12/15-Lipoxygenase Activity Increases the Degradation of Macrophage ATP-Binding Cassette Transporter G1

Melissa H. Nagelin, Suseela Srinivasan, Jianyi Lee, Jerry L. Nadler, Catherine C. Hedrick

Objective—The purpose of this study was to evaluate the effect of 12/15-lipoxygenase (12/15LO) in macrophage ABCG1 expression and function associated with cholesterol efflux.

Methods and Results—12/15LO was stably overexpressed in J774 macrophages. 12/15LO-overexpressing macrophages had a 30% reduction in HDL-mediated cholesterol efflux, corresponding with significantly reduced ABCG1 protein expression. Treatment of 12/15LO-overexpressing macrophages with a 12/15LO ribozyme to reduce 12/15LO restored HDL-mediated efflux and ABCG1 protein expression. Treating macrophages with 12/15LO unsaturated fatty acid substrates or eicosanoid products also reduced HDL-mediated cholesterol efflux. Additionally, both 12/15LO overexpression in macrophages and incubation of macrophages with eicosanoids reduced ABCG1 protein, but not mRNA, expression. However, incubation of macrophages with linoleic or arachidonic acids significantly reduced both ABCG1 mRNA and protein expression, suggesting that 12/15LO substrates and eicosanoid products differentially regulate ABCG1 expression. 12/15LO fatty acids did not decrease ABCG1 translation; however, 12/15LO fatty acids increased ABCG1 degradation when blocked by cycloheximide. ABCG1 degradation may be regulated through posttranslational modifications. Treatment with the 12/15LO eicosanoid product 12SHETE increased serine phosphorylation of ABCG1.

Conclusions—We conclude that serine phosphorylation may increase the degradation rate of ABCG1, and as a result cause macrophage cholesterol accumulation. These findings provide evidence that 12/15LO activity in the vessel wall contributes to atherogenesis by impairing the macrophage ABCG1 cholesterol efflux pathway. (Arterioscler Thromb Vasc Biol. 2008;28:1811-1819)

Key Words: lipoxygenase □ ABCG1 □ macrophage □ fatty acid □ eicosanoid

Atherosclerosis is a chronic inflammatory disease characterized by monocyte recruitment to the arterial wall and their subsequent accumulation as lipid-laden macrophages, or “foam” cells. Oxidation of low-density lipoprotein (LDL) plays an essential role in the early events leading to foam cell formation, and the enzyme 12/15-lipoxygenase (12/15LO) has been implicated in LDL oxidation.1,2

12/15LO is a nonheme iron–containing dioxygenase which incorporates molecular oxygen stereospecifically to arachidonic and linoleic acid to produce the hydroperoxy fatty acids 12S- and 15S-hydroxyeicosatetraenoic acids (12SHETE/15SHETE) and 13S-hydroxyoctadecadienoic acid (13SHODE), respectively.3,4 The 12/15-lipoxygenase family consists of “platelet-type” 12-lipoxygenase and “leukocyte-type” 12/15-lipoxygenase. Based on the ratios of 12S- and 15SHETE produced from arachidonic acid, the enzyme is designated 15-lipoxygenase (15LO) in humans and leukocyte 12-lipoxygenase in mice and pigs. Murine leukocyte 12/15LO and human 15LO proteins are highly homologous.5

12/15LO activation has been implicated in the pathogenesis of atherosclerosis and diabetes. We and others have shown that mice deficient in 12/15LO are protected from the development of atherosclerosis,6,7 whereas mice that overexpress the enzyme are more prone to lesion development.8 The mechanisms by which 12/15LO regulates atherogenesis remain unknown, but may be attributable to inflammatory actions of 12/15LO eicosanoids on the endothelium and macrophages in the vessel wall or attributable to its ability to oxidize LDL.1,8,9 12/15LO protein has been localized within atherosclerotic lesions in both rabbits and humans.10,11 In vitro data from Kuhn et al suggested a role for 12/15LO in the oxidation of LDL,12 whereas Cathcart and colleagues demonstrated that human monocytes generate superoxide that mediates the oxidation of LDL in an enzyme-dependent manner.2

Initially in lesion development monocytes are recruited to the vessel wall, where they differentiate into macrophages and upregulate scavenger receptors such as CD36 and SR-A to facilitate the uptake of oxidized LDL.13 Conversely, differentiated macrophages also upregulate members of the ATP binding cassette transporter family (ABC transporters),...
namely ABCA1 and ABCG1, to mediate the transfer or efflux of modified cholesterol moieties to high density lipoproteins (HDL). HDL transports this cholesterol back to the liver for subsequent conversion to bile and removal from the body during the process of reverse cholesterol transport (RCT). ABCA1-mediated removal of cholesterol to lipid-poor HDL particles has been extensively studied. Fatty acids can regulate ABCA1 expression and function. Oram and colleagues have demonstrated that unsaturated fatty acids, such as arachidonic and linoleic acid, inhibit cholesterol efflux from macrophages by enhancing the protein degradation and serine phosphorylation of ABCA1. Moreover, apolipoprotein A-I (apoA-I) binds and cross-links ABCA1 to stabilize and protect the transporter from degradation.

