Micro-RNA 21 inhibition of SMAD7 enhances fibrogenesis via leptin-mediated NADPH oxidase in experimental and human nonalcoholic steatohepatitis

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Dattaroy D, Pourhoseini S, Das S, Alhasson F, Seth RK, Nagarkatti M, Michelotti GA, Diehl AM, Chatterjee S. Micro-RNA 21 inhibition of SMAD7 enhances fibrogenesis via leptin-mediated NADPH oxidase in experimental and human nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 308: G298–G312, 2015. First published December 11, 2014; doi:10.1152/ajpgi.00346.2014.—Hepatic fibrosis in nonalcoholic steatohepatitis (NASH) is the common pathophysiological process resulting from chronic liver inflammation and oxidative stress. Although significant research has been carried out on the role of leptin-induced NADPH oxidase in fibrogenesis, the molecular mechanisms that connect the leptin-NADPH oxidase axis in upregulation of transforming growth factor (TGF)-β signaling have been unclear. We aimed to investigate the role of leptin-mediated upregulation of NADPH oxidase and its subsequent induction of micro-RNA 21 (miR21) in fibrogenesis. Human NASH livers and a high-fat (60% kcal) diet-fed chronic mouse model, where hepatoprotectin bromochloromethane was used to induce NASH, were used for this study. To prove the role of the leptin-NADPH oxidase-miR21 axis, mice deficient in genes for leptin, p47phox, and miR21 were used. Results showed that wild-type mice and human livers with NASH had increased oxidative stress, increased p47phox expression, augmented NF-κB activation, and increased miR21 levels. These mice and human livers showed increased TGF-β, SMAD2/3-SMAD4 colocalizations in the nucleus, increased immunoreactivity against Col1α1, and α-SMA with a concomitant decrease in protein levels of SMAD7. Mice that were deficient in leptin or p47phox had decreased oxidative stress, increased p47phox expression, augmented NF-κB activation, and increased miR21 levels. These mice and human livers showed increased TGF-β, SMAD2/3-SMAD4 colocalizations in the nucleus, increased immunoreactivity against Col1α1, and α-SMA with a concomitant decrease in protein levels of SMAD7. Mice that were deficient in leptin or p47phox had decreased oxidative stress, increased p47phox expression, augmented NF-κB activation, and increased miR21 levels. Further knockout mice had decreased colocalization events of SMAD2/3-SMAD4 in the nucleus, increased SMAD7 levels, and decreased fibrogenesis. Taken together, the studies show the novel role of leptin-NADPH oxidase induction of miR21 as a key regulator of TGF-β signaling and fibrogenesis in experimental and human NASH.

nonalcoholic fatty liver disease; ob/ob; NF-κB; SMAD7; SMAD2/3 colocalization; transforming growth factor-β

PATHOGENESIS OF NONALCOHOLIC STEATOHEPATITIS (NASH) is not clearly understood and perceived to comprise an inflammatory phase of high circulatory leptin, increased oxidative stress, elevated inflammatory cytokines resulting in hepatocellular injury, and subsequent progression into fibrosis (12). Most NASH pathophysiology is accompanied by late-stage fibrosis (34, 38, 43). Hepatic fibrosis in NASH most likely occurs from chronic liver inflammation associated with a rise in proinflammatory cytokines and oxidative stress (27). Fibrosis is closely linked to accumulation of extracellular matrix (ECM) proteins, mainly type I collagen, which also can occur in many chronic liver diseases (27). The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes can lead to condition known as cirrhosis (9). Because NASH might arise almost always from a preexisting condition of obesity, type 2 diabetes, and insulin resistance, the higher circulatory levels of adipose tissue cytokines leptin and TNF-α have been predicted to play a significant role in hepatic fibrogenesis (14). We and others have shown in rodent models of NASH that higher leptin is closely associated with fibrosis in NASH (7, 19, 36).

Leptin, a product of the ob gene, is synthesized in the liver and the adipose tissue (30). We have shown that mice fed with a high-fat diet and challenged with low doses of hepatotoxins display increases in both hepatic and circulatory levels of leptin (3, 35). An early study by Honda et al. (11) showed that leptin deficiency is responsible for resistance to thioacetamide-induced fibrogenesis, whereas subsequent studies have highlighted the role of leptin in stellate cell activation, leptin-induced transforming growth factor (TGF)-β production via Kupffer cells, and leptin signaling-induced collagen production and ECM formation (3, 8, 11, 33, 39, 40).

