

Unsaturated Fatty Acids Phosphorylate and Destabilize ABCA1 through a Phospholipase D2 Pathway*

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Abnormal high density lipoprotein (HDL) metabolism among patients with diabetes and insulin resistance may contribute to their increased risk of atherosclerosis. ATP-binding cassette transporter ABCA1 mediates the transport of cholesterol and phospholipids from cells to HDL apolipoproteins and thus modulates HDL levels and atherogenesis. Unsaturated fatty acids, which are elevated in diabetes, impair the ABCA1 pathway in cultured cells by destabilizing ABCA1 protein. Here we examined the cellular pathway that mediates the ABCA1 destabilizing effects of fatty acids. The long-chain acyl-CoA synthetase inhibitor triacsin C completely reversed fatty acid-induced ABCA1 destabilization, indicating that fatty acids need to be activated to their CoA derivatives to enhance ABCA1 degradation. Unsaturated but not saturated fatty acids stimulated phospholipase D (PLD) activity, the PLD inhibitor 1-butanol prevented the unsaturated fatty acid-induced reduction in ABCA1 levels, and the PLD2 activator mastoparan markedly reduced ABCA1 protein levels, implicating a role for PLD2 in the ABCA1 destabilizing effects of fatty acids. Unsaturated fatty acids and mastoparan increased phosphorylation of ABCA1 serines. PLD2 small interfering RNA abolished the ability of unsaturated fatty acids to inhibit lipid transport activity, to reduce protein levels, and to increase serine phosphorylation of ABCA1. The diacylglycerol analog oleoylacetyl-glycerol also reduced ABCA1 protein levels and increased its serine phosphorylation, suggesting that PLD2-generated diacylglycerols promote the destabilizing phosphorylation of ABCA1. These data provide evidence that intracellular unsaturated acyl-CoA derivatives destabilize ABCA1 by activating a PLD2 signaling pathway.

Population studies have revealed an inverse relationship between plasma high density lipoprotein (HDL)² levels and risk for cardiovascular disease, implying that factors associated with HDL metabolism are atheroprotective. One of these factors is ATP-binding cassette transporter A1 (ABCA1), an integral membrane protein that mediates the transport of cellular cholesterol and phospholipids to lipid-deficient HDL apolipoproteins (1, 2). ABCA1 is a member of a large family of ABC transporters and contains two ATP-binding domains and two six-helix transmembrane domains (3). Mutations in ABCA1 cause Tangier disease (4–7), which is characterized by a severe HDL deficiency, rapid

clearance of apoA-I from the plasma, deposition of sterols in tissue macrophages, and prevalent atherosclerosis (8, 9). Lipidation of apoA-I by ABCA1 is the key rate-limiting step in generating plasma HDL (10–12).

ABCA1 expression is tightly regulated. In macrophages, cholesterol loading markedly increases ABCA1 mRNA and protein levels (7, 13), consistent with a transporter that functions to export excess cholesterol. The transcription of this gene is regulated by nuclear liver X receptors (LXR α and LXR β) and retinoid X receptor (RXR) (14–17), which form heterodimers that are activated by oxysterols and retinoic acid, respectively (18). Analogs of cAMP are also known to activate ABCA1 gene transcription by mechanisms distinct from the LXR/RXR system (19–21).

Patients with diabetes have an elevated risk of cardiovascular disease. It is possible that diabetes-associated metabolic factors impair ABCA1 expression or activity and contribute to the higher mortality rate among those patients. Our previous studies showed that long-chain unsaturated fatty acids but not saturated fatty acids decrease ABCA1 expression in murine macrophages by increasing its protein degradation rate (22). Furthermore, we also showed that LXR agonists induce stearyl-CoA desaturase in macrophages and convert long chain saturated fatty acids into monounsaturated fatty acids that promote ABCA1 degradation (23).

One of the signaling pathways regulated by fatty acids is phospholipase D (PLD). PLD is a ubiquitous enzyme that catalyzes the hydrolysis of phosphatidylcholine to generate the second messenger phosphatidic acid and choline (24). Two PLD genes have been cloned in mammalian cells, *PLD1* (25) and *PLD2* (26). PLD2 basal activity is about 1000-fold higher than PLD1 (27). Unsaturated fatty acids and the wasp venom amphipathic α -helical peptide, mastoparan, selectively activate PLD2 (28, 29).

In the current study, we examined the mechanism by which unsaturated fatty acids destabilize ABCA1 protein. We found that linoleate-induced ABCA1 degradation was associated with increased PLD2 activity and phosphorylation of ABCA1 serines. Inhibiting PLD2 with 1-butanol or depleting PLD2 with siRNA reduced or abolished the ability of linoleate to promote ABCA1 phosphorylation and degradation. Thus, unsaturated fatty acids activate a PLD2 signaling pathway that phosphorylates ABCA1 serines and destabilizes the protein.

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 (30). ApoA-I was purified from HDL and delipidated as described previously (30). LDL was acetylated by the method of Goldstein *et al.* (31).

Cell Culture and Lipid Efflux—Baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 were generated as described previously (32). Murine J774 and RAW 264.7 macrophages

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² The abbreviations used are: HDL, high density lipoprotein; LXR, liver X receptor; RXR, retinoid X receptor; PLD, phospholipase D; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; LDL, low density lipoprotein; 8-Br-cAMP, 8-bromo-cAMP; DAG, diacylglycerol; ABCA1, ATP-binding cassette transporter A1; BHK, baby hamster kidney; siRNA, small interfering RNA; PIPES, 1,4-piperazinediethanesulfonic acid.

and ABCA1-expressing BHK cells 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 μ Ci/ml [3 H]cholesterol (PerkinElmer Life Sciences) was added to the growth medium (BHK cells) or to DMEM/BSA containing 50 μ g/ml acetylated LDL (J774 and RAW 264.7 macrophages) 24 h immediately preceding treatments (32, 22). Washed cells were then incubated for 16 h with medium containing 5 mg/ml BSA in the presence or absence of 125 μ M fatty acids (molar ratios to BSA of 0–1.8) or 100 μ M diacylglycerol (DAG) analogs. 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 the medium for J774 and RAW 264.7, and 10 nM mifepristone was added to the medium for ABCA1-expressing BHK. To label phospholipids, 1 μ Ci/ml [methyl- 3 H]choline chloride (PerkinElmer Life Sciences) was added to this medium followed by two washes of cells (33).

