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Unsaturated Fatty Acids Repress the Expression of ATP-Binding Cassette Transporter A1 in HepG2 and FHs 74 Int Cells

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Abstract

Adenosine triphosphate-binding cassette transporter A1 (ABCA1) plays a critical role in the formation and metabolism of high-density lipoproteins (HDLs). Adenosine triphosphate-binding cassette transporter A1 in the liver and small intestine, in particular, accounts for approximately 90% of plasma HDL cholesterol. Therefore, any alterations in the hepatic and intestinal expression of ABCA1 could have a large impact on HDL biogenesis. We tested the hypothesis that ABCA1 expression is regulated differentially by different types of fatty acids in the liver and small intestine. Human hepatoma HepG2 and human small intestine epithelial FHs 74 Int cells were used as an in vitro model. Cells were incubated with saturated and unsaturated fatty acids in the presence or absence of T0901317, a synthetic agonist of liver X receptor. Unsaturated fatty acids decreased ABCA1 protein levels at 100 $\mu\text{mol/L}$ of concentration regardless of the agonist with a minimal effect on messenger RNA abundance. Incubation of HepG2 and FHs 74 Int cells with rottlerin, a protein kinase C δ (PKC δ) inhibitor, increased ABCA1 protein but did not abolish linoleic acid-induced decrease in ABCA1 protein levels. Depletion of PKC δ using small interfering RNA showed decreased ABCA1 protein levels in control, palmitic acid-, and linoleic acid-treated cells, but the repressive effect of linoleic acid was sustained. In conclusion, our results indicate that unsaturated fatty acids regulate ABCA1 expression in HepG2 and FHs 74 Int cells at the posttranscriptional level, and PKC δ is likely to be involved in maintaining ABCA1 protein levels.

Keywords: ABCA1, fatty acids, HepG2 cells, FHs 74 Int cells

Abbreviations: apoA-I, apolipoprotein A-I; ABCA1, adenosine triphosphate-binding cassette transporter A1; CVD, cardiovascular disease; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LXR, liver X receptor; mRNA, messenger RNA; PKC δ , protein kinase C δ ; qPCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA.

1. Introduction

Adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) is a membrane transporter that mediates the efflux of phospholipid and free cholesterol to lipid-free or lipid-poor apolipoprotein A I (apoA-I), the major protein constituent in high-density lipoprotein (HDL) particles, contributing to HDL biogenesis.¹⁻³ The significant role of ABCA1 in HDL metabolism is underscored by the finding that mutations in ABCA1 gene lead to near absence of plasma HDL cholesterol concentrations in patients with Tangier disease and familial HDL deficiencies⁴ as well as in mice.^{5,6} In particular, deletion of ABCA1 in the liver and intestine decreased plasma HDL cholesterol concentrations by approximately 80% and 30%, respectively, indicating that ABCA1 in these tissues are quantitatively most important for maintaining plasma HDL cholesterol levels.^{7,8}

Transcription of ABCA1 is under the control of liver X receptor (LXR), a major transcription factor for genes important in cholesterol metabolism in response to cellular cholesterol levels.⁹ In addition, ABCA1 expression is also regulated by fatty acids. Human macrophages treated with linoleic acid (18:2) showed lower ABCA1 expression compared with those incubated with palmitic acid (16:0).¹⁰ Adenosine triphosphate-binding cassette transporter A1 messenger RNA (mRNA) levels in HepG2 and RAW 264.7 macrophages were also decreased by unsaturated fatty acids, with a reduction in ABCA1 promoter activity.¹¹ Mutation or deletion of direct repeat 4, the LXR/retinoid X receptor heterodimer binding element, in the promoter of ABCA1 abolished the suppressive effects of unsaturated fatty acids on the gene expression, suggesting that the inhibition is probably mediated through mechanisms involving LXR/retinoid X receptor.¹² Posttranscriptional regulation of ABCA1 by unsaturated fatty acids in macrophages has also been demonstrated. Wang et al.¹³ and Wang and Oram¹⁴⁻¹⁶ found that unsaturated fatty acids increased ABCA1 protein degradation in macrophages by activating phospholipase D2 and, subsequently, protein kinase C δ (PKC δ), which, in turn, phosphorylates ABCA1 serine residues for protein degradation. In Caco-2 cells, posttranscriptional regulation of ABCA1 expression by unsaturated fatty acids was also suggested with a modest fatty acid-induced suppression of the gene transcription.¹⁷