Although leptin has been found to play a distinct role in the stellate cell activation and TGF-β production, molecular mechanisms involving reactive oxygen species, especially the role of NADPH oxidase, were revealed recently (27). NADPH oxidase, both the phagocytic and nonphagocytic isoforms, has been detected in the liver cell types (26). Hepatic stellate cells have been found to express the NADPH oxidase isoform 2, and deletion of one of its cytosolic subunits has resulted in decreased fibrosis in rodent models of NASH (27). Further published reports from our laboratory have identified the role of peroxynitrite, a highly reactive nitroso species formed by superoxide and nitric oxide in NASH (3). We further showed that NADPH oxidase was crucial for peroxynitrite formation that was again dependent on leptin (3).

It is also critical that high circulatory levels of leptin accompanied by leptin resistance are common in patients with liver fibrosis (20). Quiescent hepatic stellate cells in humans express very low levels of p47phox, a regulatory subunit of NADPH oxidase, but they are highly activated in culture-activated stellate cells isolated from patients with liver fibrosis (2).
Although significant research has been carried out on leptin-induced NADPH oxidase in fibrogenesis, the molecular mechanisms that connect the leptin-NADPH oxidase axis in upregulation of TGF-β signaling have been unclear. Reactive oxygen species production by NADPH oxidase, primarily in the form of superoxide or hydrogen peroxide, has been shown to induce nuclear translocation of NF-κB (42). Our unpublished reports indicate that NF-κB activation led to epigenetic modulations in the form of upregulation of micro-RNA 21 (miR21) in NASH. miR21 is a small noncoding RNA that has been found to have a distinct role in inflammation, and its regulatory functions in NASH pathophysiology are slowly emerging (25, 44).

miR21, upon induction, targets several proteins either by binding entirely to the complementary sequence of mRNA of the target protein, resulting in a nonfunctional mRNA, or binding partially to cause a translational defect (28). Regulatory protein SMAD7, which plays an important role in TGF-β signaling, is a target of miR21 and has been shown to reverse the regulatory effect of this protein (6, 17). Importantly, TGF-β signaling is crucial to the fibrogenesis in NASH and has been shown conclusively in many studies (13, 41).

In this study, we test the hypothesis that high circulating leptin-induced NADPH oxidase-NF-κB activation causes miR21-mediated SMAD7 inhibition, resulting in increased TGF-β signaling. The resultant inhibition of SMAD7, a regulatory SMAD, leads to uninterrupted TGF-β function, causing fibrogenesis in NASH. The results obtained via the use of a rodent model of NASH, human tissues, and transgenic mice lacking leptin, p47phox, and miR21 show that leptin-induced NADPH oxidase causes activation of NF-κB and subsequent upregulation of miR21. Lack of miR21 led to significantly less fibrogenesis, TGF-β production, and downstream signaling of the TGF-β pathway.

**MATERIALS AND METHODS**

**Obese mice.** Adult male, pathogen-free, 6-wk-old mice with C57BL/6J background (Jackson Laboratories, Bar Harbor, ME) were used as model for diet-induced obesity. The animals were fed with high-fat diet (60% kcal) from 6 wk to 16 wk to develop diet-induced obesity (DIO). After completion of 16 wk, all experiments were conducted. Mice that contained disrupted ob gene (leptin) (B6.V-Lepob/J) (Jackson Laboratories) (ob/ob), another group of leptin knockout (KO) mice (ob/ob) treated with leptin (leptin-supplemented group, ob/ob + leptin) with disrupted p47 phox gene (B6.129S2-Ncf1tm1shl N14) (Taconic, Cranbury, NJ) (p47 phox KO), and mice with disrupted mir21 gene (B6;129S6-Mir21atm1Yoli/J) (miR21 KO) were fed with a high-fat diet and treated identically to DIO mice. The mice were housed one per cage in a temperature-controlled room at 23–24°C with a 12-h:12-h light/dark cycle with ad libitum access to food and water. All animals had been treated in strict accordance with the NIH guidelines for Care and Use of Laboratory Animals and local IACUC standards. All experiments were approved by the institutional review board at NEIHs, Duke University, and the University of South Carolina.

**Induction of liver injury in obese mice (toxin model).** Bromochloromethane (BDCM) (2.0 mmol/kg, diluted in corn oil) was administered through intraperitoneal injection in 16-wk-old DIO mice (n = 6). Liver tissues were collected and pooled after 24-h exposure of BDCM [DIO + BDCM (24 h)] and after 48-h exposure of BDCM [DIO + BDCM (48 h)].

The other group of DIO mice was administered two doses of BDCM (1.0 mmol/kg, diluted in corn oil) per week for 1 wk (DIO + BDCM 1 w) and for 4 wk (DIO + BDCM 4 w) (n = 4). Sixteen-week-old high-fat diet-fed DIO mice, administered with 100 ng GdCl₃ per day via intraperitoneal route for 4 wk, were also manufactured with BDCM (1.0 mmol/kg, diluted in corn oil) through the intraperitoneal route (GdCl₃, treated). Sixteen-week-old high-fat diet-fed gene-specific KO mice (ob/ob, p47 phox KO, miR21 KO) were administered with BDCM (1.0 mmol/kg, diluted in corn oil) through the intraperitoneal route (n = 3). DIO mice were treated with corn oil (diluent of BDCM) to use as a control. After completion of the treatments, all mice were killed for liver tissue for further experiments. The tissues were pooled and used for the study.

