Co-translational inhibition of apolipoprotein B100 synthesis by cyclosporin A in the human hepatoma cell line HepG2

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Cotranslational Inhibition of ApoB-100 Synthesis by Cyclosporin A in the Human Hepatoma Cell Line HepG2

Allard Kaptein, Elly C.M. de Wit, Hans M.G. Princen

Abstract Treatment of patients with cyclosporin A (CsA) increases low-density lipoprotein (LDL) cholesterol levels. We investigated whether an elevated hepatic secretion of apolipoprotein (apo) B-100–containing lipoproteins is responsible for the increase of LDL by using the human hepatoma cell line HepG2. Addition of CsA to the culture medium of HepG2 cells resulted in a dose- and time-dependent decrease in the secretion of apoB-100. Maximal inhibition (-50%), which was obtained at 5 μmol/L CsA, was achieved within 8 hours. The secretion of apoA-I, albumin, and [^{35}S]methionine-labeled proteins was not affected by CsA. The reduced accumulation of apoB-100 in the culture medium could not be explained by changes in the uptake and degradation of LDL by HepG2 cells treated with CsA. In addition, [^{35}S]methionine incorporation studies indicated that synthesis and/or secretion of newly synthesized apoB-100 decreased in the presence of CsA. CsA did not affect the apoB-100 mRNA level, indicating that CsA regulates the secretion of apoB-100 at the cotranslational or posttranslational level. The decreased secretion of apoB-100 was accompanied by a diminished secretion of triglycerides (-47%), cholesterol (-18%), and cholesteryl esters (-27%) in the presence of CsA. In contrast, the intracellular concentrations and the total amount of these lipids present in the culture medium and cells were not changed. This indicates that a possible limited availability of one of these lipids was not responsible for the decreased secretion of apoB-100 by CsA. Pulse-chase experiments showed that the amount of intracellular apoB-100 was already decreased by 50% after the 10-minute pulse period and that CsA did not affect the intracellular processing of apoB-100 once it was fully synthesized. Short pulse incubations in the presence of [^{35}S]methionine showed a decrease in the intracellular amount of labeled apoB-100 after an incubation of only 2 through 4 minutes, indicating that the translation was not affected but that inhibition of the apoB-100 secretion by CsA occurred at the cotranslational level. Our results suggest that the elevated plasma LDL levels observed in patients treated with CsA are not caused by hepatic overproduction of apoB-100–containing lipoproteins. (*Arterioscler Thromb.* 1994;14:780-789.)

Key Words • apolipoprotein B-100 • cyclosporin A • transferrin • cotranslational regulation • HepG2 • apolipoprotein A-I

Cyclosporin A (CsA) is one of the most effective immunosuppressive drugs available and is widely used in supporting engraftments of kidney, liver, heart, lung, and bone marrow transplants. CsA is also used in an increasing number of autoimmune diseases (eg, uveitis, psoriasis, rheumatoid arthritis, type I diabetes mellitus, nephrotic syndrome).[1,2] The compound, a cyclic undecapeptide, selectively inhibits the production of interleukin-2 by activated T lymphocytes and prevents activation of resting T cells by this lymphokine.[1,3] Several adverse reactions to CsA have been reported (for review, see References 1 through 3). The drug elevates the serum cholesterol level in renal and heart transplant recipients, primarily by an increase in the low-density lipoprotein (LDL) cholesterol level.[4-7] CsA also enhances the concentration of apolipoprotein (apo) B-100,[7] the sole protein of LDL. Increased levels of LDL cholesterol and apoB-100 are strongly associated with the development of atherosclerosis and the incidence of coronary heart disease.[8-10] Arterial disease is a leading cause of death in long-term survivors of renal[11,12] and heart[13] transplantation. Deviations in lipoprotein levels as a consequence of CsA treatment have been suggested as adversely affecting cardiovascular morbidity in transplant recipients.[4,5,7]

The mechanism of the increase in LDL cholesterol and apoB-100 levels in patients treated with CsA is not understood. The increase may be caused by an enhanced secretion of apoB-containing lipoproteins by the liver.[14] In humans apoB-100 is synthesized and secreted only by the liver.[15] Owing to the limited availability of human liver, the human hepatoma cell line HepG2 has been used frequently as a model to study the apolipoprotein and lipoprotein synthesis in human hepatocytes. This cell line synthesizes a number of apolipoproteins[16] and is a suitable model for investigating the regulation of the synthesis and/or secretion of apoB-100 in response to lipoproteins,[17] fatty acids,[18-22] butyric acid,[23] hormones,[19,21,24-26] and drugs currently administered in clinical practice.[21,27,28]

In this article we describe the effect of CsA on the secretion of apoB-100 in HepG2 cells and show that CsA inhibits apoB-100 secretion at the cotranslational level. These findings suggest that the increased serum LDL cholesterol levels in patients after long-term CsA therapy do not result from an increased secretion of apoB-100–containing lipoproteins by the liver.

Methods

Materials

CsA powder was a gift from Sandoz Ltd. Tran ^{35}S label (>1000 Ci/mmol) containing both [^{35}S]methionine and [^{35}S]cys-

teine was obtained from ICN Biomedicals Inc. Sodium [^{125}I]iodide (15 mCi/µg iodine) and [2-^{14}C]acetate (55 mCi/mmol) were purchased from Amersham International. Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were purchased from Flow Laboratories. Fetal bovine serum (FBS), penicillin, and streptomycin were from Boehringer Mannheim.

Cell Culture

The established HepG2 cell line, derived from a human liver tumor, was obtained from Dr B.B. Knowles, Wistar Institute of Anatomy and Biology, Philadelphia, Pa. The cells were cultured,[23,29] and incubations in the presence of CsA were performed in DMEM containing 10% (vol/vol) FBS or 10% (vol/vol) lipoprotein-depleted serum (LPDS). LPDS was prepared from heat-inactivated FBS by density-gradient ultracentrifugation.[30] A 50-mmol/L stock solution of CsA was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Immediately before use CsA was diluted in culture medium such that the DMSO concentration did not exceed 0.01% (vol/vol). All incubation media were adjusted for the same DMSO concentration. Except when otherwise stated, the effect of CsA was studied over a 24-hour period. At the end of the incubation period, medium was collected and centrifuged for 20 seconds at 8000g in a Heraeus centrifuge (Biofuge A) to remove detached cells and debris. The supernatant was frozen immediately on dry ice and stored at −20°C until measurement of apoA-I and apoB-100 concentrations. Cells were washed three times with cold (4°C) phosphate-buffered saline (PBS: sodium/potassium phosphate buffer, 11 mmol/L, pH 7.5, containing 150 mmol/L NaCl) and harvested by scraping in water, and cellular protein was determined.

