Impact of cholesterol metabolism and the LXRα-SREBP-1c pathway on nonalcoholic fatty liver disease

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Abstract. We previously studied fatty acid metabolism in the liver of nonalcoholic fatty liver disease (NAFLD) and reported the activation of the LXRα-SREBP-1c pathway in hepatocytes. LXRα regulates cholesterol metabolism as well as fatty acid metabolism, and its agonistic ligands are oxysterols. Moreover, there is some evidence that excess cholesterol intake is involved in the onset of NAFLD. Therefore, in this study, we examined the expression of cholesterol metabolism-associated genes in the NAFLD liver by real-time PCR. Expression of LXRα and ACAT1 was up-regulated in NAFLD and this was more noticeable in non-obese rather than in obese patients. Although the expression of the LDL receptor, which acts on cholesterol uptake, and of SREBP-2, a positive key regulator of cholesterol, was suppressed, the expression of enzymes that promote cholesterol synthesis was uniformly increased in NAFLD. Gene expression of apoB100 and microsomal triglyceride transfer protein, which are associated with VLDL secretion, and ABCG5, which is involved in cholesterol excretion, was significantly elevated in NAFLD. Because cholesterol accumulates in hepatocytes in NAFLD liver, cholesterol uptake and synthesis should be physiologically down-regulated. However, cholesterol synthesis was activated in NAFLD liver, meaning that cholesterol metabolism is dysregulated in NAFLD. Over-production of cholesterol may lead to an increased level of oxysterols, activation of LXRα and SREBP-1c, and enhanced fatty acid synthesis.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent causes of liver dysfunction, and its incidence is markedly increasing. More than 10% of patients with NAFLD develop nonalcoholic steatohepatitis (NASH), characterized by inflammatory cell-infiltration and ballooning of hepatocytes in the liver, which eventually causes liver cirrhosis and hepatocellular carcinoma (1). However, the mechanisms involved in the pathogenesis of NAFLD in humans have not been thoroughly investigated. To control the initiation and progression of NAFLD, it is necessary to understand the precise mechanism of regulation of lipogenesis in the human liver; therefore, fatty acid metabolism in hepatocytes is of considerable interest.

We comprehensively investigated the hepatic expression pattern of fatty acid metabolism-associated genes in patients with NAFLD (2-6). We found that despite increased uptake of free fatty acids and intracellular accumulation of fatty acids and triglycerides, the expression of sterol regulatory element-binding protein-1c (SREBP-1c), a key positive regulator of fatty acid synthesis, was still up-regulated, and the expression of AMP-activated protein kinase (AMPK), a negative regulator of fatty acid synthesis, was down-regulated. This led to the activation of fatty acid oxidation, reactive oxygen species production, and synthesis of phospholipids and

triglycerides. This expression pattern of SREBP-1c and AMPK in NAFLD liver suggests the breakdown of feedback regulation from the increased level of intracellular fatty acids. This phenomenon may be derived from increased visceral fat volume and insulin resistance because SREBP-1c and AMPK expression is affected by adipocytokines, insulin signaling and glucose concentration (4,7). However, many NAFLD patients are not obese and do not exhibit increased visceral fat volume or insulin resistance. It has also been demonstrated that the activity of SREBPs, which are produced in the endoplasmic reticulum (ER) as membrane-bound precursors and which form a complex with SREBP-cleavage activating protein (Scap), is post-transcriptionally regulated by intra-cellular cholesterol levels (7-10). In other words, when cholesterol levels in the ER are too low, Scap escorts SREBPs to the Golgi apparatus where the SREBPs are proteolytically processed to yield active fragments that can enter the nucleus and act as transcriptional factors. When intracellular cholesterol levels are sufficient, the SREBP/Scap complex is held in the ER by insulin-induced genes (Insigs), and the production of active SREBP fragments is inhibited. Moreover, SREBP-1c gene expression is positively regulated by the liver X receptor α (LXRα), whose activator ligands are oxysterols (1-14).

Considering the above, hepatic cholesterol metabolism, which has not been extensively investigated in NAFLD patients, needed to be examined in detail. We previously compared the nutritional characteristics between obese and non-obese NAFLD patients and found that dietary over-intake of cholesterol might be a critical factor for the induction and progression of NAFLD (15). Therefore, in the present study, we examined the expression of cholesterol metabolism-associated genes in NAFLD liver and investigated the role of cholesterol metabolism in the onset of NAFLD.

