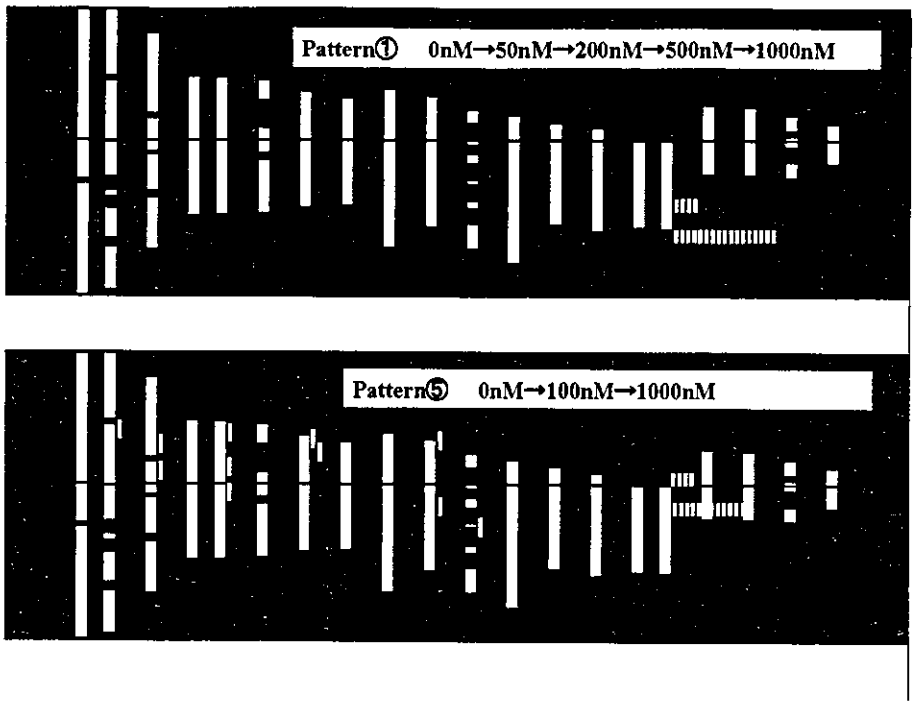

Figure 2 Chromosome ideogram pattern in DR1000L1 and L5 cell pools. The short bar corresponded to the amplified gene location.

As shown in Figure 2, the amplified location was distributed in pattern 5. On the other hands, the amplified location is concentrated only one chromosome in pattern 1. From the comparison with patterns, the pattern 1 cell pool show the higher specific production rate and was stable for long-term cultivation. Obviously, it should be compared with another increasing pattern results, but it was estimated that this chromosome location is one of the suitable site for gene-amplification. In order to pass over the long-term gene amplification process, we are now trying to construct the universal cell line which was integrated the gene-amplified targeting vector on the suitable chromosome location for gene recombination system.

ACKNOLEDGEMENT

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α1-Syntrophin Modulates Turnover of ABCA1*

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ABCA1 (ATP-binding cassette transporter A1) mediates the release of cellular cholesterol and phospholipid to form high density lipoprotein. Functions of ABCA1 are highly regulated at the transcriptional and post-transcriptional levels, and the synthesized ABCA1 protein turns over rapidly with a half-life of 1–2 h. To examine whether the functions of ABCA1 are modulated by associated proteins, a yeast two-hybrid library was screened with the C-terminal 120 amino acids of ABCA1. Two PDZ (PSD95-Discs large-ZO1) proteins, α1-syntrophin and Lin7, were found to interact with ABCA1. Immunoprecipitation revealed that α1-syntrophin interacted with ABCA1 strongly and that the interaction was via the C-terminal three amino acids SYV of ABCA1. Co-expression of α1-syntrophin in human embryonic kidney 293 cells retarded degradation of ABCA1 and made the half-life of ABCA1 five times longer than in the cells not expressing α1-syntrophin. This effect is not common among PDZ-containing proteins interacting with ABCA1, because Lin7, which was also found to interact with the C terminus region of ABCA1, did not have a significant effect on the half-life of ABCA1. Co-expression of α1-syntrophin significantly increased the apoA-I-mediated release of cholesterol. ABCA1 was co-immunoprecipitated with α1-syntrophin from mouse brain. These results suggest that α1-syntrophin is involved in intracellular signaling, which determines the stability of ABCA1 and modulates cellular cholesterol release.

Cholesterol is not catabolized in the peripheral cells and, therefore, is mostly released and transported to the liver for conversion to bile acids to maintain cholesterol homeostasis. The same pathway may also remove cholesterol that has pathologically accumulated in cells, such as at the initial stage of atherosclerosis. The assembly of high density lipoprotein (HDL)[1] particles by lipid-free apolipoproteins with cellular lipid has been recognized as one of the major mechanisms for the cellular cholesterol release (1, 2). ApoA-I-mediated cholesterol efflux is a major event in "reverse cholesterol transport," a process that generates HDL and transports excess cholesterol from the peripheral tissues, including the arterial wall, to the liver for biliary secretion. The importance of ABCA1 in this active cholesterol-releasing pathway for regulating cholesterol homeostasis became apparent with the finding that it is impaired in the cells from patients with Tangier disease, a genetic deficiency of circulating HDL (3, 4). Tangier disease is caused by mutations in ABCA1. ABCA1 mutations are also a cause of familial HDL deficiency and are associated with premature atherosclerosis (5, 6).

Cholesterol is a prerequisite for cells, but, at the same time, the hyper-accumulation of cholesterol is harmful to cells. Therefore, the expression of ABCA1 is highly regulated at both the transcriptional and post-transcriptional level. The transcription of ABCA1 is regulated by the intracellular oxysterol concentration via the LXR/RXR nuclear receptor (7), and the synthesized ABCA1 protein turns over rapidly with a half-life of 1–2 h (8–10). However, the post-translation regulatory mechanism of ABCA1 is unclear. We analyzed the associated proteins that could be involved in the post-translational regulation of ABCA1. By yeast two-hybrid screening with the C-terminal 120 amino acids of ABCA1, two PDZ (PSD95-Discs large-ZO1)-binding proteins, α1-syntrophin and Lin7, were found to interact with ABCA1. Immunoprecipitation confirmed the association of α1-syntrophin and ABCA1 via its C-terminal amino acids. The importance of this interaction in the regulation of ABCA1 function was examined.

EXPERIMENTAL PROCEDURES

Materials—The anti-ABCA1 monoclonal antibody KM3073 was generated against the first extracellular domain of the human ABCA1 protein in rats. Anti-ABCA1 monoclonal antibody KM3110 was generated against the C-terminal 20 amino acids of ABCA1 in mice. Anti-ABCA1 polyclonal antibody, previously described (11), was used for immunostaining. Affinity-purified antibody specific for α1-syntrophin was prepared using recombinant proteins. Human α1-syntrophin (amino acids 169–346) was fused to glutathione *S*-transferase (GST) in the pGEX vector (Amersham Biosciences) and to the maltose-binding protein (MBP) in the pMAL-c2 vector (New England Biolabs, Inc.). The GST-α1-syntrophin protein was used as an antigen. Obtained rabbit antiserum was affinity purified with the column coupled with the MBP-α1-syntrophin fusion protein. Anti-FLAG epitope monoclonal antibody M2 was purchased from Sigma. Human apoA-I was a gift from Dr. Shinji Yokoyama, Nagoya City University Graduate School of Medical Sciences.

Animals—16-week-old α1-syntrophin (−/−) (12) and wild-type C57BL/6 mice were used in this study. The animals were allowed *ad libitum* access to food and drinking water. Mice carrying mutations were identified by Southern blot analysis as described (12).

Yeast Two-hybrid Library Screening—The Matchmaker Two-hybrid

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[1] The abbreviations used are: HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; HEK293, human embryonic kidney 293; LXR/RXR, liver X receptor/retinoid X receptor; PBS, phosphate-buffered saline; PDZ, PSD95-Discs large-ZO1.

