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Differential Regulation of ABCA1 and Macrophage Cholesterol Efflux By Elaidic and Oleic Acids

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Abstract

Trans fatty acid consumption is associated with an increased risk of coronary heart disease. This increased risk has been attributed to decreased levels of HDL cholesterol and increased levels of LDL cholesterol. However, the mechanism by which *trans* fatty acid modulates cholesterol transit remains poorly defined. ATP-binding cassette transporter A1 (ABCA1)-mediated macrophage cholesterol efflux is the rate-limiting step initiating apolipoprotein A-I lipidation. In this study, elaidic acid, the most abundant *trans* fatty acid in partially hydrogenated vegetable oil, was shown to stabilize macrophage ABCA1 protein levels in comparison to that of its *cis* fatty acid isomer, oleic acid. The mechanism responsible for the disparate effects of oleic and elaidic acid on ABCA1 levels was through accelerated ABCA1 protein degradation in cells treated with oleic acid. In contrast, no apparent differences were observed in ABCA1 mRNA levels, and only minor changes were observed in Liver X receptor/Retinoic X receptor promoter activity in cells treated with elaidic and oleic acid. Efflux of both tracers and cholesterol mass revealed that elaidic acid slightly increased ABCA1-mediated cholesterol efflux, while oleic acid led to decreased ABCA1-mediated efflux. In conclusion, these studies show that *cis* and *trans* structural differences in eighteen carbon n-9 monoenoic fatty acids variably impact cholesterol efflux through disparate effects on ABCA1 protein degradation.

Keywords

Trans Fatty Acid; Macrophages; Cholesterol Efflux; Cholesterol Transport Proteins; Mass Spectrometry

Introduction

Epidemiological studies have suggested that increasing the daily consumption of *trans* fatty acids by 2% of daily energy intake leads to increased risk of cardiovascular disease including coronary artery disease and atherosclerosis [1,2]. *Trans* fatty acid consumption has been associated with increased plasma LDL cholesterol, decreased HDL cholesterol and an increased ratio of total cholesterol to HDL cholesterol, which are all indicators of higher risk of cardiovascular disease [3,4]. Despite the epidemiological evidence that *trans* fatty acids impact blood cholesterol levels and increase risk of cardiovascular disease, the associated mechanisms remain to be thoroughly elucidated. One mechanism may involve changes in lipoprotein secretion from the liver, which is supported by the findings that *trans* fatty acids compared to *cis* fatty acids increase apo B-containing lipoprotein secretion from HepG2 cells [5].

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Plasma lipoprotein cholesterol levels and the accumulation of cholesterol-laden macrophages in the vascular wall are highly dependent on the dynamic regulation of cholesterol biosynthesis and the transit of cholesterol from tissues to the liver via reverse cholesterol transport (RCT) [6-10]. RCT is initiated by ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux from macrophages to apoA-I, ultimately leading to the transport of cholesterol by lipoproteins to the liver for excretion in the bile [7,11]. Impairment of RCT could lead to increased atherogenesis [12]. Alternatively, accelerating RCT has been explored as a therapeutic target to reduce atherosclerotic lesions [13]. Previous studies have shown that *cis* unsaturated fatty acids and saturated fatty acids differentially regulate cholesterol efflux [14,15]. While unsaturated fatty acids have been shown to impair macrophage cholesterol efflux by increasing ABCA1 protein degradation [15], the effects of *trans* fatty acid on ABCA1-mediated macrophage cholesterol efflux are not fully understood.

Due to the importance in understanding the role of dietary lipids including *trans* fatty acids on lipid metabolism, the present studies were performed to compare the effects of *cis* and *trans* fatty acids on cholesterol efflux from macrophages. These studies show that oleic acid, but not elaidic acid treatments, result in decreased levels of ABCA1 protein due to accelerated ABCA1 degradation, which is accompanied by decreased ABCA1-mediated cholesterol efflux to apoA-I. These findings indicate that diminished circulating HDL levels in humans consuming *trans* fats may not be mediated by alterations in cholesterol efflux to apoA-I, but through other mechanisms such as that described for LDL and VLDL secretion from liver [5].