Patients and methods

Patients and samples for real-time PCR. For real-time PCR, sample tissue was obtained by liver biopsy from 33 patients (male/female 16/17) with histologically diagnosed NAFLD, including 14 with NASH, who were admitted to the Kyushu University Hospital and Kyushu Medical Center between 2005 and 2007. Written informed consent was obtained from all the patients for this investigation.

For the analysis of LXRα and acyl-CoA: cholesterol acyltransferase 1 (ACAT1) expression, patients were divided into obese (n=23, male/female 11/12) and non-obese (n=10, male/female 5/5) groups according to a body mass index (cut off point: 25 kg/m^2). As a control, normal liver tissue was obtained from 10 living donors of liver transplantation whose liver function tests and histological findings were completely normal. We performed real-time RT-PCR and compared gene expression between the NAFLD and normal liver (100%).

Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1.0 μg RNA with GeneAmp™ RNA PCR (Applied Biosystems, Branchburg, NJ, USA) using random hexamers. Real-time RT-PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 μl)

contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM MgCl$_2$, 0.5 μM of the upstream and downstream PCR primers, and 2 μl of first-strand cDNA as a template. To control variations in the reactions, all PCR data were normalized against ß-actin expression. The PCR primers for amplification of the ATP binding cassette (ABC) G5 were forward 5'-ATTGTGGTTCTCACCATTCACCAG-3' and reverse 5'-GGTTTGAATGTTCAGGACAAGGGTA-3'; the primers for ACAT1 were forward 5'-GCAGCGAAGAGGCTCAATG-3' and reverse 5'-GCAGCATATACAGGAGCAATTGG-3'; the primers for apoB100 were forward 5'-TCAAGAGTTACAGCAGATCCATCAA-3' and reverse 5'-TCAGAATGGAAGTCCTTAAGAGCAA-3'; the primers for farnesyl-diphosphate synthase (FDPS) were forward 5'-GCATGTATCTACCGCCTGCTGA-3' and reverse 5'-TCCAGGGTCTGCCCAATCTC-3'; the primers for HMG-CoA reductase (HMGR) were forward 5'-GCCTGGCTCGAAACATCTGAA-3' and reverse 5'-CTGACCTGGACTGGAAACGGATA-3'; the primers for HMG-CoA synthase (HMGS) were forward 5'-GTATGCCCTGGTAGTTGCAGGAG-3' and reverse 5'-TGTTGCATATGTGTCCCACGAA-3'; the primers for LDL receptor (LDLR) were forward 5'-CAACGGCTCAGACGAGCAAG-3' and reverse 5'-AGTCACAGACGAACTGCCGAGA-3'; the primers for LXRα were forward 5'-GCCGAGTTTGCCTTGCTCA-3' and reverse 5'-TCCGGAGGCTCACCAGTTTC-3'; the primers for micro-somal triglyceride transfer protein (MTP) were forward 5'-AGCACCTCAGGACTGCGAAGA-3' and reverse 5'-CAGAGGTGACAGCATCCACCA-3'; the primers for Niemann-Pick C1 like 1 (NPC1L1) were forward 5'-TCTGTGGAGTTTGTGTCCCACATTA-3' and reverse 5'-GTTGGTCATGGCCACACCTG-3'; the primers for SREBP-2 were forward 5'-ACAACCCATAATATCATTGAGAAACG-3' and reverse 5'-TTGTGCATCTTGGCGTCTGT-3'; the primers for squalene synthase (SS) were forward 5'-CGTGCAGTGCCTGAATGAACTTA-3' and reverse 5'-GGCAGCCAAAGTGGCAATG-3'; the primers for ß-actin were forward 5'-TGGCACCCAGCACAATGAA-3' and reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA -3'.

Statistical analysis. For statistical analysis, continuous variables were compared using the Mann-Whitney U-test, and categorical variables were compared using the Chi-square test or Fisher's test. P<0.05 was considered statistically signifi-cant. The results are expressed as means ± SD.

Results

Expression pattern of LXRα and ACAT1. Previously, we reported that, in NAFLD patients, the expression of SREBP-1c and LXRα was highly increased and that up-regulation of LXRα was closely associated with SREBP-1c overexpression (6). LXRα is a sterol sensor, and increased intracellular cholesterol drives the production of oxysterols, which are agonists for LXRα (7). Because cholesterol over-intake is more characteristic of non-obese NAFLD patients (15), mRNA expression of LXRα was re-evaluated by real-time PCR and compared between the obese and non-obese NAFLD patients (see Patients and methods). The characteristics of the patients are shown in Table I. As a result, the LXRα levels

Table I. Background of the patients.

