Research paper

Suppression of ABCA1 by unsaturated fatty acids leads to lipid accumulation in HepG2 cells

Yanhong Yang\(^a\), Ying Jiang\(^b\), Yutong Wang\(^a,\star\), Wei An\(^d,\star\star\)

\(^a\) Department of Cell Biology, Municipal Laboratory for Liver Protection and Regulation of Regeneration, Capital Medical University, Beijing 100069, China
\(^b\) Department of Pathophysiology, Capital Medical University, Beijing 100069, China

**Abstract**

Abnormal lipid metabolism may contribute to the pathogenesis of non-alcoholic steatohepatitis (NASH). ATP-binding cassette transporter A1 (ABCA1) mediates the transport of cholesterol and phospholipids from cells to HDL apolipoproteins. We previously reported that unsaturated fatty acids destabilise ABCA1 in murine macrophages and ABCA1-transfected baby hamster kidney cells by increasing its protein degradation. Here, we examined the correlation between ABCA1 and hepatic lipids. In HepG2 cells, unsaturated but not saturated fatty acids suppressed ABCA1 protein levels by promoting its protein degradation. Over-expression of ABCA1 resulted in a decrease of cellular fatty acids and triglycerides, while repression by ABCA1 siRNA increased both cellular fatty acids and triglycerides. Rats with NASH also showed lower ABCA1 protein levels in liver cells, compared with that of the normal rats. These data indicate that steatosis is associated with a decrease in ABCA1 protein expression leading to an increase in lipid storage in hepatocytes. And it further suggests that this effect could be due to an excess of unsaturated fatty acids.

© 2010 Elsevier Masson SAS. All rights reserved.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is now one of the most common liver diseases worldwide. It is characterised by macrovesicular steatosis or fatty liver and has recently been increasingly recognised to be the hepatic manifestation of insulin resistance and metabolic syndrome [1–3]. Some patients with steatosis develop superimposed necroinflammatory activity with a nonspecific inflammatory infiltrate, hepatic cellular ballooning, and non-alcoholic steatohepatitis (NASH) [4]. In 2005, Kleiner et al. proposed a system for scoring histologic findings in patients with NASH, which included features such as steatosis, lobular inflammation, and ballooning [5]. The current working model explaining the pathogenesis of NASH is the “two-hit” hypothesis, first proposed by Day and James [6], with the first “hit” being steatosis and the second “hit” being oxidative stress and inflammation, ultimately resulting in NASH [7]. More recent work suggests that the second hit could also be related to natural killer T (NKT) cell deficiency [8], regulatory T cell dysregulation [9] or the modification of the intestinal microbiota [10].

Excessive accumulation of lipid within hepatocytes has been considered to be an important factor to trigger hepatic inflammatory reactions [11], therefore, efficient transportation or delivery of lipid may be a key to NASH prevention. Lipidation of apolipoprotein A-I (apoA-I) by ATP-binding cassette transporter A1 (ABCA1) is the rate-limiting step in reverse cholesterol transport and generating plasma high density lipoprotein (HDL) [12,13]. ABCA1 is an integral membrane protein that mediates the transport of cellular cholesterol and phospholipids to lipid-deficient HDL apolipoproteins [14,15]. ABCA1 expression is highly regulated. Cholesterol loading markedly increases ABCA1 mRNA and protein levels [16,17], which is consistent with the function of ABCA1 as a transporter to export excess cholesterol. The transcription of ABCA1 gene is regulated by nuclear liver X receptors (LXRs) and retinoid X receptor (RXR) [18–20]. ABCA1 protein degradation rate is also regulated. ApoA-I increases ABCA1 protein stability by decreasing the phosphorylation of threonine residues at 1286 and 1305 in ABCA1, which directs calpain proteolysis [21], and by activating protein kinase C [22]. Protein kinase A, protein kinase 2 and Janus kinase 2 have also been shown to regulate ABCA1 activity [23–25]. Free fatty acids (FFAs) are also able to regulate ABCA1 stability. Previous studies demonstrated that long chain unsaturated fatty acids, but not saturated fatty acids,
decrease ABCA1 expression in murine macrophages and ABCA1 over-expressing baby hamster kidney (BHK) cells by increasing its protein degradation rate without affecting its mRNA level [26]. Furthermore, unsaturated fatty acids accelerate ABCA1 protein turnover through a signalling pathway involving the activation of phospholipase D2 (PLD2) [27] and protein kinase C delta (PKCδ) [28].

