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REGULATION OF ATP BINDING CASSETTE TRANSPORTER A1 AND G1 EXPRESSION BY FATTY ACIDS

Chai Siah Ku
University of Nebraska at Lincoln, csku2@huskers.unl.edu

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by

Chai Siah Ku

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Chai Siah Ku, M.S.
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Advisor: Ji-Young Lee

High density lipoprotein (HDL) is an atheroprotective element which is critical in the reverse cholesterol transport (RCT) pathway to eliminate excess cholesterol from the periphery of the body. A low plasma HDL cholesterol levels indicate a higher risk of getting cardiovascular diseases (CVD). Formation and metabolism of HDL largely rely on ATP binding cassette transporter A1 (ABCA1) and G1 (ABCG1) that are transporters to facilitate the efflux of cellular cholesterol to outside of cells. ABCA1 in the liver and small intestine is the major contributing factor for HDL formation. On the other hand, ABCA1 and ABCG1 present in macrophages contribute to cellular cholesterol homeostasis and protection against foam cell formation in artery. The objective of the study was to elucidate the regulatory mechanism for the expression of ABCA1 and ABCG1by fatty acids in HepG2 (a human hepatoma cell line), FHs 74 Int (a human small intestine epithelial cell line) and RAW264.7 macrophages. In all these cell lines, unsaturated fatty acids, but not saturated fatty acids, repressed the expression of ABCA1 and ABCG1 at the transcriptional and/or post-transcription levels in a cell type specific manner. The results of this study suggest the following mechanisms for the repressive effect of unsaturated fatty acids on the ABCA1 and ABCG1 expression: 1) ABCA1 in

HepG2 and FHs 74 Int cells is inhibited by unsaturated fatty acids at the post-transcriptional level via calpain-independent mechanism; 2) ABCA1 and ABCG1 in RAW264.7 macrophages are repressed by unsaturated fatty acids at the transcriptional level by a liver X receptor-dependent mechanism and at the post-transcriptional level by increasing protein degradation.

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Introduction

Type 2 Diabetes is one of the deadliest diseases in the United States, affecting 8% or 17.9 million people in 2008 while another 57 million are at the border line of diabetes according to American Diabetes Association. Among the complications of diabetes, cardiovascular disease (CVD) is the top leading cause of morbidity and mortality in the diabetic subjects [1, 2]. Inverse relationship between plasma high density lipoprotein (HDL) cholesterol concentrations and the CVD risk has been reported in many human studies, indicating that plasma HDL is an atheroprotective element [3, 4]. HDL removes excess cholesterol from the peripheral tissues to the liver for ultimate excretion from the body and this process is called "reverse cholesterol transport (RCT)" [5-7].

HDL formation and maturation is very important as they affect the efficiency of RCT pathway. Formation of nascent HDL begins with the association of apolipoprotein A1 (apoA-1), a major protein component of HDL particles consisting up to 70% of HDL proteins, with phospholipids and free cholesterol [8-10]. The formation of nascent HDL in the initial stage of RCT highly depends on the cholesterol efflux from intracellular spaces to extracellular acceptors via ATP binding cassette transporter A1 (ABCA1) [10, 11]. ABCA1 promotes the efflux of cellular phospholipids and cholesterol to lipid-free apoA-I to form nascent HDL [10, 12]. On the other hand, maturation of HDL depends on cholesterol efflux from peripheral tissue and ATP binding cassette G1 (ABCG1) to mature HDL particle for further lipidation. Significant role of ABCG1 in cholesterol and lipid metabolism was supported by observations of a massive accumulation of cholesterol and neutral lipids in the macrophages and liver of *Abcg1* knockout mice as well as a significant reduction in the cholesterol efflux to HDL when ABCG1 was knocked down

in macrophages by RNA interference [13, 14]. ABCA1 in the liver and intestine contribute to 80% and 20% of the HDL biogenesis, respectively, while cholesterol efflux in macrophages through ABCA1 and ABCG1 is important for cellular cholesterol homeostasis as well as inflammation[15].

Fatty acids have been suggested to alter the expression of the ABC transporters. In particular, it has been shown that ABCA1 and ABCG1 expression is suppressed by unsaturated fatty acids (UFA), but not by saturated fatty acids in macrophages. In addition, patients with type 2 diabetes have 80% lower ABCG1 expression at both mRNA and protein levels in macrophages with impaired cholesterol efflux to HDL [16]. The studies indicate an important link for the elevated CVD risk in type 2 diabetes where low plasma HDL cholesterol levels are commonly observed.

The objective of this study was to elucidate molecular mechanisms for the regulation of ABCA1 and ABCG1 in HepG2, FHs 74Int cells and RAW264.7 macrophages by fatty acids. The results of this study suggest that ABCA1 and ABCG1 expression is repressed by unsaturated fatty acidsat the transcriptional as well as post-transcriptional levels in a cell type-specific manner.

Literature Review

I. Obesity and Chronic Disease,

Obesity. Obesity is defined as a body mass index (BMI) of greater than 30.0 kg per m^2. In 2007-2008, the age-adjusted prevalence of obesity in the United States was 33.8% and the corresponding prevalence estimates for overweight and obesity combined were 68.0%[17].

Numerous studies reported elevated lipid contents in the circulation in obesity contribute to the development of cardiovascular diseases (CVD) in 3 different ways: impaired insulin dependent glucose uptake, increased production of pro-inflammatory markers including C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), and alterations in secretion of adipose-derived signaling molecules called adipokines [2, 18]. Free fatty acids (FFA) and adipokines synthesized in adipocytes have been reported to play an important role in glucose homeostasis in the body [19]. In particular, studies have demonstrated that the elevated levels of circulating free fatty acids can cause peripheral insulin resistance in both animals and human while acute lowering of FFA enhanced glucose uptake in the insulin-dependent periphery tissue [19-23]. In addition, pro-inflammatory cytokines can interact with oxidized low density lipoprotein (Ox-LDL) and trigger a series of inflammatory responses within arterial wall, leading to the development of atherosclerosis[24]. Meanwhile, late observation of various pro-atherogenesis and also cardioprotective adipokines from different tissue including foam cells due to obesity might contribute to occurrence of CVD in obesity [25]. The close relationship between obesity and CVD is further evidenced by marked delay in the

development of type 2 diabetes and amelioration of CVD in obese subjects who take antiobesity agents and weight loss by 31 -53 percent compared to placebo[26, 27].

CVD. CVD, as defined by the World Health Organization (WHO) as a group of cardiovascular disorders includes coronary heart disease (CHD), cerebrovascular disease, hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. According to the American Heart Association (AHA), there is a total of approximately 81.1 million of the population in the United States diagnosed at least one or more CVD in year 2006 [28]. Major risk factors for CVD are tightly linked to metabolic syndrome, which is a concurrence of disturbed glucose and insulin metabolism, overweight and abdominal fat distribution (obesity), dyslipidemia, and hypertension, as defined by thee WHO, National Cholesterol Education Program Adult Treatment Panel III (NCEP-APIII) and the International Diabetes Federation [29, 30]. Although the definition of metabolic syndrome is still in dispute among scholars and authoritative organizations, it is generally accepted that metabolic syndrome is close related to likelihood of CVD development [31].

Type 2 Diabetes Mellitus. American Diabetes Association reported a total of 3.6 million children and adults or7.8% of the population in the United States are diabetic and diabetes was the seventh leading cause of death in 2006 [28]. In 2004, CVD was noted on 68% of diabetes-related death certificates while 16% of diabetes-related death certificates were due to stroke among people aged 65 years or older [1, 2, 28]. Adults with diabetes have heart disease death rates and occurrence of stroke about 2 to 4 times higher than

adults without diabetes. By far, CVD is the most detrimental complication among all diabetes related complication among diabetic subjects [1].

As a part of metabolic syndrome, development of type 2 diabetes occurs more prominently in obese subjects compared with lean individuals; however, genetic background and environmental predisposition such as physical inactivity are undeniable contributing factors to type 2 diabetes. Extensive accumulation of fat droplets in the adipocytes of obese subjects may lead to impaired responsiveness of skeletal muscle to insulin and failure of insulin secretion by pancreatic β cells [22]. Weight loss therapy coupled with drug therapy, such as metformin, a type of oral anti-hyperglycemic agent has been shown to efficiently maintaining euglycemia and hence prevent type 2 diabetes [27].

Patients with diabetes and insulin resistance are at higher risk of developing CVD or CHD [32]. Excessive macronutrient intake especially foods rich in saturated fat and refined carbohydrates, has been shown to induced oxidative and inflammatory stress and to be associated with the induction of inflammation such as increased expression of suppressor of cytokine signaling-3 (SOCS-3), a mediator that interfers with insulin signal transduction [33].

II. Lipids and lipoprotein Metabolism.

Dietary lipids. Several studies have shown that excessive intake of foods rich in saturated fatty acids induces oxidative and inflammatory stress by generating reactive oxygen species (ROS) while caloric restriction is effective in preventing inflammation[33].

Dietary lipids from both animal sources and plant sources abundantly contain triacylglycerols (TAGs) that consist of glycerol and saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), *trans*-fatty acids, phospholipids, and sterols. Both dietary cholesterol and denovo synthesized cholesterol are transported mainly in lipoproteins in the circulation. As the pre-cursor of bile acids, vitamin D and various hormones such as corticosteroids and sex hormones[15] , cholesterol is very crucial for normal physiological functions of the cells and endocrine system. In addition, fluidity and permeability of mosaic structure of cell membrane rely on cellular cholesterols level.

Approximately ~300-500mg of cholesterol is consumed per day from animal products while another 1000-1600mg per day of cholesterol is made by endogenous synthesis. VLDL contains 10–15% of the total serum cholesterol, LDL cholesterol typically makes up 60–70% of the total serum cholesterol while HDL cholesterol normally makes up 20–30 % of the total serum cholesterol [28].

Dyslipidemia is commonly observed in obese and diabetic patients and the subjects have elevated levels of plasma TAG, total cholesterol, LDL cholesterol and reduced high density liporprotein (HDL) cholesterol concentrations [26, 34].NCEP-APIII has defined dyslipidemia as having a total TAG levels of 150mg/dl or higher, total cholesterol levels of 200mg/dl or higher, LDL cholesterol 130mg/dl or higher, and HDL cholesterol of 40mg/dl or lower. Plasma LDL cholesterol-lowering therapy with various drugs such as statins, HMG-CoA reductase inhibitors, can reduce incidence of CVD. In contrast, low plasma HDL cholesterol levels, which is inversely connected with the CVD risk, is another good predictor of CVD [28].

