Proteosomal Inhibition Promotes ATP-Binding Cassette Transporter A1 (ABCA1) and ABCG1 Expression and Cholesterol Efflux From Macrophages In Vitro and In Vivo

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Objective—ATP-binding cassette transporter A1 (ABCA1) and ABCG1 are key molecules in an initial step of reverse cholesterol transport (RCT), a major antiatherogenic property of high-density lipoprotein (HDL). The ubiquitin-proteasome system (UPS) mediates nonlysosomal pathways for protein degradation and is known to be involved in atherosclerosis. However, little is known about the effects of the UPS on these molecules and overall RCT. We therefore investigated whether UPS inhibition affects ABCA1/G1 expression in macrophages and RCT in vitro and in vivo.

Methods and Results—Various proteasome inhibitors increased ABCA1/G1 expression in macrophages, translating into enhanced apolipoprotein A-I– and HDL-mediated cholesterol efflux from macrophages. ABCA1 and ABCG1 were found to undergo polyubiquitination in the macrophages and HEK293 cells overexpressing these proteins, and pulse-chase analysis revealed that proteasome inhibitors inhibited ABCA1/G1 protein degradation. In vivo experiments, the proteasome inhibitor bortezomib increased ABCA1/G1 protein levels in mouse peritoneal macrophages, and RCT assays showed that it significantly increased the fecal (54% increase compared with saline) and plasma (23%) appearances of the tracer derived from intraperitoneally injected 3H-cholesterol-labeled macrophages.

Conclusion—The present study provided evidence that the UPS is involved in ABCA1/G1 degradation, thereby affecting RCT in vivo. Therefore, specific inhibition of the UPS pathway might lead to a novel HDL therapy that enhances RCT. (Arterioscler Thromb Vasc Biol. 2011;31:1980-1987.)

Key Words: ABC Transporter ■ Macrophages ■ HDL ■ reverse cholesterol transport ■ ubiquitin-proteasome system

High-density lipoprotein (HDL) removes cholesterol pathologically accumulated in atherosclerotic lesion macrophages and transports it back to the liver for subsequent conversion to bile in a process called reverse cholesterol transport (RCT). ATP-binding cassette transporter A1 (ABCA1) and ABCG1 play essential roles in cholesterol efflux from macrophages and HDL formation by acting in a sequential manner: ABCA1 generates nascent HDL particles from lipid-poor apolipoprotein A-I (apoA-I), which then facilitate cholesterol efflux via ABCG1, followed by formation of mature HDL particles. Deletion of both ABCA1 and ABCG1 in macrophages reportedly accelerated atherosclerotic lesion development as compared with deletion of either ABCA1 or ABCG1, indicating that ABCA1 and ABCG1 have a synergic role in antiatherogenesis.

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Evidence regarding the regulation of ABCA1/G1 expression, such as via transactivation by ligand-activated liver X receptor/retinoid X receptor heterodimer, has been accumulating. ABCA1 and ABCG1 have been emerging as therapeutic targets for the treatment of atherosclerotic diseases because enhancement of their expression can promote RCT by increasing cholesterol efflux from macrophages and raising HDL levels. However, strategies using liver X receptor ligands suffer from major drawbacks, including development of fatty liver and dyslipidemia by activating sterol regulatory element binding protein-1c.

An alternative approach would be to modulate the post-translational regulation of ABCA1 expression. In this regard, among the various systems reported to date, the ubiquitin-proteasome system (UPS) constitutes a major nonlysosomal pathway for intracellular degradation of proteins, in which it plays an essential role in regulating many cellular processes. Indeed, aberrations in the UPS have recently been connected with the pathogenesis of several human protein degradation disorders (eg, cancer and neurodegenerative diseases). The UPS is therefore considered to be an important target for drug
discovery. Although ABCA1 has recently been reported to undergo protein degradation via the UPS, it remains unclear whether an increase in endogenous ABCA1 expression due to proteasomal inhibition in macrophages can lead to enhanced cholesterol efflux and consequently an overall increase in RCT in vivo. Furthermore, little is yet known about the posttranslational regulation of ABCG1.

Here, we demonstrated, for the first time, that both ABCA1 and ABCG1 were polyubiquitinated and degraded via the UPS, and treatment with proteasome inhibitors enhanced ABCA1/G1 protein expression in macrophages, which in turn promoted RCT in vitro and in vivo.

Materials and Methods
An expanded Methods section is available in the supplemental materials, available online at http://atvb.ahajournals.org. It includes detailed information regarding the following: materials, cell culture protocols, Western blot analyses, real-time quantitative reverse transcription–polymerase chain reaction, determination of cholesterol efflux, generation of recombinant adenoviruses encoding synthetic microRNAs targeting mouse ABCG1, microRNA-mediated inhibition of ABCG1 in RAW264.7 (RAW) cells by adenoviral vectors, construction of expression plasmids encoding ABCA1 and ABCG1, pulse-chase assays, immunoprecipitation and detection of polyubiquitinated ABCA1 and ABCG1, determination of lipid levels, lipoprotein fractionation and protein expression in the macrophages and liver of mice treated with bortezomib, in vivo macrophage RCT studies, and statistical analysis.

