THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 7, Issue of February 15, pp. 5692–5697, 2002
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Printed in U.S.A.

Unsaturated Fatty Acids Inhibit Cholesterol Efflux from Macrophages by Increasing Degradation of ATP-binding Cassette Transporter A1*

Received for publication, October 16, 2001, and in revised form, December 10, 2001
Published, JBC Papers in Press, December 12, 2001, DOI 10.1074/jbc.M109977200

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Abnormal high density lipoprotein metabolism may contribute to the increased atherosclerosis associated with diabetes and insulin resistance. The ATP-binding cassette transporter ABCA1 mediates cholesterol transport from tissue macrophages to apoA-I, the major high density lipoprotein protein component. Because fatty acids are elevated in diabetes, we examined the effects of fatty acids on ABCA1 activity in cultured macrophages. Results showed that unsaturated fatty acids markedly inhibited ABCA1-mediated cholesterol and phospholipid efflux from macrophages when ABCA1 was induced by a cAMP analog. This was accompanied by a reduction in the membrane content of ABCA1 and a decrease in apoA-I binding to whole cells and to ABCA1. In contrast, saturated fatty acids had no effect on these processes. Fatty acids did not alter ABCA1 mRNA abundance or incorporation of methionine into ABCA1, indicating that decreased *ABCA1* transcription, enhanced mRNA decay, or impaired translation efficiency did not account for these inhibitory effects. Unsaturated fatty acids, however, increased ABCA1 turnover when protein synthesis was blocked by cycloheximide. We conclude that unsaturated fatty acids reduce the macrophage ABCA1 content by enhancing its degradation rate. These findings raise the possibility that an increased supply of unsaturated fatty acids in the artery wall promotes atherogenesis by impairing the ABCA1 cholesterol secretory pathway in macrophages.

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in diabetes (1, 2). Patients with type 2 diabetes have disorders in lipid metabolism, including elevated serum triglycerides and below normal levels of high density lipoprotein (HDL)[1] (2–4). Low HDL levels are a major risk factor for cardiovascular disease (5), raising the possibility that abnormal HDL metabolism plays a role in the increased atherosclerosis associated with diabetes.

It is widely believed that HDL protects against atherosclerosis by promoting clearance of excess cholesterol from macrophages of the artery wall. This clearance is facilitated by an ATP-binding cassette transporter called ABCA1 (6, 7), which mediates transport of cellular cholesterol and phospholipids to lipid-poor HDL apolipoproteins. Mutations in ABCA1 cause Tangier disease (8–11), a severe HDL deficiency syndrome characterized by deposition of sterols in tissue macrophages and prevalent atherosclerosis (12, 13). It is therefore feasible that factors associated with diabetes impair the activity of this lipid secretory pathway, leading to accumulation of sterols in arterial macrophages and to enhanced atherogenesis.

ABCA1 expression by macrophages is highly regulated. Cholesterol loading of macrophages markedly increases ABCA1 mRNA abundance and protein levels (11, 14), consistent with a transporter that functions to rid cells of excess cholesterol. This gene regulation is mediated by nuclear liver X receptors (LXRα and LXRβ) and retinoid X receptor (RXR) (15–18), which form heterodimers that are activated by oxysterols and retinoic acid, respectively (19). Analogs of cAMP also activate *ABCA1* transcription by mechanisms distinct from the LXR/RXR system (20–22).

There is a close link between cholesterol and fatty acid metabolism. A family of transcription factors called sterol regulatory element-binding proteins (SREBPs) regulate genes involved in both sterol and fatty acid synthesis as well as internalization of lipoprotein cholesterol (23–25). Sterols and fatty acids can function separately or together to feedback-repress different SREBP isoforms. Moreover, sterol ligands for LXR activate transcription of both *ABCA1* and *SREBP-1c* (15, 16, 26, 27), and fatty acids can antagonize these ligands (28).

One of the lipid abnormalities in type 2 diabetes is elevated fatty acids (29, 30). Moreover, macrophages in atherosclerotic lesions produce lipoprotein lipase (31), which can generate fatty acids from triglycerides. Macrophage production of this enzyme has been reported to be atherogenic (32–34) and to be induced in diabetes (35). Thus, arterial macrophages may be exposed to abnormally high levels of fatty acids in diabetes and other metabolic disorders.

In the current study, we examined the effects of fatty acids on ABCA1 activity in cultured macrophages. We found that treatment of cells with unsaturated fatty acids suppressed ABCA1-mediated cholesterol secretion from macrophages by increasing the degradation rate of ABCA1. These results support the concept that an increased supply of fatty acids in the artery wall could impair clearance of excess cholesterol from macrophages and promote atherogenesis.