Individuals with obesity and type 2 diabetes are at high risk of cardiovascular disease (CVD).¹⁸⁻²⁵ Particularly, lowered plasma HDL cholesterol concentrations by approximately 15% to 30% in type 2 diabetic individuals are associated with an increased risk of CVD.^{26,27} The decreased plasma HDL cholesterol levels in diabetic subjects could contribute to their high CVD risk. High-density lipoprotein can deliver excess cholesterol from the periphery to the liver for ultimate excretion from the body. This process called "reverse cholesterol

transport" is believed to be a major atheroprotective property of HDL.²⁸⁻³⁰ Therefore, any disturbance in this process could facilitate cholesterol accumulation and foam cell formation in the arterial wall, a primary hallmark of atherosclerosis. High plasma free fatty acid concentrations are typically associated with obesity, insulin resistance, and type 2 diabetes³¹⁻³⁵ and are known to have a large impact on insulin resistance and diabetic dyslipidemia.³⁶⁻⁴⁶ Therefore, the objective of this study was to evaluate the effect of various fatty acids on the expression of ABCA1 in the liver and intestine, which are primary sites for HDL formation, using HepG2, a human hepatoma cell line, and FHs 74 Int cells, a human small intestine epithelial cell line. To achieve the goal, we tested the hypothesis that ABCA1 expression is regulated differentially by different types of fatty acids in the liver and small intestine. FHs 74 Int cell line has received very little attention to study metabolic events occurring in small intestine. In contrast to Caco-2 cell line that is most commonly used, FHs 74 Int cell line may have advantages over Caco-2 cells, as they are derived from fetal human small intestinal epithelial cells, whereas Caco-2 cells are generated from large intestine.

2. Methods and materials

2.1. Cell culture and fatty acid preparation

HepG2 cells and FHs 74 Int cells were purchased from ATCC (Manassas, Virginia, USA). The cells were maintained in minimal essential medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, 1 \times vitamins, and 2 mmol/L L-glutamine in a humidified chamber at 37°C with 5% CO₂. For FHs 74 Int cells, 30 ng/mL epidermal growth factor and 10 μ g/mL insulin were additionally supplemented to cell culture medium. All cell culture supplies were purchased from MediaTech (Manassas, Virginia, USA).

Fatty acid and bovine serum albumin (BSA) complex (BSA to fatty acid molar ratio of 2.5) was prepared, as previously described.⁴⁷ Briefly, 2 mmol/L of fatty acid-poor and endotoxin-free BSA (Calbiochem, EMD Chemicals, Gibbstown, New Jersey, USA) was prepared in phosphate-buffered saline. Sodium salts of fatty acids (Nu-Chek-Prep, Inc., Elysian, Minnesota, USA) were dissolved in the BSA stock solution to a concentration of 5 mmol/L. After purging with N₂, the solution was sonicated in a water bath until it became clear to form the BSA/fatty acid complex (1:2.5 molar ratio of BSA to fatty acid). The complex was sterilized through a Millex-GV 0.22 μ mol/L filter unit (Millipore, Billerica, Massachusetts, USA) and diluted with cell medium to reach a final concentration of 100 μ mol/L. HepG2 cells were incubated with or without 10 μ mol/L of T0901317 (Sigma-Aldrich, St. Louis, Missouri, USA), an LXR agonist, in dimethyl sulfoxide (DMSO) for 18 hours, after which cells were treated with BSA only (control) or 100 μ mol/L of a fatty acid for 12 hours. For the experiments with rottlerin (Santa Cruz Biotechnology, Santa Cruz, California), a PKC δ inhibitor, cells were preincubated with 5 or 10 μ mol/L rottlerin in DMSO for 2 hours and, subsequently, were incubated with BSA or 100 μ mol/L fatty acids such as palmitic acid (16:0) and linoleic acid (18:2) for 12 hours. Control cells were incubated with the same amount of DMSO for a vehicle control, in addition to BSA. Cells were cultured in a complete medium devoid of FBS when they were treated with fatty acid to eliminate any potential contamination of fatty acids present in FBS.