Previous studies on ABCA1 were mainly focused on its relationship with cardiovascular diseases, however, its role in the pathogenesis of NASH as a lipid transporter remains unknown. Based on previous results demonstrating that unsaturated fatty acids suppressed ABCA1 in macrophages, we then examined the effects of fatty acids on ABCA1 in hepatocytes. In the current study, we found that treatment of HepG2 cells with unsaturated fatty acids suppressed ABCA1-mediated cholesterol secretion from hepatocytes by increasing the degradation rate of ABCA1. Furthermore, inhibition of ABCA1 expression facilitated the accumulation of free fatty acids and triglycerides in hepatocytes. These results suggest that an increased supply of fatty acids could impair clearance of excess cholesterol and phospholipids from hepatocytes and promote the formation of NASH.

2. Materials and methods

2.1. Materials

HepG2 cells were obtained from American Type Culture Collection (ATCC). Total RNA extraction reagent TRIzol, reverse transcription kit, Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA). Fatty acid-free bovine serum albumin, 22(R)-hydroxycholesterol, 9-cis-retinoic acid, and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). ApoA-I was obtained from Millipore (Billerica, MA). Methanol, butanol, hexane, and iso-propanol were acquired from Beijing Modern Eastern Biologicals (Littleton, CO) and Shanghai Kang Chen Biotech (Shanghai, China), respectively.

2.2. Animals

All protocols for animal care and experiments were approved by the Ethical Committee of Capital Medical University Beijing. Male Sprague-Dawley rats (110–130 g) were fed a standard diet (5% of energy derived from fat, 23% from protein, 55% from carbohydrate, 6% from fiber, 1.2% from calcium, 0.8% from phosphorus and 5% from water) or a high-fat diet (5% of energy derived from fat, 23% from protein, 55% from carbohydrate, 6% from fiber, 1.2% from calcium, 0.8% from phosphorus and 5% from water) or a high-fat diet (5% of energy derived from fat, 23% from protein, 55% from carbohydrate, 6% from fiber, 1.2% from calcium, 0.8% from phosphorus and 5% from water) or a high-fat diet (5% of energy derived from fat, 23% from protein, 55% from carbohydrate, 6% from fiber, 1.2% from calcium, 0.8% from phosphorus and 5% from water). Male Sprague-Dawley rats (110–130 g) were fed a standard diet (5% of energy derived from fat, 23% from protein, 55% from carbohydrate, 6% from fiber, 1.2% from calcium, 0.8% from phosphorus and 5% from water) or a high-fat diet (5% of energy derived from fat, 23% from protein, 55% from carbohydrate, 6% from fiber, 1.2% from calcium, 0.8% from phosphorus and 5% from water).

2.3. Cell culture and cholesterol efflux

HepG2 cells were maintained in DMEM containing 10% fetal bovine serum or incubated in serum-free DMEM plus 1 mg/ml fatty acid–free bovine serum albumin (DMEM/BSA). Washed cells were incubated for 16 h with medium containing 5 mg/ml BSA in the presence or absence of 125 mM fatty acids (molar ratios to BSA of 0 and 1.8). Fatty acids were added from a stock solution bound to BSA at a 3.5 molar ratio and were adjusted to lower ratios by adding fatty acid–free BSA. To induce ABCA1 expression, 10 μM 22(R)-hydroxycholesterol and 10 μM 9-cis-retinoic acid were added in combination to this medium.

