Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease

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Abstract. Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent causes of abnormal liver dysfunction, and its prevalence has markedly increased. We previously evaluated the expression of fatty acid metabolism-related genes in NAFLD and reported changes in expression that could contribute to increased fatty acid synthesis. In the present study, we evaluated the expression of additional fatty acid metabolism-related genes in larger groups of NAFLD (n=26) and normal liver (n=10) samples. The target genes for real-time PCR analysis were as follows: acetyl-CoA carboxylase (ACC) 1, ACC2, fatty acid synthase (FAS), sterol regulatory element-binding protein 1c (SREBP-1c), and adipose differentiation-related protein (ADRP) for evaluation of de novo synthesis and uptake of fatty acids; carnitine palmitoyltransferase 1a (CPT1a), long-chain acyl-CoA dehydrogenase (LCAD), long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α (HADHA), uncoupling protein 2 (UCP2), straight-chain acyl-CoA oxidase (ACOX), branched-chain acyl-CoA oxidase (BOX), cytochrome P450 2E1 (CYP2E1), CYP4A11, and peroxisome proliferator-activated receptor (PPAR)α for oxidation in the mitochondria, peroxisomes and microsomes; superoxide dismutase (SOD), catalase, and glutathione synthetase (GSS) for antioxidant pathways; and diacylglycerol O-acyltransferase 1 (DGAT1), PPARγ, and hormone-sensitive lipase (HSL) for triglyceride synthesis and catalysis. In NAFLD, although fatty acids accumulated in hepatocytes, their de novo synthesis and uptake were up-regulated in association with increased expression of ACC1, FAS, SREBP-1c, and ADRP. Fatty acid oxidation-related genes, LCAD, HADHα, UCP2, ACOX, BOX, CYP2E1, and CYP4A11, were all overexpressed, indicating that oxidation was enhanced in NAFLD, whereas the expression of CTP1a and PPARα was decreased. Furthermore, SOD and catalase were also overexpressed, indicating that antioxidant pathways are activated to neutralize reactive oxygen species (ROS), which are overproduced during oxidative processes. The expression of DGAT1 was up-regulated without increased PPARγ expression, whereas the expression of HSL was decreased. Our data indicated the following regarding NAFLD: i) increased de novo synthesis and uptake of fatty acids lead to further fatty acid accumulation in hepatocytes; ii) mitochondrial fatty acid oxidation is decreased or fully activated; iii) in order to complement the function of mitochondria (β-oxidation), peroxisomal (β-oxidation) and microsomal (ω-oxidation) oxidation is up-regulated to decrease fatty acid accumulation; iv) antioxidant pathways including SOD and catalase are enhanced to neutralize ROS overproduced during mitochondrial, peroxisomal, and microsomal oxidation; and v) lipid droplet formation is enhanced due to increased DGAT expression and decreased HSL expression. Further studies will be needed to clarify how fatty acid synthesis is increased by SREBP-1c, which is under the control of insulin and AMP-activated protein kinase.

Introduction

Nonalcoholic fatty liver disease (NAFLD), which is characterized by triglyceride accumulation in hepatocytes (hepatic steatosis), is one of the most frequent causes of
abnormal liver function (1-3). The prevalence of NAFLD in the general population is estimated to be between 14 and 24% (4-6), and it has markedly increased in all segments of the population including children. Furthermore, nonalcoholic steatohepatitis (NASH), a severe form of NAFLD which is accompanied by hepatitis and fibrosis (7), can progress to cirrhosis and hepatic failure (8). It has been reported that >20% of patients with NASH develop cirrhosis, half of which subsequently die of liver failure (8). NAFLD is often associated with obesity and/or insulin resistance; however, the precise cause of NAFLD remains unclear. It is important, therefore, to characterize lipid metabolism, particularly fatty acid metabolism, in NAFLD.

Fatty acids in the liver are derived from de novo synthesis and plasma-free fatty acids. Acetyl-CoA is an essential substrate for de novo synthesis and is ultimately converted to stearic acid (C18:0), which also can be desaturated to oleic acid in hepatocytes. Plasma fatty acids are also actively taken up by a specific transporter. Up-regulation of synthesis and/or uptake can result in fatty acid accumulation. Fatty acids in hepatocytes are metabolized by either of 2 pathways; oxidation to generate ATP (e.g. β-oxidation in the mitochondria) or esterification to produce triglycerides, which are either incorporated into lipoproteins for export or stored as lipid droplets within the hepatocytes. Defects in one or both of these pathways can lead to hepatic steatosis.

