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

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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 may play 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 (SREBP) 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, steroid 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-1 was purified from HDL,
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FIG. 1. Oleate inhibits cholesterol efflux. J774 macrophages were incubated for 24 h with 50 μg/ml acetylated LDL plus [3H]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 μM 8-Br-cAMP was added to the oleate medium. ApoA-I-mediated [3H]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) [3H]cholesterol (mean 91,565 cpm/well).

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 [3H]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. 2A) and phospholipid (Fig. 2B) efflux and reduced cell-surface apoA-I binding (Fig. 2C). 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 [3H]choline into total phospholipids.
Unsaturated Fatty Acids Inhibit ABCA1

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 [3H]cholesterol ([CH]cholesterol, UC, A) and [3H]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 [3H]cholesterol and [3H]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 125I-apoA-I binding was assayed after subsequent 2-h incubations at 0 °C. Octanole was not tested for effects on binding. Each value is the mean ± S.D. of quadruplicate. 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 carni	ine 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 cytoxic.

Oleate and linoleate inhibited apoA-I-mediated cholesterol and phospholipid efflux in a concentration-dependent manner (Fig. 3A). 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. 3B). In contrast, linoleate maximally suppressed cholesterol efflux after only 4 h, indicating that it inhibited this pathway more acutely than oleate.

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 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
 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 [35S]-apoA-I and cross-linking with DSS. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and [35S]-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.

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 [35S]methionine, and measured incorporation of radiolabel into membrane ABCA1 and apoA-I binding activity of ABCA1.

B. D. macrophages were treated as in C followed by 2-h incubations at 0 °C with [35S]-apoA-I and cross-linking with DSS. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and [35S]-labeled ABCA1 was detected by PhosphorImaging.

Unsaturated 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 [35S]methionine (100 μCi/ml) for 15 min; ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and [35S]-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.

6B). 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. 6C). 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 apoAI-mediated cholesterol and phospholipid efflux from J774 macrophages by 50–70%, whether we induced ABCA1 with LXRxRXR 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 LXR 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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