Roles of phosphatidylinositol 3-kinase and osteopontin in steatosis and aminotransferase release by hepatocytes treated with methionine-choline-deficient medium

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The cellular and molecular mechanisms involved in the evolution of experimental NASH in the MCD diet model are incompletely understood. With regard to human NASH, it is generally believed that obesity and insulin resistance result in hepatic steatosis, which provides the environment for increased oxidative stress and peroxisomal injury (7, 27, 32). Similarly, there is evidence of oxidative stress in the MCD diet model and other models of experimental NASH (14, 18, 28, 38). In both instances, it is thought that the induction of oxidative stress results in liver injury and fibrosis that characterize NASH. We, however, found in the MCD model of experimental NASH that increased OPN expression preceded the induction of collagen 1 mRNA expression by days and preceded significant oxidative stress and fibrosis by weeks (30). In addition, intact leptin signaling is considered to be essential for hepatic fibrosis to occur (15). We showed that increased OPN expression in MCD diet-fed db/db mice is accompanied by parallel increases in serum leptin and in the hepatic expression of short-form leptin receptors (31). Furthermore, leptin stimulates OPN expression by cultured mouse hepatocytes, a signal that appears to involve short-form leptin receptors (31). Leptin has also been shown to activate phosphatidylinositol (PI3)-kinase in primary cultures of rat hepatocytes (23, 33, 35). Activation of PI3-kinase is implicated in the control of cell growth and apoptosis in several organ systems including the liver (10, 17, 25, 29, 34). Moreover, OPN knockout mice fed MCD diet show lower serum alanine aminotransferase (ALT) activity and develop markedly less liver fibrosis than do wild-type mice but show similar degrees of hepatic steatosis (30). Finally, we have shown that MCD diet fed to obese and Type 2 diabetic db/db mice exhibits greater increases in OPN expression than do outbred db/m littermates, which parallels the accelerated NASH they express when fed the MCD diet (31). Taken together, these findings suggest a role for OPN as a hepatocyte-derived Th1 cytokine in the pathogenesis of NASH.

The administration of a diet deficient in methionine and choline (MCD diet) to mice produces experimental steatohepatitis that simulates human nonalcoholic steatohepatitis (NASH) (14, 36). We have demonstrated in this model that osteopontin (OPN) mRNA in cells and protein expression in medium were significantly increased during 6–24 hours of incubation. MCD medium treatment also resulted in activation of PI3-kinase by 30 minutes and its downstream target p-Akt within 1 hour of incubation. Steatosis was associated with increased expression of microsomal triglyceride transfer protein (MTTP) mRNA and increased ALT release with over expression of ALT mRNA, all of which were completely prevented by inhibition of PI3-kinase (LY294002). Blocking OPN signaling by treating with anti-OPN or anti-β3-integrin antibody prevented the increased ALT release while only partially prevented the increased ALT mRNA expression, but had no effect on either steatosis or MTTP expression. In conclusion, incubation of cultured hepatocytes with MCD medium results in cellular steatosis and OPN dependent ALT release. PI3-kinase plays a central role in signaling the MCD medium-induced steatosis and increased OPN expression, whereas OPN appears to play a role in signaling hepatocyte ALT release but not steatosis.
OPN expression and in the development of steatosis, steatohepatitis, and fibrosis in experimental NASH is undefined.

In the present study, we examined the short-term effect of exposing cultured hepatocytes to MCD culture medium with regard to the development of steatosis and release of ALT into the medium and determined whether oxidative injury and/or cell death resulted from exposure to MCD medium. Finally, we examined the expressions of OPN and PI3-kinase and assessed their roles in signaling the MCD medium-induced hepatocyte steatosis and ALT release.

MATERIALS AND METHODS

Materials. DMEM/Ham’s F-12 medium and identical medium that was manufactured to be completely deficient of methionine and choline (MCD medium) were purchased from Invitrogen (Carlsbad, CA). The mouse hepatocyte cell line AML-12 was obtained from American Type Culture Collection (Bethesda, MD). Monoclonal anti-OPN antibody M111B10 was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). Polyclonal antibody to PI3-kinase p85 subunit was purchased from Upstate Biotechnology (Lake Placid, NY). Phospho-Akt and Akt antibodies were from Cell Signaling Technology (Beverly, MA). β-Actin antibody was obtained from Sigma (St. Louis, MO). Neutralizing monoclonal antibody to β3-integrin (OPN receptor, CD-61) was purchased from BD Biosciences Pharmingen (Mountain View, CA), and a specific inhibitor of PI3-kinase (LY294002) was purchased from Calbiochem (La Jolla, CA). All other reagents were of high chemical grade from Sigma.

