Verapamil Increases the Apolipoprotein-Mediated Release of Cellular Cholesterol by Induction of ABCA1 Expression Via Liver X Receptor-Independent Mechanism

Shogo Suzuki, Tomoko Nishimaki-Mogami, Norimasa Tamehiro, Kazuhide Inoue, Reijiro Arakawa, Sumiko Abe-Dohmae, Arowu R. Tanaka, Kazumitsu Ueda, Shinji Yokoyama

Objective—Release of cellular cholesterol and phospholipid mediated by helical apolipoprotein and ATP-binding cassette transporter (ABC) A1 is a major source of plasma HDL. We investigated the effect of calcium channel blockers on this reaction.

Methods and Results—Expression of ABCA1, apoA-I–mediated cellular lipid release, and HDL production were enhanced in cAMP analogue-treated RAW264 cells by verapamil, and similar effects were observed with other calcium channel blockers. The verapamil treatment resulted in rapid increase in ABCA1 protein and its mRNA, but not the ABCG1 mRNA, another target gene product of the nuclear receptor liver X receptor (LXR). By using the cells transfected with a mouse ABCA1 promoter–luciferase construct (−1238 to +57bp), verapamil was shown to enhance the transcriptional activity. However, it did not increase transcription of LXR response element-driven luciferase vector.

Conclusions—The data demonstrated that verapamil increases ABCA1 expression through LXR-independent mechanism and thereby increases apoA-I–mediated cellular lipid release and production of HDL. (Arterioscler Thromb Vasc Biol. 2004;24:519-525.)

Key Words: calcium channel blocker ■ verapamil ■ ABCA1 ■ HDL ■ cholesterol ■ apolipoprotein ■ macrophage

It is a well-known fact that the risk of cardiovascular disease inversely correlates with the plasma level of high-density lipoprotein (HDL).1,2 The background hypothesis for this finding is that HDL functions to transport cholesterol from somatic cells to the liver for its conversion to bile acids; therefore, it is believed that HDL also removes cholesterol pathologically accumulated in the cells of arterial walls as an initial stage of atherosclerosis.3 HDL removes cellular cholesterol by two independent mechanisms: bidirectional exchange of cholesterol molecules between cell surface and HDL, in which cholesterol acyl-esterification on HDL creates its net efflux from the cells, and the interaction of helical apolipoproteins, perhaps dissociated from HDL with cellular surface to generate new HDL particles with the cellular lipid.4 The latter reaction, initially described for macrophages5 and then for other types of cells,6 was found defective in the cells from the patients with genetic HDL deficiency, Tangier disease,7,8 and in those treated with HDL-lowering drug probucol.9,10 Therefore, the reaction is assumed as a main source of plasma HDL. Mutations in ATP-binding cassette transporter (ABC) A1, one of the ABC superfamily members, were identified in Tangier disease and other genetic HDL deficiencies to indicate that this membrane protein is a key for generation of plasma HDL.11–13 Forced expression of ABCA1 led to the increase of apolipoprotein-mediated lipid release from cells,14,15 and its overexpression in mice resulted in a mild elevation of HDL cholesterol,16,17 implicating that expression level of this protein is a rate-limiting factor for production of plasma HDL. Accordingly, the increase of ABCA1 expression has been shown to protect the animals against atherosclerosis in certain limited conditions.18,19

Expression of ABCA1 gene is transcriptionally regulated. Loading of cholesterol in cells increases the ABCA1 expression and facilitates removal of excess cholesterol from cells.20 This reaction is mediated by the oxysterol-activated nuclear receptor, liver X receptor (LXR), which directly enhances ABCA1 gene transcription.21–23 The ABCA1 mRNA level is also increased by differentiation of THP-1 cells by phorbol ester24 or stimulation of RAW264 cells and macrophages by

