Cyclosporin A Traps ABCA1 at the Plasma Membrane and Inhibits ABCA1-Mediated Lipid Efflux to Apolipoprotein A-I

Wilfried Le Goff, Dao-Quan Peng, Megan Settle, Gregory Brubaker, Richard E. Morton, Jonathan D. Smith

Objective—ABCA1 mediates cellular cholesterol and phospholipid efflux to apolipoprotein A-I and other apolipoprotein acceptors. In this study, we analyzed the effect of the immunosuppressant cyclosporin A on the ABCA1-mediated lipid effluxes reactions.

Methods and Results—Cyclosporin A acted as a potent inhibitor of ABCA1 activity in several cell lines. Using the RAW264.7 mouse macrophage cell line, in which ABCA1 and its associated cholesterol efflux activity are inducible by cAMP analogues, cyclosporin A inhibition of cholesterol efflux to apolipoprotein A-I was rapidly reversible after its removal from the culture media, implying that ABCA1 levels were not drastically reduced by cyclosporin A. In fact, cyclosporin A treatment decreased ABCA1 turnover and yielded a 2-fold increase in cell-surface ABCA1. Despite the increase in cell-surface ABCA1, cyclosporin A decreased apolipoprotein A-I uptake, resecretion, and degradation in RAW cells. Finally, consistent with the inhibition of ABCA1 in vitro, cyclosporin A treatment induced a 33% reduction of high-density lipoprotein (HDL) levels in mice.

Conclusion—ABCA1 inhibition by cyclosporin A supports a role for ABCA1 endocytic trafficking in ABCA1-mediated lipid efflux and could explain in part the low HDL levels observed in some patients with transplants. (Arterioscler Thromb Vasc Biol. 2004;24:2155-2161.)

Key Words: macrophage ■ cholesterol ■ high-density lipoprotein

High plasma high-density lipoprotein (HDL) levels are associated with a decreased incidence of atherosclerosis. This anti-atherogenic property of HDL is probably mediated in part by its capacity to remove excess cholesterol from foam cells. The ATP-binding cassette transporter ABCA1, which mediates cholesterol and phospholipid efflux to lipid-poor HDL apolipoproteins, plays a key role in the elimination of cholesterol from macrophages in the artery wall. Mutations in ABCA1 cause Tangier disease, a disorder characterized by very low HDL levels, cholesterol deposition in macrophages, and premature atherosclerosis. Macrophage ABCA1 has specifically been shown to have an anti-atherogenic role in mouse models of atherosclerosis, as observed in bone marrow transplantation studies in which ABCA1-deficient marrow donors led to larger aortic lesions than wild-type donors.1,2

Macrophage ABCA1 expression is highly regulated, and its transcription is markedly increased by cholesterol loading via the nuclear liver X receptor and retinoic X receptor,3 and in rodent cells by analogs of cAMP through an unknown mechanism.4,5 ABCA1 protein and its activity are also regulated by post-translational mechanisms. The turnover of ABCA1 protein is very rapid (≈1 hour). Apolipoprotein A-I (apoAI) treatment of cells decreases the turnover of ABCA1,6 whereas free cholesterol loading in macrophages lowers ABCA1 protein levels and activity by increasing ABCA1 degradation.7 ABCA1 turnover appears to be regulated by several independent phosphorylation mechanisms, because apoAI-mediated stabilization of ABCA1 has been reported to be associated with both increased and decreased phosphorylation.6,8,9 Specific dephosphorylation of a PEST sequence in ABCA1 blocks ABCA1 degradation by calpain and leads to an increase in cell-surface ABCA1.9,10 In contrast, unsaturated fatty acids markedly inhibit ABCA1-mediated cholesterol efflux to apoAI in macrophages by increasing ABCA1 turnover and subsequently decreasing cell-surface ABCA1 and apoAI binding to cells.11

Cyclosporin A (CsA) is a strong immunosuppressant known to be a substrate for, and a noncompetitive inhibitor of, P-glycoprotein (P-gp), which is itself an ABC transporter (ABCB1).12 Several studies have implicated P-gp in cellular cholesterol metabolism and trafficking.13–15 In rat astrocytes, Ito et al have reported that apoAI induces translocation of cholesterol and phospholipids to the cytosol and that these reactions are suppressed by CsA.16 Thus, we initiated a series of studies to determine whether CsA alters ABCA1 activity. In the current study, we provide evidence that CsA is a potent inhibitor of ABCA1 and that CsA reduces ABCA1 turnover and increases total and cell-surface ABCA1 in the RAW264.7 cells. In contrast, CsA strongly decreases apoAI uptake, resecretion, and degradation. Furthermore, CsA reduces

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plasma HDL cholesterol levels in mice, a result in agreement with decreased plasma HDL cholesterol and apoAI levels observed in some studies of transplantation patients.17,18 Thus, our findings suggest that CsA therapy may have unintended consequences on ABCA1-mediated cholesterol efflux and reverse cholesterol transport.

