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Hypocholesterolemic Effects of the LDL Receptor Gene Transcriptional Upregulator CP-230821

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CP-230821 is a novel, potent LDL receptor gene transcriptional upregulator which decreases total plasma cholesterol level. Interestingly, this plasma LDL decrease does not alter hepatic lipid contents. A series of experiments was undertaken to study the molecular biology of this phenomenon. Twelve hours after CP-230821 treatment, the transcriptional activity and mRNA level of the LDL receptor gene in HepG2 cells were increased by 264% and 426%, respectively. Although treatment with the HMG-CoA reductase inhibitor compactin also increased LDL receptor gene transcription and mRNA, CP-230821 did not increase the level of HMG-CoA reductase gene transcription or mRNA. These results indicate that LDL receptor gene activity may play an important role in the decrease of plasma LDL level. These results further suggest that the LDL receptor gene and the HMG-CoA reductase gene are not strictly coordinately controlled.

Key words: CP-230821, hamster, HepG2 cells, HMG-CoA reductase, LDL receptor.

Cholesterol homeostasis in animal cells is maintained by balancing extracellular cholesterol uptake through LDL receptors with *de novo* intracellular cholesterol biosynthesis (*1*). Consequently, both the LDL receptor gene and cholesterol biosynthesis enzyme genes, such as that of the rate-limiting enzyme, HMG-CoA reductase, are under strict control at both the transcriptional and translational levels (*2-5*). Two persistent questions concerning this control are how tight this control is, and whether these genes are coordinately expressed.

Partial evidence for the coordinate control of this system stems from the discovery of sterol regulatory element binding proteins (SREBPs), membrane-bound transcription factors that regulate expression of the LDL receptor gene and genes for many enzymes in the cholesterol biosynthesis pathway, including HMG-CoA synthase, HMG-CoA reductase, farnesyl pyrophosphate synthase, and squalene synthase (*6*). In its inactive state, an SREBP is a protein anchored to the membrane of the endoplasmic reticulum. Upon cholesterol deprivation, the amino-terminal portion of SREBP is proteolytically cleaved and liberated in a two-step process, enabling its entry into the nucleus and hence its binding to sterol-response elements, SREs. This cleavage and activation is halted when cells are overloaded with sterols, such as by incubation with LDL or a mixture of cholesterol and 25-hydroxycholesterol (*6*). Recent evidence indicates that the SREBP cleavage-activating protein (SCAP) enhances SREBP cleavage from the ER membrane in a sterol-dependent manner (*6*). However, it is still undetermined whether there is a nonsterol-related mechanism regulating LDL receptor gene expression (*7-9*).

A previous report indicated that CP-230821 is a novel, potent upregulator of LDL receptor gene transcription (*10*). This compound stimulates LDL receptor promoter activity and increases LDL receptor protein level in the human hepatoma cell line HepG2 (*10, 11*). However, the precise mechanism of this effect is not known. Recently, it was suggested that CP-230821 may non-specifically stimulate intracellular tyrosine kinase(s) (*12*). Indeed, questions remain concerning the effect of LDL receptor upregulation on other regulatory genes involved in cholesterol metabolism, and possible side effects such as excessive accumulation of cholesterol in the cells.

In an effort to answer these questions, the effects of CP-230821 on plasma and hepatic lipid levels in hamsters (*Mesocricetus auratus*) and the transcriptional activity and mRNA levels of both the LDL receptor gene and HMG-CoA reductase gene in HepG2 cells were studied.

MATERIALS AND METHODS

Materials—Compound CP-230821, [2-pyridinecarbaldehyde 4-(2,3-dihydro-1,4-benzodioxin) thiosemicarbazone], was synthesized at Yamanouchi Pharmaceutical (Tokyo).

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Abbreviations: DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HDL, high-density lipoprotein; HMG-CoA, DL-3-hydroxy-3-methylglutaryl-Coenzyme A; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; RLU, a relative light emission unit; RPA, ribonuclease protection assay; SREBPs, sterol regulatory element binding proteins.