Received August 26, 2003; revision accepted December 15, 2003.
From the Department of Biochemistry, Cell Biology, and Metabolism (S.S., R.A., S.A.-D., S.Y.), Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya, Japan; the Department of Biochemistry and Metabolism (T.N.-M., N.T., K.I.), National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan; and the Laboratory of Cellular Biochemistry (A.R.T., K.U.), Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan.
Correspondence to Shinji Yokoyama, Biochemistry, Cell Biology, and Metabolism, Nagoya City University Graduate School of Medical Sciences, Kawasaki-mi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. E-mail syokoyam@med.nagoya-cu.ac.jp
Shogo Suzuki and Tomoko Nishimaki-Mogami contributed equally to this work.
The present affiliation of Shogo Suzuki is Chubu National Hospital, Gengo 36-3, Morio-cho, Ohbu 474-8511, Japan.
© 2004 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000117178.94087.ba
cAMP analogues.25–27 Transforming growth factor-β25 and bacterial lipopolysaccharide29 reportedly upregulate the ABCA1 mRNA, whereas interferon-gamma downregulates it.30 However, the protein level of ABCA1 in the cells is regulated by modulation of its degradation rate. Apolipoproteins increase the ABCA1 by interfering with its proteolytic degradation,31 and unsaturated fatty acid apparently enhances its decay.32

We previously reported that calmodulin inhibitors increased the apolipoprotein A-I (apoA-I)–mediated cellular lipid release,33 indicating that calcium-related signaling plays a role in regulation of the ABCA1–mediated cellular lipid release. Calcium channel blockers are widely used for the treatment of hypertension and other cardiovascular problems. In addition to their anti-hypertensive and anti-arrhythmic effects, these drugs are implicated for independent anti-atherosclerotic effects,34,35 including improved survival rate of patients undergoing cardiac transplantation.36 Mechanisms for such beneficial effects, if any, are not established, but a few reports indicated elevation of plasma HDL in patients using verapamil.37–39 Verapamil reportedly reduced free and esterified cholesterol accumulation in thoracic aorta of cholesterol-fed rabbits.40 On the basis of these implications, we investigated the effect of verapamil on the generation of HDL by the ABCA1–apoA-I pathway. We discovered that verapamil increased apoA-I–mediated lipid release from the cell and thereby produces more HDL, by increasing the levels of ABCA1 mRNA and protein. We demonstrated that ABCA1 mRNA was increased by verapamil by mechanisms distinct from the LXR-dependent system.

Methods

Cell Culture and Measurement of Lipid Efflux to Apolipoprotein A-I

RAW264 cells41 were obtained from Riken Gene Bank (Tsukuba, Japan) and maintained in Dulbecco modified Eagle medium (DMEM)/F-12 (1:1) containing 10% fetal calf serum. The lipid efflux measurements were performed as previously described.26,33 The cells were subcultured in 6-well plates and treated with 300 μmol/L dibutyryl cAMP (dBcAMP) in DMEM/F-12 (1:1) containing 0.1% bovine serum albumin for 18 hours, and for additional time in the same medium in the presence or absence of verapamil (Wako Pure Chemicals, Tokyo, Japan), and in d-verapamil and l-verapamil (kind gifts from Knoll AG, Ludwigshafen, Germany), nifedipine, and nicardipine (Wako) for 6 hours. For measurement of lipid release, the cells were incubated in 0.1% bovine serum albumin–DMEM/F-12 (1:1) containing 0 or 10 μg/mL human apoA-I isolated from plasma HDL fraction or 0 to 1.5% 2-hydroxypropyl-β-cyclodextrin (Sigma) for 6 hours. Lipid was extracted from the medium and the cells with chloroform/methanol (2:1, volume/volume [v/v]) and hexane/isopropanol (3:2, v/v), respectively; cholesterol and choline-phospholipid were determined by a specific enzymatic method for each lipid, respectively.26,33 The medium was centrifuged at 1.64×10⁶g for 24 hours, and the bottom fraction was analyzed by density gradient ultracentrifugation as previously described.5 The lipid mass and density were determined for each fraction collected from the bottom. Alternatively, THP-1 cells (Riken Gene Bank, Tsukuba, Japan) were maintained in RPMI1640 containing 10% fetal bovine serum in humidified atmosphere of 5% CO₂ and 95% air. Differentiation of THP-1 monocytes into macrophages was induced by culturing the cells at the density of 3×10⁶ cells/well in a 6-well plate in the presence of 3.2×10⁻⁷ M of phorbol 12-myristate 13-acetate (PMA) (Wako Pure Chemical) for 72 hours.32 The cells were cultured in RPMI1640 and 0.2% bovine serum albumin for 24 hours, and they were used for the cholesterol release experiments by incubating with apoA-I (10 μg/mL) for 24 hours in the presence of verapamil (40 μmol/L).

