

# Overexpression of Human 15(S)-Lipoxygenase-1 in RAW Macrophages Leads to Increased Cholesterol Mobilization and Reverse Cholesterol Transport

Ginny L. Weibel, Michelle R. Joshi, Eric T. Alexander, Peijuan Zhu, Ian A. Blair, George H. Rothblat

**Objective**—The purpose of this study was to determine the effect of 15-lipoxygenase-1 (15-LO-1) on cholesterol mobilization from macrophages.

**Methods and Results**—Overexpression of human 15-LO-1 in RAW mouse macrophages led to enhanced cholesterol efflux, increased cholesteryl ester (CE) hydrolysis, and increased reverse cholesterol transport (RCT). Efflux studies comparing 15-LO-1 overexpressing cells to mock-transfected RAW macrophages resulted in a 3- to 7-fold increase in cholesterol efflux to apolipoprotein A-I and a modest increase in efflux to HDL. Additional experiments revealed an increase in mRNA and protein levels of ABCA1 and ABCG1 in the RAW expressing 15-LO-1 compared to controls. Efforts to examine whether the arachidonic acid metabolite of 15-LO-1, (15S)-hydroxyeicosatetraenoic acid (HETE), was responsible for the enhanced efflux revealed this eicosanoid metabolite did not play a role. Enhanced steryl ester hydrolysis was observed in 15-LO-1 overexpressing cells suggesting that the CE produced in the 15-LO-1 expressing cells was readily mobilized. To measure RCT, RAW macrophages overexpressing 15-LO-1 or mock-transfected cells were cholesterol enriched by exposure to acetylated low-density lipoprotein and [<sup>3</sup>H]-cholesterol. These macrophages were injected into wild-type animals and RCT was measured as a percent of injected dose of <sup>3</sup>H appearing in the feces at 48 hours. We found 7% of the injected <sup>3</sup>H in the feces of mice that received macrophages overexpressing 15-LO-1 and 4% in the feces of mice that received mock-transfected cells.

**Conclusions**—These data are consistent with a model in which overexpression of human 15-LO-1 in RAW macrophages promotes RCT through increased CE hydrolysis and ABCA1-mediated cholesterol efflux. (*Arterioscler Thromb Vasc Biol.* 2009;29:837-842.)

**Key Words:** macrophage ■ lipoxygenase ■ reverse cholesterol transport ■ ABCA-1

Lipoxygenases are classified based on the position of polyunsaturated fatty acid oxygenation. The major product of human 15-LO-1 is (15S)-HETE, which is produced from the oxidation of arachidonic acid at C-15. 15-LO-1 also produces lesser amounts of (12S)-HETE from arachidonic acid. In contrast, the major metabolite from leukocyte-type 12/15-LO (found in mouse, pig, and rabbit) is (12S)-HETE. 15-LO-1 also has activity against the fatty acyl component of cholesteryl esters (CE) and phospholipids.<sup>1</sup>

The role of 15-LO-1 in atherosclerosis has been controversial. Fibroblasts and J774 macrophages, overexpressing 15-LO-1 and 12/15-LO, respectively, are capable of oxidizing LDL, a process leading to foam cell formation, as well as enhanced monocyte chemoattractant activity, which strongly suggests a proatherogenic role.<sup>2,3</sup> Published studies show mice deficient in 12/15-LO are protected from atherosclerosis in apolipoprotein E (apoE)-deficient mice.<sup>4,5</sup> However, transgenic rabbits overexpressing 15-LO-1 exhibited a 45% atten-

uation in aortic lesion area, clearly demonstrating an anti-atherogenic role.<sup>6</sup> Furthermore, Merched et al<sup>7</sup> demonstrated an atheroprotective role for 12/15-LO through the local production of potent antiinflammatory lipid mediators (lipoxin A<sub>4</sub>, resolvin D1 and protectin D1) in apoE-deficient mice. Recent epidemiological studies provide strong evidence for an antiatherogenic role of 15-LO-1. Wittwer et al<sup>8</sup> provide evidence of a polymorphism that occurs in 15-LO-1 resulting in higher 15-LO-1 activity in humans and is atheroprotective. Assimes et al<sup>9</sup> report on a variant of 12/15-LO occurring in humans that results in decreased expression of 12/15-LO but does not offer atheroprotection. Elucidating the mechanisms underlying these conflicting data will make it possible to more rigorously assess the role of 15-LO-1 to atherosclerotic disease progression in humans.

Here we focus on the effects of one member of the LO family, human 15-LO-1, and its role in macrophage foam cells. 15-LO-1 is present in human macrophage cells<sup>10</sup> and is

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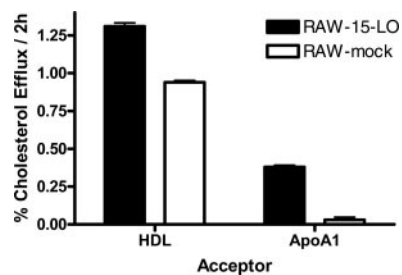
From the Division of Gastroenterology and Nutrition (G.L.W., M.R.J., E.T.A., G.H.R.), The Children's Hospital of Philadelphia, and the Centers for Cancer Pharmacology and Excellence in Environmental Toxicology (P.Z., I.A.B.), University of Pennsylvania School of Medicine.

Correspondence to Ginny L. Weibel, The Children's Hospital of Philadelphia, 3615 Civic Center Blvd, ARC1102, Philadelphia, PA 19104-4399. E-mail weibel@email.chop.edu

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**Figure 1.** Cholesterol efflux to HDL and apoA-I is increased with expression of 15-LO. Macrophages were radiolabeled with [ $^3$ H]-FC. Efflux to 20  $\mu$ g/mL apoA-I or 25  $\mu$ g/mL HDL was determined. Aliquots of media was removed from all wells, filtered, and counted by liquid scintillation counting to determine [ $^3$ H]-cholesterol released in the media. [ $^3$ H]-cholesterol counts in the media were then compared to the counts in the cells before the efflux period to determine percent release.

associated with neutral lipid bodies in various cell types.<sup>11,12</sup> The absence of the reticulocyte-type 15-LO-1 in mice provides a clean model to specifically study the effects of human 15-LO-1. Therefore, we chose this model to further define the role of macrophage 15-LO-1 in atherosclerosis.

## Methods

A detailed Methods section can be found with the Data Supplement (available online at <http://atvb.ahajournals.org>).

### Cell Culture

RAW 267.4 (American Type Tissue Culture Collection, Manassas, VA) murine macrophages stably transfected to overexpress human 15-LO-1 (RAW-15-LO)<sup>4</sup> and mock-transfected (RAW-mock) cells were routinely grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS, 50  $\mu$ g/mL gentamicin, and 500  $\mu$ g/mL geneticin.