EXPERIMENTAL PROCEDURES

Lipoproteins and ApoA-I—LDL and HDL were prepared by sequential ultracentrifugation in the density range 1.019–1.063 and 1.125–1.21 g/ml, respectively, and HDL was depleted of apoE and apoB by heparin-agarose chromatography (36). ApoA-I was purified from HDL,

* This work was supported by National Institutes of Health Grants HL18645, HL55362, and DK02456. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HDL, high density lipoprotein; LXR, liver X receptor; RXR, retinoid X receptor; SREBPs, sterol regulatory element-binding proteins; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DSS, 2,2-dimethyl-2-silapentanesulfonic acid; LDL, low density lipoprotein; 8-Br-cAMP, 8-bromo-cAMP.

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delipidated, and labeled with ^{125}I as described previously (36). LDL was acetylated by the method of Goldstein *et al.* (37).

Cell Culture and Lipid Efflux—Murine J774 and RAW 264.7 macrophages were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum or incubated in serum-free DMEM plus 1 mg/ml fatty acid-free bovine serum albumin (DMEM/BSA). To radiolabel cellular cholesterol, 1 μCi/ml [^{3}H]cholesterol (PerkinElmer Life Sciences) was added with 50 μg/ml acetylated LDL to DMEM/BSA during the 24 h immediately preceding treatments (21). Cells were then washed once with PBS containing 1 mg/ml BSA (PBS/BSA) and incubated for 16–20 h with medium containing 5 mg/ml BSA and 0 to 0.25 mM fatty acids (molar ratios to BSA of 0–3.5). Fatty acids were added from a stock solution bound to BSA at a 3.5 molar ratio and were adjusted to lower ratios by adding fatty acid-free BSA. To induce ABCA1, 0.5 mM 8-Br-cAMP was added to this medium. To label phospholipids, 1 μCi/ml [*methyl*-^{3}H]choline chloride (PerkinElmer Life Sciences) was added to this medium followed by two washes of cells (38). In some experiments, ABCA1 was induced by adding 10 μM 22(*R*)-hydroxycholesterol plus 10 μM 9-*cis*-retinoic acid to the acetylated LDL medium.

To measure lipid efflux, cells were incubated with DMEM/BSA with or without 5 μg/ml apoA-I for 2 h at 37 °C and chilled on ice, and the medium was collected and centrifuged to remove detached cells. For cholesterol efflux, the medium was counted for ^{3}H, and the cells were assayed for free and esterified [^{3}H]cholesterol after isolation by thin layer chromatography (38). For phospholipid efflux, medium and cellular choline-labeled phospholipids were extracted in chloroform:methanol and assayed for ^{3}H radioactivity (38). ApoA-I-mediated lipid efflux was calculated as the percent total ^{3}H-lipid released into the medium after subtraction of values obtained in the absence of apoA-I.

Cell-surface and ABCA1 Binding of ApoA-I—For the whole-cell binding assay, cells were incubated for 2 h at 0 °C with HEPES-buffered DMEM/BSA containing 1 μg/ml ^{125}I-apoA-I plus or minus 200 μg/ml unlabeled apoA-I and then washed at 0 °C twice with PBS/BSA and twice with PBS (38, 39). Cell-associated radioactivity and cell protein were measured after digestion in 0.2 N NaOH. Results are expressed as nanograms of ^{125}I-apoA-I per mg of cell protein after subtraction of values in the presence of unlabeled HDL. For the ABCA1 binding studies, cholesterol-loaded cells were incubated for 16 h with 8-Br-cAMP followed by 6-h incubations with 8-Br-cAMP minus or plus fatty acids. After an additional 2-h incubation with 5 μg/ml ^{125}I-apoA-I at 0 °C, cells were washed twice with ice-cold PBS, incubated for 30 min at 0 °C with PBS containing 1 mg/ml DSS (cross-linking agent), washed twice with cold PBS containing 20 mM glycine, and extracted with detergent for ABCA1 immunoprecipitation and SDS-PAGE (21).