Lipoproteins. Digested lipid component is absorbed in its simplest form into small intestine and packages into chylomicron, the largest lipoprotein in the circulation, to be transported in circulation. After chylomicrons losing TAG by lipoprotein lipase (LPL), resulting chylomicrons remnants are transported to the liver. In the liver, very low density lipoprotein (VLDL) is formed and functions to transport lipids in the circulation. VLDL becomes low density lipoprotein (LDL) after TAG in the particles is hydrolyzed by LPL. Most od LDL is recognized by LDL receptor (LDLR) primarily in liver and removed from the circulation.

Chylomicrons are the largest but lowest density lipoproteins with a density of less than 0.95g/mL. Their major function is to transport dietary lipids, TAG in particular. Therefore, it is normally abundantly found in circulation at a postprandial state. It contains ~85% of TAG, 9% of phospholipids, 3% of cholesterol ester, 2% of protein, and 1% of free cholesterol. Although apolipoprotein B-48 (apoB-48) is the main protein in chylomicron particles, chylomicrons also contain apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE. Chylomicrons leave enterocytes through lymphatic systemand by pass the hepatic portal vein. TAG in chylomicrons is generally used or stored in the peripheral tissues responsive to insulin. The chylomicron remnant is cleared from circulation to the liver.

VLDL is assembled in the live with apoB-100 and other lipids. It is the second largest lipoprotein class found in the human circulation, with a density of 0.95 to 1.006 g/mL. It is a TAG-rich lipoprotein, containing 50% TAG, 18% phospholipid, 12% cholesteryl ester, 10% proteins and 7% free cholesterol. ATP III identifies elevated serum

VLDL cholesterol levels as a the surrogate marker for elevated atherogenic remnants in persons with triglycerides ≥200 mg/dL[28].

LDL is the second smallest lipoprotein with a density of 1.019 to 1.063 g/mL. It contains 38% cholesteryl ester, 24% protein, 20% phospholipid, 8% free cholesterol and only 10% of TAG. The only apolipoprotein found in LDL particles is ApoB-100. Framingham Heart Study conducted by National Heart, Lung and Blood Institution demonstrated that high levels of plasma LDL cholesterol promote the development of CVD and atherosclerosis. The process is generally triggered by LDL oxidation in the intima of artery. Oxidation has a potential to make the apoB-100 undergo oxidative scission, further modify LDL confirmation, and disallow it to escape from intima [24].

HDL is the smallest and densest lipoproteins class found in the circulation. It participates in the RCT pathway that removes excess cholesterol from peripheral tissue to the liver for excetion from the body. It contains 55% of protein particles, 24% phospholipids, 15% cholesterol ester, 4% of TAG and only 2% of free cholesterol. The major apolipoproteins found in HDL particles are 75% apoA-I and 25% apoA-II, while the rest of the apolipoproteins are make up by apoA-IV, apoC-I, apoC-II, apoC-III and apoE [8, 35].

The formation of nascent HDL is started from lipidation of lipid free apoA-I in a combination with phospholipid and cholesterol effluxed through ATP binding cassette transporter A1 (ABCA1). ApoA-1 knockout mice showed accelerated atherosclerosis development compared with wild-type mice while the opposite protective effect was found when ApoA-I was overexpressed in mice. Nascent HDL picks up more cholesterol from peripheral tissue via ABCA1 and ABCG1 in the peripheral tissues and become

mature. Several factors contribute to low HDL cholesterol levels including elevated serum TAG, overweight and obesity, physical inactivity, cigarette smoking, type 2 diabetes, certain drugs and genetic factors. High HDL cholesterol levels are considered as a negative risk factor for CVD and has been suggested as a treatment goal in preventing CVD by ATP II [28].

III. Atherosclerosis and ATP Binding Cassette Transporters

***Development of Atherosclerosis*.** Among all types of CVD, atherosclerosis has raised most attention because it is highly related to dietary habits and lifestyle. Atherosclerosis is a condition pictured by narrowing of artery lumen due to a build-up of cholesterol-rich plaque in the intima of artery in respond to artery inflammation. The plaque contains soft, lipid-rich cores and when it ruptures, it is quickly covered by thin and inflammatory caps of fibrous tissues, causing a blockage of artery lumen in a very short time. This leads to death of acute coronary syndromes and strokes [36]. In general, atherogenesis happens in a normal physiological condition through out the lifespan. However, the prevalence has switched to younger generation in recent year [28]. In addition to dyslipidemia, other contributing factors to atherogenesis include gender, family history, cigarette smoking, hypertension, improper dietary intake, and physical inactivity.

Atherogenesis is a multiple stages process. When excess LDL particles are circulating in the body, they tend to be oxidized in the intima of artery [24]. Minimally modified LDL can increase leukocyte-endothelial adhesion and secretion of monocyte chemotactic protein-1 (MCP-1) and stimulate transformation of monocyte to

macrophages [24, 34]. Other pro-inflammatory cytokines and molecules include CRP, interleukins (ILs), and TNFα. OxLDL is not recognized by LDLR but it is taken up to the macrophages via the scavenger receptor. Cholesterol-loaded macrophages become foam cell and further trigger the migration of monocytes into intima, aggravating the foam cell formation [24]. Foam cells also contain small but significant amounts of various lipid oxidation products, which are potent mediators of macrophage function.

Reverse Cholesterol Transport (RCT). RCT is a vital process where excess free cholesterol is being transported from the peripheral tissues by HDL to the liver for secretion into bile and ultimate excretion from the body [7]. It is initiated when apoA-I interacts with serum phospholipids and free cholesterol via ABCA1 to form nascent HDL. Formation of nascent HDL triggers cholesterol efflux in the macrophages and other peripheral tissues via ABCA1 and ABCG1and eventually become mature HDL particles. Free cholesterol is normally esterified by lecithin:cholesterol acyltransferatse (LCAT) to be incorporated in the core of HDL. LCAT is necessary for extracellular cholesterol metabolism by facilitating cholesterol uptake into HDL in a more stable form and maintain a low plasma free cholesterol levels [37]. Phospholipid transfer protein (PLTP) is involved in the exchange of phospholipids between TAG-rich lipoproteins and HDL, contributing to transformation between HDL3 and HDL2. It has been reported that PLTP enzyme activity is higher in diabetic and obese patient and is correlated with high plasma TAG and low plasma HDL cholesterol levels[38]. Cholesterol ester transfer protein (CETP) allows exchange of cholesterol ester between HDL and LDL [7]. Up-regulation of the expression of macrophage ABCA1 and ABCG1 by activating liver X receptor

(LXR), a major transcription regulator for the ABC transporters, with its agonist is one way to increase RCT activity [39].

ATP binding Cassette transporter A1 and G1. There are at least 48 ABC transporter proteins have been recognized and categorized into seven families from A to G according to their sequence, functions, locations and similarities [40].Some of them are involved in lipid transport and ABC A transporters and ABC G transporters are most well-known for lipid transportand metabolism. [40]

ABCA1 is a LXR responsive gene and plays a critical role in cholesterol efflux from cells to extracellular acceptors such as lipid free apoA-1, forming nascent HDL [7]. It is a 240 kDa protein and a full transporter with 12 membrane spanning domains. ABCA1 is regulated when LXR α or β binds to LXR responsive element in the ABCA1 promoter together with an obligate heterodimer partner retinoid X receptor (RXR) [39]. Mutations in ABCA1 result in Tangier disease, a severe HDL deficiency characterized by accumulation of cholesterol in tissues rich in macrophages due to the impaired ability of cholesterol efflux [6, 41, 42]. The Tangier patients are generally diagnosed with pre-mature atherosclerosis. ABCA1 is highly expressed in most of cell types, including hepatocytes, enterocytes, adipocytes, skeletal muscle cells and macrophages.

ABCG1 plays an important role in cholesterol efflux to mature HDL particles, which is a key step in the RCT pathway for the prevention of atherosclerosis. It is a half size transporter and therefore has to form a homodimer or heterodimer with another ABCG transporter to be functional [40]. In general, the molecular weight of ABCG1 is about 70 kDa [13]. Studies have shown that macrophages with knockdown of ABCG1

have a significant reduction in cholesterol efflux to HDL [43]. *Abcg1* knockout mice exhibited massive accumulation of cholesterol in the macrophages and lung when they were fed a high fat-high cholesterol diet compare to the wild-type [14]. In addition, cholesterol efflux to HDL was increased in wild type mouse macrophages by T0901317, an LXR agonist, but not in *abcg1* knockout macrophages [14].

Patients with type 2 diabetes have 80% lower ABCG1 expression at both mRNA levels and protein level in macrophages with impaired cholesterol efflux to HDL [16]. ABCG1 is regulated by cellular cholesterol content, evidenced by an increased expression in both mRNA and protein levels in the present of acetylated LDL, and down-regulation with HDL3 treament in monocyte-derived macrophages [13, 44].

Expression of ABCA1 and ABCG1 is very important in HDL formation as well as cellular cholesterol homeostasis. Elevated cholesterol level within cells has been shown to upregulate the expression of ABCA1 and ABCG1 and vice versa [45]. Up-regulation of ABCA1 and ABCG1 expression can be achieved by the activation of LXR, which is confirmed by the observations that expression of ABCA1 and ABCG1 was elevated by LXR agonists in various cell lines [39]. Moreover, LXR agonists contribute to redistribution of ABCG1 from intracellular membrane to the plasma membrane, consequently facilitating cholesterol efflux [39]. Endotoxin, an LXRα down-regulator, has been shown to decrease mRNA and protein expression of ABCA1 and ABCG1 [46]. In addition, ABCA1 and ABCG1 expression is also positively induced by peroxisome proliferator-activated receptor (PPAR), fibric acids, cyclic AMP phosphorylated protein kinase A (cAMP/PKA) pathway in a LXR/RXR-dependent or independent manner [39]. Recent studies indicate that PEST sequence in murine ABCA1 is responsible for its

phosphorylation and thus promote protein degradation in a calpain dependent manner [45, 47].