We previously evaluated the expression of genes related to fatty acid metabolism and reported that de novo synthesis of fatty acids was up-regulated in spite of their accumulation in hepatocytes of patients with NAFLD (9). In this study, using more samples from NAFLD and control livers, we further investigated fatty acid metabolism in NAFLD by re-evaluating the expression of genes involved in de novo synthesis, uptake, oxidation, antioxidant pathways, and triglyceride synthesis and catalysis.

Patients and methods

Tissue samples were obtained by liver biopsy from 26 patients with histologically diagnosed NAFLD, including 4 patients with NASH, who were admitted to the Kyushu University Hospital between 2004 and 2006. As a control, normal liver was also obtained by biopsy from 10 men whose liver function tests and histological findings were completely normal. Real-time RT-PCR was performed as previously reported (9). 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, Tokyo, Japan), according to the manufacturer’s instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR-Green 1, 4 mM MgCl₂, 0.5 μM of the upstream and downstream PCR primers, and 2 μl of the first-strand cDNA as a template. The target genes and their primers are shown in Table I. To control for variations in the reactions, all PCRs were normalized against β-actin expression. All results are shown as the mean ± SEM. Comparisons were made by the Mann-Whitney U test.

Results

Expression of genes related to de novo synthesis and uptake of fatty acids. In the process of fatty acid synthesis, acetyl-CoA carboxylase (ACC) converts acetyl-CoA, an essential substrate of fatty acids, to malonyl-CoA. Fatty acid synthase (FAS) then utilizes both acetyl-CoA and malonyl-CoA to form palmitic acid (C16:0). In NAFLD, the expression of ACC and FAS was ~2-fold and 1.5-fold higher, respectively, than that in the normal liver (Fig. 1). Both ACC and FAS are positively regulated by a transcriptional factor, sterol regulatory element-binding protein 1c (SREBP-1c) (1). In NAFLD, SREBP-1c expression was also higher than that in the normal liver (Fig. 1). In addition to de novo synthesis, fatty acids in hepatocytes are transferred from serum by adipose differentiation-related protein (ADRP, adipophilin) (10). ADRP expression in NAFLD was 2-fold higher than in the normal liver (Fig. 1).

Gene expression related to β-oxidation in mitochondria. Carnitine palmitoyltransferase 1a (CPT1a) is a regulatory...
enzyme in mitochondria that transfers fatty acids from the cytosol to mitochondria prior to β-oxidation. β-oxidation is then catalyzed by enzymes such as long-chain acyl-CoA dehydrogenase (LCAD) and long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α (HADHα). In NAFLD, CPT1α expression was decreased by 50%, and expression of LCAD and HADHα was significantly increased 6-fold and 3-fold, respectively, compared with that in the normal liver (Fig. 2).

Uncoupling protein 2 (UCP2), a mitochondrial inner-membrane protein is emerging as a potential regulator of mitochondrial reactive oxygen species (ROS) production (11). It mediates a proton leak across the inner membrane.

Table I. Primers used for analysis for expression of fatty acid metabolism-related genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'</th>
<th>Reverse primer 3'</th>
<th>GenBank™ accession no.</th>
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<td>FAS</td>
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<td>TGATGCCCAAGAGAGTGTCCG</td>
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<td>SREBP-1c</td>
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<tr>
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<td>GGGATCGCTCTCAACAGC</td>
<td>AGATTCGCCAACCATCAC</td>
<td>NM-001122</td>
</tr>
<tr>
<td>CPT1α</td>
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<td>GAGCCAGACCTGGAAGTACC</td>
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<td>LCAD</td>
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</tr>
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<td>SOD</td>
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and uncouples fuel oxidation from adenosine triphosphate (ATP) synthesis (12). UCP2 expression in NAFLD was 2-fold higher than in the normal liver.