Materials and methods

Fatty acid-bovine serum albumin (BSA) conjugate

Fatty acids were purchased from NuChek Prep (Waterville, MN). Fatty acids were conjugated with bovine serum albumin (BSA) as 5 mM stock solutions [16]. Briefly, fatty acids were saponified with NaOH at 70 C for 30 min and then mixed with 6% fatty acid-free BSA dissolved in PBS. Stock solutions were stored in aliquots at -20 C. These fatty acid-BSA conjugates were dissolved into media to desired concentrations immediately prior to use in experiments.

J774 cell ABCA1 immunoblotting

J774 macrophages were grown in 60 mm dish in RPMI (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biological) and 1% antibiotic/antimycotic reagent (Sigma). Cells were grown to over 95% confluence before treatment with different concentrations of fatty acids in RPMI supplemented with 0.2% fatty acid-free BSA, 1 M each of T0901317 and 9-*cis* retinoic acid (collectively referred to as LXR/RXR agonists). At the end of selected incubation intervals, media were discarded and cells were washed twice with PBS before being scraped into, and extracted at 4°C with, protein extraction buffer containing protease inhibitor (Roche). Cell debris was removed by centrifugation and subsequently protein in the supernatant was quantified by Bio-Rad protein assay. Sixty to eighty g of protein were loaded per lane for SDS-PAGE and subsequent immunoblot analysis. ABCA1 was detected using rabbit polyclonal anti-ABCA1 (Novus Biologicals, 1:1,000 dilution in TBS + 0.1% Tween 20) and normalized to α -tubulin levels (Santa Cruz Biotechnologies, 1:7,000 dilution in TBS + 0.1% Tween 20).

Real-time PCR

J774 cells were grown to 90% confluence before treatment in growing media. Cells were treated with either elaidic acid, oleic acid or BSA blank control for 24 h. At the end of

treatment, cells were washed twice with cold PBS before collected with Trizol (Invitrogen). RNA isolation was done following Trizol Invitrogen RNA isolation procedure. cDNAs were generated from 5 g of DNaseI-treated RNA using SuperScript III and random hexamers (Invitrogen). Real-time PCR was done with SYBR Green reagent (Roche), using a LightCycler 480 real-time PCR detection system (Roche). Quantification of mRNA levels was done by normalizing values to GAPDH and calculated using the comparative C_T method.

Regulation of Liver X response element promoted luciferase activity by elaidic acid and oleic acid

HEK293 cells were transfected with pGL3P-luciferase reporter constructs in which three copies of Liver X response element (LXRE) were inserted upstream of the SV40 promoter. Cells were simultaneously transfected with plasmids encoding human LXR- and RXR- (or empty pcDNA3 plasmids as control) as well as a plasmid encoding -galactosidase. In parallel experiments, empty pGL3P-luciferase reporter was used as a control. Sixteen hours after transfection, media was changed to DMEM containing different fatty acids at the indicated concentrations. Following 36–48 h of incubation, luciferase activity was measured using a luciferase assay system (Promega). Activities of -galactosidase was measured by o-nitrophenyl-D-galactoside (Sigma) and used to normalize luciferase activity.

ABCA1 protein degradation

J774 macrophages grown to over 95% confluence in RPMI supplemented with 10% FBS were pre-incubated with the same medium containing LXR/RXR agonists for 16 h to induce ABCA1 expression. Medium was then removed, cells were rinsed twice with RPMI medium, and then incubated in RPMI supplemented with 0.2% BSA and 100 M of specific fatty acids for 2 h. 20 g/ml cycloheximide was then added to media to stop protein synthesis. Cell lysate was collected at time 0, 30 min, 1h, 2h, and 4h with protein extraction buffer and processed for immunoblot analysis as described above.