	Obese	Non-obese	
Body mass index (kg/m²)	29.6±2.8	23.6±1.7	p<0.01
Gender (M/F)	21/21	5/7	
Age (years)	51.2±14.0	47.2±15.1	
Waist circumference (cm)	98.6±8.8	85.1±5.7	p<0.01
Visceral fat area (cm²)	111.3±32.5	79.2±39.8	
Alanine aminotransferase (IU/l)	83.8±55.6	72.4±48.4	
γ-glutamyl transpeptidase (IU/l)	67.8±53.6	60.9±16.9	
Total cholesterol (mg/dl)	207.9±36.1	229.9±29.6	
Triglycerides (mg/dl)	169.9±116.8	134.4±47.9	
HOMA-IR	4.4±3.3	2.4±1.2	
Adiponectin (μg/dl)	6.67±2.67	6.71±2.42	
TNF-α (pg/ml)	1.28±0.52	1.38±0.92	

were significantly higher in the non-obese group although the levels in the obese group were also elevated by more than 3-fold compared with those in the control group (Fig. 1A). Moreover, we also found that the expression of ACAT1, an enzyme associated with esterification of cholesterol, was greater in NAFLD liver than in normal liver and that the non-obese patients showed greater expression of ACAT1 than the obese patients (Fig. 1B).

Expression of cholesterol metabolism-associated genes in NAFLD. To analyze the cholesterol metabolism in NAFLD liver, the expression levels of cholesterol metabolism-associated genes were evaluated by real-time PCR and compared between the NAFLD and normal control groups (Fig. 2). In NAFLD, the expression levels of LDLR and SREBP-2 were greatly reduced by 90 and 50%, respectively. In contrast, the expression levels of apoB100 and MTP were enhanced by nearly 4- and 2-fold, respectively. Furthermore, expression of the ABCG5 was increased by nearly 3-fold, whereas the expression of NPC1L1 was unchanged. Gene expression levels of cholesterol synthesis-associated enzymes were also estimated (Fig. 3). The expression of HMGR, which is the first committed enzyme in the mevalonate pathway, was enhanced by nearly 2.5-fold despite low expression of LDLR and SREBP-2. The expression patterns of the other enzymes tested, HMGS, FDPS, and SS, were broadly similar to that of HMGR. Fig. 4 shows a schema for the regulation of cholesterol in hepatocytes.

Discussion

It is well known that the onset and progression of NAFLD is closely associated with increased visceral fat volume, insulin resistance and hepatocytic accumulation of fatty acids and triglycerides, resulting in oxidative stress (16). In obese NAFLD patients, such findings are relatively common. Fatty acid synthesis in hepatocytes is positively regulated by the transcription factor SREBP-1c (7). When intracellular fatty acid levels go up, AMPK, a negative regulator of lipogenesis, down-regulates SREBP-1c. Oberkofler *et al* reported that, in adipose tissue, SREBP-1c mRNA expression in obese humans was lower than that in non-obese subjects and that the expression was up-regulated after weight loss (17). On the other hand, in our previous study, SREBP-1c expression levels were 5-fold higher in the liver of NAFLD patients compared with healthy controls, and the up-regulated SREBP-1c
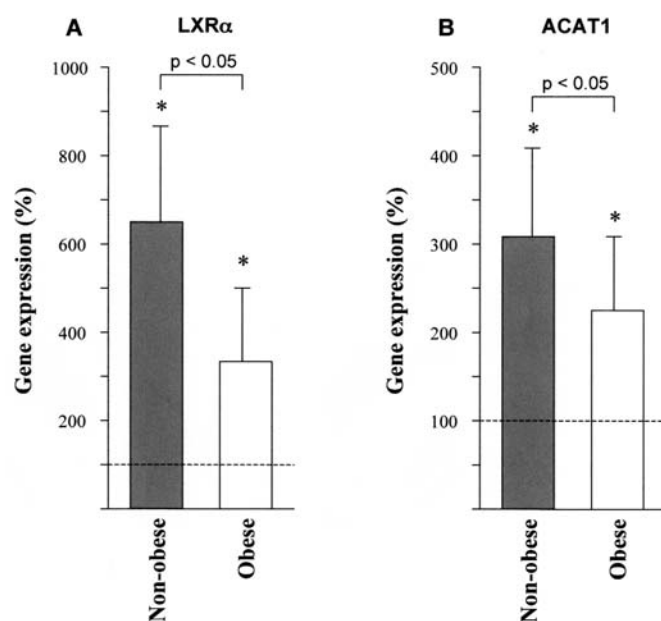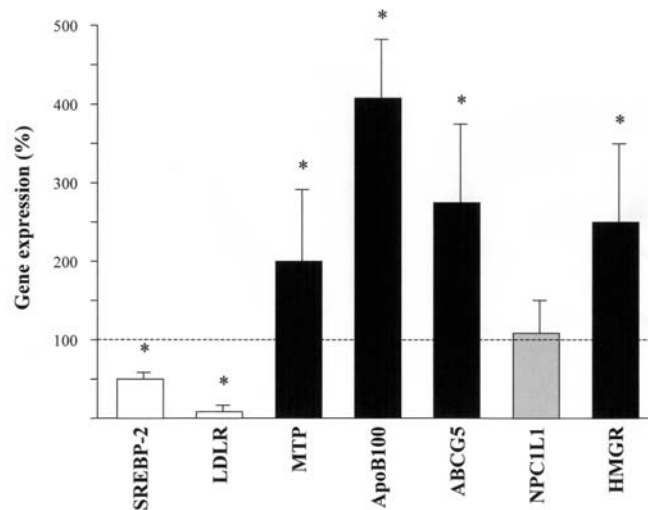