Membrane and Metabolic Labeled ABCA1—For selective labeling of plasma membrane ABCA1, macrophages were incubated for 30 min at 0 °C with PBS containing 1 mg/ml sulfo-NHS-biotin to biotinylate cell-surface proteins (11, 21, 40). For metabolic labeling, cells were incubated for 15 min at 37 °C with DMEM/BSA containing 100 μCi/ml [^{35}S]methionine (Amersham Biosciences). Cells were washed and dislodged from the dish at 0 °C in buffer containing protease inhibitors. To isolate ABCA1, cell proteins were solubilized in PBS containing 1% Triton X-100 plus protease inhibitors, and the extract was incubated overnight at 4 °C with antiserum (1:200 dilution) raised against a synthetic peptide corresponding to the deduced 22-amino acid C terminus of ABCA1 (11, 21, 40). The antibody-antigen complex was isolated by protein A-coated magnetic beads (Dynal) and electrophoresed in SDS using a 6% polyacrylamide gel. Each gel lane received immunoprecipitated protein corresponding to equal amounts of cells. [^{35}S]Methionine-labeled ABCA1 was detected on dried gels by PhosphorImaging (Cyclone, Packard Instrument Co.). To measure biotinylated ABCA1, proteins were transferred to nitrocellulose and identified with a streptavidin-horseradish peroxidase ECL assay (Bio-Rad). For immunoblots of whole membrane ABCA1, microsomal membranes were isolated from homogenized cells by ultracentrifugation; membrane proteins were solubilized in SDS buffer and resolved by SDS-PAGE, and ABCA1 was identified by immunoblot analysis (21). Equal amounts of membrane protein were added per gel lane.

Northern Blot Analysis—Total RNA was isolated from J774 cells by the single step method using Trizol Reagent (Invitrogen). mRNA was isolated from total RNA using Poly(A)Ttrack System IV (Promega). 5 μg of mRNA was loaded in each lane of a 1.2% agarose gel containing formaldehyde and formamide. RNA was then transferred to nylon membranes and cross-linked. The cDNA probes for ABCA1 and glyceraldehyde-3-phosphate dehydrogenase genes were generated by PCR and labeled with [α-^{32}P]dCTP using random hexamers (Amersham Biosciences) and Klenow (New England Biolabs). The relative abundance of ABCA1 transcripts was determined using a PhosphorImager and normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA.

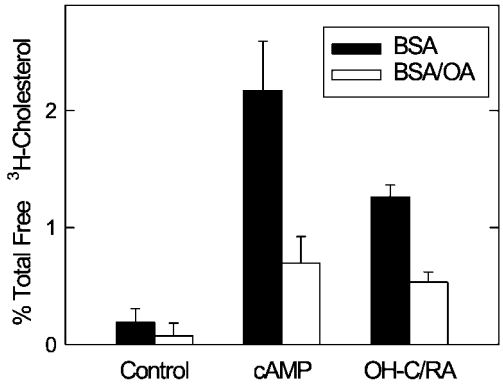

FIG. 1. **Oleate inhibits cholesterol efflux.** J774 macrophages were incubated for 24 h with 50 μg/ml acetylated LDL plus [^{3}H]cholesterol without (*Control*, *cAMP*) or with 10 μM 22(*R*)-hydroxycholesterol, 10 μM 9-*cis*-retinoic acid (*OH-C/RA*) followed by 20-h incubations with either BSA alone (5 mg/ml) or 250 μM oleate (*OA*) bound to BSA (3.5 molar ratio). Where indicated (*cAMP*), 0.5 mM 8-Br-cAMP was added to the oleate medium. ApoA-I-mediated [^{3}H]cholesterol efflux was measured during subsequent 2-h incubations as described under "Experimental Procedures." Each value is the mean ± S.D. of triplicate incubations expressed as percent total (medium plus cell) [^{3}H]cholesterol (mean 91,565 cpm/well).

ABCA1 Degradation Rate—J774 macrophages were cholesterol loaded with acetylated LDL for 24 h. After overnight incubation with DMEM/BSA containing 0.5 mM 8-Br-cAMP, cells were washed and incubated for 2 h at 37 °C in DMEM containing 5 mg/ml BSA alone or with fatty acids followed by incubations with the same medium containing 20 μg/ml cycloheximide. After the indicated times, ABCA1 was measured by immunoblot analysis.

RESULTS

Unsaturated Fatty Acids Inhibit ApoA-I-mediated Lipid Efflux—Induction of *ABCA1* expression in macrophages by either cAMP analogs (21, 22) or LXR/RXR ligands (15–17) markedly increases apoA-I-mediated cholesterol and phospholipid efflux. To examine the effects of fatty acids on this efflux, we induced *abca1* expression in cholesterol-loaded murine J774 macrophages with either 8-Br-cAMP or 22(*R*)-hydroxycholesterol/9-*cis*-retinoic acid (LXR/RXR ligands), and we measured the effects of oleate treatment on apoA-I-mediated [^{3}H]cholesterol efflux. We observed that oleate inhibited apoA-I-mediated cholesterol efflux when ABCA1 was induced by both methods (Fig. 1). Incubation with oleate for 20 h resulted in more than a 50% decrease in cholesterol efflux. Under these conditions, 30–40% of the total radiolabeled cholesterol was esterified, and this was unchanged by oleate treatment (not shown), indicating there was an adequate supply of fatty acids available for cholesterol esterification both in the absence and presence of exogenous oleate. Thus, a depletion of intracellular cholesterol by oleate could not account for the reduced cholesterol efflux.