HPLC analysis confirmed the purity was greater than 98%. The HMG-CoA reductase inhibitor compactin and 25-hydroxycholesterol were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO BRL (Tokyo). [α-^{32}P]UTP (~110 TBq/mmol) was from Amersham (Tokyo). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate) was from Cosmo Bio. (Tokyo). The TA cloning kit was purchased from Invitrogen (San Diego, CA) and the GeneAmp RNA PCR kit from Perkin-Elmer (Norwalk, CT). The Ribonuclease Protection Assay (RPA) kit (RPAII™) was purchased from Ambion (Austin, TX). The Riboprobe Gemini II kit was from Promega (Madison, WI). All other reagents were obtained from standard commercial sources.

Animal Management—Male Syrian golden hamsters (Charles River Japan, Tokyo) were kept on a 12-h light-dark cycle. After random allotment to test groups, hamsters (7 weeks age) were fed either a normal diet (CE-2, Clea Japan, Tokyo) or a high cholesterol diet (0.5% cholesterol plus 5% coconut oil in CE-2).

Drug Preparation and Administration—The animals were given either CP-230821 dissolved in 0.5% methylcellulose (CP-230821 groups: 5, 10, and 30 mg/kg/d, p.o; $n=8$ for each group) or the vehicle, 0.5% methylcellulose, alone (control group, $n=10$), once a day for 5 days. At 12 h after final treatment, hamsters were anesthetized and blood was collected from the abdominal vein.

Determination of Plasma and Hepatic Lipid Levels—Plasma was separated by centrifugation at $200\times g$ for 5 min. Plasma lipid levels including total cholesterol (TC), HDL-(HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), and phospholipids (PL) were determined using a Hitachi 736-10 Automatic Blood Chemistry Analyzer (Hitachi, Tokyo) (*13*).

After blood sampling, livers from hamsters were excised and washed immediately with ice-cold phosphate-buffered saline. The livers were weighed, frozen in liquid nitrogen and stored at −80°C until analysis. Hepatic lipid levels were determined according to the manufacturer's protocol using "Determiner TC555" (total cholesterol), "Determiner FC555" (free cholesterol), "Triglyceride G-test Wako" (triglycerides), and "Determiner PL" (phospholipids) (*14*).

Elimination of Plasma LDL Particles—Male hamsters ($n=8$) fed a normal diet were treated with CP-230821 (30 mg/kg/day) for 5 days. Before injection of DiI-LDL, hamsters were anesthetized lightly with ether.

LDLs (density 1.019 to 1.063 g/ml) were prepared from the venous blood of healthy human subjects who fasted for 12 h prior to collection, as described previously (*15*). Freshly isolated LDLs were labeled with DiI as described previously (*16*), and the protein concentration was determined (*17*). These resulting DiI-LDL solutions (200 μl/177 μg protein) were injected into the foreleg veins of hamsters in a total injection time of 5 s using a 1 ml sterile syringe equipped with an 27 gauge needle. After injection, blood samples (150 μl) were obtained from the thigh veins at the indicated times. Blood sampling at "time zero" occurred 2 min after injection. Samples were centrifuged at $1,250\times g$ at 4°C and the fluorescence intensity of DiI-LDL remaining in blood was determined using a CytoFluor II (Japan PerSeptive, Tokyo) with excitation and emission wavelengths set at 530 and 590 nm, respectively. Each fluorescence intensity value was expressed as a percentage of the time zero value.

Preparation of LDL Receptor and HMG-CoA Reductase Reporter Gene Constructs—Two oligonucleotide primers (5′-GGTACCTGATTGATCAGTGTCTATTAGGTG-3′ and 5′-GCTAGCCTCTGCCAGGCAGTGTC-3′) were synthesized on an Applied Biosystems Model 394 DMA synthesizer (Chiba) and a genomic DNA fragment of the LDL receptor promoter (−626 bp to −7 bp, the GenBank™ accession # L29401) including sterol regulatory element-1 (SRE-1) was amplified by PCR using human placental tissue genomic DNA (Clontech, CA) as a template (*18*). Likewise, two oligonucleotide primers (5′-GGTACCTATCGCCTCCGCCTAGCAGC-3′and 5′-CCAGATCTCACTAGAGGCCACCG-3′) were synthesized and a cDNA fragment of the HMG-CoA reductase promoter (−345 bp to +25 bp, the GenBank™ accession # M15959) was amplified. Each of the amplified cDNA fragments was inserted into a PCR™II vector (Invitrogen, San Diego, CA). A clone with a correct sequence was amplified and subcloned into the corresponding site of a PicaGene Basic Vector (PGV-B) (Nippon Gene, Toyama). Luciferase reporter genes consisting of either the LDL receptor promoter (LBL) or HMG-CoA reductase promoter (LBH) coupled to a luciferase gene in a PGV-B vector were constructed. A control vector (abbreviated as β-gal) (Nippon Gene) containing a full-length β-galactosidase gene driven by a chicken α-actin promoter was used to normalize the transcriptional activities of the luciferase constructs.