Figure 1. Real-time RT-PCR analysis of LXRα (A) and ACAT1 (B) gene expression in NAFLD liver. LXRα and ACAT1 expression was significantly higher in the non-obese group (BMI <25 kg/m²) than that in the obese group (BMI >25 kg/m²). *Statistically significant differences (p<0.05) compared with normal liver (100%). LXRα, liver X receptor α; ACAT1, acyl-CoA: cholesterol acyltransferase 1.

Figure 2. Real-time RT-PCR analysis of SREBP-2, LDLR, MTP, apoB100, ABCG5, NPC1L1 and HMGR gene expression in NAFLD liver. *Statistically significant differences (p<0.05) compared with normal liver (100%). SREBP-2, sterol regulatory element-binding protein-2; LDLR, LDL receptor; MTP, microsomal triglyceride transfer protein; ABCG5, ATP binding cassette G5; NPC1L1, Niemann-Pick C1 like 1; HMGR, HMG-CoA reductase.
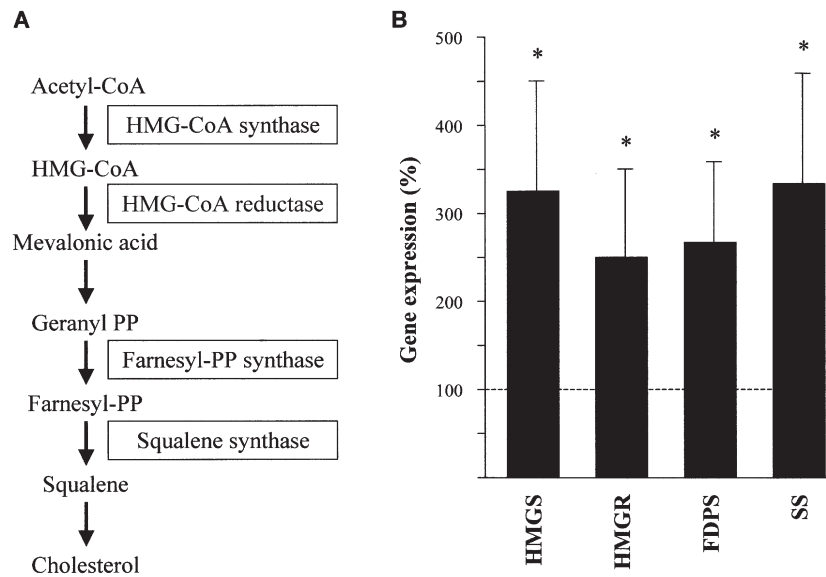
Figure 3. (A) Cholesterol synthesis pathway in hepatocytes and its related enzymes. (B) Real-time RT-PCR analysis of HMG-CoA reductase (HMGR), HMG-CoA synthase (HMGS), farnesyl-diphosphate synthase (FDPS), and squalene synthase (SS) gene expression in NAFLD liver. *Statistically significant differences (p<0.05) compared with normal liver (100%).
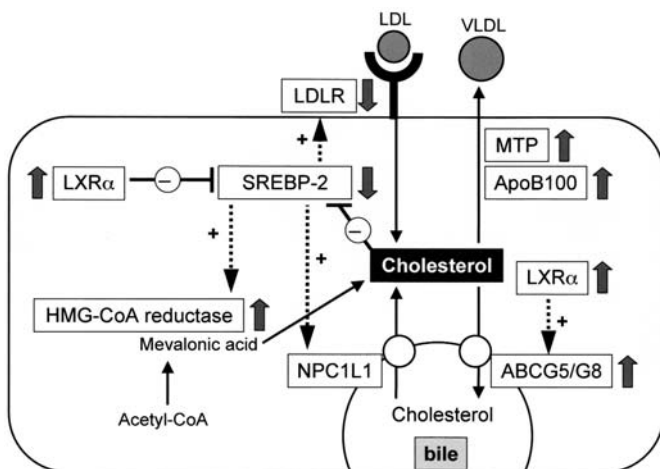