Results
Proteasome Inhibitors Increase ABCA1 and ABCG1 Protein Expression in Macrophages
We first assessed the effects of the proteasome inhibitors epoxomicin, MG132, and bortezomib on ABCA1 and ABCG1 protein levels in mouse peritoneal macrophages (MPM), RAW cells, and THP-1 macrophages. As shown in Figure 1A and 1B, the proteasome inhibitors significantly increased ABCA1 in RAW cells (2.3- to 2.4-fold), MPM (1.7- to 1.8-fold), and THP-1 macrophages (1.5- to 1.7-fold). Regarding ABCG1, we detected 2 bands (∼110 and ∼75 kDa) for ABCG1 in RAW cells and THP-1 macrophages (Figure 1A and 1C). In contrast, ∼110-kDa bands were absent or very scarce in MPM. Because ABCG1 knockdown using microRNA resulted in more marked reduction of ∼75-kDa bands (Figure 2D), we estimated ABCG1 protein levels by quantifying ∼75-kDa bands.

As shown in Figure 1A and 1B, the proteasome inhibitors promoted ABCG1 expression in the various macrophages (RAW, 1.8- to 2.6-fold; MPM, 2.4- to 3.0-fold; THP-1, 3.1- to 3.4-fold). In RAW cells, proteasome inhibitors enhanced ∼110-kDa bands in parallel with an increase in ∼75-kDa bands. Furthermore, Figure 1C shows that bortezomib treatment enhanced ABCA1 expression in a dose- and time-dependent manner. However, ABCA1/G1 mRNA levels remained unchanged with the proteasome inhibitors in RAW cells (Figure 1D), MPM, and THP-1 macrophages (data not shown), indicating that the increased protein expression was attributable to posttranscriptional regulation. In contrast, scavenger receptor class B, type I (SR-BI), another important protein involved in HDL-mediated cholesterol efflux from macrophages, was not modulated by proteasomal inhibition (Figure 1A and 1C). We did not observe cell toxicity due to proteasome inhibitors based on lactate dehydrogenase levels in the conditioned media or cell protein levels (data not shown).

We also performed Western blots analysis using a human hepatocyte cell line, HepG2. Supplemental Figure 1A and 1B shows that the proteasome inhibitors enhanced ABCA1/G1 protein expression in these cells, as had been observed in macrophages. SR-BI expression was again not affected.

Proteasome Inhibitors Promote ApoA-I- and HDL-Mediated Cholesterol Efflux From Macrophages by Increasing ABCA1/G1
Furthermore, as shown in Figure 2A, the increased ABCA1 expression due to the proteasome inhibitors translated into significant increases in apoa-I-mediated cholesterol efflux from RAW cells (MG132, 2.8-fold; epoxomicin, 3.3-fold; bortezomib, 2.5-fold). Similar results were obtained in MPM

![Figure 1](http://atvb.ahajournals.org/content/1981/S2)

**Figure 1.** Proteasome inhibitors enhance ABCA1 and ABCG1 expression. A, MPM, RAW cells, and THP-1 macrophages were lysed and subjected to a Western blot analysis 4 hours after treatment with 1 μmol/L MG132 (MG), 0.1 μmol/L epoxomicin (Epo), 0.1 μmol/L bortezomib (Bor), or the vehicle (cont). The results are from 3 separately performed experiments that yielded similar results. B, Protein levels were quantified as described in Materials and Methods. C, RAW cells treated with indicated doses of bortezomib for 4 hours or treated with 0.1 μmol/L bortezomib for indicated times were lysed and subjected to Western blot analysis. D, Total RNA was extracted from THP-1 macrophages treated with 0.1 μmol/L epoxomicin, 1 μmol/L MG132, 0.1 μmol/L bortezomib, or the vehicle for 4 hours. Real-time quantitative reverse transcription–polymerase chain reaction was performed as described in Materials and Methods. The mRNA levels of each gene were standardized for 18S ribosomal RNA levels. The results are representative of 3 or more experiments and are presented as mean±SD. *P<0.05 vs the vehicle.
ABCA1 and ABCG1 Degraded by Proteasome-Dependent Pathway

To further investigate whether ABCA1/G1 proteins are degraded by the UPS, we next performed pulse-chase experiments as additional confirmation of proteasomal degradation of ABCA1/G1 using COS-7 cells transiently transfected with FLAG-tagged expression vectors for human ABCA1/G1 (hABCA1- or hABCG1-FLAG). In the absence of MG132, the half-life for ABCA1 and ABCG1 protein degradation was 60 minutes or shorter (Figure 3A and 3B). The presence of MG132, however, increased the half-life of ABCA1 and ABCG1. Figure 3C clearly demonstrates that degradation of endogenous ABCA1 and ABCG1 proteins in THP-1 macrophages after treatment with cycloheximide was delayed in the presence of MG132. These results suggest that the UPS is involved in the degradation of both ABCA1 and ABCG1 proteins.

