Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C δ pathway

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Abstract **Abnormal HDL metabolism among patients with diabetes and insulin resistance may contribute to their increased risk of atherosclerosis. 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 increased in diabetes, impair the ABCA1 pathway in cultured cells by destabilizing ABCA1 protein. We previously reported that unsaturated fatty acids destabilize ABCA1 in murine macrophages and ABCA1-transfected baby hamster kidney cells by increasing its serine phosphorylation through a phospholipase D (PLD) pathway. Here, we examined the cellular pathway downstream of PLD that mediates the ABCA1-destabilizing effects of unsaturated fatty acids. The protein kinase C δ (PKCδ)-specific inhibitor rottlerin and PKCδ small interfering RNA completely abolished the ability of unsaturated fatty acids to inhibit lipid transport activity, to reduce protein levels, and to increase serine phosphorylation of ABCA1, implicating a role for PKCδ in the ABCA1-destabilizing effects of fatty acids.** **These data indicate that unsaturated fatty acids destabilize ABCA1 by activating a PKCδ pathway that phosphorylates ABCA1 serines.**—Wang, Y., and J. F. Oram. **Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C δ pathway.** ***J. Lipid Res.*** **2007.** 48: **1062–1068.**

Supplementary key words ATP binding cassette transporter A1 • high density lipoproteins • cardiovascular disease • diabetes

Population studies have revealed that HDL is a major cardioprotective factor. Lipidation of apolipoprotein A-I (apoA-I) by ABCA1 is the rate-limiting step in generating plasma HDL (1–3). ABCA1 is an integral membrane protein that mediates the transport of cellular cholesterol and phospholipids to lipid-deficient HDL apolipoproteins (4, 5). ABCA1 is a member of a large family of ABC transporters and contains two ATP binding domains and two six helix transmembrane domains (6). Mutations in ABCA1 cause Tangier disease (7–10), a severe HDL deficiency syndrome characterized by the deposition of sterols in tissue macrophages and prevalent atherosclerosis (11, 12).

ABCA1 expression by macrophages is highly regulated. Cholesterol loading of macrophages markedly increases ABCA1 mRNA and protein levels (10, 13), consistent with a transporter that functions to export excess cholesterol. The transcription of this gene is regulated by nuclear liver X receptors (LXRα and LXRβ) and retinoid X receptor (14–17). Analogs of cAMP are also known to activate murine ABCA1 gene transcription (18–20). The ABCA1 protein degradation rate is also regulated. ApoA-I increases ABCA1 protein stability by decreasing the phosphorylation of a sequence in ABCA1 that directs calpain proteolysis (21) and by activating protein kinase C (22). Protein kinases A, protein kinase 2, and Janus kinase 2 have also been shown to affect ABCA1 activity (23–25).

Patients with diabetes have an increased 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 these patients. Our previous studies showed that the long-chain unsaturated fatty acids palmitoleate, oleate, linoleate, and arachidonate but not the saturated fatty acids palmitate and stearate decrease ABCA1 expression in cells by increasing its protein degradation rate (26). Furthermore, we also showed that these unsaturated fatty acids accelerate ABCA1 protein turnover through a signaling pathway involving the activation of phospholipase D2 (PLD2) and the phosphorylation of ABCA1 serine residues (27). We also found that diacylglycerol (DAG) containing unsaturated but not saturated fatty acyl groups reduced ABCA1 protein levels.

In this study, we examined the mechanism by which unsaturated fatty acids destabilize ABCA1 protein. We found that linoleate-induced ABCA1 degradation was associated with increased protein kinase C δ (PKCδ) activity and the phosphorylation of ABCA1 serines. Inhibiting PKCδ with rottlerin or depleting PKCδ with small interfering RNA (siRNA) reduced or abolished the ability of linoleate to promote ABCA1 phosphorylation and degradation. Thus, unsaturated fatty acids activate a PKCδ signaling pathway

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that phosphorylates ABCA1 serine residues, which destabilizes the protein.

