zock et al. (12) prepared a lipid-rich fraction of boiled coffee by centrifugation, and administered it to 10 volunteers for 6 weeks. This treatment resulted in raised serum concentration of LDL cholesterol and triacylglycerol. Recently, Weusten-Van der Wouw et al. (13) showed that cafestol and possibly kahweol, two coffee-specific diterpenes present in coffee beans that are extracted into the lipid fraction of boiled coffee (**Fig. 1**), raised serum cholesterol levels in a controlled clinical trial. In contrast, coffee oil from which cafestol and kahweol had been removed produced no effects on serum cholesterol levels.

The mechanism by which these components raise serum cholesterol levels is unknown. A potential site of

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NaTC, sodium taurocholate; TG, triacylglycerol; CM, chylomicron; LDL, low density lipoprotein; ^{125}I-TC-LDL, ^{125}I-labeled tyramine cellobiose-labeled low density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; TLC, thin-layer chromatography.

[1]To whom correspondence should be addressed.

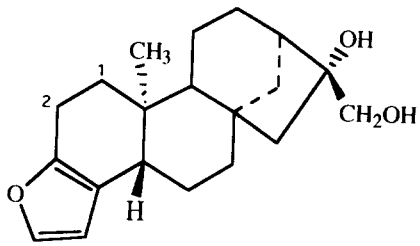

Fig. 1. Chemical structure of cafestol. Kahweol has an additional double bond between C1 and C2.

action for cafestol and kahweol may be the low density lipoprotein (LDL) receptor, which mediates the endocytic uptake of cholesterol-carrying lipoproteins containing apolipoproteins B and E. By regulating the number of cell-surface LDL receptors, cells are able to control the rate of entry of cholesterol, thereby assuring an adequate supply of the sterol and at the same time preventing its overaccumulation (14). About two-thirds of LDL removal from plasma is mediated by the LDL receptor, mainly in the liver (15). The intestine is involved in the absorption of dietary sterols and is one of the major organs of endogenous cholesterol biosynthesis. Intestinally synthesized cholesterol contributes directly to the plasma cholesterol pool to a considerable degree (16, 17). Earlier studies have shown that CaCo-2 cells express the LDL receptors, and that luminal sterols regulate this expression (18, 19). Cafestol might act like sterols in the regulation of LDL receptor activity in the intestine, and thereby contribute to the increased level of plasma cholesterol in subjects consuming boiled coffee.

In the present study, our goal was to examine possible effects of cafestol on lipid metabolism in the human intestinal cell line CaCo-2. The effects of cafestol have been compared with 25-hydroxycholesterol, which suppresses endogeneous cholesterol synthesis as well as the number of LDL receptors, in CaCo-2 cells. Thereby specific mechanisms of regulation could be better addressed and defined (19, 20).

MATERIALS AND METHODS

Chemicals

[1,2,3-^{3}H]glycerol (200 Ci/mol), [1-^{14}C]oleic acid (58 Ci/mol), [2-^{14}C]acetic acid (5 Ci/mol), chloramphenicol, D-threo-[dichloroacetyl-1,2-^{14}C] CAT assay grade (56.8 Ci/mol), [^{3}H]water (18 Ci/mol), and Na^{125}I were obtained from DuPont, NEN Products, Boston, MA. A mixture of the furanic diterpene alcohols, cafestol and kahweol, was prepared from coffee oil using the transesterification procedure as described by Bertholet (21). Cafestol in the free alcohol form was prepared from a mixture of cafestol and kahweol by hydrogenation using a palladium/calcium carbonate catalyst (21). The purity of recrystallized cafestol was greater than 99%. Oleic acid, acetic acid, 25-hydroxycholesterol, and sodium taurocholate (NaTC) were purchased from Sigma Chemical Co., St. Louis, MO. TLC plates (Silica gel F 1500) were from Schleicher and Schuell, Dassel, Germany.

Micellar solutions of fatty acids

Sodium salt solutions of oleic acid (6 mM), were mixed with NaTC (120 mM) and dissolved in chloroform–methanol 2:1 (v/v). The organic solvent was removed by rotary evaporation at room temperature. The resulting lipid film was dispersed in serum-free DME medium and the micellar solution containing fatty acid and NaTC was sonicated at 37°C for 5 min using an ultrasonic cleaner, Branson B-220. Only optically clear micellar solutions were used. The micellar solutions were diluted 10-fold in serum-free DME incubation medium to final concentrations of 0.6 mM and 12 mM of the fatty acid and sodium taurocholate, respectively (22).

Cell culture

CaCo-2 cells were utilized as an in vitro model for studying the regulation of lipid metabolism in the intestine (23, 24). Monolayer cultures of CaCo-2 cells were obtained from American Type Culture Collection, Rockville, MD, at passage no. 17, and grown in 75-cm^2 plastic flasks (Costar, Cambridge, MA) at 37°C in air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose and 3.7 g/l sodium bicarbonate) (Flow Laboratories, Irvine, Ayrshire, UK). The medium was supplemented with 20% fetal calf serum (FCS), (Gibco, Paisley, UK), L-glutamine (2 mM), insulin (10 μg/ml) (Sigma), penicillin (50 IU/ml), streptomycin (50 μg/ml), and 1% nonessential amino acids, from Flow Laboratories. The culture medium was changed every other day and the day before an experiment. For subculture, the medium was removed and the cells were detached from the culture flasks with 0.25% trypsin (Difco Laboratories, Detroit, MI) in a Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.2 g/l EDTA. Culture medium with FCS was added to stop trypsinization. Cells were suspended and seeded at approximately 4×10^4 cells/cm^2 in new flasks according to the method of Mohrmann et al. (25). Cells were grown to confluency in 1.5 ml of complete DME medium containing approximately 10^6 cells plated on the apical side of presoaked membrane filters of 3.0 μm pore size and 24.5 mm diameter (Transwell-COL, Costar). The lower well contained 2.6 ml of complete DME medium. Monolayers were harvested 2 weeks after reaching confluency (24, 26).