**Human tissues.** Human liver tissues both from patients with NASH and normal individuals were obtained from NIH repository at University of Minnesota and University of Pittsburg. Each experiment was carried out using three experimental groups and three control samples.

**Histopathology.** Liver sections were collected from each animal and fixed in 10% neutral-buffered formalin. These formalin-fixed, paraffin-embedded tissues were cut in 5-μm-thick sections. These sections were deparaffinized using standard protocols and stained with picrosirius red. Picorsirius red staining of liver sections was done by using Nova ultra Sirius red stain kit following the manufacturer’s protocol (IHC World, Woodstock, MD), and they were observed using ×20 objectives under the light microscope.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissues were cut in 5-μm-thick sections. Each section was subjected to deparaffinization using standard protocols. To describe briefly, these sections were washed with two changes of 100% xylene twice for 3 min followed by xylene, ethanol (1:1) for 3 min, and rehydrated through a series of ethanol (twice with 100%, 95%, 70%, and 50%), twice with distilled water. The sections were finally rinsed twice with PBS. Epitope retrieval of the deparaffinized sections was carried out using epitope retrieval solution and steamer (IHC World) by following the manufacturer’s protocol. The primary antibodies anti-3 nitrotyrosine, anti-TGF-β, anti-connexin tissue growth factor (CTGF), anti-extra domain A-fibronectin (EDA FN), anti-o-smooth muscle actin (SMA), and anti-Col1α1 were purchased from Abcam (Cambridge, MA) and used in recommended dilutions. Species-specific biotinylated-conjugated secondary antibody and streptavidin conjugated with horseradish peroxidase (HRP) were used from Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) to perform antigen-specific immunohistochemistry following the manufacturer’s protocols. 3,3′Diaminobenzidine (Sigma-Aldrich, St. Louis, MO) was used as a chromogenic substrate. Tissue sections were counterstained by Mayer’s hematoxylin (Sigma-Aldrich). Phosphate buffer saline was used for washing thrice between the steps. Sections were mounted in Simpo mount (GBI Laboratories, Mukilteo, WA) and observed under an ×20 objective. Morphometric analysis was done using CellSens Software from Olympus America.

**Immunofluorescence dual-labeling microscopy.** Formalin-fixed, paraffin-embedded tissues were cut in 5-μm-thick sections. Each section was subjected to deparaffinization using standard protocols. Epitope retrieval of the deparaffinized sections was carried out using epitope retrieval solution and steamer (IHC World) by following the manufacturer’s protocol. The primary antibodies anti-SMAD2/3, anti-SMAD4, anti-gp-91phox, and anti-p47phox (purchased from Cell Signaling Technology, Beverly, MA and Santa Cruz Biotechnology, Santa Cruz, CA) were used at recommended dilutions. Species-specific anti-IG secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 633 (Invitrogen, Carlsbad, CA) were used together against anti-SMAD4 and anti-SMAD2/3, respectively. Alexa Fluor 488 and Alexa Fluor 568 were used together against anti-p47phox and anti-gp91phox, respectively. The sections were mounted in a ProLong gold antifade reagent with DAPI (Life Technologies, Eugene, OR). Images were taken under ×20×60 oil objectives using an Olympus BX51 microscope.
**Quantitative real-time RT-PCR.** Real-time RT-PCR was done to measure gene expression (mRNA) levels in the liver tissue samples. Total RNA was isolated from each liver tissue by homogenization in TRIzol reagent (Life Technologies) according to the manufacturer’s instruction and was purified by using RNeasy mini kit columns (Qiagen, Valencia, CA). iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to convert 1 μg of purified RNA to cDNA following the manufacturer’s standard protocol.

qRT-PCR was performed with the gene-specific primers by using SsoAdvanced universal SYBR Green supermix (Bio-Rad) and CFX96 thermal cycler (Bio-Rad). Threshold cycle ( Ct) values for selected genes were normalized against 18S (internal control) values in the same sample. For each gene and each tissue sample, each reaction was carried out in triplicate. A liver sample from DIO mice was used as a control to compare with all other liver samples in the toxim model of NASH. The relative fold change was calculated by the 2^(-ΔΔCt) method. The sequences for the primers used for real-time PCR are as follows: human p47phox forward: GT ACCA GGGCACA GTATG, reverse: CCTGGCTTTGCTTTCATCTG; mouse p47phox forward: GTGCAGATCCGGCAACGC, reverse: TGTGCCATCGTGT CACGC.