ABCA1 is shown to be regulated by fatty acids at both transcription and post-transcription levels. Studies have shown that unsaturated fatty acids repress the expression of ABCA1 by increasing protein degradation via the activation of phospholipase D2 (PLD2) and subsequent phosphorylation on serine residues by protein kinase C (PKC) delta [12, 48, 49].

Increased ABCA1 mRNA and protein expression has been shown in human monocyte-derived macrophages in the present of acetyl salicylic acid (ASA, aspirin), indicating a potential role of ABCA1 in preventing arterial inflammation [36].

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Chapter 1

Post-transcriptional regulation of ATP-binding cassette transporter A1 (ABCA1) expression by unsaturated fatty acids in HepG2 and FHs 74 Int cells

1.1 Abstract

ATP binding cassette transporter A1 (ABCA1) plays a critical role in the formation and metabolism of high-density lipoproteins (HDL). ABCA1 in the liver and small intestine, in particular, accounts for ~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 investigated the modulation of ABCA1 expression by fatty acids in vitro using human hepatoma HepG2 and human small intestine epithelial FHs 74 Int cells. The cells were incubated with saturated and unsaturated fatty acids in the presence or absence of T0901317, a synthetic agonist of liver X receptor (LXR). Unsaturated fatty acids reduced ABCA1 protein levels at 100 μM of concentration regardless of the agonist with a minimal effect on mRNA abundanceIncubation of HepG2 and FHs 74 Int cells with rottlerin, a protein kinase C δ (PKCδ) inhibitor, increased ABCA1 protein in all treatments tested, but did not abolish linoleic acid (18:2)-induced reduction in ABCA1 protein levels. Depletion of PKCδ using small interfering RNA showed a reduction in ABCA1 protein levels in control, palmitic acid (16:0) and 18:2-treated cells, but it further aggravated the repressive effect of 18:2 on ABCA1 protein in HepG2 cells. The reduced ABCA1 protein levels by 18:2 were not abolished by calpeptin, an inhibitor of calpain protease. In conclusion, our results indicate that unsaturated fatty acids regulate ABCA1 expression in HepG2 and FHs 74 Int cells at the post-transcriptional level in a calpain protease-independent manner. PKCδ is likely to be involved in maintaining ABCA1 protein levels and potentially prevents a reduction in the protein level by unsaturated fatty acids.

1.2 Introduction

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 deficiencies [4]. Studies on targeted inactivation of *Abca1* in mice also support the role of ABCA1 in the efflux of phospholipid and free cholesterol and HDL formation [5, 6]. In particular, deletion of *Abca1* in the liver and intestine reduced plasma HDL cholesterol concentrations by ~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 to transcriptional regulation by cholesterol, ABCA1 expression can also be regulated transcriptionally by fatty acids. In human macrophages, ABCA1 expression was lower in 18:2-treated cells than cells incubated with 16:0 [10]. ABCA1 mRNA levels in HepG2 and RAW 264.7 macrophages were decreased by unsaturated fatty acids with a reduction in ABCA1 promoter activity [11]. Mutations or deletion of direct repeat 4, the LXR/retinoid X receptor (RXR) heterodimer binding element, in the promoter of *Abca1* abolished the suppressive effects of unsaturated fatty acids on ABCA1 expression [12]. These studies indicate unsaturated fatty acids, but not

saturated fatty acids, reduced ABCA1 expression by inhibiting the binding of LXR/RXR heterodimers to the *Abca1* promoter. Post-transcriptional regulation of ABCA1 by unsaturated fatty acids in macrophages has also been demonstrated. Wang and Oram [13-16] found that unsaturated fatty acids increased ABCA1 protein degradation in macrophages by activating phospholipase D_2 (PLD_2) and subsequently protein kinase C δ (PKCδ), which in turn phosphorylates ABCA1 serine residues for protein degradation. In Caco-2 cells, post-transcriptional 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 ~15-30% in type 2 diabetic individuals are associated with an increased risk of CVD in this population [26, 27]. As epidemiological studies have consistently shown a strong inverse relationship between plasma HDL cholesterol concentrations and the incidence of CVD [26-28], the reduced plasma HDL cholesterol levels in diabetic subjects could contribute to their increased CVD risk. HDL can deliver excess cholesterol from the periphery to the liver for ultimate excretion from the body. This process called “reverse cholesterol transport (RCT)” is believed to be a major athero-protective property of HDL [29-31]. 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 diabetes [32-36] and are known to have a large impact on insulin resistance and diabetic dyslipidemia [37-47]. In particular, increased free fatty acid flux to the liver contributes to hepatic insulin resistance [40-42], increased glucose output [48], steatosis [37] and high triglyceride secretion in very-low density lipoproteins [37, 43].

Given the significant role of hepatic and intestinal ABCA1 in the HDL biogenesis, understanding of the effect of fatty acids on ABCA1 in these tissues is important to determine perturbed HDL metabolism in pathological conditions such as type 2 diabetes. This study was undertaken to evaluate the effect of various fatty acids on the expression of ABCA1 in the liver and intestine using in vitro cell models, i.e., HepG2, a human hepatoma cell line, and FHs 74 Int cells, a human small intestine epithelial cell line. We found that ABCA1 expression in HepG2 and FHs 74 Int cells was post-transcriptionally repressed by unsaturated fatty acids in a calpain protease-independent manner. Our data also suggest that PKCδ may play an important role in maintaining ABCA1 protein levels and inhibit the repressive effect of unsaturated fatty acids on ABCA1 protein expression.

1.3 Materials and Methods

1.3.1 Cell culture and fatty acid preparation

HepG2 cells and FHs 74 Int cells were purchased from ATCC (Manassas, VA). The cells were maintained in Minimal Essential Medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 x vitamins and 2 mM L-glutamine in a humidified chamber at 37^{o}C with 5% CO_2. For FHs 74 Int cells, 30 ng/ml epidural growth factor and 10 μg/ml insulin were additionally supplemented to cell culture medium. All cell culture supplies were purchased from MediaTech (Manassas, VA).

Two mM of fatty acid-poor and endotoxin-free bovine serum albumin (BSA) (EMD Chemicals, Gibbston, NJ) was prepared in phosphate buffered saline (PBS). Sodium salts of fatty acids (Nu-Chek, Elysian, MN) were dissolved in the 2 mM BSA solution to a final concentration of 5 mM. The fatty acid and BSA mixture was purged

with N_2 and sonicated in warm water bath (~40^oC) until the solution became clear to form BSA/fatty acid complex (a molar ration to BAS of 1:2.5). The complex was filter sterilized and diluted with cell medium to reach a final concentration of 100 μM of fatty acid and incubated in a water bath for 1 hr prior to be added to cells. HepG2 cells were incubated with or without 10 μM of T0901317 (Sigma-Aldrich, St. Louis, MO), a LXR agonist, in dimethyl sulfoxide (DMSO) for 18 hrs, after which cells were treated with BSA only (control) or 100 μM of a fatty acid for 12 hrs. Cells were also incubated with BSA or 100 μM fatty acids (16:0 or 18:2) for 6 hrs and subsequently with 5 or 10 μM rottlerin (Santa Cruz Biotechnology, Santa Cruz, CA), a PKCδ inhibitor, in DMSO for an additional 6 hrs. Rottlerin (PKCδ inhibitor; 5 or 10 μM in DMSO; Santa Cruz Biotechnology, Santa Cruz, CA) and calpeptin (calpain protease inhibitor; 20 μg/ml in DMSO; MP Biomedicals, Solon, OH) were used to inhibit the respective enzyme. For the experiments with inhibitors, cells were preincubated with an inhibitor for 2 hrs, and subsequently with 100 μM of fatty acid for 12 hrs. Control cells were incubated with the same amount of DMSO for a vehicle control. Cells were cultured in complete medium devoid of FBS when they were treated with fatty acid to eliminate any potential contamination of fatty acids present in FBS

1.3.2 Small Interfering RNA (siRNA) Transfection

To knockdown PKCδ, HepG2 cells were transfected with Silencer® Negative Control scrambled siRNA (Ambion, Austin, TX) or siGENOME® PKCδ siRNA (Thermo Scientific, Pittsburgh, PA) using a transfection reagent DharmaFECT1 (Thermo Scientific) according to the manufacturer's protocol. In brief, 5 μl of DharmaFECT1 was

diluted into 195 μl of cell medium void of antibiotics and FBS. Scrambled control and PKCδ siRNA (2 μM) were prepared in RNase-free sterile water and 100 μl of the 2 μM siRNA solution was mixed with 100 μl of cell medium void of antibiotics and FBS. Subsequently, the media containing transfection agent and siRNA (200 μl each) were combined and incubated for 20 min at room temperature. The siRNA and transfection agent complex was then diluted to 2 ml with cell medium and added into a 6 well plate, 24 hrs after which cells were washed twice with PBS and treated with BSA or 100 μM of fatty acid for 24 hrs.

1.3.3 Total RNA Isolation and Quantitative Realtime PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer's protocol. Reverse transcription for cDNA synthesis and quantitative realtime PCR analysis were performed as previously described [49, 50]. Primers were designed according to GenBank database using the Primer Express software (Applied Biosystems, Austin, TX). The following primers were used for realtime PCR analysis: ABCA1, forward (5'-CGTTTCCGGGAAGTGTCCTA-3'), reverse (5'-GCTAGAGATGACAAGGAGGATGGA-3'); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward (5'-GTGGTCTCCTCTGACTTCAACA-3'), reverse (5'-GTTGCTGTAGCCAAATTCGTTGT-3')

1.3.4 Western Blot Analysis

Cell lysate was prepared and Western blot analysis was performed as previously described [49, 50]. Rabbit anti-ABCA1 antibody was a generous gift from Dr. John Parks

at Wake Forest University School of Medicine and PKCδ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control to normalize the data.

1.3.5 Statistical Analysis

ANOVA and Tukey's pairwise comparison with Welch's correction for unequal variance when appropriate were used to identify statistically significant differences of treatments with $P < 0.05$ considered significant by GraphPad InStat 3 (GraphPad Software Inc., La Jolla, CA). Data are expressed as mean ± SEM.