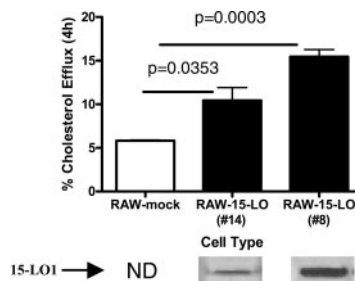
### Cholesterol Efflux

After [ $^3$ H]cholesterol labeling the cells, media containing HDL (20  $\mu$ g/mL) or apoA-I (25  $\mu$ g/mL) was added for up to 4 hours. In some experiments the ACAT inhibitor CP113818 (2  $\mu$ g/mL) was added to the media to prevent cholesterol esterification. To determine cholesterol efflux, media were sampled at indicated times, filtered, and counted by liquid scintillation counting to determine [ $^3$ H] released. [ $^3$ H]-Sterols in the media were compared to total [ $^3$ H] at time zero to determine the percent release of [ $^3$ H]cholesterol.

## Results

RAW murine macrophages stably transfected with human 15-LO-1 were used in these studies.<sup>13</sup> Our initial observations indicated that cholesterol efflux to serum was enhanced when 15-LO-1 was overexpressed (data not shown). To further study this observation, macrophages were radiolabeled for 24 hours in the presence of the ACAT inhibitor CP113818, to prevent CE from forming. Cells were exposed to media containing apoA-I or HDL. Efflux to HDL and apoA-I was higher in the cells expressing 15-LO-1 (Figure 1), indicating that expression of 15-LO-1 increases cholesterol efflux.

Because there was a dramatic increase in efflux to apoA-I (100-fold increase), we chose to use apoA-I as the extracellular acceptor in subsequent experiments. Additionally, because one of our goals was to determine the impact of 15-LO-1 expression on cellular cholesterol



**Figure 2.** Effect of the level of 15-LO expression on cholesterol efflux to apoA-I. Cholesterol efflux was measured in 2 RAW clones (#8 and #14) expressing 15-LO and mock-transfected controls. Macrophages were FC-enriched and radiolabeled by incubation with [ $^3$ H]-cholesterol and CP113818. Efflux to 20  $\mu$ g/mL apoA-I was determined as described in Figure 1. Total cell lysates were analyzed by immunoblotting for 15-LO-1 protein.

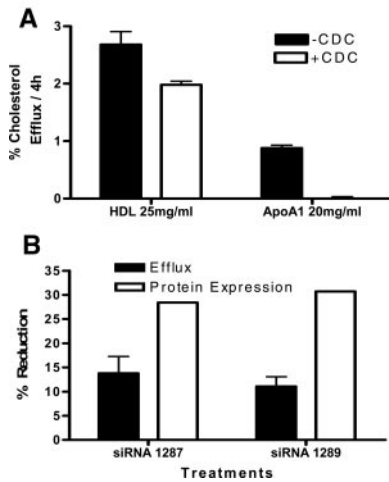
mobilization, we used macrophages enriched in CE in some of the experiments.

Cholesterol efflux to apoA-I was evaluated in 2 clones (Figure 2) and in mock-transfected cells. Figure 2 shows that 15-LO-1 expression in RAW macrophages resulted in a 2- to 3-fold increase in fractional cholesterol efflux over 4 hours. The increase in cholesterol efflux was related to the level of 15-LO-1 protein expression (measured by immunoblot), although not linearly (Figure 2). 15-LO-1 is not present in murine macrophages and was not detected in the mock-transfected control (Figure 2). Overexpression of human 15-LO-1 did not affect levels of endogenous murine 5-LO levels (data not shown).

To confirm that the increase in cholesterol efflux was attributable to 15-LO-1 expression, we used the 15-LO-1 inhibitor, cinnamyl-3,4-dihydroxy-a-cyanocinnamate (CDC).<sup>14</sup> When CDC was added to RAW-15-LO-1 cells, cholesterol efflux to HDL was reduced and efflux to apoA-I was eliminated (Figure 3A). CDC did not affect efflux in the mock-transfected cells (data not shown). We extended these studies by using small interfering RNA (siRNA) to knock-down expression of 15-LO-1 and assess the effect on cholesterol efflux. Figure 3B indicates that a 28% to 30% reduction in 15-LO-1 expression was achieved using 2 unique siRNA. This reduction in 15-LO-1 protein resulted in an 11% to 13% reduction in cholesterol efflux (Figure 3B). A scrambled siRNA (negative control) had no effect on 15-LO-1 expression or cholesterol efflux.

Because of the increase in efflux to apoA-I observed when 15-LO-1 was expressed, we investigated efflux via ABCA1, which is a major route of cholesterol efflux from macrophages.<sup>15</sup> Probuco, an inhibitor of ABCA1, attenuated cholesterol efflux from RAW-15-LO-1 cells (Figure 4). Efflux experiments conducted in the presence of the antioxidant butylated hydroxytoluene (BHT)<sup>16</sup> demonstrated that the reduction in efflux seen with Probuco was not attributable to the antioxidant properties of Probuco (Figure 4, inset).

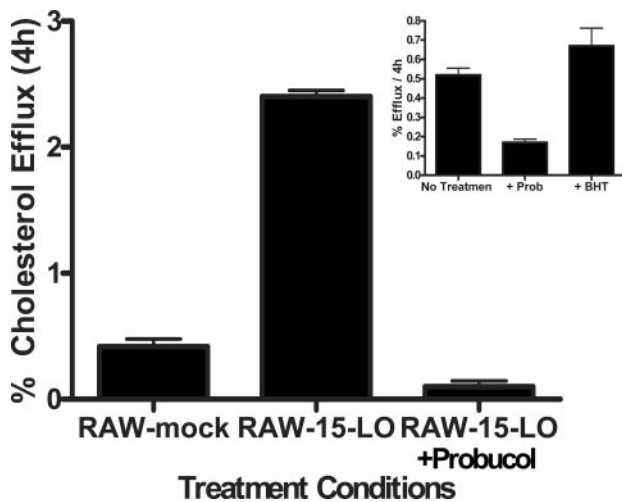
Overexpression of 15-LO-1 may result in greater ABCA1 activity. To address this, we measured cholesterol transporter message and protein levels in the RAW-15-LO-1 and RAW-mock cells. Protein and mRNA levels of ABCA1 and ABCG1 were significantly increased (10-fold and 9-fold,