2.2. Small interfering RNA transfection

To knock down PKC δ , HepG2 cells were transfected with Silencer Negative Control scrambled small interfering RNA (siRNA) (Ambion, Austin, Texas, USA) or siGENOME PKC δ siRNA (Dharmacon, Lafayette, Colorado, USA) using a transfection reagent DharmaFECT1 (Dharmacon, Lafayette, Colorado, USA) according to the manufacturer's protocol, as previously described.⁴⁷

2.3. Total RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol. Reverse transcription for complementary DNA synthesis and quantitative real-time polymerase chain reaction (qPCR) analysis were performed, as previously described.^{48,49} Primers were designed according to GenBank database using the Primer Express software (Applied Biosystems, Carlsbad, California, USA). The following primers were used for real-time polymerase chain reaction analysis: ABCA1, forward (5'-CGTTTCCGGGAAGTGTCTTA-3'), reverse (5'-GCTAGAGATGACAAGGAGGATGGA-3'); glyceraldehyde 3-phosphate dehydrogenase, forward (5'-GTGGTCTCTCTGACTTCAACA-3'), reverse (5'-GTTGCTGTAGCCAAATTCGTTGT-3').

2.4. Western blot analysis

Cell lysate was prepared, and Western blot analysis was performed, as previously described.^{48,49} Rabbit anti-ABCA1 antibody was a generous gift from Dr. John Parks at Wake Forest University School of Medicine, and PKC δ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California). β -Actin (Sigma-Aldrich, Missouri) was used as a loading control to normalize the data.

2.5. Statistical analyses

Analysis of variance and the Tukey pairwise comparison with Welch's correction for unequal variance when appropriate were used to identify statistically significant differences of treatments, with $P < .05$ considered significant by GraphPad InStat 5 (LaJolla, California, USA). Data are expressed as mean \pm SEM.

3. Results

HepG2 cells were treated with various fatty acids in the presence or absence of T0901317. In the absence of the LXR agonist, ABCA1 mRNA abundance was not significantly different in all of fatty acid-treated cells compared with control (Fig. 1A). T0901317 markedly increased ABCA1 mRNA levels by approximately 7- to 25-fold. Saturated and monounsaturated fatty acids minimally altered ABCA1 mRNA, whereas significantly higher mRNA levels were observed in cells treated with polyunsaturated fatty acids, that is, 18:2, and eicosapentaenoic acid (20:5), than control in the presence of T0901317. However, ABCA1 protein (approximately 250 kd) was noticeably decreased by unsaturated fatty acids tested, that is, 18:1, 18:2, and 20:5, compared with the control and saturated fatty acid-

treated cells, irrespective of the presence of T0901317 (Fig. 1B). In particular, polyunsaturated fatty acids decreased ABCA1 protein levels to the greatest extent. Similar posttranscriptional regulation of ABCA1 by 18:2 was observed in FHs 74 Int cells (Fig. 2A and B).