EXPERIMENTAL PROCEDURES

Lipoproteins and apoA-I

LDL and HDL were prepared by sequential ultracentrifugation in the density ranges 1.019–1.063 and 1.125–1.21 g/ml, respectively, and HDL was depleted of apoE and apoB by heparin-agarose chromatography (28). ApoA-I was purified from HDL and delipidated as described previously (28). LDL was acetylated by the method of Goldstein et al. (29).

Cell culture and lipid efflux

Baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 were generated as described previously (30). Murine RAW 264.7 macrophages 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 BSA (DMEM/BSA). To radiolabel cellular cholesterol, 1 μCi/ml [^{3}H]cholesterol (NEN Life Science Products) was added to the growth medium (BHK cells) or to DMEM/BSA containing 50 μg/ml acetylated LDL (RAW 264.7 macrophages) at 24 h immediately preceding the treatments (26, 30). Washed cells were then incubated for 6–16 h with medium containing 5 mg/ml BSA in the presence or absence of 125 μM fatty acids (molar ratios to BSA of 0:1.8). 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 RAW 264.7 and 10 nM mifepristone was added to the medium for ABCA1-expressing BHK cells.

To measure cholesterol efflux, cells were incubated with DMEM/BSA with or without 10 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. 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 (31). ApoA-I-mediated cholesterol efflux is expressed as the fraction of total free [^{3}H]cholesterol released into the medium after subtraction of values obtained in the absence of apoA-I.

Immunoblots

Cells were washed and dislodged from the dish at 0°C in buffer containing protease inhibitors. To isolate PKCδ or 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 antibody against PKCδ (Upstate) 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 PKCδ, PKCβ1, and PKCθ from RAW 264.7 cells or BHK cells, PKC proteins were isolated by immunoprecipitation with PKC-specific antibodies (Santa Cruz Biotechnology) and resolved by SDS-PAGE. For immunoblots of whole membrane ABCA1 from RAW 264.7 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 (19). Equal amounts of membrane protein were added per gel lane. For immunoblots of ABCA1 from ABCA1-expressing BHK cells, cell proteins were solubilized in PBS containing 1% Triton X-100 plus protease inhibitors, and equal amounts of protein were added per gel lane. For immunoblots of phospho-PKCδ, PKCδ was isolated by immunoprecipitation with PKCδ antibody and SDS-PAGE, and blots were probed with phospho-PKCδ [threonine-505 (Thr505)] antibody (Cell Signaling Technology).

ABCA1 serine phosphorylation

ABCA1 phosphoserines were assayed by immunoprecipitation with ABCA1 antibody followed by immunoblot analysis using phosphoserine antibody (Biomol). ABCA1 serine phosphorylation was quantitated using OptiQuant software (Packard Instrument).

siRNA transfections

PKCδ siRNA expressing plasmid pKD-PKCδ-v6 (Millipore) targets the 678–699 nucleotide region of mouse PKCδ mRNA, which is not homologous to other PKC isoforms. pKD-PKCδ-v6 and control plasmid pKD-NegCon-v1 (Millipore) were transfected into RAW 264.7 macrophages using FuGene 6 (Roche) as described in the manufacturer's protocol. Briefly, 2 μl of 0.1 mg/ml siRNA plasmid was mixed with 37.7 μl of DMEM/BSA and 0.3 μl of FuGene 6 and incubated at room temperature for 20 min. Plasmid-FuGene 6 complex was applied to 360 μl of DMEM/BSA with 10% fetal bovine serum in each well of a 24-well plate containing cultured RAW 264.7 macrophages at ~70% confluence. PKCβ1 and PKCθ (Santa Cruz Biotechnology) and scrambled negative control (Ambion) siRNAs were transfected into RAW 264.7 macrophages using siPORT™ Lipid (Ambion) as described in the manufacturer's protocol. Briefly, 1 μl of 20 μM siRNA was mixed with 41.5 μl of DMEM/BSA, and 2 μl of siPORT™ Lipid was diluted in 5.5 μl of DMEM/BSA and incubated at room temperature for 20 min. After the incubation, the diluted siPORT™ Lipid was combined with the diluted siRNA and incubated for an additional 20 min at room temperature. A total of 50 μl of siRNA-siPORT™ Lipid complex was applied to 200 μl of DMEM/BSA in each well of a 24-well plate containing cultured RAW 264.7 macrophages at ~70% confluence.

