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

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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
cAMP analogues. Transforming growth factor-β and bacterial lipopolysaccharide reportedly upregulate the ABCA1 mRNA, whereas interferon-gamma downregulates it. 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, and unsaturated fatty acid apparently enhances its decay.

We previously reported that calmodulin inhibitors increased the apolipoprotein A-I (apoA-I)-mediated cellular lipid release, 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 antiatherosclerotic effects, including improved survival rate of patients undergoing cardiac transplantation. Mechanisms for such beneficial effects, if any, are not established, but a few reports indicated elevation of plasma HDL in patients using verapamil. Verapamil reportedly reduced free and esterified cholesterol accumulation in thoracic aorta of cholesterol-fed rabbits. 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 increases 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 cells 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. 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-beta-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 quantitative reverse-transcription polymerase chain reaction (RT-PCR) using verapamil. The cells were suspended and pelleted by centrifugation at 400 g for 5 minutes, 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% diethiothreitol, and 2% lithium dodecyl sulfate. Proteins were separated by electrophoresis in polyacrylamide gel containing 0.5% sodium dodecylsulfate, and then electrophotographically 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 anti-serum and then with an anti-rabbit IgG conjugated with horseradish peroxidase (Biosouce 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 some 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 2(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'-AGGTTTGGAGATGTGTTATACAATAGTTG-3', reverse primer, 5'-CTTTTATGGAACCTTCCCCGAAA-3', probe, 5'-FAM-ACGAGATACAGCCCTCAACCTTGACC-TAMRA-3'; and ABCG1 forward primer, 5'-TCATCGTCCTGGGACATCCTTT-3', reverse primer, 5'-CAGGCCGATTTTGTATCGTA-3', probe, 5'-FAM-ATCTCCCTGCGGCTACATCGCTTTATT-TAMRA-3'. Expression data were normalized for 18S rRNA levels and were presented as fold change in the treated cells against the untreated cells.

**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 Sall (5'-GTGCAGCTCTTGGTGGTGCGACTCTT-3') and a reverse primer paired with BamHI (5'-GGATCCCTTACGCTTTTCCACATTTTGTTT-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 (pABCA1-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
RESULTS

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. 

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-1 (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 μM) 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.

Transient Transfections and Reporter Gene Assays

RAW264 cells were co-transfected with 1.3 μg of pABCA1-Luc or empty luciferase vector (pGL3) and 0.1 μg of Renilla luciferase vector (phRL-tk) (Promega) by SuperFect (Qiagen) in 24-well plates. For LXRE 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 2. Effect of nicardipine, nifedipine (A), d-verapamil, and l-verapamil (B) on apoA-I-mediated lipid release. RAW264 cells were treated with dBcAMP (300 μmol/L) for 18 hours and subsequently incubated for 6 hours with various concentrations of the calcium channel blockers in the presence of dBcAMP. Cholesterol and choline-phospholipids (PL) released into the medium during the additional 6-hour incubation with apoA-I (10 μg/mL) were analyzed. The data represent the average ± SD (n = 3) of a typical series of the 3 experiments performed.

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 l-verapamil and d-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.

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).
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. We investigated whether increased ABCA1 mRNA by verapamil is mediated by LXR. Real-time quantitative RT-PCR analysis showed that verapamil increased 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)-hydroxycholesterol, and diminished by compactin, which has reportedly depleted endogenous LXR (Figure 4A, left). 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 induction of ABCA1, ABCA1 mRNA remained unresponsive to cAMP (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. The level of ABCG1 mRNA was decreased by 22(R)-hydroxycholesterol and diminished by compactin regardless of the presence or absence of cAMP (Figure 4B). However, verapamil 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 verapamil did not upregulate the LXRE-dependent transcription.

**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, treatment of cells with an LXR ligand, 22(R)-hydroxycholesterol, increased luciferase activity (Figure 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. Verapamil treatment markedly enhanced luciferase activity (by 3.5-fold) in the presence but not in the absence of cAMP, indicating increased transcription.

**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. Because verapamil did not influence cellular cholesterol efflux by its free diffusion represented by 2-hydroxypropyl-beta-cyclodextrin-dependent cholesterol efflux, the ABCA1-mediated pathway was specifically investigated to elucidate the mechanism. We found that the rapid
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)-hydroxysterol–elicited increase and was not diminished by depletion of endogenous LXR ligands by compactin (Figure 4A). Third, expression of another LXR target gene, ABCG1, was not influenced by verapamil (Figure 4B).

ABCG1 was shown to be capable of mediating active release of cholesterol and phospholipid in macrophages. 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.

The effect of cAMP on the ABCA1 activity is by the increase of its gene transcription and by altering its specific activity through phosphorylation. 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, but another study reported unresponsiveness of the promoter construct containing the same region. 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, 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. Inhibition of the calcium-related signaling pathways was shown to downregulate various genes, but upregulation of a few genes was also reported, including interleukin-6 and the LDL receptor. 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 Research, and by a fund from the Japan Health Sciences Foundation.

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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
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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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.

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