Measurement of ApoA-I, ApoB-100, and Albumin Secretion and Analysis of ApoA-I– and ApoB-100–Containing Lipoproteins

Accumulation of apoA-I and apoB-100 in the medium of HepG2 cell cultures was measured in triplicate by using a sandwich enzyme-linked immunosorbent assay (ELISA).[23] Accumulation of albumin in the medium was measured by rocket immunoelectrophoresis using the method described by Laurell[31] using rabbit anti–human albumin antiserum (Dakopatts).

For analysis of apoA-I– and apoB-100–containing lipoproteins, culture medium was collected after a 24-hour incubation of HepG2 cells with or without 5 µmol/L CsA. After harvesting, protease inhibitors were added to the following final concentrations: 2.5 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 25 µmol/L p-hydroxymercuriphenyl sulfonic acid, 10 U/mL heparin, 10 U/mL aprotinin (Trasylol), 50 µg/mL leupeptin, and 50 µg/mL pepstatin. Culture medium (4 mL) was used for density-gradient ultracentrifugation as described by Redgrave et al.[32] After ultracentrifugation for 16 hours at 4°C, the gradient was fractionated into 0.6-mL aliquots. The density of the fractions was measured (Density Measuring Cell DMA 602M, Mettler/Paar), the fractions were dialyzed for 16 hours against PBS containing 0.1% (wt/vol) casein and 2.5 mmol/L EDTA, and the apoA-I and apoB-100 concentrations in the fractions were measured by ELISA.

Protein Synthesis

De novo synthesis of proteins was determined by measuring the incorporation of Tran ^{35}S label into the 10% (wt/vol) trichloroacetic acid–precipitable fraction of the radiolabeled culture medium and cells. The metabolically radiolabeled proteins secreted in the medium were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions by using the method of Laemmli,[33] with resolving gels containing a gradient of 4% to 20% (wt/vol) acrylamide and stacking gels of 3.5% (wt/vol) acrylamide. Protein molecular mass standards (Bio-Rad) were used for calibration of the gel. For autoradiography the gel was treated with an autoradiography enhancer (EN^{3}Hance, NEN

DuPont) in accordance with the manufacturers' instructions, dried, placed on x-ray film (Kodak X-Omat AR films: Eastman-Kodak Co), and stored at −80°C until use.

Immunoblotting

Immunoblotting was performed as described.[34] ApoB-100 and transferrin on the blot were identified by using affinity-purified rabbit anti–apoB-100 and rabbit anti-transferrin (Dakopatts) followed by an incubation with goat anti-rabbit immunoglobulin G conjugated to peroxidase.

RNA Hybridization

Total RNA was isolated from HepG2 cells by the method of Chomczynski and Sacchi.[35] ApoB-100, apoA-I, and albumin mRNA levels were analyzed by Northern blot analysis.[23]

Measurement of Receptor-Mediated Binding, Internalization, and Degradation of ^{125}I-LDL

Binding, internalization, and degradation of LDL by HepG2 cells were measured over a 3-hour period with or without 5 µmol/L CsA after an 18-hour preincubation period with or without 5 µmol/L CsA. In incubations in which ^{125}I-LDL binding, uptake, and degradation were studied in the presence of CsA, the ^{125}I-LDL was preincubated with CsA before addition to the HepG2 cell culture, since the majority of CsA in the circulation is present in LDL.[36,37]

The LDL receptor–mediated binding, internalization, and degradation of LDL were measured at 37°C.[29,30] Binding, internalization, and degradation were corrected for nonspecific binding, internalization, and degradation by performing the same experiments in the presence of excess unlabeled LDL (200 µg/mL). In addition to the binding at 37°C, the binding of ^{125}I-LDL was measured at 4°C. At this temperature LDL is not internalized.

Measurement of Intracellular and Extracellular Triglycerides, Cholesterol, and Cholesteryl Esters

After a 24-hour incubation in the presence or absence of CsA, HepG2 cells were washed five times with cold PBS. Thereafter, the cells were harvested by scraping and homogenized by sonication (Branson, 60 W for 20 seconds). Samples were taken for measurement of protein content. Lipids were extracted from the cell suspension as described by Bligh and Dyer[38] after adding 2 µg cholesterol acetate as an internal standard. The neutral lipids were separated by high-performance thin-layer chromatography on silica gel–60–precoated plates.[29]

When DMEM/LPDS was used as the culture medium, the mass of triglycerides, cholesterol, and cholesteryl esters secreted in the medium was also measured. At the end of the incubation the medium was centrifuged at 12 000 rpm for 30 minutes at 4°C to remove detached cells and cell debris. Extraction and analysis of lipids were the same as for the cell suspension. Blank culture medium was also analyzed to correct for low levels of lipids present in the LPDS (0.10, 0.20, and 0.45 µg triglycerides, cholesterol, and cholesteryl esters, respectively, were found per milliliter culture medium).

Analysis of ApoA-I, ApoB-100, Albumin, and Transferrin Synthesis

After a 16-hour preincubation in DMEM/LPDS with or without 5 µmol/L CsA, HepG2 cells were used for pulse-chase studies using Tran ^{35}S label[39] with minor modifications. After a 30-minute incubation on methionine- and cysteine-free minimum essential medium (ICN Biomedicals Inc), cells were pulsed with Tran ^{35}S label (100 µCi/mL) for 10 minutes. After the pulse period, the medium was removed, and cells were washed three times with DMEM (chase medium) at 37°C and incubated with DMEM with or without 5 µmol/L CsA for 0, 10, 20, 35, 60, or 90 minutes. At the end of the chase time cells and media were harvested separately.