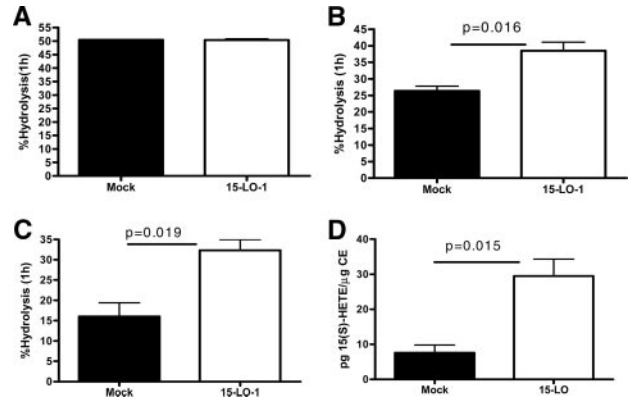
**Figure 3.** Inhibition of 15-LO reduces cholesterol efflux to apoA-I. (3A) Macrophages were radiolabeled with [<sup>3</sup>H]-FC. After an 18-hour equilibration period in 0.2% BSA±CDC (5 μg/mL), cells were exposed to media containing 25 μg/mL HDL or 20 μg/mL apo-AI for 4 hours. B, 15-LO-1 siRNA or their scrambled control were transfected into 15-LO-1 or mock-transfected RAW cells as described in Methods. Protein levels of 15-LO-1 were determined by immunoblot. Percent efflux was measured as described in Figure 1. Data are expressed as percent reduction compared to an untreated control.

respectively) in RAW-15-LO-1 cells (supplemental Figure I). The RNA and protein data were in agreement with the efflux data (Figure 4). We do not have a valid method for assessing ABCG1-mediated efflux in the RAW cells, but based on the data presented in Figure 5, we hypothesize that ABCG1 might also be contributing to the increase in cholesterol efflux and is responsible for the increase in efflux to HDL (Figure 1).

To address the possibility that a metabolite of 15-LO-1 was responsible for the observed changes in efflux and transporter



**Figure 4.** Probulcol effect on cholesterol efflux to apoA-I. Macrophages were radiolabeled with [<sup>3</sup>H]-FC. After an equilibration in 0.2% BSA, cells were treated with either BSA alone or 20 mmol/L Probulcol for 2 hours. Efflux to 20 μg/mL apoA-I was measured after 4 hours. Percent efflux was determined as described in Figure 1. Inset, RAW cells were treated as described above, but 0.5 mmol/L BHT was substituted for Probulcol.



**Figure 5.** Steryl Ester isolated from 15-LO expressing RAW are a better substrate for neutral cholesteryl ester hydrolase. A, Commercially available [<sup>3</sup>H]-cholesteryl oleate (3μCi/mL) was added to a cell homogenate (as a source of nCEH) prepared from 15-LO-1 or mock-transfected control cells and incubated for 1 hour. Hydrolysis was measured as described in Methods. B and C, RAW macrophages expressing human 15-LO or mock-transfected controls were cholesterol enriched and labeled as described in Methods. Cellular lipids were extracted using isopropanol. The lipid isolates were then added back, at constant CE mass, to a commercially available bovine pancreatic nCEH (B) or a RAW cell homogenate (as a source of nCEH; C). Hydrolysis was allowed to occur for 1 hour. Percent hydrolysis was determined as described in Methods. D, CE was isolated from 15-LO-1 expressing or mock-transfected cells by thin layer chromatography. (15S)-HETE in the isolated CE lipid fraction was measured using an EIA assay as described in Methods.

expression, we incubated (15S)- or (12S)-HETE with mock-transfected RAW cells. Control experiments were conducted to ensure the eicosanoids were incorporated into the cells (supplemental Figure IIC). (15S)-HETE and to a lesser extent (12S)-HETE are products of 15-LO-1 actions on arachidonic acid. Incubation of (15S)-HETE with parent or mock-transfected RAW cells did not alter cholesterol efflux to apoA-I (supplemental Figure IIA and IIB) or cholesterol transporter expression (supplemental Figure III). Similar results were obtained when (12S)-HETE was used (supplemental Figure IIA and IIB). These results indicate that neither (15S)- nor (12S)-HETE were responsible for the cellular changes seen in the RAW-15-LO-1 macrophages.

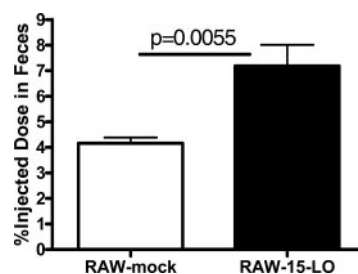
CE hydrolysis is another aspect of cholesterol metabolism that was influenced by the overexpression of 15-LO-1. Hydrolysis was significantly increased in macrophages expressing 15-LO-1 (49±0.5%/24 hours in mock transfected cells; 75±0.1%/24 hours in 15-LO-1 cells). To determine whether the observed increase in CE hydrolysis was attributable to an increase nCEH activity, homogenates of RAW-15-LO-1 and RAW-Mock cells were added to a commercially available [<sup>3</sup>H]-CE. Hydrolysis was calculated by comparing total cpm in CE before and after the hydrolysis period. There was not a significant difference in the amount of CE hydrolysis between the 2 cell types, indicating that overexpression of 15-LO-1 did not increase nCEH activity (Figure 5A). We next investigated the possibility that steryl esters produced in the RAW-15-LO-1 cells are better substrates for nCEH than that present in RAW-mock cells. To test this possibility the cellular lipids from the 2 cell lines were extracted and

incubated with a commercially available nCEH (Figure 5B) or a RAW mock-transfected cell homogenate (Figure 5C) or 15-LO-1 cell homogenate (data not shown) as source of nCEH. The lipid extract was added back to the nCEH at constant CE mass. We measured an increase in CE hydrolysis when the lipid extracted from the RAW-15-LO-1 cells was used as substrate compared to the lipid from the RAW-mock cells (Figure 5B and 5C), no matter what the source of nCEH. Thus, the steryl esters produced in 15-LO-1 expressing cells are a more suitable substrate for nCEH and therefore are more rapidly hydrolyzed than the steryl esters isolated from mock-transfected RAW cells. We next measured the amount of the oxidized fatty acid, (15*S*)-HETE in the CE lipid fraction isolated from mock-transfected or 15-LO-1 expressing cells. Figure 5D indicates a 3-fold increase in the amount of (15*S*)-HETE in the CE produced in 15-LO-1-expressing cells compared to the CE produced in mock-transfected cells.