Expression of other genes related to fatty acid oxidation. When cytosolic fatty acids accumulate due to impairment of oxidative capacity in mitochondria, alternative pathways in the peroxisomes (β-oxidation) and in microsomes (ω-oxidation) are activated. In peroxisomal β-oxidation, straight-chain acyl-CoA oxidase (ACOX) and branched-chain acyl-CoA oxidase (BOX) are responsible for the initial oxidation of very-long-chain fatty acyl-CoAs. In NAFLD, the expression of ACOX and BOX was increased 2-fold and 10-fold, respectively, compared with that in the normal liver (Fig. 3). In microsomal ω-oxidation, CYP2E1 and CYP4A11, which are inducible hepatic microsomal cytochrome P-450s, can initiate the autopropagative process of lipid oxidation. In NAFLD, the expression of CYP2E1 and CYP4A11 was significantly higher (14-fold and 4-fold, respectively) than in the normal liver (Fig. 3). Peroxisome proliferator-activated receptor (PPAR)α, a transcriptional factor, up-regulates the expression of a suite of genes that includes peroxisomal and mitochondrial β-oxidation enzymes as well as CYP4A. In NAFLD, PPARα expression was significantly decreased by 50% compared with that in the normal liver (Fig. 3).

Expression of genes related to antioxidant pathways. ROS are formed during the process of fatty acid oxidation. They are eliminated by antioxidant enzymes such as superoxide dismutase (SOD) and catalase, and by compounds such as glutathione, which is produced by glutathione synthetase (GSS). In NAFLD, the expression of SOD and catalase was increased 5-fold and 10-fold, respectively, compared with that in normal liver, whereas GSS expression was unchanged (Fig. 4).

Expression of genes related to lipid droplet formation. Fatty acids are also metabolized by esterification to produce

Figure 2. Real time RT-PCR analysis for gene expression of mitochondrial β-oxidation in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. CPT1a, carnitine palmitoyltransferase 1a; LCAD, long-chain acyl-CoA dehydrogenase; HADHα, long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α; UCP2, uncoupling protein 2.

Figure 3. Real time RT-PCR analysis for gene expression of peroxisomal or microsomal oxidation in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. ACOX, straight-chain acyl-CoA oxidase; BOX, branched-chain acyl-CoA oxidase; CYP, cytochrome P450; PPARα, peroxisome proliferator-activated receptor α.
triglycerides, which are stored within hepatocytes as lipid droplets. Diacylglycerol O-acyltransferase 1 (DGAT1) is involved in triglyceride synthesis, and its expression in NAFLD was increased 2-fold compared to that in the normal liver (Fig. 5). Expression of PPARγ, which facilitates the storage of triglycerides in NAFLD, was 1.5 times greater than that of the controls. Hormone-sensitive lipase (HSL) is a key enzyme for catalyzing triglyceride accumulation in the form of lipid droplets. The expression of HSL was decreased by 80% in NAFLD as compared with the normal liver (Fig. 5).

Discussion

We previously reported a study on the expression of genes related to fatty acid metabolism in NAFLD (9). In the present study, we evaluated a wider range of genes and used a greater number of samples from both NAFLD and normal livers. Fatty acid metabolism in hepatocytes can occur by four mechanisms: a) \textit{de novo} fatty acid synthesis and uptake of plasma-free fatty acids; b) fatty acid catalysis by oxidation in mitochondria, peroxisomes, and microsomes; c) neutralization of ROS derived from fatty acid oxidation; and d) conversion between fatty acids and triglycerides.

With respect to \textit{de novo} fatty acid synthesis, the expression of ACC1 and FAS was increased in NAFLD, while expression of ACC2 was not. It has been reported that humans (20) and mice (21) with hepatic steatosis accumulate excess oleic acid (C18:1), the end-product of \textit{de novo} fatty acid synthesis. This evidence, taken together with our results, suggests that fatty acid synthesis rates are increased in NAFLD despite the accumulation of fatty acids. In normal liver, fatty acid synthesis is positively regulated by transcriptional factor SREBP-1c, and in fatty acid overload, \textit{de novo} fatty acid synthesis is suppressed through down-regulation of SREBP-1c (1). In NAFLD, the expression of SREBP-1c was increased 2-fold, indicating that negative feedback regulation via SREBP-1c failed to occur.