Immunoblotting of ABCA1

Expression of ABCA1 protein was examined by immunoblotting as previously described.31,42 The cells were suspended and pelleted by centrifugation at 600g for 5 minutes, and re-suspended in cold 5 mmol/L Tris buffer (pH 8.5) containing 1 mmol/L benzamide and 1 mmol/L phenylmethylene sulfonil fluoride. After vortex mixing and centrifugation at 400g for 5 minutes, the supernatant was ultracentrifuged at 100 000g for 1 hour, and the precipitant was re-suspended in 50 mmol/L Tris-buffered saline (pH 7.4) containing the protease inhibitors and was used as a cell membrane fraction. The membrane fraction was treated in 360 mmol/L urea, 0.08% Triton X-100, 0.04% dithiothreitol, and 2% lithium dodecyl sulfate. Proteins were separated by electrophoresis in polyacrylamide gel containing 0.5% sodium dodecyl sulfate, and then electrophototheythered transferred to a PVDF membrane (Bio-Rad Laboratory, Hercules, Calif). After being blocked with 5% skim milk in Tris-buffered saline, the membrane was incubated for 2 hours at room temperature with a specific rabbit antisemur and then with an anti-rabbit IgG conjugated with horseradish peroxidase (Biosource International) for 1 hour. ABCA1 was visualized by using an ECL substrate kit (Amersham Pharmacia).

Measurement of mRNA Levels

The messenger RNA level of ABCA1 was determined by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR). The cells were incubated in the medium containing 0.1% bovine serum albumin in the presence or absence of 300 μmol/L dBcAMP for 18 hours. To examine the effect of cellular cholesterol synthesis, 50 μmol/L compactin along with 50 μmol/L mevalonic acid were added to the medium for the same samples. The cells were further incubated for 6 hours in the presence or absence of 300 μmol/L dBcAMP, 30 μmol/L verapamil, 50 μmol/L compactin (along with 50 μmol/L mevalonic acid), and 22(R)-hydroxycholesterol (2 μg/mL). Total RNA was extracted from cells by using the Qiagen (Chatsworth, Calif) RNeasy Mini Kit, and DNAase was treated according to the manufacturer’s protocol (Qiagen). The TaqMan one-step RT-PCR Master Mix Reagent Kit was used to determine relative expression levels of mRNA using the ABI Prism 7700 sequence detection system (Applied Biosystems). Primer/probe sequences used were as follows: ABCA1 forward primer, 5’-AGTTTTGGAGATGTATACATAATGTG-3’, reverse primer, 5’-CTTTTGGAGACCTTCCGGAAA-3’, probe, 5’-FAM-AAGATAAGCGGCTCACAACCTGACC-TAMRA-3’; and ABCG1 forward primer, 5’-TTCATGCTCTGGGACATCCTT-3’, reverse primer, 5’-CAGCCCGAGTTTGTATCTCGA-3’, probe, 5’-FAM-ATCTCCCTGGGCTCATCGCTTATT-TAMRA-3’. Expression data were normalized for 18S rRNA levels and were presented as fold change in the treated cells against the untreated controls.

Construction of Luciferase Reporter Genes

The 5’-flanking region of mouse ABCA1 gene (−1238/+219, relative to the transcription start site) was prepared by PCR using mouse normal ES genomic DNA as a template and a forward primer paired with SalI (5’-GTCGACCATCTTTGTTGGCAGACCTTC-3’) and a reverse primer paired with BamHI (5’-GGATCCTTCAATTGTTGGCAGACCTTC-3’) and a reverse primer paired with BamHI (5’-GGATCCTTCAATTGTTGGCAGACCTTC-3’) and a reverse primer paired with BamHI (5’-GGATCCTTCAATTGTTGGCAGACCTTC-3’) and a reverse primer paired with BamHI (5’-GGATCCTTCAATTGTTGGCAGACCTTC-3’) and a reverse primer paired with BamHI (5’-GGATCCTTCAATTGTTGGCAGACCTTC-3’). The PCR product was sub-cloned into pCR2.1 (Invitrogen). A fragment (−1238/+57) was excised and inserted into pGL3 Basic vector (Promega) to generate ABCA1 promoter–luciferase reporter construct (pABC1-Luc). LXR response element (LXRE)–driven luciferase reporter vector (pLXRE-tk-Luc) was constructed by inserting complementary oligonucleotides containing 2 copies of LXREs and 2 copies of LXREb from the sterol response element binding protein-1c promoter and overhangs for KpnI and BglII into an upstream of the thymidine kinase (tk) promoter. The mutant reporter vector
Verapamil Increases Lipid Release by ApoA-I