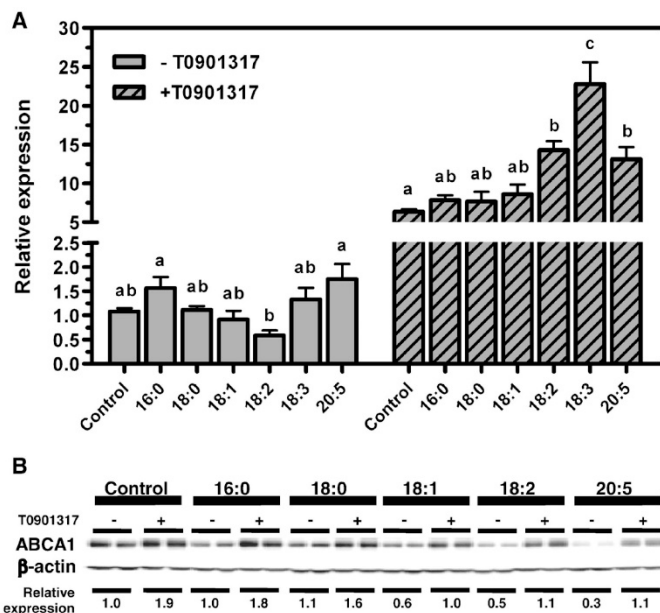


Figure 1. Unsaturated fatty acids inhibited ABCA1 expression at the posttranscriptional level in HepG2 cells. Cells were incubated with or without an LXR agonist T0901317 (10 μ mol/L) for 18 hours and, subsequently, with BSA only (control) or fatty acids (100 μ mol/L) complexed with BSA for 12 hours. A, Adenosine triphosphate-binding cassette transporter A1 mRNA abundance by qPCR. Values are expressed as means \pm SEM; $n = 6$. Bars without a common letter are significantly different; $P < .05$. B, Adenosine triphosphate-binding cassette transporter A1 protein by Western blot analysis with β -actin as a loading control. A representative blot of 2 separate experiments is shown. Densitometry analysis was conducted using β -actin for normalization, and ABCA1 protein expression levels relative to control without T0901317 treatment are shown as numbers below the gel image. Values of 2 lanes of the same treatment are averaged.

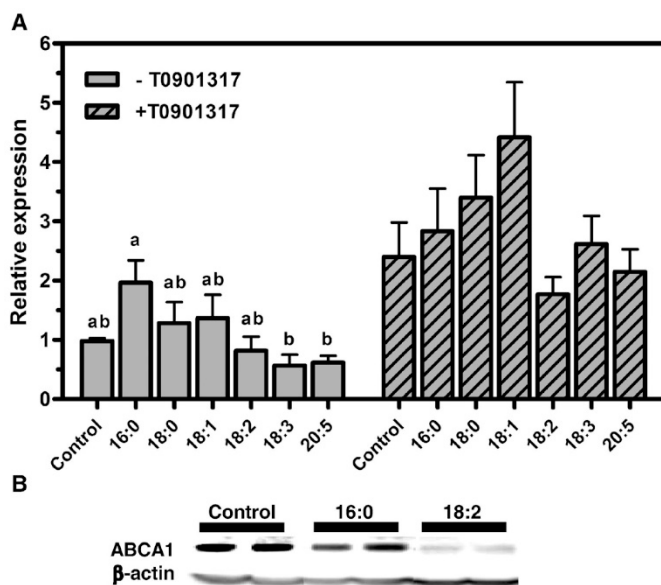


Figure 2. Unsaturated fatty acids inhibited ABCA1 expression at the posttranscriptional level in FHs 74 Int cells. Cells were without or with T0901317 (10 μ mol/L) for 18 hours and, subsequently, with BSA only (control) or fatty acids (100 μ mol/L) complexed with BSA for 12 hours. **A**, Adenosine triphosphate-binding cassette transporter A1 mRNA abundance by qPCR. Values are expressed as means \pm SEM; $n = 6$. Bars without a common letter are significantly different; $P < .05$. There was no statistical significance detected between groups in the presence of T901317. **B**, Adenosine triphosphate-binding cassette transporter A1 protein of cells treated with fatty acids in the presence of T0901317 is visualized by Western blot analysis, with β -actin as a loading control. A representative blot of 2 separate experiments is shown.