1.4 Results

1.4.1 Down-regulation of ABCA1 protein levels by unsaturated fatty acids

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 (Figure 1A). T0901317 markedly increased ABCA1 mRNA levels by ~7-25-fold. Saturated and monounsaturated fatty acids minimally altered ABCA1 mRNA whereas significantly higher mRNA levels were observed in cells treated with poly unsaturated fatty acids, i.e., oleic acid (18:1), 18:2 and eicosapentaenoic acid (20:5), than control. However, ABCA1 protein (~250 Kda) was noticeably reduced by unsaturated fatty acids tested, i.e., 18:1, 18:2 and 20:5, compared with control and saturated fatty acid-treated cells irrespective of the presence of T0901317 (Figure 1B). In particular, polyunsaturated fatty acids reduced ABCA1 protein levels to the greatest extent. Similar results were observed in FHs 74 Int cells (Figure 2A & B).

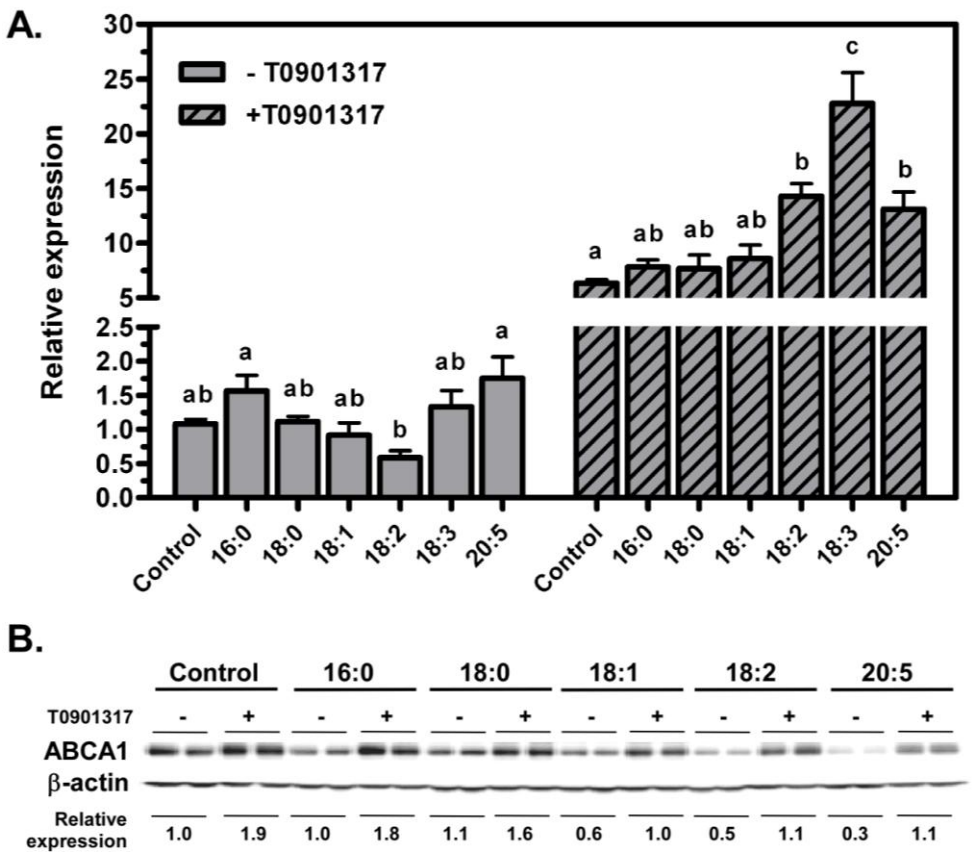

Figure 1 Unsaturated fatty acids inhibited ABCA1 expression at the post-transcriptional level in HepG2 cells. Cells were incubated with or without a LXR agonist T0901317 (10 μM) for 18 hrs and subsequently with BSA only (control) or fatty acids (100 μM) complexed with BSA for 12 hrs. **A**. ABCA1 mRNA abundance by quantitative realtime PCR. Values are Means ± SEM, n = 6. Bars without a common letter are significantly different, $P < 0.05$. **B**. ABCA1 protein by Western blot analysis with β-actin as a loading control. A representative blot of two 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 two lanes of the same treatment are averaged.

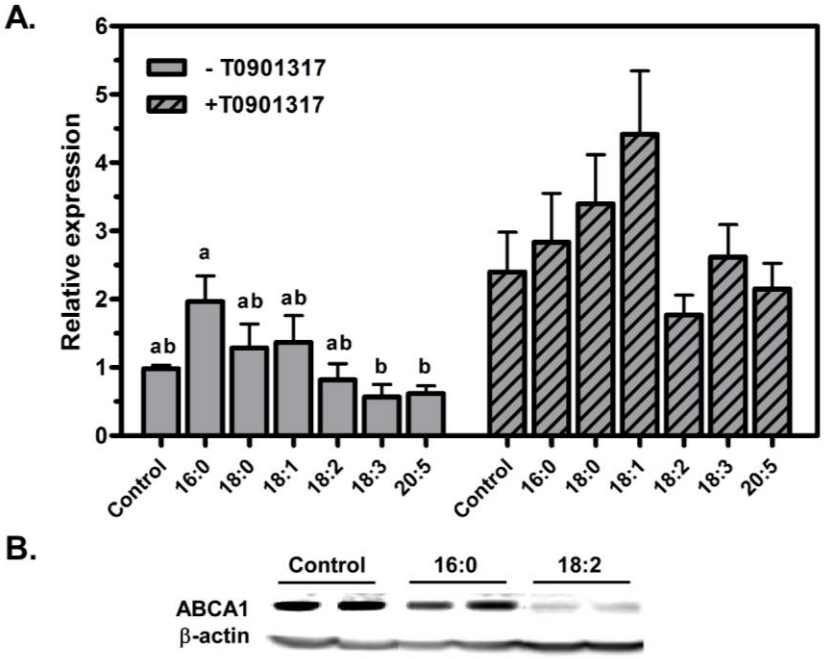

Figure 2 Unsaturated fatty acids inhibited ABCA1 expression at the post-transcriptional level in FHs 74 Int cells. Cells were without or with T0901317 (10 μM) for 18 hrs and subsequently with BSA only (control) or fatty acids (100 μM) complexed with BSA for 12 hrs. **A**. ABCA1 mRNA abundance by quantitative realtime PCR. Values are Means ± SEM, n = 6. Bars without a common letter are significantly different, $P < 0.05$. **B**. ABCA1 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 two separate experiments is shown.

1.4.2 Involvement of PKCδ in the post-transcriptional regulation of ABCA1 expression by fatty acids

Degradation of ABCA1 is an important regulatory mechanism for its activity [51, 52]. Post-transcriptional regulation of ABCA1 by unsaturated fatty acids is suggested to result from destabilization of ABCA1 protein through its phosphorylation by PKCδ in macrophages [16]. To evaluate if the same mode of action exists in HepG2 and FHs 74 Int cells, cells were incubated with 16:0 or 18:2 in the presence of 5 or 10 μmol/L of rottlerin, a PKCδ 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 (Figure 3A) and FHs 74 Int cells (Figure 3B). To further verify the involvement of PKCδ in the degradation of ABCA1 protein by unsaturated fatty acids, HepG2 cells were transfected with control scrambled or PKCδ siRNA to knockdown PKCδ and subsequently, the cells were incubated with 16:0 and 18:2. Depletion of PKCδ by ~70-90% by siRNA was not able to abolish the repressive effect of 18:2 on ABCA1 protein and even aggravated the inhibition (Figure 4). Interestingly, knockdown of PKCδ reduced ABCA1 protein in all treatments, suggesting that PKCδ might play a role in maintaining ABCA1 protein levels.

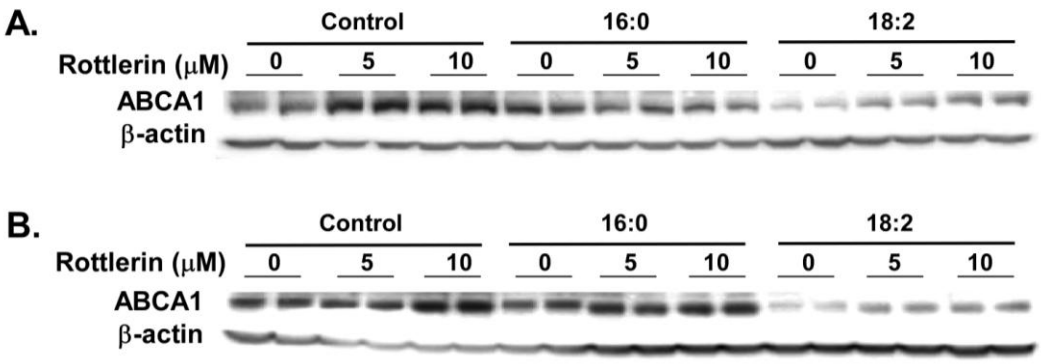

Figure 3 Rottlerin increased ABCA1 protein but could not reverse unsaturated fatty acid-induced down-regulation of ABCA1 protein expression in HepG2 and FHs 74 Int cells. HepG2 (**A**) and FHs 74 Int (**B**) cells were preincubated with rottlerin (5 and 10 μM) for 2 hrs, after which they were treated with BSA only (control), 16:0 or 18:2 (100 μM) for 12 hrs. ABCA1 protein expression was measured by Western blot analysis using β-actin as a loading control. A representative blot of two separate experiments is shown

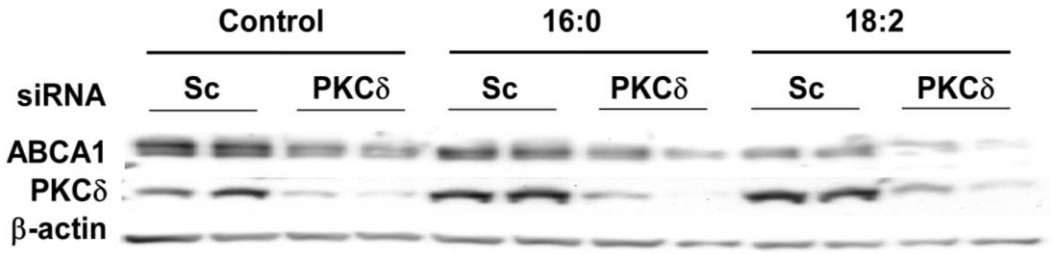

Figure 4 Depletion of PKCδ failed to abolish reduced ABCA1 protein levels by unsaturated fatty acids in HepG2 cells. Cells were transfected with 100 nM of scrambled control (Sc) or PKCδ siRNA for 24 hrs and subsequently incubated with BSA only (control), 16:0 or 18:2 (100 μM) for 24 hrs. ABCA1 and PKCδ protein levels were determined by Western blot analysis using β-actin as a loading control. A representative blot of two separate experiments is shown

1.4.3 Role of calpain protease in the modulation of ABCA1 protein levels by fatty acids

Phosphorylation in the PEST sequence of ABCA1 is shown to increase ABCA1 protein degradation by calpain protease [53, 54]. To assess whether this mechanism is involved in reduced ABCA1 protein levels by unsaturated fatty acids, HepG2 cells were treated with calpeptin, a calpain inhibitor. Although calpeptin induced a general increase in ABCA1 protein levels, it could not reverse reduced ABCA1 protein by 18:2 (Figure 5), suggesting that repressed ABCA1 protein by unsaturated fatty acids is not likely calpain protease-dependent.