We assessed the specificity of the inhibitory effects of fatty acids on apoA-I-mediated cholesterol efflux by incubating cells with different unsaturated and saturated fatty acids. We also measured their effects on choline-labeled phospholipid efflux and apoA-I binding to cells, two other processes dependent on *ABCA1* expression (21, 41, 42). The unsaturated fatty acids palmitoleate, oleate, linoleate, and arachidonate decreased apoA-I-mediated cholesterol (Fig. 2*A*) and phospholipid (Fig. 2*B*) efflux and reduced cell-surface apoA-I binding (Fig. 2*C*). In contrast, the saturated fatty acids octanoate, palmitate, and stearate had no significant effect on lipid efflux or apoA-I binding. Thus, only unsaturated fatty acids inhibited these ABCA1-mediated processes. These different fatty acids had no effect on incorporation of [^{3}H]choline into total phospholipids

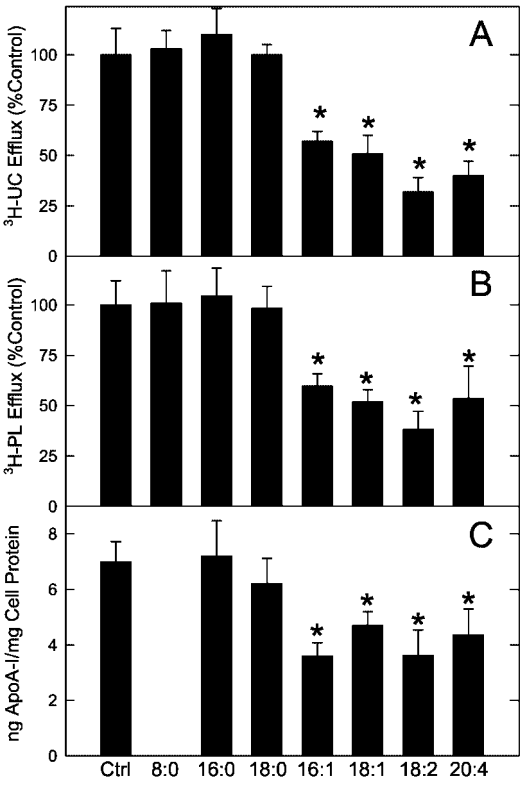

FIG. 2. **Different unsaturated fatty acids inhibit lipid efflux and apoA-I binding.** *A* and *B*, cholesterol-loaded macrophages were incubated for 16 h with 8-Br-cAMP and either 5 mg/ml BSA alone or BSA plus the indicated fatty acids (BSA molar ratio of 1.8), and apoA-I-mediated [^{3}H]cholesterol (*UC*, *A*) and [^{3}H]choline-labeled phospholipid (*PL*, *B*) efflux were measured during subsequent 2-h incubations as described under "Experimental Procedures." Each value represents the mean ± S.D. of 3–6 incubations expressed as percent control lipid efflux values (1331 and 817 cpm per well for [^{3}H]cholesterol and [^{3}H]phospholipid, respectively). *C*, cholesterol-loaded macrophages were incubated for 20 h with 8-Br-cAMP in the presence or absence of different fatty acids (BSA molar ratio of 3.5), and ^{125}I-apoA-I binding was assayed after subsequent 2-h incubations at 0 °C. Octanoate was not tested for effects on binding. Each value is the mean ± S.D. of quadruplicates. *Asterisks* indicate significant ($p < 0.03$) differences from controls.

(not shown), presumably because endogenous supplies of fatty acids were sufficient for maximum production of choline-labeled phospholipids. We observed the same fatty acid specificity for inhibition of cholesterol efflux from RAW264.7 cells, another murine macrophage line (data not shown). The carnitine palmitoyltransferase inhibitor perhexiline had no effect on the fatty acid inhibition of cholesterol efflux (data not shown), indicating that it does not require mitochondrial uptake and oxidation. None of these fatty acids changed the cell protein content per well, indicating that they were not cytotoxic.

Oleate and linoleate inhibited apoA-I-mediated cholesterol and phospholipid efflux in a concentration-dependent manner (Fig. 3*A*). At the same concentrations, linoleate inhibited lipid efflux more effectively than oleate. This inhibition occurred over a low (0.5) to high (1.5) physiologic range of plasma unsaturated fatty acid to albumin molar ratio.