To measure cholesterol efflux, cells were incubated with DMEM/BSA with or without 10 μg/ml apoA-I at 37 °C for 4 h and chilled on ice, then the medium was collected and centrifuged to remove detached cells. Cholesterol was extracted from the medium and the cells with methanol/chloroform (1:2) and hexane/isopropanol (1:1), respectively. Cholesterol content was assayed following the manufacturer’s guide. ApoA-I–mediated cholesterol efflux is expressed as the fraction of cholesterol released into the medium after subtraction of values obtained in the absence of apoA-I.

2.4. Western blot

Cells were washed and dislodged from the dish at 0 °C in buffer containing protease inhibitors. Cellular proteins were solubilised in phosphate-buffered saline containing 1% Triton X-100 plus protease inhibitors and resolved by SDS-PAGE. The expression of ABCA1 and GAPDH was identified by Western blot analysis using antibodies against ABCA1 and GAPDH, respectively [30].

2.5. Determination of fatty acids and triglycerides

Lipid mass analysis was conducted as previously described [31]. HepG2 cells were cultured in 6-well plates at 80% confluency in DMEM. Total cellular lipids were extracted by hexane/isopropanol (1:1). The chloroform/methanol extract was back-extracted with water to remove contaminating proteins. The amounts of free fatty acids and triglycerides were measured by colorimetric enzyme assays using test reagents for serum lipids, according to the manufacturer’s protocol.

2.6. Plasmid transfection

ABCA1 shRNA plasmid and control plasmid were designed and constructed by Shanghai GenePharma Co. CMV-ABCA1, shABCA1 and the control plasmid were transfected into HepG2 hepatocytes using FuGene HD as described in the manufacturer’s protocol. The target sequences of ABCA1 shRNA were as follows: (1) 5’-GCT TCC AGG AGA AGT CCT ATG-3’; (2) 5’-GGA ACT GGA CAA TGC AGA ACC-3’; (3) 5’-GTC GCT CAA AAT GAT TCT-3’; (4) 5’-GCT TCA CAC TCA AGA TCT TGC-3’.

2.7. Quantitative real-time polymerase chain reaction

Total RNA from HepG2 hepatocytes or rat liver was extracted using TRIzol. Two micrograms of total RNA were reverse transcribed into cDNA using Superscript II RT. Gene expression was quantified using a SYBR Green PCR kit with 18S rRNA as the control. Quantitative RT-PCR was performed with a Prism 7300 sequence detecting system. The primer sequence was as previously described [32,33].
2.8. Statistical analysis

The results of multiple observations are presented as mean ± SEM. The data were analysed with statistics software SPSS 11.5 (SPSS Inc, Chicago, IL, USA) by a nonparametric analysis of variance test. Difference were considered as significant if \( p < 0.05 \).

3. Results

3.1. Reduction of ABCA1 protein by unsaturated fatty acids

It was previously reported that when ABCA1 was induced by 8-Br-cAMP, unsaturated, but not saturated fatty acids, inhibited apoA-I-mediated lipid efflux from J774 macrophages [26]. To examine the effects of fatty acids in hepatocytes, we incubated HepG2 cells with 22(R)-hydroxycholesterol and 9-cis-retinoic acid for 16 h in the absence or presence of different fatty acids and measured the apoA-I-mediated cholesterol efflux and ABCA1 protein levels. Similar to the previous results obtained from J774 macrophages, the unsaturated fatty acids such as palmitoleate, oleate and linoleate decreased apoA-I-mediated cholesterol efflux by 40–60\% (Fig. 1A) and decreased ABCA1 protein levels by 60–70\% (Fig. 1B,C). In contrast, saturated fatty acids palmitate and stearate had little or no effect on apoA-I-mediated cholesterol efflux and ABCA1 protein levels.