Degradation of ABCA1 is an important regulatory mechanism for its activity.^{50,51} Post-transcriptional regulation of ABCA1 by unsaturated fatty acids is suggested to result from destabilization of ABCA1 protein through its phosphorylation by PKC δ in macrophages.¹⁶ To evaluate if the same mode of action exists in HepG2 and FHs 74 Int cells, the cells were incubated with 16:0 or 18:2 in the presence of 5 or 10 μ mol/L of rottlerin, a PKC δ inhibitor. Although rottlerin tended to increase ABCA1 protein in most treatments, it did not abolish the repressive effect of 18:2 on ABCA1 protein in HepG2 cells (Fig. 3A) and FHs 74 Int cells (Fig. 3B). To further verify the involvement of PKC δ in the degradation of ABCA1 protein by unsaturated fatty acids, HepG2 cells were transfected with control scrambled or PKC δ siRNA to knockdown PKC δ ; and, subsequently, the cells were incubated with 16:0 and 18:2. Depletion of PKC δ by approximately 70% to 90% by siRNA was not able to abolish the repressive effect of 18:2 on ABCA1 protein (Fig. 4). Interestingly, knockdown of PKC δ decreased ABCA1 protein in all treatments, suggesting that PKC δ might play a role in maintaining ABCA1 protein levels in the cell line.

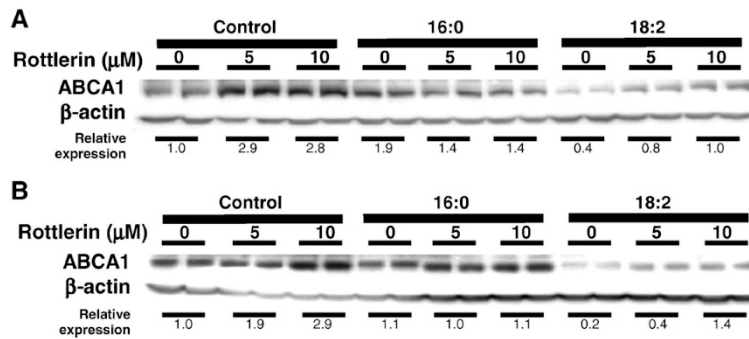


Figure 3. Rottlerin increased ABCA1 protein but could not reverse unsaturated fatty acid-induced down-regulation of ABCA1 protein expression in HepG2 and FHs 74 Int cells. HepG2 (A) and FHs 74 Int (B) cells were preincubated with rottlerin (5 and 10 μmol/L) for 2 hours, after which they were treated with BSA only (control), 16:0 or 18:2 (100 μmol/L) for 12 hours. Adenosine triphosphate-binding cassette transporter A1 protein expression was measured by Western blot analysis using β-actin as a loading control. A representative blot of 2 separate experiments is shown, and relative ABCA1 protein levels to control with no rottlerin treatment based on densitometry analysis are listed.

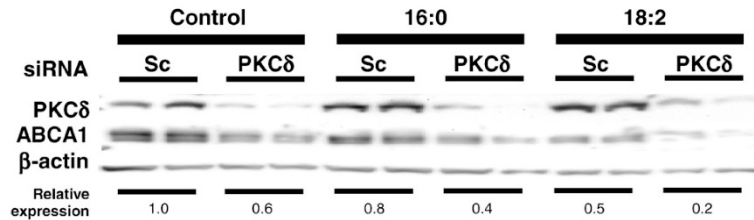


Figure 4. Depletion of PKCδ failed to abolish decreased ABCA1 protein levels by unsaturated fatty acids in HepG2 cells. Cells were transfected with 100 nmol/L of scrambled control or PKCδ siRNA for 24 hours and subsequently incubated with BSA only (control), 16:0 or 18:2 (100 μmol/L) for 24 hours. Adenosine triphosphate-binding cassette transporter A1 and PKCδ protein levels were determined by Western blot analysis using β-actin as a loading control. A representative blot of 2 separate experiments is shown, and relative ABCA1 protein levels to scrambled control based on densitometry analysis are listed. Sc indicates scrambled control.