Cholesterol efflux assays

Cholesterol efflux from J774 cells in the presence and absence of fatty acids was screened using a conventional tritiated cholesterol-based efflux assay [17-20], and was delineated in molecular detail with a recently developed deuterated cholesterol-based assay [21]. J774 cells were plated to over 95% confluence in 6-well plates in growth media. The following day, media was replaced with RPMI media containing 1% fetal bovine serum, 1% antibiotic/antimycotic reagent and either 15 g/ml of [d_7]-cholesterol or [3H]-cholesterol (1 Ci/ml) (with 15 g/ml of unlabeled cholesterol added). Labeling was also performed in the presence of LXR/RXR agonists. Cells were labeled for 24 h and were washed once with RPMI media with 0.2% BSA prior to incubation with equilibration media, which contains 0.2% fatty acid-free BSA in RPMI and 1% of antibiotic/antimycotic reagent together with indicated concentration of fatty acid-BSA conjugate in the presence of LXR/RXR agonists. Equilibration lasted for 18 h followed by the efflux interval. Cells were washed twice with RPMI media containing 0.2% BSA before efflux. Efflux was initiated by adding either 0.2% BSA in RPMI media or 20 g/ml apoA-I in the same media for 6 h. For efflux experiment with [d_7]-cholesterol, media was collected at the end of the efflux step into 15 ml conical and centrifuged at 2000 rpm for 5 min at 4 °C to spin down cell debris, supernatant was collected before further analysis. Cells were scraped off plates with 1.5 ml PBS (750 l twice) after two washes with PBS. Samples were stored at -80 °C until further analysis. To analyze lipid composition of both media and cell samples, a modified Bligh-Dyer lipid extraction [22] was performed on cell samples in the presence of internal standards (0.4 g of 17:0 CE, 0.4 g of [d_7]-17:0 CE and 4 g of [d_4]-cholesterol). Similarly, 700 μ l of media samples was extracted by a modified Bligh-Dyer lipid extraction with 0.125 μ g of [d_7]-17:0 CE, 0.125 μ g

of 17:0 CE and 0.6 μg of [*d*₄]-cholesterol as internal standards. For [³H]-cholesterol-labeled cells, intracellular radiolabel was determined by incubating cells with 1 ml isopropanol for 1h with gentle agitation at room temperature, followed by scraping cells and measuring radioactivity with liquid scintillation spectrometry. THP-1 cells were grown in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum (Atlanta Biological) and 1% antibiotic/antimycotic reagent (Sigma). For [³H]-cholesterol efflux assay, cells were plated at a density of 10⁶ cells per well of 6-well plate in the same growing medium with the addition of 100 nM of phorbol 12-myristate 13-acetate (PMA) that triggers THP-1 monocyte differentiation to macrophages that stick to the bottom of cell culture wells following a 40h treatment interval. Subsequently these differentiated cells were used for [³H]-cholesterol efflux assays under similar conditions as those described for J774 cells.

Quantification of cholesterol and CE using electrospray ionization mass spectrometry (ESI-MS)

Free cholesterol was derivatized to its cholesteryl acetate derivative to aid in ionization under ESI-MS conditions. Direct-infusion ESI-MS of long chain CE and cholesteryl acetate was performed using a Thermo Fisher TSQ Quantum Ultra with Xcalibur data acquisition software in positive ion mode. Samples were analyzed at a flow rate of 3.5 $\mu\text{l}/\text{min}$. Other parameters for CE analysis were set at spray voltage = 3800 V, sheath gas = 8 (arbitrary units), ion sweep gas pressure = 0.5 (arbitrary units), and capillary temperature = 270 °C. In MS/MS mode, the collision energy for analysis of CE molecular species was set at 18 eV for cholesteryl acetate and 25 eV for long chain CE molecular species. Spectra for MS/MS scan modes (NL 368.5 and 375.5 for CE and [*d*₇]-CE, respectively) [21,23]; and product ion (PI) *m/z* 83 for acetylated cholesterol derivative [21] were acquired over 3-5 min with a scan rate of 0.5 scans/s.