Accumulation of Ubiquitinated ABCA1 and ABCG1 in the Presence of Proteasome Inhibitors

The above observations led us to hypothesize that degradation of ABCA1 and ABCG1 proteins is mediated by ubiquitination, a common process in which ubiquitin is covalently attached to the lysine residues of substrate proteins for subsequent degradation.16,17

As shown in Figure 4A, polyubiquitinated ABCA1-FLAG was detected in the presence of epoxomicin, MG132, and lactacystin, another proteasome inhibitor. Immunoblot analysis with anti-ABCA1 antibodies revealed that the steady state level of ABCA1-FLAG was increased in the presence of epoxomicin (Figure 4A, compare lanes 5 and 6). However, the band of polyubiquitinated ABCA1-FLAG detected with anti-ABCA1 antibodies was barely visible (Figure 4A, lower panel), a finding consistent with that of a previous study18 in which detection of ubiquitin-modified proteins at the steady state was difficult. Similarly, polyubiquitinated ABCG1 was also evident in the presence of the proteasome inhibitors (Figure 4B, upper panel). ABCG1 protein levels visualized by immunoblotting demonstrated that ABCG1-FLAG expression was enhanced by MG132 or lactacystin (Figure 4B, lower panel, compare lanes 5 and 7 to 8). Similar results were
Polyubiquitination of Endogenous ABCA1 and ABCG1

As the results up to this point had not totally excluded the possibility that the ubiquitination of ABCA1/G1 proteins was due to exogenous overexpression in COS-7 or HEK293 cells, we qualified polyubiquitinated ABCA1/G1 proteins using the cell lysates from THP-1 macrophages and MPM treated with MG132. Figure 4C and 4D shows that polyubiquitinated ABCA1 and ABCG1 were detected in THP-1 macrophages, and steady-state levels of ABCA1/G1 were increased by MG132. Regarding MPM, similar results were obtained for both ABCA1 and ABCG1 (Figure 4E and 4F). These observations indicate that endogenous ABCA1 and ABCG1 proteins in macrophages indeed undergo polyubiquitination.

Bortezomib Enhances ABCA1 and ABCG1 Expression in Peritoneal Macrophages In Vivo

Next, we used bortezomib to further extend these in vitro observations into in vivo circumstances. We chose bortezomib as it is the only proteasome inhibitor approved for a human disease (drug-resistant multiple myeloma). Consistent with our in vitro observations, the subcutaneous injection of bortezomib (0.5 mg/kg) or the vehicle into male C57BL6/J mice produced a modest but significant increase in ABCA1 and ABCG1 expression 5 days afterward in MPM as compared with saline (Figure 5A). In contrast, there was no change in SR-BI expression in MPM. As shown in Figure 5B, bortezomib also enhanced ABCA1 expression in the liver. In contrast, bortezomib attenuated SR-BI expression in the liver, especially the 57-kDa nonglycosylated form, which was not detected in MPM or other macrophage cell lines. In contrast to the in vitro experiment using HepG2 cells, we could not detect ABCG1 protein expression in mouse livers despite using 2 primary antibodies for ABCG1 for detection.

The fact that bortezomib increased ABCA1 and decreased SR-BI expression in mouse livers had not translated into modulation of total cholesterol and HDL cholesterol (HDL-C) after acute administration (72 hours after injection) of bortezomib (Supplemental Table). We therefore investigated longer term effects of bortezomib on plasma lipids: mice were injected with bortezomib every 7 days, and blood samples were obtained before and 3 to 21 days after the initial injection. Figure 5B and 5C and Supplemental Figure II show that there were again no differences in total cholesterol, HDL-C, and triglycerides levels between a bortezomib group and a saline group. There were also no differences in cholesterol levels in lipoprotein fractions separated by fast protein liquid chromatography between the 2 groups (Figure 5D).

Systemic Proteasome Inhibition by Bortezomib Promotes Macrophage RCT In Vivo

Finally, we performed an in vivo RCT assay to assess whether increased cholesterol efflux from macrophages due to proteasomal inhibition translates into promoted overall RCT in vivo. As shown in Figure 6A, bortezomib produced a significant increase in plasma 3H-tracer levels, of 20%, at 24 hours after injection, resulting in a 1.5 fold-increase in fecal excretion of the 3H-tracer compared with the vehicle (Figure 6D). In contrast, bortezomib treatment did not affect 3H-tracer levels in the liver or the bile (Figure 6B and 6C). To further investigate whether promotion of overall RCT by bortezomib treatment was attributable to increased cholesterol efflux from macrophages, 3H-cholesterol and acetylated low-density lipoprotein–loaded RAW cells were pretreated with bortezomib (0.1 μmol/L for 4 hours) before intraperitoneal injection, and then an in vivo RCT assay was performed. The results revealed that bortezomib pretreatment resulted in increased 3H-tracer levels in plasma (24 hours, Figure 6E) and feces (Figure 6F), similar to in vivo systemic administration of bortezomib. These results indicate that the enhancement of the initial step of RCT (cholesterol efflux from macrophages) indeed translated into increased overall RCT in mice in vivo.