Cells were washed three times with PBS at 4°C and homogenized in 1 mL NET buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L tris[hydroxymethyl]aminomethane, pH 7.4, 0.0625 mol/L sucrose, 0.5% Triton X-100, and 0.5% sodium deoxycholate), and protease inhibitors were added as described above. The medium was centrifuged for 20 seconds at 8000*g* (Heraeus centrifuge, Biofuge A) to remove detached cells and debris. The supernatant was mixed (1:1) with 2× NET buffer, and protease inhibitors were added. Aliquots of 500 μL cell extracts or 900 μL culture medium were combined with an excess of monospecific affinity-purified goat anti-human apoA-I, rabbit anti-human apoB-100,[23] and rabbit anti-albumin antiserum (Dakopatts) and incubated for 16 hours at 4°C. Then 75 μL of a 50% solution of protein G–Sepharose (Pharmacia) in NET buffer was added and incubated for 45 minutes at 4°C and 30 minutes at room temperature. The antigen-antibody–protein G–Sepharose complex was pelleted by centrifugation for 2 minutes at 4000 rpm, the supernatant was aspirated, and the pellet was resuspended in 500 μL NET buffer and washed by centrifugation through a 30% sucrose solution in 500 μL NET buffer. After two additional washes with NET buffer, 50 μL sample buffer was added, and the samples were boiled for 5 minutes and subjected to electrophoresis as described above. ApoB-100, apoA-I, and albumin were localized on the dried gels after autoradiography. Bands containing apoB-100, apoA-I, and albumin were sliced from the gel, and the gel fragments were solubilized in 1 mL of an NH_3/H_2O_2 solution (0.94% [wt/vol] NH_3 and 7.5% [vol/vol] H_2O_2) for 16 hours at 55°C and counted for radioactivity.

For the pulse-labeling experiments, HepG2 cells were incubated with or without 5 μmol/L CsA in the presence of Tran ^{35}S label (100 μCi/mL) for 2, 4, 7, or 10 minutes after a 16-hour preincubation as described above for the pulse-chase studies. Analysis of the labeled apoB-100, apoA-I, and albumin present in the cells was performed as described above. Transferrin was also immunoprecipitated from the cell homogenate.

Statistical Analysis

Statistical significance of differences was calculated by using Student's *t* test for paired data with the level of significance as $P<.05$.

Results

Effects of CsA on ApoB-100 and ApoA-I Secretion

The addition of increasing amounts of CsA resulted in a decrease in the secretion of apoB-100 in the medium of HepG2 cell cultures without affecting the apoA-I secretion (Fig 1). The decrease in the apoB-100 secretion was observed both in DMEM/FBS and DMEM/LPDS but was more pronounced in DMEM/LPDS. In both media a significant decline in apoB-100 secretion was observed with 5 μmol/L CsA. With 2 μmol/L CsA the decrease reached significance only when DMEM/LPDS was used. From the changes in cell morphology and loss of cells from the culture dishes, we determined that CsA concentrations ≥10 μmol/L were cytotoxic to HepG2 cell cultures. Therefore, further experiments were performed at a concentration of 5 μmol/L CsA. At this concentration no adverse effect was observed on the cell morphology; on the amount of cell protein present in the culture dishes at the end of the incubation; or on apoA-I, albumin, and total protein synthesis (see below).

In Fig 2 the time course of the effect of CsA on the secretion of apoB-100 and apoA-I by HepG2 cells is shown. With control medium, apoB-100 and apoA-I accumulation increased linearly with time over the
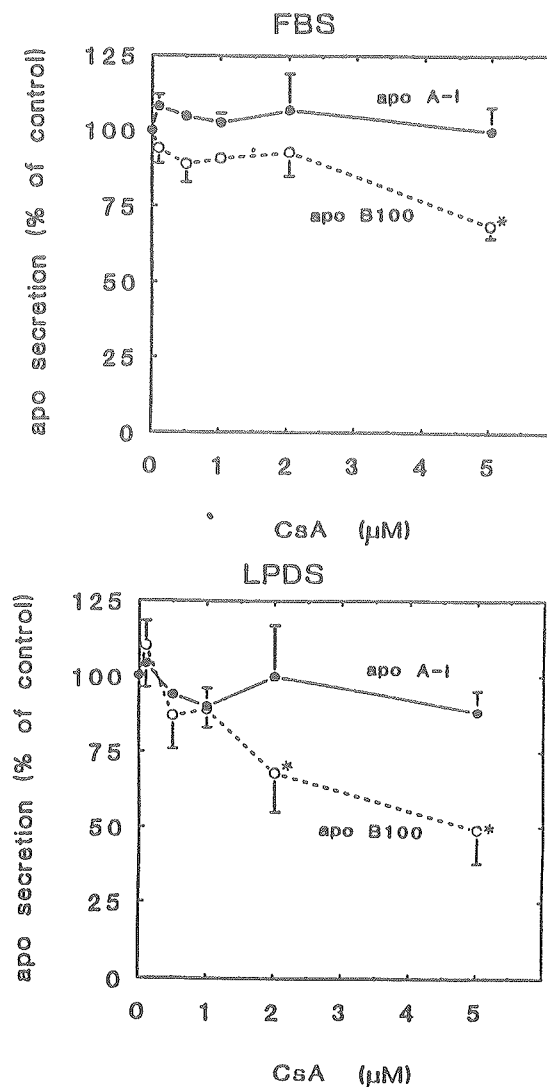

FIG 1. Line graphs showing effects of various cyclosporin A (CsA) concentrations on the secretion of apolipoprotein (apo) A-I and apoB-100 in the medium of HepG2 cell cultures. HepG2 cells were incubated with CsA in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum (FBS) or 10% (vol/vol) lipoprotein-depleted serum (LPDS). Apolipoprotein concentrations were measured as described in "Methods." Results were normalized for cell protein in culture dishes and are mean±SD for three independent experiments. ApoA-I (●) production by HepG2 cells on DMEM/FBS and DMEM/LPDS was 4.5±0.4 and 6.0±0.4 μg/24 h per milligram cell protein, respectively. ApoB-100 (○) production by HepG2 cells on DMEM/FBS and DMEM/LPDS was 5.6±0.4 and 5.4±0.8 μg/24 h per milligram cell protein, respectively (each mean±SD for three experiments). *$P<.05$ between control and treated cells.

24-hour period of the experiment (data not shown). A significant decrease in apoB-100 secretion was observed after a 4-hour incubation with 5 μmol/L CsA present in the medium. Maximal inhibition of the apoB-100 secretion was reached after incubation for 8 hours in the presence of 5 μmol/L CsA. In subsequent experiments the effect of CsA on HepG2 cell cultures was studied over a 24-hour period.