RESULTS

PKCδ is required for fatty acid inhibition of ABCA1

We previously demonstrated that the long-chain unsaturated fatty acids palmitoleate, oleate, linoleate, and arachidonate decreased apoA-I-mediated lipid efflux from murine macrophages and ABCA1-transfected BHK cells (27) by increasing ABCA1 degradation through a PLD2-DAG pathway that increased ABCA1 serine phosphorylation. Because this implicated a PKC pathway, we screened PKC isoform-specific inhibitors for their abilities to reverse the destabilizing effects of unsaturated fatty acids. Inhibitors of PKCα/β1 (Gö 6976; EMD Biosciences), PKCζ (myristoylated PKC$_\zeta$ pseudosubstrate inhibitor), or PKCθ (myristoylated PKCθ pseudosubstrate inhibitor) had no effect on linoleate-inhibited apoA-I-mediated cholesterol efflux from ABCA1-expressing BHK cells when they were incubated for 6 h with or without fatty acid or inhibitor (data not shown). The PKCδ-specific inhibitor rottlerin, however, completely abolished the ability of linoleate to inhibit apoA-I-mediated cholesterol efflux (**Fig. 1A**), reduce ABCA1 protein levels (Fig. 1B), and increase ABCA1 serine phosphorylation (Fig. 1C). Rottlerin also blocked the inhibitory effects of linoleate on apoA-I-mediated cho-