RAW264 cells do not react to apolipoproteins to release cellular lipid, and dBcAMP markedly induces the apoA-I–mediated lipid release.26 When the cAMP-treated cells were further incubated with verapamil for 6 hours, release of cholesterol and choline-phospholipids by apoA-I increased by 4-fold and 2-fold, respectively (Figure 1A). Density gradient centrifugation analysis of the medium showed that this increase was attributed to enhancement of generation of HDL particles (Figure 1B). In contrast, diffusion-mediated cholesterol efflux to 2-hydroxypropyl-beta-cyclodextrin was unchanged by the verapamil treatment (Figure 1C). The increase of the apoA-I–mediated cholesterol release was accompanied by a reciprocal decrease in the cellular cholesterol level (Figure 1D). The similar results were observed with human monocytic cell line cells, THP-1, after differentiated to the macrophage-like stage with PMA (Table I, available online at http://atvb.ahajournals.org). The apoA-I–mediated release of cellular cholesterol was increased by a factor of 2 or more. Unlike RAW264, cell cholesterol also increased in this cell line.

Verapamil Increases Lipid Release Through Induction of ABCA1

As shown in Figure 3A, increase of lipid release reached maximum after the 6-hour incubation with verapamil. Immunoblotting analysis of the cell membrane at the 6-hour incubation demonstrated that verapamil and nifedipine increased the ABCA1 protein level (Figure 3B). RT-PCR

Calcium Channel Blockers Increase Lipid Release

The effect of other calcium channel blockers, nicardipine and nifedipine,44 was examined. Figure 2A shows that both compounds increased release of cholesterol and phospholipids mediated by apoA-I. Because the verapamil is a mixture of its stereo isomers, the effect of d-verapamil and l-verapamil was examined separately. As shown in Figure 2B, l-verapamil increased release of cholesterol and phospholipids more efficiently than did d-verapamil at concentrations lower than 10 μmol/L. Because l-verapamil is known as a more potent calcium channel blocker than d-verapamil,44 the effect of verapamil on the cholesterol release is likely to be associated with its activity of blocking calcium channels.

Results

Transgenic Transfections and Reporter Gene Assays

RAW264 cells were co-transfected with 1.3 μg of pABCA1-Luc or empty luciferase vector (pG3L) and 0.1 μg of Renilla luciferase vector (pRL-tk) (Promega) by SuperFect (Qiagen) in 24-well plates. For LXR activation studies, 0.75 μg of pLXRE-tk-Luc or pLXREmut-tk-Luc and 0.75 μg of pSV-β-galactosidase control vector (Promega) were used. Three hours after transfection, cells were treated with or without 300 μmol/L dBcAMP for 6 to 18 hours, and subsequently with or without 30 μmol/L verapamil and the indicated reagents for 6 to 12 hours. Luciferase and β-galactosidase activities were determined in cell lysate. The firefly luciferase activity was standardized for either the Renilla luciferase or the β-galactosidase activity in each sample.

Figure 1. Verapamil enhances apoA-I–mediated lipid release in cAMP-treated RAW 264 cells. RAW264 cells were treated with dBcAMP (300 μmol/L) for 18 hours and subsequently incubated for 6 hours with 0 to 40 μmol/L verapamil in the presence of the same concentration of dBcAMP. The cells were further incubated with apoA-I (10 μg/mL) (A, B, D) or 0% to 1.5% 2-hydroxypropyl-beta-cyclodextrin (C) for 6 hours. A, Effect of verapamil on the apoA-I–mediated release of phospholipid and cholesterol. B, Density gradient analysis of the culture medium obtained from cells treated with or without verapamil (10 μmol/L) in the presence of apoA-I (10 μg/mL). C, Cholesterol efflux to 2-hydroxypropyl-beta-cyclodextrin. D, Cellular levels of total cholesterol and free cholesterol in the presence of apoA-I. The data represent the average±SD (n=3) of a typical series of the 5 experiments performed except for density gradient analysis in which each data point represent a single assay point.