4. Discussion

Of the various complications associated with type 2 diabetes, CVD is the leading cause of morbidity and mortality in diabetic subjects with 2- to 8-fold higher risk than the nondiabetic population.⁵²⁻⁵⁴ As increased plasma free fatty acid concentrations and influx into tissues have been suggested to play a role in diabetic dyslipidemia^{31-33, 36-38} and ABCA1 in the liver and intestine is the major factor to maintain plasma HDL cholesterol levels,^{7,8} our current study was undertaken to investigate the effect of fatty acids on the hepatic and intestinal ABCA1 expression. We found that unsaturated fatty acids, polyunsaturated fatty

acids in particular, repressed ABCA1 expression in HepG2 and FHs 74 Int cells at the post-transcriptional level and that PKC δ could play an important role in maintaining ABCA1 protein levels.

Adenosine triphosphate-binding cassette transporter A1 facilitates the efflux of phospholipid and free cholesterol to lipid-free or lipid-poor apoA-I, which is the critical step for HDL biogenesis.^{2,3} In vitro interaction of apoA-I with ABCA1 produces heterogeneous-sized, pre- β HDL subpopulations, which have a different metabolic fate depending on the degree of lipidation, with less lipidated HDL being rapidly removed by kidney.^{55,56} Tissue site of ABCA1 expression is also important for the production of HDL, as dysfunctional ABCA1 in the liver and intestine led to decreased plasma HDL cholesterol concentrations by approximately 80% and 30%, respectively.^{7,8} The studies emphasize a pivotal role of ABCA1 in HDL biogenesis by lipidating newly secreted apoA-I in the liver and intestine, which are exclusive sites of apoA-I secretion. Accordingly, lack of interaction of apoA-I with ABCA1 in the liver and intestine due to diminished ABCA1 expression could possibly lower the production of nascent HDL, consequently reducing plasma HDL cholesterol levels. In the present study, ABCA1 protein levels were significantly lowered by unsaturated fatty acids but not by saturated fatty acids without reducing its mRNA abundance in HepG2 and FHs 74 Int cells. The posttranscriptional inhibition of ABCA1 expression has also been shown in other cells.^{11,13,14,17} Considering the critical role of hepatic and intestinal ABCA1 in HDL biogenesis, the diminished ABCA1 expression by unsaturated fatty acids could have a negative impact on plasma HDL cholesterol concentrations.

Although much attention has been given to the changes in plasma total and low-density lipoprotein (LDL) cholesterol concentrations in response to dietary fatty acids, studies have shown that dietary fatty acids can alter plasma HDL cholesterol levels. Isoenergetic substitution of n-3 polyunsaturated fatty acids for saturated fatty acids decreased plasma concentrations of total cholesterol, LDL cholesterol, and HDL cholesterol in nonhuman primates⁵⁷ and humans.⁵⁸ African green monkeys fed atherogenic diets containing polyunsaturated fatty acids had significantly decreased plasma concentrations of total cholesterol, HDL cholesterol, and apoA-I compared with saturated and monounsaturated fatty acid-fed animals.⁵⁹ In addition, a recent study in humans reported that plasma HDL cholesterol concentrations were lower when subjects were on a polyunsaturated fatty acid-rich diet compared with a saturated fatty acid-rich diet.⁶⁰ Therefore, studies exist to support an HDL cholesterol-lowering effect of unsaturated fatty acid and polyunsaturated fatty acids in particular, relative to saturated fatty acids. We found that all unsaturated fatty acids decreased ABCA1 protein levels in HepG2 cells but that polyunsaturated fatty acids, such as 18:2 and 20:5, induced the greatest reduction. Our data suggest that the decreased plasma HDL cholesterol concentrations by unsaturated fatty acids compared with saturated fatty acids could be, at least in part, attributed to diminished hepatic and intestinal ABCA1 expression, lowering nascent HDL formation.