Lipoprotein isolation and labeling

LDL was isolated from freshly prepared plasma by sequential ultracentrifugation in a Centricon T-2060 ultracentrifuge in the density range 1.019–1.063 g/ml, in a TFT 70.38 rotor for 24 h at 43,000 rev/min and 10°C, as described by Havel, Eder, and Bragdon (27). The final preparations were extensively dialyzed against phosphate-buffered saline (PBS: 0.15 M NaCl, 20 mM NaH_2PO_4 and 1 mM EDTA; pH 7.4). One aliquot of LDL was labeled with ^{125}I-labeled tyramine cellobiose (^{125}I-TC-LDL) (28). More than 97% of the radioactivity was precipitated by 10% (w/v) trichloroacetic acid. The advantage of labeling a protein with radioiodinated tyramine cellobiose is that the degradation products are trapped in the organelles where degradation takes place. The final specific activities of ^{125}I-TC-LDL were 200–500 cpm/ng protein. Before use, the ^{125}I-labeled lipoproteins were diluted with unlabeled LDL to a specific activity of 50 cpm/ng protein. LDL was stored in the presence of EDTA under nitrogen at 4°C, and used within 1–2 weeks.

LDL uptake and degradation studies

CaCo-2 cells were grown on filter membranes until 2 weeks after reaching confluency. Twenty four h prior to commencing the experiments, the medium was replaced with DME medium supplemented with human lipoprotein-deficient serum (LPDS) to maximize LDL receptor activity (29). The cells were then washed and preincubated for 18 h with LPDS-DMEM containing either control (ethanol, 0.5%), cafestol (2–50 μg/ml), cafestol:kahweol:isokahweol (48:47:5, 5–50 μg/ml), or 25-hydroxycholesterol (5–10 μg/ml). Thereafter, the cells were incubated up to 24 h with the respective diterpenes or sterols. Incubations, in the presence of ^{125}I-TC-LDL (10 μg LDL/ml, 50 cpm/ng protein), were done by adding the labeled LDL to the lower well.

After incubation, the cells were placed on ice, extensively washed, and the homogenized cell fractions were precipitated by an equal volume of trichloroacetic acid (20%, w/v). Uptake and degradation of labeled LDL were calculated from the trichloroacetic acid-insoluble and soluble fractions. In the text "uptake of LDL" is used synonymously with "cell-associated LDL".

Little information is available concerning eventual toxic effects on cells of the diterpenes. Concentrations beyond 20 μg/ml of cafestol may have deleterious effects on HepG2 cells (A. C. Huggett, unpublished results). In the present study no difference of morphological patterns of the cells was observed by phase contrast microscopy. No cell loss (evaluated as cellular protein content) was observed after incubation for five days in the presence of cafestol (20 μg/ml), as compared to cells incubated without the diterpene (unpublished observations). Furthermore, no decrease in fatty acid oxidation and secretion of triacylglycerol during 24 h, indicated no toxic effect of cafestol on the cells (unpublished data).

Measurement of cell-associated and secreted lipids

The culture medium was removed and the remaining cells were rinsed twice by serum-free DME medium to wash off unattached and damaged cells. The monolayers were then preincubated with 5% FCS-supplemented DMEM with either control (ethanol, 0.5%), cafestol (2–50 μg/ml) or 25-hydroxycholesterol (10 μg/ml) for 18 h. Thereafter, the cells were incubated in serum-free DME medium containing the respective compounds (concentrations and incubation periods are indicated in legends to tables and figures), micellar oleic acid (0.6 mM), [1,2,3-^{3}H]glycerol (13 μCi/ml), [1-^{14}C]oleic acid (1 μCi/ml), [^{3}H]water (9 mCi/ml), or [2-^{14}C]acetic acid (3 μCi/ml). Radioisotopes were added to the upper chamber in the cell culture system. The cells were scraped off the filter membranes into PBS. The medium was collected from the inside and outside of the tissue culture inserts and treated further as explained below.

Lipid extraction and thin-layer chromatography

Lipids from cells and media were extracted with chloroform–methanol 2:1 (v/v). The homogenized cell fraction was mixed with 20 volumes of chloroform–methanol 2:1 (v/v) (30). Four volumes of a 0.9% sodium chloride solution (pH 2) was added and the mixture was allowed to separate into two phases. The organic phase was dried under a stream of nitrogen at 40°C. Medium devoid of cellular debris was added to 4 volumes of chloroform–methanol 2:1 (v/v) and 2% serum as unlabeled carrier for the lipids. The water phase of the medium extract was reextracted once with 4 volumes of chloroform–methanol 2:1 (v/v), and the combined organic phases were further treated in the same way as for the cells. The residual lipid extract was redissolved in 200 μl hexane and separated by thin-layer chromatography (TLC) using hexane–diethyl ether–acetic acid 65:35:1 (v/v/v) as developing solvent. The various lipids were finally identified by iodine (Sigma Chemical Co.), and the TLC foils were cut into 8 ml of liquid scintillation fluid and counted in a scintillation spectrometer (Packard, TRI-CARB 1900 TR).

RNA analysis

Total RNA was extracted by the guanidinium isothiocyanate method with centrifugation through a CsCl gradient and subsequent phenol/chloroform extractions (31). Concentration of RNA was determined by absorption at 260 nm. Twenty μg total RNA was denatured for 15 min at 50°C in 50% formamide and 6%

formaldehyde followed by 15 min on ice, and then transferred to a 1% agarose gel in 0.05 M MOPS, pH 7.0, 1 mM EDTA, pH 7.5, and 2.2 M formaldehyde for size fractionation. The presence of equal amounts of RNA in each lane was ensured by inspection after ethidium bromide staining. RNA was then transferred with a Pharmacia vacuum blotter (Pharmacia Biotechnology, Oslo, Norway) to a Zeta-Probe membrane from Bio-Rad.