**Methods**

**Cell Culture and Lipid Efflux Assays**

Cells were grown at 37°C in 5% CO2 in Dulbecco modified Eagle medium (DMEM), or RPMI medium for THP1 cells, supplemented with 10% fetal bovine serum and penicillin streptomycin. Lipid efflux to apoAI (Biosdesign, Saco, Me) or methyl-β-cyclodextrin (Sigma) from cholesterol-loaded RAW264.7 cells was performed as previously described,19,20 with specific modifications for different cell lines mentioned in the figure legends. The percentage of cholesterol or choline phospholipid (PL) efflux was calculated as 100×(medium dpm)/(medium dpm + cell dpm).

**Total and Cell-surface ABCA1 Quantification**

RAW264.7 cells were grown in 100 mm² dishes (surface biotinylation) or 6-well plates (total ABCA1 content) to 80% confluence and incubated in DMEM supplemented with 50 mM/L glucose, 2 mM/L glutamine, and 0.2% bovine serum albumin (DME glutamine glucose BSA) in the presence or absence of 0.3 mM/L 8Br-cAMP for 16 to 24 hours. Cells were treated for an additional 4 hours with or without 10 μM/L CsA (Sigma). To measure cell-surface ABCA1, plasma membrane proteins were purified via cell-surface biotinylation. Cells were incubated for 30 minutes on ice with phosphate-buffered saline (PBS) containing 1 mg/mL sulfo-NHS-biotin (Pierce). The PBS-washed cell pellet was lysed in 500 μL of lysis buffer (2 mM/L EDTA, 25 mM/L Tris-Phosphate pH 7.8, 1% Triton X-100, and 10% protease inhibitor). After discarding the nuclear pellet, the protein concentration was determined using a 2-tailed t test, and comparison of 3 or more groups was performed using ANOVA with Newman-Keuls post-test. All statistics were performed using Prism software from GraphPad (San Diego, Calif).

**Statistics**

Data are shown as mean ± SD. Comparison of 2 groups was performed by a 2-tailed t test, and comparison of 3 or more groups was performed by ANOVA with Newman-Keuls post-test. All statistics were performed using Prism software from GraphPad (San Diego, Calif).

**Results**

CsA Inhibits ABCA1-Mediated Lipid Efflux

A recent study has reported that apoAI induces translocation of free cholesterol (FC) and PL to an intracellular site in rat astrocytes and that this translocation is suppressed by CsA.16 This observation suggests that CsA might inhibit ABCA1 activity, because FC and PL efflux to apoAI are mediated by ABCA1. To address this, we examined the effects of CsA on lipid efflux in RAW264.7 cells, a murine macrophage cell line in which cAMP analogues lead to a 50-fold induction of ABCA1 mRNA and a robust induction of FC and PL efflux to apoAI.5 We first analyzed the effect of CsA on FC efflux from RAW264.7 cells to methyl-β-cyclodextrin, which strips FC from the plasma membrane, and when used in moderate doses for short time periods it can be used as an indicator of plasma membrane FC content.19 We observed that 10 μM/L CsA, added 2 hours before the efflux period, and again during the 30-minute efflux period, did not affect FC efflux to methyl-β-cyclodextrin, indicating that CsA did not alter the FC content in plasma membrane in RAW264.7 cells (Figure 1A). In contrast, 10 μM/L CsA led to an almost complete inhibition of 8Br-cAMP–induced ABCA1-mediated FC and PL efflux to apoAI, when added during the 4-hour lipid efflux
Thus, CsA appeared to be a very potent inhibitor of ABCA1 in RAW264.7 cells. In a dose-response experiment, we calculated the IC50 for CsA inhibition of ABCA1-mediated FC efflux as being 5.1 μmol/L in RAW264.7 cells (data not shown). To assess whether CsA inhibition of cholesterol efflux might be mediated by P-gp inhibition, we tested 2 other P-gp inhibitors, verapamil and ketoconazole, and neither inhibited cholesterol efflux from ABCA1-induced RAW cells (data not shown). In a separate experiment using 8Br-cAMP–treated RAW264.7 cells, we examined the effect of a 2-hour 10 μmol/L CsA pretreatment on the time course of FC efflux after washout of the CsA. As shown in Figure 1D, the time course of FC efflux to apoAI in CsA-pretreated cells was similar to that in nonpretreated cells. Thus, FC efflux to apoAI is fully and rapidly restored when CsA was removed from the culture medium, suggesting that CsA did not drastically reduce ABCA1 levels and that ABCA1 inhibition by CsA may be post-transcriptional.