Results

Alterations in ABCA1 expression in J774 cells treated with fatty acids

ABCA1 is the principal mediator responsible for the lipidation of apoA-I during cholesterol efflux. Accordingly, studies were designed to determine the impact of elaidic acid versus oleic acid treatments on ABCA1 protein levels. While keeping the total fatty acid treatment fixed at either 100 or 250 M and varying the ratio of elaidic acid and oleic acid it was evident that there was a concentration dependent decrease in ABCA1 protein associated with increasing concentrations of oleic acid (Figures 1A-D). While these studies varying the elaidic and oleic acids levels, mimic the potential ratios of these fatty acids from diets enriched with *trans* fatty acids, additional studies demonstrated that oleic acid specifically reduces ABCA1 protein, while elaidic acid does not have this specific effect (Figures 1E and F). Further studies examined the effect of other *trans* fatty acids on ABCA1 protein levels. These studies show that while elaidic acid does not reduce ABCA1 protein levels other *trans* fatty acids do have an impact in reducing ABCA1 protein levels (Figures 1G & H). In fact, both 9*t*,12*t*-18:2, and 9*c*,12*t*-18:2 treatments reduced ABCA1 levels to that observed with oleic acid treatment. Also the major *trans* fat in dairy products, vaccenic acid (11*t*-18:1), did not significantly reduce ABCA1 levels, while its *cis* analog significantly reduced ABCA1 levels.

Disparate ABCA1 protein degradation in the presence of elaidic and oleic acids

To address the possibility that ABCA1 degradation is altered by elaidic acid, J774 cells were incubated with, or without, fatty acids for 2 h, followed by incubations with the fatty acids supplemented with cycloheximide. Data shown in Figure 2A and 2B demonstrate that oleic acid treatment significantly reduced ABCA1 protein levels after 1h of cycloheximide treatment, whereas elaidic acid had no effect. The disparate degradation of ABCA1 elicited

by treatments with elaidic and oleic acid were maintained at all time points examined in these ABCA1 protein stability assays at incubations up to 4h. In fact, following 4h of treatment with oleic acid in the presence of cycloheximide, ABCA1 levels were decreased greater than 90% of the t=0 levels, while levels of ABCA1 in cells treated with either no fatty acid or elaidic acid were both reduced only 40%.

Elaidic acid and oleic acid do not alter ABCA1 mRNA in macrophages

Further studies examined whether elaidic and oleic acids alter the mRNA levels of ABCA1. Both elaidic and oleic acid (100 M) did not significantly alter whole cell levels of ABCA1 mRNA, suggesting that elaidic acid and oleic acid do not alter ABCA1-mediated cholesterol efflux by modulating ABCA1 transcription (Figure 3A). Additional experiments showed that oleic acid modestly decreases LXR promoter activity using a luciferase activity assay in transfected HEK293 cells (Figure 3B). In comparison, elaidic acid does not alter LXR promoter activity compared to treatments with no fatty acid (Figure 3B).

Elaidic and oleic acids differentially alter J774 macrophage cholesterol efflux

Since elaidic and oleic acid disparately alter ABCA1 protein levels, further experiments were performed to show that these changes functionally alter cholesterol efflux. Both ABCA1 protein levels, and the efflux of cholesterol from J774 cell macrophages, were compared in cells pretreated with either elaidic acid or oleic acid. Initial experiments examined cholesterol efflux from J774 cells that were radiolabeled with [³H]-cholesterol. For these experiments, cells were first labeled with [³H]-cholesterol for 24 h in the presence of LXR/RXR agonists, and then were subsequently treated with either elaidic acid, oleic acid or no fatty acid supplementation (BSA alone) in the presence of LXR/RXR agonists for 18 h. As shown in Figure 4A, elaidic acid treatment resulted in a slightly higher apoA-I-mediated cholesterol efflux than control treatment, while cells treated with oleic acid displayed a significantly reduced efflux. Parallel studies to the efflux studies with the same treatments including loading macrophages with unlabeled cholesterol were examined to determine ABCA1 levels under these conditions at the end of efflux conditions. As shown in Figure 4B and C, decreased amounts of ABCA1 protein were observed in cells treated with oleic acid prior to efflux (no efflux), as well as in cells subjected to efflux conditions in the presence of apoA-I.