Membranes were prehybridized at 65°C 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% sodium dodecyl sulfate) with 100 μg/ml of denaturated salmon testis DNA for 2 h. The probe, human LDL-receptor pSP 15 (32), was labeled with Megaprime DNA labeling system 1606 from Amersham, Buckinghamshare, UK, and hybridized to the membrane over night under the same conditions as described above. The membrane was washed for 15 min at room temperature 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 (Fuji medical X-ray film, Japan) the densities of the signals were determined by scanning the film with a Bio-Image scanner and analyzing it with a program from Bio-Image (Millipore, Bedford, MA). To calibrate the mRNA signal levels with an internal standard in addition to total RNA, filters were stripped and rehybridized with a probe for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), which is regarded as a "housekeeping gene," as expression of this gene often remains refractory to many common gene inducing agents (33).

DNA transfection, β-galactosidase and reporter CAT assay

CaCo-2 cells were plated at 1.2×10^4 cells/cm^2 in 100-mm dishes and transfected 2–3 days postplating (34) with 15 μg of DNA (a synthetic, active and a synthetic, mutant, inactive sterol regulatory element 1 (SRE-1) promoter). The SRE-1 promoter (35) was kindly provided by Dr. J. L. Goldstein and Dr. M. S. Brown, University of Texas Health Science Center, Dallas, TX). Briefly, 15 μg of test plasmid DNA, 2 μg of SR α Lac 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 (NaCl, 280 mM/$Na_2HPO_4 \cdot 2H_2O$, 1.5 mM/HEPES, 50 mM). The precipitate was allowed to form over 30 min at room temperature and thereafter the precipitated solution was added dropwise to each monolayer. The cells were incubated for 20 h with the DNA and then washed twice with 10 ml of warm PBS and refed with 10 ml of DME medium containing calf lipoprotein-deficient serum (5 mg protein/ml) in the presence or absence of cafestol (10 μg/ml) or 25-hydroxycholesterol (5 μg/ml). After incubation for 48 h the cells were harvested for measurement of β-galactosidase (36) and CAT activity (37).

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 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 $NaHPO_4$ (60 mM)/NaH_2PO_4 (40 mM)/KCl (10 mM)/$MgSO_4 \cdot 7H_2O$ (1 mM). 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.

Measurement of CAT activity. An aliquot of the supernatant was incubated for 1 h at 37°C in a final volume of 200 μl containing 4 μl of [^{14}C]chloramphenicol and 25 μl of acetyl CoA (2.59 mg/ml). Ice-cold ethyl acetate (1 ml) was than added and the mixture was 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 reaction products were separated by TLC using chloroform–methanol 95:5 (v/v) as developing solvent. After autoradiography (Fuji medical X-ray film, Japan) the radioactive spots were cut out and counted in a scintillation counter.

Enzyme assays and protein measurements

Total cholesterol and free cholesterol were assayed using Bio Mérieux and Boehringer Mannheim enzyme kits, respectively, after Folch extraction of the cells. Samples were taken for protein determination, using bovine serum albumin as a reference protein (38).

Statistical analysis

All values are reported as the mean ± SD of indicated samples and number of experiments. Comparison of different treatments was evaluated by *t*-test (two-tailed).

RESULTS

Metabolism of low density lipoprotein

Cafestol significantly increased the amount of ^{125}I-TC-LDL associated with the CaCo-2 cells by approximately 25 and 50% after incubations for 12 and 24 h, respectively, as compared to cells incubated with control medium (**Fig. 2**). During the initial 6 h of incubation the rate of uptake and degradation of LDL was slightly, but not significantly, greater in cells incubated with cafestol than control media. However, 25-hydroxycholesterol

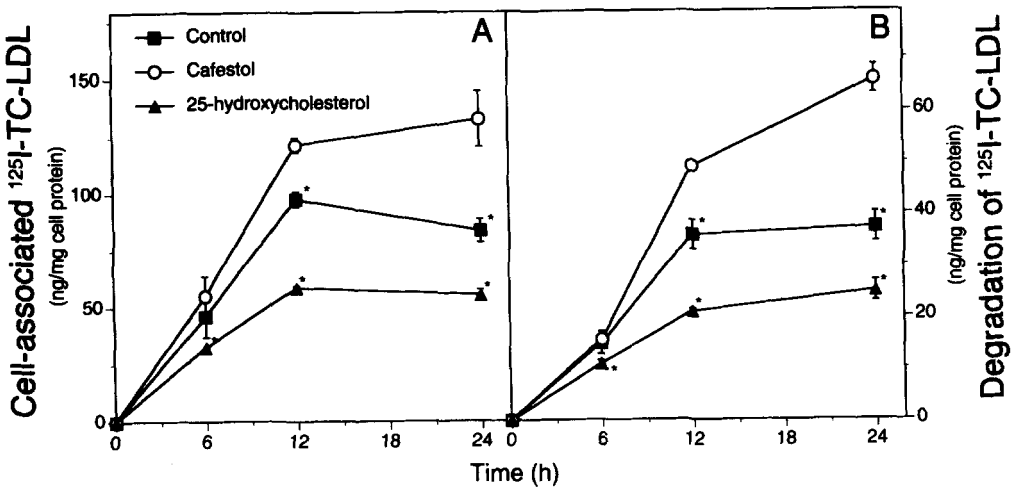

Fig. 2. Cell-association and degradation of ^{125}I-TC-LDL. Two weeks after the cells reached confluency, the medium was replaced with DMEM containing human lipoprotein-deficient serum (LPDS, 5 mg protein/ml) and incubated for 24 h. The cells were then washed and preincubated for 18 h with LPDS-DMEM containing either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) dissolved in 0.5% ethanol. Thereafter, the cells were incubated for up to 24 h with the respective agents. Incubations were performed in the presence of ^{125}I-TC-LDL (10 μg LDL/ml; 50 cpm/ng protein) added to the basolateral lower well. Data represent means ± SD of nine cultures. * Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.03$.