Much less is known about the regulation of ABCG1, and the role of this transporter in the development of atherosclerosis remains controversial. ABCG1 mediates the removal of cholesterol to mature HDL particles and facilitates the clearance of esterified cholesterol from macrophages. Uehara et al have demonstrated that unsaturated fatty acids suppress ABCG1 gene transcription. In the current study, we show that fatty acids of the 12/15LO pathway decrease HDL-mediated cholesterol efflux from macrophages. This reduction in cholesterol efflux is associated with enhanced degradation and serine phosphorylation of ABCG1. These novel results support the concept that 12/15LO activity in the vessel wall impairs the clearance of excess cholesterol from macrophages through regulation of ABCG1, thereby contributing to the development of macrophage foam cell formation.

Materials and Methods

For detailed methodology, please see the Data Supplement, available online at http://atvb.ahajournals.org. J774a.1 (J774) cells were cultured in DMEM containing 10% fetal bovine serum (FBS). J774 cells stably overexpressing porcine leukocyte 12/15LO (Plox) and mock-transfected (Mock) J774 cells were kind gifts of Dr Tamhiro Yoshimoto (Kanazawa University School of Medicine, Kanazawa, Japan).

Results

12/15LO Activity Reduces HDL-Mediated Cholesterol Efflux

Macrophages in atherosclerotic lesions have been shown to express high levels of 12/15LO. Thus, we assessed the role of 12/15LO and its eicosanoid products in regulating macrophage function associated with RCT. We used J774 macrophages overexpressing porcine leukocyte 12/15LO (Plox) or mock-transfected (Mock) control cells. We have previously shown that Plox macrophages have increased production of fatty acid hydroxy derivatives and a 3-fold increase in 12SHETE production compared to Mock controls. In the current study, Mock and Plox macrophages were labeled with [3H]cholesterol, and the effects of 12/15LO overexpression on HDL- or apoA-I–mediated [3H]cholesterol efflux were measured. Tall and colleagues have reported that HDL is the preferred cholesterol acceptor from ABCG1 whereas lipid free apoA-I is the preferred cholesterol acceptor from ABCA1. 12/15LO-overexpressing Plox macrophages had a significant 30% reduction in HDL-mediated cholesterol efflux compared to Mock controls (Figure 1A). No significant change in apoA-I–mediated cholesterol efflux between the 2 groups was observed, suggesting that 12/15LO activity preferentially modulates ABCG1-mediated cholesterol efflux.

To further determine that 12/15LO activity is directly responsible for the reduction in cholesterol efflux, we inhibited expression of 12/15LO in Mock and Plox macrophages using an adenovirus expressing a ribozyme to 12/15LO (AdRz). We have previously used this ribozyme to reduce 12/15LO expression by 60%. The 12/15LO ribozyme restored HDL-mediated cholesterol efflux in Plox macrophages to levels similar to control (Figure 1A), indicating that 12/15LO is able to regulate HDL-mediated cholesterol efflux.

12/15LO Activity Reduces ABCG1 Cellular Expression

Because ABCG1 is known to regulate HDL-mediated cholesterol efflux, we assayed the effects of 12/15LO activity on ABCG1 expression. We confirmed that 12/15LO protein was overexpressed in Plox macrophages (Figure 1B). Plox macrophages had a significant 60% reduction in ABCG1 protein expression (Figure 1B). No significant change in ABCA1 protein expression was observed (Figure 1B). Inhibition of 12/15LO in Plox macrophages with the 12/15LO ribozyme prevented the downregulation of ABCG1 protein expression (Figure 1C). These data suggest that the reduction in ABCG1 expression and cholesterol efflux to HDL is regulated by 12/15LO activity.

12/15LO Eicosanoids Reduce Cholesterol Efflux to HDL

When macrophages are presented with an amount of cholesterol that exceeds which can be incorporated into cellular membranes, the excess cholesterol is stored in macrophages as cholesteryl esters. The enzyme acyl-coenzyme A (CoA): cholesterol acyltransferase (ACAT) esterifies excess cholesterol with long chain fatty acids, and the resulting cholesteryl esters are stored in the cytoplasm as neutral lipid droplets. To examine whether fatty acid treatment enhanced cholesteryl ester formation, we treated J774 macrophages with arachidonic or linoleic acid for 16 hours and measured esterified cholesterol accumulation (Wako Chemicals USA Inc). Treatment with arachidonic or linoleic acids did not significantly alter macrophage cholesteryl ester content (data not shown). We next incubated J774 macrophages with fatty acid precursors and eicosanoid products of the 12/15LO pathway and measured cholesterol efflux. J774 macrophages were labeled with [3H]cholesterol and incubated with arachidonic and linoleic acids. Subsequently, HDL– and apoA-I–mediated cholesterol efflux was measured. Incubation of J774 macrophages with arachidonic or linoleic acid for 16 hours decreased HDL-mediated cholesterol efflux by 43% (Figure 1D), compared to control. Arachidonic and linoleic acids also significantly reduced apoA-I–mediated cholesterol efflux by 77% and 87%, respectively. These data are consistent with the findings of Oram and colleagues indicating that unsaturated fatty acids downregulate ABCA1 expression and function.