To measure lipid efflux, cells were incubated with DMEM/BSA with or without 10 μ g/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 (33). For phospholipid efflux, medium and cellular choline-labeled phospholipids were extracted in chloroform:methanol and assayed for 3 H radioactivity (33). ApoA-I-mediated lipid efflux is expressed as the fraction of 3 H-labeled lipid released into the medium after subtraction of values obtained in the absence of apoA-I.

Immunoblot—Cells were washed and dislodged from the dish at 0 °C in buffer containing protease inhibitors. To isolate PLD2 or ABCA1, cell proteins were solubilized in phosphate-buffered saline containing 1% Triton X-100 plus protease inhibitors, and the extract was incubated overnight at 4 °C with antibody against PLD2 or ABCA1 (Novus Biologicals). 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. For immunoblots of PLD2 from RAW 264.7 cells, PLD2 protein was isolated by immunoprecipitation with PLD2 antibody and resolved by SDS-PAGE. For immunoblots of whole membrane ABCA1 from J774 cells, 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 (20). Equal amounts of membrane protein were added per gel lane. For immunoblots of ABCA1 from ABCA1-expressing BHK cells, cell proteins were solubilized in phosphate-buffered saline containing 1% Triton X-100 plus protease inhibitors and equal amounts of protein were added per gel lane.

ABCA1 Phosphorylation—Cells were incubated with phosphate-free/BSA containing [32 P]orthophosphate (0.5 mCi per 60-mm plate) for 2 h to label the endogenous adenosine triphosphate pool (34) followed by incubations with DMEM in the absence or presence of fatty acids. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and 32 P-ABCA1 was visualized by autoradiography. ABCA1 phosphoserines, phosphothreonines, and phosphotyrosines were assayed by immunoblot analysis of immunoprecipitated ABCA1 using phosphoserine (Biomol), phosphothreonine, and phosphotyrosine (Santa Cruz Biotechnology) antibodies that recognize a broad range of serine-, threonine-, and tyrosine-phosphorylated proteins, respectively. ABCA1 serine phosphorylation was quantitated using OptiQuant software (Packard Instrument).

PLD Activity—PLD activity was determined as described (29). BHK cells were incubated in 24-well plates with 2 μ Ci/ml [3 H]myristic acid for 90 min. Cells were then incubated in PIPES-buffered medium in the presence of 1% ethanol for 10 min before the treatment. Radiolabeled phosphatidylethanol was isolated and quantified as described previously (35). The samples were dried under nitrogen and dissolved in 100 μ l of a mixture of chloroform/methanol (2:1) of which 25 μ l was placed on a silica gel-plated sheet for thin layer chromatography in a mixture of chloroform/methanol/glacial acetic acid (65:15:2) (v/v/v). Regions containing phosphatidic acid and phosphatidylethanol were counted for 3 H radioactivity after iodine staining.

PLD2 siRNA Transfection—PLD2 siRNA (Ambion) was transfected into RAW 264.7 macrophages using siPORTTM Lipid (Ambion) as described in the manufacturer's protocol. Briefly, 1 μ l of 20 μ M siRNA was mixed with 41.5 μ l of DMEM/BSA, and 2 μ l of siPORTTM Lipid was diluted in 5.5 μ l of DMEM/BSA and incubated at room temperature for 20 min. After the incubation, the diluted siPORTTM Lipid was combined with the diluted siRNA and incubated for an additional 20 min at room temperature. Total 50 μ l of siRNA-siPORTTM Lipid complexes was applied to 200 μ l DMEM/BSA in each well of a 24-well plate containing cultured RAW 264.7 macrophages at ~70% confluence.

RESULTS

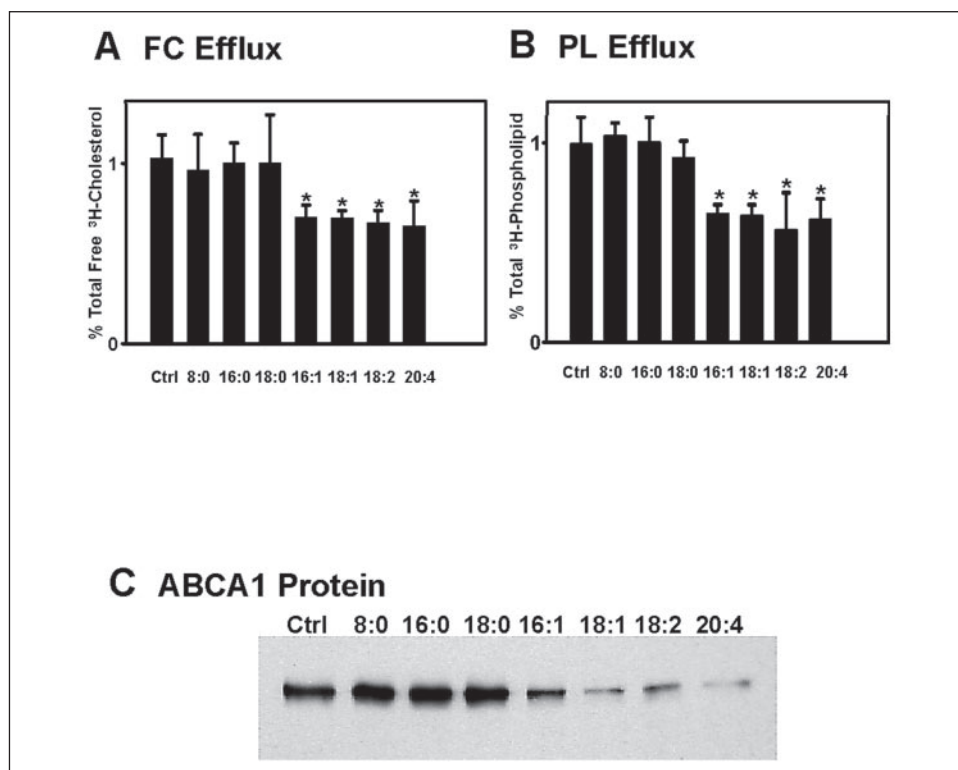
Long-chain Unsaturated Fatty Acids Inhibit ApoA-I-mediated Lipid Efflux in ABCA1-expressing BHK Cells—We previously demonstrated that long-chain unsaturated fatty acids decreased apoA-I-mediated lipid efflux in J774 macrophages in the presence of cAMP analog (22) or LXR/RXR ligands (23) by increasing ABCA1 degradation. To examine the effects of fatty acids in ABCA1-expressing BHK cells, we incubated BHK cells with mifepristone for 16 h in the presence or absence of different fatty acids. To avoid direct interactions of fatty acids with apoA-I, we performed lipid efflux assays during subsequent 2-h incubations with apoA-I but in the absence of fatty acids. Because ABCA1 is continually synthesized during this period, this assay provides a minimum estimate of the damaging effects of fatty acids on ABCA1 function.