decreased the rate of LDL uptake and degradation markedly. This was evident at all time points examined. The rate of cell-association of labeled LDL was linear up to 12 h incubation in the presence of cafestol, 25-hydroxycholesterol, or control medium. The levelling off of the cafestol-mediated increase in LDL metabolism could hardly be due to metabolism of cafestol in CaCo-2 cells since only 1–2% of the labeled cafestol that was added in the presence of oleic acid, was esterified into cafestol-oleate after incubation for 24 h (data not shown).

A mixture of the diterpenes cafestol–kahweol–isokahweol 48:47:5 promoted a modest increase in the rate of uptake and degradation for all concentrations above 5 μg/ml, as compared to pure cafestol. Maximum rate of uptake and degradation of LDL was achieved with 30 μg/ml of cafestol–kahweol–isokahweol and pure cafestol (**Fig. 3**). There was a lower rate of uptake and degradation of LDL with 50 μg/ml of each of the lipid fractions.

To examine whether the cafestol-mediated increase in the rate of uptake of LDL was due to an increase in

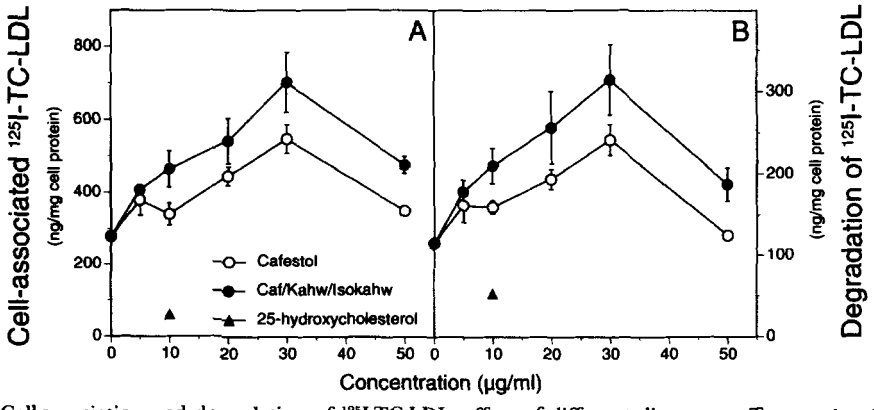

Fig. 3. Cell-association and degradation of ^{125}I-TC-LDL, effect of different diterpenes. Two weeks after the cells reached confluency, the medium was replaced with DMEM containing human lipoprotein-deficient serum (LPDS, 5 mg protein/ml) and incubated for 24 h. The cells were then washed and preincubated for 18 h with LPDS-DMEM containing either control (ethanol, 0.5%), cafestol (0.5–50 μg/ml), or cafestol:kahweol:isokahweol (0.5–50 μg/ml), dissolved in 0.5% ethanol. Thereafter, the cells were incubated for 24 h with the respective agents. Incubations were performed in the presence of ^{125}I-TC-LDL (10 μg LDL/ml; 50 cpm/ng protein) added to the basolateral lower well. Data represent means ± SD of triplicate cultures. This experiment was repeated three times with similar results.

the available number of cell-surface LDL receptors, binding experiments at 4°C were performed. Specific binding of radiolabeled LDL was increased by 21% and reduced by 30% after preincubation with cafestol and 25-hydroxycholesterol for 18 h, respectively, as compared to control cells (data not shown). Specific binding represents the difference between total binding and nonspecific binding.

Cellular cholesterol metabolism

Cafestol had no significant effect on incorporation of [^{14}C]acetate into labeled unesterified cholesterol, as compared to control cells, whereas 25-hydroxycholesterol caused a 80% decrease after 24 h of incubation (**Fig. 4A**). However, cafestol promoted approximately a 2-fold higher secretion of labeled unesterified cholesterol than control cells after 24 h of incubation (Fig. 4B). In contrast, 25-hydroxycholesterol exposure led to a significantly lower secretion of unesterified cholesterol (Fig. 4B). Cafestol contributed to a small decrease in cell-associated as well as secreted cholesteryl ester up to 24 h. On the other hand, 25-hydroxycholesterol promoted a similar incorporation of [^{14}C]acetate into cell-associated cholesteryl ester and an increase in secreted cholesteryl ester, as compared to the control medium (Fig. 4C and D). Moreover, addition of cafestol to the cells resulted in a significant decrease in the incorporation of [^{14}C]acetate into cell-associated and secreted triacylglycerol by approximately 50% after both 6 and 24 h of incubation (Fig. 4E and F).

When the cell suspension was assayed for mass of unesterified and esterified cholesterol, unesterified cholesterol content (per mg cell protein) was unaltered by cafestol, but reduced by 25-hydroxycholesterol (**Table 1**). A significant enhancement of cholesteryl ester was found in cells incubated with 25-hydroxycholesterol. By contrast, cafestol treatment decreased the cellular cholesteryl ester content slightly, but not significantly (Table 1).