To assess the role of 12/15LO eicosanoids on cholesterol efflux, J774 macrophages were labeled with [3H]cholesterol...
and treated with 12SHETE, 13SHODE, or 15SHETE. Subsequently, HDL- and apoA-I–mediated cholesterol efflux was measured. 12SHETE and 13SHODE significantly reduced cholesterol efflux to HDL by 23% and 13%, respectively (Figure 1E). Treatment with 15SHETE produced a 10% reduction in HDL-mediated cholesterol efflux, although this reduction was not statistically significant (Figure 1E). No significant change in apoA-I–mediated cholesterol efflux was observed, indicating that products of the 12/15LO pathway preferentially regulate ABCG1-mediated cholesterol efflux.

Eicosanoid Products Regulate ABCG1 Expression in Macrophages

We next examined whether 12/15LO fatty acids regulate ABCG1 protein expression. Oram and colleagues have previously described the role of unsaturated fatty acids in reducing ABCA1 membrane content. In a similar manner, we found that arachidonic and linoleic acid significantly reduced ABCG1 protein expression within 8 hours and continuing through 24 hours compared to control (eg, time 0; Figure 2A).

To determine whether specific 12/15LO products regulated ABCG1 expression, we incubated J774 macrophages with 12/15LO eicosanoids for up to 24 hours. Treatment with the 12/15LO eicosanoids also reduced ABCG1 protein expression; however, these eicosanoids reduced ABCG1 expression at much earlier times than did arachidonic and linoleic acids (Figure 2B). Compared to control (eg, time 0), 12SHETE significantly reduced ABCG1 protein expression from 1 to 4 hours, 13SHODE significantly reduced ABCG1 protein expression from 30 minutes to 2 hours, and 15SHETE significantly reduced ABCG1 expression from 4 to 8 hours (Figure 2B). Thus, 12/15LO eicosanoids seem to produce more rapid downregulation of ABCG1 than did arachidonic and linoleic acids.

12/15LO Activity Does Not Affect ABCG1 mRNA Expression

Based on the findings of Uehara and colleagues indicating that unsaturated fatty acids suppress ABCA1 and ABCG1 gene transcription, we examined the effects of 12/15LO on ABCG1 mRNA expression. Plox macrophages had no change in relative ABCG1 mRNA levels compared to Mock controls (supplemental Figure IA). Interestingly, ABCA1 mRNA was significantly reduced in 12/15LO-overexpressing macrophages by 40% (Figure 1A). However, the reduction in ABCA1 mRNA does not appear to translate to decreased ABCA1 protein expression or decreased apoA-I-mediated cholesterol efflux (Figure 1A and 1B).

Treatment of J774 macrophages with arachidonic or linoleic acid significantly reduced ABCG1 and ABCA1 mRNA expression (supplemental Figure IB), consistent with the findings of Uehara and colleagues. However, treatment of J774 macrophages with 12/15LO eicosanoids resulted in no changes in ABCG1 or ABCA1 mRNA levels (supplemental Figure IC). These results suggest that the inhibitory effects of