Similar to our previous results with 8-Br-cAMP-treated J774 macrophages, unsaturated fatty acids palmitoleate, oleate, linoleate, and arachidonate significantly decreased apoA-I-mediated [3 H]cholesterol (Fig. 1A) and [3 H]phospholipid (Fig. 1B) efflux in ABCA1-transfected BHK. Measurements of ABCA1 protein levels prior to the lipid efflux assays showed a much greater reduction in ABCA1 protein in response to treatment with these fatty acids (Fig. 1C). On the other hand, saturated fatty acids octanoate, palmitate, and stearate had no significant effect on either lipid efflux or ABCA1 levels. These results show that unsaturated fatty acids have the same inhibitory effect in both cAMP-treated murine macrophages and ABCA1-transfected BHK cells.

Formation of CoA Derivatives Is Required for Fatty Acid Inhibition of ABCA1—To test whether free fatty acids need to be metabolized to inhibit ABCA1, we used triacsin C to inhibit long-chain acyl-CoA synthetase, which converts free fatty acids into acyl-CoA as the first step of fatty acid metabolism. Both J774 (Fig. 2, A–C) and ABCA1-transfected BHK cells (Fig. 2, D–F) were incubated with or without triacsin C in the presence or absence of linoleate for 6 h, and apoA-I mediated lipid efflux and ABCA1 protein were measured. Triacsin C completely reversed the inhibitory effects of linoleate on apoA-I mediated cholesterol (Fig. 2, A and D) and phospholipid (Fig. 2, B and E) efflux and ABCA1 protein levels (Fig. 2, C and F). These results indicate that the free fatty acids have to be transported into the cell and metabolized to inhibit ABCA1.

Unsaturated Fatty Acids Increase ABCA1 Serine Phosphorylation—Since it has been shown that phosphorylation plays an important role in

FIGURE 1. Unsaturated fatty acids inhibit lipid efflux and reduce ABCA1 protein levels. A and B, ABCA1-expressing BHK cells were incubated for 16 h in DMEM with 10 nM mifepristone 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 (A) and [3 H]choline-labeled phospholipids (B) efflux were measured during subsequent 2-h incubations as described under "Experimental Procedures." Each value represents the mean \pm S.D. of triplicates. Results are representative of three similar experiments. Asterisks indicate significant ($p < 0.05$) differences from controls. C, ABCA1 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. FC, free cholesterol; PL, phospholipid; Ctrl, control.



ABCA1 regulation (36–38), we tested the effect of different fatty acids on ABCA1 phosphorylation. ABCA1-expressing BHK cells were labeled with 32 P and then incubated with or without different fatty acids for 40 min (Fig. 3A). Unsaturated fatty acids palmitoleate, oleate, and linoleate significantly increased 32 P incorporation into ABCA1 while saturated fatty acids palmitate and stearate had no or little effect, indicating unsaturated but not saturated fatty acids increase ABCA1 phosphorylation. We used phosphoamino acid-specific antibodies to determine whether fatty acids increased phosphorylation of either serine or threonine, two amino acids shown previously to be targets for signaling pathways that alter ABCA1 stability or activity (36, 38). Immunoblots of immunoprecipitated ABCA1 showed that linoleate treatment increased phosphorylation of serine but not threonine (Fig. 3B). ABCA1 tyrosine phosphorylation was also unaffected by linoleate (data not shown). Quantitation by computer analyses revealed that the phosphoserine content of ABCA1 was doubled by linoleate treatment (Fig. 3C).

Time courses revealed that linoleate increased ABCA1 serine phosphorylation within 20 min of treatment (Fig. 3D). After 60 min, there was no difference in the phosphoserine content of ABCA1 in cells treated without or with linoleate, perhaps because of increased degradation of the phosphorylated protein.

Unsaturated Fatty Acids Impair ABCA1 through a PLD2 Pathway—Because fatty acids can activate protein kinases through different signaling pathways, we examined the possibility that phospholipases play a role in the fatty acid-induced phosphorylation and destabilization of ABCA1. J774 cells were incubated with or without different inhibitors of phospholipase pathways in the presence or absence of linoleate for 6 h, and cholesterol efflux was measured (Fig. 4A). The inhibitory effect of linoleate was largely reversed by the PLD inhibitor 1-butanol but not by its isoform 2-butanol, which does not inhibit PLD (39). Neither the phospholipase A2 inhibitor ONO-RS-082 nor the phospholipase C inhibitor D609 affected linoleate inhibition of ABCA1. These results

suggest that a PLD pathway is involved in the fatty acid-mediated impairment of ABCA1 activity.

We also tested the effects of modulators of PLD on fatty acid-induced ABCA1 inhibition in ABCA1-transfected BHK cells. Similar to what we observed in J774 cells, 1-butanol reversed the ability of linoleate to reduce ABCA1 cholesterol efflux activity (Fig. 4B) and protein levels (Fig. 4C). As with linoleate, treatment with the PLD2-specific activator mastoparan alone reduced apoA-I-mediated cholesterol efflux and ABCA1 protein levels, whereas the control mastoparan-17, which does not activate PLD, had no effect. Thus, PLD2 appears to be the PLD isoform that is involved in the fatty acid destabilizing effects on ABCA1.

We measured PLD activity and ABCA1 serine phosphorylation in response to these various treatment protocols. Linoleate treatment caused a $\sim 100\%$ increase in PLD activity, which was prevented by 1-butanol but not by 2-butanol. Mastoparan, but not mastoparan-17, increased PLD activity by more than 4-fold, and this was further increased by addition of linoleate (Fig. 5A). The saturated fatty acid stearate had no effect on PLD activity. The time course for the stimulatory effect of linoleate on PLD activity showed that linoleate increased PLD activity within 5 min, with the maximum effect occurring after 15 min of treatment (Fig. 5B). Linoleate increased serine phosphorylation when added alone or to medium containing either 2-butanol or mastoparan-17, but it had no effect in the presence of 1-butanol (Fig. 5C). Mastoparan alone increased ABCA1 serine phosphorylation, and this was modestly increased by addition of linoleate. These results suggest that unsaturated fatty acids increase phosphorylation of ABCA1 serines by activating a PLD2 pathway.