A Luciferase Transient Transfection Assay—Transfection was conducted as reported previously (*19*). The cell line HepG2 (American Type Culture Collection, Rockville, MD) was selected as a source of model hepatocytes with a cholesterol homeostasis similar to that of human liver cells. These cells exhibit near-normal cholesterol biosynthesis, bile acid synthesis, and secretion of a number of liver-specific proteins, such as lipoproteins (*20*). Cells were seeded into a 96-well plate at a density of 1.5×10^5 cells per well. The cells were incubated for 12 h at 37°C until they formed a monolayer. The medium was then aspirated and the cells were washed with DMEM.

DEAE-dextran (250 μg/ml) plus the reporter constructs (LBL or LBH plus β-gal, 3 μg each/ml) were dissolved in DMEM, and added to the cultures, which were then incubated for 6 h. At that time, 10% FCS-DMEM containing chloroquine (30 μg/ml) was added and the cells were incubated for 12 additional hours. The transfected cells were washed with fresh medium, and 10% FCS-DMEM containing a test compound (CP-230821 or compactin 10^{-5} M) was added. The cells were incubated up to 33 h further. Dimethyl sulfoxide (DMSO) 0.1% was added as a control treatment. At the indicated times, cell lysates were prepared and the luciferase activity (RLU, a relative light emission unit) in the transfected cells was determined.

Determination of mRNA Levels in HepG2 Cells Using RPA—Both LDL receptor gene and HMG-CoA reductase gene mRNA levels were determined by means of a ribonuclease protection assay (RPA) as described previously (*4*). Briefly, sample RNA was mixed with a ^{32}P-labeled riboprobe in hybridization buffer, then the mixtures were denatured and hybridized. After incubation of the hybridized samples with RNase, the protected fragments were

analyzed by using denaturing polyacrylamide gel electrophoresis containing urea. mRNA levels were determined by estimating the photostimulated luminescence (PSL) of the corresponding mRNA band (LDL receptor, 298 bp; HMG-CoA reductase, 247 bp) with a bio-imaging analyzer system BAS-2000 (Fuji Film, Tokyo).

All data were normalized using the glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH, 283 bp) level present in the same sample (*4*). All experiments were performed in triplicate.

Protein Determination—Determination of protein concentration was done using the method of Bradford (*17*).

Statistical Analysis—Statistical significance of differences within each group was calculated by using the paired Student's *t* test.

RESULTS

Hypocholesterolemic Effects of CP-230821—CP-230821 was previously found to be a transcriptional upregulator of the LDL receptor gene (*10*). To investigate the hypolipidemic effect of CP-230821, male hamsters fed either a high cholesterol diet or a normal diet were orally treated for 5 days at 5, 10, or 30 mg/kg/d. As shown in Fig. 1a, a 30 mg/kg/d dose of CP-230821 decreased the total plasma cholesterol (TC), LDL-cholesterol (LDL-C), triglycerides (TG), and phospholipids (PL) levels by 50, 62, 54, and 25%, respectively, in animals with high dietary cholesterol. However, the HDL-cholesterol (HDL-C) level was not significantly affected (15%) even in animals fed a high cholesterol diet. Hamsters fed a normal diet (Fig. 1b), and treated with CP-230821 at a dose of 30 mg showed a decrease only in total plasma cholesterol and LDL-cholesterol levels by 30 and 47%, respectively. These results show that CP-230821 has significant lipid-lowering effects in both high-cholesterol and normal diet groups. These results show the potential systemic effect of this compound under both dietary conditions.

In Vivo Elimination of Plasma LDL Particles in Hamsters—As shown in Fig. 2, DiI-LDLs injected into hamsters treated with 30 mg/kg CP-230821 for 5 days disappeared faster than DiI-LDLs injected into the non-treated group, showing a 32% decrease at 8 h. This result indicates that the decrease of plasma LDL-cholesterol in the CP-230821-treatment group is due to a stimulation of the clearance rate of LDL particles.