To examine the species specificity of ABCA1 inhibition by CsA, we induced ABCA1 in differentiated human macrophage THP-1 cells with 22-hydroxy cholesterol and 9-cis retinoic acid. Similar to what we observed in RAW264.7 cells, the addition of 10 μmol/L CsA in the medium during the 4-hour chase period significantly reduced FC efflux to apoAI in THP-1 cells (Figure 2; \( P < 0.001 \)). We also observed CsA inhibition of FC efflux to apoAI in mouse J774 macrophages and rat McA-RH7777 hepatocytes (data not shown). Thus, CsA inhibition of ABCA1 activity is not cell type-specific or species-specific.

CsA Increases Cell-surface ABCA1 and Decreases Its Turnover

Total and cell-surface ABCA1 were measured as described in the Methods section to determine whether ABCA1 inhibition by CsA results from a decrease of ABCA1 levels or an alteration of ABCA1 trafficking. As previously shown, 8Br-cAMP treatment of RAW267.4 cells induced total ABCA1 protein, which was increased an additional 33% \( (P < 0.05) \) by the 4-hour treatment with 10 μmol/L CsA (Figure 3A). More strikingly, the 4-hour CsA treatment led to a significant 2-fold increase in cell-surface ABCA1 \( (P < 0.05) \). To confirm this
result, we analyzed the effect of CsA on the ABCA1 distribution in ABCA1–GFP stably transfected HEK293 cells.20 As shown in the Figure 3B (left), and as previously reported,24 ABCA1 is mainly localized in intracellular vesicles and at the cell surface. The 4-hour treatment with 10 \( \mu \text{mol/L} \) CsA led to an apparent increase in ABCA1 at the plasma membrane (Figure 3B, right), confirming the results of the cell-surface biotinylation experiments. Thus, ABCA1 inhibition by CsA is not caused by a reduction of ABCA1 levels but instead it is associated with an increase of cell-surface ABCA1.

To address the possibility that the CsA-mediated ABCA1 immobilization at the plasma membrane affects ABCA1 turnover, RAW264.7 cells were stimulated with 8Br-cAMP and incubated with cycloheximide to inhibit protein synthesis in the presence or absence of CsA. Analysis of remaining cellular ABCA1 by Western blot showed that ABCA1 turnover was substantially slower in the CsA-treated cells with an ABCA1 half-life of 2.6 hours compared with 0.9 hours in the control-treated cells (Figure 4).

CsA Inhibits ApoAI Uptake
To elucidate the mechanism by which CsA inhibits ABCA1-mediated FC and PL to apoAI, we tested the possibility that CsA could affect apoAI trafficking in RAW264.7 cells. To address this, apoAI uptake, ressecretion, and degradation were assayed by the incubation of RAW264.7 cells with \[^{125}\text{I}]\text{apoAI}\) for 1 hour at 37°C in the presence or absence of CsA. After extensive washing with HDL to remove cell surface-bound \[^{125}\text{I}]\text{apoAI}\), the cells were chased for an additional 90 minutes at 37°C, with or without CsA, in the presence of HDL to compete for reuptake of any rescreted apoAI. Pretreatment with 8Br-cAMP significantly induced cellular uptake, ressecretion, and degradation of total \[^{125}\text{I}]\text{apoAI}\) \( (P<0.001)\) in RAW264.7 cells as compared with untreated cells (Figure 5A), thus confirming our earlier observations.21 Interestingly, these cAMP inductions were strongly reduced by the 10 \( \mu \text{mol/L} \) CsA treatment \( (79.6\% \text{ and } 75.2\%, -90\%, \text{ respectively}; P<0.001)\). Thus, ABCA1 inhibition by CsA is accompanied by alterations in apoAI endocytosis, ressecretion, and degradation by RAW264.7 cells. This result was confirmed by the use of fluorescently labeled apoAI. Cell uptake of apoAI, in a pattern consistent with endocytosis, was stimulated by 8Br-cAMP induction of ABCA1, as we and others have previously demonstrated.20,25 This uptake was reduced to background levels by CsA (Figure 5B).