Specificity of the Inhibition of ApoB-100 Secretion by CsA

The finding that CsA did not affect the secretion of apoA-I by HepG2 cells indicated that the inhibitory
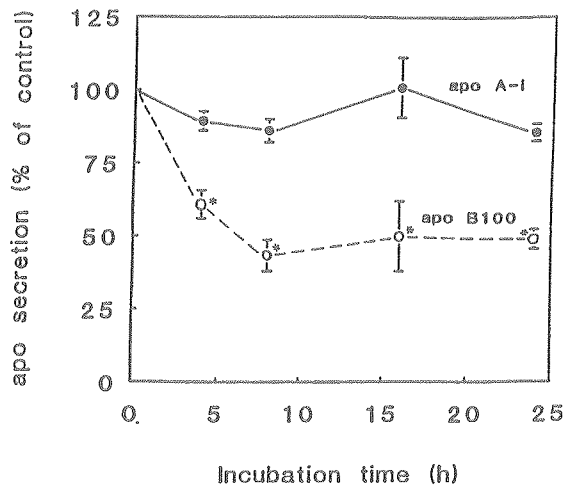
FIG 2. Line graph showing time course of the effect of cyclosporin A (CsA) on apolipoprotein (apo) A-I and apoB-100 secretion in the medium of HepG2 cells. HepG2 cells were cultured on Dulbecco's modified Eagle's medium/lipoprotein-depleted serum with and without 5 μmol/L CsA. Apolipoprotein concentrations were measured as described in "Methods." Results were normalized for cell protein in culture dishes and are mean±SD for three independent experiments. ApoA-I (●) and apoB-100 (○) production by HepG2 cells in control medium was 0.29±0.06 and 0.21±0.05 μg/mg cell protein per hour, respectively. *$P<.05$ between control and treated cells.
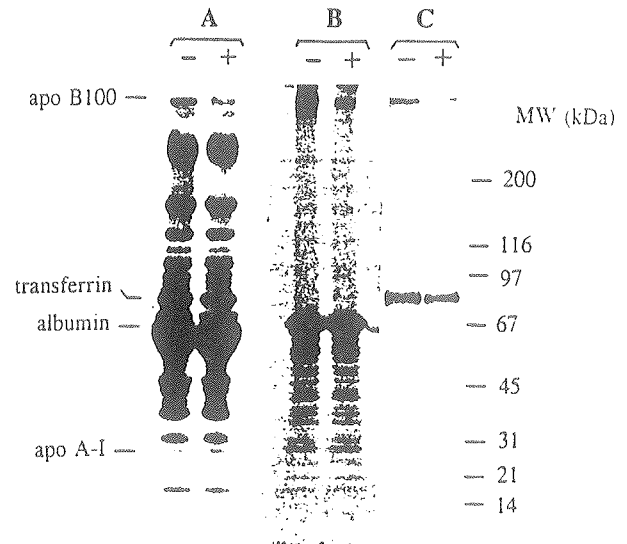

FIG 3. Autoradiograph showing effect of cyclosporin A (CsA) on the pattern of metabolically radiolabeled proteins secreted in the medium of HepG2 cell cultures. HepG2 cells were incubated in Dulbecco's modified Eagle's medium/lipoprotein-depleted serum with or without 5 μmol/L CsA containing 20 μCi Tran ^{35}S label/mL. Both culture medium (A) and the immunoprecipitate of the culture medium (B), obtained by using anti-human apolipoprotein (apo) B-100, anti-human apoA-I, and anti-human albumin, were applied to the gel. C, Immunoblot of the metabolically radiolabeled medium after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showing apoB-100 and transferrin. + or − indicates incubation with or without CsA, respectively. The position of the molecular mass standards are indicated on the right; on the left side the positions of apoB-100, apoA-I, albumin, and transferrin are indicated.

effect of CsA on the secretion of apoB-100 was not part of a general effect of CsA on protein synthesis. To further exclude this possibility, we measured the secretion of albumin and total protein. No effects were found on the secretion of albumin or total de novo synthesized proteins (Table 1) or on the cellular content of de novo synthesized proteins (data not shown) after incubation in DMEM/FBS or DMEM/LPDS with 5 μmol/L CsA. To explore the possibility that CsA may alter the pattern of the proteins secreted in the culture medium, the medium of a metabolic labeling experiment with Tran ^{35}S label was separated by SDS-PAGE and auto-radiographed (Fig 3A). HepG2 cells incubated with CsA secreted a pattern of proteins in the culture medium similar to that of control cells. By specific immunoprecipitation (Fig 3B) and immunoblotting (Fig 3C) the protein with molecular mass of approximately 500 kd. which showed a lower intensity on the autoradiograph after incubation with CsA, was identified as apoB-100. A decreased secretion of transferrin was also observed in the presence of 5 μmol/L CsA.

Since HepG2 cells can bind and internalize LDL,[30] a stimulation of the uptake and degradation might have

caused the decreased accumulation of apoB-100 in the culture medium. However, CsA inhibited the binding (−31% at 4°C and −38% at 37°C), internalization (−22%), and degradation (−23%) of LDL by HepG2 cells (Table 2).

These results indicated that the inhibitory effect of CsA on apoB-100 secretion by HepG2 cells was rather specific and that the secretion of newly synthesized apoB-100 was affected.

Effect of CsA on ApoB-100, ApoA-I, and Albumin mRNA Levels

The effect of CsA on the apoB-100, apoA-I, and albumin mRNA levels was determined. CsA did not affect the mRNA level of apoB-100, apoA-I, or albumin (Fig 4), indicating that the decrease in apoB-100 secretion by CsA must be regulated at the translational level or the cotranslational or posttranslational level.

TABLE 1. Effect of Cyclosporin A on the Secretion of Albumin and ^{35}S-Labeled Proteins in HepG2 Cells

	DMEM/FBS	DMEM/FBS+5 μmol/L CsA	DMEM/LPDS	DMEM/LPDS+5 μmol/L CsA
Albumin secretion, μg/mg cell protein per 24 h	42.6±4.5	43.1±0.8	38.3±8.7	33.0±4.1
[35]S Incorporation in secreted proteins, % of control	100	89±11	100	98±14

DMEM indicates Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPDS, lipoprotein-depleted serum; and CsA, cyclosporin A. Results are mean±SD for three independent experiments. No statistically significant difference between control and CsA-treated cells was observed.