Further experiments employed [*d*₇]-cholesterol labeling, and measurements of cholesterol and CE mass using mass spectrometry to further evaluate alterations in cholesterol efflux elicited by pretreatment with either elaidic or oleic acid. This technique allows the simultaneous measurement of labeled cholesterol and unlabeled cholesterol mass and the metabolism of cholesterol to CE. Similar to our findings using [³H]-cholesterol to measure efflux, data shown in Figure 5A demonstrate that the efflux of natural cholesterol from J774 cells to apoA-I containing media is slightly elevated in cells pretreated with elaidic acid, but reduced in cells pretreated with oleic acid. The concomitant efflux of [*d*₇]-cholesterol from cells was unaltered in cells pretreated with elaidic acid (Figure 5B). Interestingly, in these studies the combined efflux of [*d*₇]-cholesterol and unlabeled cholesterol was very similar to that of the efflux measured using [³H]-cholesterol with only a modest increase in efflux in cells pretreated with elaidic acid and a more striking depression in efflux elicited by pretreatment with oleic acid (Figure 5C). Since there is variable responsiveness in measured cholesterol efflux in different cell lines [24], we also examined cholesterol efflux in THP-1 cells differentiated into macrophage-like cells. Oleic acid, and not elaidic acid also suppressed human macrophage THP-1 cell [³H]-cholesterol efflux (Figure 5D). The majority of the mass of total cholesterol in the media from efflux studies in J774 cells was free cholesterol while in the cell approximately one fourth of the total cholesterol was esterified in the CE pool. Data shown in Figure 6A and B illustrate that cells pretreated with

oleic acid had significantly more 18:1 CE in them following efflux compared to those pretreated with elaidic acid. Additionally, 18:1 enrichment of CE was observed in both [*d*₇] (Panel B) and unlabeled (Panel A) CE pools of cells pretreated with either elaidic acid or oleic acid in comparison to cells that were pretreated with BSA only (the negative control). The predominant CE molecular species in these cells under all conditions was 18:1 CE. We also examined the time course of elaidic acid and oleic acid incorporation into both [*d*₇] and unlabeled CE pools following the [*d*₇]-cholesterol labeling period without an efflux interval. These data show that oleic acid incorporation into the CE pool is significantly greater following 6h of labeling, but this difference is reduced at 18h (Figure 7). It should be noted that the content of [*d*₇] and unlabeled CE at 18h is the condition at the beginning of efflux conditions.

Discussion

The association of *trans* fat consumption and risk of cardiovascular disease has been shown in multiple epidemiological studies and meta-analysis [1,2,4,25]. *Trans* fat consumption is associated with decreased levels of plasma HDL cholesterol and increased levels of LDL cholesterol, as well as increased ratio of total cholesterol level to HDL cholesterol level, which are all risk factors of cardiovascular disease [3,4]. Furthermore, the adverse effect of *trans* fat in the diet on increasing risk of cardiovascular diseases is considered to be at least as great as that of saturated fat in the diet [26]. Despite the epidemiological evidence for deleterious effects of *trans* fats on cardiovascular health, the mechanisms by which *trans* fats cause these effects remain elusive. The ABCA1-mediated macrophage cholesterol efflux pathway is considered as a critical anti-atherogenic process and is the rate-limiting step in HDL-mediated reverse cholesterol transport [7,11,13]. Considering that *trans* fat consumption is associated with decreased HDL cholesterol levels, we initially designed this study to determine if *trans* fat has any effect on this particular pathway. Our results show that elaidic acid and oleic acid disparately regulate ABCA1 protein levels through different effects on ABCA1 protein degradation. Neither elaidic acid nor oleic acid significantly altered ABCA1 at a transcriptional level. The disparate effects of elaidic acid and oleic acid on ABCA1 protein levels result in parallel functional changes in cholesterol efflux to apoA-I.

Others have shown that *cis* unsaturated fatty acids accelerate ABCA1 protein degradation through a mechanism involving PKC-mediated ABCA1 phosphorylation [15,27]. Several other studies have investigated ABCA1 regulation by fatty acids, mostly focused on *cis* unsaturated fatty acids and saturated fatty acids. These other studies suggested that *cis* fatty acids including both monounsaturated and polyunsaturated fatty acids reduce ABCA1 protein level to different extent [15,28]. The present study similarly shows that *cis* unsaturated fatty acid leads to ABCA1 protein degradation, while further demonstrating the critical nature of the *cis* configuration in this mechanism since ABCA1 is stable in cells treated with elaidic acid.