CsA Reduces HDL Cholesterol Levels in Mice
Inactivating ABCA1 mutations lead to severe HDL deficiency in humans and mice. If ABCA1 inhibition by CsA occurs in vivo, then one would expect that CsA would reduce plasma HDL levels. To address this, C57BL/6 mice were injected intraperitoneally with 50 mg/kg per day CsA for 6 days and plasma HDL cholesterol levels were analyzed.
cells, and HEK293 cells, in which it was shown to abolish degradation, and residual cell were calculated as previously described. Vanadate and its derivatives have been reported to either mediate lipid efflux in RAW264.7 cells, smooth muscle mediated FC efflux to apoAI. Glyburide inhibits ABCA1-transporters have been demonstrated to inhibit ABCA1-mediated lipid efflux to apoAI. Compared with those compounds, we show that CsA, a substrate and inhibitor of the ABC transporter family member P-gp (ABCB1), is a very potent inhibitor of ABCA1 activity, with a 10 μmol/L dose leading to 90% inhibition of both FC and PL efflux to apoAI, whereas we previously demonstrated that 1 mmol/L glyburide or vanadate were required to observe a similar inhibitory effect. Probucol also acts as an ABCA1 inhibitor, with half maximal inhibition of ~20 μmol/L. We found that the IC50 for ABCA1 inhibition by CsA was ~5 μmol/L; therefore, CsA appears to be the most potent ABCA1 inhibitor described.

The mechanism by which CsA inhibits ABCA1 provides new clues in the understanding of the ABCA1-mediated lipid efflux to apolipoprotein acceptors. It has previously been shown that treatments that increase ABCA1 levels by decreasing its degradation are associated with increased lipid efflux to apoAI. For example, ceramides increase cell-surface ABCA1 and ABCA1-mediated FC efflux in CHO cells. ABCA1 interaction with apoAI or synthetic amphipathic helical peptides prevents ABCA1 degradation and leads to the increase of ABCA1 levels and lipid efflux activity. Phospholipid transfer protein can also bind to ABCA1 and decrease its turnover. ABCA1 contains a C-terminal PDZ-binding domain, and cotransfection of ABCA1 with the PDZ protein α1-syntrophin markedly decreases ABCA1 turnover. In contrast, unsaturated fatty acids inhibit ABCA1-mediated FC efflux to apoAI in macrophages by increasing ABCA1 turnover and decreasing cell-surface ABCA1. FC loading of macrophages also increases ABCA1 turnover, which can be blocked by a proteosome inhibitor. Surprisingly, CsA inhibition of ABCA1 was accompanied by a decrease of ABCA1 turnover and a subsequent increase of total and cell-surface ABCA1 in RAW264.7 cells. This is similar to what was recently observed by the inhibition of human fibroblast ABCA1 by probucol. Although we did not specifically assess the effect before and after treatment by fast protein liquid chromatography. No differences in the body weights were detected in the control and treated animals before and after the study. As shown in Figure 6, a 6-day treatment with 50 mg/kg per day CsA induced a ~33% (P<0.01) decrease of plasma HDL cholesterol levels in treated mice (n=5), whereas injection of vehicle in control mice (n=5) did not induce any significant changes. These findings suggest that CsA can inhibit ABCA1 activity in vivo.

Discussion

In this study, we report that CsA acts as a strong inhibitor of ABCA1 with CsA leading to increased ABCA1 at the plasma membrane and reduced apoAI uptake, resecretion, and degradation. Several inhibitors known to affect various ABC transporters have been demonstrated to inhibit ABCA1-mediated FC efflux to apoAI. Glyburide inhibits ABCA1-mediated lipid efflux in RAW264.7 cells, smooth muscle cells, and HEK293 cells, in which it was shown to abolish apoAI binding to the cells and cross-linking to ABCA1. Vanadate and its derivatives have been reported to either inhibit or have no significant effect on ABCA1-mediated lipid efflux to apoAI. Compared with those compounds, we show that CsA, a substrate and inhibitor of the ABC transporter family member P-gp (ABCB1), is a very potent inhibitor of ABCA1 activity, with a 10 μmol/L dose leading to 90% inhibition of both FC and PL efflux to apoAI, whereas we previously demonstrated that 1 mmol/L glyburide or vanadate were required to observe a similar inhibitory effect. Probucol also acts as an ABCA1 inhibitor, with half maximal inhibition of ~20 μmol/L. We found that the IC50 for ABCA1 inhibition by CsA was ~5 μmol/L; therefore, CsA appears to be the most potent ABCA1 inhibitor described.