TABLE 2. Effect of Cyclosporin A on the Binding, Internalization, and Degradation of LDL by HepG2 Cell Cultures

	DMEM/FBS	DMEM/ FBS+5 μmol/L CsA
LDL binding (4°C), ng LDL protein/mg cell protein	30.2±2.1	20.9±1.4*
LDL binding (37°C), ng LDL protein/mg cell protein	30.4±5.6	18.9±3.5*
LDL internalized, ng LDL protein/mg cell protein per 3 h	230.0±14.1	178.3±6.2*
LDL degraded, ng LDL protein/mg cell protein per 3 h	101.4±10.0	78.0±5.2*

LDL indicates low-density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and CsA, cyclosporin A. HepG2 cells were preincubated in DMEM/FBS with or without 5 μmol/L CsA followed by incubation with ^{125}I-labeled LDL (10 μg/mL) with or without CsA (final concentration in the binding assay, 5 μmol/L CsA). Comparable findings were made in experiments using DMEM/lipoprotein-depleted serum. Data are corrected for nonspecific binding, internalization, and degradation. Each value represents mean±SD of quadruplicate determinations.
*$P<.05$ different from control incubations.

Effect of CsA on Intracellular and Extracellular Lipid Levels

Since apoB-100 is secreted by hepatocytes only when assembled with lipids into a lipoprotein (for review, see References 40 and 41), the availability of these lipids is important for the secretion of apoB-100. The levels of triglycerides, free cholesterol, and cholesteryl esters were measured; no effect of CsA was seen on the cellular content of these lipids (Table 3). The amount of these lipids secreted by the HepG2 cells in the presence of LPDS was also measured. Concomitantly with the diminished secretion of apoB-100, a decrease in the secretion of triglycerides, free cholesterol, and cholesteryl esters was found. The amount of the lipids secreted by HepG2 cells, however, was only a small fraction of the cellular content of the lipids (see Table 3 legend). The total mass of lipids (secreted and cellular amounts) was not affected by CsA.
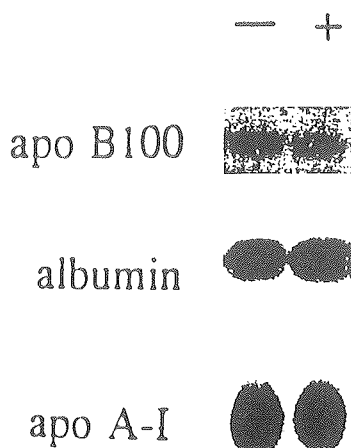

FIG 4. Autoradiograph of a Northern blot hybridization with [32P]-labeled probes for apolipoprotein (apo) B-100, apoA-I, and albumin mRNA with 6 μg total RNA applied to the gel. The hybridized blot was exposed for the appropriate times to detect the different mRNAs. Total RNA was isolated from HepG2 cells incubated in the presence (+) or absence (−) of 5 μmol/L cyclosporin A.

The fall in the secretion of triglycerides is comparable in magnitude to the effect of CsA on the apoB-100 secretion, whereas the secretion of free cholesterol and cholesteryl esters was less inhibited by CsA. This may indicate that the composition of the apoB-100–containing lipoproteins secreted by the HepG2 cells was affected. To investigate this, we separated HepG2 cell culture medium after a 24-hour incubation with or without 5 μmol/L CsA by density-gradient ultracentrifugation (Fig 5). The majority of apoB-100 is present in a lipoprotein particle with a buoyant density (1.02 to 1.06 g/mL). When HepG2 cells were incubated in the presence of CsA (5 μmol/L), the distribution of apoB-100 in the density gradient was similar to that of the control incubation except that 50% less apoB-100 was found.

Analysis of ApoB-100 Synthesis and Secretion

These results suggest that the decrease in the secreted amount of triglycerides, cholesterol, and cholesteryl esters resulted from a decline in apoB-100 secretion. To investigate the level of regulation of apoB-100 secretion in more detail, pulse-chase experiments were performed. HepG2 cells preincubated with or without 5 μmol/L CsA were pulse labeled for 10 minutes with Tran ^{35}S label and chased for 0, 10, 20, 35, 60, and 90 minutes in medium without label. Cells and media were analyzed for ^{35}S incorporation in apoA-I, apoB-100, and albumin (Figs 6 and 7). The synthesis of ^{35}S-labeled albumin and apoA-I by HepG2 cells and the secretion of these proteins in the culture medium were not affected by CsA. CsA decreased the amount of ^{35}S-labeled apoB-100 secreted by the HepG2 cells. After a 90-minute chase period in control medium, 21% of the pulse-labeled apoB-100 was found in the medium. In the presence of 5 μmol/L CsA the apoB-100 secretion was 50% of the control incubation. In addition, the intracellular amount of ^{35}S-labeled apoB-100 was already decreased by 50% after the 10-minute pulse period when incubated with CsA. Comparing the ratio between total (in cells and medium) labeled apoB-100 of CsA-treated and control cells during the chase period showed that the later steps in the intracellular assembly

TABLE 3.   Effect of Cyclosporin A on the Amount of Intracellular and Secreted Lipids

	Lipid Concentration, % of Control	
	DMEM/ FBS+5 μmol/L CsA	DMEM/ LPDS+5 μmol/L CsA
Triglycerides, intracellular	108±4	106±10
Free cholesterol, intracellular	109±7	103±13
Cholesteryl ester, intracellular	96±8	95±7
Triglycerides, secreted	. . .	53±12*
Free cholesterol, secreted	. . .	82±9*
Cholesteryl ester, secreted	. . .	73±8*

DMEM indicates Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPDS, lipoprotein-depleted serum; and CsA, cyclosporin A. Hep G2 cells were incubated in DMEM/FBS or DMEM/LPDS with or without 5 μmol/L CsA. Mass of triglycerides, free cholesterol, and cholesteryl esters was measured as described in "Methods." Results were normalized for cell protein and are mean±SD for four independent experiments. Cellular contents of triglycerides, free cholesterol, and cholesteryl esters for these four experiments were 87.4±31.3, 13.1±3.4, and 16.3±2.5 μg/mg cell protein, respectively, in DMEM/FBS and 88.1±23.2, 15.1±3.1, and 12.7±2.4 μg/mg cell protein, respectively, in DMEM/LPDS. The secretion rate of triglycerides, free cholesterol, and cholesteryl esters, measured only in DMEM/LPDS, was 3.4±0.3, 2.2±0.2, and 0.51±0.02 μg/24 h per milligram cell protein, respectively.
*Significant difference between control and CsA-treated cells.

or processing of apoB-100–containing lipoproteins were not affected by CsA (Fig 7).