Hepatocyte cell culture. AML-12 cells were grown in 1:1 mixture of DMEM-Ham’s F-12 medium supplemented with 10% fetal bovine serum and antibiotics (growth medium) as previously described (30). Cells were subcultured in growth medium until the cultures reached 70–80% confluence, at which time they were made quiescent by incubation for 24 h in serum-free DMEM-Ham’s F-12 medium. Quiescent cells were then exposed either to control growth medium or MCD medium supplemented with identical glucose and serum concentrations. As a measure of oxidative injury, thiobarbituric acid-reactive substances (TBARs) were measured in cell homogenates by a routine spectrophotometric kit procedure (Zeptometrix, Buffalo, NY) and expressed as nanomoles of malondialdehyde (MDA) produced per milligram of protein.

Real-time PCR analysis. Total RNA was isolated from cultured hepatocytes using TRIzol reagent (GIBCO-BRL, Grand Island, NY). One microgram of total RNA was reverse transcribed using iSCRIPT cDNA synthesis kit from Bio-Rad Laboratories, Hercules, CA. Real-time PCR was performed using 4 μl total cDNA in a 50-μl reaction volume containing QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) with the specific primers for mouse OPN and the housekeeping gene GAPDH (Integrated DNA Technologies, Coralville, IA). The primer sequences used are as follows: OPN: forward 5'-CAG CCT GCA AGA TTC TA-3'; reverse 5'-GCC CGA GGA GTG TCT CTT 3'; microsomal triglyceride transfer protein (MTTP): forward 5'-GTG GAG GAA TCA TGG TGA-3'; reverse 5'-TGA TCT TAG GTG TAC TTT GGC CC-3'; ALT2: forward 5'-CAG ACC CAG ACA ACA TTG ACC TG-3'; reverse 5'-GTC GTG GAT CTG ACG TGC C-3'; reverse 5'-TGC CGT CAT CTG CAC AAC CTT C-3'. Amplification was performed in duplicate for each sample in an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA) with a hold for 2 min at 50°C, denaturation for 10 min at 95°C, followed by 40 PCR cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 60 s. The relative amount of mRNA was calculated using GAPDH as the endogenous control.

Western blot analysis. Immunoreactive levels of PI3-kinase, p-Akt, and Akt in the cell lysates and of OPN in conditioned medium were assessed by Western blot analysis as previously described (30). Briefly, cells were scraped off the flask in cold PBS and centrifuged. The cell pellet was dissolved 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) containing protease inhibitor cocktail (Calbiochem, La Jolla, CA). For gel electrophoresis, samples of cell homogenate containing 25–50 μg protein were applied. For measurement of protein in conditioned medium, in every experiment, 4.0 ml medium were concentrated to 0.5 ml (8-fold concentration), and samples containing 0.5 μl of concentrated medium were applied. Proteins were resolved by 10% SDS polyacrylamide gel electrophoresis under reducing conditions and then transferred overnight onto nitrocellulose membranes by electrophoresis. Specific primary antibodies to OPN, PI3-kinase p85 subunit, phospho-Akt, and Akt were used to detect their expressions. β-Actin antibody was used to confirm equal protein loading among samples. The bound primary antibodies were detected with a respective horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution, Amersham, Arlington Heights, IL) and visualized with an enhanced chemilumi-
nescence method. Protein expression levels were determined by densitometry.

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.

RESULTS

Effect of MCD medium on hepatocyte steatosis, medium ALT, cell viability, apoptosis, and oxidative injury. We first examined the effect of MCD medium on hepatocyte steatosis. Incubation of cultured hepatocytes with MCD medium resulted in progressive increases in cell triglyceride content beginning at 6 h with a maximal threefold increase observed at 24 h (Fig. 1A). Oil Red O staining of MCD medium-treated hepatocytes for 18 h showed a marked increase in neutral fat within hepatocytes compared with cells incubated with control medium (Fig. 1B).