Verapamil Increases Lipid Release by ApoA-I

(pLXREmut-tk-Luc) was constructed with oligonucleotides containing 2 copies of mutant LXREa and LXREb (Figure 5C).
hydroxycholesterol had an additive effect.

of ABCA1, and the combination of verapamil and 22(R)-
hydroxycholesterol was still capable of potentiating induction
expression of ABCA1 mRNA (Figure 4A, right), 22(R)-
hydroxycholesterol had an additive effect. Even when cAMP
markedly enhanced the
nation of verapamil and 22(R)-hydroxycholesterol was not dimin-
ished by compactin, and the combi-
nation of verapamil and 22(R)-hydroxycholesterol increased luci-
ferase activity (Figure 5B). Thus, the data clearly demonstrated
that verapamil did not upregulate the LXR-dependent transcription.

Figure 3. Verapamil rapidly enhances apoA-I–mediated lipid
release through an induction of ABCA1. RAW264 cells were
washed with dBcAMP (300 μmol/L) for 24 hours. Verapamil
(10 μmol/L) was included in the medium during the last 1 to 18
hours. The cells were further incubated with apoA-I (10 μg/mL)
for 6 hours, and the lipid released into the medium was ana-
alyzed (A). The data represent the average±SD (n=3) of a typical
series of three experiments. Immunoblotting analysis of ABCA1
(B) and RT-PCR analysis of ABCA1 mRNA (C) in cells treated
with or without verapamil (10 μmol/L) or nifedipine (30 μmol/L)
for 6 hours.

analysis showed the increase of ABCA1 mRNA by the
verapamil treatment (Figure 3C).

Increased ABCA1 Expression by Verapamil Is
Independent of LXR

Expression of ABCA1 has been shown to be stimulated by
LXR activation.21–23 We investigated whether increased
ABCA1 mRNA by verapamil is mediated by LXR. Real-time
quantitative RT-PCR analysis showed that verapamil in-
creased ABCA1 mRNA level in cAMP-treated and untreated
cells (Figure 4A). In the cAMP-untreated cells, ABCA1
mRNA was increased by an LXR ligand, 22(R)-hydroxychole-
sterol, and diminished by compactin, which has reportedly
depleted endogenous LXR.24,25 The effect of verapamil was not
diminished by compactin, and the combination of verapamil and
22(R)-hydroxycholesterol had an additive effect. Even when cAMP
markedly enhanced the expression of ABCA1 mRNA (Figure 4A, right), 22(R)-
hydroxycholesterol was still capable of potentiating induction
of ABCA1, and the combination of verapamil and 22(R)-
hydroxycholesterol had an additive effect.

ABCG1 is also known as LXR-responsive gene.26,27 The
level of ABCG1 mRNA was increased by 22(R)-hydroxycho-
lesterol and diminished by compactin regardless of the
presence or absence of cAMP (Figure 4B). However, verap-
amil did not increase but rather slightly decreased the
expression of ABCG1 mRNA.

In the parallel experiments, we examined the effect of
verapamil on LXRE-dependent transcriptional activity. In
cells transfected with an LXRE-driven luciferase-reporter
vector (LXRE-tk-Luc), but not in cells with a mutant LXRE-
containing reporter vector (LXREmut-tk-Luc), luciferase
activity was induced by 22(R)-hydroxycholesterol and
decreased by depleting endogenous ligand with compactin
(Figure 5). Whereas verapamil slightly induced the reporter
gene expression in the cAMP-treated cells, similar extent of
induction was observed even when LXRE was mutated
(Figure 5B). Thus, the data clearly demonstrated that verap-
amil did not upregulate the LXRE-dependent transcription.

Figure 4. Verapamil induces ABCA1 mRNA but not that of
ABCG1 through LXR-independent mechanisms. RAW264 cells
were cultured in the presence or absence of dBcAMP
(300 μmol/L) and compactin (comp) (50 μmol/L, along with
50 μmol/L mevalonic acid) for 18 hours and subsequently
in the preses of cAMP and 22(R)-hydroxycholesterol (22(R)-HC) (2 μg/mL), as
indicated. Total RNA was isolated from the cells and relative
mRNA levels of ABCA1 (A) and ABCG1 (B) were measured with
TaqMan one-step RT-PCR analysis. Data were normalized to
18S rRNA levels. The values represent the average±SD relative
to the untreated cells (taken as 1) from 3 independent experi-
ments performed in duplicate.