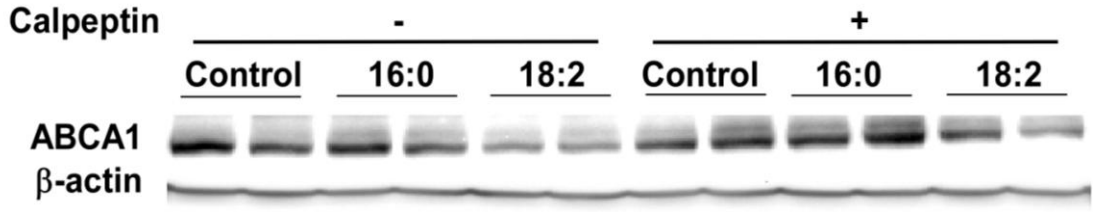

Figure 5 Inhibition of calpain protease did not reverse the repressive effect of unsaturated fatty acids on ABCA1 protein in HepG2 cells. Cells were preincubated with a calpain inhibitor calpeptin (20 μg/ml) for 2 hrs and subsequently treated with BSA only (control) or 100 μM of 16:0 or 18:2 for 12 hrs. Western blot analysis was conducted for ABCA1 and β-actin

1.5 Discussion

Obesity and type 2 diabetes are currently significant health issues in the United States and other Westernized countries. Of the various complications associated with type 2 diabetes, CVD is the leading cause of morbidity and mortality in diabetic subjects with 2-8-fold higher risk than the non-diabetic population [55-57]. Although low plasma HDL cholesterol concentrations in obese and diabetic individuals could be a major mediator for atherogenesis, the mechanisms underlying perturbed HDL metabolism are not clearly understood. As increased plasma free fatty acid concentrations and influx into tissues have been suggested to play a role in diabetic dyslipidemia [32-34, 37-39] 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 in a calpain-protease independent manner. In addition, our data suggest that PKCδ could play an important role in maintaining ABCA1 protein expression and potentially inhibit down-regulation of the protein by unsaturated fatty acids.

ABCA1 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 [58, 59]. Tissue site of ABCA1 expression is also important for the production of HDL as dysfunctional ABCA1 in the liver and intestine

led to reduced plasma HDL cholesterol concentrations by ~80% and ~30%, respectively [7, 8]. Studies using animal models of Tangier disease, such as the WHAM chicken and *Abca1*$^{-/-}$ mice, support that apoA-I secretion from the liver and intestine is not rate limiting in HDL formation, but rather modifications in apoA-I after secretion are important in maintaining plasma HDL cholesterol levels [5, 60]. Additionally, initial lipidation of apoA-I via the interaction with ABCA1 is shown to be a critical preliminary step in HDL formation presumably by producing nascent HDL [61, 62]. 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-1 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 post-transcriptional inhibition of ABCA1 expression has also been shown in macrophages [13, 14], HepG2 [11] and Caco-2 cells [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. Isocaloric substitution of n-3 polyunsaturated fatty acids for saturated fatty acids

decreased plasma concentrations of total cholesterol, LDL cholesterol and HDL cholesterol in non-human primates [63] and humans [64]. 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 acids-fed animals [65]. 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 [66]. Therefore, studies exist to support a 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 reduced ABCA1 protein levels in HepG2 cells, but 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 h in murine macrophage-like cells and differentiated THP-1 cells [52, 67], suggesting that post-transcriptional 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 PLD_2 and subsequently PKCδ, which resulted in the serine phosphorylation and destabilization of ABCA1 [15, 16]. However, we found that neither rottlerin nor depletion of PKCδ 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 PKCδ knockdown lowered protein levels independent of types of fatty acids. In addition, PKCδ siRNA treatment aggravated the repressive effect of 18:2 on ABCA1 protein levels in HepG2 cells. Our results indicate that PCKδ is not involved in facilitating ABCA1 protein degradation by unsaturated fatty acids in HepG2 and FHs 74 Int cells. Rather, PKCδ is likely to play a role in maintaining ABCA1 protein expression and inhibition of PKCδ could be involved in reduced ABCA1 protein levels by unsaturated fatty acids. 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 (unpublished data), we do not think the contradiction is due to different cell types used in two studies. More investigation is necessary to explain the differences.

Although rottlerin has been widely used as a selective PKCδ inhibitor, there is a recent debate on its selectivity for PKCδ as it inhibits other kinases [68]. Increased ABCA1 expression by rottlerin but decreased expression by PKCδ siRNA shown in our study favor that rottlerin is not a PKCδ-specific inhibitor. Phosphorylation of ABCA1 can alter its protein degradation or activity depending on sites of phosphorylation. Phosphorylation in the PEST sequence of ABCA1 is shown to increase the protein degradation by calpain [53, 54] whereas ABCA1 protein levels are positively correlated to its phosphorylation in THP-1 cells [51]. 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.

Phosphorylation of Thr-1286 and Thr-1305 in the PEST sequence of ABCA1 increases protein degradation by calpain [53, 54]. In the present study, although calpeptin increased ABCA1 protein levels, it could not reverse the reduced ABCA1 protein by 18:2. This suggests that protein degradation by unsaturated fatty acids may not be a calpain protease-dependent. Interestingly, ABCA1 is known to be ubiquitinated and lactacystin, a proteosome inhibitor, increased the protein in macrophages [53, 69], suggesting that a proteasomal degradation pathway exists for ABCA1 protein. Whether this mechanism is involved in the post-transcriptional regulation of ABCA1 by unsaturated fatty acids in HepG2 and FHs 74 Int cells needs to be further investigated. 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 athero-protective role of unsaturated fatty acids, polyunsaturated fatty acids in particular, compared with saturated fatty acids. Athero-protection of polyunsaturated fatty acids, however, is attributable to their effects on very-low density lipoprotein, LDL and triglyceride metabolism, anti-inflammation and anti-thrombosis [63, 70-72]. 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 pathological conditions require our better understanding of the modulation of ABCA1 expression by fatty acids.

1.6 Literature Cited

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Chapter 2

Repression of ATP binding cassette transporter A1 (ABCA1) and ABCG1 expression by unsaturated fatty acids in RAW 264.7 macrophages

2.1 Abstract

Reverse cholesterol transport (RCT), a process to deliver excess cholesterol from the periphery to the liver for ultimate excretion from body, is a major athero-protective property of high-density lipoproteins. As major transporters for cholesterol efflux in macrophages, ATP-binding cassette transporter A1 (ABCA1) and ABCG1 play a pivotal role in the RCT. We investigated molecular mechanisms for the regulation of ABCA1 and ABCG1 expression by fatty acids in RAW 264.7 macrophages. Cells were incubated with fatty acids including palmitic (16:0), oleic (18:1), linoleic (18:2), linolenic (18:3), eicosapentaenoic (20:5) and docosahexaenoic (22:6) acid in the absence or presence of T0901317, a liver X receptor (LXR) agonist.. Unsaturated fatty acids (UFA), but not saturated fatty acids, significantly reduced ABCA1 and ABCG1 mRNA levels in the absence of the LXR agonist. The repression by UFA, however, was abolished by T0901317, suggesting a potential LXR-dependent repression of basal ABCA1 and ABCG1 expression by UFA. Treatment with trichostatin A, a histone deacetylase inhibitor, not only increased the basal ABC transporter expression but abrogated the transcriptional repression by UFA. Although repressive effect of ABCA1 and ABCG1 mRNA by UFA was abolished by T0901317, protein levels remained diminished by UFA, which suggests post-transcriptional repression of the ABC transporters by UFA. Chemical and genetic deficiency of protein kinase δ (PKCδ) potentiated the repressive effect of 18:0 on ABCA1 and ABCG1. Calpeptin, a calpain protease inhibitor, could not counteract 18:2-induced repression of the protein levels. In conclusion, UFA diminished ABCA1 and ABCG1 expression in macrophages by two distinct mechanisms in RAW 264.7 macrophages: LXR-dependent transcriptional repression of basal expression

possibly via modifications in histone acetylation state; and calpain-independent post-transcriptional inhibition.

2.2 Introduction

Dyslipidemia, an underlying condition for several chronic diseases such as coronary heart disease (CHD) and type 2 diabetes, is characterized by high plasma concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides as well as low plasma high-density lipoprotein (HDL) cholesterol levels (1). Since the hypothesis that high levels of plasma HDL cholesterol are protective against CHD was initially proposed in the early 1950s (2), epidemiological studies have consistently shown a strong inverse relationship between plasma HDL cholesterol levels and the incident of CHD (3-6). The anti-atherogenic effect of HDL is due in part to the ability of HDL to promote cholesterol efflux from cells and to participate in reverse cholesterol transport (RCT) (7-9). In the RCT, excess cholesterol is transported from the periphery to the liver for ultimate excretion from body and HDL function as the primary acceptor of cellular free cholesterol (10).