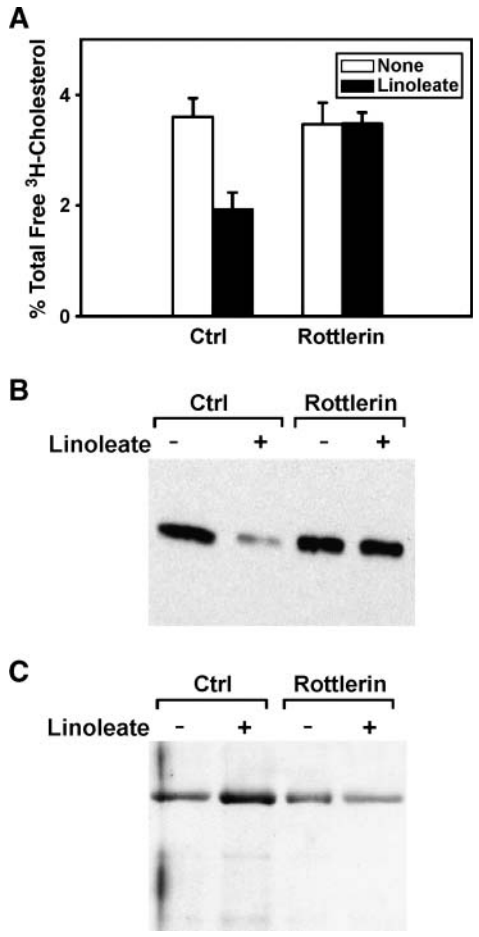

Fig. 1. The protein kinase C δ (PKCδ) inhibitor rottlerin prevents ABCA1 impairment by unsaturated fatty acids. A: ABCA1-expressing baby hamster kidney (BHK) cells were incubated for 16 h in DMEM with 10 nM mifepristone and 1 mg/ml BSA, then incubated for 6 h in DMEM with 10 nM mifepristone and either 5 mg/ml BSA alone or BSA plus 125 μM linoleate (BSA molar ratio of 1.8) in the presence or absence of 5 μM rottlerin, and apolipoprotein A-I (apoA-I)-mediated [^{3}H]cholesterol efflux was measured during subsequent 2 h incubations as described in Experimental Procedures. Each value represents the mean ± SD of three incubations. B: ABCA1 protein levels were assayed by immunoblot analysis. C: ABCA1-expressing BHK cells were incubated for 40 min in DMEM with either 5 mg/ml BSA alone or BSA plus linoleate (BSA molar ratio of 1.8) in the presence or absence of 5 μM rottlerin. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and blots were probed with phosphoserine antibody. All results are representative of three similar experiments. Ctrl, control.

lesterol efflux from J774 macrophages (data not shown). The maximum inhibitory effects of rottlerin were <5 μM with both ABCA1-transfected BHK cells and J774 macrophages. These results suggest that PKCδ mediates the ABCA1-destabilizing effects of fatty acids.

To confirm that PKCδ activation is important for the ABCA1-destabilizing effects of unsaturated fatty acids, we knocked down PKCδ expression with a specific siRNA and measured the effects of linoleate on ABCA1 activity and levels. We used siRNAs for PKCβ1 and PKCθ as positive controls. Because of their higher transfection efficiency, we used murine RAW 264.7 macrophages for these studies. Transfections with PKC siRNAs decreased PKCδ, PKCβ1, and PKCθ levels by >80%, and linoleate treatment had no effect on PKC protein levels (**Fig. 2A**). PKCδ siRNA abolished the ability of linoleate to inhibit apoA-I-mediated cholesterol efflux (Fig. 2B), to reduce ABCA1 protein levels (Fig. 2C), and to increase the phosphorylation of ABCA1 serines (Fig. 2D). In contrast, knocking down either PKCβ1 or PKCθ had no effect on the ability of linoleate to inhibit ABCA1 cholesterol efflux activity (Fig. 2B), reduce ABCA1 protein (Fig. 2C), or increase ABCA1 serine phosphorylation (Fig. 2D). Thus, ablating PKCδ expression completely blocks the ABCA1-inhibitory and -phosphorylating effects of linoleate, indicating that PKCδ is required for these fatty acid effects.

Unsaturated fatty acids impair ABCA1 via PKCδ activation

It was demonstrated that PKCδ activation is linked to phosphorylation at Thr505 (32). To test whether unsaturated fatty acids activate PKCδ, we incubated ABCA1-expressing BHK cells for 30 min with stearate, linoleate, dioctanoylglycerol, or oleoylacetylglycerol and measured PKCδ phosphorylation. In the absence of inducers of stearoyl-CoA desaturase 1, the saturated fatty acid stearate has no effect on ABCA1 stability (26, 27). We also showed previously that dioctanoylglycerol had no affect on ABCA1 activity, levels, or phosphorylation, whereas oleoylacetylglycerol, which contains an unsaturated fatty acyl side chain, mimicked the effects of unsaturated fatty acids on these parameters (27). Phospho-Thr505 immunoblot analyses of immunoprecipitated PKCδ revealed that linoleate and oleoylacetylglycerol markedly increased PKCδ phosphorylation at Thr505, whereas the saturated fatty acid stearate and dioctanoylglycerol failed to increase phospho-Thr505 (**Fig. 3**). These data indicate that unsaturated fatty acids and a DAG containing an unsaturated fatty acyl side chain induce PKCδ activity.

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 through a PLD2 pathway. Here, we provide evidence that this occurs by increasing the phosphorylation of ABCA1 serines through a PKCδ signaling pathway.