TABLE 1. Effect of cafestol and 25-hydroxycholesterol on mass of unesterified and esterified cholesterol in CaCo-2 cells

	Control	Cafestol	25-OHcholesterol
		μg/mg cell protein	
Unesterified cholesterol	35.8 ± 2.3	35.5 ± 1.1	31 ± 1.9[a]
Cholesteryl ester	4.5 ± 0.8	3.9 ± 0.1	6.4 ± 0.4[a]

Two weeks after the cells reached confluency, the cells were washed and preincubated for 18 h with DMEM containing human lipoprotein-deficient serum (LPDS, 5 mg protein/ml) containing either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (25-OH-cholesterol) (10 μg/ml) dissolved in 0.5% ethanol. Thereafter, the cells were incubated in LPDS-DMEM with micellar oleic acid (0.6 mM) in the presence of control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) for 24 h. Data represent means ± SD of seven cultures.

[a]Represents significant difference between 25-hydroxycholesterol and control medium at $P \leq 0.05$.

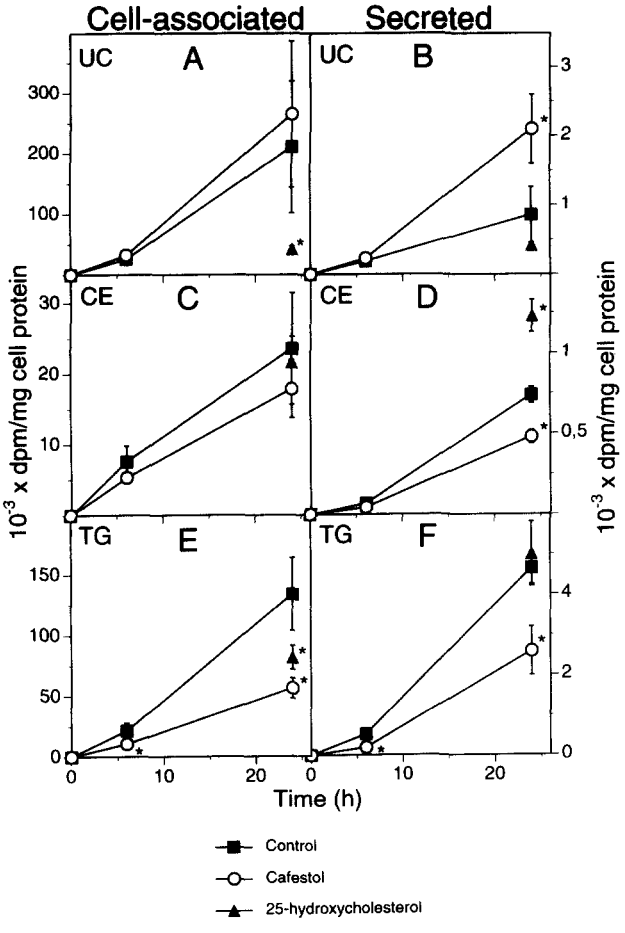

Fig. 4. Time-course of incorporation of [2-^{14}C]acetate into cell-associated and secreted lipids. Two weeks after the cells reached confluency, the medium was replaced with DMEM containing 5% FCS, supplemented with either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml), dissolved in 0.5% ethanol, and incubated for 18 h. Thereafter, the cells were incubated in serum-free DMEM with [2-^{14}C]acetic acid (100 μM, 3 μCi/ml) in the presence of control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) for 6 and 24 h. Unesterified cholesterol (UC), cholesteryl ester (CE), and triacylglycerol (TG) from cells and basolateral media were measured. Data represent means ± SD of nine cultures. * Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.05$.

Metabolism of [1-^{14}C]oleic acid

CaCo-2 cells were incubated with labeled oleic acid in addition to either cafestol or 25-hydroxycholesterol for 24 h (**Table 2**). Incorporation of [1-^{14}C]oleic acid into cholesteryl ester was slightly reduced in the presence of cafestol, whereas 25-hydroxycholesterol promoted a 3-fold increase as compared to control cells. There were corresponding findings in the medium. The rate of cell-association and secretion of triacylglycerol, cholesteryl ester, and acid-soluble radioactivity was linear up to 12 h of incubation in the presence of cafestol, 25-hydroxycholesterol, or control medium (data not

shown). There was a similar appearance of labeled triacylglycerol in the cells after incubation with control medium, cafestol, or 25-hydroxycholesterol up to 24 h. Secretion of labeled triacylglycerol was reduced to 25% of the control by supplementation with cafestol and to 70% of control with 25-hydroxycholesterol. The acid-soluble activity of micellar fatty acids was doubled in cells exposed to cafestol, whereas 25-hydroxycholesterol-exposed cells were similar to the control cells.

At 10 μg/ml of cafestol supplementation to the medium, the trichloroacetic acid-soluble activity reached a maximum. For the rate of cell association, as well as secretion of labeled triacylglycerol and cholesteryl ester, saturation was achieved at a similar concentration (data not shown).

Incorporation of [^{3}H]water into cholesterol and triacylglycerol

The problems associated with introducing labeled fatty acids into pools of potentially different sizes were minimized by incubating the CaCo-2 cells with tritiated water in the presence of oleic acid, as tritium enters the lipid pathways at early steps, due to rapid equilibration of [^{3}H]water with the cellular water phase. Cafestol, as well as 25-hydroxycholesterol, had no significant effect on incorporation of [^{3}H]water into cell-associated and secreted unesterified cholesterol, as compared to control cells after 6 h of incubation (**Table 3**). However, cafestol promoted a significant reduction in cell association and secretion of labeled cholesteryl ester than in control cells. In contrast, 25-hydroxycholesterol exposure led to a significantly higher cell association and secretion of cholesteryl ester. Cells incubated with cafestol and 25-hydroxycholesterol in the presence of oleic acid showed an incorporation of tritium into cellular triacylglycerol similar to control cells. However, cells exposed to cafestol led to a 30% lower secretion of labeled triacylglycerol than was observed in cells incubated with control medium (Table 3).

