Data Supplement

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 [^{35}S]methionine for 20 minutes. ABCG1 was immunoprecipitated, and ^{35}S-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[1].

Cellular Cholesterol Efflux Measurements. Cholesterol efflux assays were performed as described, with minor modification[2]. 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 [^{3}H]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 125μM 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[3,4]. The efflux medium was then removed and a 100 μl aliquot was taken for ^{3}H 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 ^{3}H radioactivity determination. Results are expressed as [^{3}H]cholesterol in medium/([^{3}H]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 Na_3VO_4 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 H_2O 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'-

CCCGGAAACGCAAGTCC-3'; ABCG1 Forward 5'-TTCCCCTGGAGATGAGTGTC-3' and Reverse 5'-CAGTAGGCCACAGGGAACAT-3'; LXR alpha Forward 5'-GGATAGGGTTGGAGTCAGCA-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 ΔC_t 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 $p<0.05$[5].

References

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3. Nichols AB, Ravenscroft C, Lamphiear DE, Ostrander LD, Jr. Independence of serum lipid levels and dietary habits. The Tecumseh study. *JAMA* 1976;236:1948-1953.

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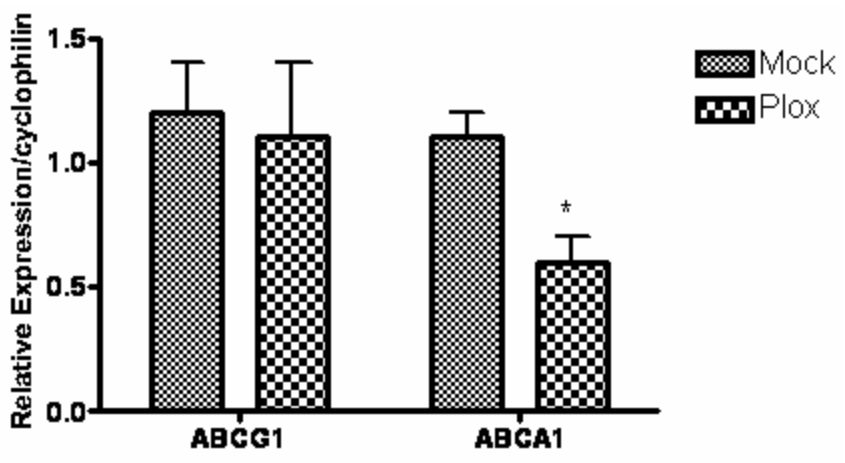
A



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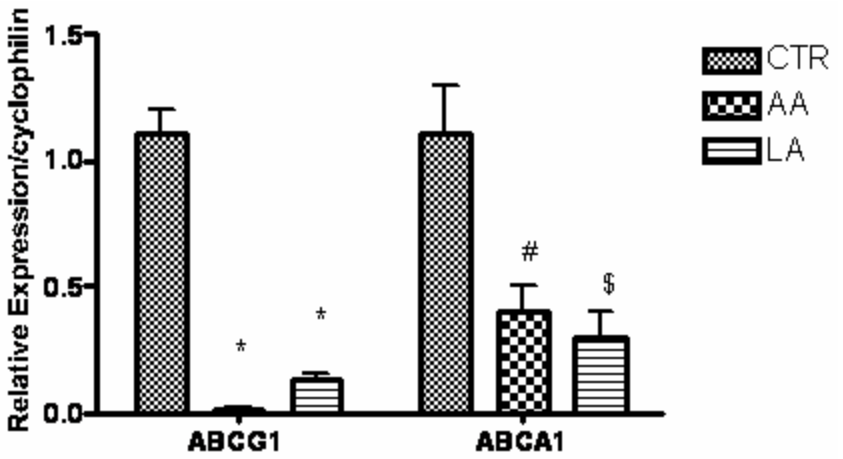

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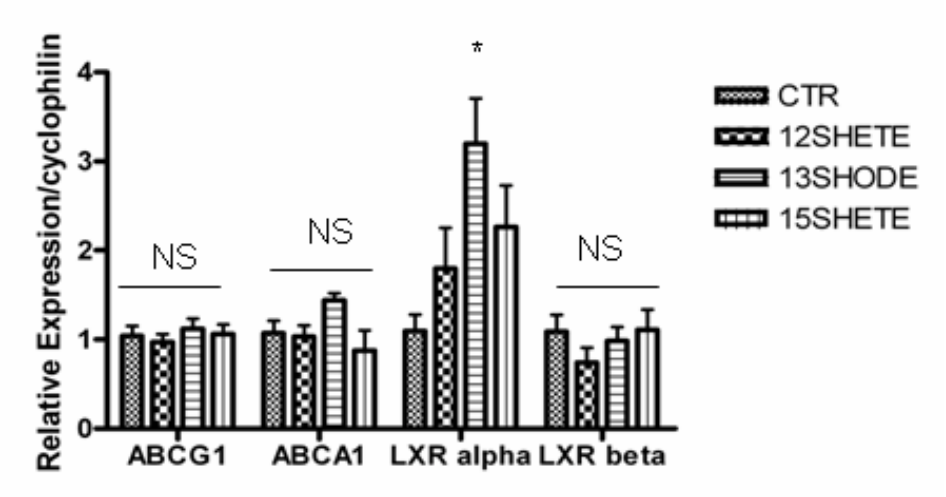
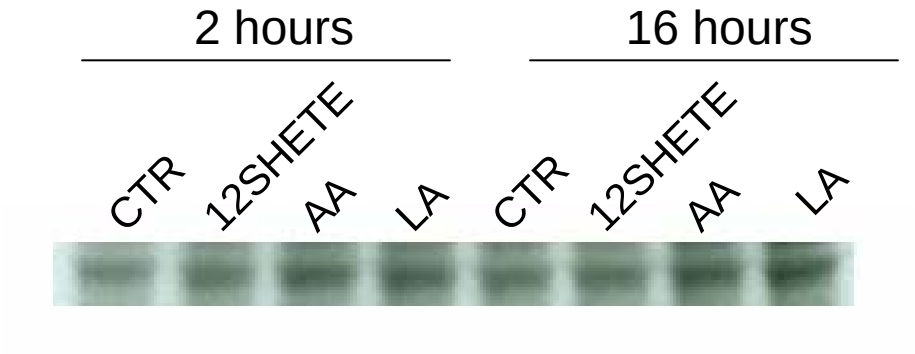


Supplementary Figure I



Supplementary Figure II