Figure 4. Schema of cholesterol metabolism in hepatocytes. Wide gray arrows indicate changes in gene expression in NAFLD liver. ABCG5/G8, ATP binding cassette G5/G8; LDLR, LDL receptor; LXRα, liver X receptor α; MTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick C1 like 1; SREBP-2, sterol regulatory element-binding protein-2.

contributes to increased *de novo* synthesis of fatty acids, even in hepatocytes with fatty acid accumulation (2-6). Up-regulated insulin receptor substrate-1 (IRS-1) is associated with up-regulation of SREBP-1c, and suppression of SREBP-1c by AMPK fails in NAFLD (2-6). However, many NAFLD patients are not obese and many of them do not exhibit insulin resistance or increased visceral fat volume (Table I). These data compel us to question what triggers and maintains the disease in non-obese patients. To explore this problem, we examined the nutritional assessment of NAFLD patients and compared findings between obese and non-obese patients (15). Unsurprisingly, total energy and carbohydrate intake was significantly higher in obese patients. Interestingly,

a characteristic finding was that dietary cholesterol intake was significantly higher in non-obese patients than in obese patients whose cholesterol intake was also increased compared with normal controls (15). This is an important clue since LXRα, a sterol sensor that regulates intracellular cholesterol levels, can induce SREBP-1c expression and activation (7).

It has been reported that SREBP-1c is positively regulated through the transcription factor LXRα which forms a hetero-dimer with retinoid X receptor α (18). Since stimulation of the peroxisome proliferator activated receptor α (PPARα) increases LXRα expression (19,20), the accumulated fatty acids in the NAFLD liver, which are endogenous PPARα agonists, may increase LXRα expression. In addition, insulin increases LXRα expression (21); therefore, the lipogenic effect of insulin in the NAFLD liver may be partially mediated via the LXRα pathway. LXRα expression is controlled by auto-regulation because the LXRα gene promoter contains functional LXR response elements (22). Ligands of LXRα include the cholesterol derivatives oxysterols. We previously reported that LXRα expression is highly up-regulated, in addition to SREBP-1c, and shows a strong positive correlation with SREBP-1c in NAFLD patients (6). Moreover, in the non-obese group, whose dietary cholesterol intake was greater than in the obese group, the LXRα levels were significantly higher than in the obese group (Fig. 1A). These results indicate that cholesterol excess, which ultimately results in elevated oxysterols, stimulates SREBP-1c synthesis through an LXRα-dependent mechanism, particularly in non-obese NAFLD patients. SREBP-1c may be primarily up-regulated by LXRα in NAFLD although, as shown in our previous reports (2-6), insulin signaling and intracellular glucose levels also affect SREBP-1c expression. We assumed that the up-regulation of SREBP-1c is mainly attributable to the activation of LXRα for which the natural ligands are oxysterols.

theless, it may be that cholesterol itself and certain ...ls inhibit proteolytic processing of the inactive SREBP-1c precursor to its mature active form, which works in the nucleus (8). However, the post-transcriptional regulation at sufficient intracellular cholesterol levels may be considered as a transient short-time regulation under physiological situations; therefore, it is reasonable to assume that the post-transcriptional mechanism cannot function appropriately in chronic diseases such as NAFLD. Accordingly, cholesterol excess seems to be positively associated with the initiation and progression of NAFLD. Indeed, in animal models, it has been reported that a high cholesterol diet causes NASH without obesity (23-25). Therefore, it is plausible that a person with a high cholesterol intake could develop non-obese NAFLD. Up-regulation of LXRα is needed to regulate intracellular cholesterol levels; however, it also up-regulates lipogenesis via SREBP-1c resulting in steatosis, which is apparently deleterious for hepatocytes. Generally, most intracellular cholesterol is esterified with fatty acids by ACAT1. In our study, ACAT1 expression was greater in NAFLD, particularly in non-obese patients than in the normal controls (Fig. 1B). Therefore, it is likely that an abundance of cholesterol promotes fatty acid production for its esterification.