System 3 from Clontech was used following the manufacturer's instructions. The ABCA1 C terminus region coding for 120 amino acids was cloned from cDNA (13) in pGBKT7. The yeast strain AH109, transformed with pGBKT7/ABCA1-C120, was mated with the yeast strain Y187, which had been pretransformed with a human bone marrow cDNA library. The plasmids, purified from β-galactosidase positive clones, were transformed into *Escherichia coli* and sequenced.

Cellular Lipid Release Assay—Cells were subcultured in poly-L-Lys-coated 6-well plates at a density of 1.0×10^6 cells in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. After 24 h, cells were transfected with ABCA1 and/or FLAG-α1-syntrophin using LipofectAMINE (Invitrogen). After 24 h of incubation, the cells were washed with phosphate-buffered saline (PBS) and incubated in 0.02% bovine serum albumin in Dulbecco's modified Eagle's medium with 10 μg/ml apoA-I. The lipid content in the medium was determined after 24 h incubation as described previously (14).

Immunoprecipitation Analysis—HEK293 cells, transiently expressing ABCA1, were lysed with PBS containing 1% Triton X-100 and protease inhibitors (100 μg/ml 4-(amidino)-phenylmethanesulfonyl fluoride hydrochloride (pAPMSF)), 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Equal amounts of total protein were incubated with 5 μg of anti-FLAG antibody M2 for 1 h at 4 °C. Brain of normal mice and mice lacking α1-syntrophin (α1-*Syn*$^{-/-}$) (12) was homogenized in ice-cold homogenization buffer (10 mM sodium phosphate, 0.4 M NaCl, 5 mM EDTA, pH 7.8, and the protease inhibitors). The particulate fraction was pelleted by centrifugation (12,000 × *g* for 10 min), resuspended in 10 volumes of homogenization buffer, and recentrifuged. Washed pellets were solubilized in homogenization buffer containing 1% Triton X-100 and incubated on ice for 30 min. The suspension was centrifuged again, and the supernatant was then incubated with anti-α1-syntrophin rabbit polyclonal antibody for 100 min at 4 °C. The immunocomplexes were incubated with protein G-Sepharose (Sigma) for 1 h and washed four times with homogenization buffer containing 1% Triton X-100. The bound proteins were separated by SDS-PAGE (7%) and analyzed by immunoblotting using the anti-ABCA1 antibody KM3073 or KM3110.

Immunostaining—HEK293 cells were co-transfected with ABCA1 and FLAG-tagged α1-syntrophin or FLAG-tagged Lin7 using LipofectAMINE. The cells were fixed in 4% paraformaldehyde and 5% sucrose in PBS$^+$ (PBS with 0.87 mM $CaCl_2$ and 0.49 mM $MgCl_2$) for 30 min and permeabilized for 5 min in 0.4% Triton X-100 in PBS$^+$. The cells were blocked with 10% goat serum diluted with PBS$^+$. This was followed by incubation with anti-ABCA1 polyclonal antibody and anti-FLAG M2 antibody. The cells were then stained with Alexa 488-labeled anti-rat IgG antibody and Alexa 564-labeled anti-mouse IgG antibody (Molecular Probes) as secondary antibodies. The fluorescence images were obtained using an Axiovert microscope (Carl Zeiss) equipped with a MicroRadiance confocal laser-scanning microscope (Bio-Rad).

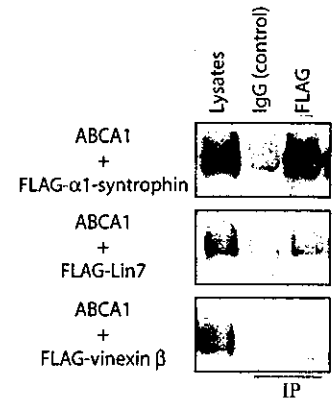

FIG. 1. ***In vivo* association of ABCA1 with α1-syntrophin.** HEK293 cells were co-transfected with human ABCA1 and FLAG-tagged α1-syntrophin, FLAG-tagged Lin7, or FLAG-tagged vinexin β. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody. Immunocomplexes and cell lysates (5%) were subjected to immunoblotting using Anti-ABCA1 monoclonal antibody KM3110, generated against the C-terminal 20 amino acids of ABCA1. Mouse IgG was used as a negative control. The data are representative of three independent experiments.

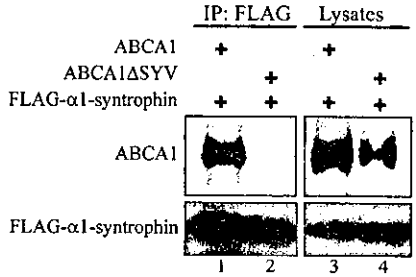

FIG. 2. **ABCA1 interacts with α1-syntrophin via the C-terminal three amino acids.** HEK293 cells were co-transfected with ABCA1 or ABCA1ΔSYV, in which the C-terminal three amino acids were trimmed, and with FLAG-tagged α1-syntrophin. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody. Immunocomplexes and cell lysates (5%) were subjected to immunoblotting using the anti-ABCA1 monoclonal antibody KM3073, generated against the first extracellular domain of the human ABCA1, and an anti-FLAG antibody. The data are representative of two independent experiments.

RESULTS

ABCA1 Interacts with Two PDZ-binding Proteins—To search for proteins that are associated with the C-terminal region of ABCA1, a fusion construct of the Gal4 DNA-binding domain with the C-terminal 120 amino acids of human ABCA1 was used as bait for two-hybrid screening. The identified genes contained two PDZ-containing proteins, α1-syntrophin (10 clones) and Lin7 (two clones). To determine whether the interaction between ABCA1 and α1-syntrophin or Lin7 occurs *in vivo*, we transfected FLAG-tagged α1-syntrophin, FLAG-tagged Lin7, or FLAG-tagged vinexin β (15) (as a negative control) together with ABCA1 into HEK293 cells. Lysates prepared from transfected cells were immunoprecipitated with anti-FLAG antibody, and precipitates were evaluated by immunoblotting with anti-ABCA1 antibody. As shown in Fig. 1, ABCA1 was co-immunoprecipitated with FLAG-tagged α1-syntrophin or FLAG-tagged Lin7, but not with FLAG-tagged vinexin β. ABCA1 was not precipitated with mouse IgG control from any of the lysates in which the expression of ABCA1 (Fig. 1) and FLAG-tagged proteins (data not shown) were detected by immunoblotting. More than 25% of the ABCA1 expressed in HEK293 was roughly estimated to be co-immunoprecipitated with FLAG-tagged α1-syntrophin, suggesting strong interaction between ABCA1 and α1-syntrophin (Fig. 2). The interaction between ABCA1 and Lin7 seemed to be weak, because the amount of precipitated ABCA1 with Lin7 was much less than that with α1-syntrophin. The amount of ABCA1 in lysates was consistently higher when co-expressed with α1-syntrophin than with other proteins.

ABCA1 Interacts with α1-Syntrophin via the C-terminal Three Amino Acids—ABCA1 contains the amino acid sequence ESYV at the C terminus, which has been described as a binding target for syntrophin PDZ domains (16). To determine whether the C-terminal three amino acids SYV are important for the interaction, ABCA1ΔSYV, in which these amino acids were trimmed, was co-expressed with FLAG-tagged α1-syntrophin in HEK293 cells. Although the expression of ABCA1ΔSYV was detected in the lysates, no ABCA1ΔSYV was co-precipitated with FLAG-tagged α1-syntrophin. These results suggest that the interaction is mediated with the C-terminal three amino acids SYV of ABCA1 and α1-syntrophin PDZ domains.