Discussion

Plasma concentrations of HDL-C have strong inverse correlations with risk of atherosclerotic cardiovascular disease.19
Although the exact mechanisms by which HDL exerts protective effects against development of atherosclerosis remain elusive, HDL has been postulated to facilitate the efflux of cholesterol from peripheral tissues and transport it back to the liver in a process called RCT.\textsuperscript{1,2} Recently, researchers have identified several key molecules that work in concert on cholesterol efflux from macrophages, the initial step in RCT, which include ABCA1, ABCG1, and SR-BI.\textsuperscript{3,4,20} Lipid-poor apoA-I is a good substrate for ABCA1-mediated cholesterol efflux from cells,\textsuperscript{4,20} and the resulting HDL is in turn an acceptor for ABCG1- and SR-BI-mediated cholesterol efflux.\textsuperscript{4,20,21} Based on these observations, great interest has been focused on research to develop novel therapeutic strategies that increase the expression of these cholesterol transporters.

Here, we demonstrated that ABCA1 and ABCG1 undergo protein degradation through polyubiquitination in the UPS. We further showed, for the first time, that inhibition of the UPS using proteasome inhibitors enhanced both apoA-I- and HDL-mediated cholesterol efflux from macrophages by in-

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** ABCA1 and ABCG1 undergo polyubiquitination. A and B, COS-7 cells were transiently transfected with either FLAG-tagged hABCA1 (A)/hABCA1G1 (B) or mock plasmids and cultured in the presence or absence of proteasome inhibitors (epoxomicin, 0.1 μmol/L; MG132, 1 μmol/L; lactacystin, 1 μmol/L) for 4 hours. The cells were then lysed and immunoprecipitated (IP) using the M2 anti-FLAG antibody. The immunoprecipitate was probed by immunoblotting (IB) using antibodies against ABCA1 (A), ABCG1 (B), or polyubiquitin (polyUb). THP-1 macrophages (C and D) or MPM (E and F) were incubated in the presence or absence of MG132 (MG, 1 μmol/L) for 3 hours, lysed, and immunoprecipitated using ABCA1 (C and E) or ABCG1 (D and F) antibodies. The immunoprecipitate was probed by immunoblotting using antibodies against ABCA1 (C and E), ABCG1 (D and F) or against polyubiquitin. Cont indicates control.
creasing ABCA1/G1 expression. Bortezomib, one of these proteasome inhibitors, promoted overall RCT in vivo. Although evidence for the role of the UPS in ABCA1 protein degradation is very scarce, Wang et al.\textsuperscript{22} have reported that ABCA1 protein is ubiquitinated in the presence of lactacystin and Azuma et al.\textsuperscript{12} observed proteasomal degradation of ABCA1 and interaction between ABCA1 and the COP9 signalosome, a key molecule controlling protein ubiquitination and deubiquitination. However, as their observations were limited to exogenously overexpressed ABCA1, it remains unclear whether endogenous ABCA1 undergoes the same type of degradation in macrophages. Moreover, such previous studies did not look at whether an increase in ABCA1 protein due to proteasomal inhibition can increase cholesterol efflux. The present study built on the previous findings by demonstrating that (1) both exogenous (Figure 4A) and endogenous ABCA1 protein (Figure 4C and 4E) were polyubiquitinated and degraded by proteasomes, and (2) proteasome inhibitors enhanced ABCA1 protein expression, resulting in promoting apoA-I-mediated cholesterol efflux from macrophages (Figure 2A). It is worth noting that increasing ABCA1 protein by UPS inhibition preserves its function, because the UPS is considered to be essential for protein quality control. Recently, Arakawa et al.\textsuperscript{23} demonstrated a novel strategy for enhancing cholesterol efflux from macrophages involving inhibition of ABCA1 degradation. They found that the compounds derived from the hypolipidemic drug probucol increased ABCA1 expression in macrophages and the liver, and raised HDL levels in plasma, which in turn attenuated atherosclerosis in rabbits. Although no detailed mechanisms for the decrease in ABCA1 degradation due to these compounds are known, it is interesting that not only was ABCA1 protein increased by them, but its function in the efflux of cholesterol from macrophages to apoA-I was also maintained.