Studies examining the role of *trans* fat in regulating ABCA1-mediated cholesterol efflux are conflicting [29,30]. In contrast to our findings and those of Buonacorso and co-workers [30], the study by Fournier and co-workers [29] showed that *trans* fatty acids reduced ABCA1-mediated cholesterol efflux without altering its protein or transcript levels. One difference in our study with this previous study [29] is that we first labeled cells with cholesterol and then treated the cells with fatty acids. In contrast to the study design described herein, the previous study of Fournier et al. [29] combined cholesterol and fatty acid incubation into one step. It is possible that the presence of fatty acids could cause different levels of cholesterol uptake by macrophages therefore the reduced cholesterol efflux at further steps could be caused by the reduced initial uptake of labeling cholesterol. Also there may be

subtle difference in the expression of ABCA1 in these two studies as we used LXR/RXR agonists whereas the previous study used cAMP. Interestingly, we did observe a modest disparate LXR promoter activity with elaidic acid and oleic acid treatments. Our studies also revealed that mRNA levels of ABCA1 were not altered by elaidic acid, which is similar to that previously reported [29].

Alterations in cholesterol efflux in cells treated with either elaidic acid or oleic acid were assessed by measuring efflux of either [^3H]-, [d_7]- or unlabeled cholesterol from J774 cells, as well as [^3H]-cholesterol from THP-1 cells. Each of these efflux assays led to the same conclusion that elaidic acid does not suppress cholesterol efflux while oleic acid does suppress cholesterol efflux. Experiments assessing intracellular and media mass measurements of unlabeled and [d_7]-cholesterol and CE were interesting. J774 cells pretreated with elaidic acid contained less CE (both natural 18:1 CE and [d_7] CE) than the cells pretreated with oleic acid after efflux, which may partially explain the reduced efflux out of cells with oleic acid treatment. Data showing that the incorporation of oleic acid into CE pools is greater than that of elaidic acid (Fig. 7) suggest that the larger pool of CE in oleic acid treated cells following efflux conditions reflects the differences in CE pools prior to efflux. Although this is the most likely explanation for the difference in CE pools following efflux conditions in elaidic acid and oleic acid treated cells, it still is possible that this difference may reflect differential subcellular localization of the CE pool, reduced hydrolysis rates of this CE pool, differences in the regulation of ACAT enzyme activity, an overall diminished flux of cholesterol out of the cell, or any combination of these.

Collectively, we found that a single difference in the double bond configuration between elaidic acid and oleic acid renders drastic differences in their regulation on ABCA1-mediated cholesterol efflux. Oleic acid decreased ABCA1 protein level by increasing its degradation, whereas elaidic acid seemed to stabilize ABCA1 protein level. In concordance with their regulation on ABCA1 protein level, elaidic acid and oleic acid demonstrated disparate regulation on macrophage cholesterol efflux with elaidic acid slightly increasing cholesterol efflux while oleic acid reduces cholesterol efflux. Since *trans* fat consumption has been associated with decreased HDL cholesterol level, our observation with elaidic acid on regulation ABCA1-mediated cholesterol efflux pathway seems paradoxical. However, it should be appreciated that the increase in cholesterol efflux with elaidic acid treatment is fairly modest, and HDL levels are largely modulated by liver-mediated apoA-1 secretion and HDL lipidation. It should also be noted that *trans* fat feeding accelerates liver biosynthetic pathways leading to oleic acid production [31]. Thus, elaidic acid may have an indirect deleterious impact on atherosclerosis and reverse cholesterol transport via the accelerated biosynthesis of oleic acid, which decreases ABCA1 mediated efflux. It is also likely that elaidic acid reduces plasma HDL levels by impacting hepatic apoA1 synthesis or HDL synthesis [32]. Taken together, the impact of altered fat in the diet (e.g., high *trans* fat, monounsaturated fatty acid and saturated fat) on liver lipid biosynthesis and lipoprotein production, and the interaction of these pathways with therapeutic agents designed to improve cardiovascular health through targeting lipid biosynthetic pathways certainly should have profound end-results on atherosclerosis progression and/or regression.