To elucidate whether CsA inhibits the synthesis or cotranslational processing of apoB-100, short pulse-labeling experiments were performed. HepG2 cells, preincubated for 16 hours in DMEM/LPDS with or without 5 μmol/L CsA, were pulse labeled for 2, 4, 7, or 10 minutes in methionine- and cysteine-free medium containing Tran ^{35}S label. Cells were harvested and analyzed for ^{35}S incorporation in apoB-100, apoA-I, albumin, transferrin, and total cellular protein (Fig 8). A decreased amount of ^{35}S-labeled apoB-100 was observed in HepG2 cells treated with CsA, but only after
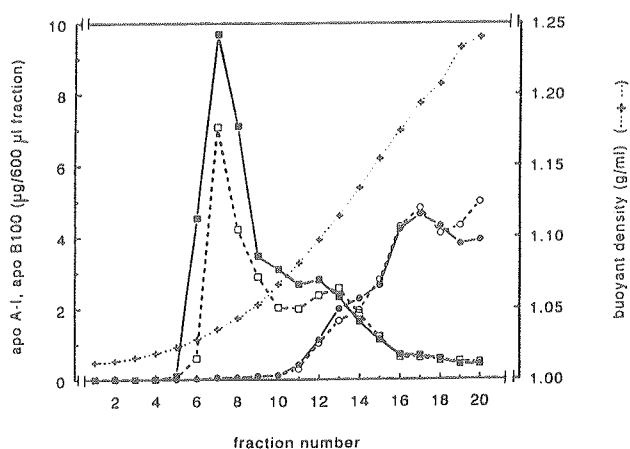

FIG 5.   Line graph showing effect of cyclosporin A (CsA) on the density gradient pattern of lipoproteins secreted by HepG2 cell cultures. HepG2 cells were incubated in the presence or absence of 5 μmol/L CsA. Density-gradient ultracentrifugation was performed as described in "Methods." Apolipoprotein (apo) A-I concentration in gradient fractions of incubations with (o) or without (●) CsA and apoB-100 concentration in gradient fractions of incubations with (□) or without (■) CsA were measured by enzyme-linked immunosorbent assay after dialysis against phosphate-buffered saline containing 0.1% casein and 2.5 mmol/L EDTA.
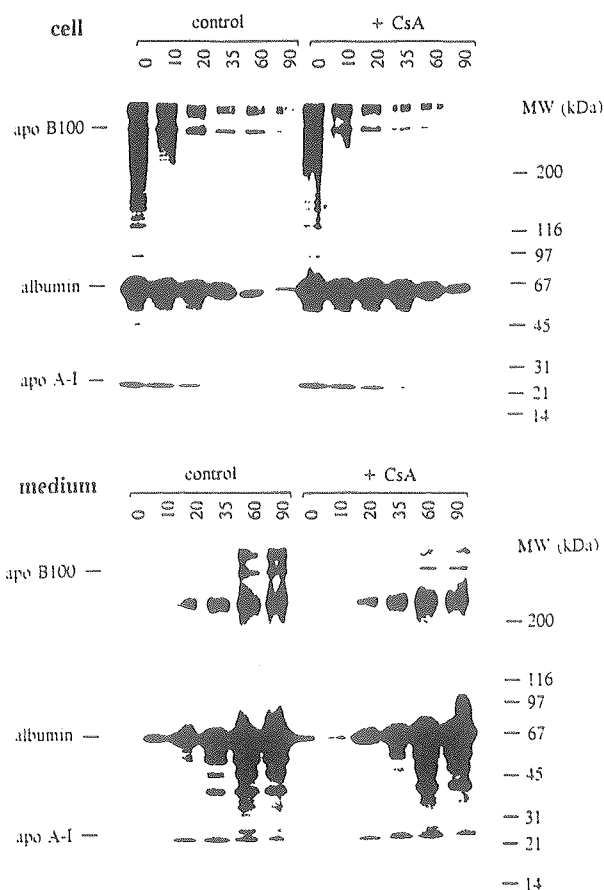

FIG 6.   Apolipoprotein (apo) B-100, apoA-I, and albumin were immunoprecipitated from cells (top) and media (bottom) of pulsed-chased control and cyclosporin A (CsA) (5 μmol/L)–treated cells, separated by gel electrophoresis (4% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis), and autoradiographed. Incubation conditions and chase times are indicated at the top of the autoradiographs.
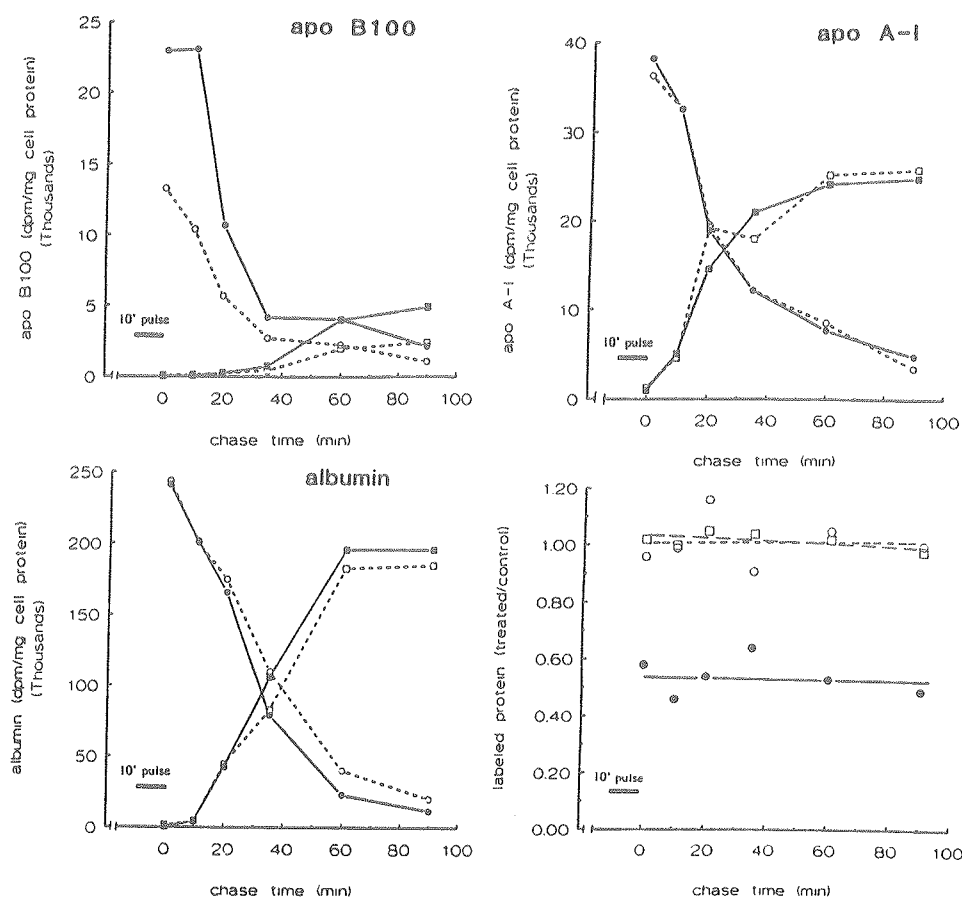
FIG 7. Line graphs showing pulse-chase analysis of the effect of cyclosporin A (CsA) on de novo synthesized apolipoprotein (apo) B-100, apoA-I, and albumin. Bands from Fig 6 were sliced and counted after solubilization of the gel. o and ● in the graphs for apoB-100, apoA-I, and albumin are data of immunoprecipitated ^{35}S-labeled apoB-100, apoA-I, and albumin present intracellularly in HepG2 cells with or without CsA (5 μmol/L) treatment, respectively. □ and ■ depict data for the secreted amounts of ^{35}S-labeled apoB-100, apoA-I, and albumin by HepG2 cells treated with or without CsA, respectively. Lower right, Proportion between total radiolabeled (in cells and medium) apoB-100 (●), apoA-I (o), and albumin (□) in HepG2 cells treated with 5 μmol/L CsA and in untreated control cultures.