In addition to an increase in \textit{de novo} fatty acid synthesis, fatty acid uptake from serum can contribute to accumulation of fatty acids in NAFLD. ADRP has been suggested to play a role in fatty acid transport, although its function is not fully understood. It has been reported that ADRP-knockout mice
are resistant to the development of fatty liver (10). In NAFLD, the expression of ADRP was increased 2-fold and our findings are in agreement with the results of immunohistochemical studies in NAFLD by Motomura et al (13). It is very intriguing that not only de novo fatty acid synthesis was increased but also fatty acid uptake from serum, despite the excess hepatic accumulation of fatty acids in NAFLD.

Oxidation of fatty acids occurs mainly in the mitochondria. In this study, the expression of CPT1a was decreased, whereas that of LCAD, HADHα, and UCP2 was increased. CPT1a is a membrane transporter of fatty acids (acyl-CoA) from the cytoplasm into the mitochondrial matrix and is a primary regulatory enzyme involved in mitochondrial β-oxidation. The down-regulation of CPT1a expression in NAFLD is attributable to an increase in malonyl-CoA (14), since the expression of ACC1 which converts acetyl-CoA to malonyl-CoA, was increased (Fig. 1). Therefore, it is possible that β-oxidation in the mitochondria might be reduced in NAFLD. On the other hand, increased expression of LCAD and HADHα suggests enhancement of β-oxidation. Furthermore, up-regulation of UCP2 expression suggests that excess ROS production occurs by increased β-oxidation because UCP2 potentially reduces ROS production (15,16). When β-oxidation reaches maximal levels, a lack of unesterified CoA could inhibit CPT and thereby prevent further entry of acyl groups to the mitochondria, thus serving as an intramitochondrial control on β-oxidation (17). β-Oxidation in mitochondria was at maximal levels, thereby resulting in decreased expression of CPT1a.

When there is an excess of fatty acids in hepatocytes, alternative pathways of fatty acid oxidation are activated, such as β-oxidation in peroxisomes and ω-oxidation in the endoplasmic reticulum (microsomes). The peroxisomal acyl-CoA oxidases ACOX and BOX are the first and rate-limiting enzymes of β-oxidation pathways in peroxisomes (18), and their expression was increased in this study, indicating that peroxisomal β-oxidation is compensatively enhanced in NAFLD. In the endoplasmic reticulum, ω-oxidation by CYP2E1 and CYP4As occurs (19). We demonstrated that the expression of both CYP2E1 and CYP4A11 was up-regulated in NAFLD. Increased activity of CYP2E1 in the liver is associated with factors commonly observed in NAFLD; obesity, diabetes, and hyperlipidemia. CYP4As are also assumed as key intermediaries in adaptive responses to the perturbations of hepatic lipid metabolism that accompany fasting, diabetes, and overnutrition. Recent evidence obtained from CYP2E1-null mice demonstrates that there is a compensatory increase in CYP4A activity (20). Therefore, in NAFLD, situations where CYP2E1 is fully activated may lead to increased CYP4A11 expression. On the whole, it appears that accumulation of fatty acids in NAFLD enhances oxidation not only in mitochondria but also in peroxisomes and microsomes.

PPARα is a major transcriptional activator of genes involved in mitochondrial β-oxidation, such as peroxisomal ACOX and microsomal CYP4As (18,21). In our study, the expression of PPARα was unexpectedly reduced in NAFLD. When we compared the expression of ACOX to that of BOX, which is not regulated by PPARα, we found that the enhancement of expression of ACOX was less than that of BOX (2-fold vs. 10-fold, respectively) (Fig. 3). Similarly, CYP4A11 showed less enhancement of expression than did CYP2E1 (4-fold vs. 14-fold, respectively). The difference in the expression of oxidation enzymes in peroxisomes and microsomes might be attributable to the decreased expression of PPARα. We initially found that HOMA-IR, an index of insulin resistance, was negatively correlated with PPARα expression, suggesting that the decreased expression of PPARα may be attributable to insulin resistance, which often accompanies NAFLD. Recent studies have demonstrated that
PPARα agonists reduce hepatic steatosis in animal models (22,23). Further study will be needed to clarify the mechanism of down-regulation of PPARs and the effects of PPARs activation as a treatment for NAFLD.