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Abbreviations

ESI Electrospray ionization

MS	Mass spectrometry
CE	Cholesteryl esters
LXR	Liver X receptor
RXR	Retinoic X receptor
RCT	Reverse cholesterol transport
ABCA1	ATP-binding cassette transporter A1
apoA-I	apolipoprotein A-I

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Figure 1.

Alterations in macrophage ABCA1 protein level by elaidic acid (*t*-18:1) and oleic acid (*c*-18:1). J774 macrophages were treated with selected amounts of *t*-18:1 and *c*-18:1 at a total fatty acid concentration of 100 μM for 24 h in the presence of LXR/RXR agonists. A representative immunoblot of these experiments for cellular ABCA1 protein is shown in A. Panel B shows densitometry analysis performed on 3 independent experiments of immunoblots under conditions shown in A. Panel C shows a representative ABCA1 protein immunoblot from J774 macrophage cell protein in cells treated with different combinations *t*-18:1 and *c*-18:1 at a total fatty acid concentration of 250 M. Panel D shows densitometry analysis of 3 independent experiment as shown in Panel C. Panels E and F show similar analyses of ABCA1 protein in J774 cells treated with selected concentrations of either *t*-18:1 or *c*-18:1. Similarly, Panels G and H show analyses of J774 cell ABCA1 protein in cells treated with the indicated *cis* and *trans* fatty acids at a concentration of 250 M (note that for these panels, *t*-18:1 and *c*-18:1 are indicated as 9*t*-18:1 and 9*c*-18:1, respectively). Values are means ± S.E.M. for three independent measurements. For some S.E.M. values the error bars are within the symbol.

Figure 2.

ABCA1 protein levels with protein degradation assay. J774 macrophages were first incubated for 16 h with LXR/RXR agonists to induce ABCA1 level, and then were incubated with either *t*-18:1, *c*-18:1, or blank control for 2 h followed by addition of cycloheximide. A representative immunoblot of cellular ABCA1 from treatments at indicated time points after addition of cycloheximide is shown in Panel A. Panel B shows relative protein levels compared to that at time zero ($t=0$) after densitometry analysis from three independent experiments. At each time point the amount of ABCA1 protein was compared to that at the beginning of this degradation assay. Values are means + S.E.M. ** and *** indicate $p < 0.01$ and 0.001 , respectively, for differences between *t*-18:1 and *c*-18:1 treatments.

Figure 3.

ι -18:1 and ϵ -18:1 have no effects on ABCA1 mRNA level and LXR element activity. Cells were treated as indicated with 100 μ M of either ι -18:1, ϵ -18:1 or both fatty acids in the presence or absence of LXR/RXR agonists. Real-time PCR analysis of macrophage J774 ABCA1 mRNA levels (Panel A) were normalized to GAPDH mRNA and expressed as fold-change over the blank control treatment without the presence of LXR/RXR agonists. Data shown in Panel B are from HEK293 cells that were co-transfected with LXRE-luciferase plasmids and plasmids encoding human LXR- and RXR-. 16 hours after transfection, media was replaced with DMEM media containing 100 μ M of either ι -18:1, ϵ -18:1 or BSA blank control. Luciferase activity was measured after 48 hours of fatty acid incubation and normalized to -galactosidase activity. Values are means + S.E.M. for three independent measurements. * and ** indicate $p < 0.05$ and 0.01 , respectively, for differences between indicated conditions.

Figure 4.