Figure 1. 12/15LO activity reduces cholesterol efflux to HDL. A, Mock-transfected (Mock) or 12/15LO-overexpressing (Plox) J774 macrophages were incubated with recombinant adenovirus expressing either the 12/15LO ribozyme (AdRz) or control LacZ vector (AdLacZ). Total cell lysates were analyzed by immunoblotting for ABCG1, ABCA1, or 12/15LO. Right, Densitometry of immunoblots normalized to beta actin. Data represent the mean±SE of 3 experiments (*significantly lower than Mock P<0.006; #significantly lower than Mock P<0.05 by ANOVA). B, Left, Total cell lysates were analyzed by immunoblotting for ABCG1, ABCA1, or 12/15LO. Right, Densitometry of immunoblots normalized to beta actin. Data represent the mean±SE of 4 experiments (*significantly lower than Mock P<0.02 by ANOVA). C, Mock or Plox macrophages were incubated with recombinant adenovirus expressing either the 12/15LO ribozyme (AdRz) or control LacZ vector (AdLacZ). Total cell lysates were analyzed by immunoblotting for ABCG1. Results are representative of 2 similar experiments. D, HDL- or apoA-I–mediated cholesterol efflux was measured in J774 macrophages treated with vehicle control (CTR) or 500 nmol/L 12SHETE, 13SHODE, or 15SHETE. Data represent the mean±SE of 3 experiments (*significantly lower than CTR P<0.001; #significantly lower than CTR P<0.04 by ANOVA).
Figure 2. 12/15LO activity reduces cellular ABCG1. Cell lysates were analyzed by immunoblotting for ABCG1 at each time point. Each time point is compared to control (CTR, eg, time 0) for statistical evaluation. A, Top, J774 macrophages were incubated with vehicle control (CTR) or with arachidonic (AA) or linoleic (LA) acid (125 μmol/L) for up to 24 hours. Bottom, Densitometry of immunoblot normalized to beta actin. Data represent the mean±SE of 3 experiments (*significantly lower than CTR P<0.04 by ANOVA). B, Top, J774 macrophages were incubated with vehicle control (CTR) or with the indicated eicosanoids (500 nmol/L) for up to 24 hours. Bottom, Densitometry of immunoblot normalized to beta actin. Data represent the mean±SE of 3 experiments (*significantly lower than CTR P<0.05 by ANOVA).
Figure 3. 12/15LO products enhance ABCG1 degradation. A, Left, J774 macrophages were treated with either vehicle control (CTR), arachidonic (AA), or linoleic (LA) acids for 2 hours before the addition of cycloheximide for the indicated time points. Cell lysates were analyzed by immunoblotting for ABCG1. Right, Densitometry of immunoblot normalized to beta actin. Data represent the mean±SE of 3 experiments (*significantly lower than CTR 60 minutes $P<0.002$ by ANOVA). B, Left, J774 macrophages were treated with cycloheximide and either vehicle control (CTR), 12SHETE, or 15SHETE for the indicated time points. Cell lysates were analyzed by immunoblotting for ABCG1. Right, Densitometry of immunoblots normalized to beta actin. Data represent the mean±SE of 3 experiments (*significantly lower than CTR 30 minutes $P<0.003$; #significantly lower than CTR 60 minutes $P<0.05$ by ANOVA).
12/15LO activity on HDL-mediated cholesterol efflux and ABCG1 protein expression are not attributable to inhibition of ABCG1 transcription or message stability. These data also indicate that unsaturated fatty acids and 12/15LO eicosanoids regulate ABCG1 expression differently. Thus, unsaturated fatty acids transcriptionally regulate ABCG1 expression whereas 12/15LO eicosanoids regulate ABCG1 expression posttranslationally.

The 12/15LO product, 13SHODE, is a ligand for peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ has been shown to induce the expression of liver X receptor α (LXRα) thereby stimulating the expression of ABCA1 and ABCG1, leading to enhanced cholesterol efflux. Addition of 13SHODE to J774 macrophages resulted in a significant increase in LXR–α expression (supplemental Figure IC); however, 13SHODE treatment did not significantly induce ABC transporter expression, indicating that 13SHODE may regulate ABCA1 and ABCG1 posttranslationally.

12/15LO Products Enhance ABCG1 Degradation
We hypothesized that 12/15LO activity may reduce ABCG1 protein expression through decreased translation or increased protein degradation. To measure the effect of 12/15LO on translation, J774 macrophages were treated with 12SHETE, arachidonic, or linoleic acids for 2 or 16 hours. Cells were labeled with [35S]methionine for 20 minutes, and [35S]methionine incorporation into immunoprecipitated ABCG1 was measured. Treatment with 12/15LO fatty acids did not change methionine incorporation into ABCG1 (supplemental Figure II), indicating that reduced translation efficiency cannot account for the 12/15LO-mediated decrease in ABCG1 expression.

To address the possibility that the 12/15LO pathway regulates ABCG1 degradation, we treated J774 macrophages with 12/15LO fatty acids in the presence of cycloheximide to arrest protein synthesis. We pretreated J774 macrophages with arachidonic or linoleic acids for 2 hours before the addition of cycloheximide. Arachidonic but not linoleic acid significantly reduced ABCG1 protein expression after treatment with cycloheximide for 60 minutes (Figure 3A). To assess the role of the 12/15LO eicosanoids on ABCG1 degradation, we treated J774 macrophages with 12SHETE or 15SHETE immediately before the addition of cycloheximide. 12SHETE or 15SHETE also reduced ABCG1 protein expression, but seemed to have a more rapid effect than arachidonic acid. 12SHETE and 15SHETE significantly reduced ABCG1 expression after treatment with cycloheximide for 30 or 60 minutes, respectively (Figure 3B). These data are consistent with those of Figure 2, which shows rapid degradation of ABCG1 with 12/15LO eicosanoids compared to unsaturated fatty acids.

12/15LO Products Enhance ABCG1 Serine Phosphorylation
We hypothesized that enhanced degradation of ABCG1 may be associated with posttranslational modifications. Oram and colleagues determined that enhanced degradation of ABCA1 is associated with serine phosphorylation. To measure phosphorylation changes in ABCG1, we used phosphoamino acid-specific antibodies to determine whether 12/15LO eicosanoids regulate serine, threonine, or tyrosine phosphorylation. Immunoblots of immunoprecipitated ABCG1 revealed that serine, threonine, and tyrosine phosphorylation occurs at a basal state (Figure 4). 12SHETE treatment significantly enhanced serine, but not threonine or tyrosine, phosphorylation (Figure 4). ABCG1 serine phosphorylation was induced within 15 minutes after 12SHETE treatment and continued through 1 hour of exposure. Thus, serine phosphorylation occurs concurrently with 12/15LO eicosanoid-regulated degradation of ABCG1 (Figure 3B) and may account for the enhanced degradation rate.