Co-localization of the ABCA1 and PDZ-containing Proteins α1-Syntrophin and Lin7—ABCA1 is mainly localized to plasma membrane but is also substantially expressed in intracellular compartments (11, 17–20). To determine whether ABCA1 and α1-syntrophin or Lin7 are co-localized in cells, ABCA1 was co-transfected with FLAG-tagged α1-syntrophin or FLAG-tagged Lin7 into HEK293 cells. The subcellular localization of these proteins was examined under a confocal laser scanning microscope. α1-Syntrophin resided mainly on plasma mem-

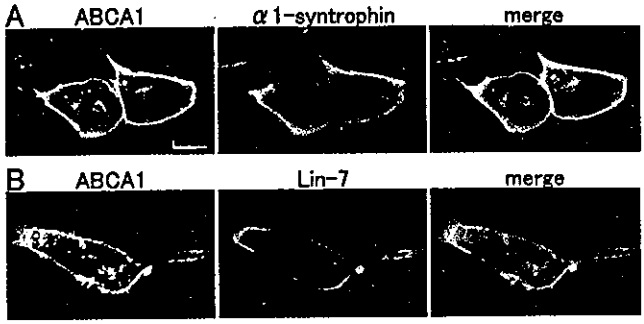

FIG. 3. **Co-localization of ABCA1 and PDZ-containing proteins, α1-syntrophin and Lin7.** HEK293 cells were co-transfected with ABCA1 and FLAG-tagged α1-syntrophin or FLAG-tagged Lin7. The cells were fixed in 4% paraformaldehyde and 5% sucrose, permeabilized in 0.4% Triton X-100, and then doubly stained with anti-ABCA1 polyclonal antibody (*left*) and anti-FLAG antibody (*middle*). A merged image of the staining (*green*, ABCA1; *red*, α1-syntrophin) is also shown (*right*). The data are representative of three independent experiments. *Bar*, 10 μm

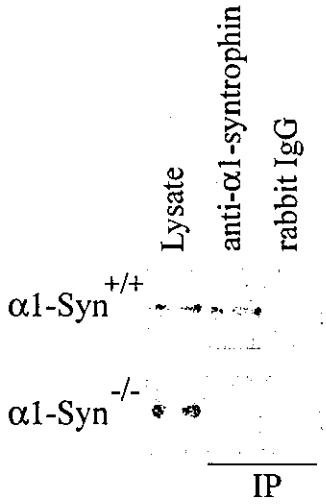

FIG. 4. **Physiological association of mouse ABCA1 with α1-syntrophin.** Brain lysates prepared from normal mouse or α1-syntrophin (−/−) were immunoprecipitated (*IP*) with anti-α1-syntrophin antibody. Immunocomplexes and cell lysates (1%) were subjected to immunoblotting using anti-ABCA1 antibody KM3110. Rabbit IgG was used as a negative control. The data are representative of three independent experiments.

brane, where it co-localized with ABCA1 (Fig. 3*A*). Lin7 also localized mainly on plasma membrane and appeared not to be uniformly distributed but rather clustered in a specific region of plasma membrane in some cells. In those regions, high expression of ABCA1 and the formation of filopodia were observed (Fig. 3*B*).

Interaction of α1-Syntrophin with ABCA1 in Mouse Brain—Among syntrophin isoforms, α1-syntrophin is mainly expressed in brain, skeletal muscle, and heart in mouse (21). To examine whether ABCA1 and α1-syntrophin interact physiologically, we tried co-immunoprecipitation of these two proteins from mouse brain. Lysates prepared from mouse brain were immunoprecipitated with anti-α1-syntrophin antibody, and precipitates were evaluated by immunoblotting with anti-ABCA1 antibody. As shown in Fig. 4, mouse ABCA1 was co-immunoprecipitated with α1-syntrophin, but not with control IgG. This interaction was confirmed to be specific, because ABCA1 was not precipitated from brain of α1-*Syn*$^{-/-}$ mice (Fig. 4).

α1-Syntrophin Modulates Turnover of ABCA1—Syntrophins have been reported to be involved in protein stability. For example, interaction with β2-syntrophin controls the degradation of ICA512, which connects insulin secretory granules to the utrophin complex and the actin cytoskeleton, by calpain (22), and the stability of AQP4 (23) and neuronal nitric-oxide synthase (12) is suggested as being controlled by α1-syntrophin. The amount of ABCA1 in lysates was consistently higher on co-expression with α1-syntrophin than with other proteins as shown in Fig. 1. Therefore, we examined the effect of α1-syntrophin on the stability of ABCA1. FLAG-tagged α1-syntrophin or FLAG-tagged Lin7 was transiently co-expressed with ABCA1 in HEK293 cells. At 48 h after transfection, the medium was replaced with 10% fetal bovine serum/Dulbecco's modified Eagle's medium containing 100 μg/ml cycloheximide, and cellular protein synthesis was inhibited to block supply of the newly synthesized ABCA1. After the indicated times, the amount of ABCA1 was measured by immunoblotting (Fig. 5*A*). After the inhibition of cellular protein synthesis, 80% of ABCA1 was degraded in 7 h, and the half-life was about 2 h as reported previously (10). Thus, ABCA1 protein turns over rapidly in HEK293 cells. When ABCA1 was co-expressed with α1-syntrophin, only ~30% of ABCA1 was degraded in a 7-h treatment with cycloheximide, and the half-life was estimated to be 10 h (Fig. 5*B*). Lin7, which is a PDZ protein and also binds to the C terminus region of ABCA1, did not show a significant effect on the half-life of ABCA1. The half-life of ABCA1ΔSYV was scarcely affected by co-expression of α1-syntrophin (data not

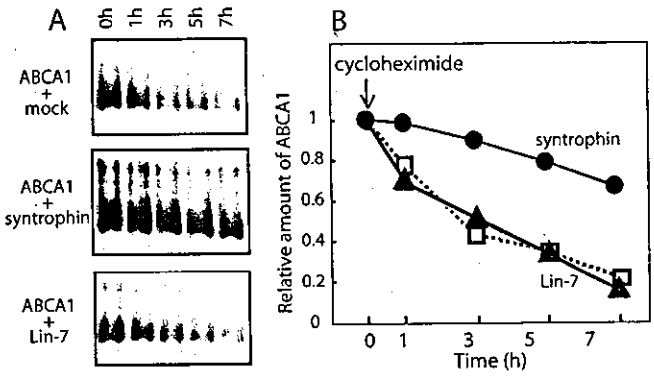

FIG. 5. **α1-Syntrophin modulates turnover of ABCA1.** *A*, HEK293 cells were co-transfected with ABCA1 and vector (*mock*), FLAG-tagged α1-syntrophin, or FLAG-tagged Lin7. At 48 h after transfection, 100 μg/ml of cycloheximide was added to block protein synthesis. After the indicated times, cell lysates were subjected to immunoblotting using Anti-ABCA1 monoclonal antibody KM3110. *B*, quantitation of ABCA1 levels. Values are expressed as fold increase with respect to the amount of ABCA1 just before adding cycloheximide. □, mock transfected; ●, co-transfected with α1-syntrophin; ▲, co-transfected with Lin7. The data are representative of two experiments with similar results.

shown). These results suggest that α1-syntrophin decreases ABCA1 protein degradation by interacting with the C terminus three amino acids of ABCA1.

α1-Syntrophin Increases apoA-I-mediated Cholesterol Efflux by ABCA1—To analyze the functional consequences of decreased ABCA1 protein degradation in the presence of α1-syntrophin, the apoA-I-mediated release of cholesterol was examined from HEK293 cells transiently cotransfected with ABCA1 and α1-syntrophin (Fig. 6). Human ABCA1 transiently expressed in HEK293 cells supported the apoA-I-mediated release of cholesterol as previously reported with ABCA1-green fluorescent protein (13). Co-expression of α1-syntrophin significantly increased the apoA-I-mediated release of cholesterol, although expression of α1-syntrophin alone did not affect it.