Unsaturated fatty acids reduced ABCA1 protein levels in both murine macrophages and ABCA1-transfected BHK cells. Inhibition of PKCδ with the PKCδ-specific inhibitor rottlerin or reducing PKCδ levels with siRNA reversed the ability of linoleate to increase ABCA1 serine phosphorylation and reduce ABCA1 protein levels. Linoleate increased the phosphorylation of PKCδ Thr505, which is associated with enzyme activity. These observations strongly suggest that phosphorylation of ABCA1 serines through a PKCδ pathway is responsible for the fatty acid-induced destabilization of this transporter. We are in the

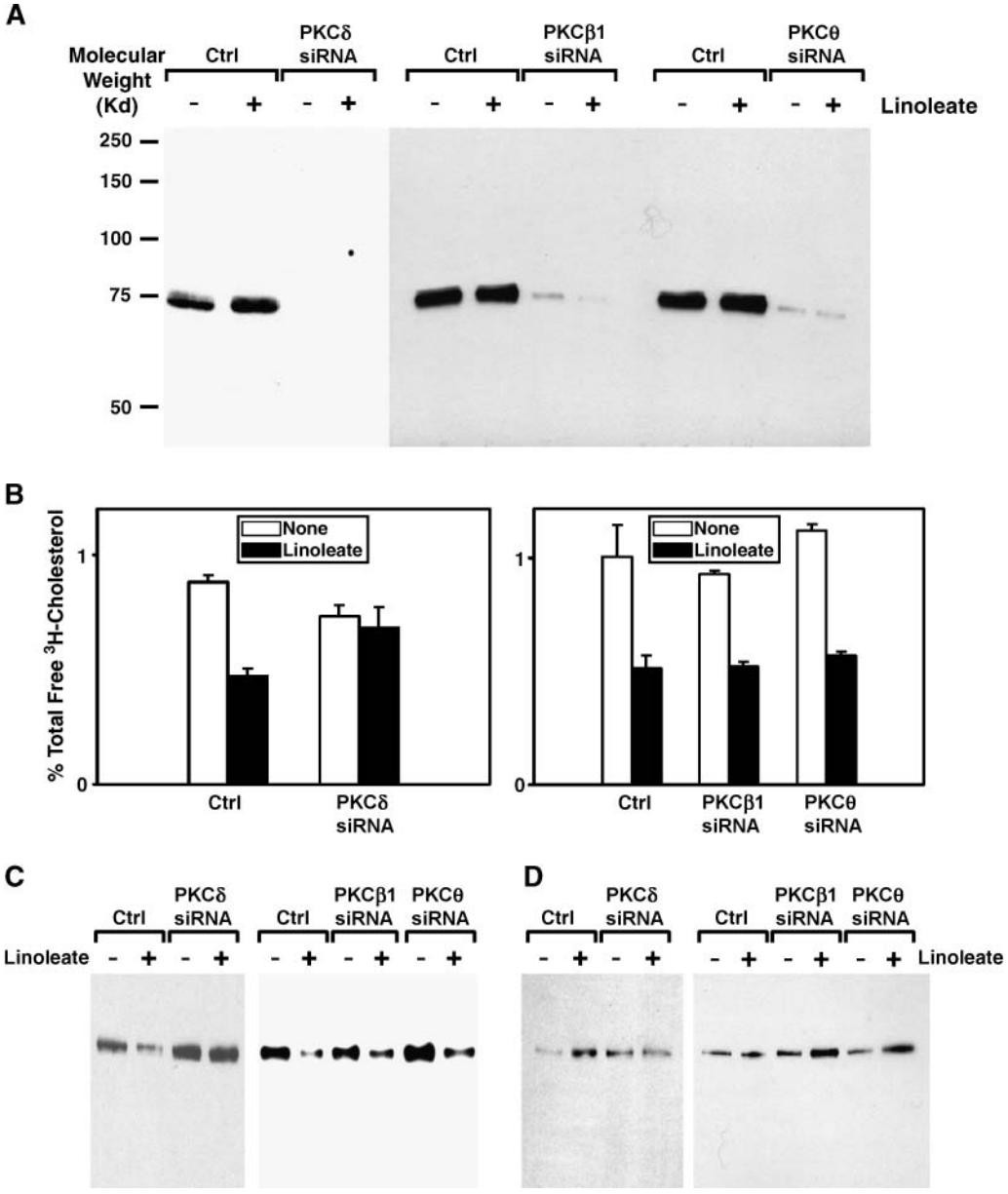

Fig. 2. PKCδ small interfering RNA (siRNA) blocks linoleate-mediated effects on ABCA1. A: RAW 264.7 macrophages were transfected with mouse PKCδ siRNA expression plasmid or control plasmid (Ctrl), or cells were transfected with PKCβ1, PKCθ, or their scrambled (Ctrl) siRNAs as described in Experimental Procedures. PKCδ, PKCβ1, and PKCθ protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. B: Cholesterol-loaded siRNA-transfected 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 [^{3}H]cholesterol efflux was measured during subsequent 2 h incubations. Each value is the mean ± SD of triplicate determinations. Results are representative of three similar experiments. C: Membrane ABCA1 protein levels were assayed by immunoblot analysis. Results are representative of three similar experiments. D: Cells transfected with 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 three similar experiments.

process of preparing ABCA1 serine mutants to identify the residues that are the targets of PKCδ.

Our previous results implied that the fatty acid-stimulated PLD2 pathway activates a protein kinase that targets ABCA1 for proteolysis. PLD generates several important signaling molecules that activate protein kinases, including phosphatidic acid, lysophosphatidic acid, and DAG. We had obtained results suggesting that DAG containing unsaturated fatty acyl groups mediates the ABCA1-destabilizing effects of PLD2 (27). Here, we further demonstrate that a DAG containing an unsaturated fatty acyl group activates PKCδ. These findings suggest that the