To confirm that PLD2 activation is important for the ABCA1 destabilizing effects of unsaturated fatty acids, we knocked down PLD2 expression and measured the effects of linoleate on ABCA1 activity and levels. Because of their higher transfection efficiency, we used murine RAW 264.7 macrophages for these studies. Linoleate treatment did not increase PLD2 protein levels (Fig. 6A), consistent with an effect on activ-

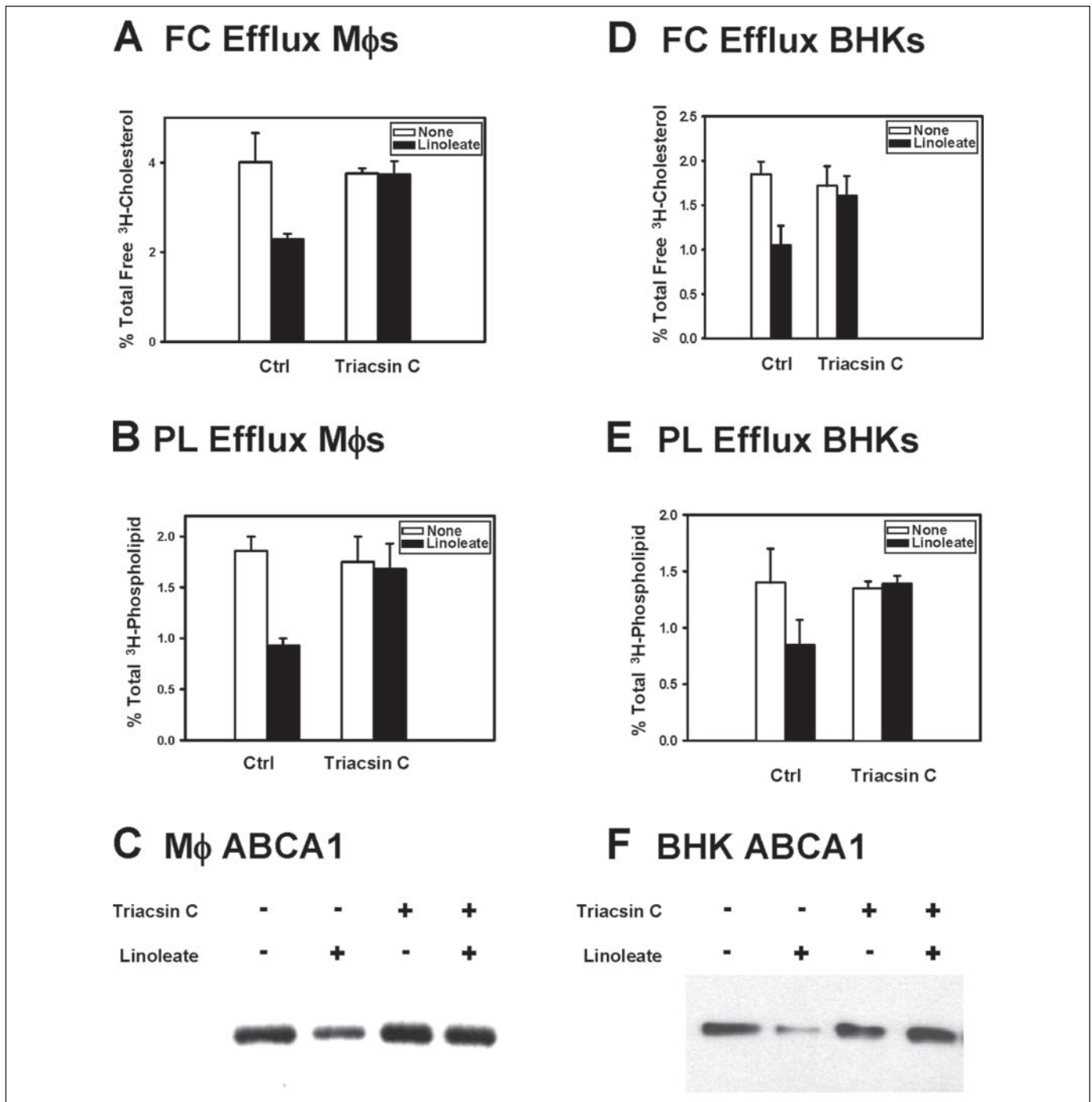


FIGURE 2. CoA acylation is required for fatty acid inhibition of ABCA1. A and B, cholesterol-loaded J774 macrophages were incubated for 16 h with 0.5 mM 8-Br-cAMP and 1 mg/ml BSA, then incubated for 6 h with 0.5 mM 8-Br-cAMP and either 5 mg/ml BSA alone or BSA plus 125 μ M linoleate (BSA molar ratio of 1.8) in the presence or absence of 10 μ M triacsin C. ApoA-I-mediated [3 H]cholesterol (A) and [3 H]phospholipid (B) efflux were measured during subsequent 2-h incubations. Each value is the mean \pm S.D. of triplicates. Results are representative of two similar experiments. C, membrane ABCA1 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. D and E, ABCA1-expressing BHK cells were incubated for 16 h with 10 nM mifepristone and 1 mg/ml BSA, then incubated for 6 h with 10 nM mifepristone and either 5 mg/ml BSA alone or BSA plus 125 μ M linoleate (BSA molar ratio of 1.8) in the presence or absence of 10 μ M triacsin C. ApoA-I-mediated [3 H]cholesterol (D) and [3 H]phospholipid (E) efflux were measured during subsequent 2-h incubations. Each value is the mean \pm S.D. of triplicates. Results are representative of two similar experiments. F, ABCA1 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. FC, free cholesterol; PL, phospholipid; Ctrl, control.

ity rather than expression. PLD2 siRNA transfection decreased PLD2 expression by more than 80%. PLD2 siRNA also abolished the ability of linoleate to inhibit apoA-I-mediated cholesterol efflux (Fig. 6B), to reduce ABCA1 protein levels (Fig. 6C), and to increase phosphorylation of ABCA1 serines (Fig. 6D). Thus, ablating PLD2 expression completely blocks the ABCA1 inhibitory and phosphorylating effects of linoleate, clearly indicating that PLD2 is required for these fatty acid effects.