**miR21 expression levels in liver tissues.** Total miRNA was isolated from liver tissue by homogenization in Qiazol (Qiagen) following the manufacturer’s instructions. The purification was done by using miRNeasy mini kit columns (Qiagen). Purified miRNA (1,000 ng) was converted to cDNA using miScript cDNA synthesis kit (Qiagen) following the manufacturer’s protocol. qRT-PCR was performed with miRNA-specific primers (Qiagen) using miScript SYBR Green PCR master mix (Qiagen) and CFX96 thermal cycler (Bio-Rad). Ct values for the selected gene were normalized against RNU6-2 (internal miR expression control) values in the same sample.

**Western blotting.** Thirty milligrams of each liver tissue were homogenized in 500 μl of RIPA buffer (Sigma-Aldrich) in the presence of phosphatase and protease inhibitor (Pierce, Rockford, IL) by using dounce homogenizer. After centrifuging the homogenate, we collected the supernatant for further experiments. Thirty micrograms of protein from each sample were loaded on 4 – 12% Bis-Tris gradient gels (Invitrogen) for SDS-PAGE. By using precast nitrocellulose/filter paper sandwiches (Bio-Rad) and Trans-Blot Turbo transfer system (Bio-Rad), proteins were transferred to nitrocellulose membrane. Blots were blocked with 5% nonfat milk solution. Primary antibody against SMAD7, leptin, and β-actin (purchased from Abcam) were used at recommended dilutions and incubated overnight at 4°C. Species-specific secondary antibody conjugated with HRP was used. Pierce ECL Western Blotting substrate (Thermo Fisher Scientific, Rockford, IL) was used. The blot was developed by using BioMax MS Films and cassettes (with intensifying screen, Kodak). Densitometry analysis of the images was done using Lab Image 2006 Professional 1D gel analysis software from KAPLEAN Bioimaging Solutions (Liepzig, Germany).

**NF-κB transcription factor binding assay.** Nuclear fractions of each liver sample were isolated by using NF-κB assay kit and following manufacturer’s standard protocol (Five photon Biochemicals, San Diego, CA). DNA binding activity of the transcription factor NF-κB present in those nuclear extracts was detected using the ELISA method (Abcam NF-κB p65 Transcription Factor Assay Kit) following the manufacturer’s standard protocol.

**Statistical analyses.** In vivo experiments were repeated two to three times with an average of three mice per group with other in vivo experiments having four to six mice per group (n = 3/4/6 wherever applicable; data from each group of mice were pooled). The statistical analysis of data was done by ANOVA followed by Bonferroni post hoc correction for performing intergroup comparisons. Quantitative data from Western blots, depicted by the relative intensity of the bands, were analyzed by performing Student’s t-test. *P < 0.05 and ##P < 0.01 are considered statistically significant.

**RESULTS**

Leptin induces NADPH oxidase subunit p47phox and membrane association with gp91phox in NASH of both mice and humans. We have shown previously that leptin induces p47phox in early steatohepatitis, leading to the formation of peroxynitrite and corresponding inflammation and Kupffer cell activation (3). To prove the role of leptin in induction of p47phox in fibrogenesis of NASH, experiments were performed in both a mice model of NASH and human tissue samples from patients with NASH. Results showed that the DIO + BDCM group that shows typical NASH lesions had a 1.6-fold increase in p47phox mRNA expression compared with the DIO group alone (P < 0.05) (Fig. 1A). Leptin KO (ob/ob) mice showed a significant decrease in p47phox expression compared with the DIO + BDCM group (P < 0.05), whereas leptin supplementation in leptin KO (ob/ob + leptin) mice resulted in a significant upregulation of p47phox mRNA compared with only leptin KO (ob/ob) mice (P < 0.01). Human NASH (Hu NASH) tissues showed a significant increase in p47phox mRNA expression compared with controls (Hu CTRL) (P < 0.05) (Fig. 1B). p47phox subunit of NADPH oxidase requires its association with its membrane counterpart gp91phox for activation of this enzyme. We studied the membrane association of these two subunits by immunofluorescence microscopy in both mice and human tissue samples. Results were analyzed based on the number of colocalization/overlay events (shown by yellow) per 300 cells counted (Fig. 1, C and D). Results showed that the DIO + BDCM group had a significant increase in the colocalization events compared with the DIO only group (P < 0.01) (Fig. 1, C and D). Leptin KO (ob/ob) mice had a significant decrease in the number of colocalization events compared with the DIO + BDCM group (P < 0.01). Leptin-supplemented leptin KO mice (ob/ob + leptin) had a significant increase in the colocalization events compared with only leptin KO (ob/ob) mice (P < 0.05) (Fig. 1, C and D). Human NASH samples showed a significant increase in the p47phox/gp91phox colocalization compared with human controls (Fig. 1, C and D) (P < 0.05). The above results suggest that leptin induced p47phox expression in NASH and aided in the membrane association of p47phox and gp91phox that assumes significance in causing oxidative stress in NASH.