Turnover of ABCA1 protein is rapid with a half-life of less than 1 hour in murine macrophage-like cells and differentiated THP-1 cells,^{51,61} suggesting that posttranscriptional regulation could be an important determinant for its function. Unsaturated fatty acids, but not saturated fatty acids, were shown to increase ABCA1 protein degradation in macrophages and baby hamster kidney cells by activating phospholipase D2 and, subsequently,

PKC δ , which resulted in the serine phosphorylation and destabilization of ABCA1.^{15,16} However, we found that neither rottlerin nor depletion of PKC δ by siRNA abolished the repressive effect of 18:2 on ABCA1 protein levels in HepG2 and FHs 74 Int cells. Instead, rottlerin treatment tended to increase the transporter protein, whereas PKC δ knockdown lowered protein levels independent of types of fatty acids. Our results indicate that PKC δ is not involved in facilitating ABCA1 protein degradation by unsaturated fatty acids in HepG2 and FHs 74 Int cells and that it is likely to play a role in maintaining ABCA1 protein expression. The reasons for the contradictory observations between our results and previously reported data are not clear. However, as we found the similar response in RAW 264.7 macrophages (manuscript in submission), we do not think the contradiction is because of different cell types used in the 2 studies. More investigation is necessary to explain the difference.

Although rottlerin has been widely used as a selective PKC δ inhibitor, there is a recent debate on its selectivity for PKC δ , as it inhibits other kinases.⁶² Increased ABCA1 expression by rottlerin but decreased expression by PKC δ siRNA shown in our study favor that rottlerin is not a PKC δ -specific inhibitor. Phosphorylation of ABCA1 can alter its protein degradation or activity depending on sites of phosphorylation. Phosphorylation in the PEST sequence (proline, glutamic acid, serine, and threonine) of ABCA1 is shown to increase the protein degradation by calpain,^{63,64} whereas ABCA1 protein levels are positively correlated to its phosphorylation in THP-1 cells.⁵⁰ Given that rottlerin treatment enhanced ABCA1 proteins, we speculate that kinases susceptible to the inhibition by rottlerin could be present to lower ABCA1 protein in HepG2 and FHs 74 Int cells. Identification of the kinases and phosphorylation sites in ABCA1 is important to increase ABCA1 expression and, consequently, plasma HDL cholesterol concentrations.

In summary, the present study demonstrated that unsaturated fatty acids lower ABCA1 expression in HepG2 and FHs 74 Int cells. The inhibition by unsaturated fatty acids could have a large impact on HDL formation, lowering plasma HDL cholesterol concentrations. As plasma HDL cholesterol levels are inversely related to the risk of CVD, one could argue that the potential unfavorable effect of unsaturated fatty acid on ABCA1 contradicts a well-known atheroprotective role of unsaturated fatty acids, polyunsaturated fatty acids in particular, compared with saturated fatty acids. Atheroprotection of polyunsaturated fatty acids, however, is attributable to their effects on very low-density lipoprotein, LDL, and triglyceride metabolism, anti-inflammation, and antithrombosis.^{57,65-67} These benefits could override the inhibition of hepatic and intestinal ABCA1 expression by polyunsaturated fatty acids in protecting against atherosclerosis. The repressive effect of unsaturated fatty acids on ABCA1 expression has a particular health implication in obese and type 2 diabetic subjects, whose plasma free fatty acids are commonly elevated. Increased fatty acid influx into tissues consequent to high plasma fatty acid levels is known to perturb various metabolic pathways related to insulin resistance and diabetic dyslipidemia. Proper dietary interventions for the pathologic conditions require our better understanding of the modulation of ABCA1 expression by fatty acids. In vivo study warrants verification of our in vitro findings to develop dietary strategy to achieve relative abundance of each type of fatty acids in the diet to maintain optimal health.

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