ALT release was determined by measuring its activity in the concentrated conditioned medium. As shown in Fig. 2, treatment with MCD medium resulted in a twofold increase in medium ALT by 18 h, which then declined slightly by 24 h of examination. Cell viability, as determined by trypan blue exclusion, and number of adherent cells were not affected by incubating with MCD medium for 6–24 h (data not shown). Furthermore, no apoptosis could be detected by DNA laddering at any time point (data not shown). TBARs were measured in cells exposed to MCD or control medium for 24 h to assess oxidative injury. Incubating in MCD medium produced no significant difference in TBARs, 0.24 ± 0.10 vs. 0.14 ± 0.04 nmol/mg protein for control medium (P = not significant), indicating no significant increase in global oxidative injury induced by MCD medium.

Role of OPN in MCD medium-induced steatosis and ALT release. We examined the effect of MCD medium on OPN mRNA and protein expression. Exposure to MCD medium induced significant increases in OPN mRNA levels beginning at 6 h with an ~10-fold increase observed at 18–24 h of examination (Fig. 3). Similarly, significant increases in OPN protein expression were observed at 6 h of treatment and remained elevated for up to 24 h of incubation (Fig. 4).

To assess the possible role of OPN in MCD medium-induced hepatocyte fat accumulation and ALT release, an
anti-OPN antibody or a neutralizing antibody to OPN receptor β3-integrin was used. Cultures were exposed to MCD or control medium for 18 h in the absence or presence of either anti-OPN antibody or β3-integrin-neutralizing antibody followed by measurement of cell triglyceride content and medium ALT. Treatment with either anti-OPN or β3-integrin receptor antibody completely prevented the increase in medium ALT induced by the MCD medium (Fig. 5A). In contrast, OPN or β3-integrin receptor antibody had no significant effect on steatosis induced by the MCD medium (Fig. 5B). These antibodies had no significant effect on medium ALT or triglyceride content in cells incubated with control medium (data not shown).

**Effect of MCD medium on PI3-kinase and p-Akt activities.**

We determined the effect of MCD medium on PI3-kinase and its downstream target p-Akt by Western blot analysis. As shown in Fig. 6A, MCD medium induced a significant increase in PI3-kinase beginning at 1 h of incubation, which remained elevated through 12 h of examination and declined to control levels at 18 h of examination. Treatment with MCD medium produced a sustained increase in p-Akt levels during 1–18 h of incubation (Fig. 6B). Immunoreactive levels of Akt protein were not altered during this incubation time, indicating the specificity of the changes in p-Akt induced by the MCD medium (Fig. 6B).

**Role of PI3-kinase in the regulation of OPN expression and in the development of fat accumulation and ALT release.**

Subsequent studies examined the role of PI3-kinase in MCD medium-induced stimulation of OPN expression, steatosis, and ALT release. Cultures were exposed to MCD or control medium for 18 h in the absence or presence of a specific inhibitor of PI3-kinase (LY294002, 10 or 25 μM), and OPN expression, medium ALT, and cell triglyceride content were assessed.

PI3-kinase inhibitor blocked the MCD medium-induced increases of OPN mRNA expression in a dose-dependent manner, with 25 μM inhibitor completely preventing the effect (Fig. 7A). The inhibitor also blocked the increase in OPN protein expression induced by the MCD medium (Fig. 7B). Moreover, the PI3-kinase inhibitor prevented any MCD medium-induced increases in medium ALT (Fig. 8A) and cell triglyceride content (Fig. 8C). The inhibitor had no effect in cultures incubated with control medium (data not shown).