DISCUSSION

In this study, we identified α1-syntrophin as a protein interacting strongly with ABCA1 via the C-terminal three amino

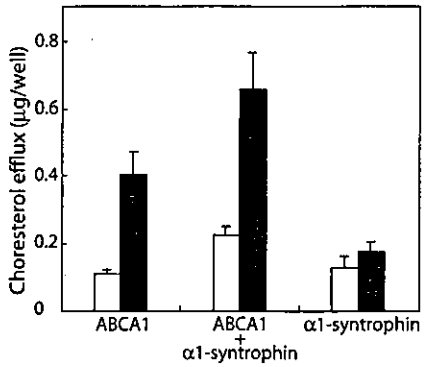

FIG. 6. **Effects of α1-syntrophin co-expression on apoA-I-mediated cholesterol transport.** The cholesterol content of the medium in 6-well plates containing HEK293 cells transiently transfected with ABCA1 alone, ABCA1 and α1-syntrophin, and α1-syntrophin alone was measured after 24 h incubation in the presence (*black bars*) or absence (*white bars*) of 10 μg/ml of apoA-I.

acids SYV of ABCA1. Co-expression of α1-syntrophin retarded degradation of ABCA1 and made the half-life of ABCA1 in HEK293 cells five times longer than in the cells not expressing α1-syntrophin. This effect is not common among PDZ-containing proteins interacting with ABCA1, because Lin7, which also binds to the C terminus region of ABCA1, did not show a similar effect. Co-expression of α1-syntrophin significantly increased the apoA-I-mediated release of cholesterol. Because this interaction was observed in mouse brain, α1-syntrophin could be involved in lipid homeostasis in brain.

Mammalian cells have developed sophisticated mechanisms to ensure adequate cellular cholesterol levels, because cholesterol plays a critical role in several important cell functions, including protein trafficking, membrane vesiculation, and signal transduction, and, at the same time, hyper-accumulation of cholesterol is harmful for cells. Plasma membrane cholesterol content, for example, is regulated through a feedback mechanism controlled by sterol regulatory element binding protein-2 (SREBP-2) (24, 25). To eliminate excess cholesterol from the cell, expression of ABCA1, a key molecule for apoA-I-mediated cholesterol efflux, is stimulated by intracellular oxysterol via the LXR/RXR nuclear receptor (26). The synthesized ABCA1 protein turns over rapidly with a half-life of 1–2 h (8, 10) to cancel cholesterol efflux by ABCA1. Because co-expression of α1-syntrophin retarded degradation of ABCA1 and made the half-life of ABCA1 in HEK293 cells five times longer than in the cells not expressing α1-syntrophin, α1-syntrophin is expected to be involved in intracellular signaling, which determines the stability of ABCA1.

Recently, it has been proposed that ABCA1 is regulated in two different ABCA1 degradation pathways under various cellular conditions: (i) a basal calpain degradation pathway that is turned off by interaction with apolipoproteins (9, 10); and (ii) a ubiquitin-proteasome pathway that is activated by marked free cholesterol loading (27). A sequence rich in proline, glutamate, serine, and threonine (PEST sequence) just before the second membrane spanning domain of ABCA1 (amino acid residue 1283–1306) is involved in regulating the calpain degradation of ABCA1 (10). Although the nature of the apoA-I-ABCA1 interaction is not fully understood, conformational alteration of ABCA1 through the PEST sequence may be induced by its direct or indirect interaction with apoA-I, which may render ABCA1 resistant to proteolysis by calpain. Because ABCA1 was ubiquitinated as well when co-expressed with α1-syntrophin (data not shown), α1-syntrophin seems not to affect ABCA1 degradation in an ubiquitin-proteasome pathway. Binding of α1-syntrophin to the C terminus of ABCA1 may cause a conformational alteration similar to that caused by apoA-I binding and render ABCA1 resistant to proteolysis by calpain.

Syntrophins are a family of five proteins (α1, β1, β2, γ1, and γ2,) containing two pleckstrin homology domains, a PDZ domain, and a C-terminal syntrophin-unique region (28). Analysis of α-*Syn*$^{-/-}$ mouse has demonstrated that perivascular localization of AQP4 in brain requires α1-syntrophin (23) and that the stability of AQP4 (23) and neuronal nitric-oxide synthase (12) decreases in the absence of α1-syntrophin. β2-syntrophin was also reported to interact with ABCA1 and was proposed to participate in the retaining of ABCA1 in cytoplasmic vesicles by forming a ABCA1-β2-syntrophin-utrophin complex (29). It is possible that α1-syntrophin is also involved in endocytotic recycling of ABCA1. Extracellular lipid-free apoA-I may first interact with ABCA1 on plasma membrane, but it is not clear whether the formation of HDL takes place extracellularly or if intracellular events, such as endocytotic recycling, are involved (30). It is intriguing that apoA-I and α1-syntrophin have a similar effect on ABCA1 turnover. A mutation of ABCA1 that causes Tangier disease (W590S) does not affect apoA-I binding or initial ATP binding/hydrolysis but results in a defect in lipid efflux (11, 31, 32). Because apoA-I failed to affect calpain degradation of ABCA1-W590S in HEK293 cells (10), additional signals following apoA-I binding to ABCA1 are speculated to be necessary for the subsequent inhibition of calpain degradation. It is possible that PDZ-containing proteins such as α1-syntrophin are involved in intracellular signaling, which determines the stability of ABCA1.

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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 also 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 macrophages[5] 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 ester[24] or stimulation of RAW264 cells and macrophages by

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cAMP analogues.[25–27] Transforming growth factor-β[28] and bacterial lipopolysaccharide[29] 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 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[41] 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-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 a specific enzymatic method for each lipid, respectively.[26,33] The medium was centrifuged at $1.64\times10^5 g$ 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% CO_2 and 95% air. Differentiation of THP-1 monocytes into macrophages was induced by culturing the cells at the density of 3.0×10^6 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.[31] 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 phenylmethane sulfonyl 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 dodecylsulfate, and then electrophoretically 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 antiserum 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 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′-AGGTTTGGAGATGGTTATACAATAGTTG-3′, reverse primer, 5′- CTTTTAGGACACTTCCCGGAAA-3′, probe, 5′FAM-ACGAATAGCAGGCTCCAACCCTGACC-TAMRA3′; and ABCG1 forward primer, 5′-TTCATCGTCCTGGGCATCTT-3′, reverse primer, 5′-CAGCCCGGATTTTGTATCTGA-3′, probe, 5′FAM-ATCTCCCTGCGGCTCATCGCCTATTT-TAMRA3′. 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 tailed with SalI (5′-GTCGACTTCTGGTGTTGGCACTCTTC-3′) and a reverse primer tailed with BamHI (5′-GGATCCT-CTTACCTGTTTTCCACTTTGCTGTTTG-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 LXREa and 2 copies of LXREb from the sterol response element binding protein-1c promoter[43] and overhangs for *Kpn*I and *Bgl*II into an upstream of the thymidine kinase (tk) promoter. The mutant reporter vector

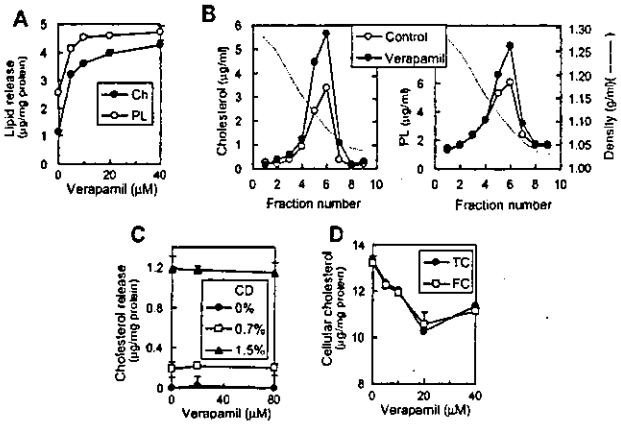

Figure 1. Verapamil enhances apoA-I–mediated lipid release in cAMP-treated RAW 264 cells. RAW264 cells were treated with dBcAMP (300 μmol/L) for 18 hours and subsequently incubated for 6 hours with 0 to 40 μmol/L verapamil in the presence of the same concentration of dBcAMP. The cells were further incubated 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.