Hepatic Lipid Contents in Hamsters Treated with CP-230821—To examine whether this decrease in plasma cholesterol level results in increased hepatic lipid accumulation, the hepatic lipid contents of CP-230821 treated or non-treated groups were determined. Hamsters fed a normal diet were treated with CP-230821 (30 mg/kg/d) for 5 days. As shown in Table I, the hepatic lipid contents, including total and free cholesterol, triglycerides and phospholipids, in CP-230821-treated hamsters with a

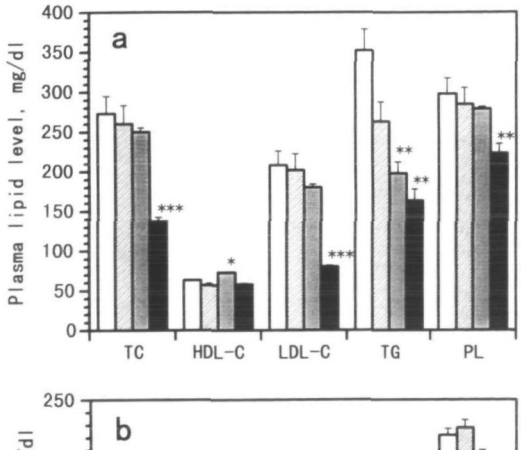

Fig. 1. **Effect of CP-230821 on plasma lipid levels in hamsters.** Hamsters were fed a high cholesterol diet (a) or a normal diet (b) for 5 days. Either CP-230821 (▨, 5 mg; ▩, 10 mg; ■, 30 mg/kg/d) or 0.5% methylcellulose solution (□, control group) was simultaneously administered with this diet. After treatment, plasma lipid levels (mg/dl) were measured as described in "MATERIALS AND METHODS." TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TG, triglycerides; PL, phospholipids. Statistical significance *vs.* control group: $*p<0.05$; $**p<0.01$, $***p<0.001$.

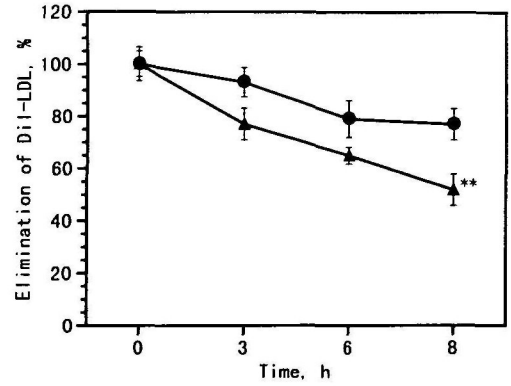

Fig. 2. **CP-230821 stimulates elimination of LDL particles from blood.** Male hamsters were administered either CP-230821 (▲, 30 mg/kg/d) or the vehicle alone (●, control group) for 5 days. After treatment, a DiI-LDL solution (200 μl) was intravenously injected, and the amount of fluorescence remaining in the blood was determined at the times indicated. Statistical significance *vs.* control group: $**p<0.01$.

TABLE I. **Effect of CP-230821 on hepatic lipid contents in hamsters.** Male hamsters were administered CP-230821 (30 mg/kg/d) or the vehicle alone for 5 days. After treatment, hepatic lipid contents ([a]μg/mg protein) were determined as described in "MATERIALS AND METHODS." [b]LW/BW: Liver weight (g) to body weight (g) ratio. No significant differences were found.

Parameter	Control	CP-230821
Total cholesterol[a]	17.2± 3.4	15.2±4.3
Free cholesterol[a]	13.1± 1.7	13.3±2.9
Triglycerides[a]	48.1±11.5	46.6±8.0
Phospholipids[a]	92.8± 9.7	95.1±8.4
LW/BW ratio[b]	3.9± 0.2	3.8±0.1

decreased blood cholesterol level showed no change when compared to the control group. Liver and body weight in hamsters showed no change after 5 days of treatment with CP-230821.