Because PLD2 can activate protein kinases through generation of DAG, we tested the effects of different membrane-permeable DAG analogs on ABCA1. We found that 1-oleoyl-2-acetyl-*sn*-glycerol decreased apoA-I mediated cholesterol efflux (Fig. 7A), inhibited ABCA1 protein (Fig. 7B), and increased ABCA1 serine phosphorylation (Fig. 7C). Meanwhile, DAGs with saturated acyl groups (1,2-dioctanoyl-*sn*-glycerol and 1,2-dipalmitoyl-*sn*-glycerol) had no effect, suggesting that

FIGURE 3. Linoleate promotes ABCA1 serine phosphorylation. A, ABCA1-expressing BHK cells were ^{32}P -labeled and incubated for 40 min with either 5 mg/ml BSA alone or BSA plus 125 μM indicated fatty acids (BSA molar ratio of 1.8). ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and ^{32}P -labeled ABCA1 was detected by autoradiography. Results are representative of two similar experiments. B, ABCA1-expressing BHK cells were incubated for 40 min with either 5 mg/ml BSA alone or BSA plus 125 μM linoleate (BSA molar ratio of 1.8). ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and blots were probed with phosphoserine or phosphothreonine antibodies. Arrows indicate mature ABCA1 based on molecular weight standards. Results are representative of two similar experiments. Ab, antibody. C, ABCA1 serine phosphorylation was quantitated by measuring the band intensity in immunoblots ($n = 3$). Results are representative of three similar experiments. Asterisks indicate significant ($p < 0.01$) differences from controls. D, cells were treated and assayed as in B, except cells were incubated for 0–60 min without or with linoleate. Results are representative of two similar experiments.

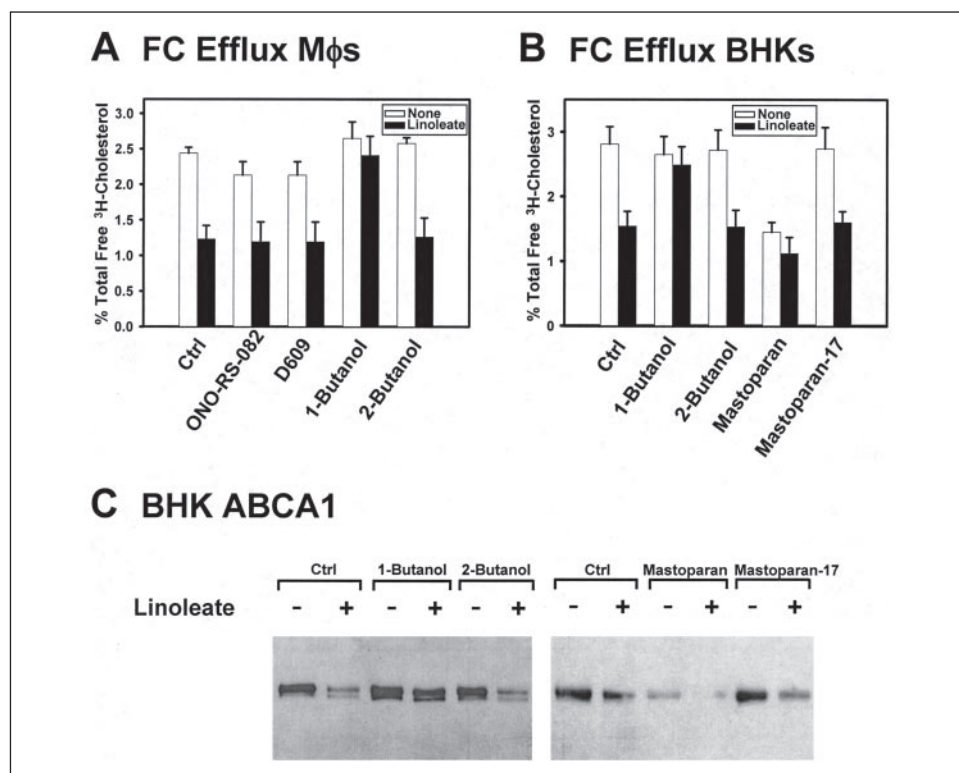
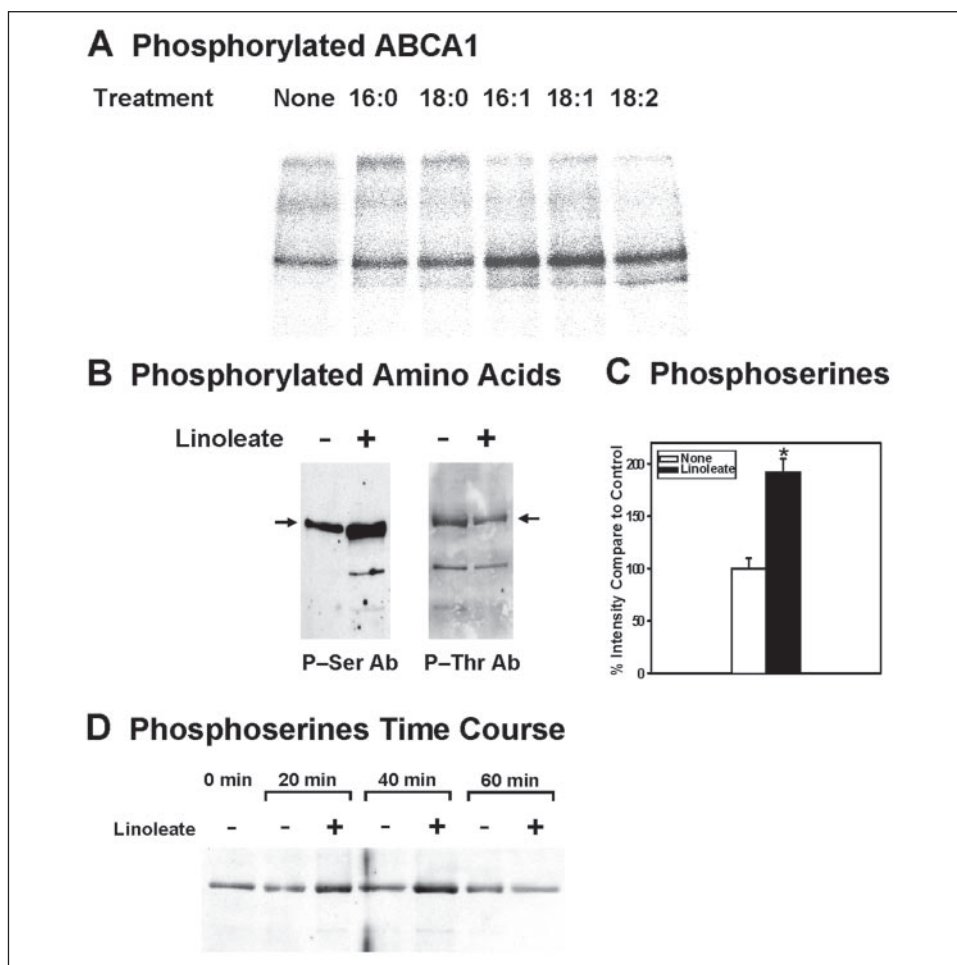