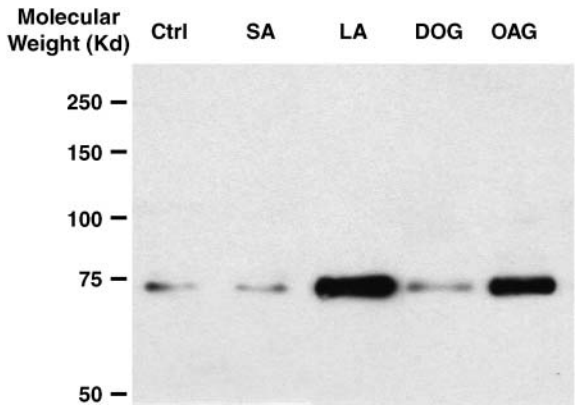

Fig. 3. Linoleate and an oleoyl-containing diacylglycerol (DAG) activate PKCδ. ABCA1-expressing BHK cells were incubated for 30 min in DMEM with 5 mg/ml BSA alone or BSA plus 125 μM stearic acid (SA), 125 μM linoleate (LA), 100 μM 1,2-dioctanoyl-*sn*-glycerol (DOG), or 100 μM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). PKCδ was isolated by immunoprecipitation with a PKCδ antibody and SDS-PAGE, and blots were probed with anti-PKCδ (threonine-505) antibody. Results are representative of three similar experiments. Ctrl, control.

PLD2 pathway serves to generate DAG subspecies enriched in unsaturated fatty acids that function as signaling molecules to activate PKCδ, thus increasing serine phosphorylation and the degradation of ABCA1.

Previous studies have shown that oleate stimulates PKCδ translocation in RAW 264.7 macrophages (33). PKCδ belongs to a novel PKC subfamily of serine/threonine kinases that includes PKCδ, ε, η, and θ. They are maximally activated by DAG without requiring calcium (34). It has been shown that in murine macrophages, the glucocorticoid-induced tumor necrosis factor receptor stimulated the secretion of matrix metalloproteinase 9 via the activation of PKCδ and PLD (35), linking these two pathways together. Here, our results demonstrated that linoleate can activate PLD2 and PKCδ to phosphorylate its downstream target ABCA1.

Our current and previous studies suggest the following model for the ABCA1-destabilizing effects of fatty acids (**Fig. 4**). 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 PKCδ, which phosphorylates serine residues in ABCA1 that target ABCA1 for proteolysis. PKCδ can further activate PLD2 to form a positive feedback loop (35).