Verapamil Increases Promoter Activity of ABCA1

To determine whether increased ABCA1 mRNA level is
resulted from enhanced gene transcription, we examined the
effect of verapamil on promoter activity of ABCA1. A mouse
ABCA1 promoter–luciferase construct (–1238/+57) was
prepared and transfected into RAW264 cells. As expected by
the presence of a consensus binding site for LXR/RXR in this
promoter region,28 treatment of cells with an LXR ligand,
22(R)-hydroxycholesterol, increased luciferase activity (Fig-
ure 6). This promoter region was unresponsive to cAMP
stimulation, whereas cAMP greatly increased ABCA1
mRNA level (by 60-fold) (Figure 4A), which is consistent
with our previous report.29 Verapamil treatment markedly
enhanced luciferase activity (by 3.5-fold) in the presence but
not in the absence of cAMP, indicating increased transcrip-
tion.

Discussion

In the present study, we report that generation of HDL
mediated by apoA-I and ABCA1 in RAW 264 cells is
enhanced by verapamil, which has been indicated to cause a
significant increase in plasma HDL in several previous
reports.37–39 Because verapamil did not influence cellular
cholesterol efflux by its free diffusion represented by
2-hydroxypropyl-beta-cyclodextrin-dependent cholesterol ef-
flux, the ABCA1-mediated pathway was specifically inves-
tigated to elucidate the mechanism. We found that the rapid
Verapamil activates mouse ABCA1 promoter (from -1238 to +57 bp). RAW 264 cells were transfected with ABCA1 promoter-luciferase construct (pABC1-Luc) or empty luciferase vector (pGL3) in the presence of a Rennia luciferase plasmid as a reference. Cells were treated with or without dBcAMP (300 μmol/L) for 6 hours and subsequently treated for 12 hours with or without verapamil (30 μmol/L) or 22(R)-hydroxysterolol (22(R)-HC) (2 μg/mL), as indicated, in the presence or absence of dBcAMP. Cellular firefly luciferase activity was measured and normalized to Rennia luciferase activity. The values represent the average±SD from 3 independent experiments performed in triplicate.

Figure 5. Verapamil (ver) does not increase LXRE-dependent transcriptional activity. RAW264 cells were transfected with a luciferase reporter plasmid containing 4 copies of LXREs upstream of the thymidine kinase promoter (LXRE-tk-Luc) or a plasmid-containing mutated LXRE complex (LXREmut-tk-Luc) in the presence of pSV-β-galactosidase as a reference plasmid. Cells were treated as described in Figure 4. Luciferase activity in dBcAMP-ununtreated (A) and dBcAMP-treated (B) cells were measured and normalized to the β-galactosidase activity. The values represent the average±SD relative to untreated cells (taken as 1) from 2 independent transfections performed in triplicate. C. Sequences of 2 LXREs derived from the mouse sterol response element binding protein-1c promoter and mutated LXREs (LXREMUT). Mutated bases are underlined and the LXREs are indicated as bold letters.

Enhancement of lipid release by verapamil was accompanied by elevation of the mRNA level and an increase in ABCA1 protein. Furthermore, a region of ABCA1 promoter (−1238/+57) was shown to respond to verapamil treatment, demonstrating that verapamil upregulated ABCA1 at the transcriptional level.

Induction of ABCA1 mRNA is primarily mediated by the activation of the nuclear receptor LXR.21–23 However, the following findings in this article indicate that LXR is not involved in the ABCA1 induction by verapamil. First, verapamil did not enhance transcription of the LXRE-driven luciferase (Figure 5). Second, upregulation of ABCA1 mRNA by verapamil was additive to 22(R)-hydroxysterolol-elicted increase and was not diminished by depletion of endogenous LXR ligands by compactin (Figure 4A). Third, expression of another LXR target gene, ABCG1,45,46 was not influenced by verapamil (Figure 4B).