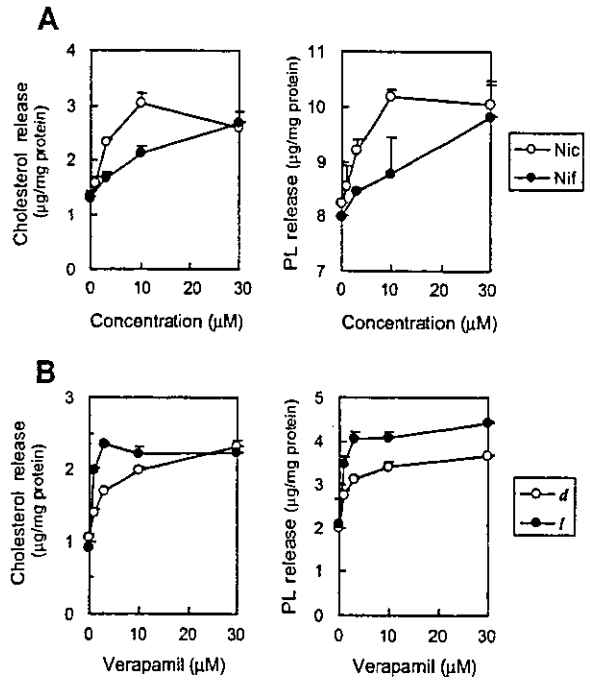

Figure 2. Effect of nicardipine, nifedipine (A), *d*-verapamil, and *l*-verapamil (B) on apoA-I–mediated lipid release. RAW264 cells were treated with dBcAMP (300 μmol/L) for 18 hours and subsequently incubated for 6 hours with various concentrations of the calcium channel blockers in the presence of 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.

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

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 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.

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.[26] When the cAMP-treated cells were further incubated with verapamil for 6 hours, release of cholesterol and choline-phospholipid 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.

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). RT-PCR

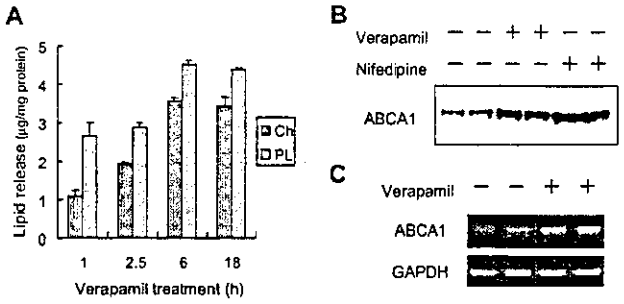

Figure 3. Verapamil rapidly enhances apoA-I–mediated lipid release through an induction of ABCA1. RAW264 cells were treated with dBcAMP (300 μmol/L) for 24 hours. Verapamil (10 μmol/L) was included in the medium during the last 1 to 18 hours. The cells were further incubated with apoA-I (10 μg/mL) for 6 hours, and the lipid released into the medium was analyzed (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 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[45] (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 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.[46,47] The level of ABCG1 mRNA was increased 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 LXR-dependent transcription.

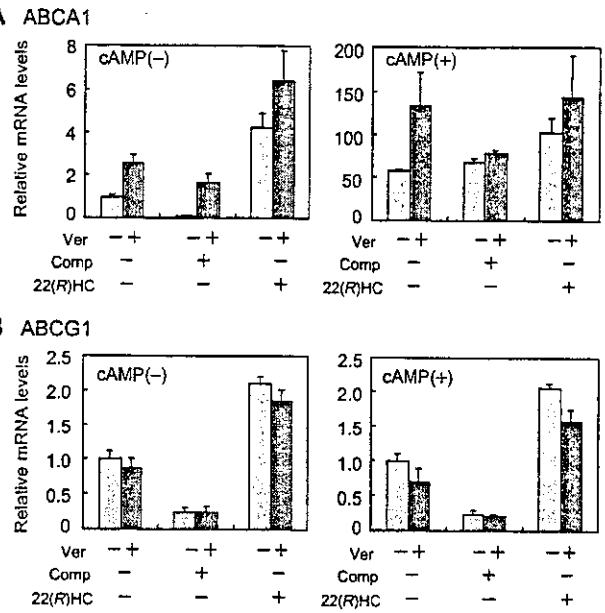

Figure 4. Verapamil induces ABCA1 mRNA but not that of ABCG1 through LXR-independent mechanisms. RAW264 cells were cultured in the presence or absence of dBcAMP (300 μmol/L) and compactin (comp) (50 μmol/L along with 50 μmol/L mevalonic acid) for 18 hours and subsequently treated for 6 hours with or without verapamil (ver) (30 μmol/L), compactin, or 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 experiments 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,[48] 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.[26] 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.[37–39] 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

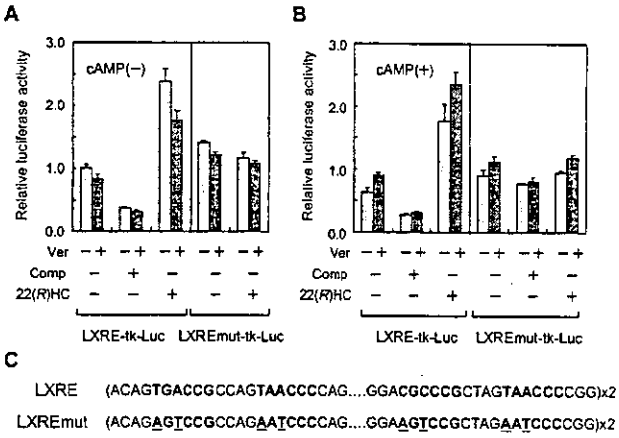

Figure 5. Verapamil (ver) does not increase LXRE-dependent transcriptional activity. RAW264 cells were transfected with a luciferase reporter plasmid containing 4 copies of LXREs upstream of the thymidine kinase promoter (LXRE-tk-Luc) or a plasmid-containing mutated LXRE complex (LXREmut-tk-Luc) in the presence of pSV-β-galactosidase as a reference plasmid. Cells were treated as described in Figure 4. Luciferase activity in dBcAMP-untreated (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

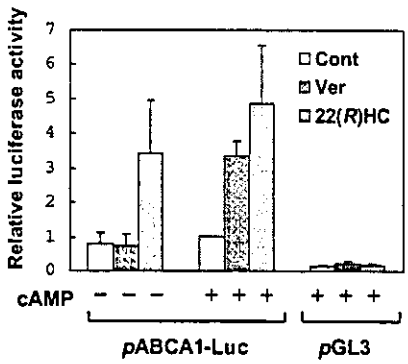

Figure 6. Verapamil activates mouse ABCA1 promoter (from −1238 to +57 bp). RAW264 cells were transfected with ABCA1 promoter-luciferase construct (*p*ABCA1-Luc) or empty luciferase vector (*p*GL3) in the presence of a Rennila luciferase plasmid as a reference. Cells were treated with or without dBcAMP (300 μmol/L) for 6 hours and subsequently treated for 12 hours with or without verapamil (30 μmol/L) or 22(*R*)-hydroxycholesterol (22 (*R*)-HC) (2 μg/mL), as indicated, in the presence or absence of dBcAMP. Cellular firefly luciferase activity was measured and normalized to Renilla luciferase activity. The values represent the average±SD from 3 independent experiments performed in triplicate.