We have even less knowledge of the posttranslational regulation of ABCG1 than we have about that of ABCA1. In
this regard, Nagelin et al recently reported that 12/15-lipoxygenase increased ABCG1 degradation in macrophages through p38- and c-Jun NH2-terminal kinase 2-dependent mechanisms.\textsuperscript{24,25} They found that MG132 and lactacystin canceled the inhibitory effects of eicosanoids produced by lipoxygenase on ABCG1 protein levels but they did not address the issue of whether UPS is involved in this degradation mechanism. In the present study, we demonstrated for the first time that both exogenous (Figure 4B) and endogenous (Figure 4D and 4F) ABCG1 underwent polyubiquitination and were subsequently targeted for degradation via the UPS. Similar to the case of ABCA1, there had been an increase in ABCG1 protein due to proteasomal inhibition and its function was also maintained, and resulting in enhancing HDL-mediated cholesterol efflux from macrophages (Figure 2B).

Interestingly, Nagelin et al\textsuperscript{24} demonstrated that ABCG1 serine phosphorylation was induced during degradation by eicosanoids. Regarding an association between protein phosphorylation and substrate recognition by the UPS, 1×β has been extensively investigated.\textsuperscript{26} Phosphorylation generally targets 1×β for ubiquitination and degradation by the 26S proteasome, leading to nuclear translocation of the nuclear receptor-κB, which in turn transactivates various inflammatory genes. These researchers observed that proteasomal inhibition canceled ABCG1 degradation by eicosanoids, and lactacystin induced the accumulation of phosphorylated ABCG1.\textsuperscript{25} We speculate that phosphorylation induced by eicosanoids triggers ABCG1 degradation via the UPS. Because phosphorylation of ABCA1 also accelerates the degradation of its protein,\textsuperscript{27} there might be similar, if not identical, machinery involving ABCA1 and ABCG1 degradation via the UPS. Additional studies are therefore needed to explore the precise mechanisms for ABCA1/G1 protein regulation by the UPS.

A major strength of this study is that we were able to extend in vitro observations into in vivo circumstances, by demonstrating that UPS inhibition by bortezomib promoted overall RCT in mice. Furthermore, similar to systemic administration, intraperitoneal injection of RAW cells pretreated with it resulted in increased tracer counts in plasma and feces as compared with the vehicle (Figure 6E and 6F). These results indicate that enhanced cholesterol efflux from macrophages primarily contributes to promotion of overall RCT.

In this experiment, we observed an increase in ABCA1 and a decrease in SR-BI protein levels in the liver (Figure 5A). However, there was no change in SR-BI expression in macrophages (Figure 1A) and HepG2, a human hepatocyte cell line (Supplemental Figure I). Although the precise reasons remain unclear, several differences, such as species or in vitro in vivo setting, might be involved in bortezomib-mediated changes in SR-BI expression in mouse livers.

Increased ABCA1 and reduced SR-BI expression in the liver are believed to raise plasma HDL levels.\textsuperscript{28–30} However, these changes did not accompany an increase in plasma HDL concentrations. At present, the exact mechanism for the lack of an association is unclear. Several possibilities deserve discussion. First, this may be due to a species difference: mice (HDL animals) without cholesterol ester transfer protein versus humans (low-density lipoprotein animals) with cholesterol ester transfer protein. In this regard, future studies using animals with cholesterol ester transfer protein (eg, hamsters) will be needed to clarify this issue. Second, other, as-yet-unknown mechanisms may offset the effects of ABCA1/SR-BI changes. Third, our in vivo experiment assessed the effect of a single injection of bortezomib, which may differ from its chronic effects.

ABCA1 and ABCG1 play a pivotal role in cholesterol efflux from macrophages which in turn can regulate overall RCT as observed in the present study, and thus strategies that increase ABCA1/G1 expression have drawn much attention as novel HDL therapies to reduce cardiovascular morbidity and mortality. A promising target in this has been liver X receptor ligands because they activate ABCA1/G1 transcription. However, there would be a major drawback with such gain-of-function strategies, involving an off-target effect due to complex cross-talk among transcriptional factors, and in this regard, liver X receptor ligands have caused fatty liver and dyslipidemia by activating sterol regulatory element binding protein-1c.\textsuperscript{7} In contrast, a UPS strategy could provide selective inhibition, in particular protein degradation, because E3 ubiquitin ligases are, in general, specific to proteins, as described below. Therefore, the identification of E3 ligases for ABCA1 and ABCG1 warrants future study.

Although systemic proteasomal inhibition produced a clear increase in RCT in vivo in the present study, previous studies yielded mixed results. Herrmann et al\textsuperscript{31} reported that long-term treatment with a proteasome inhibitor promoted the development of early atherosclerotic lesions in coronary arteries from hypercholesterolemic pigs. On the other hand, other studies have suggested that proteasomal inhibition may be beneficial against atherosclerotic plaque progression and complications.\textsuperscript{32} For example, MG132 reportedly enhanced endothelial dependent relaxation, which was associated with an increase in endothelial nitric oxide synthase protein and its activity.\textsuperscript{33} Meiners et al\textsuperscript{34} demonstrated that local application of MG132 resulted in significant inhibition of intimal hyperplasia in the rat carotid artery by inhibiting degradation of 1×β followed by nuclear factor-κB inactivation. However, overall, the favorable effect of inhibiting the UPS on atherogenesis is likely to be conditional and appears to depend on multiple factors, such as proteasome inhibitor dosage, cell type, and way of administration.\textsuperscript{35} In this regard, though a proteasome inhibitor reportedly induced apoptosis in rapidly proliferating tumor cells, the same dose of the drug had a protective property with respect to apoptosis in differentiated and quiescent cells,\textsuperscript{36} indicating the complexity of an association between proteasomal inhibition and cell fate.