Discussion
12/15-lipoxygenase (12/15LO) is expressed in high levels in macrophages found in early atherosclerotic lesions. 12/15LO has been implicated in the pathogenesis of diabetes and atherosclerosis. Studies in mice have clearly shown that 12/15LO activity contributes to the atherosclerotic burden. Moreover, studies have reported increased 12/15LO activity in patients with Type 2 diabetes, including increased renal excretion 12/15LO eicosanoid metabolites. The 12/15LO pathway is upregulated by glucose, growth factors, and cytokines, so it is quite likely that this enzyme plays a
significant role in the cardiovascular complications associated with diabetes. We have previously shown that 12/15LO activity is upregulated in diabetic db/db mice, and that these diabetic mice have reduced macrophage ABCG1 expression and function. In the current study, we demonstrate for the first time that 12/15LO activity in macrophages increases the degradation of ABCG1. ABCG1 is a key regulator of cholesterol efflux from peripheral cells to HDL in RCT. Thus, factors that reduce ABCG1 expression and function will impact macrophage foam cell formation and cholesterol homeostasis in the arterial wall, and may contribute to the accelerated cardiovascular disease observed in patients with Type 2 diabetes.

12/15LO overexpression in macrophages significantly reduces HDL-mediated but not apoA-I-mediated cholesterol efflux, and this reduction in efflux correlates with a significant reduction in ABCG1, but not ABCA1, protein expression (Figure 1). Moreover, arachidonic and linoleic acids, and 12/15LO eicosanoid products also reduced HDL-mediated cholesterol efflux (Figure 1). Treatment of macrophages with 12/15LO eicosanoids did not reduce cholesterol efflux to apoA-I, indicating a preferential role in targeting ABCG1 (Figure 1).

Although the reduction in cholesterol efflux may appear modest, we have previously found that a 20% reduction in cholesterol efflux can lead to a 4-fold increase in cholesteryl ester accumulation. Rader and colleagues have demonstrated that knockdown of ABCG1 levels in J774 macrophages by approximately 50% results in a significant reduction in RCT. Thus, the changes in macrophage cholesterol efflux in response to fatty acids or 12/15LO products would have a significant impact on RCT.

12/15LO activity in macrophages had no effect on ABCG1 mRNA expression (supplemental Figure 1). Uehara and colleagues reported that unsaturated fatty acids can suppress ABCG1 and ABCA1 gene expression. Consistent with their findings, we observed downregulation of ABCG1 and ABCA1 mRNA expression by linoleic and arachidonic acids (supplemental Figure 1). Interestingly, neither 12/15LO overexpression in macrophages nor incubation of macrophages with 12/15LO eicosanoids produced significant changes in ABCG1 mRNA expression (supplemental Figure 1). Both ABCA1 and ABCG1 gene expression are regulated by the nuclear hormone receptor, liver X receptor (LXR). It has been previously shown that unsaturated fatty acids antagonize the activation of LXR–α by oxysterols. Thus arachidonic and linoleic acids might regulate ABCG1 and ABCA1 gene transcription through inhibition of LXR–α activation. Regulation of LXR–α is incompletely understood, and studies have demonstrated important roles for phosphorylation and acetylation of LXR–α in regulating the activity of this transcription factor. Postranslational modifications of LXR–α may account for upregulation of LXR–α expression by 13SHODE with no corresponding upregulation of ABC transporter mRNA expression (supplemental Figure 1). Studies to examine the effects of 12/15LO eicosanoids on LXR activation are underway in the laboratory.

12/15LO could reduce ABCG1 protein expression through decreased message translation or enhanced protein degradation. 12/15LO fatty acids or eicosanoids did not alter translation efficiency as determined by methionine incorporation into de novo ABCG1 protein synthesis (supplemental Figure II). Treatment of J774 macrophages with 12SHETE in the presence of cycloheximide indicated that protein degradation appears to be the likely mechanism by which 12/15LO products regulate ABCG1 levels (Figure 3). Surprisingly, unsaturated fatty acids and 12/15LO eicosanoids had differential effects on ABCG1 protein expression. Incubation of macrophages with 12/15LO eicosanoids produced a rapid reduction in ABCG1 protein expression within 30 minutes to 1 hour which continued through 4 to 8 hours, whereas incubation of macrophages with either arachidonic or linoleic acids reduced ABCG1 expression only after approximately 8 hours of incubation (Figure 2). The time needed to convert fatty acid substrate into eicosanoid product might account for the disparity in ABCG1 expression. Arachidonic and linoleic acid substrates also produced a more lasting effect in reducing ABCG1 expression that continued 16 to 24 hours after treatment, whereas levels of ABCG1 returned to control (eg, time 0) levels by 16 to 24 hours of treatment with 12/15LO eicosanoids. Because cells rapidly metabolize eicosanoids, the return of ABCG1 levels to control levels is most likely attributable to the metabolism of the eicosanoid and subsequent loss of regulation. Alternatively, the return of ABCG1 levels to control levels may be attributable to attempts of the macrophage to restore ABCG1 expression and cholesterol homeostasis in a compensatory manner.