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*)-hydroxycholesterol–elicited 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 transcription[26] 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 *l*-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-6[53] 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

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RESEARCH ARTICLE

3,3′,4,4′,5-Pentachlorobiphenyl Inhibits Drug Efflux Through P-Glycoprotein in KB-3 Cells Expressing Mutant Human P-Glycoprotein

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The effects on the drug efflux of 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126), the most toxic of all coplanar polychlorinated biphenyls (Co-PCBs), were examined in KB-3 cells expressing human wild-type and mutant P-glycoprotein in which the 61st amino acid was substituted for serine or phenylalanine (KB3-Phe^{61}). In the cells expressing P-glycoproteins, accumulations of vinblastine and colchicine decreased form 85% to 92% and from 62% to 91%, respectively, and the drug tolerances for these chemicals were increased. In KB3-Phe^{61}, the decreases in drug accumulation were inhibited by adding PCB-126 in a way similar to that with cyclosporine A: by adding 1 μM PCB-126, the accumulations of vinblastine and colchicine increased up to 3.3- and 2.3-fold, respectively. It is suggested that PCB-126 decreased the drug efflux by inhibiting the P-glycoprotein in KB3-Phe^{61}. Since there were various P-glycoproteins and many congeners of Co-PCBs, this inhibition has to be considered a new cause of the toxic effects of Co-PCBs.

INTRODUCTION

Among the group of polychlorinated biphenyls (PCBs), coplanar PCBs (Co-PCBs) are known to be highly toxic and indestructible congeners [1, 2]. Co-PCBs had been produced as useful artificial lubricants for the chemical industry and in the manufacture of a variety of daily-use products until their production and use were prohibited. These chemicals have thoroughly polluted the environment, and may have accumulated via the food chain and bioaccumulation in humans and animals, especially in their lipid-rich organs [2, 3, 4, 5]. In the environment, 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) which is the most toxic congener of Co-PCBs is also highly polluting the environment [1, 6]. Like dioxin, the mixture of Co-PCBs or the metabolites of Co-PCBs seems to produce either estrogenic or antiestrogenic effects [7, 8]. Co-PCBs were the agonists for arylhydrocarbon receptors (AhR) [1, 9], and they inhibited the estrogenic response via crosstalk between AhR and estrogen receptors [10, 11]. Actually, Co-PCBs have acted as environmental endocrine disruptors, exerting adverse biological effects even at a very low dose [6, 12]. They also disturbed normal fetal and neonatal development, producing malformations and the onset of cancer [4, 6, 13, 14].

Drug transport systems such as P-glycoprotein are present on the cell membrane to exclude toxic xenobiotics [15] and to transport metabolites such as steroid hormones [16]. P-glycoprotein is capable of transporting a variety of structurally unrelated chemicals [17] and can induce multidrug resistance during cancer chemotherapy [18, 19]. The P-glycoprotein-mediated transport of chemicals was inhibited by other chemicals. Cyclosporine A, for example, competed with vinblastine for a site to bind to P-glycoprotein, and inhibited the transport of this substrate [20]. The most hydrophobic steroid hormone, progesterone, blocked vinblastine transport, although progesterone itself was not transported. In a series of steroid hormones, it was revealed that the more hydrophobic the congeners, the more potent their interference with the transport of other chemicals [21]. One of the congeners of Co-PCBs, 3,3′,4,4′-tetrachlorobiphenyl (PCB-77), was not transported by human P-glycoprotein, but accumulated abundantly in cells as can be expected due to its lipophilic nature [22]. Co-PCBs easily enter cells and may accumulate in the cell membrane due to their lipophilicity. P-glycoprotein in the cell membrane may be exposed to Co-PCBs, which may modify the transport of other drugs and metabolites in the same way as do hydrophobic steroid hormones and cyclosporine A, thus altering the effects of those substances.

TABLE 1. Drug tolerance for vinblastine and colchicine in KB3-Vec and KB3-MDR1s, and the ratios for the tolerance of KB3-Vec to KB3-MDR1s (KB3-MDR1s/KB3-Vec).

	KB3-MDR1s			
	KB3-Vec	KB3-His^{61}	KB3-Phe^{61}	KB3-Ser^{61}
Vinblastine (ID_{50}, nM)	3.4	26	30	40
(KB3-MDR1s/KB3-Vec)	(1)	(7.6)	(8.8)	(11.8)
Colchicine (ID_{50}, nM)	8.8	46	859	43
(KB3-MDR1s/KB3-Vec)	(1)	(5.2)	(97.6)	(4.9)

Note. Inhibition dose for 50% cell growth (ID_{50}) by adding chemicals was expressed as the index of drug tolerance.

Though P-glycoprotein has a low specificity for substrates, it was found to contain many mutations of amino acids that modulated substrate specificity [23, 24]. Such mutations might be involved in the modification of binding and transporting mechanisms. The toxicity of drugs was reduced in the transformant cells expressing P-glycoprotein, probably due to an enhancement of the drug efflux. These drug tolerances varied among the transformant cells in which the His 61 position (His^{61}) in the predicted transmembrane domain 1 of human P-glycoprotein was replaced with a variety of amino acids [24]. Accordingly, the effect of Co-PCBs might differ among the mutants of P-glycoproteins. The possibility of a modification in P-glycoprotein by binding with Co-PCBs must be examined. Here, we investigated the effect of PCB-126 on the efflux of vinblastine and colchicine in KB-3 cells transfected with multidrug resistant (MDR1) genes which code both wild and His^{61} mutant P-glycoproteins.

MATERIALS AND METHODS

Chemicals

For radioactive tracers, we used [G-^{3}H]-vinblastine sulphate (470 GBq/mmol) (Amersham Pharmacia Biotech, NJ, USA) and [^{3}H]-colchicine (2,830 GBq/mmol) (Perkin Elmer Japan Co Ltd, Tokyo, Japan). As the representative of Co-PCBs, PCB-126 (Kanto Kagaku, Tokyo, Japan) was used, and cyclosporine A (Sigma, Mo, USA) was used as the inhibitor of P-glycoprotein.

Cell culture

The transformant cells of human cultured cells, that is, KB3-1 expressing with wild-type human P-glycoprotein (KB3-His^{61}) and the mutant P-glycoprotein, in which His^{61} was substituted with serine (KB3-Ser^{61}) or phenylalanine (KB3-Phe^{61}), were prepared as reported in [24]. As the control, KB3-1 transfected with the transfection vector pHaMAIRESneo (KB3-Vec) was also used. These cells were maintained in medium 199 supplemented with 10% fetal calf serum (FCS) in an atmosphere of 5% CO_2 at 37°C, and the transformant cells were maintained in the same medium with appropriate reagents; KB3-His^{61} and KB3-Ser^{61} were maintained with 100 μM vinblastine and KB3-Phe^{61} with 60 μM colchicine.

Drug tolerance of cells

The cells were seeded in 96-well microplates at a density of 10^5/well in a 500-μL medium identical to that of the maintenance culture except for varied concentrations of vinblastine and colchicine, and then incubated for 4 days. The viability of these cells was then measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Wako Co Ltd, Osaka, Japan). The inhibition dose for 50% cell growth (ID_{50}) was calculated as the index for drug tolerance.

Drug accumulation and its inhibition

For a determination of the cellular accumulation of the chemicals, coverslips with a 24-well multidish (Nalgen Nunc International, Ill, USA) were used. Cells were seeded at a density of 5×10^6/well in a 750-μL maintenance medium on the dish and incubated in 5% CO_2 at 37°C. After 4 days, the medium was replaced with a fresh medium without colchicine, and the cells were incubated for 6 hours. The medium was again replaced with a 750-μL fresh medium containing either 11 nM [^{3}H]-vinblastine (5.16 kBq/mL) or 11 nM [^{3}H]-colchicine (31.1 kBq/mL). The coverslips were removed from the 24-well dish after a 1-, 2-, and 3-hours incubation. These cells were washed 3 times with PBS, and treated with a lysing solution (cell culture lysis reagent, Promega, Wis, USA) overnight. Radioactivity was then measured by a liquid scintillation counter. The accumulation was indicated as pmol/well.