FIGURE 4. PLD is required for linoleate-mediated inhibition of ABCA1. A, cholesterol-loaded J774 macrophages were incubated for 6 h with 0.5 mM 8-Br-cAMP and either 5 mg/ml BSA alone or BSA plus linoleate (BSA molar ratio of 1.8) in the presence or absence of 10 μM ONO-RS-082, 50 $\mu\text{g/ml}$ D609, 0.4% (v/v) 1-butanol, or 0.4% 2-butanol. ApoA-I-mediated [^3H]cholesterol efflux was measured during subsequent 2-h incubations. Each value is the mean \pm S.D. of triplicates. B, ABCA1-expressing BHK cells were incubated for 6 h with 10 nM mifepristone and either 5 mg/ml BSA alone or BSA plus 125 μM linoleate (BSA molar ratio of 1.8) in the presence or absence of 0.4% 1-butanol, 0.4% 2-butanol, 30 μM mastoparan, or 30 μM mastoparan-17. ApoA-I-mediated [^3H]cholesterol efflux was measured during subsequent 2-h incubations. Each value is the mean \pm S.D. of triplicates. C, immunoblot analysis of ABCA1 in BHK cells. Results are representative of two similar experiments. FC, free cholesterol; Ctrl, control.

FIGURE 5. PLD activity affects ABCA1 phosphorylation. A, ABCA1-expressing BHK cells were labeled with 2 $\mu\text{Ci}/\text{ml}$ [^3H]myristic acid for 90 min. Cells were then incubated in PIPES-buffered medium in the presence of 1% ethanol for 10 min before 15-min treatments with the indicated PLD inhibitor or activator in the presence or absence of BSA bound linoleate (BSA molar ratio of 1.8) or stearate (BSA molar ratio of 1.8). PLD activity was determined as described under "Experimental Procedures". Each value represents the mean \pm S.D. of triplicates. Results are representative of three similar experiments. Asterisks indicate significant ($p < 0.05$) differences from controls (*Ctrl*). B, cells were treated and assayed as described for A, except the cells were incubated for 5–25 min without or with linoleate. Each value represents the mean \pm S.D. of triplicates. Results are representative of two similar experiments. C, ABCA1-expressing BHK cells were incubated for 16 h with 10 nM mifepristone and 1 mg/ml BSA, then incubated for 40 min with either 5 mg/ml BSA alone or BSA plus 125 μM linoleate (BSA molar ratio of 1.8) in the presence or absence of indicated activator or inhibitor. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and phosphoserines were identified by immunoblot analysis. Results are representative of three similar experiments.

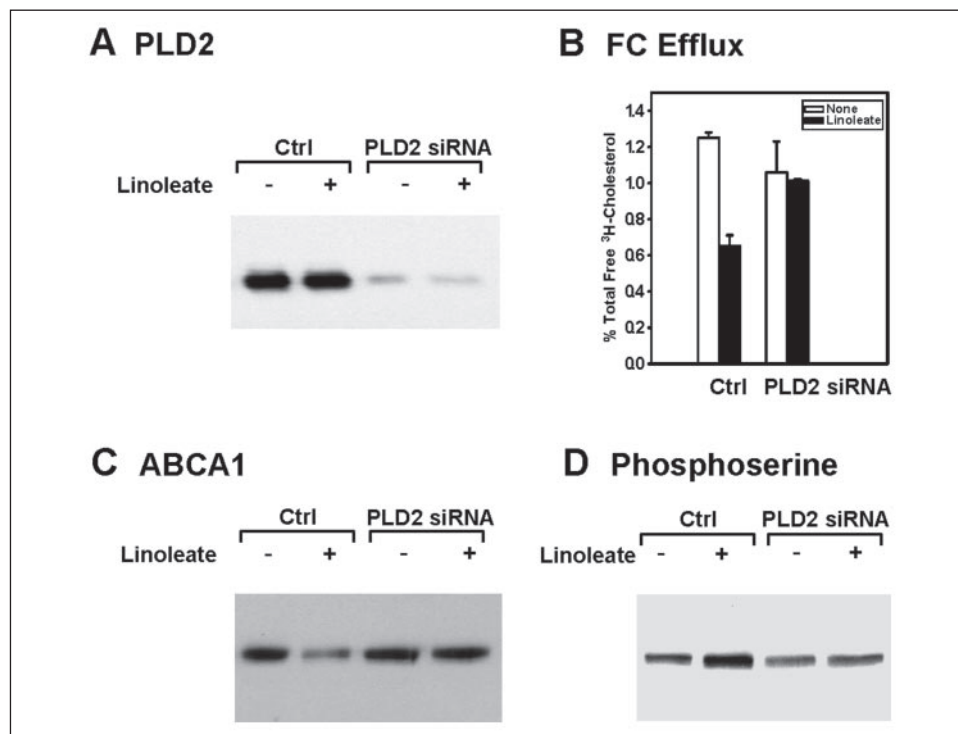
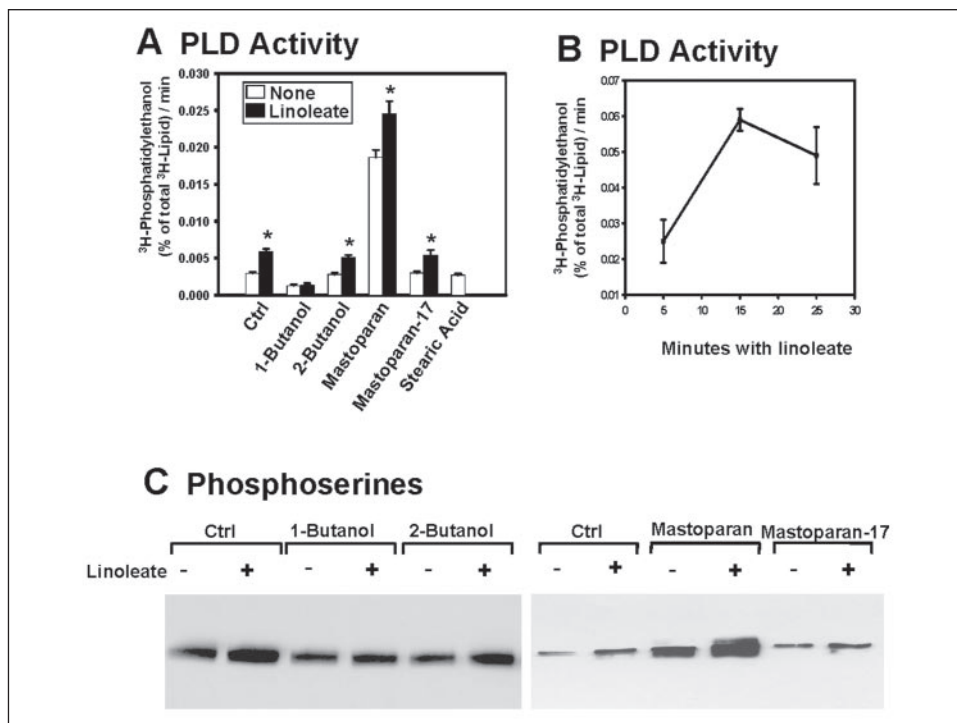