Next, we evaluated cholesterol metabolism in the NAFLD liver. Cholesterol metabolism can occur via three mechanisms; first, as we have shown, the cholesterol-LXRα-SREBP-1c signaling pathway leading to fatty acid synthesis; second, *de novo* cholesterol synthesis, its uptake from serum, and output into serum or bile; and third, bile acid synthesis and its secretion. Our question was whether physiological cholesterol metabolism is disturbed or not in NAFLD liver. Serum LDL-cholesterol is taken into hepatocytes via the LDLR. For secretion, cholesterol is packed into lipoproteins together with triglycerides and apoB100 by MTP, and secreted into serum as VLDL. Cholesterol is also secreted into bile via ABCG5/ABCG8. SREBP-2 is a key factor for cholesterol synthesis, which simultaneously stimulates gene expression of LDLR and HMGR. The accumulation of cholesterol in hepatocytes and LXRα combine to down-regulate SREBP-2 activity, and thus decrease cholesterol synthesis and uptake (7).

In our study, SREBP-2 and LDLR expression was down-regulated in NAFLD liver (Fig. 2), which could be explained by cholesterol overload and LXRα activation. Surprisingly, the expression of HMGR showed a contradictory result and was enhanced by nearly 2.5-fold (Fig. 3), despite the low expression levels of LDLR and SREBP-2. Moreover, the expression of other enzymes associated with cholesterol synthesis such as HMGS, FDPS and SS, was up-regulated in NAFLD liver (Fig. 3). These data indicate that the regulation of cholesterol synthesis and uptake is impaired in NAFLD liver. At present, we cannot explain the exact reason for this discrepancy. More studies are needed for the precise understanding of the system that regulates cholesterol. The increased expression of apoB100 and MTP in NAFLD liver (Fig. 2) may be involved in the response to overload of cholesterol and triglycerides with VLDL secretion. ABCG5 gene expression is increased by a high cholesterol diet and is mediated by the LXRα pathway (26-29). Cholesterol loading of hepatocytes results in the increased formation of oxysterols,

which stimulate LXRα and enhance cholesterol efflux into bile through ABCG5/ABCG8 transporters. Therefore, up-regulation of ABCG5 expression in our study is an expected result, while NPC1L1 expression was unchanged between NAFLD and normal liver (Fig. 2).

NPC1L1 is a sterol transporter that is present in the proximal jejunum and on the canalicular aspect of hepatocytes (26). It is essential for the absorption/reabsorption of cholesterol and of other plant sterols from the intestine and liver. In animal models, it has been reported that knockout of NPC1L1 or treatment with ezetimibe, an inhibitor of NPC1L1-dependent cholesterol transport, provides resistance against hepatic steatosis (30,31). With regard to treatment options, lowering of intrahepatic cholesterol levels may be beneficial for treating NAFLD. From this point of view, ezetimibe and statins, HMGR inhibitors, can be used as a new therapeutic option for NAFLD. It is expected that the inhibition of NPC1L1-dependent absorption of dietary and biliary cholesterol will decrease intracellular cholesterol levels and down-regulate/inactivate LXRα, resulting in an improvement of steatohepatitis. We are now running a pilot study of ezetimibe to treat NAFLD patients. Although only a preliminary result, ezetimibe significantly lowered the serum levels of alanine aminotransferase and γ-glutamyl transpeptidase after administration for 4 months (data not shown).

In this study, we obtained the following findings in NAFLD patients. i) Cholesterol over-intake is a potent inducer of NAFLD, which is primarily achieved via the LXRα-SREBP-1c pathway. ii) LXRα was overexpressed in the liver of NAFLD patients, and the expression was positively correlated with SREBP-1c expression at least at mRNA levels. iii) Physiological cholesterol metabolism in the liver may be, at least partially, disturbed in NAFLD liver. iv) Some modulators of cholesterol metabolism, such as ezetimibe and statins, may have therapeutic potential for NAFLD.

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