Because our data demonstrated increased cholesterol mobilization in macrophages is attributable to overexpression of human 15-LO-1, we next evaluated the effect overexpression of 15-LO-1 has on RCT *in vivo*. The model of RCT used in these studies is discussed in detail elsewhere.<sup>17</sup> Briefly, donor macrophages are cholesterol-enriched and labeled *ex vivo* and then injected into the peritoneum of host animals. [<sup>3</sup>H]Cholesterol moves from the macrophages in the peritoneum (periphery) through the plasma compartment to the liver where the [<sup>3</sup>H]cholesterol is eliminated in the feces. In the present studies, cholesterol enriched RAW macrophages overexpressing 15-LO-1 or mock-transfected cells were used as the donor macrophages. The loading efficiency was similar in both cell types (83.77±1.79 μg total cholesterol/mg protein (73.7% FC, 26.3% CE) in the 15-LO-1 cells and 80.01±0.60 μg total cholesterol/mg protein (69.1% FC, 30.9% CE) in the mock-transfected cells). The cells were injected *ip* into wild-type mice, and plasma (retro-orbital bleed) and feces (cumulative) were collected over 48 hours. After this period liver and bile samples were collected. All samples were analyzed for <sup>3</sup>H content as described in Methods. There were no significant differences in plasma, liver, and bile <sup>3</sup>H content (data not shown) for the experimental period. However, there were significantly more fecal <sup>3</sup>H counts in the animals injected with RAW-15-LO-1 macrophages compared to the animals receiving RAW-mock-controls (Figure 6). Because the movement of radiolabeled sterol from the injected macrophages to the feces reflects the efficiency of RCT, the increased <sup>3</sup>H in the feces demonstrates that 15-LO-1 expression in macrophages increases RCT.

## Discussion

The initiation and progression of atherosclerosis is a complex process involving multiple pathways and risk factors. Many studies have investigated cellular and serum pro- or anti-atherogenic factors and often find that the line between a certain factor promoting or reversing atherosclerosis is blurred. Such is the case with 15-LO-1. Some of the conflicting results in the literature might be attributable to species differences, isoenzyme positional specificity for oxidation, or expression pattern.<sup>1,7</sup> (15*S*)-HETE is the major product of human reticulocyte 15-LO-1. It arises



**Figure 6.** Expression of 15-LO in macrophages results in greater RCT. RAW macrophages overexpressing 15-LO or mock-transfected cells were cholesterol enriched with 50 μg/mL acLDL and 5 μCi/mL [<sup>3</sup>H]-cholesterol in DMEM + 1% FBS for 48 hours and then injected into wild-type mice as described in Methods. Feces was collected over 48 hours and analyzed for <sup>3</sup>H content as described in Methods.

from the oxidation of arachidonic acid at C-15 to yield (15*S*)-hydroperoxyeicosatetraenoic acid (HPETE), which then undergoes a 2-electron reduction to (15*S*)-HETE. 15-LO-1 also produces smaller amounts of (12*S*)-HETE through the intermediate formation of (12*S*)-HPETE.<sup>18</sup> Products of LO have been shown to be both pro- and antiatherogenic.<sup>1</sup> This study focuses on one particular component of the complex disease of atherosclerosis, the macrophage.

Foam cells of both macrophage and smooth muscle origins are responsible for a large portion of the lipid volume in atherosclerotic plaques.<sup>19</sup> Mobilization of foam cell lipid is key to plaque regression. 15-LO-1 has been localized to macrophages in early and advanced lesions,<sup>20,21</sup> although this too is subject to debate.<sup>22</sup> The current studies focus on the effect of human reticulocyte-type 15-LO-1 has on cholesterol mobilization from macrophages, specifically; human 15-LO-1 was overexpressed in murine RAW macrophages<sup>13</sup> and used for these studies.

## Cholesterol Efflux

Our investigations revealed that cholesterol efflux to HDL and apoA-I was enhanced with the expression of human 15-LO-1. The idea that 15-LO-1 expression is responsible for increased cholesterol efflux is supported by our data demonstrating that the increase can be inhibited with CDC, a 15-LO-1 inhibitor (Figure 3), and cholesterol efflux to apoA-I is increased with expression of 15-LO-1 (Figure 2). We determined that metabolites resulting from 15-LO-1 action on arachidonic acid, (15*S*)-HETE, and (12*S*)-HETE are not responsible for the increase in efflux. Recently, Nagelin et al reported that (12*S*)-HETE but not (15*S*)-HETE reduced cholesterol efflux from J774 macrophages stably transfected with porcine 12/15-LO to HDL. The decrease in efflux is presumably attributable to increased degradation of ABCG1 expression.<sup>23</sup> Interestingly, this group also found that ABCG1 expression levels returned to control levels after 24 hours, suggesting that the decrease in ABCG1 expression is only temporary. The conflicting data in these reports may be attributable to time of incubation, cell type used for transfection, or LO species differences (porcine versus human).

## Cholesterol Transporter Expression

Polyunsaturated fatty acids (PUFAs) have been shown to decrease expression of ABCA1 and ABCG1 in macrophages

through destabilization of the ABCA1 protein<sup>24,25</sup> and inhibition of ABCA1 and ABCG1 transcription.<sup>26</sup> In the current studies, where 15-LO-1 is overexpressed, ABCA1 and ABCG1 expression is increased in the presence or absence of exogenous PUFAs. It is possible that overexpression of 15-LO-1 results in a rapid metabolism of PUFAs, thereby eliminating any effect the PUFAs may have on cholesterol transporter expression.

Expression of 12/15-LO and its linoleic acid metabolite 13-*S*-HODE has been shown to activate peroxisome proliferator-activated receptors (PPARs).<sup>27</sup> Activation of PPAR $\gamma$  and PPAR $\alpha$  affects cholesterol efflux through up-regulation of cholesterol transporters ABCG1 and ABCA1.<sup>28</sup> We report an increase in ABCG1 as well as ABCA1 protein and mRNA levels in RAW macrophages overexpressing 15-LO-1 under the experimental conditions presented in these studies. However, we did not detect 13-*S*-HODE in the 15-LO-1 RAW cells (data not shown), and the eicosanoid products of 15-LO-1, (12*S*)-HETE, or (15*S*)-HETE did not affect cholesterol efflux or transporter expression in our experiments (supplemental Figures II and III).

If oxidized metabolites of arachidonic acid were not responsible for the increase in efflux and transporter expression, the question of what is causing the increase remains. It is known that LOs can oxidize the fatty acyl component of CE contained in LDL.<sup>29</sup> Additionally, Lund et al<sup>30</sup> reported that soybean LO oxidizes the B-ring of cholesterol at C-7. The hypothesis that an oxidized sterol may be responsible for the increase in cholesterol transporter expression is reasonable considering oxysterols can modulate ABCA1, ABCG1, and nCEH expression.<sup>31–33</sup> Efforts to determine whether the human 15-LO-1 has similar activity on the B-ring of cholesterol are currently underway.