Macrophage cholesterol efflux with $[^3\text{H}]$ -cholesterol and concomitant ABCA1 protein levels in J774 cells treated with *t*-18:1 and *c*-18:1. $[^3\text{H}]$ -cholesterol efflux was quantified as described in "Materials and methods". Efflux was measured as the percentage of total lipidic tritium in the media versus total lipidic tritium in both cells and media. ApoA-I-mediated efflux represents the difference between efflux in the presence and absence (BSA only) of apoA-I in the media. For these efflux studies macrophage J774 cells were labeled with 15 $\mu\text{g}/\text{ml}$ of $[^3\text{H}]$ -cholesterol (1 μCi) for 24 h, followed by 18 h treatments with either *t*-18:1 and *c*-18:1 (100 μM) in the presence of LXR/RXR agonists. $[^3\text{H}]$ -cholesterol efflux was initiated by removing fatty acids and adding media containing either 20 $\mu\text{g}/\text{ml}$ apoA1 or

0.2% BSA RPMI media as control (basal level efflux) for 6 h (Panel A). A representative blot showing ABCA1 protein levels at different steps of the efflux assay is shown in Panel B. J774 macrophage cells were loaded with cholesterol (in the absence of [³H]-cholesterol) for 24 hours followed by 18 hours treatment with indicated fatty acid at 100 μ M. Protein was extracted at this step for immunoblot analysis for ABCA1 as shown in “no efflux” condition. Other cells were further treated with blank efflux media (ctrl efflux) or efflux media containing 20 μ g/ml apoA1 (apoA1 efflux) for 6 hours. Protein was also extracted at the end of efflux for immunoblot analysis. Panel C shows densitometry analysis of three independent determinations of indicated conditions with each condition normalized to the blank treatment under “no efflux” condition. Values represent the mean $\pm$ S.E.M. * and *** indicate $p < 0.05$ and 0.001 , respectively, for differences between indicated conditions. In panel A, *** indicates $p < 0.001$ for comparisons between efflux in cells treated with *c*-18:1 compared to that in cells treated with *t*-18:1 or no fatty acid (blank).

Figure 5. Macrophage cholesterol efflux with [*d*₇]-cholesterol. Macrophage J774 cells were labeled with [*d*₇]-cholesterol and subsequently treated with either elaidic acid (*t*-18:1) or oleic (*c*-18:1) acids (100 μ M) in the presence of LXR/RXR agonists prior to efflux intervals as described in “Materials and methods”. ApoA-I-mediated efflux was measured as described in Figure 4. Efflux of unlabeled cholesterol is shown in Panel A. Panel B shows [*d*₇]-cholesterol efflux, while Panel C shows the combined natural and [*d*₇]-cholesterol efflux. Panel D shows THP-1 [³H]-cholesterol efflux. THP-1 monocytes were differentiated with 100 nM of phorbol myristate acetate (PMA) to macrophages before efflux assays. Values represent the mean $\pm$ S.E.M. for three independent experiments. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, for comparisons between efflux in cells treated with *c*-18:1 compared to that in cells treated with *t*-18:1 or no fatty acid (blank).

Figure 6.

Cellular levels of 18:1 CE after cholesterol efflux assay labeled with [*d*₇]-cholesterol. Following the cholesterol efflux experimental interval (e.g., 6 h), both 18:1 CE and 18:1-[*d*₇]-CE was determined in cell (and media) extracts as described in “Materials and methods” and these determinations were used in part to measure cholesterol efflux. Cellular unlabeled 18:1 CE (Panel A) and 18:1-[*d*₇]-CE (Panel B) were quantified and expressed in nmol per cell culture well, and values for three independent experiments. * indicates p < 0.05 for comparisons between *c*-18:1 with *t*-18:1 treatments.

Figure 7.

Cellular levels of 18:1 CE in elaidic acid and oleic acid treated J774 cells. J774 cells were labeled with [*d*₇]-cholesterol for 24 h, followed by equilibration with RPMI media containing either *t*-18:1 or *c*-18:1 for either 2, 6, or 18 h in the presence of 1 μM of LXR/RXR agonists. Cells were collected at the end of each treatment for Bligh & Dyer lipid extraction analysis in the presence of appropriate internal standards. Values represent the mean ± S.E.M. ** indicates $p < 0.01$ for comparisons between efflux in cells treated with *t*-18:1 compared to that in cells treated with *c*-18:1.