Removal of cholesterol from macrophages present in arterial wall via the RCT is of significant importance in the prevention of atherosclerosis development. ATP binding cassette transporter A1 (ABCA1) and ABCG1 play a pivotal role in this process. ABCA1 facilitates the efflux of cellular cholesterol to extracellular acceptors, namely lipid-free or lipid-poor apolipoprotein A-I (11-14). In contrast, ABCG1 is highly expressed in macrophages and mediates the efflux of cholesterol to HDL_2 (15, 16). Deletion of macrophage *Abcg1* led to the deposition of FC and cholesteryl esters in various tissues, implicating its role in maintaining cellular cholesterol level (17, 18). Studies have shown that dysfunction of ABCA1 and ABCG1 in macrophages induces cholesterol

accumulation and accelerates atherosclerosis although some contradictory observations exist as to the role of ABCG1 in atherogenesis (19-23).

Transcription of ABCA1 and ABCG1 is primarily under the control of liver X receptors (LXR) in response to cellular cholesterol levels (24). Studies have shown that fatty acids can alter ABCA1 expression at the transcriptional and post-transcriptional levels. Post-transcriptional repression of ABCA1 expression by unsaturated fatty acids in macrophages has been demonstrated by a series of studies conducted by Wang and Oram (25-28). In their studies, unsaturated fatty acids increase ABCA1 protein degradation in macrophages by activating phospholipase D_2 (PLD_2) and subsequently protein kinase C δ (PKCδ). This, in turn, phosphorylates ABCA1 serine residues for protein degradation. Despite post-transcriptional regulation of ABCA1 by unsaturated fatty acids demonstrated by Wang and Oram, others have shown that fatty acids could alter transcription of ABCA1 and ABCG1. In human macrophages, ABCA1 and ABCG1 mRNA levels were lowered by linoleic acid compared with palmitic acid (29). ABCA1 mRNA abundance in HepG2 and RAW 264.7 macrophages was reduced by unsaturated fatty acids with diminished ABCA1 promoter activity (30). Mutations or deletion of direct repeat 4, a *cis*-acting element for LXR binding, in the promoters of ABCA1 and ABCG1 abolished the suppressive effects of unsaturated fatty acids on their transcription (31). These studies suggest that unsaturated fatty acids reduced the ABC transporter expression by an LXR-dependent mechanism.

Although studies have demonstrated that unsaturated fatty acids, but not saturated fatty acids, reduce ABCA1 and ABCG1 expression. However, limited and contradictory observations exist as to the molecular mechanisms underlying the repressive effect of

unsaturated fatty acids. This study was conducted to gain an insight into mechanisms underlying the modulation of ABCA1 and ABCG1 expression by fatty acids in RAW 264.7 macrophages. We found that unsaturated fatty acids repress the ABC transporters in macrophages at both transcriptional and post-transcriptional levels.

2.3 Materials and Methods

2.3.1 Cell culture and fatty acid preparation

Raw 264.7 macrophages were purchased from ATCC (Manassas, VA) and maintained in RPMI medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/ml streptomycin, 1x vitamins and 2 mmol/L L-glutamine in a humidified incubator at 37^{o}C with 5% CO_2.

Stock solution (2 mmol/L) of fatty acid-poor and endotoxin-free bovine serum albumin (BSA) (EMD Chemicals, Gibbston, NJ) was prepared in PBS. Sodium salts of fatty acids (Nu-Chek, Elysian, MN) were dissolved in the BSA stock solution to a final concentration of 5 mmol/L. The fatty acid and BSA mixture was purged with N_2 and sonicated in warm water bath (~40^{o}C) until the solution became clear to form BSA/fatty acid complex (a molar ratio to BSA of 1:2.5). The complex was then filter sterilized and diluted with cell medium to reach a final concentration of 100 μmol/L of fatty acid and incubated in a water bath at 37^{o}C for 1 hr prior to addition to cells.

RAW264.7 macrophages were incubated without or with 10 μmol/L T0901317 (Sigma-Aldrich, St. Louis, MO), an LXR agonist, in DMSO or 18 hrs, after which cells were treated with BSA only (control) or 100 μmol/L of fatty acid for 12 hrs. Rottlerin (PKCδ inhibitor; 5 or 10 μM in DMSO; Santa Cruz Biotechnology, Sata Cruz, CA) and

calpeptin (calpain protease inhibitor; 20 μg/ml in DMSO; MP Biomedicals, Solon, OH) were used to inhibit the respective enzyme. For the experiments with inhibitors, cells were preincubated with an inhibitor for 2 hrs, and subsequently with 100 μmol/L of fatty acid for 12 hrs. Control cells were incubated with the same amount of DMSO as a vehicle control. For experiments with trichostatin A (TSA), a pan-histone deacetylase inhibitor (HDACi), RAW 264.7 macrophages were incubated with 100 μmol/L fatty acid in the absence or presence of 500 nmol/L of TSA for 24 hrs. To evaluate protein degradation, RAW 264.7 macrophages were treated with BSA control or 100 μmol/L of 18:2 for 2 hrs, after which 20 μg/ml cycloheximide in DMSO (Acros, Thermo Fisher Scientific), a translation inhibitor, was added to halt protein synthesis. Cells were cultured in complete medium devoid of FBS when they were treated with fatty acid to eliminate any potential contamination of fatty acids present in FBS.

2.3.2 Small Interfering RNA (siRNA) Transfection

RAW 264.7 macrophages were transfected with Silencer® Negative Control scrambled siRNA (Ambion, Austin, TX) or siGENOME® PKCδ siRNA (Thermo Scientific, Pittsburgh, PA) to knockdown PKCδ using DharmaFECT1 transfection reagent (Thermo Scientific) according to the manufacturer's protocol. In brief, 5 μl of DharmaFECT1 was diluted into 195 μl of cell medium void of antibiotics and FBS. Scrambled control and PKCδ siRNA (2 μmol/L) were prepared in RNase-free sterile water and 100 μl of the 2 μmol/L siRNA solution was mixed with 100 μl of cell medium void of antibiotics and FBS. Subsequently, the media containing transfection agent and siRNA (200 μl each) were combined and incubated for 20 min at room temperature. The

siRNA and transfection agent complex was then diluted to 2 ml with cell medium and added into a 6 well plate, 24 hrs after which cells were washed twice with PBS and treated with BSA or 100 μmol/L of fatty acid for 24 hrs.

Table 2.1 Real-time PCR primers for gene expression analysis

Gene	Forward Primer	Reverse Primer
ABCA1	5'- CGTTTCCGGGAAGTGTCCTA -3'	5'- GCTAGAGATGACAAGGAGGATGGA -3'
ABCG1	5'- AGGTCTCAGCCTTCTAAAGTTCCTC -3'	5'- TCTCTCGAAGTGAATGAAATTTATCG -3'
LXRα	5'- CGACAGAGCTTCGTCCACAA -3'	5'- ACAGCTCGTTCCCCAGCAT-3'
PPARγ	5'- GCCCACCAACTTCGGAATC -3'	5'- TGCGAGTGGTCTTCCATCAC -3'
PGC-1α	5'- AAGCTGAAGCCCTCTTGCAA -3'	5'- ACTGTACCGGGCCCTCTTG -3'
PGC-1β	5'- CGCTCCAGGAGACTGAATCCAGT -3'	5' -CTTGACTACTGTCTGTGAGGC -3'
GAPDH	5'- TGTGTCCGTCGTGGATCTGA -3'	5'- CCTGCTTCACCACCTTCTTGAT -3'

2.3.3 Total RNA Isolation and Quantitative Realtime PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer's protocol. Reverse transcription for cDNA synthesis and quantitative realtime PCR analysis were performed as previously described (32, 33). Primers were designed according to GenBank database using the Primer Express software (Applied Biosystems, Austin, TX). Lists of primer sequence are available as Online Supporting Material with the online posting of this paper at http://jn.nutrition.org.

2.3.4 Western Blot Analysis

Cell lysate was prepared and Western blot analysis was performed as previously described (32, 33). Rabbit ABCA1 anti-serum was a generous gift from Dr. John Parks at Wake Forest University School of Medicine. Rabbit ABCG1 antibody was purchased from Novus Biologicals (Littleton, CO) and PKCδ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO) and used as a loading control to normalize the data.

2.3.5 Statistical Analysis

ANOVA and Tukey's pairwise comparison with Welch's correction for unequal variance when appropriate were used to identify statistically significant differences of treatments with $P < 0.05$ considered significant by GraphPad InStat 3 (GraphPad Software, Inc.). Data are expressed as mean ± SEM.

2.4 Results

2.4.1 Macrophage ABCA1 and ABCG1 expression was repressed by unsaturated fatty acids

To gain an insight into the modulation of macrophage ABCA1 and ABCG1 expression by fatty acids, RAW 264.7 macrophages were treated with various fatty acids including 16:0, 16L1, 18:1, 18:2, 18:3 and 20:5 in the absence or presence of T091317, an LXR agonist. In the absence of the LXR agonist, both ABCA1 and ABCG1 mRNA levels were significantly repressed by all the unsaturated fatty acids tested compared with the control (**Figure 1A**). However, when T0901317 was present, the repressive effect was completely abolished, suggesting the transcriptional repression of the ABC transporters by unsaturated fatty acids in macrophage is presumed to be LXR-dependent. ABCA1 (~250 KDa) and ABCG1 (~60 KDa) protein levels were also markedly reduced by unsaturated fatty acids in the absence of T0901317 in consistent with mRNA levels (**Figure 1B**). Unsaturated fatty acids, however, were able to lower the ABC transporter protein levels when the cells were treated with the LXR agonist despite no changes in mRNA abundance were observed. The data indicates a potential LXR-independent repression of ABCA1 and ABCG1 by unsaturated fatty acids expression at the post-transcriptional level.