Leptin induced NADPH oxidase activation causes peroxynitrite-mediated oxidative stress in NASH of both mice and humans. We have previously shown that peroxynitrite-mediated oxidative stress plays a crucial role in activating Kupffer cells in early steatohepatitic lesions in NASH (3). To prove that leptin induced activation of NADPH oxidase in causing oxidative stress through peroxynitrite formation, experiments were performed in vivo to estimate 3-nitrotyrosine immunoreactivity in these tissues (Fig. 2). Results showed that 3-nitrotyrosine immunoreactivity was significantly increased in the DIO + BDCM group compared with the DIO group (P < 0.05) (Fig. 2, A and B). Leptin KO (ob/ob) mice had a significant decrease in 3-nitrotyrosine immunoreactivity compared with DIO + BDCM group (P < 0.05). Leptin-supplemented leptin KO (ob/ob + leptin) mice showed a significant increase in 3-nitrotyrosine immunoreactivity compared with the DIO group (P < 0.05).
significant increase in the 3-nitrotyrosine immunoreactivity compared with leptin KO (ob/ob) mice ($P < 0.05$). Human NASH samples ($n = 3$) showed a significant increase in 3-nitrotyrosine immunoreactivity compared with human controls, as shown by morphometry analysis of the slides ($P < 0.05$) (Fig. 2B).

All morphometric analysis of the stained liver sections was expressed as a percentage area of immunoreactivity (Fig. 2B). The results suggested that leptin was significantly associated with an increase in 3-nitrotyrosine immunoreactivity, and peroxynitrite might be a prominent player in causing oxidative stress.

Leptin-induced NADPH oxidase activation mediates NF-$\kappa$B translocation to the nuclei and upregulation of miR21. NADPH oxidase-mediated oxidative stress has been shown to induce NF-$\kappa$B translocation (42). NF-$\kappa$B translocation and its binding to miR21 promoter are crucial for many inflammatory events in disease progression (37). To prove that leptin, through its activation of NADPH oxidase in vivo causes NF-$\kappa$B translocation and binding to DNA, we performed experiments with liver tissue of both mice and humans. Results showed that NF-$\kappa$B activation and DNA binding were significantly increased in the DIO + BDCM group compared with the DIO only group ($P < 0.05$) (Fig. 3A). Leptin KO (ob/ob) mice showed a significant decrease in the NF-$\kappa$B activation and binding compared with the DIO + BDCM group ($P < 0.05$). p47phox KO mice, which cannot participate in NADPH oxidase-mediated oxidative stress, also showed a significant decrease in NF-$\kappa$B activation and binding to DNA compared with the DIO + BDCM group ($P < 0.05$) (Fig. 3A). Human NASH samples from liver showed a significant increase in

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**Fig. 1.** Leptin induces NADPH oxidase subunit p47phox and membrane association with gp91phox. A: qRT-PCR analysis of p47phox mRNA expression of diet-induced obesity (DIO), DIO + bromodichloromethane (BDCM), ob/ob, and ob/ob + leptin mice samples normalized against DIO. B: qRT-PCR analysis of p47phox mRNA expression of human (Hu) nonalcoholic steatohepatitis (NASH) samples normalized against Hu control (CTRL). C: immunofluorescence dual-labeling microscopy depicting gp91phox (red)-p47phox (green) colocalization (yellow) in DIO (i), DIO + BDCM (ii), ob/ob (iii), ob/ob + leptin (iv), Hu CTRL (v), and Hu NASH (vi) liver samples taken at ×60 (oil) magnification. D: morphometric analysis of colocalization events of C, shown in colocalization events/500-cell unit. *$P < 0.05$ and #$P < 0.01$ are considered statistically significant.
NF-κB activation and binding to DNA compared with corresponding control tissues (P < 0.01) (Fig. 3B). The DIO + BDCM group had a significant increase in miR21 levels (4-fold) compared with the DIO group (P < 0.01) (Fig. 3C). Leptin KO (ob/ob) or p47phox KO mice had a significant decrease in miR21 levels compared with the DIO + BDCM group (P < 0.01) (Fig. 3C). However, leptin supplementation to leptin KO (ob/ob + leptin) mice had a significant increase in miR21 levels compared with leptin KO (ob/ob) mice and had comparable levels of miR21 with the DIO + BDCM group (P < 0.01) (Fig. 3C). Human liver samples that had NASH etiology had significantly higher miR21 levels (4.5-fold) compared with human control tissues (P < 0.01) (Fig. 3D). The above results suggested that leptin-mediated NADPH oxidase activation was significantly associated with increased miR21 levels in NASH. The results assume significance because miR21-mediated repression of its target proteins can play a major role in disease pathology.