We compared time courses for the inhibitory effects of these two fatty acids by treating cells for various times with fatty acids and measuring apoA-I-mediated cholesterol efflux during subsequent 2-h incubations. Oleate significantly decreased cholesterol efflux after only 2 h of treatment, but the maximum effect occurred after 16 h (Fig. 3*B*). In contrast, linoleate maximally suppressed cholesterol efflux after only 4 h, indicating that it inhibited this pathway more acutely than oleate.

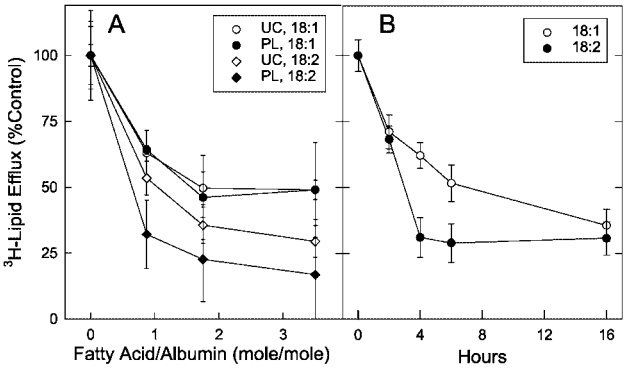

FIG. 3. **Oleate and linoleate inhibitory effects on cholesterol efflux are concentration- and time-dependent.** *A*, cholesterol-loaded and 8-Br-cAMP-treated macrophages were incubated for 16 h with 8-Br-cAMP and 5 mg/ml BSA plus 0–250 μM oleate or linoleate. *B*, macrophages were incubated for the indicated times with 8-Br-cAMP and 5 mg/ml BSA plus 125 μM oleate or linoleate (1.8 BSA molar ratio). Prior to the fatty acid treatments, cells were incubated for 16 to 0 h with 8-Br-cAMP to maintain a constant period of exposure to this inducer. ApoA-I-mediated efflux of [^{3}H]cholesterol (*UC*) and [^{3}H]choline-labeled phospholipids (*PL*) was measured during subsequent 2-h incubations. Each value is the mean ± S.D. of triplicates expressed as percent control (1152 and 879 cpm/well for [^{3}H]cholesterol and [^{3}H]phospholipid, respectively) or zero time lipid efflux values (1248 and 1189 cpm [^{3}H]cholesterol/well for oleate- and linoleate-treated cells, respectively).

Unsaturated Fatty Acids Reduce Cellular ABCA1—Because ABCA1 controls the rate of apoA-I-mediated lipid efflux, we assayed the effects of fatty acids on the cell membrane content of ABCA1. Incubating cells with unsaturated fatty acids caused a significant decrease in membrane ABCA1 (Fig. 4*A*). In contrast, saturated fatty acids had no or little effect on ABCA1 levels. As with lipid efflux, oleate and linoleate reduced ABCA1 membrane protein in a concentration-dependent manner (Fig. 4*B*). We compared the effects of stearate and linoleate on the plasma membrane content of ABCA1 by treating cells for 6 h with fatty acids, biotinylating cell-surface proteins, isolating ABCA1 by immunoprecipitation, and assaying for biotinylated (cell-surface) ABCA1 with a streptavidin probe. Results showed that linoleate, but not stearate, reduced both the total and plasma membrane content of ABCA1 (Fig. 4*C*).

We tested whether apoA-I binding to ABCA1 is reduced by unsaturated fatty acid treatment, which could account for the lower apoA-I binding to whole cells (Fig. 2*C*). For these studies, we incubated cells for 6 h with no fatty acid, stearate, or linoleate and then for 2 h at 0 °C with ^{125}I-apoA-I. ApoA-I binding to ABCA1 was determined by treating cells with the cross-linking agent DSS, isolating ABCA1 by immunoprecipitation, and detecting iodinated ABCA1 by PhosphorImaging. Results showed a single band with a molecular mass greater than 250 kDa that was markedly reduced by linoleate treatment (Fig. 4*D*). In contrast, stearate had no effect on the amount of ^{125}I-apoA-I cross-linked to ABCA1. Taken together, these studies show that unsaturated, but not saturated, fatty acids reduce the total membrane content of ABCA1 in J774 macrophages, leading to less plasma membrane ABCA1 available for interactions with apoA-I.