We anticipate that 12/15LO mediates posttranslational modifications of ABCG1, resulting in enhanced degradation. Indeed, we observed the presence of phosphorylated serine, threonine, and tyrosine residues in ABCG1 in the basal state (Figure 4). Treatment with 12SHETE enhanced ABCG1 serine, but not threonine or tyrosine phosphorylation (Figure 4). These data suggest that phosphorylation may be one potential mechanism of regulation of ABCG1 protein expression. Oram and colleagues have elegantly shown that unsaturated fatty acids reduce ABCA1 expression, at least in part through phosphorylation that enhances degradation. These investigators did not examine the effects of 12/15LO eicosanoids on ABCA1 in their study; however, our data (shown in Figure 1) indicate that 12/15LO overexpression does not significantly impact ABCA1 protein expression. We are currently working to identify the role that posttranslational modifications to ABCG1 play in regulating ABCG1 degradation by unsaturated fatty acids and eicosanoids. A direct role for serine phosphorylation in regulating ABCG1 degradation will need to be verified by mutational analysis.

Interestingly, Belkner et al demonstrated that 12/15LO-overexpressing macrophages have reduced intracellular lipid accumulation and foam cell formation attributable to reductions in SR-A expression and enhanced cholesteryl ester degradation. However, these authors found that the 12/15LO products 12SHETE and 13SHODE failed to reduce intracellular cholesterol deposition and tended to enhance lipid accumulation. Moreover, cholesterol efflux to fetal calf serum was not different between control and 12/15LO-overexpressing macrophages over 18 hours of incubation. Our study used specific acceptors (lipid free apoA-I or HDL)
to delineate the contribution of ABC transporters and shorter incubation time (4 hours), which may account for the differences between the studies. Moreover, we demonstrated that 12/15LO eicosanoids can directly regulate cholesterol efflux and ABCG1 expression. Thus, 12/15LO activity may regulate both macrophage lipid uptake and efflux in a compensatory manner, depending on the surrounding lipid environment in the arterial wall. Additional mechanistic studies are needed to delineate the “proatherogenic” versus “antiatherogenic” properties of this enzyme.

The findings of our current study have important implications. Our data indicate that 12/15LO plays a role in regulating cholesterol efflux from macrophages, thus providing a novel, important link between 12/15LO activity in the vessel wall and macrophage foam cell formation. Elevated levels of plasma free fatty acids are prevalent among subjects with metabolic syndrome and Type 2 diabetes, and these free fatty acids can accumulate in atherosclerotic lesions. The action of these fatty acids is 2-fold in the vessel wall. First, as shown by us and by Oram’s group, fatty acids have the ability to directly regulate ABCG1 and ABCA1 expression and function by enhancing ABC transporter degradation and inhibiting ABC transporter transcription. Second, these fatty acids act as substrates for the 12/15LO enzyme to convert them into eicosanoids, which cause ABCG1 degradation and reduced cholesterol efflux. We have previously reported regulation of ABCG1 transcription by chronic elevated glucose in Type 2 diabetes. Taken together, our data indicate that ABCG1 is regulated by both glucose and free fatty acids, which has important clinical implications for diabetic atherosclerosis. Understanding these novel mechanisms by which unsaturated fatty acids and products of the 12/15LO pathway enhance ABCG1 degradation will be beneficial for developing new therapies to regulate ABCG1 and ABCA1 expression in atherogenesis, especially in the setting of Type 2 diabetes.

Acknowledgments
The authors thank Dr John S. Parks (Wake Forest University) for recombinant apoA-I and helpful advice regarding cholesterol efflux, and Dr David L. Brautigan (University of Virginia) for providing invaluable advice regarding immunoprecipitation studies.

Sources of Funding
This work was supported by NIH P01 HL57598 (to J.L.N. and C.C.H.) and NIH R01 HL085790 (to C.C.H.).

Disclosures
None.