Leptin-mediated NADPH oxidase activation and its corresponding induction of miR21 repress regulatory SMAD protein in both mice and human NASH. miR21 has been found to repress many functional proteins in disease pathology (31). SMAD7, a regulatory protein in the TGF-β signaling cascade, has been shown to be a target for miR21 (6). We studied the role of the leptin-NADPH oxidase axis in mediating miR21-induced SMAD7 repression. Results showed that protein levels of SMAD7 were significantly elevated during the initial phases of NASH development (DIO + BDCM, 48 h) compared with the DIO group. The protein levels of SMAD7 decreased significantly at 1 wk and at the termination of the study (4 wk) that showed full-blown NASH symptoms and correlated well with fibrotic lesions (P < 0.05) (Fig. 4, A and C). miR21 KO mice showed a significant increase in SMAD7 protein expression compared with the DIO + BDCM group at 4 wk (P < 0.01) (Fig. 4, B and D). Leptin KO (ob/ob) mice or p47phox KO mice or DIO + BDCM mice that were depleted with macrophage toxin GdCl3 did not show any significant protein levels of SMAD7 (Fig. 4, B and D). This is not surprising because these mice did not have oxidative stress and there was no induction of SMAD7 in the initial phases (data not shown). Human NASH tissues showed a significant decrease in SMAD7 protein levels, as analyzed by Western blot compared with corresponding controls (P < 0.01) (Fig. 4, B and D). The results suggested that the increased miR21 in mice and human NASH is strongly associated with decreased SMAD7 protein. The results assume significance because SMAD7 plays a crucial role in regulating and inhibiting TGF-β signaling, crucial for fibrogenesis in NASH.

Leptin-mediated NADPH oxidase activation and corresponding induction of miR21 regulate TGF-β levels in mice and human NASH. TGF-β has been shown to be key for NASH fibrogenesis (10). To prove the role of leptin-mediated NADPH oxidase induction of miR21 in regulating TGF-β levels in NASH, experiments were performed with liver slices of mice and human NASH tissues, and we assessed the immunoreactivity by using immunohistochemistry. Results showed that the DIO + BDCM group had a significantly increased level of TGF-β compared with the DIO group (P < 0.01) (Fig. 5, A and B). Leptin KO (ob/ob), p47phox KO, and miR21 KO mice had a significant decrease in TGF-β immunoreactivity compared with the DIO + BDCM group (P < 0.01) (Fig. 5, A and B). Leptin supplementation to leptin KO (ob/ob + leptin) mice showed a significant increase in TGF-β immunoreactivity compared with leptin KO (ob/ob) mice alone (P < 0.05) (Fig. 5, A and B). Human NASH liver slices showed a significant increase in TGF-β immunoreactivity compared with control tissues, as shown by percentage area covered by the immunoreactive staining (morphometric analysis) (P < 0.05) (Fig. 5, A and B). The results suggested that leptin-mediated NADPH oxidase activation and miR21 induction play a crucial role in TGF-β levels in the livers of NASH.