### ABCA1-Mediated Efflux

Cholesterol efflux via ABCA1 is inhibited by ProbucoL.<sup>34</sup> We found that ProbucoL abolished the increase in cholesterol efflux to apoA-I in the 15-LO-1 expressing cells. To rule out the possibility that the antioxidant properties of ProbucoL might also play a role in efflux reduction, we tested another antioxidant, BHT, and found no reduction to efflux to apoA-I (Figure 4, inset).

### Steryl Ester Hydrolysis

We found that CE hydrolysis was substantially increased when 15-LO-1 was overexpressed. The increase in hydrolysis could be attributable to an increase in nCEH activity, the production of a more suitable nCEH substrate, or the formation of eicosanoid metabolites that increased CE hydrolase activity.<sup>35</sup> Our data indicate that the increase in CE hydrolysis seen with overexpression of human 15-LO-1 is not attributable to an increase in nCEH activity in RAW macrophage cells (Figure 5A). Additionally, we have found that the steryl esters extracted from 15-LO-1 overexpressing foam cells are a better substrate for nCEH compared to the steryl esters extracted from mock-transfected cells. Our data are consistent with expression of 15-LO-1 leading to the oxidation of cytoplasmic CE, an idea suggested by Belkner et al.<sup>36</sup> (15*S*)-HETE was shown to be readily incorporated as the

fatty acyl chain of CE.<sup>37</sup> Our studies indicated that there is an increase in the amount of (15*S*)-HETE in the CE fraction isolated from 15-LO-1-expressing cells compared to CE isolated from mock-transfected cells. These data, coupled with previous studies demonstrating that oxidized CE are preferentially hydrolyzed compared to nonoxidized CE,<sup>38</sup> suggest that (15*S*)-HETE is incorporated into CE creating a better substrate for nCEH.

### Reverse Cholesterol Transport

Expression of human 15-LO-1 in macrophages results in an increased rate of cellular CE hydrolysis and greater cholesterol efflux, which favors cholesterol mobilization. We therefore evaluated the effect expression of human 15-LO-1 in macrophages has on RCT. The model of RCT we used in this study has been validated and discussed in detail elsewhere.<sup>17</sup> There was no difference in the 2 groups with regard to [<sup>3</sup>H]sterol in the blood, bile, or liver. However, when human 15-LO-1 is expressed in the donor macrophages, fecal excretion is significantly higher when compared to that of mock-transfected donor macrophages. Our studies are in agreement with Merched et al who developed a macrophage-specific 12/15-LO overexpressing mouse on an apoE-deficient background.<sup>7</sup> They found a significant decrease in aortic atherosclerotic lesion area with macrophage specific 12/15-LO overexpression along with a concurrent increase in lipoxin A<sub>4</sub> production. Lipoxins are potent antiinflammatory mediators that trigger the resolution phase of atherosclerosis. Such lipid mediators may be playing a role in the RCT system and efflux studies described in the present work.

### Summary

Considering the complexity of atherosclerosis, and that the expression pattern of 15-LO-1 is not limited to peripheral foam cells, our findings do not preclude proatherogenic properties of 15-LO-1 such as oxidation of LDL. However, we propose that expression of human 15-LO-1 in macrophage cells results in increased cellular cholesterol mobilization through increased CE hydrolysis, ABCA1-mediated efflux, and ultimately increased RCT. These observations suggest that 15-LO-1 expression in peripheral foam cells is antiatherogenic.

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### Disclosures

None.

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# Arteriosclerosis, Thrombosis, and Vascular Biology



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## Overexpression of Human 15(S)-Lipoxygenase-1 in RAW Macrophages Leads to Increased Cholesterol Mobilization and Reverse Cholesterol Transport

Ginny L. Weibel, Michelle R. Joshi, Eric T. Alexander, Peijuan Zhu, Ian A. Blair and George H. Rothblat

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## EXPERIMENTAL PROCEDURES

**Materials.** Bovine serum albumin (BSA, essentially fatty acid free), heat-inactivated fetal bovine serum (FBS), gentamicin, and FC were purchased from Sigma-Aldrich (St. Louis, MO). [1,2-<sup>3</sup>H]cholesterol (51Ci/mmol) and [1,2-<sup>3</sup>H]-cholesteryl oleate (60Ci/mmol) were obtained from New England Nuclear (Waltham, MA). Organic solvents were obtained from Fisher Scientific (Pittsburg, PA). Tissue-culture flasks and plates were from Falcon and Corning (Corning, NY). Tissue culture medium was obtained from Gibco-Invitrogen (Carlsbad, CA). Human LDL (1.019 < d < 1.063 g/ml) and HDL<sub>3</sub> (1.125 < d < 1.21 g/ml) was isolated by sequential ultracentrifugation, dialyzed against 0.15 mol/l NaCl, and sterilized by filtration<sup>1</sup>. Human apoA-I was obtained from isolated human HDL as previously described<sup>2</sup>. LDL was converted to acLDL by acetylation with acetic anhydride<sup>3</sup>. Hyperfilm-MP and enhanced chemiluminescence (ECL) reagent were obtained from Amersham Biosciences (Piscataway, NJ). SiRNAs were purchased from Applied Biosystems (Ambion), Austin, TX. HiPerFect transfection reagent was purchased from Qiagen, Valencia, CA. The acyl CoenzymeA:cholesterol acyltransferase (ACAT) inhibitor Pfizer CP-113,818 was a gift from Pfizer Pharmaceuticals.

**Cell Culture.** RAW 267.4 (American Type Tissue Culture Collection, Manassas, VA) murine macrophages stably transfected to overexpress human 15-LO-1 (RAW-15-LO)<sup>4</sup> and mock-transfected (RAW-mock) cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 50 µg/ml gentamicin and 500 µg/ml geneticin. To cholesterol-label the macrophages, media containing 1% FBS, [<sup>3</sup>H]cholesterol +/- (1 µCi/ml) acLDL (25 µg protein/ml) was added to the cells for 48 h. After this, cells were incubated in media containing 0.2% BSA for 18 h to allow cellular cholesterol radiolabel to equilibrate. In certain experiments, some incubations contained 0.3mM CTP-cAMP during equilibration to stimulate the expression of ABCA1<sup>5</sup>. After equilibration, time zero cells were harvested to determine total counts/min (cpm), protein, and FC and CE mass in the cells before adding the extracellular cholesterol acceptors.