an incubation time of 2 through 4 minutes. A comparable effect was found for transferrin. In accordance with previous results, we did not observe an effect of CsA on the synthesis rate of apoA-I, albumin, or total cellular protein. This indicates that CsA does not directly inhibit the synthesis of apoB-100 but that it works indirectly by a process that is closely linked to the synthesis of apoB-100.

Discussion

We showed that CsA inhibits the secretion of apoB-100 by the human hepatoma cell line HepG2 in a dose- and time-dependent way. The secretion of apoA-I and albumin and the total amount of newly synthesized protein were not affected by CsA, indicating that the effect of CsA on apoB-100 secretion is not due to a general effect of the drug on protein synthesis and secretion by HepG2 cells. ApoB-100 secretion was inhibited significantly at concentrations (2 to 5 μmol/L) that are close to plasma levels of the drug commonly observed in patients (0.5 to 5 μmol/L).[42-44] Analysis of metabolically labeled and secreted proteins by HepG2 cells in the presence or absence of CsA showed that CsA inhibited the synthesis and/or the secretion of newly synthesized apoB-100.

We observed no differences in the apoB-100, apoA-I, or albumin mRNA levels in HepG2 cells after incubation with CsA. This indicated that the decreased de novo synthesis or secretion of apoB-100 is regulated at the translational or cotranslational/posttranslational level. Before secretion from hepatocytes, apoB-100 must be assembled with lipids into a lipoprotein. Triglycerides, cholesterol, and cholesteryl esters are re-

quired for the assembly of apoB-containing lipoproteins in hepatocytes and are involved in the regulation of apoB-100 secretion by HepG2 cells.[17-22,27,45] CsA treatment of HepG2 cells did not affect the intracellular amounts of cholesterol, cholesteryl esters, or triglycerides. However, treatment with CsA resulted in an impaired secretion of all three lipids. Since only small amounts of triglycerides, cholesterol, and cholesteryl esters were secreted, the total mass of these lipids present in cells and medium was not affected by CsA. This indicated that the inhibitory effect of CsA on apoB-100 secretion was not caused by a decrease in the availability of cholesterol, cholesteryl esters, and/or triglycerides for the assembly of apoB-100–containing lipoproteins.

The observed distribution of apoB-100–containing particles (predominantly as a constituent of an LDL-like particle) and apoA-I–containing particles (in both a dense HDL-like particle and a lipid-poor or lipid-free form) in the density gradient agreed with earlier reports.[19,46] The differences in the secretion of triglycerides, cholesterol, and cholesteryl esters from HepG2 cells treated with CsA were not reflected in changes in the buoyant density of the apoB-100–containing lipoproteins secreted. This suggests that the observed changes in lipid composition do not notably affect the density of the lipoproteins secreted by HepG2 cells and/or that more cholesterol and cholesteryl esters are secreted in apoA-I–containing HDL-like particles. The latter contention is conceivable, since triglycerides are the most abundant lipids in apoB-100–containing lipoproteins and cholesterol and cholesteryl esters in apoA-I–containing particles secreted by HepG2 cells.[19,47]
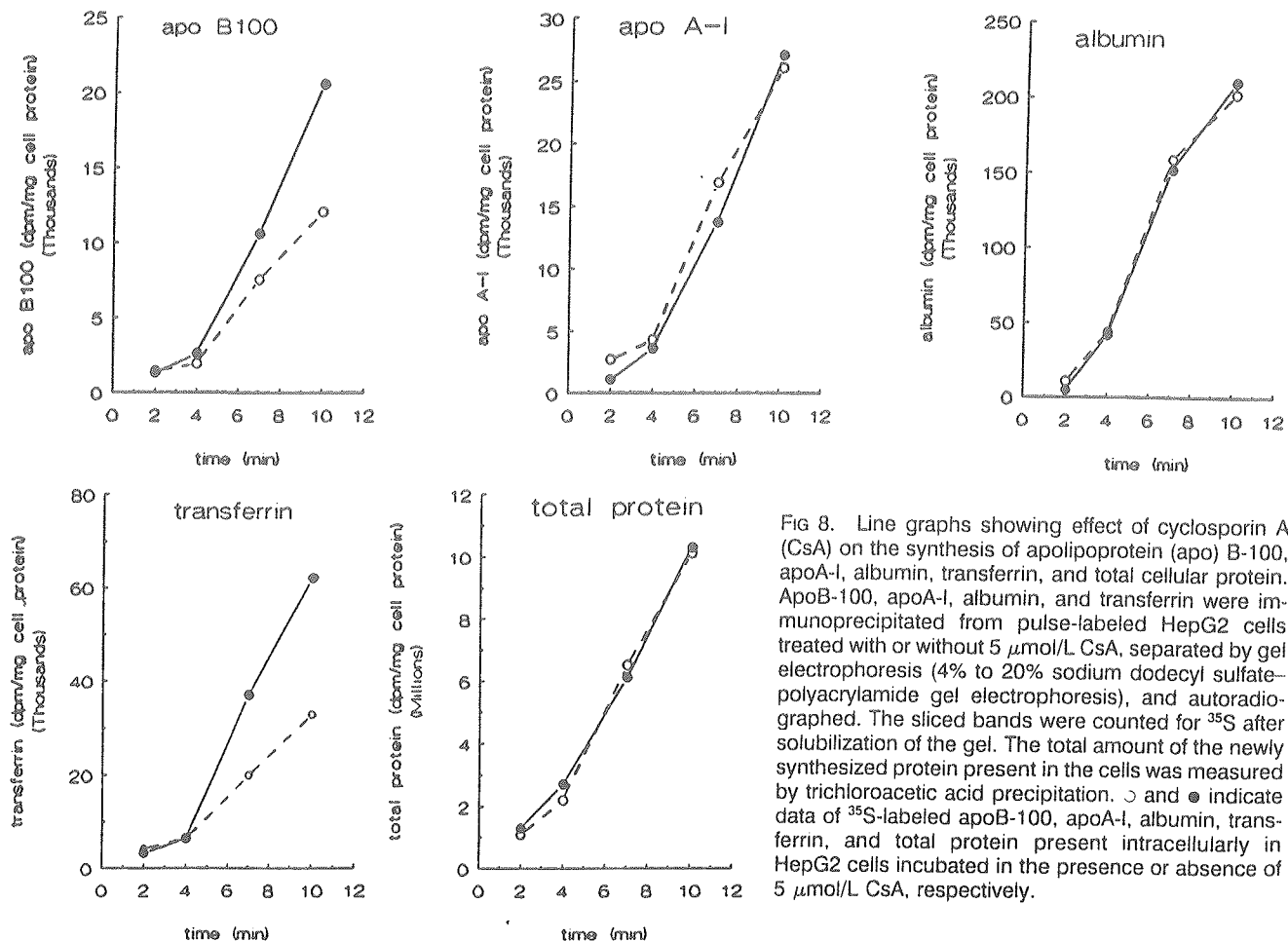
FIG 8. Line graphs showing effect of cyclosporin A (CsA) on the synthesis of apolipoprotein (apo) B-100, apoA-I, albumin, transferrin, and total cellular protein. ApoB-100, apoA-I, albumin, and transferrin were immunoprecipitated from pulse-labeled HepG2 cells treated with or without 5 μmol/L CsA, separated by gel electrophoresis (4% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis), and autoradiographed. The sliced bands were counted for ^{35}S after solubilization of the gel. The total amount of the newly synthesized protein present in the cells was measured by trichloroacetic acid precipitation. ○ and ● indicate data of ^{35}S-labeled apoB-100, apoA-I, albumin, transferrin, and total protein present intracellularly in HepG2 cells incubated in the presence or absence of 5 μmol/L CsA, respectively.