We investigated the expression of genes related to anti-oxidant pathways including SOD, catalase, and GSS, because it was expected that ROS overproduction would occur as a result of enhanced mitochondrial and peroxisomal β-oxidation and microsomal ω-oxidation, as described above. As we expected, the expression of SOD and catalase was dramatically enhanced. In contrast, the expression of GSS, which produces glutathione, was unchanged. Although we do not know precisely why GSS levels were unchanged, the antioxidant effects of glutathione can also be regulated by glutathione peroxidase, which, together with glutathione, neutralizes ROS.

Excess lipid droplet formation in NAFLD is indicative of increased triglyceride synthesis in hepatocytes, and in the present study, we observed increased expression of DGAT1. The expression of PPARγ, which is a transcriptional factor that facilitates adipogenesis by various mechanisms including induction of DGAT1 expression, was unexpectedly unchanged in NAFLD. It has been reported that the expression of PPARγ is markedly increased in fatty liver (24). Conversely, adipogenesis resulting in triglyceride storage occurs under conditions where there is a decrease in PPARα activity and fatty acid oxidation (21), implying that cross-talk occurs between PPARγ and PPARα in this study, the expression of both PPARγ and PPARα was decreased, suggesting that the cross-talk between these receptors might be impaired in NAFLD. Expression of HSL was also greatly down-regulated, indicating that lipolysis, in contrast to lipogenesis, is inhibited in NAFLD. Further study is needed to clarify the mechanisms that regulate expression of HSL and protein kinase A, which is a major regulator of HSL expression (25).

In summary, our results in patients with NAFLD indicate that: i) de novo synthesis of fatty acids is increased, although fatty acids have already been accumulated in hepatocytes, and is accompanied by increased fatty acid uptake from serum; ii) mitochondrial fatty acid oxidation is decreased or fully activated to increase fatty acid accumulation; iii) in order to complement the function of mitochondria (β-oxidation), peroxisomal (β-oxidation) and microsomal (ω-oxidation) oxidation is up-regulated; iv) antioxidant pathways including SOD and catalase are enhanced to neutralize overproduced ROS by enhanced oxidation; and v) lipid droplet formation is enhanced (Fig. 6). Four cases of histologically proven NASH were included in the present study, and the gene expression profiles did not differ between patients with NASH and those with NAFLD (data not shown). Since ROS are believed to be a major cytotoxic factor in NASH (19,26,27), it is assumed that uncompensated ROS overproduction due to enhanced oxidation might lead to the transition from simple obesity to NASH, a condition in which excessive ROS production can cause mitochondrial failure leading to apoptosis and oncrosis (necroptosis) (28-30).

Eleven of the NAFLD patients in this study, who were candidates as donors for liver transplantation received a strict low-calorie diet, exercise, and 400 mg/day bezafibrate (a ligand of PPARα) for 4-8 weeks prior to the operations (31). We found that this treatment normalized dysfunctional expression of genes related to fatty acid metabolism, i.e. ACC1 and FAS expression were decreased and CPT1α and PPARα expression were increased (data not shown). Therefore, treatments that target the expression of fatty acid metabolism-related genes may be beneficial in NAFLD.

Finally, as discussed above, several disorders of fatty acid metabolism were recognized in NAFLD, and we assume that unregulated enhancement of de novo fatty acid synthesis is a primary disorder in NAFLD. Fatty acid synthesis by ACC1 and FAS is tightly regulated by SREBP-1c and its expression is also regulated negatively by AMP-activated protein kinase (AMPK) and positively by insulin (1,32). Obesity, which is often accompanied by NAFLD, causes decreasing serum levels of adiponectin and increasing levels of TNFα. It has been reported that decreased adiponectin and/or increased TNFα activity results in decreased SREBP-1c expression (33,34). Insulin resistance, a condition in which insulin-signaling pathways are suppressed, was also commonly observed in NAFLD which presumably caused a decrease in SREBP-1c expression. We are now investigating AMPK expression and insulin-receptor substrates which are key molecules in the insulin signaling cascade affecting lipid metabolism (35).

References


