Sphingomyelin Synthase 2 Activity and Liver Steatosis
An Effect of Ceramide-Mediated Proliferator-Activated Receptor γ2 Suppression

Yue Li,* Jibin Dong,* Tingbo Ding,* Ming-Shang Kuo, Guoqing Cao, Xian-Cheng Jiang, Zhiqiang Li

Objective—Sphingolipid de novo biosynthesis is related to nonalcoholic fatty liver disease or hepatic steatosis. However, the mechanism is still unclear. Sphingomyelin synthase (SMS), using ceramide as one of the substrates to produce sphingomyelin, sits at the crossroads of sphingolipid biosynthesis. SMS has 2 isoforms: SMS1 and SMS2. SMS2 is the major isof orm in liver.

Approach and Results—To investigate the relationship between liver SMS2 activity–mediated sphingolipid changes and hepatic steatosis, we used 2 mouse models: SMS2 liver-specific transgenic and SMS2 knockout mice. We found that SMS2 liver-specific transgenic livers have lower ceramide and higher sphingomyelin, whereas SMS2 knockout livers have higher ceramide and lower sphingomyelin. We also found that liver SMS2 overexpression promoted fatty acid uptake and liver steatosis, whereas SMS2 deficiency had an opposite effect in comparison with their respective controls. Importantly, the exogenous ceramide supplementation to Huh7 cells, a human hepatoma cell line, reduced the expression of proliferator-activated receptor γ2 and its target genes, CD36 and FSP27. Proliferator-activated receptor γ reporter analysis confirmed this phenomenon. Furthermore, proliferator-activated receptor γ antagonist treatment significantly decreased triglyceride accumulation in SMS2 liver-specific transgenic mice.

Conclusions—We attributed these effects to ceramide that can suppress proliferator-activated receptor γ2, thus reducing the expression of CD36 and FSP27 and reducing liver steatosis. After all, SMS2 inhibition in the liver could diminish liver steatosis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: CD36 ■ ceramide ■ liver lipids ■ liver steatosis ■ proliferator-activated receptor γ2 ■ sphingolipids ■ sphingomyelin

There is a relationship between sphingolipid de novo biosynthesis and nonalcoholic fatty liver disease or hepatic steatosis.1 However, the mechanism is still unclear because many sphingolipids may be involved in fatty liver formation. Sphingomyelin synthase (SMS), which uses ceramide as one of the substrates to produce sphingomyelin, sits at the crossroads of sphingolipid biosynthesis. Overexpression or blockage of SMS activity should influence not only sphingomyelin but also ceramide levels.2 SMS has 2 isoforms: SMS1 and SMS2. The major isoform in the liver is SMS2.2,3

Ceramide is composed of sphingosine and a fatty acid. Ceramide can be generated through the de novo pathway.4 It also can be generated through the sphingomyelinase pathway, which breaks down sphingomyelin in the cell membranes and releases ceramide.5 Roles have been proposed for ceramide in heart disease, and it has been shown to induce apoptosis.6 Ceramide mediates an inflammatory response initiated by cytokines or oxidized low-density lipoprotein, a response that upregulates adhesion molecule expression and induces adhesion and migration of monocytes. These events are crucial in the initiation and progression of atherogenesis.7,8 Plasma ceramide may contribute to maladaptive inflammation in patients with coronary heart disease.9 It has been reported that plasma ceramide levels in apolipoprotein E knockout (KO) mice are higher than in controls.10 Plasma ceramides may also correlate with oxidized low-density lipoprotein, becoming a risk factor for atherosclerosis.10

CD36 is a member of the class B scavenger receptor family, located on cell surface lipid rafts.11 CD36 expression is increased concomitantly with hepatic TG content in different animal models of liver steatosis.12,13 CD36 is regulated by proliferator-activated receptor (PPAR) γ.14 PPARγ and CD36 mRNA expression is specifically upregulated in high-fat diet–induced liver steatosis in mice.15

PPARγ is a member of a nuclear hormone superfamily that heterodimerizes with the retinoid X receptor. These

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proteins are transcriptional regulators of genes encoding proteins involved in adipogenesis and lipid metabolism.10 PPARγ exists in 3 protein isoforms, PPARγ1, PPARγ2, and PPARγ3, which are created by alternative promoter usage and alternative splicing at the 5’ end of the gene. PPARγ3 expression was restricted to colon and adipose tissue in humans. PPARγ2 contains 30 additional amino acids at the N terminus compared with PPARγ1.13 PPARγ1 is expressed in many tissues, whereas significant PPARγ2 expression is limited in certain tissues, such as adipose tissues and the liver. Increased expression of either or both isoforms has been observed in livers of obese and insulin-resistant rodents.18,19 In fact, aberrant hepatic expression of PPARγ2 stimulates hepatic lipogenesis in a mouse model dealing with obesity, insulin resistance, dyslipidemia, and hepatic steatosis.20