**Western Blot Analysis.** Cell monolayers were lysed with a 1% Triton X-100, 0.5% Nonidet P-40, 10 mmol/l Tris buffer and homogenized through a 27-gauge needle. Equal amounts of protein were separated on 3% to 8% tris-acetate gels and transferred to polyvinylidene fluoride membrane. Blots were obtained using anti-ABCA1 (Novus Biologicals, Littleton, CO), anti-ABCG1 (Novus Biologicals), or anti-15-LO-1 (Cayman Chemicals, Ann Arbor, MI) antibodies. Chemiluminescence was used to visualize the proteins according to the manufacturer's instructions.

**Cholesterol Efflux.** After [<sup>3</sup>H]cholesterol labeling the cells, media containing HDL (20µg/ml) or apoA-I (25µg/ml) was added for up to 4 h. In some experiments the ACAT inhibitor CP113,818 (2 µg/ml) was added to the media to prevent cholesterol esterification. To determine cholesterol efflux, media were



sampled at indicated times, filtered and counted by liquid scintillation counting to determine [<sup>3</sup>H] released. [<sup>3</sup>H]-Sterols in the media were compared to total [<sup>3</sup>H] at time zero to determine the percent release of [<sup>3</sup>H]cholesterol. To determine the impact of (12S)-HETE and (15S)-HETE on sterol efflux, following the labeling period (described above), macrophages were equilibrated in media containing 0.2% BSA +/- 500 nM or 1 μM (12S)-HETE or (15S)-HETE (added as a solution in ethanol). Vehicle was added as a control to separate wells. At this point (15S)-HETE incorporation into cells was determined by EIA assay (Cayman Chemical, Ann Arbor, MI). After the pretreatment, media containing 25 μg/ml apoA-I was added for 2 hours. An ACAT inhibitor (2 μg/ml) was present in the media at all times. Efflux and cholesterol transporter expression was determined as described above.

#### *Cholesteryl Ester Hydrolysis.*

*CE Hydrolysis in Whole Cells.* Macrophages were enriched with <sup>3</sup>H-CE for 24 h by incubation with acLDL (100 μg/ml) containing [<sup>3</sup>H]-FC (1 μCi/ml). Cells were then equilibrated in BSA for 18 h. After equilibration, cells contained from 30% to 40% of the [<sup>3</sup>H]cholesterol as CE. The acceptors (see Figure legends) were added for 24 h in media containing the ACAT inhibitor CP113818 (2 μg/ml). At the end of this incubation, wells were washed with phosphate-buffered saline (PBS), allowed to air dry and extracted overnight with isopropanol. Lipids in the extracts were separated by thin layer chromatography to determine the loss of [<sup>3</sup>H] from the CE pool<sup>6</sup>. Percent hydrolysis was calculated by comparing total cpm in CE before the hydrolysis period to total cpm in CE after the hydrolysis period.

*CE Hydrolysis in Cell Homogenates.* To determine if there was a functional increase in neutral cholesteryl ester hydrolase (nCEH) when 15-LO-1 was over-expressed, CE hydrolysis was measured in cell homogenates. Homogenates of 15-LO-1 expressing and mock-transfected RAW cells were prepared by scraping the cells into DMEM-HEPES (pH=7.4) media and briefly sonicated using a microtip as previously described<sup>7</sup>. Commercially available [1,2-<sup>3</sup>H]-cholesteryl oleate (3 μCi/ml) in ethanol was added to an aliquot of cell homogenate and incubated for 1 h at 37 °C. The reactions were stopped by extraction with chloroform and methanol (1:1, v/v). Lipid extracts were analyzed as described above.

*Hydrolysis of Steryl Esters Isolated from Cells.* To determine if the steryl esters generated in the 15-LO-1 expressing cells are a preferential substrate for nCEH compared to steryl esters generated in mock-transfected cells, we isolated the lipid from both cell types and incubated with a commercially available bovine pancreatic nCEH (Sigma-Aldrich, St. Louis, MO). Macrophages were enriched with <sup>3</sup>H-CE for 24 h by incubation with acLDL (100 μg/ml) containing [<sup>3</sup>H]-FC (1 μCi/ml). Cells were then equilibrated in BSA for 18 h. Lipids were extracted from 15-LO-1 expressing and mock-transfected RAW cells with isopropanol. The lipid fractions (5 μg CE per incubation) were then incubated with a commercially available nCEH (bovine pancreatic, 1.5 U/ml final concentration, Sigma) or a mock-transfected cell homogenate (a source of nCEH, prepared as described

above) for 1 h at 37 °C. The reactions were stopped by extraction with chloroform and methanol (1:1, v/v). Lipid extracts were analyzed as described above.

*Determination of 15(S)-HETE Incorporation into Cholesteryl Ester Fraction.* Mock and 15-LO-1 RAW macrophages were seeded in 100 cm dishes. Cell monolayers were incubated in media containing 1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-cholesterol, 1% FBS, arachidonic acid (75  $\mu\text{g/ml}$  added at a 5:1 molar ratio with BSA) and acLDL (100  $\mu\text{g/ml}$ ) for 24h. Monolayers were washed with PBS and extracted with isopropanol in the presence of an antioxidant (BHT, 200  $\mu\text{g/ml}$ ). Total radioactivity incorporated was determined by LSC and cholesterol mass was determined by gas/liquid chromatography using cholesterol methyl ether as an internal standard. The remaining lipid was separated on a silica G gel-coated glass plate using a hexane:diethyl ether:formic acid (9:1:0.1) solvent system. The portion of the plate containing cholesteryl ester was scraped and extracted with chloroform:methanol (1:1). (15S)-HETE was quantitated with an EIA kit (Cayman Chemicals, Ann Arbor, MI) as per the manufacturer's instructions. Equivalent mass of CE was used for each sample.

*siRNA Transfection.* RAW mock transfected and 15-LO-1 RAW cells were plated at a density of  $2 \times 10^5$  cells in 24-well plates in 200  $\mu\text{l}$  routine growth media. ALOX15 siRNA, GAPDH positive control and negative control #1 siRNA were added to culture medium without serum and vortexed. HiPerFect transfection reagent was added to the diluted siRNA at a concentration of 3  $\mu\text{l}$  per 5 nM siRNA, vortexed and allowed to incubate for 10 minutes at room temperature. Transfection complexes were added to respective wells for a final concentration of 8 nM siRNA per well. Cells were allowed to incubate 6 hours at 37°C before labeling with 3  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-cholesterol in DMEM containing 2  $\mu\text{g/ml}$  ACAT inhibitor (Cp113818), 10% FBS and 50  $\mu\text{g/ml}$  gentamicin for 24h. Media containing 0.3 mM cAMP in 0.2% BSA and 2  $\mu\text{g/ml}$  ACAT inhibitor was then added to wells for a period of 18h.