FIGURE 6. PLD2 siRNA prevents the inhibitory effect of linoleate on ABCA1. A, RAW 264.7 macrophages were transfected with mouse PLD2 siRNA as described under "Experimental Procedures." PLD2 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. B, cholesterol-loaded PLD2 siRNA transfected RAW 264.7 and control cells were incubated for 16 h with 0.5 mM 8-Br-cAMP and either 5 mg/ml BSA alone or BSA plus 125 μM linoleate (BSA molar ratio of 1.8). ApoA-I-mediated [^3H]cholesterol efflux was measured during subsequent 2-h incubations. Each value is the mean \pm S.D. of triplicates. Results are representative of two similar experiments. C, membrane ABCA1 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. D, control cells and cells transfected with PLD2 siRNA were incubated for 40 min with either 5 mg/ml BSA alone or BSA plus linoleate (BSA molar ratio of 1.8). ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and blots were probed with phosphoserine antibody. Results are representative of two similar experiments. FC, free cholesterol; Ctrl, control.

DAG containing unsaturated fatty acyl groups mediates the ABCA1-destabilizing effects of PLD2.

DISCUSSION

Metabolic factors that modulate ABCA1 activity could have a profound impact on cholesterol transport and atherosclerosis. Our previous studies revealed that unsaturated fatty acids impair ABCA1 expression by enhancing its degradation rate. Here we provide evidence that this occurs by increasing phosphorylation of ABCA1 serines through a PLD2 signaling pathway.

Unsaturated fatty acids reduced ABCA1 protein levels in both

murine macrophages and ABCA1-transfected BHK cells. This was prevented by the acyl-CoA synthetase inhibitor triacsin C in both cell types, indicating that unsaturated fatty acids had to be converted to fatty acyl-CoA to reduce ABCA1 levels. These results also imply that linoleate destabilizes ABCA1 by a common mechanism in both cell types, consistent with a lack of cell specificity.

Unsaturated but not saturated fatty acids increased phosphorylation of ABCA1. Since ABCA1 is hyper-expressed in BHK cells, it was easier to detect small changes in ABCA1 phosphorylation in these cells. We found that ABCA1 serines and threonines were phosphorylated in the

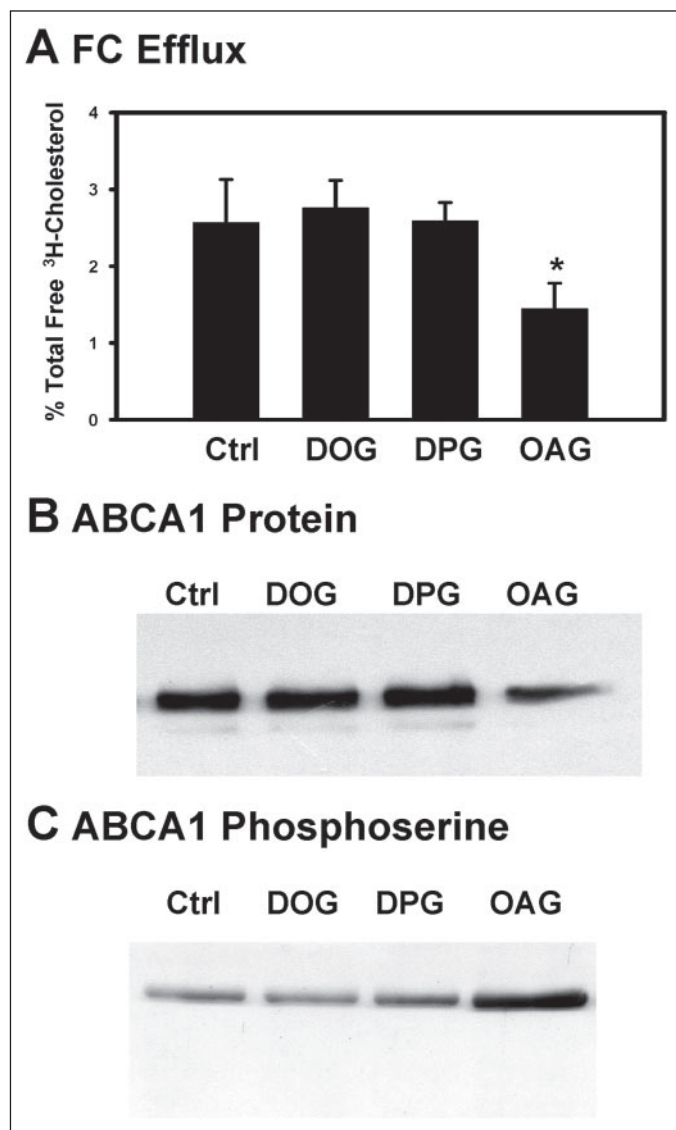


FIGURE 7. DAG-derived from unsaturated fatty acids affects ABCA1 and its serine phosphorylation. A, ABCA1-expressing BHK cells were incubated for 16 h in DMEM with 10 nM mifepristone and either 5 mg/ml BSA alone or BSA plus 100 μ M indicated DAG analog, and apoA-I-mediated [³H]cholesterol efflux was measured during subsequent 2-h incubations as described under "Experimental Procedures." Each value represents the mean \pm S.D. of triplicates. Results are representative of three similar experiments. Asterisks indicate significant ($p < 0.05$) differences from controls (Ctrl). B, ABCA1 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. C, ABCA1-expressing BHK cells were incubated for 16 h with 10 nM mifepristone and 1 mg/ml BSA, then incubated for 40 min with either 5 mg/ml BSA alone or BSA plus 100 μ M indicated DAG. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and phosphoserines were identified by immunoblot analysis. Results are representative of two similar experiments. The abbreviations are: DOG, 1,2-dioctanoyl-sn-glycerol; DPG, 1,2-dipalmitoyl-sn-glycerol; OAG, 1-oleoyl-2-acetyl-sn-glycerol.