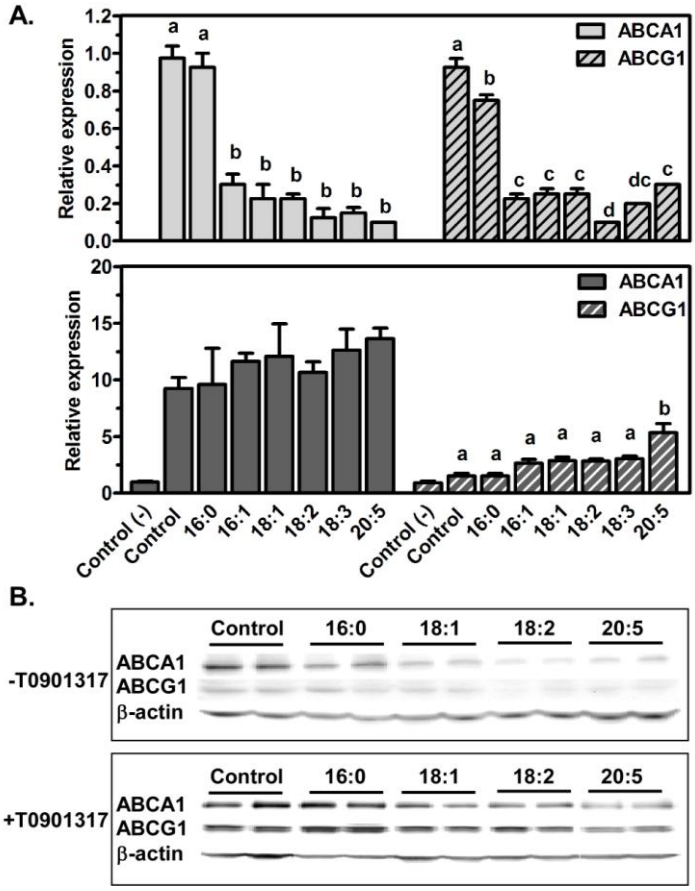

Figure 1 Regulation of ABCA1 and ABCG1 expression by fatty acids in RAW 264.7 macrophages. Cells were incubated with or without a LXR agonist T0901317 (10 μM) for 18 hrs and subsequently with BSA only (control) or fatty acids (100 μM) complexed with BSA for 12 hrs. **A**. ABCA1 mRNA abundance by quantitative realtime PCR. Values are Means ± SEM, n = 6. Bars without a common letter are significantly different, $P < 0.05$. **B**. ABCA1 protein by Western blot analysis with β-actin as a loading control. A representative blot of two separate experiments is shown.

1.4.2 Inhibition of histone deacetylase (HDAC) abolished the repressive effect of unsaturated fatty acids on ABCA1 and ABCG1 mRNA levels

Alterations in chromatin structure are an important way to regulate gene transcription. To evaluate a potential involvement of modulations in histone acetylation status in the unsaturated fatty acid-induced transcriptional repression of macrophage ABCA1 and ABCG1, RAW 264.7 macrophages were incubated with 16:0 and 18:2 in the presence of trichostatin A (TSA), a pan-histone deacetylase inhibitor (HDACi). In consistent with the previous observation shown in Figure 1, without TSA, both ABCA1 and ABCG1 mRNA levels were significantly repressed by 18:2 (**Figure 2**). However, when the macrophages were treated with TSA, mRNA levels of ABCA1 and ABCG1 was significantly increased and furthermore the repression by 18:2 was completely abolished. It is of interest that in the presence of TSA, 16:0-treated cells showed significantly lower ABCA1 and ABCG1 mRNA abundance than control and 18:2-treated cells.

TSA treatment altered the expression of other genes that have been linked to the regulation of ABCA1 and ABCG1 transcription. Whereas peroxisome proliferator-activated receptor γ (PPARγ) and PPARγ coactivator 1α (PGC-1α) expression was significantly increased by TSA along with ABCA1 and ABCG1, LXRα and PGC-1β mRNA levels were significantly decreased by TSA (**Figure 3**).

Figure 2

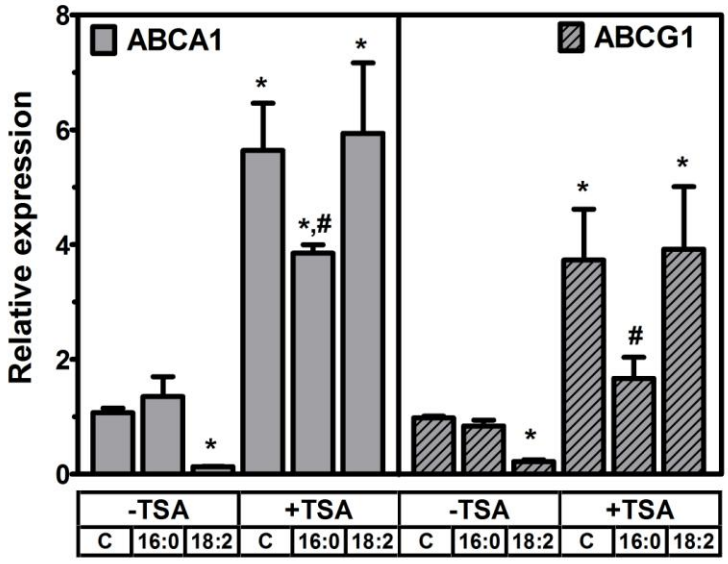

Figure 2 Effect of TSA on ABCA1 and ABCG1 expression in RAW 264.7 macrophages. The cells were incubated with 500 nmol/L TSA for 20 hrs together with fatty acids (100 μM) complexed with BSA. mRNA abundance was measured by quantitative realtime PCR. Means ± SEM, n = 6-13. *, $P < 0.05$ vs. control (-TSA). #, $P < 0.05$ vs. control (+TSA).

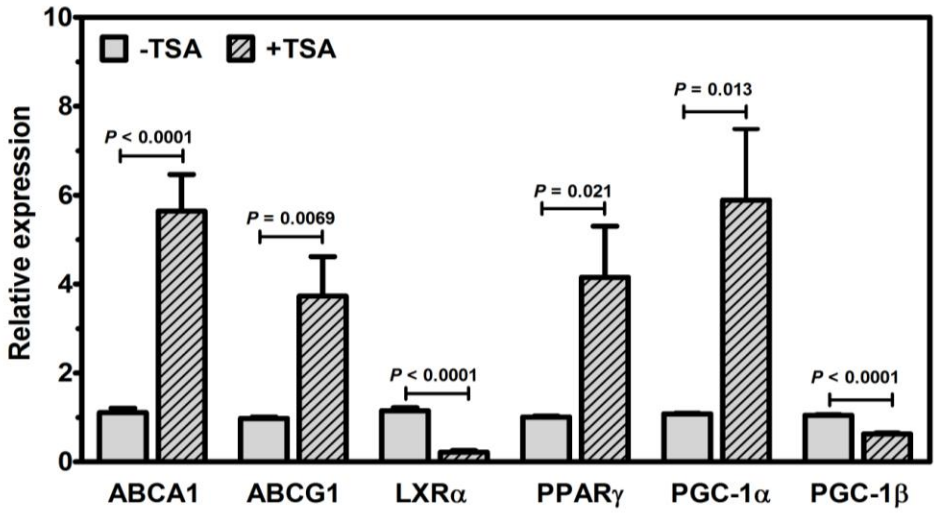

Figure 3 Effect of TSA on the expression of genes related to transcriptional regulation of ABC transporters in RAW 264.7 macrophages. The cells incubated TSA and fatty as described in figure 2 were analyzed by realtime PCR. Means ± SEM, n = 6-13.

2.4.3 Rottlerin did not abolish the unsaturated fatty acids-induced reduction in ABCA1 and ABCG1 proteins

Macrophage ABCA1 and ABCG1 protein levels were diminished by unsaturated fatty acids even with LXR activation in the condition which the repression was abolished. Post-transcriptional regulation of ABCA1 by unsaturated fatty acids was suggested to result from destabilization of ABCA1 protein through its phosphorylation by PKCδ pathway (28). To evaluate a role of PKCδ in the fatty acid regulation of ABCA1 as well as ABCG1 protein levels, we incubated RAW 264.7 macrophages with 16:0 or 18:2 in the presence of rottlerin, a commonly used PKCδ inhibitor, or after depletion of PKCδ using siRNA. In contrast to the previous report in literature (28), neither rottlerin nor PKCδ siRNA abolished the repressive effect of 18:2 on ABCA1 and ABCG1 proteins in the macrophages (**Figure 4A and 4B**). Instead, rottlerin treatment and PKCδ deficiency potentiated the repressive effect of 18:2.

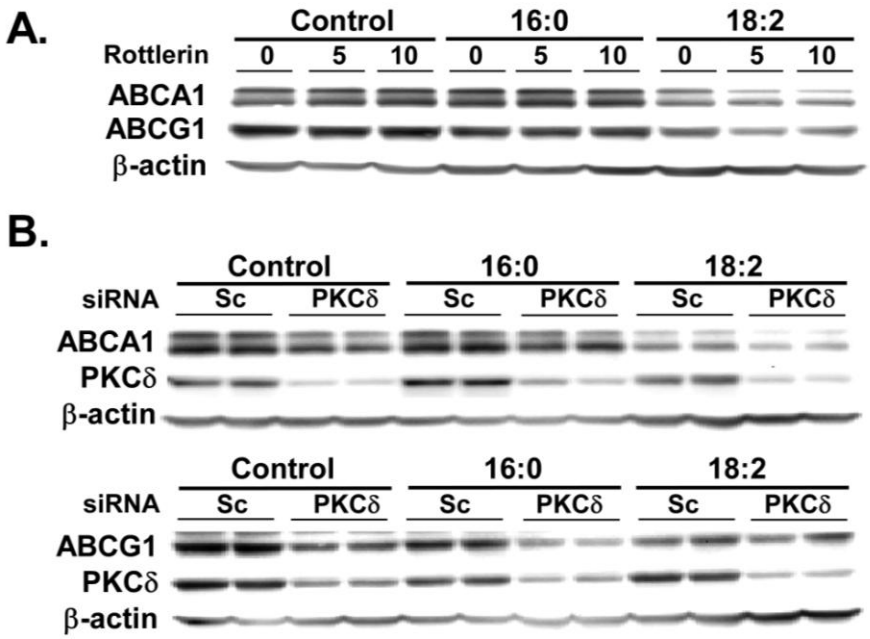

Figure 4 Role of PKCδ in ABCA1 and ABCG1 protein levels in RAW 264.7 macrophages. (**A**) Cells were pre-incubated with rottlerin (5 and 10 μM) for 2 hrs, after which they were treated with BSA only (control), 16:0 or 18:2 (100 μM) for 12 hrs. ABCA1 and ABCG1 protein expression was measured by Western blot analysis using β-actin as a loading control. A representative blot of two separate experiments is shown. (**B**) Cells were transfected with 100nM of scrambled control (Sc) or PKCδ siRNA for 24 hrs and subsequently incubated with BSA only (control), 16:0 or 18:2 (100 μM) for 24 hrs. ABCA1, ABCG1 and PKCδ protein levels were determined by Western blot analysis using β-actin as a loading control. A representative blot of two separate experiments is shown

2.4.4 Enhanced ABCA1 protein degradation by unsaturated fatty acids is calpain-independent

Degradation of ABC transporter proteins is an important regulatory means for their activity (34, 35). To assess whether reduced ABCA1 protein levels by unsaturated fatty acids is due, at least in part, to increased protein degradation, RAW 264.7 macrophages were treated with cycloheximide, a translation inhibitor, together with 18:2. ABCA1 protein degradation was faster by 18:2 compared with controls (**Figure 5A**). Phosphorylation of PEST sequence in ABCA1 protein is shown to increase the protein degradation by calpain protease (36, 37). To assess whether calpain is involved in the protein degradation of ABCA1 and ABCG1 by fatty acids, RAW 264.7 macrophages were treated with calpeptin, a calpain inhibitor. Although calpeptin induced a general increase in ABCA1 and ABCG1protein levels, it could not reverse reduced protein levels by 18:2, suggesting that the ABC transporter protein degradation by unsaturated fatty acids is not a calpain protease-dependent (**Fig 5B**).