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of CsA on ABCA1 mRNA, we observed that the removal of CsA from the medium fully and rapidly restored FC efflux to apoAI in RAW264.7 cells, a result that supports a post-transcriptional mechanism of ABCA1 inhibition. In agreement with this hypothesis, Jin et al have reported that mRNA expression of ABCA1 was not altered by CsA in THP-1 cells.34

Previous studies have suggested that the localization of ABCA1 at the plasma membrane favors the interaction with apoAI and thus stimulates lipid efflux to apoAI.4,23 However, our finding provides evidence that an increase of ABCA1 at the cell surface is not necessarily associated with a stimulation of lipid efflux, suggesting that a more complex regulatory mechanism occurs. We previously proposed and found evidence to support the hypothesis that ABCA1-dependent FC efflux to apoAI involves endocytosis and reserection of apoAI.19-21 Inhibitors of endocytosis reduced FC efflux to apoAI. Induction of ABCA1 led to markedly increased levels of cellular binding, uptake, and reserection of apoAI, and this apoAI uptake occurred in coated pits. Moreover, cholesterol efflux to apoAI and apoAI cellular uptake and degradation requires extracellular Ca2+.21 In cells stably transfected with an ABCA1–GFP fusion expression vector, ABCA1 and apoAI were colocalized in intracellular vesicles at 37°C.20

The time course and temperature dependence of ABCA1-mediated lipid efflux to apoAI are also consistent with a role for endocytosis in this process.19 The retroendocytosis hypothesis has been supported by data from other laboratories that demonstrated bidirectional transport of ABCA1 between the plasma membrane and intracellular vesicles,24 and that intracellular pools of cholesterol constituted the major cholesterol source for ABCA1-mediated FC efflux to apoAI.35,36

In this study, we observed that the CsA inhibition of ABCA1 is accompanied by a strong reduction of apoAI uptake, reserection, and degradation in RAW264.7 cells; thus, despite the increase of total and cell-surface ABCA1, CsA is able to inhibit ABCA1-mediated FC efflux by altering ABCA1 and apoAI trafficking. Thus, CsA could result in plasma membrane-bound ABCA1 that cannot bind to apoAI or ABCA1 that can bind apoAI but cannot mediate endocytosis and lipid efflux, and we were unable to distinguish between these possibilities because CsA led to ABCA1-independent binding of apoAI to cells at 4°C (data not shown).

We did not investigate the mechanism by which CsA sequesters ABCA1 to the plasma membrane; however, CsA has been demonstrated to directly bind to P-gp,77,38 and by analogy we speculate that CsA may bind directly to ABCA1 and alter its activity and cellular trafficking. Of course, other mechanisms are also possible, including calcineurin-mediated effects resulting in changes in ABCA1 phosphorylation or other post-translational modifications. Because CsA sequesters ABCA1 at the plasma membrane and decreases ABCA1 turnover, we suggest that ABCA1 turnover is in part regulated by bidirectional vesicular trafficking of ABCA1.

CsA treatment of transplantation patients is associated with an increased incidence of dyslipidemia, a risk factor for cardiovascular disease.39 We observed that a 6-day CsA treatment markedly reduced plasma HDL cholesterol levels in chow diet-fed C57BL/6 mice, a result in agreement with decreased plasma HDL cholesterol and apoAI levels observed in some studies of transplantation patients.17,18 Because the liver is the major source of plasma HDL in mice,1,2 the observed in vivo effect of CsA on HDL is probably caused by hepatic inhibition of ABCA1, consistent with ABCA1 inhibition we observed in McA-RH7777 rat hepatocytes. However, a previous study did not observe a CsA-mediated reduction of plasma HDL cholesterol levels in C57BL/6 mice that were fed an atherogenic diet.40 The lack of response to CsA in this study might be caused by the already low levels of HDL in response to the high-cholesterol cholate-containing diet, or the extended length of treatment (≥135 days), which could allow compensatory mechanisms to overcome the CsA effects on HDL.40 In conclusion, ABCA1 inhibition by CsA in vivo could contribute in part to the appearance of the dyslipidemia frequently observed in transplantation patients; thus, CsA therapy may have unintended atherogenic consequences.

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References


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