In this study, we specifically investigated diet-induced liver steatosis in both liver-specific SMS2 transgenic and SMS2 KO mice. We found that liver SMS2 overexpression promotes mouse liver steatosis, whereas SMS2 deficiency has opposite effect in comparison with controls. We explored the potential mechanisms in this study.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Table 1. Mouse Liver Lipid Measurement in SMS2 KO, SMS2LTg, and WT Mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>SMS2LTg</th>
<th>WT</th>
<th>SMS2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chow diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, μg/mg liver</td>
<td>1.0±0.1</td>
<td>1.2±0.6 0</td>
<td>8±0.2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Phospholipids, μg/mg liver</td>
<td>2.7±0.3</td>
<td>3.2±0.4</td>
<td>2.9±0.3</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>Triglyceride, μg/mg liver</td>
<td>10±2</td>
<td>15±3*</td>
<td>11±3</td>
<td>9±2</td>
</tr>
<tr>
<td>FFA, μmol/mg liver</td>
<td>7.1±0.6</td>
<td>8.5±0.4*</td>
<td>7.9±1.1</td>
<td>6.5±0.3*</td>
</tr>
<tr>
<td>Sphingomyelin, μg/mg liver</td>
<td>0.8±0.2</td>
<td>1.1±0.1*</td>
<td>0.9±0.1</td>
<td>0.6±0.1*</td>
</tr>
<tr>
<td>Ceramide, ng/mg liver</td>
<td>66±6</td>
<td>53±5*</td>
<td>72±8</td>
<td>82±9*</td>
</tr>
<tr>
<td><strong>High-fat diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, μg/mg liver</td>
<td>3.0±0.7</td>
<td>3.3±0.4</td>
<td>3.0±0.6</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>Phospholipids, μg/mg liver</td>
<td>5.1±0.3</td>
<td>5.3±0.6</td>
<td>5.4±0.5</td>
<td>5.1±0.2</td>
</tr>
<tr>
<td>Triglyceride, μg/mg liver</td>
<td>25±5</td>
<td>38±8*</td>
<td>21±4</td>
<td>11±3*</td>
</tr>
<tr>
<td>FFA, μmol/mg liver</td>
<td>11.4±1.8</td>
<td>14.1±2.0*</td>
<td>12.0±2.5</td>
<td>9.2±2.1*</td>
</tr>
<tr>
<td>Sphingomyelin, μg/mg liver</td>
<td>1.5±0.3</td>
<td>2.0±0.2*</td>
<td>1.6±0.3</td>
<td>1.2±0.2*</td>
</tr>
<tr>
<td>Ceramide, ng/mg liver</td>
<td>80±7</td>
<td>59±5*</td>
<td>82±4</td>
<td>95±6*</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=5). FFA indicates free fatty acids; KO, knockout; LTg, liver-specific transgenic; SMS2, Sphingomyelin synthase 2; and WT, wild-type.

Results
Lipid Analysis in SMS2 Liver-Specific Transgenic and SMS2 KO Mice on Chow Diet
We used LC/MS/MS to measure liver sphingolipids and used enzymatic assay to measure liver total cholesterol, total phospholipid, triglyceride, and free fatty acid. As indicated in Table 1, livers from SMS2 liver-specific transgenic (LTg) mice contained significantly more sphingomyelin and less ceramide than controls (38% and 20%; P<0.01 and P<0.05, respectively), and livers from SMS2 KO mice contained significantly less sphingomyelin and more ceramide levels than controls (33% and 14%; P<0.01 and P<0.05, respectively). We also observed that liver-free fatty acid levels were significantly increased in SMS2LTg (20%; P<0.05) and significantly decreased in SMS2 KO mice (18%; P<0.05). Triglyceride levels were significantly increased in SMS2LTg mice (50%; P<0.01) compared with controls. There was a decrease of triglyceride in SMS2 KO liver, but it was not statistically significant. There were no significant changes in total phospholipids and total cholesterol. All other liver sphingolipids have no noticeable difference (Table I in the online-only Data Supplement).

We next stained the liver section from SMS2LTg and control mice with Oil Red O and found that SMS2 overexpression in the liver promoted lipid accumulation (Figure IA and IB; Figure IA–ID in the online-only Data Supplement). We performed insulin tolerance test on SMS2LTg and control mice and found that SMS2LTg mice, with lower liver ceramide levels, have lower insulin sensitivity (Figure II in the online-only Data Supplement) than controls. Similarly, we previously reported that SMS2-deficient mice have higher insulin sensitivity.21

Liver SMS2 Overexpression Promotes, Whereas Liver SMS2 Deficiency Diminishes Diet-Induced Steatosis in Mice
We found that SMS2LTg mice had significantly larger livers (Figure 1C and 1D), whereas SMS2 KO mice had smaller livers (Figure 1E and 1F), compared with their respective controls, after 8-week high-fat, high-cholesterol diet feeding. We also noticed that SMS2LTg liver had a milk-like color (Figure 1C).

We further stained the liver frozen sections with Oil Red O, finding that SMS2 overexpression caused substantial lipid accumulation in the liver (Figure 1G and 1H; Figure IE–IH in the online-only Data Supplement), whereas SMS2 deficiency had the opposite results (Figure II and 1J; Figure III in the online-only Data Supplement).

As indicated in Table 1, after a high-fat feeding, the liver from SMS2LTg mice contained significantly higher sphingomyelin levels (33%; P<0.01), whereas the liver from SMS2 KO mice contained significantly less sphingomyelin levels (25%; P<0.01) compared with controls. Liver triglyceride levels were significantly increased in SMS2LTg mice (52%; P<0.001) and significantly decreased in SMS2 KO mice (48%; P<0.001). Liver-free fatty acid levels were significantly increased in SMS2LTg mice (24%; P<0.05) and significantly decreased in SMS2 KO mice (23%; P<0.05). There were no significant changes in total phospholipids and total cholesterol (Table 1).
We have already characterized high-density lipoprotein and apolipoprotein A-I levels in SMS2LTg and SMS2 KO mice. We did not observe changes in high-density lipoprotein cholesterol and apolipoprotein A-I. We also measured ABCA1 levels on mouse liver homogenates using Western blot, and we did not find significant changes (Figure IV in the online-only Data Supplement).

Ceramide But Not Sphingomyelin or Phosphatidylcholine Can Suppress PPARγ Expression

To gain insight into the mechanisms of how SMS2 overexpression promotes and deficiency prevents the formation of fatty livers, we examined the expression levels of the responsible genes using real-time quantitative polymerase chain reaction in the liver. We found that PPARγ2 was significantly elevated in high-fat–fed SMS2 transgenic mice (2.1-fold; \( P < 0.001 \)) but suppressed in SMS2 KO mice (69%, \( P < 0.001 \); Table 3). Consequently, CD36 and FSP27, 2 downstream effectors of PPARγ2, were dramatically increased in SMS2 transgenic mice (3.7-fold, \( P < 0.0001 \) and 5.8-fold, \( P < 0.0001 \)) and decreased in SMS2 KO mice (48%, \( P < 0.001 \) and 61%, \( P < 0.001 \); Table 3). We also found that diacylglycerol acyltransferase 1 and diacylglycerol acyltransferase 2 were significantly increased in SMS2 transgenic liver (Table 3). All other genes related to lipid metabolism had no statistically significant changes under SMS2 overexpression and deficiency (Table 3). Our observation suggested that PPARγ2 is at least one of the key factors mediating SMS2 activity–related liver steatosis.

We next sought to examine protein levels of CD36 in SMS2LTg and SMS2 KO livers. We found that SMS2LTg liver had significantly higher CD36 protein levels (7-fold, \( P < 0.001 \); Figure 2A), whereas SMS2 KO liver had significantly lower CD36 protein levels (70%, \( P < 0.01 \); Figure 2B) compared with their controls.

CD36 is located on plasma membrane lipid rafts.22 We then investigated how SMS2 overexpression or deficiency affected CD36 in lipid rafts. We isolated these rafts from the whole liver using reported protocols.23,24 CD36 was found in light CD36 (5-fold, \( P < 0.001 \); Figure 2P) and decreased in SMS2 KO mice (48%–61%, \( P < 0.001 \); Figure 2B). We found that SMS2 KO liver raft regions contained significantly more CD36 (72%, \( P < 0.001 \); Figure 2E).

To further evaluate the effect of SMS2 liver overexpression or deficiency on CD36, we examined the free fatty acid uptake. Hepatocytes from SMS2LTg or SMS2 KO mice were isolated and incubated with [14C]oleic acid. We found that SMS2LTg hepatocytes took up more free fatty acid (Figure 2F), whereas SMS2 KO hepatocytes took up less (Figure 2G) compared with their controls.

We noticed that SMS2 overexpression decreases ceramide and increases sphingomyelin in the liver homogenates, whereas SMS2 deficiency has opposite effects (Table 1). To directly examine the effect of lipid changes on PPARγ2, we examined...
whether exogenous ceramide supplementation to Huh7 cells, a hepatoma cell line, could stimulate PPARγ2 suppression. For this, we treated the cells with ceramide (10 µmol/L). As shown in Figure 3A–3C, the exogenous supplementation of ceramide in culture significantly reduced PPARγ2 and its downstream genes, CD36 and FSP27, expression levels; and PPARγ2 protein levels (Figure 3D and 3E). We found that ceramide 22:0 and 24:0 but not 16:0 are suppressors of PPARγ2 protein 1; and WT, wild-type.

**Table 2.** Mouse Liver Plasma Membrane Lipid Analysis by LC/MS/MS

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>SMS2LTg</th>
<th>WT</th>
<th>SMS2 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC, nmole/mg</td>
<td>68±8</td>
<td>65±15</td>
<td>62±10</td>
<td>60±13</td>
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<tr>
<td>SM, nmole/mg</td>
<td>26±7</td>
<td>37±6*</td>
<td>22±5</td>
<td>12±3*</td>
</tr>
<tr>
<td>Ceramide, ng/mg</td>
<td>755±90</td>
<td>702±81</td>
<td>820±108</td>
<td>783±120</td>
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<tr>
<td>DH Cer, ng/mg</td>
<td>116±29</td>
<td>129±39</td>
<td>121±31</td>
<td>133±23</td>
</tr>
<tr>
<td>Gly Cer, ng/mg</td>
<td>291±42</td>
<td>246±35</td>
<td>231±52</td>
<td>266±44</td>
</tr>
<tr>
<td>GM3, ng/mg</td>
<td>99±16</td>
<td>110±23</td>
<td>120±36</td>
<td>102±13</td>
</tr>
<tr>
<td>High-fat diet</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PC, nmole/mg</td>
<td>88±11</td>
<td>77±21</td>
<td>82±9</td>
<td>79±14</td>
</tr>
<tr>
<td>SM, nmole/mg</td>
<td>33±9</td>
<td>51±8*</td>
<td>35±6</td>
<td>22±5*</td>
</tr>
<tr>
<td>Ceramide, ng/mg</td>
<td>937±100</td>
<td>902±91</td>
<td>990±79</td>
<td>883±81</td>
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<tr>
<td>DH Cer, ng/mg</td>
<td>144±19</td>
<td>121±59</td>
<td>129±26</td>
<td>124±33</td>
</tr>
<tr>
<td>Gly Cer, ng/mg</td>
<td>308±51</td>
<td>292±41</td>
<td>330±61</td>
<td>289±50</td>
</tr>
<tr>
<td>GM3, ng/mg</td>
<td>120±30</td>
<td>99±29</td>
<td>116±22</td>
<td>105±21</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=4–5). DH Cer indicates dihydroceramide; PC, phosphatidylcholine; SM, sphingosine; and WT, wild-type.

**Table 3.** Hepatic Gene Expression in Mice After High-Fat Feeding

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>SMS2LTg</th>
<th>WT</th>
<th>SMS2 KO</th>
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<tr>
<td>TG and DAG hydrolysis</td>
<td></td>
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<tr>
<td>HSL</td>
<td>100±41</td>
<td>95±36</td>
<td>100±37</td>
<td>199±59</td>
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<tr>
<td>ATGL</td>
<td>100±31</td>
<td>136±27</td>
<td>100±43</td>
<td>109±37</td>
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<tr>
<td>FFA oxidation</td>
<td></td>
<td></td>
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<tr>
<td>AOX</td>
<td>100±28</td>
<td>145±31</td>
<td>100±44</td>
<td>113±53</td>
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<tr>
<td>CPT1</td>
<td>100±37</td>
<td>141±66</td>
<td>100±18</td>
<td>94±19</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>100±37</td>
<td>149±42</td>
<td>100±28</td>
<td>188±54</td>
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<tr>
<td>FFA uptake and transport</td>
<td></td>
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<tr>
<td>PPARγ1</td>
<td>100±11</td>
<td>114±20</td>
<td>100±23</td>
<td>81±22</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>100±40</td>
<td>209±31*</td>
<td>100±33</td>
<td>31±9*</td>
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<tr>
<td>CD36</td>
<td>100±21</td>
<td>370±56*</td>
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<tr>
<td>FSP27</td>
<td>100±27</td>
<td>582±102*</td>
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<td>39±14*</td>
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<tr>
<td>Lipid synthesis and storage</td>
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<tr>
<td>FAS</td>
<td>100±16</td>
<td>126±36</td>
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<td>SREBP1c</td>
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<td>74±10</td>
<td>100±43</td>
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<tr>
<td>DGAT1</td>
<td>100±31</td>
<td>173±36*</td>
<td>100±54</td>
<td>143±52</td>
</tr>
<tr>
<td>DGAT2</td>
<td>100±45</td>
<td>183±41*</td>
<td>100±58</td>
<td>159±37</td>
</tr>
<tr>
<td>Mitochondrial bioenergetic/lipid metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCP2</td>
<td>100±21</td>
<td>78±48</td>
<td>100±51</td>
<td>75±9</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=4). AOX indicates acetyl-CoA oxidase; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; FAS, fatty acid synthase; FAS27, fat-specific protein of 27 kDa; FFA, free fatty acids; HSL, hormone-sensitive lipase; KO, knockout; LTg, liver-specific transgenic; PPAR, peroxisome proliferator-activated receptor; SREBP, sphingolipid synthase 2; SREBP, sterol-responsive element-binding protein; UCP1, uncoupling protein 1; and WT, wild-type.

**Discussion**

SMS2 is the major SMS isoform in the liver. It contributes to >70% of the total SMS activity in liver. In this study, we demonstrated that (1) SMS2 overexpression decreases hepatic ceramide and increases sphingomyelin levels, whereas SMS2 deficiency has an opposite effect; (2) SMS2 overexpression increases, whereas SMS2 deficiency decreases liver plasma membrane sphingomyelin levels; (3) liver SMS2 overexpression promotes, whereas SMS2 deficiency prevents high-fat diet–induced triglyceride and free fatty acid accumulation in the liver; (4) SMS2 overexpression induces the levels of hepatic PPARγ2 and its downstream genes, CD36 and FSP27, whereas SMS2 deficiency reduces all these levels; (5) exogenous ceramide but not sphingomyelin or phosphatidylcholine suppresses PPARγ2, CD36, and FSP27 expression levels; and (6) PPARγ antagonist reduces triglyceride accumulation in mouse liver.

There is a relationship between sphingolipid de novo biosynthesis and hepatic steatosis. Pharmacological inhibition of serine palmitoyltransferase or glucosylceramide synthase or the genetic depletion of acid sphingomyelinase reduces hepatic triglyceride levels in mice susceptible to the development of a fatty liver. However, the mechanism is still unknown, and sometimes the results are incongruous. Previously, Mitsutake et al have reported that SMS2 deficiency in mice diminishes the development of obesity and fatty liver, and they attributed this phenomenon to the reduction of sphingomyelin...
levels in cell membrane lipid rafts. We also reported that SMS2 deficiency–mediated reduction of plasma membrane sphingomyelin increases insulin sensitivity and decreases high-fat–induced obesity. It is known that sphingomyelin levels in plasma membrane lipid rafts are important in mediating many important cell functions, such as insulin signaling, cholesterol efflux, inflammatory responses, and lipid uptake and transportation. Plasma membrane sphingomyelin-mediated effect is still observed in this study. In this study, we showed that SMS2 LTg mouse plasma membrane contained significantly more sphingomyelin (Table 2) and significantly more amount of CD36 (Figure 2D) and CD63 (Table 2) and CD36 (Figure 2E).

However, there is 1 thing which could not be fully explained by lipid raft sphingomyelin changes: why do SMS2 LTg mice have significantly higher and SMS2-deficient mice have significantly lower CD36 and FSP27, a well-known protein involved in hepatocyte lipid droplet formation and liver steatosis? We also reported that SMS2 deficiency–mediated reduction of plasma membrane sphingomyelin increases insulin sensitivity and decreases high-fat–induced obesity. It is known that sphingomyelin levels in plasma membrane lipid rafts are important in mediating many important cell functions, such as insulin signaling, cholesterol efflux, inflammatory responses, and lipid uptake and transportation. Plasma membrane sphingomyelin-mediated effect is still observed in this study. In this study, we showed that SMS2 LTg mouse plasma membrane contained significantly more sphingomyelin (Table 2) and significantly more amount of CD36 (Figure 2D) and CD63 (Table 2) and CD36 (Figure 2E).

However, there is 1 thing which could not be fully explained by lipid raft sphingomyelin changes: why do SMS2 LTg mice have significantly higher and SMS2-deficient mice have significantly lower CD36 and FSP27, a well-known protein involved in hepatocyte lipid droplet formation and liver steatosis? Previous researchers have reported that high ceramide levels contribute to the development of nonalcoholic fatty liver disease via multiple ways involved in insulin resistance, oxidative stress, inflammation, and apoptosis. Yetukuri et al even demonstrated that in ob/ob mice hepatic ceramide levels were strongly correlated with the degree of steatosis. However, what we found in this study was different from the study reported by Yetukuri et al. First of all, we showed that although SMS2 overexpression or deficiency does not change ceramide levels on liver plasma membrane (Table 2), SMS2 LTg mice had significantly lower, whereas SMS2 KO mice had higher ceramide levels in liver homogenates (Table 1). The former had severe liver steatosis with (Figure 1G and 1H; Figure IE–IH in the online-only Data Supplement) or without high-fat diet feeding (Figure 1A and 1B; Figure IA–ID in the online-only Data Supplement), whereas the latter had much less liver steatosis (Figure 1I and 1J; Figure I(I)–IL in the online-only Data Supplement). It is plausible that ceramide depletion in the liver causes steatosis, whereas ceramide accumulation has an opposite effect. Second, exogenous ceramide suppressed PPARγ2 in a dose-dependent fashion (Figure 3D and 3E). We further confirmed this by using PPARγ reporter analysis (Figure 3H). Third, it has been reported that, in primary

Does ceramide play an important role in mediating liver steatosis? Previous researchers have reported that high ceramide levels contribute to the development of nonalcoholic fatty liver disease via multiple ways involved in insulin resistance, oxidative stress, inflammation, and apoptosis. Yetukuri et al even demonstrated that in ob/ob mice hepatic ceramide levels were strongly correlated with the degree of steatosis. However, what we found in this study was different from the study reported by Yetukuri et al. First of all, we showed that although SMS2 overexpression or deficiency does not change ceramide levels on liver plasma membrane (Table 2), SMS2 LTg mice had significantly lower, whereas SMS2 KO mice had higher ceramide levels in liver homogenates (Table 1). The former had severe liver steatosis with (Figure 1G and 1H; Figure IE–IH in the online-only Data Supplement) or without high-fat diet feeding (Figure 1A and 1B; Figure IA–ID in the online-only Data Supplement), whereas the latter had much less liver steatosis (Figure 1I and 1J; Figure I(I)–IL in the online-only Data Supplement). It is plausible that ceramide depletion in the liver causes steatosis, whereas ceramide accumulation has an opposite effect. Second, exogenous ceramide suppressed PPARγ2 in a dose-dependent fashion (Figure 3D and 3E). We further confirmed this by using PPARγ reporter analysis (Figure 3H). Third, it has been reported that, in primary
cultured adipocytes, ceramide treatment reduced PPARγ expression in a time- and concentration-dependent manner. It has also been reported that, in murine mesenchymal stem cells, ceramide treatment reduced PPARγ expression and reduced levels of both triglyceride and specific fatty acids. Recently, we reported that ceramide does not cause cardiac toxicity and insulin resistance. As one of the potential mechanisms, we believe that ceramide can reduce liver steatosis through the direct suppression of PPARγ expression in the liver. Although the role of PPARγ in liver steatosis is still uncertain, our PPARγ antagonist study (Figure V in the online-only Data Supplement) suggests PPARγ is one of the factors for liver steatosis.

In line with the association between low ceramide levels and nonalcoholic fatty liver disease observed in our SMS2LTg mice, Deevska et al reported that glucosylceramide synthase (which uses ceramide to produce glucosylceramide) inhibition markedly reduced liver steatosis in mice. The linkage may be that ceramide can suppress PPARγ expression in the liver (Figure 3).

Although PPARγ was once considered an orphan receptor, native and modified polyunsaturated fatty acids have emerged as strong candidates for endogenous activators of this receptor. However, so far no endogenous suppressors of PPARγ have been reported. We hypothesized that certain species of ceramide could be native lipid suppressors for PPARγ. Our results indicated that ceramide 22:0 and 24:0 but not 16:0 can suppress PPARγ in an Huh7 cell culture system (Figure 3F, 3G, and 3I). The detailed mechanism that causes this deserves further investigation.

Another possible mechanism linking SMS2 activity and nonalcoholic fatty liver disease might be related to hepatic steatosis and improved insulin sensitivity. Bijl et al reported that glucosylceramide synthase (which uses ceramide to produce glucosylceramide) inhibition markedly reduced liver steatosis in mice. The linkage may be that ceramide can suppress PPARγ expression in the liver (Figure 3).

Figure 3. The exogenous supplementation of ceramide in culture significantly reduces proliferator-activated receptor γ2 (PPARγ2) protein levels. Huh7 cells were incubated with exogenous 0, 10, and 30 µmol/L of sphingomyelin (SM), ceramide, or phosphatidylcholine (PC) for 24 hours. Cellular PPARγ2, CD36, and FSP27 mRNA levels were measured by real-time polymerase chain reaction. A, PPARγ2 mRNA levels. B, CD36 mRNA levels. C, FSP27 mRNA levels. Cellular PPARγ2 mass was measured by Western blotting. D and E, PPARγ Western blot after treatment with exogenous 0, 10, and 30 µmol/L of sphingomyelin, ceramide, or phosphatidylcholine for 24 hours. F and G, PPARγ Western blot after treatment with exogenous 0, 10, and 30 µmol/L of ceramides C16:0, C22:0, and C24:0 for 24 hours. H and I, PPAR reporter assay. Huh7 cells were transfected with PPAR reporter, negative control, or positive control. After 24 hours of transfection, the cells were treated with exogenous sphingomyelin, ceramide, or phosphatidylcholine for 24 hours, and then dual-luciferase reporter assay was performed. H, Dual-luciferase reporter assay after treatment with exogenous 0, 20, and 40 µmol/L of sphingomyelin, ceramide, or phosphatidylcholine for 24 hours. I, Dual-luciferase reporter assay after treatment with exogenous 0, 20, and 40 µmol/L of ceramides C16:0 and C24:0 for 24 hours. Values are mean±SD (n=5; *P<0.05, **P<0.01).
cellular sphingomyelin levels. We have previously reported that SMS2 deficiency–mediated reduction of sphingomyelin in the plasma membranes leads to an improvement in tissue and whole-body insulin sensitivity, and this might be associated with less liver steatosis in these mice. However, exogenous sphingomyelin had no effect on PPARγ expression (Figure 3).

The importance of this study is the finding that increase in ceramide levels can prevent liver steatosis through suppression of PPARγ. Increasing ceramide levels in the liver can be achieved by SMS2 inhibition.

Acknowledgments

We thank Drs David Peake, Hai H. Bui, and Youyan Zhang (Eli Lilly) for their valued support and technical guidance.

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Disclosures

None.

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**Significance**

Imbalances in input, oxidation, synthesis, and output of fatty acids could contribute to nonalcoholic fatty liver disease. There is a relationship between sphingolipid de novo biosynthesis and nonalcoholic fatty liver disease. Sphingomyelin synthase overexpression reduces liver ceramide level, thus inducing liver steatosis, whereas sphingomyelin synthase 2 deficiency has opposite effect. The current study is the first one to indicate that ceramide can suppress proliferator-activated receptor γ2 and its target genes, CD36 and FSP27, thus diminishing liver steatosis. Regulation of ceramide levels through manipulation of sphingomyelin synthase activity could have a clinical impact on the treatment of nonalcoholic fatty liver disease.
Sphingomyelin Synthase 2 Activity and Liver Steatosis: An Effect of Ceramide-Mediated Proliferator-Activated Receptor γ2 Suppression

Yue Li, Jibin Dong, Tingbo Ding, Ming-Shang Kuo, Guoqing Cao, Xian-Cheng Jiang and Zhiqiang Li
Materials and Methods

Mice and diet. SMSLTg and SMS KO mice were created in our laboratory. The SMS2LTg founder animals, with C57BL/6J/CBA mixed background, were backcrossed with C57BL/6J mice five generations. The original SMS2 KO mice, with 129 mouse genetic background, were backcrossed with C57BL/6 mice six generations. All mice used were littermates, or with the same genetic background. The groups of mice (male or female at age 10 weeks) were fed either rodent chow, or a high-fat and high-cholesterol (Research Diets, Inc.) diet for 8 weeks. All procedures and protocols involving the use of animals were approved by the SUNY Downstate Medical Center Animal Care and Use Committee.

Liver lipid measurements. Total liver lipids were extracted according to a modified method from Folch et al. Briefly, snap-frozen liver tissues (~100 mg) were homogenized in 5 ml of 1 N NaOH and extracted twice with 5–10 ml of a chloroform/methanol (2:1, v/v) solution. The organic layer was dried under nitrogen gas and resolubilized in 1 ml of chloroform containing 2% Triton X-100. This extract was dried again and resuspended in 1 ml of water to achieve a final concentration of 2% Triton X-100, and then measured triglyceride concentration using kit from Thermo, measured free fatty acids, total cholesterol, and total phospholipid concentration using kits from Wako.

Histological analysis. Liver tissue samples were either embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek) and sectioned (7 μm) for neutral lipid staining using Oil Red O and a hematoxylin counterstain or fixed in 10% formalin,
embedded in paraffin, and sectioned (5 μm) for hematoxylin and eosin staining. Sections were photographed at either ×100 (hematoxylin and eosin) or ×200 (Oil Red O) magnification.

**Lipid analyses by LC MS/MS.** Ceramides comprised of a D-erythro-sphingosine backbone and a fatty acid amide were determined by a 2D LC-ESI MS/MS method. Lipid extracts from cells were injected onto a normal-phase column, where the polar lipids were retained, while the ceramide fractions were trapped on a reversed-phase column. Ceramides were eluted, separated, and detected using a triple quadruple mass spectrometer equipped with positive ion electrospray ionization (ESI) and selected reaction monitoring. Levels of PC and SM were measured via a flow injection ESI-MS/MS method. Protonated molecular ions of PC/SM species are selected by precursor ion scans of m/z 184 and the ion intensities across the flow injection profile were summed together, and after isotope correction, the quantities of each PC and SM species are then calculated relative to PC and SM internal standards.

**mRNA analyses.** RNA was isolated from cells using TriZol (Invitrogen). The mouse primers used for SMS2 RT-PCR were: Forward 5’-GGTTCCCCACAGAAACCAAGA-3’, and reverse 5’-GATGCCTGTGTTCACCAC-3’. For liver SMS2 transgene, human SMS2 mRNA was determined by real-time PCR using Taqman® Gene Expression Assay (Applied Biosystems, assay ID Hs00380453_m1). The human primers used for SMS2 were: Forward 5’-TCCAGTCTGCTCCAAAGCTC-3’ and reverse 5’-CACTCACGCAGCCAGCAGAT-3’. 18S rRNA was used as internal control. The forward
and reverse primer sequences for 18S rRNA are: 5’-AGTCCCTGCCCTTTGTACACA-3’ and 5’-GATCCGAGGGCCTCACTAAAC-3’, respectively.

**SMS activity assay.** Cells were homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5% sucrose, and a cocktail of protease inhibitors (Sigma). The homogenate was centrifuged at 5000 rpm for 10 minutes and the supernatant was mixed in assay buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, C₆-NBD-ceramide (3.3 μg/ml), and phosphotidylcholine (100 μg/ml). The mixture was incubated at 37°C for 2 hours. Lipids were extracted in chloroform: methanol (2:1, v/v), dried under N₂ gas, and separated by thin layer chromatography (TLC) Chloroform:MeOH:20% NH₄OH (14:6:1, v/v). Band intensity was quantified by Image–Pro Plus 4.5 (Media Cybernetics Inc.).

**Plasma membrane isolation.** Mouse liver (0.5 g) was homogenized and plasma membrane was isolated using a kit (Biovision). The purity of the preparation was checked by Western blot, using the cytoplasmic, total membrane, and plasma membrane fractions, with respective antibodies as we did previously.

**Lipid raft isolation.** Liver lipid rafts were isolated, using reported protocols. Briefly, 0.1 g mouse liver was homogenized in 1.5 ml of 0.5 M Na₂CO₃ (pH 11), containing protease inhibitors. The homogenates were centrifuged (1,300 g, 5 minutes) to pellet cellular debris and nuclei. One milliliter of the postnuclear supernatant was then adjusted to 45% sucrose by addition of 1 ml of 90% sucrose, 50 mM Hepes (pH 6.5), and 150 mM NaCl. A discontinuous sucrose gradient was formed by overlaying this solution with 6 ml of
35% sucrose and 3.5 ml of 5% sucrose, both in the same buffer, containing 0.25 M Na₂CO₃, and this was centrifuged at 38,000 rpm for 18 hours in a SW41 rotor (Beckman). Fractions from top to bottom (1 ml each) were collected. Lyn kinase and caveolin-1 were used to locate the raft fractions.

**Free fatty acid uptake.** Mouse hepatocytes were isolated from the liver according our reported procedure. Liver fatty acid uptake was assayed according to reported method with modification. Briefly, viable hepatocytes were identified by trypan blue exclusion and counted, and incubated for 3 h in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin mix and 1% L-glutamine. The cells were washed once with PBS, and then incubated in 1 ml of assay buffer (40 µM sodium oleate, 10 µM fatty acid-free bovine serum albumin, 5 µCi/ml [14C]oleate in phosphate-buffered saline (PBS) with 1 mM MgCl₂ and 1.2 mM CaCl₂) for 2 and 5 min at 37 °C. Assays were stopped by the addition of 1 ml of ice-cold stop solution (PBS containing 1 mM MgCl₂, 1.2 mM CaCl₂, 0.1% fatty acid-free bovine serum albumin, and 500 µM phloretin). The cells were washed three times with the above stop solution, and then lipids were extracted using Hexanes/Isopropanol (3/2, v/v). Radioactivity was analyzed by scintillation counting.

**Exogenous ceramide, sphingomyelin, and phosphatidylcholine supplementation.** Sphingomyelin (Sigma) and phosphatidylcholine (Sigma) were dissolved in absolute ethanol. Ceramde was dissolved in ethanol/dodecan (99.8/0.2, v/v) to make a 2-mM stock. Huh7 cells were incubated with exogenous 0, 10, and 30 µM sphingomyelin, ceramide or phosphatidylcholine for 24 h.
Statistical analysis. Each experiment was conducted at least 3 times. Unless otherwise indicated, data are expressed as mean ± SD. Differences between 2 groups were analyzed by unpaired 2-tailed Student’s $t$ test and among multiple groups by ANOVA followed by Student-Newman-Keuls test. A $P$ value of less than 0.05 was considered significant.
Supplement

Supplemental figure legends:

**Supplemental Fig. I. The effect of SMS2 overexpression and deficiency on liver lipid accumulation.** Liver samples were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek), frozen, and sectioned (7 μm), then were stained Oil Red O and hematoxylin. Sections were photographed at x100 and x250 magnification. Panel A-D, livers from control and SMS2 Tg mice on chow. Panel E-H, livers from control and SMS2 Tg mice on a high fat high cholesterol diet. Panel I-L, mouse livers from control and SMS2 KO mice on a high fat high cholesterol diet. This set of result is the representatives of 6 WT and 6 SMS2LTg mice, and 6 WT and 6 SMS2 KO mice.

**Supplemental Fig. II. Insulin tolerance test.** Mice were fasted for 15 h, insulin (0.75 units per kg of body weight) was injected via i.p. and then blood glucose levels were measured immediately before and 15, 30, and 60 min after insulin injection. Values are Mean ± S.E., n = 9, *P<0.05.

**Supplemental Fig. III. Liver plasma membrane purity determination.** Plasma membrane isolation from mouse liver. The purity of cytoplasm (Cyto), total subcellular organelle membranes (TM), and plasma membranes (PM) were determined by Western blot. Na⁺/K⁺ATPase is plasma membrane marker and Cytochrome C is mitochondrial marker.
Supplemental Fig. IV. Liver ABCA1 Western blotting. SDS-PAGE was performed on 4–15% SDS-polyacrylamide gradient gel using mouse liver homogenate, and the separated proteins were transferred to nitrocellulose membrane. Western blot analysis for ABCA1 was performed. Values are Mean ± S.E., N=3.

Supplemental Fig. V. PPAR-γ antagonist GW9662 ameliorate liver steatosis. PPARγ antagonist GW9662 was injected into SMS2 Tg mice (i.p., 2 mg/kg body weight per day) for four weeks, and then the mice were sacrificed and the liver triglyceride was measured. N=5, *P <0.05.

Supplemental Table I. Liver lipid measurement in SMS2 KO, SMS2LTg, and WT mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Sph</th>
<th>S-1-P</th>
<th>DHS-1-P</th>
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<tbody>
<tr>
<td></td>
<td>(ng/mg liver)</td>
<td></td>
<td></td>
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<tr>
<td>WT</td>
<td>1.9±0.2</td>
<td>0.15±0.03</td>
<td>0.18±0.01</td>
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<tr>
<td>SMS2 KO</td>
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<tr>
<td>WT</td>
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<td>0.19±0.02</td>
</tr>
<tr>
<td>SMS2LTg</td>
<td>1.8±0.1</td>
<td>0.15±0.02</td>
<td>0.17±0.03</td>
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</tbody>
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Supplemental Fig. III. Li et al.
Supplemental Fig. VI. Li et al.

WT | SMS2 Tg
---|---

**ABCA1**

**β-actin**

![Western Blot](image)

![Bar Graph](image)
Liver TG

Supplemental Fig. V. Li et al.

![Liver triglyceride levels comparison](image)