Transcriptional Activation of the LDL Receptor Gene with CP-230821 Does Not Affect the HMG-CoA Reductase Gene—The transcriptional activation effect of CP-230821 was verified using the luciferase reporter gene construct described in this paper. Figure 3 shows the time-dependent character of LDL receptor gene transcription after treatment with CP-230821. Transcriptional stimulation of this gene in HepG2 cells was also stimulated by treatment with the HMG-CoA reductase inhibitor compactin up to 24 h after treatment when compared with the control (CP-230821, 3.6-fold; compactin, 3.3-fold). However, continuous stimulation of LDL receptor gene promoter activity up to 33 h following treatment was seen only in the CP-230821 treated cells, not in those treated with compactin. In contrast, addition of 25-hydroxycholesterol completely abolished luciferase activity up to 33 h (*ca.* 82% decrease *vs.* control).

The effects of these compounds on the transcriptional activity of both the LDL receptor gene and the HMG-CoA reductase gene were also compared using the promoter vectors (LBL and LBH) described in "MATERIALS AND METHODS" (Table II). Interestingly, HMG-CoA reductase gene transcriptional activity in CP-230821-treated cells was not stimulated as was seen with the LDL receptor gene at 12 h incubation (Table II). Further incubation (24, 48, and 72 h) gave the same result (data not shown). In contrast, compactin treatment increased the transcriptional activity of both the LDL receptor gene and the HMG-CoA reductase gene. These results indicate the specificity of CP-230821 for gene transcriptional activation of LDL receptor.

Effect of CP-230821 on the LDL Receptor Gene and the HMG-CoA Reductase Gene mRNA Levels in HepG2 Cells—It was previously reported that the time-course change of mRNA levels by cholesterol depletion is different for the LDL receptor gene and the HMG-CoA reductase gene in HepG2 cells (*4*). Under cholesterol depletion conditions, HMG-CoA reductase mRNA level responded very quickly and reached a maximum level at 8-12 h after cholesterol deprivation. In contrast, the LDL receptor gene mRNA level increased slowly for up to 48 h. Since CP-230821 markedly stimulated LDL receptor gene transcription *in vitro* (Fig. 3) (*10*), mRNA levels of the LDL receptor gene and the HMG-CoA reductase gene were determined by ribonuclease protection assay (RPA). Figure 4 shows a typical pattern corresponding to the target mRNAs. Results in Table III indicate that the HMG-CoA reductase inhibitor compactin increased both the LDL receptor gene and the HMG-CoA reductase gene mRNA levels. Likewise, CP-230821 treatment dramatically increased LDL receptor mRNA level. However, the HMG-CoA reductase gene mRNA level was not changed by this treatment, similar to the response seen in the transcriptional activity assay (Table II). In contrast, the oxysterol 25-hydroxycholesterol repressed both mRNA levels (*21*).

TABLE II. **Effect of CP-230821 on the transcriptional activity of the LDL receptor gene and the HMG-CoA reductase gene.** A luciferase reporter gene construct (LBL or LBH) containing the promoter region of either the LDL receptor gene or the HMG-CoA reductase gene was transfected into HepG2 cells as described in "MATERIALS AND METHODS." After transient transfection, cells were treated with each compound for 12 h. Luciferase activity (RLU) was calculated as % *vs.* control activity. Each compound was tested at a concentration of 10^{-5} M. Statistical significance *vs.* control: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Treatment	Transcriptional activity (%)	
	LDL receptor (LBL)	HMG-CoA reductase (LBH)
Control	100.0±10.0	100.0±9.3
Compactin	217.6±38.3*	154.7±4.6**
CP-230821	263.8±66.1*	118.4±13.0
25-Hydroxycholesterol	16.3±2.4***	85.2±8.2

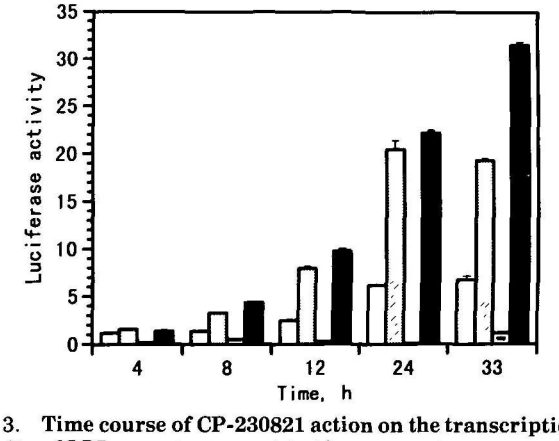

Fig. 3. **Time course of CP-230821 action on the transcriptional activity of LDL receptor gene.** A luciferase reporter gene construct (LBL) containing the promoter region of the LDL receptor gene was transfected into HepG2 cells as described in "MATERIALS AND METHODS." After transient transfection, cells were treated with each compound for the indicated times. Luciferase activity was normalized to β-gal activity. Compactin (▨), 25-hydroxycholesterol (▨), or CP-230821 (■) was tested at a concentration of 10^{-5} M each. Control group (□).