Unsaturated Fatty Acids Do Not Affect ABCA1 mRNA Abundance—We conducted Northern blot analysis to test whether the decrease in ABCA1 levels caused by unsaturated fatty acids is due to a decrease in ABCA1 mRNA. Treatment with saturated or unsaturated fatty acids did not significantly alter the cell content of ABCA1 mRNA (Fig. 5), implying that the inhibitory effects of unsaturated fatty acids were not at the level of *ABCA1* transcription or message stability.

Unsaturated Fatty Acids Enhance ABCA1 Degradation—We

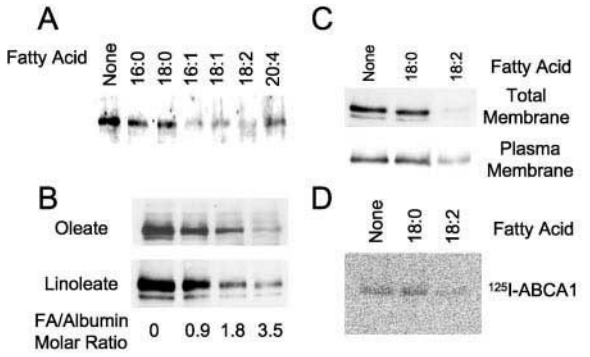

FIG. 4. **Unsaturated fatty acids reduce the membrane content and apoA-I binding activity of ABCA1.** *A*, cholesterol-loaded macrophages were incubated for 16 h with 8-Br-cAMP and 5 mg/ml BSA in the absence or presence of different fatty acids (125 μM, 1.8 BSA molar ratio). Immunoblot analysis of membrane ABCA1 was conducted as described under "Experimental Procedures." Results represent two similar experiments. *B*, same as in *A*, except that the fatty acids (*FA*) were oleate and linoleate at different concentrations (0, 62.5, 125, and 250 μM). *C*, cholesterol-loaded macrophages were incubated for 16 h with 8-Br-cAMP followed by 6 h with 8-Br-cAMP in the absence or presence of 125 μM stearate or linoleate bound to 5 mg/ml BSA. Cells were treated with sulfo-*N*-hydroxysuccinimide-biotin; ABCA1 was isolated by immunoprecipitation, and biotinylated ABCA1 was detected by streptavidin ECL. Whole membrane ABCA1 was identified as in *A* and *B*. *D*, macrophages were treated as in *C* followed by 2-h incubations at 0 °C with ^{125}I-apoA-I and cross-linking with DSS. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and ^{125}I-labeled ABCA1 was detected by PhosphorImaging.

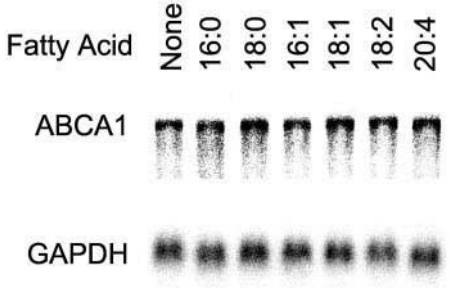

FIG. 5. **Fatty acids do not alter ABCA1 mRNA.** Macrophages were incubated for 16 h with 8-Br-cAMP and 5 mg/ml BSA minus or plus different fatty acids (125 μM). Northern blot analysis was conducted as described under "Experimental Procedures." Results are representative of two similar experiments.

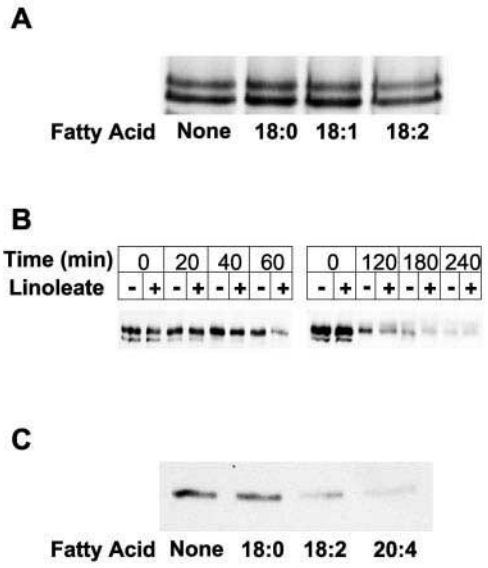

FIG. 6. **Unsaturated fatty acids do not inhibit ABCA1 synthesis but increase its degradation rate.** *A*, cholesterol-loaded macrophages were incubated for 16 h with 8-Br-cAMP in the absence or presence of stearate, oleate, or linoleate (125 μM). Cell proteins were radiolabeled with [^{35}S]methionine (100 μCi/ml) for 15 min; ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and ^{35}S-labeled ABCA1 was detected by autoradiography. Results are representative of two similar experiments. *B*, cholesterol-loaded macrophages were incubated for 16 h with 8-Br-cAMP followed by 2-h incubations with 8-Br-cAMP in the absence or presence of linoleate (125 μM, 1.8 BSA molar ratio). Cycloheximide (20 μg/ml) was added to the medium, and membrane ABCA1 levels were assayed by immunoblot analysis after additional 0–240-min incubations. The 0–60- (*left*) and 0–240-min (*right*) time courses are from separate experiments. *C*, same as in *B*, except that different fatty acids were compared, and the incubation time with cycloheximide was 60 min.