TABLE 2. Metabolism of [1-^{14}C]oleic acid in CaCo-2 cells

	Control	Cafestol	25-OHcholesterol
	10^4 x dpm/mg cell protein		
Cholesteryl ester			
Cell-associated	1.3 ± 0.2	0.90 ± 0.3	3.8 ± 0.7[a]
Secreted	0.34 ± 0.1	0.07 ± 0.03[a]	0.3 ± 0.03
Triacylglycerol			
Cell-associated	37.5 ± 6.1	32.8 ± 3.3	41.8 ± 5.6
Secreted	14.7 ± 6.7	4.0 ± 3.1[a]	10.1 ± 0.1
Acid-soluble activity	14.2 ± 5.6	28.4 ± 6.4[a]	16.2 ± 2.1

Two weeks after the cells reached confluency, the medium was replaced with DMEM containing 5% FCS, supplemented with either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (25-OHcholesterol) (10 μg/ml), dissolved in 0.5% ethanol, and incubated for 18 h. Thereafter, the cells were incubated in serum-free DMEM with micellar oleic acid (0.6 mM) and [1-^{14}C]oleic acid (1 μCi/ml) in the presence of control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) for 24 h. Data represent means ± SD of nine cultures.

[a]Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.02$.

TABLE 3. Incorporation of [^{3}H]water in CaCo-2 cells

	Control	Cafestol	25-OHcholesterol
	10^{-3} x dpm/mg cell protein		
Unesterified cholesterol			
Cell-associated	3.6 ± 0.6	3.4 ± 0.7	4.1 ± 0.6
Secreted	0.97 ± 0.1	0.99 ± 0.2	0.81 ± 0.1
Cholesteryl ester			
Cell-associated	3.6 ± 0.2	2.8 ± 0.2[a]	4.5 ± 0.2[a]
Secreted	0.79 ± 0.1	0.58 ± 0.03[a]	1.2 ± 0.2[a]
Triacylglycerol			
Cell-associated	12.1 ± 1.4	11.2 ± 1.9	12.2 ± 1.3
Secreted	1.4 ± 0.1	1.0 ± 0.2[a]	1.5 ± 0.3

Two weeks after the cells reached confluency, the medium was replaced with DMEM containing 5% FCS, supplemented with either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (25-OHcholesterol) (10 μg/ml), dissolved in 0.5% ethanol, and incubated for 18 h. Thereafter, the cells were incubated in serum-free DMEM with micellar oleic acid (0.6 mM) and [^{3}H]water (9 mCi/ml) in the presence of control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) for 6 h. Data represent means ± SD of eight cultures.

[a]Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.05$.

Cell-associated and secreted [^{3}H]triacylglycerol

It has been reported that intake of coffee lipids, cafestol and kahweol, increases the levels of serum triacylglycerol as well as cholesterol in humans (13). We investigated the effect of cafestol on the rate of cell association and secretion of [^{3}H]triacylglycerol in CaCo-2 cells (**Fig. 5**). After 6 h of incubation the generation of cell-associated and secreted triacylglycerol reached a plateau in the presence of cafestol or 25-hydroxycholesterol. Cell-associated [^{3}H]triacylglycerol levels were similar for the control cells and the cells incubated with cafestol, as well as 25-hydroxycholesterol exposure (Fig. 5A), during the incubation period. Secretion of radiolabeled triacylglycerol from cells incubated with cafestol or 25-hydroxycholesterol was reduced by approximately 80 and 50%, respectively, after 24 h of incubation, as compared to the control cells (Fig. 5B). Cell-associated and secreted [^{3}H]phospholipids were similar in cells incubated with cafestol up to 24 h, as compared to control incubations (data not shown).

Expression of mRNA for LDL receptor

CaCo-2 cells were incubated with cafestol to examine whether the regulation of the LDL receptor expression occurs at the level of transcription in the presence of this coffee lipid (**Fig. 6**). Incubation of CaCo-2 cells with 20 μg/ml of cafestol promoted a more than 3-fold up-regulation of LDL receptor mRNA, as compared to control cells. In contrast, treatment of the cells with

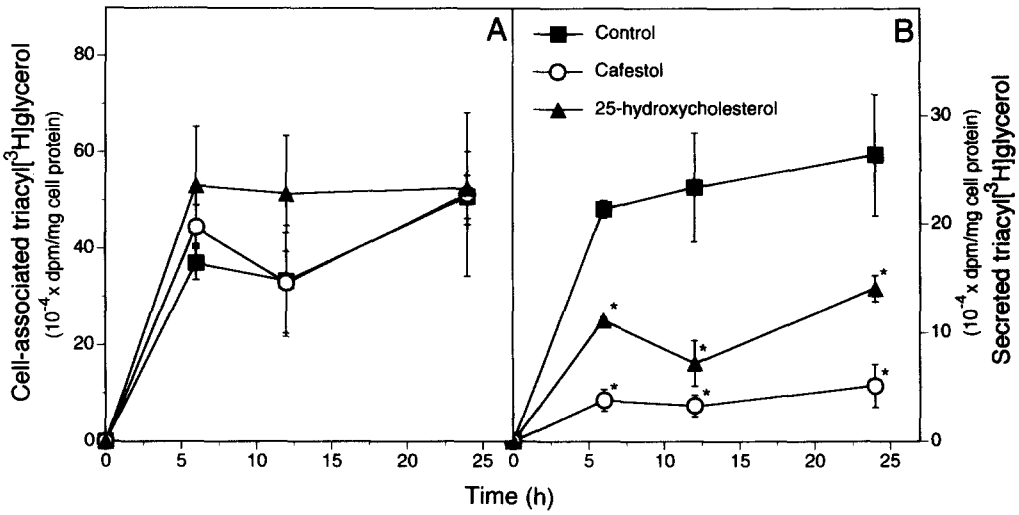

Fig. 5. Time-course of cell-associated and secreted triacyl[^{3}H]glycerol. Two weeks after the cells reached confluency, the medium was replaced with DMEM containing 5% FCS, supplemented with either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) dissolved in 0.5% ethanol, and incubated for 18 h. Thereafter, the cells were incubated in serum-free DMEM containing oleic acid (0.6 mM) and [1,2,3-^{3}H]glycerol (13 μCi/ml) in the presence of control (ethanol, 0.5%), cafestol (20 μg/ml), and 25-hydroxycholesterol (10 μg/ml) up to 24 h to measure cell-associated (A) and secreted (B) labeled triacylglycerol. Data represent means ± SD of six cultures. * Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.001$.