*Protein and Cholesterol Determination.* Lipids were extracted from monolayers with isopropanol or lipid droplets isolated from cholesterol-enriched cells using chloroform:methanol (1:1) containing cholesteryl methyl ether (Sigma) as an internal standard. Total and free cholesterol was quantified by gas-liquid chromatography<sup>8</sup>. Total phospholipid was quantified by the method of Rouser *et al.*<sup>9</sup>. Triacylglyceride was quantified using a commercially available kit (Sigma). Protein was measured by the method of Markwell *et al.*<sup>10</sup>.

*Animals.* Wild-type C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were fed a standard chow diet *ad libitum* before and during the study. On the day of injection, animals were divided into groups (N=6) and allowed unlimited access to food and water. RAW-15-LO-1 or RAW-mock transfected cells which were previously cholesterol enriched and labeled by incubation with [ $^3\text{H}$ ]cholesterol (1  $\mu\text{Ci/ml}$ ) and acLDL (100  $\mu\text{g}$  protein/ml) were

injected (typically  $4.5 \times 10^6$  cells containing  $7.5 \times 10^6$  cpm in 0.5 ml DMEM) intraperitoneally (ip) as described previously<sup>11</sup>. Blood was collected at 6 h, 24 h, and 48 h, by retro-orbital bleed and plasmas were used for liquid scintillation counting (LSC). Feces were collected continuously from 0 to 48 h and stored at 4 °C before extraction. At 48 h after injection, mice were exsanguinated and perfused with cold PBS, and portions of the liver were removed and flash-frozen for lipid extraction.

*Fecal Lipid Extraction.* Feces collected from 0 to 48 h were weighed and soaked in water (1 ml water/100 mg feces) overnight at 4 °C. The next day, an equal volume of ethanol was added, and the samples were homogenized using a Tissue Terror (model # 985370, Biospec Products Inc.). A 200 $\mu$ l aliquot of each homogenized fecal sample was counted in a LSC to measure <sup>3</sup>H-total sterols.

*Data analysis.* Data are from representative experiments and are expressed as mean +/- standard deviation for an N=3 unless otherwise stated. Statistical test for significance was performed using an unpaired t test with a 95% confidence interval.

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Figure 1

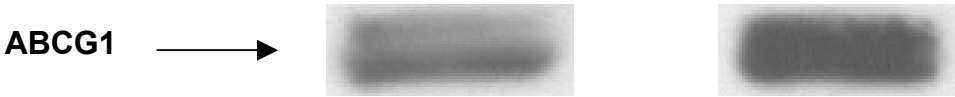
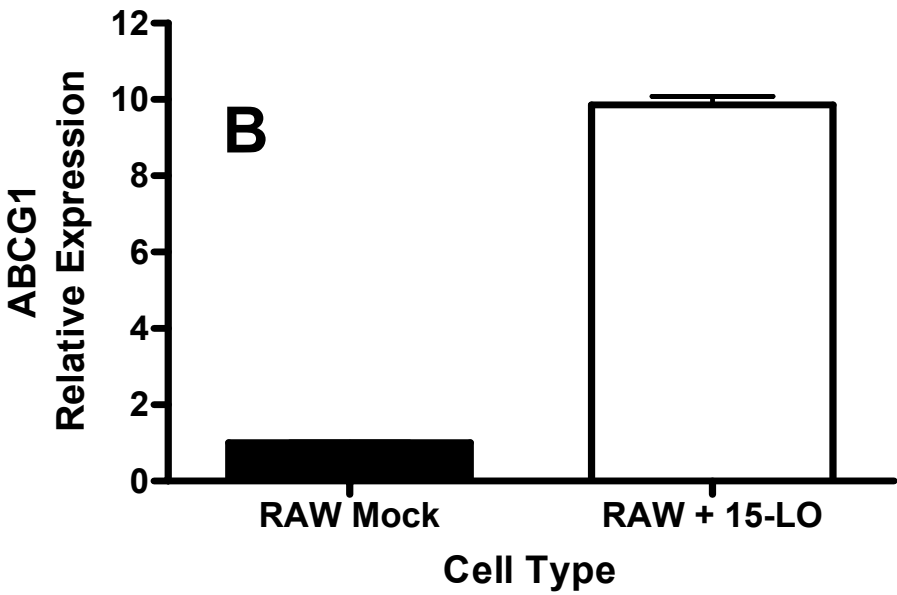
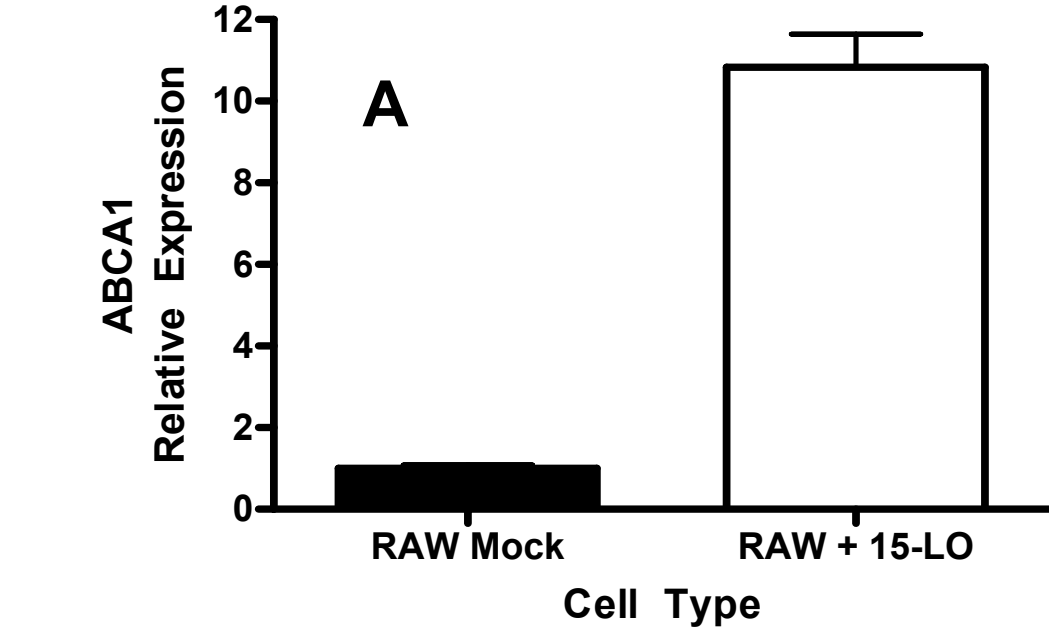