ABCG1 was shown to be capable of mediating active release of cholesterol and phospholipid in macrophages.49 However, in the present study, we provide evidence that two genes, ABCA1 and ABCG1, are differentially regulated. We demonstrated that treatment of RAW264 cells with cAMP markedly increased the ABCA1 mRNA, whereas the ABCG1 mRNA was unchanged. In addition, verapamil enhanced expression of ABCA1 but not ABCG1. These findings were apparently coincidental with the previous report of differential regulation of ABCA1 and ABCG1 gene shown in the lipopolysaccharide-stimulated THP-1 cells.29

The effect of cAMP on the ABCA1 activity is by the increase of its gene transcription28 and by altering its specific activity through phosphorylation.50,51 However, a role of cAMP is somewhat puzzling in the present data. Verapamil induced the transcription of the ABCA1 gene in the presence of cAMP, with respect to the increase of the ABCA1 mRNA and the results of the reporter gene assay. However, verapamil increased the mRNA even in the absence of cAMP, whereas it did not enhance the reporter gene transcription in the absence of cAMP. In addition, the reporter gene did not respond to cAMP in the conditions given. However, the reporter gene assay may not always give a consistent result for the effect of cAMP. An earlier study showed limited responsiveness of the conserved region of human ABCA1 promoter to cAMP stimulation,52 but another study reported unresponsiveness of the promoter construct containing the same region.22 Thus, the effect of cAMP requires more studies to understand its underlying mechanisms. Nevertheless, our results with RAW264 cells without cAMP treatment and with the differentiated THP-1 cells indicate that the effect of calcium channel blockers can be independent of cAMP for the endogenous ABCA1 gene.

Verapamil is a well-known, widely used calcium channel blocker. We showed that other calcium channel blockers, nicardipine and nifedipine, also caused enhancement in apoA-I-mediated lipid release, so that this effect is likely to be related to inhibition of calcium channel itself. This view was further supported by the finding that 1-verapamil, a more potent calcium channel blocker,44 was more effective in enhancing lipid release than d-verapamil. The results were also consistent with our previous findings that calmodulin inhibitors increased the apoA-I-mediated cellular lipid release from the same cell-line cells.33 Inhibition of the calcium-related signaling pathways was shown to downregulate various genes, but upregulation of a few genes was also reported, including interleukin-653 and the LDL receptor.54 Involvement of protein kinase C was suggested for the latter case. Further investigation is required to clarify exact mechanism by which verapamil and other calcium channel blockers induce ABCA1 expression and potentially increase HDL.
This should include obtaining the evidence that the reaction is generally observed in other types of cells and identification of specific sites of the promoter for upregulation of transcription. The findings would open a new path to seek technology to enhance the HDL production and thereby prevent atherogenesis.

Acknowledgments

This work was supported in part by grants-in-aid from Ministry of Science, Education, Technology, Culture, and Sports of Japan, by a grant (MF-16) from the Organization for Pharmaceutical Safety and Science, Education, Technology, Culture, and Sports of Japan, by a fund from the Japan Health Sciences Foundation.

References

prevention of coronary artery disease in heart-transplant recipients. 
Verapamil Increases the Apolipoprotein-Mediated Release of Cellular Cholesterol by Induction of ABCA1 Expression Via Liver X Receptor-Independent Mechanism
Shogo Suzuki, Tomoko Nishimaki-Mogami, Norimasa Tamehiro, Kazuhide Inoue, Reijiro Arakawa, Sumiko Abe-Dohmae, Arowu R. Tanaka, Kazumitsu Ueda and Shinji Yokoyama

Arterioscler Thromb Vasc Biol. 2004;24:519-525; originally published online January 15, 2004; doi: 10.1161/01.ATV.0000117178.94087.ba
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/24/3/519

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2004/03/01/24.3.519.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Table I

Cellular cholesterol release from differentiated THP-1 cells. See the text for the detail of experimental conditions. The data represent mean ± S.E. for three samples.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol released to the medium (µg/mg cell protein)</th>
<th>Cellular cholesterol (µg/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.35 ± 0.02</td>
<td>40.7 ± 1.0</td>
</tr>
<tr>
<td>ApoA-I (10 µg/mL)</td>
<td>1.31 ± 0.10</td>
<td>30.9 ± 0.2</td>
</tr>
<tr>
<td>Verapamil (40 µM)</td>
<td>0.41 ± 0.10</td>
<td>50.6 ± 0.8</td>
</tr>
<tr>
<td>Verapamil + apoA-I</td>
<td>2.61 ± 0.08</td>
<td>46.1 ± 1.1</td>
</tr>
</tbody>
</table>