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Arterioscler Thromb Vasc Biol. 2008;28:1811-1819; originally published online July 17, 2008; doi: 10.1161/ATVBAHA.108.167908
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplementary Figure Legends

FIGURE I. 12/15LO activity does not alter ABCG1 mRNA expression. Macrophage total RNA was isolated and analyzed by quantitative real-time PCR for murine ABCG1, ABCA1, LXRα, or LXRβ. Panel A. Mock and Plox macrophages. Data represent the mean +/- SE of 8 samples (*significantly lower than Mock p<0.006 by ANOVA). Panel B. J774 macrophages were treated with either vehicle control (CTR) or with arachidonic (AA) or linoleic acids (LA). Data represents mean +/- SE of 8 samples (*significantly lower than CTR p<0.0001; †significantly lower than CTR p<0.002; $significantly lower than CTR p<0.0005 by ANOVA). Panel C. J774 macrophages were treated with either vehicle control (CTR) or with 12SHETE, 13SHODE, or 15SHETE. Data represents mean +/- SE of 8 samples (*significantly higher than CTR p<0.003 by ANOVA).

FIGURE II. 12/15LO fatty acids do not alter ABCG1 translation. J774 macrophages were treated with either vehicle control (CTR), 12SHETE, arachidonic (AA), or linoleic acid (LA) for 2 or 16 hours. Cells were labeled with [35S]methionine for 20 minutes. ABCG1 was immunoprecipitated, and 35S-labeled ABCG1 was detected by autoradiography. Results are representative of 2 similar experiments.

Materials and Methods

Chemicals and Reagents. FBS was obtained from HyClone (Logan, UT, USA). HDL was from Intracel (Frederick, MD, USA). NuPAGE 4-12% denaturing gels, MOPS running and transfer buffers, and nitrocellulose was from Invitrogen (Carlsbad, CA, USA). Mouse anti-ABCG1 antibody was from Novus Biologicals, Inc. Mouse anti-ABCA1 antibody and human lipid-free apoA-I were kind gifts from John Parks, Ph.D. at Wake Forest University. Mouse anti-beta actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RNeasy Mini kit was from
Qiagen (Valencia, CA, USA). Arachidonic acid, linoleic acid, 12SHETE, 13SHODE, and 15SHETE were purchased from BioMol (Plymouth Meeting, PA, USA). Cycloheximide was from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Recombinant Adenoviral Delivery. Mock or Plox macrophages were infected at a multiplicity of infection of 50 for 48 hours with the recombinant adenoviral vectors, AdRz (expresses 12/15LO ribozyme) or AdLacZ control as we have described previously.

Cellular Cholesterol Efflux Measurements. Cholesterol efflux assays were performed as described, with minor modification. J774 macrophages were plated in 12-well plates at a density of 6x10^5 cells/well. To measure effects cholesterol efflux, cells were radiolabeled with 2 µCi/ml of [3H]cholesterol for 16 hours in DMEM containing 10% fetal bovine serum (FBS). After radiolabeling, cells were washed 3 times with PBS. Cells were incubated with 0.2% fatty acid free BSA (FAFBSA) in DMEM in the presence of 125 μM linoleic acid, arachidonic acid, or vehicle control for 16 hours or the indicated eicosanoids (500nM) or vehicle control for 2 hours. Cells were washed and allowed to equilibrate for an additional 2 hours in 0.2% FAFBSA. Cholesterol efflux was conducted for 4 hours at 37°C in media containing: 1) 0.2% FAFBSA, 2) 0.2% FAFBSA + 15 μg/ml lipid-free human apolipoprotein A-I (apoA-I), or 3) 0.2% FAFBSA + 50μg protein/ml of human HDL. Human apoA-I and HDL were isolated as described previously. The efflux medium was then removed and a 100 μl aliquot was taken for 3H radioactivity determination. Adherent cells were rinsed three times with cold PBS, cells were dried, and isopropanol was added for overnight extraction at room temperature. A 100 μl aliquot of the extract was taken for 3H radioactivity determination. Results are expressed as [3H]cholesterol in medium/([3H]cholesterol in medium + cell) X 100%. Specific efflux to apoA-I or HDL was calculated by subtracting non-specific efflux in the presence of 0.2% FAFBSA only.

Immunoblotting for ABCA1, ABCG1, and 12/15LO. J774 macrophages were incubated in 0.2% FAFBSA in DMEM in the absence or presence of 125μM linoleic or arachidonic fatty acids,
500nM eicosanoids (HETEs and HODEs), or vehicle control as indicated in the Figure Legends. RIPA buffer (50mMTrisHCl (pH 8.0), 150mM NaCl, 1% Igepal, 10mM NaF, 2mM Na₃VO₄ containing Sigma protease inhibitor cocktail) was added to cells after incubation to generate whole cell lysates. Lysates were sonicated and protein was quantified via a protein assay kit (BioRad, Hercules, CA, USA).