It was previously demonstrated that unsaturated fatty acids stimulate ABCA1 degradation in the presence of a cAMP analogue or LXR/RXR ligands in J774 macrophages [28,34]. To identify the mechanism by which unsaturated fatty acids reduce ABCA1 protein levels in HepG2 cells, we measured the effects of fatty acids on ABCA1 mRNA expression and degradation. We conducted quantitative real-time PCR analysis to test whether the reduction of ABCA1 protein caused by unsaturated fatty acids is due to a decrease in ABCA1 mRNA. Treatment with saturated or unsaturated fatty acids did not significantly alter the cellular content of ABCA1 mRNA (Fig. 2A), implying that the inhibitory effects of unsaturated fatty acids were not at the level of ABCA1 transcription or mRNA stability. To address the possibility that ABCA1 degradation rates are affected by fatty acids, we incubated 22(R)-hydroxycholesterol/9-cis-retinoic acid-treated HepG2 cells with or without linoleate for 2 h, added cycloheximide to arrest protein synthesis, and monitored changes in ABCA1 protein levels during the cycloheximide treatment. After the preincubation with linoleate for 2 h, there was no detectable difference in ABCA1 content between the treated and control cells (Fig. 2B,C). However, after treatment with cycloheximide for 1–3 h, the linoleate-treated cells had lower ABCA1 levels, compared with the control cells. These results show that linoleate reduces ABCA1 levels by enhancing the rate of ABCA1 degradation in these LXR/RXR-activated hepatocytes.

3.2. Alteration of cellular free fatty acids and triglycerides by ABCA1

We have demonstrated that unsaturated fatty acids affect ABCA1 stability. Furthermore, as a cellular lipid transporter, ABCA1 expression may also have a significant impact on cellular lipid levels. To test this hypothesis, we altered ABCA1 expression levels in HepG2 hepatocytes either by transfection of an ABCA1-expressing
plasmid or an ABCA1 mRNA interference (RNAi) plasmid. Seventy-two hours after transfection, the ABCA1 protein level was measured for each shRNA construct, but the cholesterol efflux was measured only for shRNA2. Of the four different ABCA1 RNAi sequences, one (ABCA1 shRNA2) appeared to be the most effective at reducing ABCA1 protein levels (Fig. 3B). As expected, transfection of the ABCA1-expressing vector significantly increased ABCA1 protein levels (Fig. 3A,B) and cholesterol efflux (Fig. 3C), compared with the control cells. RNAi resulted in a marked decrease in the same two categories. To evaluate the impact of ABCA1 expression on cellular lipids, cellular free fatty acid (Fig. 3D) and triglyceride (Fig. 3E) content were also measured in cells transfected with CMV-ABCA1, ABCA1 shRNA2 or their control plasmids. Consistent with the role of ABCA1 as a cholesterol and phospholipid transporter, when compared with control cells, ABCA1 over-expression caused a 40% and 43% decrease in cellular free fatty acids and triglycerides, respectively, while ABCA1 RNAi resulted in a 72% and 52% increase in cellular free fatty acids and triglycerides, respectively. These data indicate that the alteration ABCA1 protein level leads to the modification of the cellular lipid storage.

3.3. Reduction of hepatic ABCA1 level in rats with NASH

To further demonstrate that reduction of ABCA1 levels is one of the major factors that lead to lipid accumulation within hepatocytes in vivo, we measured hepatic ABCA1 in rats with NASH, a previously established animal model [29]. Rats were fed either with a normal diet, for the control group, or with a high-fat diet for 22 weeks to develop NASH (Table 1 and Fig. 4A). Hepatic ABCA1 levels were then measured in these rats. Similar to the results obtained from cultured HepG2 cells, there was no significant difference in ABCA1 mRNA levels between control and rats with NASH (Fig. 4B). However, rats with NASH showed significant reduction in ABCA1 protein levels (Fig. 4C). This evidence indicates that steatosis is associated with a decrease in ABCA1 protein expression leading to an increase in lipid storage in hepatocytes. And it further suggests that this effect could be due to an excess of unsaturated fatty acids.