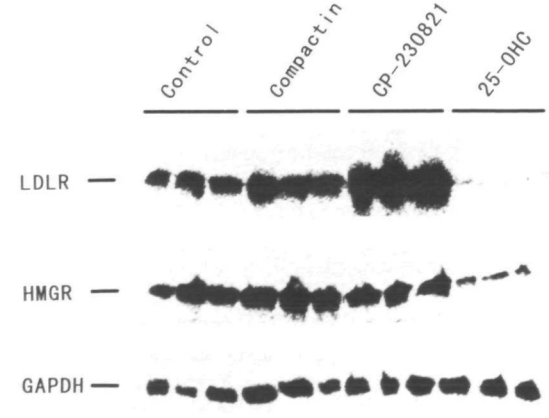

Fig. 4. **Quantitative analysis of mRNA levels of the LDL receptor gene and the HMG-CoA reductase gene in HepG2 cells.** HepG2 cells were treated with or without compactin, CP-230821, or 25-hydroxycholesterol (25-OHC). After treatment, total RNA was isolated and the mRNA levels were determined by RPA as described in "MATERIALS AND METHODS." A typical pattern of the protected fragments in each sample ($n=3$) separated by 8 M urea-PAGE is shown. LDLR, LDL receptor; HMGR, HMG-CoA reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE III. **Effect of CP-230821 on mRNA levels of the LDL receptor gene and the HMG-CoA reductase gene in HepG2 cells.** HepG2 cells were treated with compactin at 10^{-6} M, CP-230821 at 10^{-5} M, 25-hydroxycholesterol at 10^{-5} M, or DMSO (control). LDL receptor (at 12 h) and HMG-CoA reductase (at 8 h) mRNA levels were determined by RPA. Each mRNA level was normalized to the GAPDH mRNA level. Statistical significance *vs.* control: $^{*}p<0.05$; $^{**}p<0.01$.

Treatment	mRNA level (%)	
	LDL receptor	HMG-CoA reductase
Control	100.0±15.7	100.0±8.3
Compactin	138.1±10.2*	145.8±12.7*
CP-230821	425.7±31.9**	103.5±16.9
25-Hydroxycholesterol	43.2±3.6**	34.0±4.5**

Additional data obtained from TaqMan™ PCR analysis using an ABI PRISM7700 (Perkin Elmer Applied Biosystems, Tokyo) showed that treatment with CP-230821 increased the mRNA level of SREBP-1abc (*ca.* 100% increase) without affecting SREBP-2 during a short incubation (3-6 h). The SREBP-2 mRNA level in CP-230821-treated cells decreased during further incubation (*ca.* 30% decrease at 12-24 h). Previous studies found different patterns of SREBP mRNA levels between CP-230821 and compactin treatments (*22*). These results indicate that CP-230821 increases LDL receptor expression at both the transcriptional and the mRNA level, but does not affect HMG-CoA reductase gene expression.

Taken together, all these results indicate that CP-230821 stimulates hepatic LDL receptor activity at the gene transcription, mRNA expression and plasma LDL uptake activity levels, which may in turn promote the elimination of plasma LDLs.