**Relative roles of PI3-kinase and OPN signaling in expressions of MTTP and ALT mRNA.** To determine whether MCD medium-induced activation of PI3-kinase may be working through sterol regulatory element-binding protein (SREBP-1) to cause increased transcription of MTTP, we measured MTTP mRNA in cells exposed to MCD medium for 18 h in the absence or presence of inhibitor. Compared with control medium, exposure to MCD medium resulted in an ~2.5-fold increase in MTTP mRNA expression, which was completely blocked by inhibition of PI3-kinase activation but not by
blocking OPN signaling by inclusion of anti-OPN antibody or β3-integrin receptor-neutralizing antibody (Fig. 9A). To examine the possibility that OPN was feeding back to the hepatocytes to induce ALT synthesis as a possible mechanism of increased ALT release, we measured ALT mRNA expression. As shown in Fig. 9B, incubation with MCD medium resulted in an approximately fourfold increase in ALT mRNA expression vs. control medium. Inhibition of PI3-kinase activation completely eliminated the MCD medium-induced overexpression of ALT mRNA, whereas incubating with either anti-OPN antibody or β3-integrin-neutralizing antibody reduced ALT mRNA expression somewhat, to approximately threefold the values seen with control medium (Fig. 9B).

**DISCUSSION**

Our results demonstrate that incubating cultured mouse hepatocytes with MCD medium causes cellular steatosis and increased release of ALT. The MCD medium-induced increases in triglyceride formation and ALT release were accompanied by the activation of PI3-kinase and markedly increased OPN expression. An inhibitor of PI3-kinase prevented the increases in OPN expression, cellular steatosis, and ALT release. Antibodies to OPN or its receptor blunted the increase in ALT release but had no effect on steatosis induced by the MCD medium. Together, these findings indicate that the activation of PI3-kinase plays an important role in the development of hepatocyte steatosis and induction of OPN expression in cultured hepatocytes exposed to MCD medium. OPN, in turn, is potentially involved in progression from steatosis to the phenotype of NASH in the MCD model.

Obesity and its complications including nonalcoholic fatty liver disease and NASH are enormous public health problems and the focus of intense research. Among the experimental models most successfully used in the study of NASH is feeding mice an MCD diet. This feeding regimen results in hepatic steatosis within a few days, elevated serum ALT by 1 wk, histological inflammation by 2 wk, and hepatic fibrosis by 4 wk (30). Here, we showed that cultured mouse hepatocytes exposed to MCD medium demonstrate the early effects observed in MCD diet-fed mice, namely, hepatocyte steatosis and re-
lease of ALT. This is potentially a worthy in vitro model to study the signaling mechanisms involved in fatty liver disease.

We have shown that hepatocyte-derived OPN plays an important role in the pathogenesis of experimental NASH. MCD diet induces a marked increase in the hepatic expression of OPN in A/J mice, which precedes increased serum ALT and liver collagen I expression (30). OPN knockout mice fed the MCD diet exhibit significantly lower hepatic collagen I expression than WT mice and develop essentially no liver fibrosis (30). Increased OPN protein expression in MCD diet-fed animals is localized mainly to hepatocytes, and TNF-α, TGF-β, and leptin stimulate OPN expression in cultured hepatocytes (30). These findings suggested a paracrine role for hepatocyte-derived OPN in activating stellate cells to produce collagen and cause fibrosis in experimental NASH. In the current study, exposing cultured hepatocytes to MCD medium produced significant stimulation of OPN mRNA and protein expression. This effect was analogous to the OPN expression in MCD diet experimental NASH (30, 31). Moreover, neutralizing antibody to OPN or its β3-integrin receptor was able to block the MCD medium-induced ALT release. These findings suggest that exposure to MCD medium induces the synthesis and secretion of OPN by hepatocytes, which then acts in an

Fig. 8. Effect of PI3-kinase inhibitor on MCD medium-induced increase in ALT release (A) and cell triglyceride content (B) in cultured hepatocytes. Cultures were exposed to MCD medium for 18 h with and without the inhibitor of PI3-kinase (LY294002; 10 μM), and medium ALT and cell triglyceride levels were assessed (means ± SE, n = 3 for each measurement). *P < 0.01 vs. control; **P < 0.01 vs. MCD; and P = NS vs. control.