To further investigate the biochemical background of the inhibition of apoB-100 synthesis and/or secretion by CsA in HepG2 cells, pulse-chase experiments were conducted. After a 10-minute pulse and a 90-minute chase period with control cells, total ^{35}S-labeled apoB-100 recovered from the initial apoB-100 synthesized was 30% from cells and medium and 21% from the culture medium alone. These data showed that the major part of synthesized apoB-100 is not secreted but intracellularly degraded, which agrees with other studies in HepG2 cells[20,48,49] and rat hepatocytes.[39,50] The recovery of labeled apoA-I and albumin (cells and medium) was 85% to 90% of initial apoA-I and albumin synthesized, indicating there is almost no intracellular degradation of these two proteins.[39,48,51] Treatment of HepG2 cells with CsA resulted in a 50% decrease in secretion of ^{35}S-labeled apoB-100 after the 90-minute chase period. The decrease in radiolabeled apoB-100 was already observed after the 10-minute pulse period. Since the synthesis of apoB-100 in HepG2 cells takes 14 minutes,[52] our data indicated that CsA either directly inhibits the translation of apoB-100 or affects a process involved in the cotranslational processing of apoB-100. Short pulse-labeling experiments showed that CsA did not inhibit the synthesis of apoB-100 at the translational level. A decrease in the cellular amount of newly synthesized apoB-100 in HepG2 cells treated with CsA was observed only after an incubation of 2 through 4 minutes, indicating regulation at the cotranslational level.

CsA binds with high affinity to cyclophilin,[53] a peptidyl-prolyl *cis-trans* isomerase,[54,55] and inhibits this enzyme, which catalyzes the folding of nascent proteins transferred into the endoplasmic reticulum.[51,56,57] We suggest that inhibition of cyclophilin by CsA may inhibit the correct folding of apoB-100 during biosynthesis, leading to increased intracellular degradation. This would result in reduced apoB-100 and consequently lipid secretion from HepG2 cells by CsA. A similar effect of CsA on protein folding occurs during the intracellular maturation of transferrin in HepG2 cells.[51] We also observed a decreased secretion of transferrin in HepG2 cells treated with CsA. In pulse-labeling experiments (Fig 8) a comparable time course of the effect of CsA on the synthesis of apoB-100 and transferrin was observed, suggesting that a similar mechanism is involved in the intracellular maturation of both proteins.

Peptidyl-prolyl *cis-trans* isomerase activates protein disulfide isomerase,[57] another enzyme involved in the cotranslational processing of proteins. Protein disulfide isomerase, in turn, forms a complex with microsomal triglyceride transfer protein.[58] Lack of the latter protein in humans leads to abetalipoproteinemia.[59] Our data suggest an important role of peptidyl-prolyl *cis-trans* isomerase in apoB-100 maturation. Hence, secretion of apoB-100 appears to be dependent on the various components (so-called chaperons) that are involved in the process of proper folding of the protein and assembly with lipids into a lipoprotein.

The suggestion[14] that the increased plasma LDL levels in patients treated with CsA may be caused by hepatic

overproduction of apoB-100–containing lipoproteins is not supported by our data. However, the results obtained with HepG2 cells may not properly reflect what happens in vivo in the liver. A major difference is that HepG2 cells secrete apoB-100 as a constituent of a lipoprotein with the density of LDL,[19,46] possibly as a result of a reduced amount of smooth endoplasmic reticulum,[46] which may lead to a defective lipoprotein assembly and secretion.[21] In vivo apoB-100 is secreted by the liver predominantly as a constituent of very-low-density lipoprotein. Future research will be necessary to elucidate whether CsA in vivo also affects the hepatic production of apoB-100–containing lipoproteins. Processes other than the synthesis of apoB-100 may cause the elevated LDL level in patients treated with CsA. CsA inhibited the binding, internalization, and degradation of LDL in HepG2 cells (Table 2). We suggest that the latter observation and our previous finding that CsA specifically inhibits the alternative pathway in bile acid synthesis[60,61] may be related to the elevated levels of apoB-100–containing lipoproteins in vivo.

Acknowledgments

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