Leptin-mediated NADPH oxidase activation and corresponding induction of miR21 in NASH are crucial for regulation of SMAD2/3-SMAD4 assembly in the nucleus, a key event in TGF-β signaling pathway. TGF-β signaling through SMAD2/3 is crucial for collagen deposition and fibrogenesis...
It has been shown previously that SMAD2/3 binds with SMAD4 and translocates to the nucleus. The nuclear colocalization of the SMAD2/3-SMAD4 heterodimer complex is a key event in the TGF-β functional pathway. TGF-β induces heteromeric complexes of SMAD2, 3, and 4, and their concomitant translocation to the nucleus, which is required for efficient TGF-β signal transduction (23). To study the role of leptin-mediated NADPH oxidase activation and miR21 induction in TGF-β signal transduction pathway, experiments were performed using liver tissue slices. Immunoreactivity to SMAD2/3 and SMAD4 and their nuclear colocalizations was assessed by immunofluorescence microscopy. Results showed that the number of colocalization events in the nucleus were significantly higher in the DIO/BDCM group compared with the DIO group (*P < 0.01) (Fig. 6, A and C). The colocalization events in the leptin KO (ob/ob), p47phox KO, and miR21 KO were significantly decreased compared with the DIO + BDCM group (P < 0.01 and P < 0.05). Leptin supplementation to leptin KO (ob/ob + leptin) mice significantly increased the number of nuclear colocalization events compared with leptin KO (ob/ob) mice alone (*P < 0.05 and #P < 0.01). Human NASH samples showed significant increase in SMAD2/3 and SMAD7 nuclear colocalizations compared with human liver controls (P < 0.05) (Fig. 6, A and C). The results were analyzed based on the number of nuclear colocalizations per 300 cells (Fig. 6C). Oil micrographs (×60) of the DIO + BDCM group in the mouse model and human NASH liver are shown in Fig. 6B. The results show that increased TGF-β-induced signaling through SMAD2/3 and SMAD4 is regulated by leptin-mediated NADPH oxidase and its subsequent upregulation of miR21.
Leptin-mediated NADPH oxidase activation and subsequent miR21 induction regulate the levels of TGF-β signaling proteins. CTGF and EDAFN were evaluated in a mice model of NASH and human NASH livers. In fibrotic liver, CTGF is constantly expressed in activated hepatic stellate cells and acts downstream of TGF-β to modulate ECM production (18). On the other hand, FN is a major component of the ECM and occurs in two main forms: plasma and cellular FN. The latter includes the alternatively spliced domain A (EDA) (15). EDAFN has been shown to participate in TGF-β-mediated fibroblast differentiation and fibrogenesis (1). To prove the role of leptin-mediated NADPH oxidase activation and subsequent induction of miR21 in TGF-β signaling, immunohistochemical analysis of the mice liver and human liver tissues was performed. Results showed that there was a significant increase in the immunoreactivity of CTGF and EDAFN in the DIO + BDCM group compared with the DIO group (P < 0.01 and P < 0.05) (Fig. 7A, ii, and 7B, ii) (Fig. 7, C and D, for morphometric analysis). On the other hand, leptin KO (ob/ob), p47phox KO, and miR21 KO mice had a significant decrease in the CTGF and EDAFN levels compared with the DIO + BDCM group (Fig. 7A, iii, v, and vi) (Fig. 7B, iii, v, and vi) (Fig. 7, C and D for morphometric analysis) (P < 0.05). Leptin supplementation in leptin KO (ob/ob + leptin) mice showed a significant increase in the levels of immunoreactivity of both these crucial proteins (P < 0.05) (Fig. 7A, iv, and 7B, iv) (Fig. 7, C and D, for morphometric analysis). Human NASH livers had a significant increase in the immunoreactivity of CTGF and EDAFN compared with control livers (P < 0.05) (Fig. 7A, viii, and 7B, viii) (Fig. 7, C and D, for morphometric analysis). The above results show that leptin-mediated NADPH oxidase activation and subsequent miR21 inhibition of SMAD7 are closely associated with increased TGF-β signaling, resulting in higher CTGF and EDAFN levels in NASH.

Leptin-mediated NADPH oxidase activation and miR21 induction cause stellate cell activation and collagen deposition in NASH. Stellate cell activation is a key event for NASH progression (36). α-SMA is a biomarker for stellate cell activation (5). Importantly, increased fibrogenesis and TGF-β signaling result in higher ColIα1 levels in tissues. To show the role of leptin-mediated NADPH oxidase in stellate cell activation and fibrogenesis, immunohistochemical analyses of mice and human liver tissues were performed. Results showed that immunoreactivity to α-SMA and ColIα1 was significantly high in the DIO + BDCM group compared with the DIO group (P < 0.01) (Fig. 8A, ii, and 8B, ii) (morphometric analysis Fig. 8, C and D). Leptin KO (ob/ob), p47phox KO, and miR21 KO mice had significantly decreased immunoreactivity of α-SMA and ColIα1 compared with the DIO + BDCM group (P < 0.01) (Fig. 8A, iii, v, and vii; 8B, iii, v, and vi) (morphometric analysis Fig. 8, C and D). Leptin supplementation in leptin KO (ob/ob + leptin) mice showed a significant increase in the levels of
both α-SMA and Col1α compared with the leptin KO mice (ob/ob) group alone (P < 0.05) (Fig. 8A, iv, and 8B, iv) (morphometric analysis Fig. 8, C and D). Human NASH liver exhibited a significant increase in α-SMA and Col1α, shown by increased immunoreactivity to these proteins and morphometric analysis of the tissue staining (P < 0.05) (Fig. 8A, viii, and 8B, viii) (morphometric analysis Fig. 8, C and D). The results suggested that leptin-mediated NADPH oxidase and the subsequent induction of miR21 regulated the stellate cell activation and corresponding collagen deposition in both mice and human NASH livers.