Fig. 9. Effect of MCD medium with and without inhibition of PI3-kinase (LY294002; 10 μM) and OPN signaling (using OPN or β3-integrin antibodies) on microsomal triglyceride transfer protein (MTTP) mRNA expression (A) and ALT mRNA expression (B). Cultures exposed to MCD medium for 18 h had increased expression of MTTP mRNA (A) and ALT mRNA (B). Inhibition of PI3-kinase activation completely blocked this effect, whereas blocking OPN signaling with antibodies had no effect on the MCD-induced overexpression of MTTP mRNA and only partial reduction in the expression of ALT mRNA (mean ± SE, n = 3 for each measurement). *P < 0.01 vs. control; **P < 0.01 vs. MCD.
autocrine fashion to cause an increase in the hepatocyte release of ALT. This is also analogous to in vivo experimental NASH, in which OPN knockout mice show substantially lower serum ALT than WT littermates when fed the MCD diet (30). In this study, we showed that the MCD medium induced, OPN-dependent ALT release into the medium is not associated with increased oxidative injury, cell necrosis, or apoptosis. Although not examined in this study, hepatic fibrosis in experimental NASH is probably also affected by hepatocyte secretion of OPN, which then acts on stellate cells to increase collagen synthesis (16).

We sought to determine whether signaling through PI3-kinase was responsible for enhanced OPN expression in this model of NASH. We found that MCD medium increases the expression of PI3-kinase and its downstream target Akt. Moreover, a specific inhibitor of PI3-kinase completely prevented the MCD medium-induced increases in OPN expression. Our findings are consistent with recent observations where PI3-kinase has been shown to mediate the EGF-induced OPN expression by HepG2 cells (39), high glucose-induced increase in OPN expression in renal proximal tubular cells (11), and in the metastatic behavior of breast cancer cells (20). Together, these findings suggest a potential role of PI3-kinase-dependent OPN expression in the pathogenesis of NASH.

We found that PI3-kinase inhibition prevented MCD medium-induced hepatocyte steatosis and ALT release. In contrast, antibodies to either OPN or its receptor prevented the ALT release but had no effect on steatosis induced by the MCD medium. These results complement our in vivo findings where OPN knockout and WT mice on MCD diet developed similar degrees of steatosis, but the knockout mice exhibited significantly reduced serum ALT levels and no hepatic fibrosis (30). Of interest, citrus flavonoid naringenin causes decreased VLDL secretion via activation of PI3-kinase, which in turn enhances the expression of SREBP-1 (4). Increased expression of SREBP-1 causes reduced expression of MTTP, which reduces movement of triglyceride into microsomes leading to reduced lipidation and secretion of VLDL (3). Abnormal lipidation and synthesis of VLDL are considered to play a significant role in the development of steatosis (5, 6), and polymorphisms of MTTP have been implicated as risk factors in human NASH (2, 22). We found that MCD medium-induced increased expression of MTTP was eliminated by blocking PI3-kinase activation, suggesting that signaling through SREBP-1 is involved in the development of MCD-associated steatosis.

It appears that PI3-kinase-dependent increased OPN expression is involved in release of ALT from cultured hepatocytes exposed to MCD medium. Preventing increased ALT release by blocking OPN signaling is quite analogous to the in vivo model of experimental NASH (30). However, we found that treatment with MCD medium evoked increased expression of ALT mRNA. Blockade of PI3-kinase activation completely inhibited the increased expression of ALT mRNA and accumulation of ALT in the medium. Blocking OPN signaling completely blocked the MCD medium-induced accumulation of ALT in the medium but only partially reduced the overexpression of ALT mRNA. In addition, we could find no evidence of cellular oxidative injury, cell death, or apoptosis. These somewhat surprising findings suggest that increased PI3-kinase-dependent ALT transcription is a response to exposure to the MCD medium and is at least in part responsible for the increased accumulation of ALT in the medium. The differential effect of blocking OPN signaling on ALT mRNA expression and accumulation in the medium suggests that mechanisms other than increased synthesis are involved in the loss of ALT into the medium. Taken together, these provocative findings suggest that the increase in serum ALT in in vivo experimental NASH may not necessarily reflect hepatocyte injury or death but may be in part an adaptive response to metabolic challenge.

In summary, our studies show that treating cultured hepatocytes with MCD medium causes hepatocyte steatosis, induction of OPN synthesis, and increased ALT loss. Activation of PI3-kinase appears to be directly involved in all of these effects of exposure to MCD medium. PI3-kinase-dependent increases in OPN expression appear to play an important role in signaling release of ALT and potentially act as a hepatocyte-derived signal for fibrogenesis in experimental NASH. The model used in these studies should provide the opportunity to further dissect the pathogenic mechanisms of experimental fatty liver disease.

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