basal state in these transfectants. Linoleate treatment approximately doubled the phosphoserine content of ABCA1 without affecting phosphothreonine levels. Inhibition of PLD2 with the PLD inhibitor 1-butanol or reducing PLD2 levels with siRNA reversed the ability of linoleate to increase ABCA1 serine phosphorylation and reduce ABCA1 protein levels. Activation of PLD2 by mastoparan also increased ABCA1 serine phosphorylation and reduced ABCA1 protein levels. Serine phosphorylation by linoleate lagged behind activation of PLD2, with maximum effects occurring after 40 and 15 min, respectively. These observations strongly suggest that phosphorylation of ABCA1 serines through a PLD2 pathway is responsible for the

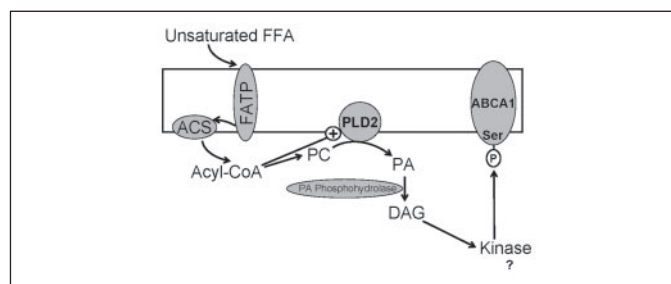


FIGURE 8. A model for the inhibition of ABCA1 by unsaturated fatty acids. The abbreviations used are: FFA, free fatty acid; FATP, fatty acid transfer protein; ACS, acyl-CoA synthetase; PC, phosphatidylcholine; PA, phosphatidic acid.

fatty acid-induced destabilization of this transporter. A direct role of serine phosphorylation in this process will need to be verified by mutational analyses.

Previous studies had identified a PLD activity in mammalian cells that was activated by oleate. Since cloning of the two PLD genes, studies showed that PLD2 but not PLD1 could be activated by unsaturated fatty acids such as oleate, linoleate, and arachidonate but not by saturated fatty acids such as palmitate and stearate (28, 40). This fatty acid activation profile is the same as that observed for destabilization of ABCA1 (22), consistent with a role of PLD2 in the effects of fatty acids on ABCA1. Here we show that linoleate but not stearate increased PLD activity in BHK cells, implying that unsaturated fatty acids are activating PLD2 in these cells. Further support for this idea was provided by results showing that mastoparan, a wasp venom protein known to specifically activate PLD2 (29), dramatically increased PLD activity. The involvement of the PLD2 isoform in ABCA1 destabilization was confirmed by the observations that mastoparan alone markedly reduced ABCA1 protein levels and that selective ablation of PLD2 abolished the ability of linoleate to reduce ABCA1 activity and protein levels.

Our results imply that the fatty acid-stimulated PLD2 pathway activates a protein kinase that targets ABCA1 for proteolysis. We have not yet identified the protease, protein kinase, or signaling molecules involved in this PLD2 pathway. ABCA1 phosphorylation was shown to increase ABCA1 degradation by calpain, but this pathway does not play a role in the ABCA1 destabilizing effects of fatty acids (23). Treating cells with different classes of protein kinase inhibitors have so far failed to block the ABCA1 inhibitory effects of linoleate. PLD generates several important signaling molecules that activate protein kinases, including phosphatidic acid, lysophosphatidic acid, and DAG. We found that DAG containing unsaturated but not saturated fatty acyl groups reduced ABCA1 protein levels and increased ABCA1 serine phosphorylation, consistent with DAG mediating the ABCA1-destabilizing effects of PLD2. These findings suggest that the PLD2 pathway serves to generate DAG subspecies enriched in unsaturated fatty acids that have selective signaling functions.

Based on the current and previous results, we propose the following model (Fig. 8) for the ABCA1 destabilizing effects of fatty acids. Unsaturated fatty acids are transported into the cell by fatty acid transfer protein and converted to their acyl-CoA derivatives, some of which are incorporated into pools of phosphatidylcholine-containing unsaturated acyl side chains. These acyl-CoAs also activate PLD2, which acts as a feed-forward mechanism to generate phosphatidic acid and DAG enriched in unsaturated acyl groups. These DAG subspecies activate a specific protein kinase, which phosphorylates serine residues in ABCA1 that target ABCA1 for proteolysis.

The involvement of a signaling pathway indicates that the fatty acid-induced down-regulation of ABCA1 has a biological function. Fatty

acids destabilize ABCA1 over a fatty acid to albumin molar ratio in the high physiologic range, and enhanced ABCA1 degradation occurs when cells are exposed to fatty acids for only 2 h (22). When ABCA1 is induced by LXR ligands, saturated fatty acids also become ABCA1 destabilizers because they are converted to unsaturated fatty acids by an LXR-induced enzyme (23). Thus, the activity of the ABCA1 pathway in cholesterol loaded cells is likely to become rapidly suppressed when they are exposed to moderately high levels of the most common fatty acids, oleate and palmitate. One possible reason for this suppression is to retain a reservoir of cholesterol and phospholipids in anticipation of new membrane synthesis. This is particularly relevant to unsaturated fatty acids, which have been shown to have mitogenic effects (41). It is also possible that an intracellular pool of excess cholesterol and phospholipids is required for formation of triglyceride-rich lipid droplets formed by the influx of fatty acids. In support of this idea is the observation that adipocytes accumulate both triglycerides and free cholesterol (42).

The current study has important therapeutic implications for treating cardiovascular disease. It has been reported that ABCA1 protein levels are poorly correlated with mRNA levels among different mouse tissues (43), implying that posttranscriptional regulation plays an important role in determining ABCA1 expression. Thus, an understanding of the mechanisms by which fatty acids increase ABCA1 degradation will shed new light on designing therapeutic interventions that enhance the activity of this cholesterol removal pathway and prevent atherosclerosis. Furthermore, therapeutic interventions designed to treat dyslipidemias and diabetes may have an additional atheroprotective benefit by enhancing the ABCA1-dependent cholesterol removal pathway.

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