Proteins destined for degradation by proteasomes are recognized and ubiquitinated in a process that requires a conserved cascade of enzymatic reactions. Together with ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2, E3 ubiquitin ligases promote the transfer of ubiquitin to specific protein substrates. Their substrate specificities lead us to believe that targeting the active site of E3 enzymes or their interaction with substrates would enable the creation of selective drugs with fewer side effects compared with systemic proteasomal inhibition.\textsuperscript{10} Besides E3 ubiquitin ligases, deubiquitinating enzymes are also an emerging ther-
apatic target because they have a catalytic pocket for targeted binding of small molecules.\textsuperscript{11} Identification of E3 ligases or deubiquitinating enzymes specific for ABCA1/G1 could provide a new insight into the posttranslational regulation of these transporters, as well as in drug discovery.

In conclusion, the present study demonstrated that ABCA1 and ABCG1 underwent polyubiquitination with subsequent degradation via the UPS and that proteasomal inhibition resulted in increased cholesterol efflux from macrophages and promoted overall RCT in vivo by increasing functional ABCA1 and ABCG1 expression. These findings may ultimately provide the basis for a novel therapeutic strategy for atherosclerotic diseases in humans.

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Disclosures

None.

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Supplemental Material

Materials and Methods

Materials

Epoxomicin and lactacystin were purchased from the Peptide Institute (Osaka, Japan), MG132 from Calbiochem (San Diego, CA), and bortezomib from Sequoia Research Products (Berkshire, UK). Human apoA-I was purchased from Sigma (St. Louis, MO). HDL was isolated by sequential ultracentrifugation and acetylated LDL (AcLDL) was prepared according to the methods previously reported.\textsuperscript{1, 2}

Cell Cultures

THP-1 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 (Sigma) containing 10% fetal bovine serum (FBS). The differentiation of THP-1 monocytes into macrophages was induced in the presence of 200 nmol/L of PMA for 72 hr. RAW264.7 (RAW), COS-7 and HEK293 cells (Riken Cell Bank) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. All cultures were kept in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C. Mouse peritoneal macrophages (MPM) were harvested from the peritoneal cavity of mice as described previously.\textsuperscript{2, 3} Briefly, for in vitro experiments, C57BL/6 mice (Clea
Japan, Tokyo, Japan) were injected intraperitoneally with 10% thioglycollate (Difco, Detroit, MI). Three days later, the mice were euthanized and macrophages collected by lavage of the peritoneum with ice-cold phosphate buffered saline (PBS). For in vivo experiments, resident macrophages were collected without injection of thioglycollate. Cells were seeded on 12-well plates at a density of $2 \times 10^6$ cells/well in DMEM plus 10% FBS.

**Western Blot Analyses**

Cells were harvested and protein extracts prepared as previously described. They were then subjected to Western blot analyses (NuPAGE Novex 4-12% Bis-Tris Gel, Invitrogen, Carlsbad, CA; 25 µg protein per lane) using rat anti-ABCA1 antiserum, and rabbit anti ABCG1 (Novus Biologicals Littleton, CO, USA and Santa Cruz, Santa Cruz, CA)-, scavenger receptor class B, type I (SR-BI)-(Novus Biologicals) and β-actin (Santa Cruz))-specific antibodies. The proteins were visualized and quantified using a chemiluminescence method (ECL Plus Western Blotting Detection System; GE Healthcare UK Ltd) and the NIH image analysis software program.

**Real-time Quantitative RT-PCR**

At the indicated hours after treatment with epoxomicin, MG132 and bortezomib, total RNA was extracted from the cells, and first-strand cDNA was synthesized from the
total RNA (250 ng) by placing in a Reverse Transcription Reagent (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with a Perkin–Elmer 7900 PCR machine, TaqMan PCR master mix and FAM-labeled TaqMan probes (Assays-on-Demand, Applied Biosystems) for human or mouse ABCA1, ABCG1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression data were normalized for GAPDH levels.