25-hydroxycholesterol caused a 30% decrease of LDL receptor mRNA expression. The signal levels were calibrated with that of the G3PDH transcript (Fig. 6).

Effect of cafestol and 25-hydroxycholesterol on CAT activity in SRE-1 promoter transfected cells

We transfected CaCo-2 cells with the active and mutated promoter regions of the SRE-1 coupled to the reporter gene CAT to investigate whether cafestol regulated the LDL receptor expression at the transcriptional level by interacting with the SRE-1 promoter (**Fig. 7**). SRE-1 is a conditional positive element that enhances LDL receptor gene transcription in the absence of sterols (35, 39–41). When cafestol (10 μg/ml) was incubated with the transfected cells, there was a 20% up-regulation of CAT activity in the cells transfected with the active promoter, whereas 25-hydroxycholesterol (5 μg/ml) abolished the CAT activity. In cells transfected with the mutant promoter construct, there was no rise of CAT activity in any incubations.

DISCUSSION

Consumption of boiled coffee is reported to raise serum cholesterol levels in humans (1, 7–9, 42–45). In the present study we investigated how the human intestinal cells, CaCo-2 cells, responded to a coffee lipid, cafestol, that has recently been identified as one of the major cholesterol-raising components of boiled coffee (13). The intestine might contribute to the increased levels of lipids in plasma in individuals consuming boiled coffee. A significant decrease in the rate of uptake and degradation of radiolabeled LDL in HepG2 cells and human skin fibroblasts was observed after cafestol exposure as shown in current experiments (A. C. Rustan, A. C. Huggett, B. Halvorsen, J. Lieberg, K. Dranger, T. Ranheim, C. A. Drevon, M. S. Nenseter, E. N. Christiansen, and R. Blomhoff, unpublished results).

The principal effect of cafestol in CaCo-2 cells was to promote an increased rate of uptake and degradation of LDL (Fig. 2), whereas the rates of secretion of cholesteryl ester and triacylglycerol were decreased in the presence of cafestol (Tables 2, 3; Figs. 4, 5). Interestingly, a mixture containing the coffee lipids cafestol and kahweol, in approximately equal proportions together with a small amount of isokahweol, was slightly more active than pure cafestol in producing an increased uptake and degradation of LDL (Fig. 3). Our results indicate that the increase in the rate of uptake and degradation of LDL observed after incubation with cafestol in CaCo-2 cells might be due to an increased number of LDL receptors on the cell surface.

Derivatives of cholesterol are potent regulators of intracellular cholesterol metabolism (46), and it has been reported that oxygenated sterols stimulate the formation of cholesteryl esters in CaCo-2 cells, as well as in cultured human skin fibroblasts, rat hepatocytes, and several other cell types (14, 20, 47–49). In our present study, 25-hydroxycholesterol increased the rate of secretion of cholesteryl ester from CaCo-2 cells, while cafestol reduced the secretion of cholesteryl ester, as compared to control cells (Table 3, Fig. 4). These find-

ings confirmed the responsiveness of the cells to oxysterols, agents known to regulate cholesterol biosynthesis (19, 20).

A relationship between coffee lipids and high triacylglycerol levels was documented (12), and this was even more marked in studies using purified coffee lipids than in studies using boiled coffee (13). However, we observed that cafestol caused a reduced secretion of triacylglycerol from CaCo-2 cells (Tables 2, 3; Figs. 4, 5). Furthermore, the reduced secretion of triacylglycerol was accompanied with an increase in the trichloroacetic acid-soluble activity (Table 2). This might be due to an increase in a ligand-dependent transcription factor similar to the peroxisome proliferator-activated receptor (PPAR) that might increase peroxisomal fatty acid oxidation (50).

The expression of the LDL receptor in CaCo-2 cells is suggested to be partly regulated by the level of luminal sterols, and this regulation occurs, at least to some extent, at the level of transcription (19). Incubation of CaCo-2 cells with cafestol caused an increase in the rate of uptake and degradation of LDL and the LDL receptor mRNA levels as compared to untreated cells, suggesting that cafestol may alter the transcription of the LDL-receptor (Figs. 2, 6). On

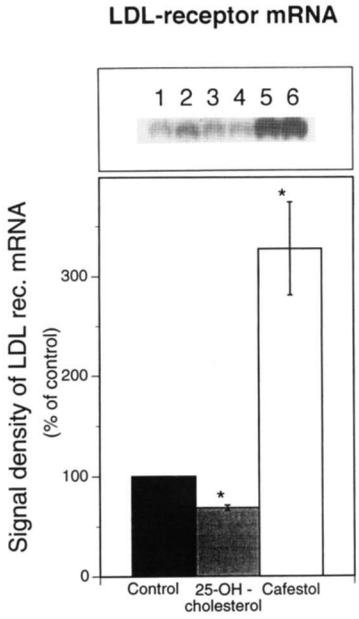

Fig. 6. LDL receptor mRNA levels. Two weeks after the cells reached confluency, the medium was replaced with DMEM containing human lipoprotein-deficient serum (LPDS, 5 mg protein/ml) and incubated for 24 h. The medium was replaced with LPDS-DMEM supplemented with either control (ethanol, 0.5%), cafestol (20 μg/ml), or 25-hydroxycholesterol (10 μg/ml) dissolved in 0.5% ethanol. After 24 h incubation at 37°C, total RNA was extracted from the cells and Northern blots were prepared using 20 μg total RNA per lane. mRNA was quantified by scanning the autoradiograms and standardized with an internal standard, G3PDH. Control (lanes 1–2); 25-hydroxycholesterol (25-OHcholesterol) (lanes 3–4); cafestol (lanes 5–6). Data represent means ± SD of four cultures. *Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.02$.