To examine the effect of PCB-126 on drug accumulation, PCB-126 and the inhibitor of p-glycoprotein, cyclosporine A, were added to the incubation medium. One hundred pM to 10 μM PCB-126 or cyclosporine A was added to the medium, and the accumulations of vinblastine and colchicine were measured.

Statistics

The student's t-test was employed to examine the statistical significance of the accumulation between cell groups.

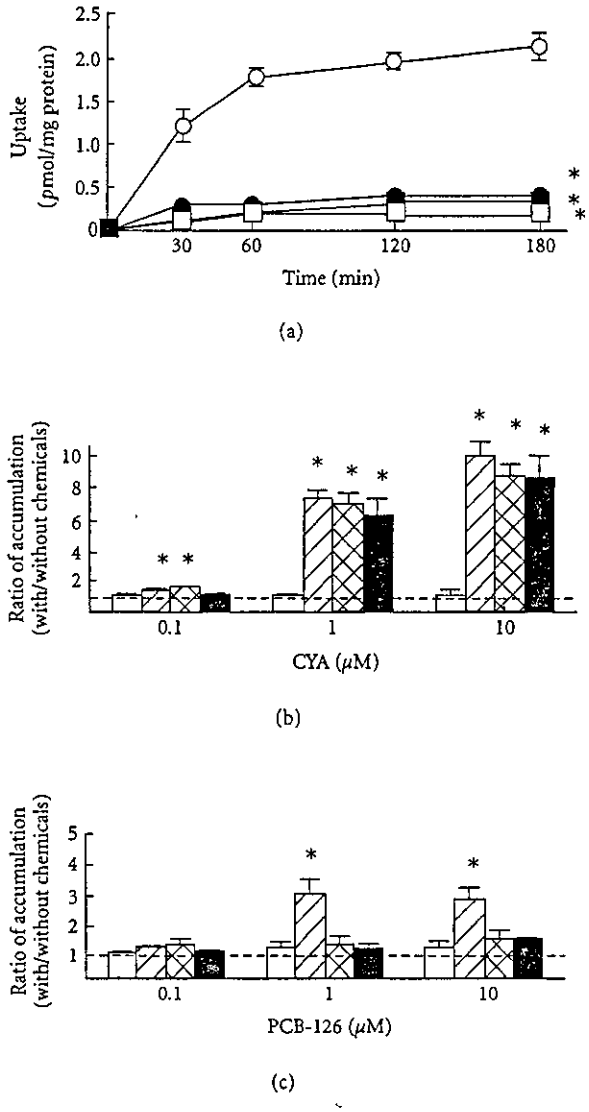

FIGURE 1. (a) Uptake of vinblastine in KB3-1 expressing with wild-type human P-glycoprotein (KB3-His^{61}, filled circles), mutant P-glycoprotein in which His^{61} was replaced with phenylalanine (KB3-Phe^{61}, open squares) and serine (KB3-Ser^{61}, filled squares), and transfected with transfection vector (KB3-Vec, open circles). (b) The ratios between the accumulations of vinblastine with and without cyclosporine A (CYA) and (c) PCB-126 (with/without CYA or PCB-126) in KB3-Vec (open columns), KB3-His^{61} (cross-hatched columns), KB3-Phe^{61} (hatched columns), and KB3-Ser^{61} (filled columns). *, $P < .05$ compared to KB3-Vec. Means and SD of 4 experiments.

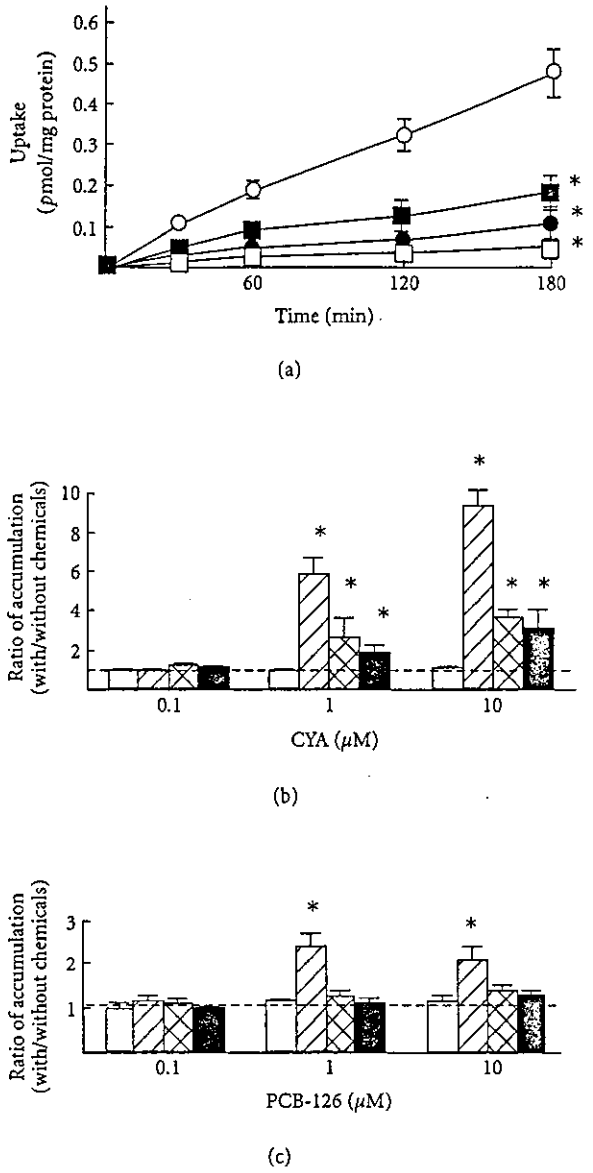

FIGURE 2. (a) Uptake of colchicine in KB3-1 expressing with wild-type human P-glycoprotein (KB3-His^{61}, filled circles), mutant P-glycoprotein in which His^{61} was replaced with phenylalanine (KB3-Phe^{61}, open squares) and serine (KB3-Ser^{61}, filled squares), and transfected with transfection vector (KB3-Vec, open circles). (b) The ratios between the accumulations of colchicine with and without cyclosporine A (CYA) and (c) PCB-126 (with/without CYA or PCB-126) in KB3-Vec (open columns), KB3-His^{61} (cross-hatched columns), KB3-Phe^{61} (hatched columns), and KB3-Ser^{61} (filled columns). *, $P < .05$ compared to KB3-Vec. Means and SD of 4 experiments.

RESULTS AND DISCUSSION

Table 1 indicates the drug tolerances of KB3-Vec, KB3-His^{61}, KB3-Ser^{61}, and KB3-Phe^{61} in the medium with vinblastine or colchicine. The ID_{50} increased in all the cell groups expressing P-glycoprotein (KB3-MDR1s), KB3-His^{61}, KB3-Ser^{61}, and KB3-Phe^{61}, except for KB3-Vec. As for vinblastine, the ID_{50} increased around 10-fold in all KB3-MDR1s except for KB3-Vec, and the order of drug tolerances was KB3-Ser^{61} > KB3-Phe^{61} > KB3-His^{61}. With colchicine, the ID_{50} increased around 5-fold in KB3-His^{61} and KB3-Ser^{61}, and 98-fold in KB3-Phe^{61}. The increase in the ID_{50} for colchicine in KB3-Phe^{61} was extremely

high, indicating a special relation between the side chain of Phe^{61} and the relatively smaller substrate, colchicine. Taguchi et al showed an inverse relation between the bulk of the side chain of the 61st amino acid in P-glycoprotein and the molecular weight of the drug; the P-glycoprotein, which has a large side chain in the 61st amino acid, indicated less sensitivity to the smaller molecular weight of the chemicals, and vice versa [24].