**Determination of Cholesterol Efflux**

Cholesterol efflux experiments were performed as previously described.\(^1,2,4\) THP-1 macrophages or RAW cells were radiolabeled with \(^3\)H-cholesterol (5 µCi/mL) pre-equilibrated with AcLDL (50 µg/mL) in the media containing 0.2 % bovine serum albumin (BSA) for 24 hr, and in the case of RAW cells and MPM, in the presence of 0.3 mmol/L 8-bromoadenocine 3’,5’-cyclic monophosphate. The cells were washed twice with PBS and incubated for 24 hr in DMEM containing 0.2 % BSA in the presence or absence of the proteasome inhibitors. They were again washed with PBS and incubated in DMEM containing 0.2 % BSA in the presence or absence of apoA-I (10 µg/mL) or HDL (50 µg/mL) for 24 hr. The percentage cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and the cells.
Cloning and Generation of Recombinant Adenoviruses Encoding for Synthetic microRNAs Targeting Mouse ABCG1

The pre-microRNA (miR) sequences for mouse ABCG1 were designed using an RNAi designer on-line tool, Invitrogen's RNAi Designer. Three different double-stranded oligo duplexes encoding desired miR target sequences were selected and cloned into pcDNA6.2/EmGFP-GW vector (Invitrogen).

The sequences of one of the most efficient oligo duplexes are as follows: top sequence 5′- TGC TGT ATC CTT TCT TCT TCC ACC AGG TTT TGG CCA CTG ACT GAC CTG GTG GAA AGA AAG GAT A -3′ and bottom sequence 5′- CCT GTA TCC TTT CTT TCC ACC AGG TCA GTC AGT GGC CAA AAC CTG TGG AGA GAA GGA AC -3′. The sequences of the negative control oligo duplex are as follows: top sequence 5′-GCT AAA GTA TGC CGT GAG CGT TTG CCA TGA TGA GTC CCA GCA TAC TTT-3′ and bottom sequence 5′-CCT AAA GTA TGC TGG GAC TCA TCA TGG CAA ACG CTC CGC CAG ACA TTC-3′.

The miR flanking sequence was transferred into pDONR221 vector (Invitrogen) to generate entry vectors, which were used to generate adenoviruses encoding for synthetic miRNAs (Ad-miR-mABCG1 and Ad-miR-neg) using the ViraPower Adenoviral Expression System (Invitrogen), according to the manufacturer's protocol.
adenovirus titer in plaque-forming units was determined by a plaque formation assay following infection of HEK293 cells. The multiplicity of infection (MOI) was defined as the ratio of the total number of plaque-forming units to the total number of cells that were infected.

**MicroRNA-Mediated Inhibition of ABCG1 in RAW Cells by Adenoviral Vectors**

For in vitro infection, RAW cells were seeded in 6 or 12 well dishes in DMEM containing 10% FBS. At 80% confluent, cells were infected with 100 MOI of Ad-miR mABCG1 or Ad-miR neg. After 24 hr of infection, the medium was replaced with DMEM containing 0.2% BSA and cells were used for cholesterol efflux assays or protein expression analysis.

**Construction of Expression Plasmids Encoding ABCA1 and ABCG1**

To obtain human ABCA1 and ABCG1 open reading frames, RT-PCR was performed using first strand cDNA derived from human monocyte-derived macrophages as a template and the specific primers. To generate FLAG-tagged expression vectors for human ABCA1 (hABCA1-FLAG) and ABCG1 (hABCG1-FLAG), the PCR products with FLAG epitope sequences in the carboxyl-terminus were ligated in pcDNA3.1 (+) (Invitrogen). All constructs were verified by DNA sequencing.

**Pulse-chase Assays**
In the pulse-chase experiments, COS-7 cells transfected with hABCA1-FLAG or hABCG1-FLAG were cultured under DMEM without cysteine and methionine (Sigma) for 60 min to deplete cells of methionine and cysteine. $^{35}$S-labeled methionine/cysteine mix was added to the DMEM (100 µCi/mL) which was incubated for 60 min, followed by a chase period for the indicated times during which it was cultured under DMEM supplemented with 1.5 mg/mL L-methionine and 0.5 mg/mL L-cysteine, in the absence or presence of MG132 (1 µmol/L). The cell lysates were subjected to immunoprecipitation by incubating with anti-FLAG M2 antibodies (Sigma). The precipitates were then washed, and eluted by incubation in SDS sample buffer. Finally, the eluted proteins were separated by SDS-PAGE, and the gel was subjected to fluorography. The bands were quantified using an image analysis system and the NIH image analysis software program.

**Immunoprecipitation and Detection of Polyubiquitinated ABCA1/ABCG1**

Forty-eight hr after transfection with hABCA1-FLAG or hABCG1-FLAG, COS-7 cells were incubated for 4 hr in the presence or absence of the proteasome inhibitors, and then lysed. To collect FLAG-tagged proteins, anti-FLAG M2-agarose (Sigma) was added to the cell lysates, and they were gently rotated overnight at 4 °C. After washing, the agarose preparation was boiled with SDS-PAGE sample buffer, and the supernatants
were subjected to Western blot analysis with an anti-ubiquitin antibody (Santa Cruz) 
and ABCA1- and ABCG1-specific antibodies.

Endogenous ABCA1 and ABCG1 in the THP-1 macrophages and MPM were 
immunoprecipitated with the specific antibodies using a Seize X Protein G 
Immunoprecipitation Kit (Pierce, Rockford, IL) according to manufacturer’s 
instructions.