DISCUSSION

CP-230821 increased LDL receptor activity at both the transcriptional activity and the mRNA levels (Fig. 3, Tables II and III), stimulating elimination of LDL particles from blood (Fig. 2), and thereby causing a marked decrease of plasma cholesterol *in vivo* (Fig. 1). The decreased plasma cholesterol does not seem to accumulate in the liver of hamsters treated with CP-230821 (Table I). These results suggest that this compound does not induce the development of fatty liver *in vivo*. Additional data indicate that the HMG-CoA reductase gene is not coordinately upregulated by treatment with CP-230821 *in vitro* (Tables II and III). The *in vitro* effect of this compound is very interesting since both the LDL receptor gene and the HMG-CoA reductase gene are coordinately regulated to effect intracellular cholesterol homeostasis (*23*). Further evidence suggests that CP-230821, but not compactin, does not inhibit cholesterol biosynthesis activity *in vitro* when [^{14}C]HMG-CoA or [^{14}C]acetyl-CoA is used as a substrate (data not shown). This effect may be a desirable characteristic for hypocholesterolemic drugs, since HMG-CoA reductase inhibitors such as compactin induce the enzymes *in vitro* and *in vivo* (*1, 24*). Additionally, these reductase-inhibiting drugs reduce the number of components essential for the health and proliferation of cells (*25*). However, the possibility remains that plasma LDL-cholesterol may be taken up into other tissues such as blood vessel walls, stimulating the acceleration of atherosclerosis. Consequently, further evaluation of the tissue distribution of blood LDL-cholesterol after CP-230821 treatment is needed.

It was previously reported that HOE-402 stimulates the LDL receptor pathway by increasing LDL receptor mRNA levels, hence decreasing the plasma LDL-cholesterol level, in heterozygous Watanabe heritable hyperlipidemic rabbits, but not in homozygous LDL receptor-defective animals (*26*). This emphasizes the necessity of a functional LDL receptor system for hypolipidemic action for this class of drugs. Ashton *et al.* (*27*) also reported that the novel nonsteroidal compound RPR102359 upregulates LDL receptor expression at the protein level, but does not affect cholesterol biosynthesis activity in HepG2 cells. They hypothesized that this compound may be a SREBP antagonist since the effect was observed even in the presence of 25-hydroxycholesterol. Current thinking suggests that SREBPs are upregulators of LDL receptor gene and HMG-CoA reductase gene transcription. The mature forms of SREBPs are produced by proteolytic cleavage of the immature SREBP proteins that are bound to the endoplasmic reticulum membrane (*6*). To clarify further the mechanism of LDL receptor upregulation by compounds such as RPR102359 and CP-230821, it is necessary to determine the mechanisms of proteolytic cleavage of the membrane-bound SREBPs, especially their suppression by oxysterols such as 25-hydroxycholesterol.

In addition, the results obtained using RT-TaqMan™ PCR analysis indicate that the SREBP-2 mRNA level in HepG2 cells did not increase in CP-230821-treated cells during a 24 h incubation, while the LDL receptor mRNA levels did increase. In contrast, the SREBP-1 mRNA level in CP-230821-treated cells increased rapidly, then subsided to the control level at 12 h. These data indicate that the dramatic increase of LDL receptor mRNA level caused by CP-230821 treatment may be due to the induction of SREBP-1 rather than SREBP-2. In *in vivo* experiments, cholesterol depletion in hamsters caused by feeding of bile acid-binding resin and the cholesterol synthesis inhibitor mevinolin elicited a 3-fold increase in the LDL receptor mRNA in parallel with the SREBP-2 mRNA level, but there was no significant change in the amount of mRNA for SREBP-1 (*28*). This indicates that SREBP-2 is responsible for the increased transcription following sterol depletion and that SREBP-1 may be the signal for basal transcription of the LDL receptor gene and the HMG-CoA reductase gene. These results, taken together with our data, suggest that there may be other factor(s) which transcriptionally upregulate the LDL receptor gene independently of the control of HMG-CoA reductase gene transcription.

In contrast, Yagi *et al.* (*29*) found that non-oxysterol inhibitors specifically target the HMG-CoA reductase gene promoter in HepG2 cells. However, none of these compounds inhibits the binding of LDL to its receptor, despite reducing the synthesis of HMG-CoA reductase. These results illustrate functional separation in the regulation of the LDL receptor gene and the HMG-CoA reductase gene (*1-3*).

In summary, the LDL receptor gene upregulator CP-230821 effectively lowers plasma LDL-cholesterol in hamsters fed either high-cholesterol or normal diets. This decrease was due to elimination of plasma LDL particles through LDL receptor activation. Apart from having

pharmacologic implications, identification of the targets specific for LDL receptor gene upregulation may provide a basis for finding additional hypocholesterolemic drug candidates.

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