Proteins were separated by SDS-PAGE and transferred to nitrocellulose and blocked for 1 hour with 2.5% milk-Tris-buffered saline + 1% Tween 20 (TBST) at room temperature. Fifty micrograms of whole cell lysate was used to detect all proteins. ABCA1 and ABCG1 antibodies (1:500 dilution) were incubated with the blot at 4°C overnight in 2.5% Milk-TBST. The 12/15LO antibody (1:1000 dilution) was incubated with the blot at 4°C overnight in 2.5% Milk-TBST. ABCA1, ABCG1, and 12/15LO blots were then incubated with HRP conjugated anti-rabbit secondary antibody (1:2000 dilution) in 2.5% Milk-TBST for 1 hour at room temperature. The beta actin antibody (1:10000 dilution) was incubated with the blot for 1 hour at room temperature in 2.5% Milk-TBST. Beta actin blots were then incubated with HRP conjugated anti-mouse secondary antibody (1:5000 dilution) in 2.5% milk-TBST for 1 hour at room temperature. Proteins were visualized using chemiluminescence and normalized to beta actin as gel loading control. Densitometry was performed using Stratagene Zero D-Scan densitometry software.

Quantitative Real-Time PCR. J774 macrophages were treated with 0.2% FAFBSA with vehicle control, 125μM arachidonic or linoleic acid for 8 hours, or 500nM 12/15LO eicosanoids (HETEs and HODEs) for 4 hours. Total cellular RNA was collected from J774 macrophages using the RNeasy Micro kit (Qiagen) following the manufacturer’s protocol. One microgram of cDNA was synthesized using the Iscript cDNA synthesis kit (Bio-Rad). Total cDNA was diluted 1:10 in H₂O and 4μl were used for each real-time condition using a Bio-Rad MyIQ Single Color Real-Time PCR Detection System and iQ SYBR Green supermix (Bio-Rad). Primer sequences are as follows: ABCA1 Forward 5’-GGTTTGGAGATGGTTATACAATAGTTGT-3’ and Reverse 5’-
CCCGGAACGCAAGTCC-3’; ABCG1 Forward 5’-TTCCCTGGAGATGAGTGTGC-3’ and Reverse 5’-CAGTAGGCCACAGGGAACAT-3’; LXR alpha Forward 5’-GGATAGGGTTGGAGATGAGTGTGC-3’ and Reverse 5’-GCTCAGGAGCTGATGATCCA-3’ and Reverse 5’-GGAGCGCCTGTTACACTGTT-3’; LXR beta Forward 5’-GCTCAGGAGCTGATGATCCA-3’ and Reverse 5’-GCGCTTGATCCTCGTGTAG-3’; Cyclophilin Forward 5’-TGGAGAGCACCAAGACAGACA-3’ and Reverse 5’-TGCCGGAGTCGACAATGAT-3’.

Samples were normalized to cyclophilin using the ΔCt method.

*Metabolic Labeling and Immunoprecipitation of ABCG1.* J774 macrophages were incubated with vehicle control, 12SHETE (500nM), arachidonic or linoleic acid (125μM) for 2 or 16 hours in 0.2% FAFBSA in DMEM. Cells were incubated with 200 μCi/mL [35S]methionine (New England Nuclear) for 20 minutes at 37°C in 0.2% FAFBSA in DMEM. Cells were lysed with RIPA buffer and lysates were incubated overnight at 4°C with antibody against ABCG1 (Novus Biologicals). The antibody-antigen complex was isolated by protein A-sepharose beads (Invitrogen) and resolved by SDS-PAGE. Each gel lane received equal amounts of immunoprecipitated protein. The gels were dried, and [35S]methionine was detected by autoradiography.

*ABCG1 Degradation Rate.* J774 macrophages were washed and incubated with 0.2% FAFBSA in DMEM. Arachidonic or linoleic acid (125μM) or vehicle control was added 2 hours prior to the addition of cycloheximide (20μg/mL) for the indicated time points, or 12SHETE (500nM) or vehicle control was added immediately prior to the addition of cycloheximide (20μg/mL) for the indicated time points. Cells were lysed with RIPA buffer and analyzed by SDS-PAGE and immunoblotting for ABCG1.

*Immunoprecipitation of ABCG1.* J774 macrophages lysates were prepared with RIPA buffer. Lysates were incubated overnight at 4°C with antibody against ABCG1 (Novus Biologicals). The antibody-antigen complex was isolated by protein A-sepharose beads (Invitrogen) and resolved.
by SDS-PAGE. Each gel lane received equal amounts of immunoprecipitated protein. ABCG1 phosphorylation was assayed by immunoblot analysis using phosphoserine (BioMol), phosphothreonine, and phosphotyrosine (Santa Cruz Biotechnology) antibodies that recognize a broad range of serine-, threonine-, and tyrosine-phosphorylated proteins.

**Statistical Analysis.** Data for all experiments comparisons between groups was performed using analysis of variance (ANOVA) methods using the Statview 6.0 software program. Data is graphically represented as the mean +/- SE, in which the mean consists of a minimum of three experiments performed in triplicate. Comparisons between groups and tests of interactions were performed assuming a two-factor analysis with the interaction term testing each main effect with the residual error testing the interaction. All comparisons were made using Fisher’s least standard difference procedure, so that multiple comparisons were performed at the alpha = 0.05 level only if the overall F-test from the ANOVA is significant at p<0.05.

**References**


Supplementary Figure I
Supplementary Figure II