Determination of Lipid Levels, Lipoprotein Fractionation and Protein Expression 
in the Macrophages and Liver of Mice Treated with Bortezomib

Six-week-old male C57BL/6 mice were treated with 0.5 mg/kg of bortezomib or the 
vehicle (saline) by single subcutaneous injection. Before and 72 hr after injection, blood 
samples were obtained from the tail vein under anesthesia. Total and HDL cholesterol 
levels in plasma were determined by enzymatic measurement using commercially 
available kits (WAKO, Osaka, Japan).

We also investigated longer term effects of bortezomib on plasma lipids: the mice 
were injected with bortezomib every 7 days and blood samples were obtained before 
and 3-21 days after the initial injection. For lipoprotein fractionation analysis, equal 
volumes of plasma samples were pooled from mice for each group (a total volume of 
400 µl). Lipoproteins were fractionated by fast protein liquid chromatography (FPLC)
using a Superose 6 10/300 GL FPLC column (Amersham Biosciences, Piscataway, NJ). Fractions (500 µl) were collected and used for cholesterol measurement. After 5 days of treatment, resident peritoneal macrophages and livers were isolated. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996), and had been approved by the Internal Animal Care and Use Committee of the National Defense Medical College.

in Vivo Macrophage RCT Studies

RAW cells were cultured under RPMI 1640 supplemented with 10% FBS, and then radiolabeled with $^3$H-cholesterol (5 µCi/mL) pre-equilibrated with AcLDL (50 µg/mL) for 48 hr. These foam cells were then washed, equilibrated, detached with cell scrapers, resuspended in RPMI 1640, and pooled before being injected into mice. Before the initial experiments, seven-week-old male C57BL/6 mice had been divided into 2 groups (6 mice per group) and treated with 0.5 mg/kg of bortezomib or the vehicle by single subcutaneous injection. In another experiment to assess the effect of bortezomib on in vivo RCT (ex vivo experiments), we treated AcLDL-loaded RAW cells with 0.1 µmol/L of bortezomib for 4 hr just before injection of the cells, and did not perform any treatment with the mice.
On the day of injection of the cholesterol-loaded cells, the mice were caged individually with unlimited access to food and water. The $^3$H-cholesterol-labeled and AcLDL-loaded RAW cells (typically $5.0 \times 10^6$ cells containing $7.5 \times 10^6$ counts per minute (cpm) in 0.5 mL RPMI 1640) were injected intraperitoneally as described previously. Blood was obtained at 24 and 48 hr and plasma subjected to liquid scintillation counting (LSC). Feces were collected continuously from 0 to 48 hr and stored at 4°C before being counted. At 48 hr after injection, mice were exsanguinated and livers and bile were removed.

**Liver, Bile and Fecal Analyses**

Liver lipids were extracted as described previously. Briefly, a 50-mg piece of liver tissue was homogenized in water, and lipids were extracted with a 2:1 (vol/vol) mixture of chloroform/methanol. The lipid layer was collected, evaporated, resuspended in a 3:2 (vol/vol) mixture of hexane/isopropanol, and counted in an LSC. The bile was directly counted in an LSC. The total feces collected from 0 to 48 hr were weighed and soaked in distilled water (1 mL water per 100 mg feces) overnight at 4°C. An equal volume of ethanol was added the next day, the samples were homogenized and 200 µL of the homogenized samples was counted in an LSC. Results were expressed as percentage of cpm injected.
Statistical Analysis

The Student’s t-test was performed as appropriate. A p value of less than 0.05 was considered to be statistically significant. Values are expressed as mean ± SD.

References


Supplemental Figure I. Proteasome inhibitors enhance ABCA1 and ABCG1 expression in HepG2 cells. A, The cells were lysed and subjected to a Western blot analysis 4 hr after treatment with 1 µmol/L of MG132 (MG), 0.1 µmol/L of epoxomicin (Epo), 0.1 µmol/L of bortezomib (Bor), or the vehicle (Cont). The results are from 3 separately performed experiments that yielded similar results. B, Protein levels were quantified as described in Methods. The results are representative of 3 experiments and presented as mean ± SD. * p<0.05 vs. the vehicle.
Supplemental Figure II. Proteasome inhibitor bortezomib does not affect plasma triglycerides (TG) levels in mice. Mice were treated with either 0.5 mg/kg/day of bortezomib or the vehicle (saline) by subcutaneous injection every 7 days and blood samples were collected before and 7, 14, 21 days after the treatments. TG levels in plasma were determined as described in Methods.
Supplemental Table. Effect of bortezomib on plasma lipids in mice.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Bortezomib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>72 hr</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>78.1±4.3</td>
<td>79.7±4.3</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>59.2±7.7</td>
<td>65.7±6.5</td>
</tr>
</tbody>
</table>

Data were expressed as mean±SD. TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol.