Obese and diabetic \textit{db/db} mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin

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Obese and diabetic \textit{db/db} mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. \textit{Am J Physiol Gastrointest Liver Physiol} 287: G1035–G1043, 2004. First published July 15, 2004; doi:10.1152/ajpgi.00199.2004.—Obesity and type 2 diabetes are associated with nonalcoholic steatohepatitis (NASH), but an obese/diabetic animal model that mimics human NASH remains undefined. We examined the induction of steatohepatitis and liver fibrosis in obese and type 2 diabetic \textit{db/db} mice in a nutritional model of NASH and determined the relationship of the expressions of osteopontin (OPN) and leptin receptors to the pathogenesis of NASH. \textit{db/db} mice and the corresponding lean and nondiabetic \textit{db} mice were fed a diet deficient in methionine and choline (MCD diet) or control diet for 4 wk. Leptin-deficient obese and diabetic \textit{ob/ob} mice fed similar diets were used for comparison. MCD diet-fed \textit{db/db} mice exhibited significantly greater histological inflammation and higher serum alanine aminotransferase (ALT) levels than \textit{db/db} and \textit{ob/ob} mice. Trichrome staining showed marked pericellular fibrosis in MCD diet-fed \textit{db/db} mice but no significant fibrosis in \textit{db/db} or \textit{ob/ob} mice. Collagen I mRNA expression was increased 10-fold in \textit{db/db} mice, 4-fold in \textit{db/ob} mice, and was unchanged in \textit{ob/ob} mice. RNA expressions of OPN, TNF-\alpha, TGF-\beta, and short-form leptin receptors (Ob-Ra) were significantly increased in \textit{db/db} mice compared with \textit{db/ob} or \textit{ob/ob} mice. Parallel increases in OPN and ObRa protein levels were observed in \textit{db/db} mice. Cultured hepatocytes expressed only Ob-Ra, and leptin stimulated OPN mRNA and protein expression in these cells. In conclusion, our results demonstrate the development of an obese/diabetic experimental model for NASH in \textit{db/db} mice and suggest an important role for Ob-Ra and OPN in the pathogenesis of NASH.

obesity; diabetes; insulin resistance; osteopontin; fibrosis

NONALCOHOLIC STEATOHEPATITIS (NASH) is commonly associated with obesity, type 2 diabetes, and the metabolic syndrome (1, 41, 45, 48). However, several studies examining the pathogenesis of NASH have employed lean and nondiabetic strains of mice fed a diet deficient in methionine and choline (MCD diet). We and others have demonstrated that this model produces steatohepatitis and liver fibrosis that is histologically similar to human NASH (11, 13, 23, 25, 26, 46). In addition, mice lacking methionine adenosyltransferase have been shown to develop steatohepatitis similar to human NASH (31, 34, 47). The molecular signaling mechanisms that lead to the activation of inflammation and fibrosis in these models of NASH remain poorly defined. Several studies suggest that peroxidative injury may play a role in the development of steatohepatitis in human and in experimental NASH (11, 26, 47, 48). However, we (46) recently assessed oxidative stress during the progression of steatosis to steatohepatitis in MCD diet-fed A/J mice and found that peroxidative injury occurs late in developing steatohepatitis. We have identified an important role for the Th1 proinflammatory cytokine osteopontin (OPN) early in disease progression in this dietary model of NASH (46). We showed that OPN is synthesized by hepatocytes, and its expression is markedly increased early in the development of steatohepatitis (46). The MCD diet-induced liver injury and fibrosis was blunted in OPN knockout mice, indicating a key role for OPN in signaling the progression of liver injury and fibrosis in this nutritional model of NASH (46).

The importance of obesity and diabetes/insulin resistance in the pathogenesis of NASH has not been clearly established. This is, in part, due to the lack of an appropriate animal model of obesity and diabetes that mimics the pathology of human NASH. Obese and diabetic \textit{ob/ob} mice, which are leptin-deficient, develop steatohepatitis but not liver fibrosis when fed a MCD diet (27). These observations and others have suggested an essential role for leptin in liver fibrosis. Studies involving animal models of toxicity and stellate cell cultures suggest that leptin signaling permits, induces, and/or augments hepatic fibrosis (17, 19, 44, 49, 53). As a result of the absence of leptin signaling, the information derived from experiments involving \textit{ob/ob} mice may not be applicable to the understanding of the pathogenesis of human NASH. No animal model of NASH has been described that combines the features of hyperleptinemia, obesity, insulin resistance, diabetes, and hepatic fibrosis.

The \textit{db/db} mouse is hyperleptinemic and develops obesity and severe type 2 diabetes partly due to a functional defect in the long-form leptin receptor (Ob-Rb), which plays a significant role in the regulation of food intake and the control of body weight (5, 28, 32, 50). Recent studies show that \textit{db/db} mice express functional short-form leptin receptors (Ob-Ra) in the brain and kidneys, where they are involved in leptin-induced stimulation of interleukin-1\beta expression and/or collagen synthesis (16, 18). Potential roles for Ob-Ra in the pathogenesis of liver disease in \textit{db/db} mice have not been examined.
Primary cultures of hepatocytes have been shown to express Ob-Ra but not Ob-Rb (8, 59). Ob-Ra appears to be involved in leptin-induced activation of phosphoinositol 3-kinase in these cells (59). The roles of Ob-Ra and OPN in the progression of NASH have not been examined in a diabetic model. Of interest, OPN has been shown to play an important role in the renal and vascular complications of obesity and diabetes (12, 37, 54). We also reported that hyperglycemia-induced proliferation and collagen synthesis in cultured mesangial and vascular smooth muscle cells is mediated by increased expression of OPN (51, 52). Based on these evidence, we hypothesized that db/db mice may develop marked hepatic inflammation and fibrosis in a nutritional model of NASH and that this enhanced effect would involve leptin, Ob-Ra, and OPN.

The present study examined the development of steatohepatitis and liver fibrosis in db/db mice fed the MCD diet in an attempt to develop a murine model to study the pathogenesis of NASH under conditions of obesity and type 2 diabetes. In addition, we determined the relationship of leptin, leptin receptors, and OPN to the development of hepatic fibrosis in this model of NASH and in cultured hepatocytes.

**MATERIALS AND METHODS**

**Animals and experimental protocol.** Female db/db, ob/ob, and db/mice from the C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). Six mice each of db/db, db/m, and ob/ob at age 10–12 wk were fed for 4 wk either MCD diet or control diet (MCD diet reconstituted with methionine and choline; ICN Biochemicals, San Diego, CA). Body weights were recorded at the start and the end of the experimental period. Blood was collected by cardiac puncture, and livers were rapidly excised, rinsed in ice-cold saline, and weighed. Aliquots of liver were snap frozen in liquid nitrogen and kept at −80°C until being analyzed. A portion of each liver was fixed in 10% formalin for histology. Alanine aminotransferase levels were determined in fresh serum using a spectrophotometric procedure (Sigma Diagnostics, St. Louis, MO).

**Histology and immunohistochemistry.** Formalin-fixed liver tissue was processed, and 5-μm-thick paraffin sections were stained with hematoxylin and eosin (H&E) and Masson’s trichrome for histolog-

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**Table 1. Effect of MCD diet on body and liver weight, liver triglyceride content, and serum levels of leptin in db/m, db/db, and ob/ob mice**

<table>
<thead>
<tr>
<th>Measurements</th>
<th>db/m Control</th>
<th>db/m MCD</th>
<th>db/db Control</th>
<th>db/db MCD</th>
<th>ob/ob Control</th>
<th>ob/ob MCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>24.7±1</td>
<td>15.1±1*</td>
<td>49.6±3</td>
<td>42.4±2</td>
<td>59.4±2</td>
<td>42.8±1*</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>1.16±0.11</td>
<td>1.17±0.10</td>
<td>2.10±0.12</td>
<td>3.0±0.13</td>
<td>3.10±0.03</td>
<td>2.41±0.15</td>
</tr>
<tr>
<td>Liver/body wt, ×100</td>
<td>4.67±0.1</td>
<td>7.75±1.0*</td>
<td>4.16±0.3</td>
<td>7.16±0.2*</td>
<td>5.21±0.1</td>
<td>5.55±0.2</td>
</tr>
<tr>
<td>Liver TG content, mg/g liver</td>
<td>30±6</td>
<td>121±7*</td>
<td>46±3</td>
<td>91±7*</td>
<td>124±15</td>
<td>200±10*</td>
</tr>
<tr>
<td>Serum leptin, ng/ml</td>
<td>3.89±0.30</td>
<td>6.73±0.20*</td>
<td>18.9±1.0</td>
<td>52.7±3.0*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3–5 mice. *P < 0.01 compared with respective control value. MCD, diet deficient in methionine and choline; TG, triglyceride; ND, not detected.

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**Fig. 1. Representative photomicrographs showing the effect of methionine- and choline-deficient (MCD) diet on liver histology in db/m, db/db, and ob/ob mice.** Mice were fed the MCD or control diet for 4 wk, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Arrows point to lobular inflammation. Original magnification, ×100; n = 6.
Table 2. Effect of MCD diet on the degree of hepatic inflammation and fibrosis in db/m, db/db, and ob/ob mice

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Control</th>
<th>db/m MCD</th>
<th>db/db MCD</th>
<th>ob/ob MCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>0.0 ± 0.0</td>
<td>2.0 ± 0.2</td>
<td>3.0 ± 0.3*</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Central vein fibrosis</td>
<td>0.12 ± 0.05</td>
<td>0.10 ± 0.01</td>
<td>0.47 ± 0.12*</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Portal tract fibrosis</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.43 ± 0.10*</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

Severity of hepatic inflammation was scored on a scale of 0–3 as described in METHODS. Central vein (central/pericentral) and portal tract (portal/peripheral) fibrosis were scored using morphometric analysis as described in METHODS and is expressed as a percentage of total cross-sectional area representing fibrosis. Values are means ± SE of 3–5 mice. *P < 0.01 compared with respective values in controls, db/m MCD and ob/ob MCD.

OPN immunostaining was performed using a Vectastain Elite ABC kit, 3,3'-diaminobenzidine (Vector laboratories, Burlingame, CA), and a monoclonal anti-OPN antibody MPIIIIB10 (Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA) as described (46).

Measurement of liver triglyceride content and serum levels of glucose, insulin, and leptin. Liver samples were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 1 μM PMSF. Triglyceride content in the liver homogenates was measured using a spectrophotometric kit procedure (Thermo DMA, Arlington, TX). Serum levels of glucose were measured by glucose oxidase. Serum insulin and leptin concentrations were determined by radioimmunoassay using kit procedures (Linco Research, St. Charles, MO).

Real-time PCR analysis. Total RNA was isolated from liver samples using TRIzol reagent (GIBCO-BRL, Grand Island, NY). One microgram of total RNA was reverse-transcribed using 50 units of SuperScript II RNaseH- reverse transcriptase and 50 ng of random hexamers (Invitrogen, Carlsbad, CA) and was analyzed by real-time PCR as described (46). The primer sequences used are as follows: collagen I forward 5'-AGT TTC AGC TTT GTG GAT CAC CTC-3', reverse 5'-TCC CTC GAC TCC TAC ATC TTC-3'; OPN forward 5'-CAG CCT GCA CCC AGA TCA TCA-3', reverse 5'-GCC GAA GGA GAT TCT GCT TCT-3'; TNF-α forward 5'-CAG CCT GCT TGA TCT ACA TCA-3', reverse 5'-GCC TAC AGG AGT GTC ACT GTA-3'; TGF-β forward 5'-CTC TCC TAC TCC TCA TGG CCA-3', reverse 5'-GTC TCT CTT AAA GTC AAT GTA-3'; and GAPDH forward 5'-GTC GTG GAT CTG ACG TGC C-3', reverse 5'-TGC CTG CTG CAC CAC CTT C-3'. The mRNA expression of Ob-Ra and Ob-Rb was analyzed by RT-PCR using the following primers:

Ob-Ra/Ob-Rb forward 5'-ACA CTG TTA ATT TCA CAC CAG AG-3', Ob-Rb reverse 5'-AGT CAT TCA AAC CAT AGT TTA GG-3'; and Ob-Rb reverse 5'-TGG ATA AAC CCT TGC TCT TCA-3'.

Western blot analysis. Protein expression of OPN and leptin receptors were determined by Western blot analysis as previously described (46). Briefly, liver samples were homogenized in a lysis buffer (50 mM Tris·HCl, pH 7.4, containing 150 mM NaCl, 25 mM EDTA, 5 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, and 1 mM DTT), and samples containing 10–25 μg protein were resolved by SDS-PAGE and immunoblotting. OPN protein levels were assessed with anti-OPN antibody MPIIIIB10. Leptin receptor protein expression was determined with mouse monoclonal antibody sc-8391 that recognizes all forms of leptin receptors (Santa Cruz Biotechnology, Santa Cruz, CA).

Hepatocell cell culture. A mouse hepatocyte cell line AML-12 was obtained from The American Type Culture Collection (Manassas, VA). These cells exhibit differentiated, nontransformed hepatocyte phenotype and have been previously used in numerous studies of hepatocyte injury (24, 46). Cultures were grown in 1:1 mixture of Dulbecco’s Modified Eagle’s Medium/Ham’s Nutrient Mixture F-12 (DME/Ham’s F-12 medium) supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 nmol dexemethasone, and antibiotics as previously described (46). Cultures were maintained in 75-cm² flasks in their growth medium in a 5% CO₂, 95% air incubator environment at 37°C. To assess the effect of leptin on OPN expression, cells were subcultured in 100-mm dishes in growth medium until the cultures reached 70–80% confluence. Cells were then made quiescent by incubation for 24 h in serum- and hormone-free DME/Ham’s F-12 medium. Quiescent cultures were exposed for 18 h in the serum-free medium in the absence or presence of recombinant mouse leptin (10–1,000 ng/ml) at 37°C. Total RNA was isolated and analyzed for the mRNA expressions of OPN by real-time PCR and for leptin receptors by RT-PCR. In separate experiments, cultured hepatocytes were treated with leptin (100 ng/ml) for 1–18 h, and at the end of each time point conditioned medium was collected and analyzed for OPN protein expression by Western blotting as previously described (46).

Statistical analysis. Comparison between groups was performed using Student’s t-test for unpaired samples or by ANOVA. A P value of <0.05 was considered statistically significant.
increased significantly in db/db mice. db/db mice on control diet had severe hyperglycemia (serum glucose = 449 ± 69 mg/dl) and hyperinsulinemia (serum insulin = 8.81 ± 1.1 ng/ml in db/db vs. 3.89 ± 0.30 ng/ml in db/m mice). Table 1 shows the results of body and liver weights, liver triglyceride (TG) content, and serum levels of leptin. The MCD diet-fed db/m and ob/ob mice had significant decreases in body weight compared with mice fed control diet, whereas the body weights of db/db mice were unaffected. The liver weight relative to body weight increased significantly in db/m and db/db mice fed the MCD diet but was unchanged in ob/ob mice. db/db mice on control diet had 50% higher levels of liver TG than db/m mice, whereas ob/ob mice had much higher liver TG levels than db/db mice. MCD diet caused significant increases in liver TG levels in all mice. Serum leptin levels were significantly elevated in db/db (18.9 ± 1 ng/ml) vs. db/m (3.89 ± 0.30 ng/ml) mice on control diets. Serum leptin was undetectable in ob/ob mice. MCD diet resulted in significant increases in serum leptin levels in both db/m and db/db mice.

Effect of MCD diet on body and liver weight, liver triglyceride content, and serum levels of leptin. The db/db mice on control diet had severe hyperglycemia (serum glucose = 449 ± 69 mg/dl) and hyperinsulinemia (serum insulin = 8.81 ± 1.1 ng/ml in db/db vs. 3.89 ± 0.30 ng/ml in db/m mice). Table 1 shows the results of body and liver weights, liver triglyceride (TG) content, and serum leptin levels in db/m, db/db, and ob/ob mice. The MCD diet-fed db/m and ob/ob mice had significant decreases in body weight compared with mice fed control diet, whereas the body weights of db/db mice were unaffected. The liver weight relative to body weight increased significantly in db/m and db/db mice fed the MCD diet but was unchanged in ob/ob mice. db/db mice on control diet had 50% higher levels of liver TG than db/m mice, whereas ob/ob mice had much higher liver TG levels than db/db mice. MCD diet caused significant increases in liver TG levels in all mice. Serum leptin levels were significantly elevated in db/db (18.9 ± 1 ng/ml) vs. db/m (3.89 ± 0.30 ng/ml) mice on control diets. Serum leptin was undetectable in ob/ob mice. MCD diet resulted in significant increases in serum leptin levels in both db/m and db/db mice.

Effect of MCD diet on collagen deposition. Trichrome staining and elastin staining of control and MCD diet-fed mice were stained with Masson’s trichrome stain. Long arrow points to perivenular and small arrows to pericentral fibrosis in db/db mice. Original magnification, ×400; n = 6.

RESULTS

Effect of MCD diet on body and liver weight, liver triglyceride content, and serum levels of leptin. The db/db mice on control diet had severe hyperglycemia (serum glucose = 449 ± 69 mg/dl) and hyperinsulinemia (serum insulin = 8.81 ± 1.1 ng/ml in db/db vs. 3.89 ± 0.30 ng/ml in db/m mice). Table 1 shows the results of body and liver weights, liver triglyceride (TG) content, and serum leptin levels in db/m, db/db, and ob/ob mice. The MCD diet-fed db/m and ob/ob mice had significant decreases in body weight compared with mice fed control diet, whereas the body weights of db/db mice were unaffected. The liver weight relative to body weight increased significantly in db/m and db/db mice fed the MCD diet but was unchanged in ob/ob mice. db/db mice on control diet had 50% higher levels of liver TG than db/m mice, whereas ob/ob mice had much higher liver TG levels than db/db mice. MCD diet caused significant increases in liver TG levels in all mice. Serum leptin levels were significantly elevated in db/db (18.9 ± 1 ng/ml) vs. db/m (3.89 ± 0.30 ng/ml) mice on control diets. Serum leptin was undetectable in ob/ob mice. MCD diet resulted in significant increases in serum leptin levels in both db/m and db/db mice.

Effect of MCD diet on liver fibrosis. Trichrome staining and collagen I mRNA expression were assessed as indices of liver fibrosis. As shown in Fig. 3 and Table 2, animals on control diet show insignificant levels of hepatic fibrosis. MCD diet had no effect on the development of central vein or portal tract fibrosis in db/m and ob/ob mice. In contrast, MCD diet-fed db/db mice showed 5-fold increase in central/pericentral vein fibrosis and 14-fold increase in portal/periportal fibrosis when compared with controls and MCD diet-fed db/m and ob/ob mice.
Effect of MCD diet on the mRNA expression of osteopontin TNF-α and TGF-β in db/db, db/db, and ob/ob mice as determined by real-time PCR analysis

<table>
<thead>
<tr>
<th>Measurements</th>
<th>db/m Control</th>
<th>db/m MCD</th>
<th>db/db Control</th>
<th>db/db MCD</th>
<th>ob/ob Control</th>
<th>ob/ob MCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteopontin</td>
<td>1±0.20</td>
<td>4.5±1.00*</td>
<td>3.0±0.61</td>
<td>10.0±1.5*</td>
<td>1.2±0.20</td>
<td>1.4±0.32</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1±0.11</td>
<td>3.3±0.43*</td>
<td>0.4±0.07</td>
<td>8.2±1.3*</td>
<td>0.9±0.11</td>
<td>6.9±1.7*</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1±0.21</td>
<td>1.4±0.20</td>
<td>0.5±0.06</td>
<td>4.0±0.70*</td>
<td>0.8±0.07</td>
<td>2.1±0.36</td>
</tr>
</tbody>
</table>

Hepatic mRNA expression is standardized against internal GAPDH and is expressed as fold differences between MCD diet-fed mice and respective mice on the control diet. Values are means ± SE of 3–8 mice. *p < 0.01 compared with respective control value.

Effect of MCD diet on OPN protein expression. OPN is present as a native 78-kDa protein in various tissue and cells, whereas the 66-kDa OPN is a secreted biologically active form of OPN that is overexpressed in many pathological conditions (10, 37). Similar to our previous observations in A/J mice (46), significant constitutive levels of 78-kDa and 66-kDa OPN protein were observed in db/mice on control diet (Fig. 5A). The 66-kDa OPN expression was fivefold higher in db/db mice than in db/mice on control diet, whereas the expression of 78-kDa protein was unchanged (Fig. 5). MCD diet had no effect on 78-kDa OPN and caused a threefold increase in 66-kDa protein in db/mice. In contrast, MCD diet produced a fourfold increase in 78-kDa and ninefold increase in 66 kDa OPN protein expression in db/db mice (Fig. 5). However, the diet had no significant stimulatory effect on OPN protein expression in ob/ob mice (data not shown). Immunohistochemistry showed predominant localization of OPN in hepatocytes of db/db mice fed control diet, which was significantly increased in MCD diet-fed mice (Fig. 6). Some OPN staining was also observed in the inflammatory cells. Specificity of the immunostaining for OPN was determined by incubations with nonspecific IgG, which showed no significant staining of OPN (Fig. 6).

Effect of MCD diet on leptin receptor expression. mRNA for Ob-Ra and Ob-Rb was constitutively expressed in the livers of db/mice and db/db mice (Fig. 7A). MCD diet produced a fivefold increase in Ob-Ra mRNA expression in db/mice and a sevenfold increase in db/db mice (Fig. 7A). The diet also caused a threefold increase in Ob-Rb mRNA expression in db/mice, whereas it had no effect in db/db mice. Western blot analysis (Fig. 7B) showed increases in Ob-Rb and Ob-Ra protein expression in both db/mice and db/db mice fed the MCD diet. Ob-Rb is nonfunctional in db/db mice (5, 28). The expression of functional short-form leptin receptors, which has multiple isoforms including Ob-Ra, was markedly increased in MCD diet-fed db/db mice compared with its expression in db/mice (Fig. 7B).

Effect of leptin on OPN expression in cultured hepatocytes. Treatment of cultured hepatocytes with leptin for 18 h resulted in significant increases in OPN mRNA expression in a dose-dependent manner (Fig. 8A). We recently reported that hepatocytes in culture produce significant amounts of a secreted form of 66-kDa OPN, which is stimulated three- to fourfold by TNF-α and TGF-β (46). We examined the effect of leptin on OPN expression in cultured hepatocytes and compared it with that of TNF-α and TGF-β. Incubations with leptin (100 ng/ml) for 1–18 h resulted in increased OPN protein expression in a time-dependent fashion, and a threefold increase in 66-kDa OPN protein expression was observed at 18 h (Fig. 8B). Thus
a similar magnitude of the effect on OPN protein expression is observed by leptin, TNF-α, and TGF-β in cultured hepatocytes.

Subsequently, we determined the mRNA expression of Ob-Ra and Ob-Rb in control and leptin-treated (100 ng/ml) cultured hepatocytes. Normal mouse liver was used as a positive control in RT-PCR analysis. Hepatocytes in culture exhibited significant mRNA expression of Ob-Ra under control conditions (Fig. 8C, left), but no Ob-Rb mRNA expression was detected (Fig. 8C, right). Incubation with leptin for 18 h had no significant effect on Ob-Ra mRNA expression (Fig. 8C, left).

DISCUSSION

The present study shows that the MCD diet-fed db/db mouse is valuable experimental model of NASH in association with obesity, insulin resistance, and type 2 diabetes. These mice exhibited accelerated steatohepatitis and liver fibrosis when compared with corresponding lean and nondiabetic db/m mice and leptin-deficient obese and type 2 diabetic ob/ob mice. In addition, db/db mice fed the MCD diet showed substantial increases in serum leptin and the hepatic mRNA and protein expressions of Ob-Ra and Ob-Rb.

Fig. 6. Immunohistochemistry of OPN expression in db/db mice. Control and MCD diet-fed db/db mice were examined for OPN protein localization by immunohistochemistry. Arrows point to OPN localization in hepatocytes and inflammatory cells. MCD diet-fed liver sections incubated with control IgG had no significant OPN staining. Original magnification, ×100; n = 3.

Fig. 7. Effect of MCD diet on leptin receptor mRNA and protein expression in db/m and db/db mice. A: total RNA was isolated and analyzed for the long-form (Ob-Rb) and short-form (Ob-Ra) leptin receptor mRNA expression by RT-PCR. GAPDH was used as a housekeeping gene. B: leptin receptor protein expression was assessed by Western blotting with a mouse monoclonal antibody that recognizes all forms of leptin receptors. β-actin antibody was used to confirm equal protein loading among samples; n = 6.

Fig. 8. Effect of leptin on OPN mRNA expression (A), OPN protein expression (B), and Ob-Ra and Ob-Rb mRNA expression (C) in cultured hepatocytes. A: quiescent cultures were exposed in the absence or presence of mouse recombinant leptin (10–1,000 ng/ml) for 18 h followed by the measurements of OPN mRNA expression by real-time PCR. B: OPN protein expression was assessed during 1–18 h of incubation with 100 ng/ml leptin by Western blot analysis, and β-actin was used as a housekeeping gene for comparison. C: mRNA expressions of Ob-Ra and Ob-Rb were assessed from control and leptin-treated (100 ng/ml) cultured hepatocytes by RT-PCR analysis, and normal mouse liver sample was used as a positive control; n = 4.
expressions of Ob-Ra and OPN. We also demonstrated that leptin stimulates OPN expression in cultured hepatocytes, which appears to involve Ob-Ra because Ob-Rb are not expressed in these cells. Together, these findings suggest that enhanced leptin signaling through Ob-Ra in association with increased OPN expression may contribute to marked hepatic fibrosis in this experimental model of NASH.

The db/db mouse represents an animal model that uniquely mimics the metabolic syndrome associated with NASH in humans. They are obese and exhibit severe hyperglycemia, insulin resistance, and hyperleptinemia while on control diet. However, they have only modestly increased liver TG content compared with db/m mice and do not spontaneously develop steatohepatitis or liver fibrosis. Feeding db/db and db/m mice with MCD diet produced similar degrees of hepatic steatosis, and ob/ob mice developed more severe steatosis. Both db/m and ob/ob mice exhibited significant reductions in body weight when fed the MCD diet, but db/db mice appeared more resistant and had a minimal loss in body weight. MCD diet caused a threefold increase in serum leptin levels in db/db mice, findings that are consistent with observations of high serum leptin levels reported in most studies of patients with NASH (7, 55). However, others have shown no significant changes in serum leptin levels between patients with NASH and simple steatosis (4).

MCD diet-fed db/db mice developed accelerated liver injury, inflammation, and fibrosis when compared with their corresponding heterozygous nonobese and nondiabetic db/m mice. The pattern of inflammation and fibrosis in MCD diet-fed db/db mice observed in our studies is very similar to that seen in human NASH (1, 3). In contrast, consistent with a recent report (27), ob/ob mice that are leptin deficient develop moderate lobular inflammation and no detectable fibrosis on the MCD diet. These findings demonstrate the importance of obesity, diabetes, and leptin in the pathogenesis of experimental NASH and highlight the value of the MCD diet-fed db/db mouse model in the study of obese and diabetic NASH.

We hypothesize that hepatic leptin signaling is maintained in the db/db mouse based on evidence of functional Ob-Ra in other tissues of this strain (16, 18). If Ob-Ra are also expressed in the liver and can bind leptin to activate downstream pathways, the hyperleptinemia that these animals exhibit would make them susceptible to accelerated liver injury and fibrosis when fed the MCD diet. Numerous studies have shown an important role for leptin in hepatic fibrogenesis (17, 19, 27, 44, 49, 53). Leptin has been shown to induce collagen I mRNA expression in cultured rat stellate cells (44, 49). Carbon tetrachloride and thioacetamide-induced hepatic fibrosis in mice can be enhanced by the administration of leptin (17, 19). Moreover, MCD diet and carbon tetrachloride, which are known to induce liver fibrosis, failed to cause hepatic fibrosis in leptin-deficient ob/ob mice (27). Our results show that MCD diet causes marked increases in both the mRNA and protein expression of Ob-Ra in db/db mice and also increases serum leptin levels. The protein expression of Ob-Rb, which is nonfunctional in db/db mice, was also increased. The marked increases in the expression of functional Ob-Ra and serum leptin levels in db/db mice suggest that enhanced leptin signaling through Ob-Ra receptors may contribute to accelerated hepatic fibrosis in this model of NASH.

OPN plays an important role in inflammatory and noninflammatory diseases in various organ systems, including bone, kidney, and vasculature, as well as autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, and autoimmune myocarditis (10, 21, 37, 58). OPN is a glycosylated phosphoprotein secreted by a variety of immune cells as well as epithelial, endothelial, and smooth muscle cells (10, 37). It is expressed in smooth muscle cells and macrophages in vascular injury including atherosclerosis (6, 20, 29). OPN is found in macrophages and renal epithelial cells during tubulointerstitial inflammation of the kidney resulting from a variety of factors including ischemia, hypoxia, angiotensin II-induced injury, and glomerulonephritis (14, 36, 38, 43, 51). OPN is also a key cytokine in granulomatous inflammation (40). Although the many functions of OPN have not been completely defined, it is involved in macrophage recruitment during inflammation, acts as a survival or mitogenic factor for epithelial and vascular cells, and is associated with renal extracellular matrix synthesis and fibrosis (30, 33, 39, 42, 51, 52). OPN is also implicated in cardiac and pulmonary fibrosis as well as numerous types of cancers, including hepatocellular carcinoma (2, 9, 15, 35, 57).

In the liver, carbon tetrachloride induces OPN expression in Kupffer cells and macrophages (22). We have shown that OPN expression is induced early in the progression of liver injury and fibrosis in MCD diet-fed A/J mice (46). OPN-null mice showed less liver injury and fibrosis than the wild-type mice, directly implicating OPN in the pathogenesis of NASH in this nutritional model. We also found increased hepatic expression of OPN in patients with biliary atresia, which correlates with portal duct proliferation and fibrosis (56).

The present study shows significantly greater constitutive expression of OPN mRNA and protein in db/db vs. db/m mice on control diet, which was predominantly localized to hepatocytes. These results are consistent with other animal models of diabetes and cultured cells where diabetes and/or hyperglycemia have been shown to increase OPN expression in the kidney and the vasculature (12, 37, 51, 52). In our study, MCD diet markedly increased OPN mRNA and protein expression in db/db mice in parallel to the increases in collagen I mRNA expression. In contrast, the leptin-deficient ob/ob mice that do not develop liver fibrosis on the MCD diet had no effect on OPN expression. Together, these results suggest that the interaction of leptin and OPN may be key signaling events in the development of liver fibrosis in NASH. Moreover, leptin stimulated OPN mRNA and protein expression in cultured hepatocytes, which express only Ob-Ra. Our results are consistent with other reports where the expression of Ob-Ra but not Ob-Rb is observed in primary cultures of hepatocytes (8, 59). Given our findings in MCD diet-fed OPN-null mice, which defined a key role for OPN in hepatic fibrosis in experimental NASH (46), it seems likely that increased OPN expression via leptin signaling through Ob-Ra contribute to marked liver fibrosis in the insulin resistant/diabetic db/db mouse model of NASH. Since increased expressions of TNF-α and TGF-β in MCD diet-fed db/db mice were also observed in our studies, defining the interactions of these cytokines with OPN in the signaling cascade of hepatic fibrosis will be of considerable interest.

In summary, our studies show that db/db mice develop marked steatohepatitis and liver fibrosis when fed a MCD diet, thus providing a nutritional model of NASH that closely mimics human NASH, where obesity and diabetes/insulin...
resistance play key permissive roles. The diet increased the expression of Ob-Ra and OPN in db/db mice. In addition, leptin increased OPN expression in cultured hepatocytes, which expresses only Ob-Ra, suggesting that enhanced leptin signaling via Ob-Ra and OPN may play a role in accelerated hepatic fibrosis in NASH.

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