4. Discussion

Metabolic factors that modulate ABCA1 activity could have a profound impact on cholesterol and phospholipid transport. Our

3.3. Reduction of hepatic ABCA1 level in rats with NASH

To further demonstrate that reduction of ABCA1 levels is one of the major factors that lead to lipid accumulation within hepatocytes in vivo, we measured hepatic ABCA1 in rats with NASH, a previously established animal model [29]. Rats were fed either with a normal diet, for the control group, or with a high-fat diet for 22 weeks to develop NASH (Table 1 and Fig. 4A). Hepatic ABCA1 levels were then measured in these rats. Similar to the results obtained from cultured HepG2 cells, there was no significant difference in ABCA1 mRNA levels between control and rats with NASH (Fig. 4B). However, rats with NASH showed significant reduction in ABCA1 protein levels (Fig. 4C). This evidence indicates that steatosis is associated with a decrease in ABCA1 protein expression leading to an increase in lipid storage in hepatocytes. And it further suggests that this effect could be due to an excess of unsaturated fatty acids.

4. Discussion

Metabolic factors that modulate ABCA1 activity could have a profound impact on cholesterol and phospholipid transport. Our

Table 1
Liver index and serum lipid profile.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>NASH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver index</td>
<td>0.028 ± 0.001</td>
<td>0.045 ± 0.004*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>0.2 ± 0.1</td>
<td>2.6 ± 1.5*</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.1 ± 0.4</td>
<td>3.9 ± 1.7*</td>
</tr>
</tbody>
</table>

Data are mean ± SD for 6 animals per group. The liver index was calculated as the liver weight divided by the weight of the rat. Asterisks indicate statistically significant (p < 0.05) differences from the control group.
previous studies revealed that in murine macrophages and ABCA1 over-expressing baby hamster kidney cells, unsaturated fatty acids impair ABCA1 expression by enhancing its degradation rate through a PLD2 and PKC\( \delta \) signalling pathway. Here, we provide evidence that this decrease in ABCA1 expression also occurs in hepatocytes and that ABCA1 also therefore regulates cellular fatty acid content.

Unsaturated fatty acids suppressed apoA-I-mediated cholesterol efflux and decreased ABCA1 protein levels from HepG2 hepatocytes by 40–50\%, and this decrease was due to the increase in the ABCA1 degradation rate. Hepatic FFA and triglyceride content were significantly decreased in cells with ABCA1 over-expression and markedly increased after inhibition of ABCA1 with RNAi. These observations show an association between FFAs and hepatic ABCA1, strongly suggesting that a decrease in protein levels of ABCA1 by FFAs may contribute to the development of NASH. Hepatic ABCA1 protein levels in rats with NASH were also decreased, while mRNA level remained unchanged. These results show that hepatic lipid accumulation in NASH is associated with a low ABCA1 protein level and suggest that unsaturated fatty acids could, in part, lead to this decrease in ABCA1.

These studies have important clinical implications. Type 2 diabetes (TIID) and insulin resistance are characterised by elevated fatty acids, low plasma HDL levels, and prevalent NASH [35–37]. Patients with TIID have a selective increase in linoleate levels in serum lipids [38]. Our findings raise the possibility consequently that unsaturated fatty acid especially linoleate could contribute in part to FFA and triglyceride storage in hepatocytes.

It is usually believed that cis-unsaturated fatty acids have a beneficial effect by lowering plasma LDL levels [39]. Saturated (palmitate and stearate), but not unsaturated (oleate and linoleate), fatty acids were able to induce apoptosis by increasing caspase-3 activity and DNA fragmentation in hepatoma cells [40]. However, our results suggest that unsaturated fatty acids may promote hepatic steatosis by impairing the lipid-removal pathway in hepatocytes. This is consistent with the observation that the condition of hypercholesterolemia was aggravated in obese rats, as shown by elevated levels of total cholesterol and triglycerides by dietary PUFA treatment [41]. Therefore, with a high concentration of unsaturated fatty acids, its beneficial effect will be countered and may have some negative impact on the ABCA1 pathway in hepatocytes.

The current study also has important therapeutic implications for treating NASH. Understanding the mechanisms by which fatty acids increase ABCA1 degradation will be useful in the design of therapeutic interventions that enhance the activity of this lipid-removal pathway and prevent formation of NASH.