then examined the possibilities that unsaturated fatty acids either inhibit translation or enhance degradation of ABCA1. To measure their effects on translation, we pretreated cells for 16 h with or without fatty acids, incubated cells for 15 min with [^{35}S]methionine, and measured incorporation of radiolabel into immunoisolated ABCA1. This short term incubation with [^{35}S]methionine more extensively labeled the lower band of the ABCA1 doublet frequently seen by immunoblot analysis (Fig. 6*A*), consistent with this band being a precursor form (*e.g.* nonglycosylated) of ABCA1. Pretreating cells with stearate, oleate, or linoleate did not significantly affect incorporation of methionine into either band (Fig. 6*A*). Therefore, inhibition of translation efficiency cannot account for the fatty acid-induced decrease in ABCA1 levels.

To address the possibility that ABCA1 degradation rates are affected by fatty acids, we incubated cells with or without linoleate for 2 h, added cycloheximide to arrest protein synthesis, and monitored changes in ABCA1 levels during the cycloheximide treatment. During the first 40 min of cycloheximide treatment, only the lower band of the ABCA1 doublet disappeared, probably due to conversion of precursor to mature protein, and this was unaffected by linoleate treatment (Fig. 6*B*). After 60 min with cycloheximide, the mature form of ABCA1 progressively decreased so that it was barely detectable by 240 min. Linoleate treatment markedly reduced ABCA1 levels at the 60-, 120-, and 180-min time points. At the 60-min time point, both linoleate and arachidonate decreased ABCA1 levels, whereas stearate had no effect (Fig. 6*C*). These results indicate that unsaturated, but not saturated, fatty acids enhance the degradation rate of preformed ABCA1.

DISCUSSION

Metabolic factors that modulate ABCA1 activity are likely to have a major impact on disposal of tissue cholesterol and susceptibility to atherosclerosis. Previous studies (6, 7) revealed that *ABCA1* expression is positively regulated at the level of transcription by both sterols and cAMP analogs. Here we show that unsaturated fatty acids impair the activity of this lipid removal pathway by reducing the cellular content of ABCA1.

Oleate, the most abundant plasma fatty acid, suppressed apoA-I-mediated cholesterol and phospholipid efflux from J774 macrophages by 50–70%, whether we induced ABCA1 with LXR/RXR ligands or a cAMP analog. We observed similar effects with other macrophage lines, including murine RAW264.7 and human THP-1 cells (data not shown). Monounsaturated (palmitoleate and oleate), diunsaturated (linoleate), and polyunsaturated (arachidonate) fatty acids all inhibited lipid efflux from cAMP-treated macrophages, whereas saturated fatty acids (octanoate, palmitate, and stearate) had no effect. Thus, these inhibitory effects have broad specificity for unsaturated fatty acids. Linoleate tended to inhibit lipid efflux more effectively than oleate, suggesting that the degree of unsaturation is also an important factor. These inhibitory effects were associated with a reduced membrane content of ABCA1 and a de-

crease in apoA-I binding to whole cells and to ABCA1. Both oleate and linoleate progressively inhibited cholesterol and phospholipid efflux and reduced ABCA1 levels with increasing ratios of fatty acid to albumin, and this was over a physiologic range for plasma unsaturated fatty acids.

Unsaturated fatty acids appeared to reduce the membrane content of ABCA1 by increasing its degradation. Fatty acids did not change ABCA1 mRNA abundance in cAMP-treated macrophages, indicating they had no net effect on mRNA synthesis and degradation rates. The conclusion that unsaturated fatty acids can reduce ABCA1 activity without inhibiting transcription was further supported by results showing that they decreased cholesterol efflux from transfected baby hamster kidney cells (43) forced to overexpress ABCA1 (data not shown). Fatty acids also did not appear to alter ABCA1 translation efficiency, as evidenced by no effect on methionine incorporation into newly synthesized ABCA1. When protein synthesis was blocked by cycloheximide, however, acute treatment of cells with unsaturated fatty acids enhanced the rate of turnover of preformed ABCA1. Thus, with cAMP-treated J774 macrophages, protein degradation was the only step identified in the ABCA1 biosynthetic pathway affected by fatty acids, making this the likely mechanism for the reduced ABCA1 levels.