Figure 2

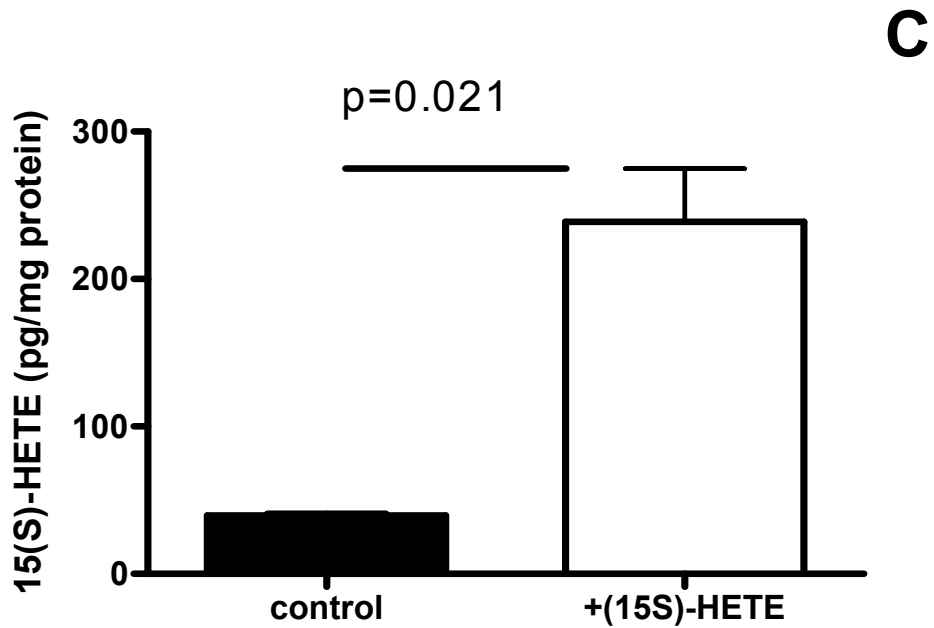
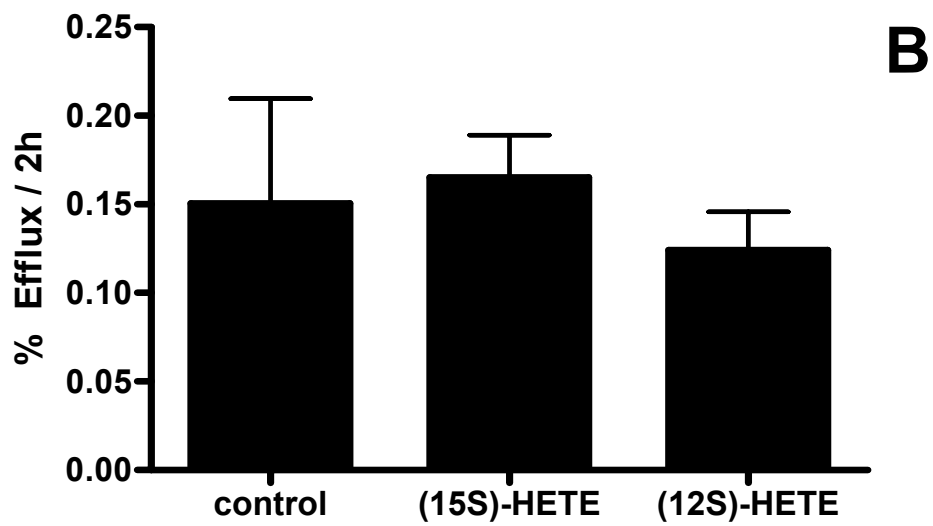
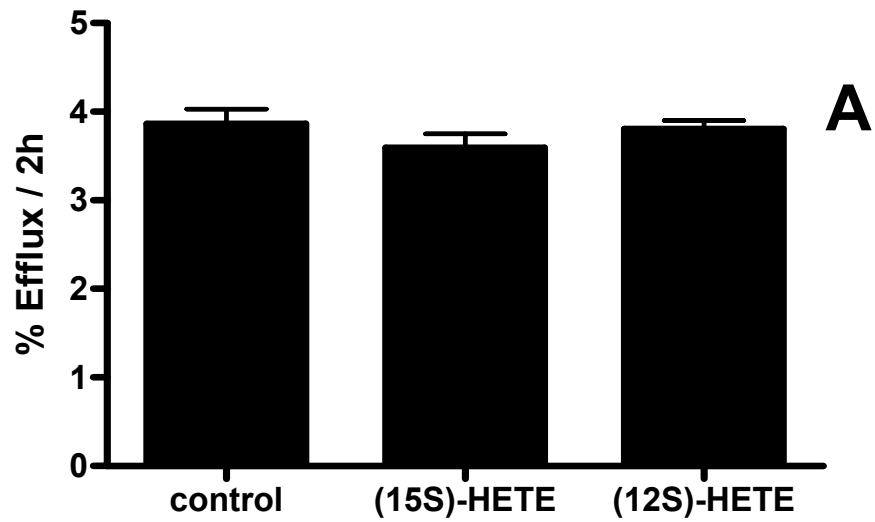
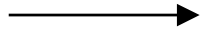


Figure 3

**ABCG1**



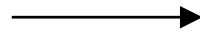
**a**

**b**

**c**

**d**

**ABCA1**



**a**

**b**

**c**

**d**

**Figure 1. ABCA1 & ABCG1 mRNA levels in RAW mock-transfected and RAW overexpressing 15-LO macrophages.** Purified RNA was amplified into cDNA with random primers. Real-Time was performed on 15 ng cDNA/well. Cell lysates were analyzed by immunoblot. 20µg of total cell protein was added to each lane.

**Figure 2. Effect of 15-LO metabolites on efflux to HDL3 or apoA-I.** Mock-transfected RAW Macrophages were radiolabeled for 24 hours with [<sup>3</sup>H]-cholesterol. Following an equilibration period, cells were treated with BSA alone or 500 nM of (15S)-HETE, or (12S)-HETE for 4 hours. Efflux to 50 µg/ml HDL3 (**A**) or 20 µg/ml apoA-I (**B**) was measured at 2 hours. Total incorporation of (15S)-HETE into cells was measured by EIA before the efflux period (**C**)

**Figure 3. Effect of 15-LO metabolites on ABCA1 and ABCG1 protein expression.** Mock-transfected RAW cells were treated as described in Figure 2. Cell lysates were analyzed by immunoblot. 20µg of total cell protein was added to each lane. Lanes correspond to cell treated with **A** (12S)-HETE; **B** (15S)-HETE; **C** no treatment; **D** Positive control cell lysate.