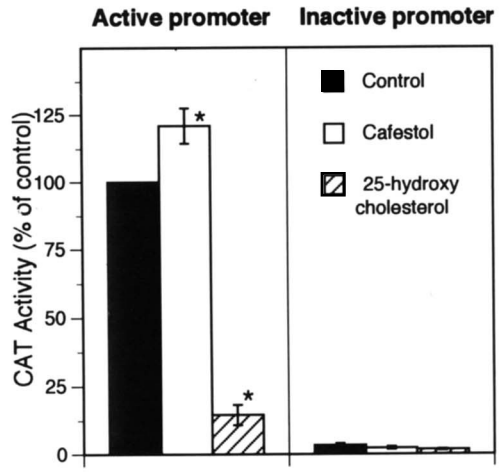

Fig. 7. Chloramphenicol acetyltransferase (CAT) activity in cells transfected with SRE-1. CaCo-2 cells were grown on filter membranes and cultured for 2 weeks after reaching confluency. The cells were transfected with active and mutated promoter regions of the SRE-1 coupled to CAT gene for 20 h. Thereafter, the cells were washed and incubated for 48 h with LPDS-DMEM containing either control (ethanol, 0.5%), cafestol (10 μg/ml), or 25-hydroxycholesterol (5 μg/ml) dissolved in 0.5% ethanol. Data represent means ± SD of triplicate cultures. This experiment was repeated three times, demonstrating similar results. * Represents significant difference between cafestol or 25-hydroxycholesterol and control medium at $P \leq 0.02$.

the other hand, incubation of the CaCo-2 cells with 25-hydroxycholesterol produced a reduction in the rate of uptake and degradation of LDL and the LDL receptor mRNA levels. Furthermore, incubation with cafestol enhanced the rate of uptake of LDL by approximately 20% in P19 cells, a murine embryonic carcinoma cell line, 3T3 cells, a murine fibroblast cell line, and in J774 cells, a murine macrophage-like cell line, whereas 25-hydroxycholesterol decreased the rate of uptake of LDL in all these cell lines (data not shown). These findings are in contrast to studies in HepG2 cells and human skin fibroblasts, where cafestol reduced the rate of uptake and degradation of LDL (A. C. Rustan et al., unpublished results). Srivastav et al. (51) examined the cell-specific regulation of the LDL receptor by 25-hydroxycholesterol and palmitate in CaCo-2 cells and HepG2 cells. They found that 25-hydroxycholesterol reduced the LDL receptor activity by transcriptional mechanisms in both cell lines. Palmitate, however, decreased expression of the LDL receptor activity by a post-transcriptional mechanism in CaCo-2 cells.

A sterol regulatory element-1 (SRE-1) is located in the promoter region of the gene for the LDL receptor (41). The promoter region is able to bind sterol regulatory element binding protein-1 (SREBP-1), and thereby regulates the transcription of the genes for the LDL receptor and HMG-CoA synthase (35, 39, 40). When CaCo-2 cells were transfected with the SRE-1 promoter coupled to the gene for CAT, we observed an up-regulation of CAT activity (Fig. 7). Incubation with 25-hydroxycholesterol

inhibited CAT activity in CaCo-2 cells transfected with the SRE-1 promoter. This is consistent with findings in normal CaCo-2 cells reported by Field et al. (19), where cells were incubated with micelles containing 25-hydroxycholesterol, and the rate of the LDL receptor synthesis was significantly decreased. Moreover, 25-hydroxycholesterol decreased the rate of uptake and degradation of LDL in HepG2 cells and human skin fibroblasts (46, 47). Cafestol may act differently than 25-hydroxycholesterol in the processing of SREBPs in different cell lines. This might be due to phosphorylation of the amino terminal portion of SREBP-1, thus affecting its activation (39). It might also be caused by an inadequate shielding of the proteolytic site on the SREBP-1 precursor, or influence other proteins (chaperones) assisting the active SREBP-1 fragment in maintaining its conformation (41).

We observed that the rate of uptake of LDL was increased and that the output of lipids was decreased by CaCo-2 cells after incubation with cafestol. How this might be relevant to the increasing serum lipid levels reported in humans consuming boiled, unfiltered coffee (1, 7, 8, 9, 42–45) is somewhat confusing. The increase in LDL receptor activity and LDL receptor mRNA levels suggests that CaCo-2 cells incubated with cafestol lack or have a perceived lack of cholesterol. However, the mass of both unesterified cholesterol and cholesteryl ester was unchanged in cells treated with cafestol, as compared to control cells. On the other hand, 25-hydroxycholesterol decreased unesterified cholesterol, while cholesteryl ester was increased (Table 1).

Our study and the studies in HepG2 cells and human skin fibroblasts (A. C. Rustan et al., unpublished results) indicate that cafestol and 25-hydroxycholesterol may influence cholesterol metabolism differently in the examined cell lines. As far as we know, cafestol is the only nutrient that influences regulation of cholesterol metabolism differently in alternative cell lines.

In summary, we have observed that incubation of cafestol with cultured CaCo-2 cells promoted an enhanced rate of uptake and degradation of LDL, possibly due to an increase in the transcription of the LDL receptor mRNA. Furthermore, cafestol appears to reduce secretion of both cholesteryl ester and triacylglycerol in the cells.

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