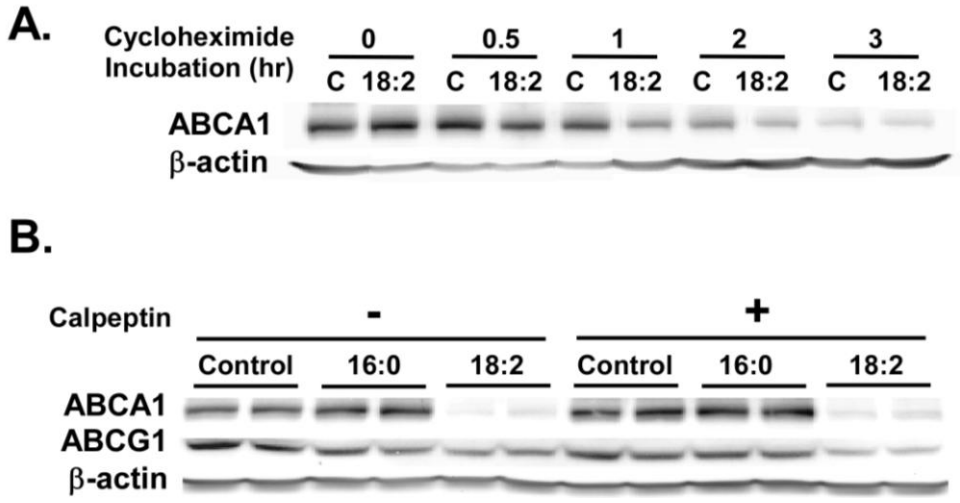

Figure 5 Inhibition of calpain protease did not reverse the repressive effect of unsaturated fatty acids on ABCA1 protein in HepG2 cells. (**A**) Cells were treated with BSA control or 100 μmol/L of fatty acids for 2 hrs and subsequently 20 μg/ml cycloheximide was added. Cellular proteins were obtained 0, 0.5, 1, 2 and 3 hrs after the addition of cycloheximide for Western blot analysis. (**B**) RAW 264.7 macrophages were preincubated with a calpain inhibitor calpeptin (20 μg/ml) for 2 hrs and subsequently treated with BSA only (control) or 100 μM of 16:0 or 18:2 for 12 hrs. Western blot analysis was conducted for ABCA1 and β-actin.

2.5 Discussion

ABCA1 and ABCG1 in macrophages play a pivotal role in maintaining cellular cholesterol homeostasis by effluxing cholesterol to extracellular acceptors such as lipid-free or lipid-poor apoA-I and HDL, respectively. Therefore, disturbance of the ABC transporter functions in macrophages, particularly in the arterial wall, could facilitate foam cell formation and consequently atherogenesis. Elevated plasma free fatty acid concentrations in obesity have been associated with insulin resistance and type 2 diabetes (38-42). As cardiovascular disease is the leading cause of morality and mortality among complications associated with type 2 diabetes (43-45), it is critical to understand mechanisms for accelerated atherosclerosis development by diabetic factors. The goal of this study was to understand molecular regulatory mechanisms for the regulation of macrophage ABCA1 and ABCG1 by fatty acids. We found that expression of ABCA1 and ABCG1 in macrophages are repressed by unsaturated fatty acids via two distinct mechanisms, i.e., LXR-dependent transcriptional repression and increased calpain-independent protein degradation.

Several studies have reported that fatty acids modulate the expression of ABCA1 and ABCG1 (25-31, 46, 47). The detailed regulatory mechanisms, however, remain largely enigmatic and contradictory. We observed that unsaturated fatty acids repressed mRNA abundance of ABCA1 and ABCG1 in RAW 264.7 macrophages and the repression was abolished by an LXR agonist. The data indicate unsaturated fatty acids may inhibit the basal expression of the ABC transporters possibly in an LXR-dependent manner in macrophages. Involvement of LXR in the transcriptional repression of ABCA1 and ABCG1 in macrophages was also suggested by Uehara et al. (30, 31). Of importance

is that unliganded LXR is suggested to repress the basal expression of the ABC transporters by recruiting co-repressors, such as nuclear receptor corepressor (NCoR) and silencing mediators for retinoid and thyroid hormone receptors (SMRT), in macrophages but not in the liver (48). As we observed the transcriptional repression of the basal ABCA1 expression by unsaturated fatty acids only in macrophages but not in HepG2 cells (manuscript in preparation), it can be presumed that unsaturated fatty acids could enhance recruitment of the co-repressor complex to unliganded LXR and/or its activity in the promoters of macrophage ABCA1 and ABCG1.

Exchange of co-repressor with co-activator is widely used to switch function of transcription factors from active repression to transcriptional activation. Transcription factors that have been shown to interact with a co-repressor complex for transcriptional repression include activator protein 1 (49-51), nuclear factor κB (50-52), thyroid hormone receptor (53-55) and LXRα (48). NCoR and SMRT are essential components of co-repressor complex and mediate active transcriptional repression via activating histone deacetylase (HDAC) in the complex (49, 56, 57). HDAC3 is known to regulate ABCA1 and ABCG1 expression as a component of a co-repressor complex containing NCoR/SMRT (48, 58). Interestingly, we found that TSA, a pan-HDACi, markedly increased the ABC transporter mRNA and abolished the repressive effect of unsaturated fatty acids on the transporter expression in RAW 264.7 macrophages. The result suggests that basal transcription of ABCA1 and ABCG1 may be repressed by a HDAC and unsaturated fatty acid could further inhibit the transcription by modulating histone deacetylation in the promoters of the ABC transporters. Several lines of evidence exist to support potential roles of fatty acids, such as short-chain FA (e.g., butyrate) and 9-

hydroxystearic acid, in modulating chromatin structure (59, 60). However, whether fatty acids commonly present in the diet could utilize this mode of transcriptional regulation is largely unknown. It should be further investigated whether long-chain fatty acids utilize HDAC to alter gene expression.

Turnover of ABCA1 protein is rapid with a half-life of less than 1 hr in macrophages (35, 61), suggesting that post-transcriptional regulation could be an important determinant for its function. We found that unsaturated fatty acids reduced ABCA1 and ABCG1 protein levels in macrophages. Although T0901317 abolished the repression of the ABC transporter mRNA levels by unsaturated fatty acids, protein levels of both transporters remained to be diminished by unsaturated fatty acids compared with saturated fatty acids, suggesting that post-transcriptional repression of ABCA1 and ABCG1 is likely to exist in addition to the transcriptional inhibition. Reduced ABCA1 protein levels by 18:2 were at least partly due to faster protein degradation as demonstrated by the experiment using cycloheximide. It has been suggested that unsaturated fatty acids induce ABCA1 protein degradation by activating PLD_2 and PKCδ pathway that phosphorylates serine residues of ABCA1 for degradation (25-28). We, however, found PKCδ may function in an opposite direction as its depletion by siRNA and chemical inhibition using rottlerin reduced ABCA1 and ABCG1 proteins and failed to reverse the repressive effect of unsaturated fatty acids. The reason for the contradictory observations is not clear and further study is necessary to identify the role of PKCδ in the post-transcriptional regulation of the ABC transporters by fatty acids.

Studies have shown that phosphorylation of ABCA1 can alter its protein degradation or activity depending phosphorylation sites. ABCA1 protein levels are

positively correlated to its phosphorylation in THP-1 cells (34). In contrast, phosphorylation of Thr-1286 and Thr-1305 in the PEST sequence of ABCA1 increases the protein degradation by calpain (36, 37). When calpain protease was inhibited by calpeptin in our study, 18:2 was still able to lower ABCA1 and ABCG1 protein levels in RAW 264.7 macrophages, suggesting that elevated the protein degradation by 18:2 may not be mediated through calpain. ABCA1 is known to be ubiquitinated and lactacystin, a proteosome inhibitor, increased the protein in macrophages (36, 62), suggesting that a proteasomal degradation pathway exists for ABCA1 protein. Whether this mechanism is involved in the post-transcriptional regulation of ABCA1 and ABCG1 by unsaturated fatty acids in RAW 264.7 macrophages needs to be further investigated.

In summary, we found that unsaturated fatty acids repress the expression of ABCA1 and ABCG1 in macrophages at the transcription and post-transcription levels. As macrophage ABCA1 and ABCG1 play a pivotal role in removal of cellular cholesterol, which can prevent foam cell formation, the inhibitory function of unsaturated fatty acids in the transporter expression could have a negative impact on atherogenesis. One could argue that the potential unfavorable effect of unsaturated fatty acid on ABCA1 and ABCG1 contradicts a well-known athero-protective role of unsaturated fatty acids, polyunsaturated fatty acids in particular, compared with saturated fatty acids. Athero-protection of polyunsaturated fatty acids, however, is attributable to their effects on very-low density lipoprotein, LDL and triglyceride metabolism, anti-inflammation and anti-thrombosis (63-66). These benefits could override the inhibition of ABCA1 and ABCG1 expression by unsaturated fatty acids in protecting against atherosclerosis. Understanding of mechanisms by which fatty acids modulate the ABC transporters in macrophages is

necessary to develop a therapeutic means to enhance beneficial effect of unsaturated fatty acids in the prevention of atherosclerosis development.

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