This model implies that the fatty acid-induced down-regulation of ABCA1 has a biological function. Fatty acids acutely destabilize ABCA1 over a fatty acid-to-albumin molar ratio in the high physiologic range (26). Multiple unsaturated fatty acids have the same effects, including the most abundant one, oleate. In the presence of LXR ligands, which are generated when cells overaccumulate cholesterol, the saturated fatty acids palmitate and stearate also become ABCA1 destabilizers because they are converted to unsaturated fatty acids by the LXR-induced enzyme stearoyl-CoA desaturase 1 (36). Thus, the activity of the ABCA1 pathway in cholesterol-loaded cells, such as atherogenic macrophages, is likely to become rapidly suppressed when they are exposed to moderately high levels of the most common fatty acids, oleate and palmitate.

Although the physiologic reason for this suppression is unknown, it is consistent with other studies showing cross-regulation of the fatty acid and sterol metabolic pathways (37–39). There are several possible reasons why unsaturated fatty acids would inhibit ABCA1. The incorporation of unsaturated fatty acids into phospholipids may promote membrane synthesis, which would require

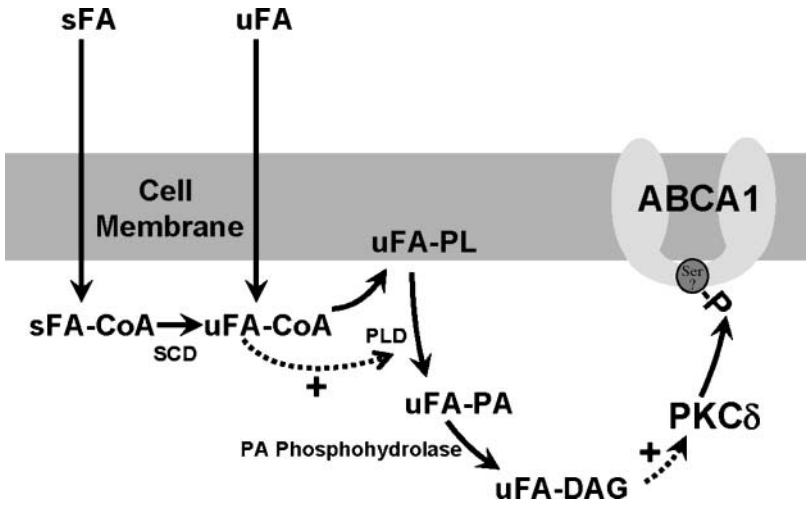

Fig. 4. A model for the inhibition of ABCA1 by unsaturated fatty acids. PA, phosphatidic acid; PC, phosphatidylcholine; PL, phospholipid; PLD, phospholipase D; SCD, stearoyl-CoA desaturase; Ser, serine; sFA, saturated fatty acid; uFA, unsaturated fatty acid.

an increased supply of cholesterol. Membrane ABCA1 might also play a direct role in the formation of membrane lipid rafts. Recent studies have shown that overexpressing ABCA1 in cells disrupts membrane lipid rafts (40), whereas ablating ABCA1 expression increases the lipid raft content (41). Reducing ABCA1 levels could be one of the mechanisms by which unsaturated fatty acids modulate membrane lipid rafts. It is also possible that inhibiting ABCA1 serves to suppress some other as yet unidentified ABCA1 function that plays a role in fatty acid metabolism.

The current study has important therapeutic implications for treating cardiovascular disease. A mouse study showed that ABCA1 mRNA and protein levels in tissues were highly discordant (42), consistent with the importance of posttranscriptional events in regulating ABCA1 levels. Thus, an understanding of the mechanisms by which fatty acids increase ABCA1 degradation will be helpful for designing therapeutic interventions that enhance the activity of this cholesterol-removal pathway and prevent atherosclerosis.

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