This fatty acid-stimulated degradation of ABCA1 presumably involves proteolytic processing. ABCA1 is recycled rapidly between the plasma membrane and late endosomes and lysosomes (44), which are likely sites for ABCA1 degradation. Fatty acids might disrupt ABCA1 trafficking so as to direct a higher fraction of the protein to these intracellular compartments. Unsaturated fatty acids can elicit intracellular signals (45), which may play a role in altering ABCA1 trafficking or proteolysis. Changes in membrane fluidity caused by an altered fatty acid composition of phospholipids could also destabilize ABCA1, leading to its increased degradation. Membrane fluidization has been shown to impair the activity of another ABC transporter (46–48).

The current study reveals yet another link between cholesterol trafficking and fatty acid metabolism. Previous studies have shown that LXR/RXR ligands induce both ABCA1 (15–18) and SREBP-1c (26, 27), a transcription factor that regulates several enzymes of fatty acid synthesis and desaturation. Unsaturated, but not saturated, fatty acids reduce the mRNA levels of SREBP-1a and SREBP-1c and inhibit proteolytic processing of these isoforms (28, 49, 50). We found a similar specificity for the inhibitory effects of fatty acids on ABCA1. The reason for this association between regulation of sterol trafficking and fatty acid metabolism is unclear, but it may reflect homeostatic mechanisms that modulate the physical properties of membranes.

A recent study (28) showed that unsaturated fatty acids can act as competitive antagonists of LXR in cultured rat hepatoma and human embryonic kidney cell lines. This appears to account for much of the fatty acid-inhibited transcription of *SREBP-1c*. Because LXR ligands induce ABCA1, unsaturated fatty acids could also inhibit *ABCA1* transcription in oxysterol-treated cells, a possibility we have yet to test in detail. We found, however, that oleate was no more effective in inhibiting cholesterol efflux from cells treated with LXR/RXR ligands than from cells treated with 8-Br-cAMP, which activates *ABCA1* transcription by an LXR-independent mechanism (15, 40). This may be because fatty acids do not block LXR activation in the presence of high concentrations of LXR ligand (28). Nevertheless, these findings suggest that unsaturated fatty acids have the potential for reducing cellular ABCA1 levels by multiple mechanisms, similar to what has been described for SREBPs (28, 49, 50).

These studies have important clinical implications. Type 2 diabetes and insulin resistance are characterized by elevated fatty acids, low plasma HDL levels, and prevalent cardiovascular disease (2, 3, 29). In addition, arterial macrophages produce lipoprotein lipase (31), which can locally generate fatty acids. Macrophage lipoprotein lipase has been reported to be atherogenic (32–34) and to be induced in diabetes (35). Type 2 diabetic patients have a selective increase in oleate levels in serum lipids (30). Our findings raise the possibility that impaired ABCA1-mediated cholesterol secretion from macrophages may contribute to the enhanced atherosclerosis associated with metabolic disorders that elevate fatty acid levels in the artery wall.

Nutritional studies have shown that different fatty acids have diverse effects on lipoprotein metabolism. It is believed that substituting dietary saturated fatty acids with *cis*-unsaturated fatty acids protects against cardiovascular disease by lowering plasma LDL levels (51). Our results suggest that, although reducing atherogenic particles, these dietary manipulations may suppress cholesterol efflux from macrophages. This may partially explain why a meta-analysis of clinical trials showed only a small cardiovascular risk benefit with modified dietary fat intake (52). Moreover, one of the cellular enzymes induced by LXR activators is stearoyl-CoA desaturase (27), which converts saturated fatty acids to monounsaturated fatty acids. This implies that cells with activated LXRs have the ability to generate ABCA1 inhibitors from saturated fatty acids. Thus, changes in total fatty acid supply rather than composition may have the greatest impact on the ABCA1 pathway in cholesterol-loaded macrophages.

The current study also has important implications about therapeutic approaches for treating cardiovascular disease. Based on their ability to stimulate *ABCA1* transcription, LXRs have become attractive targets for drug development. These nuclear receptors, however, also stimulate fatty acid production and desaturation. Our findings predict that this will counteract the transcriptional activation of *ABCA1* by reducing the actual ABCA1 protein content. Thus, an understanding of the mechanisms by which fatty acids increase ABCA1 degradation will be critical for designing therapeutic interventions that maximize the activity of this cholesterol removal pathway.

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