Figure 1a shows the vinblastine uptake as a function of time in KB3-Vec and of reductions in the uptake in KB3-MDR1s. The uptakes of vinblastine in KB3-Vec hyperbolically increased during a 3-hour incubation as reported in [22], and the accumulations after that incubation in KB3-His^{61}, KB3-Ser^{61}, and KB3-Phe^{61} were reduced by 85%, 88%, and 92%, respectively, compared to KB3-Vec. Thus, under such conditions, most of the drug might have been transported to an extracellular site by the P-glycoproteins which were expressed by transfection with each MDR1 gene. These excretions might contribute to the increase in ID_{50} in KB3-MDR1s.

Figures 1b and 1c show the effects of cyclosporine A (b) and PCB-126 (c) on the accumulations of vinblastine after a 3-hours incubation in KB3-Vec and KB3-MDR1s; the ratios between the accumulations with and without cyclosporine A or PCB-126 in each cell group were compared. An increase in the ratios indicates an increased inhibition against the drug efflux due to the addition of cyclosporine A or PCB-126. The ratios were increased by adding more than 1 μM cyclosporine A in all KB3-MDR1s, while there was no clear increase in the ratio in KB3-Vec. The ratios in KB3-His^{61}, KB3-Ser^{61}, and KB3-Phe^{61} increased 7.0-, 6.3-, and 7.5-fold, respectively, by adding 1 μM cyclosporine A, and those accumulations increased 9.2-, 9.1-, and 10.3-fold, respectively, by adding 10 μM cyclosporine A. Though the increase in the ratio was less than 2-fold by adding 0.1 μM cyclosporine A, those in KB3-Phe^{61} and KB3-His^{61} were increased significantly. The ratio in KB3-Phe^{61} was relatively higher than in the other cell groups, though the difference among KB3-MDR1s was less than 20%. By adding 0.1, 1 and 10 μM PCB-126, the ratio in KB3-Phe^{61} increased 1.4-, 3.3-, and 2.8-fold, respectively, thus revealing an inhibition of the drug efflux by the addition of PCB-126. PCB-126 might have had an effect on the mutant P-glycoprotein in KB3-Phe^{61}, thus inhibiting the drug efflux. The ratios in KB3-His^{61} and KB3-Ser^{61} also increased around 1.5-fold, but their effect was no different from that in KB3-Vec.

Figure 2a shows the colchicine uptake as a function of time in KB3-Vec and of reductions in the uptakes in KB3-MDR1s. The colchicine uptake displayed a somewhat linear function compared to the vinblastine uptake in all cell groups. The accumulations after a 3-hours incubation in KB3-His^{61}, KB3-Ser^{61}, and KB3-Phe^{61} were reduced, respectively, by 75, 62, and 91% compared to that in KB3-Vec. Thus, under those conditions, KB3-Phe^{61} demonstrated the highest ability to transport colchicine, while KB3-Ser^{61} showed the lowest.

Figures 2b and 2c show the effects of cyclosporine A (b) and PCB-126 (c) on the accumulations during a 3-hours incubation in KB3-Vec and KB3-MDR1s. The ratios between accumulations with and without these chemicals in KB3-His^{61}, KB3-Ser^{61}, and KB3-Phe^{61} increased 2.6-, 2.0-, and 5.8-fold by adding 1 μM cyclosporine A, and increased 3.6-, 2.9-, and 9.2-fold by adding 10 μM cyclosporine A, respectively, while there was no such effect in KB3-Vec. The cell group which showed the largest reduction in drug accumulation, KB3-Phe^{61}, also showed the largest increase in accumulation when the inhibitor for P-glycoprotein was added. This might be due to an inhibition of the efflux through P-glycoprotein as reported in [21]. These effects of cyclosporine A on drug accumulation were considered due to a modification of P-glycoprotein by its binding [20]. By adding 1 and 10 μM PCB-126, the ratios in KB3-Phe^{61} increased 2.3- and 2.1-fold, respectively, indicating an inhibition of the drug efflux. The ratios in KB3-His^{61} and KB3-Ser^{61} increased slightly, though there was no significant difference from that in KB3-Vec. Thus, PCB-126 might have bound with Phe^{61} mutant P-glycoprotein to inhibit the efflux of colchicine.

In all KB3-MDR1s, accumulations of vinblastine were equally reduced, and cyclosporine A inhibited these reductions to almost the same degree among KB3-MDR1s. However, the reductions and inhibitions in the accumulation of colchicine varied among cell groups. Previously, one of the present authors revealed that the cells expressing mutant P-glycoprotein which possessed a larger 61st amino acid gained a tolerance to various chemicals, whereas, in the cells expressing the mutant P-glycoprotein which possessed a smaller 61st amino acid, the tolerance for large chemicals was superior to that for small chemicals [24]. These results were explained as being due to the difference in the bulk of the side chain of the 61st amino acid in P-glycoprotein, rather than to the electron charge or hydrophilicity in the side chain. In this experiment, KB3-Phe^{61} showed a superior ability for excretion in both vinblastine and colchicine, and the excretion in KB3-Phe^{61} was more potently inhibited by cyclosporine A and PCB-126 compared to the other cells. These results showed that the Phe^{61} mutant P-glycoprotein in which the side chain of the 61st amino acid was larger than that in the other mutants was less specific for substrates. Therefore, it might have reacted to a variety of substrates with almost the same affinity. However, the wild type, His^{61} P-glycoprotein, and Ser^{61} mutant P-glycoprotein were somewhat specific for substrates, and their affinity was predominant in the larger substrate, vinblastine. The Phe^{61} mutant P-glycoprotein might have the ability to bind with a variety of chemicals dissimilar to one another in structure and molecular size. Thus, PCB-126 might also bind to the Phe^{61} mutant P-glycoprotein and inhibit the efflux of vinblastine and colchicine, while being unable to react with the other P-glycoproteins.

The contamination level of Co-PCBs and the concentrations used in this experiment will now be discussed. As a result of their locations on the food chain, several marine mammals showed high levels of contamination with PCBs, that is, up to around 5 mg/kg in their blubber [25]. In humans who consumed high levels of fish, the total content of lipid-adjusted plasma PCBs increased up to 770 μg/kg [26], and PCB-126 accumulated up to 1.4 μg/kg in plasma lipid [4]. Among workers employed in capacitor manufacturing, the concentrations of high- and low-chlorinated PCBs in adipose tissue increased up to 165 and 414 μg/kg, respectively [27]. PCBs are highly lipophilic and may accumulate in the lipid bilayers of the cell membrane. In the present experiment, the effect of Co-PCBs was revealed at levels of 0.1 to 10 μM PCB-126, that is, 30 to 3000 μg/kg of PCB-126. Therefore, in populations exposed to an environment highly contaminated with PCBs, the concentrations of PCBs or Co-PCBs in their cell membranes would probably increase to around the concentrations found in this experiment.

In conclusion, it was indicated that PCB-126 inhibited the drug efflux through the mutant P-glycoprotein in KB3-Phe^{61}. The interaction of PCB-126 with P-glycoprotein was modified by the mutation of the 61st amino acid of the protein. There have been many amino acid mutations that modulated substrate specificity in P-glycoprotein [28]. Therefore, there may be P-glycoproteins which are affected by some congener(s) of Co-PCBs as revealed in this experiment. Though the precise mechanism of toxicity is still unknown, P-glycoprotein plays a role both in the extrusion of xenobiotics and in the transport of steroid hormones [16, 21]. Consequently, not only the toxicities of xenobiotics but also the effects of steroid hormones may be disturbed by Co-PCBs. In particular, the regulation of brain differentiation in an embryo by steroid hormones seems to be influenced by Co-PCBs. In addition to the reported mechanism for toxicities [1, 7, 8, 9, 10, 11, 12], the effect of Co-PCBs on P-glycoprotein has to be considered as a new cause of the toxic effects of these chemicals.

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