**Leptin-mediated NADPH oxidase activation and miR21 induction cause fibrogenesis in NASH.** Analysis of picrosirius staining for collagen showed increased reactivity in the DIO + BDCM group, especially in the perportal regions, compared with the DIO group (Fig. 9A, ii). Leptin KO (ob/ob), p47phox KO, or miR21 KO mice showed a minimal fibrotic stain compared with the DIO + BDCM group (Fig. 9A, iii, v, and vi). Leptin supplementation sharply increased perportal staining of picrosirius red (Fig. 9A, iv), whereas human tissues for NASH showed higher picrosirius red staining compared with controls (Fig. 9A, viii). To show a correlation between the levels of tissue leptin and fibrotic stage, leptin immunoreactivity in mouse liver homogenates at 24 h, 48 h, 1 wk, and 4 wk was assessed by Western blot followed by normalizations against β-actin (Fig. 9, B and C) and by picrosirius red staining depicting fibrosis at 24 h, 48 h, 1 wk, and 4 wk in the DIO + BDCM group (Fig. 9D). Results showed that increased leptin concentrations correlated well with perportal and bridging fibrosis at 4 wk following the toxin exposure in an underlying condition of steatosis (NASH model).

**DISCUSSION**

In this study, we report for the first time the role of leptin-mediated NADPH oxidase in inducing miR21 in a preclinical NASH model as well as in human patients with NASH. We also show the mechanistic role of miR21 in repressing SMAD7, a crucial regulator of TGF-β signaling, thus modulating fibrogenesis in NASH (Fig. 10). Significantly, the miR21 modulation of TGF-β signaling, fibrogenesis, and NASH progression were mediated by NADPH oxidase in our present...
study. The study assumes huge clinical significance because the role of NADPH oxidase has been shown in fibrogenesis by us and others, but the link between NADPH oxidase-based oxidative stress signaling through posttranscriptional epigenetic changes and TGF-β signaling remained largely elusive in vivo.

An earlier report from our laboratory showed that leptin-mediated NADPH oxidase activation activated Kupffer cells largely through the formation of peroxynitrite in an early stage of steatohepatitic injury (3). Through the present study, we advanced our understanding about the role of leptin-mediated NADPH oxidase-based oxidative stress in a full-blown NASH model and in human NASH livers. The present study used a rodent model of NASH that had a hepatotoxin BDCM as a second hit (36). All our investigations were carried out at a time point where NASH symptoms were confirmed using clinical indicators. NADPH oxidase subunit p47phox induction and subsequent activation were observed in these liver tissues (Fig. 1, A–D). The induction and subsequent activation of NADPH oxidase were dependent on the presence of leptin because leptin KO mice (ob/ob) or leptin supplementation (100 ng, daily for 4 wk) in leptin KO (ob/ob) mice had opposite outcomes (Fig. 1). On the basis of our previous reports in the same model, we also established the nature of the oxidizing species because we observed significant increase in 3-nitrotyrosine immunoreactivity, a measure for generation of highly reactive peroxynitrite (Fig. 2, A and B).

Highly reactive oxidizing species have been shown to induce changes in the epigenetic foci and cause increases in the expression of noncoding RNAs (16). There are several miRNAs that are known to be induced in an inflammatory condition (31, 37). NASH has been shown to induce several miRNAs with specific functions targeted toward its progression (4, 21, 29). miR21 is induced in an inflammatory microenvironment and has been shown to be induced in disease pathophysiology following the
Fig. 7. Leptin-mediated NADPH oxidase activation and subsequent induction of miR21 regulate TGF-β signaling proteins. Connective tissue growth factor (CTGF) and extra domain A-fibronectin (EDAFN) in mice and human NASH. A: CTGF immunoreactivity as shown by immunohistochemistry in liver slices from the DIO (i), DIO+BDCM (ii), ob/ob (iii), ob/ob + leptin (iv), p47phox KO (v), miR21 KO (vi), Hu CTRL (vii), and Hu NASH (viii) groups, respectively. Images were taken at ×20 magnification. B: EDAFN immunoreactivity as shown by immunohistochemistry in liver slices from the DIO (i), DIO+BDCM (ii), ob/ob (iii), ob/ob + leptin (iv), p47phox KO (v), miR21 KO (vi), Hu CTRL (vii), and Hu NASH (viii) groups, respectively. Images were taken at ×20 magnification. C: morphometric analysis of the immunoreactivity of A. D: morphometric analysis of the immunoreactivity of B. *P < 0.05 and #P < 0.01 are considered statistically significant.
Fig. 8. Leptin-mediated NADPH oxidase activation and miR21 induction cause stellate cell activation and collagen deposition in NASH. A: α-smooth muscle actin (SMA) immunoreactivity as shown by immunohistochemistry in liver slices from the DIO (i), DIO + BDCM (ii), ob/ob (iii), ob/ob + leptin (iv), p47phox KO (v), miR21 KO (vi), Hu CTRL (vii), and Hu NASH (viii) groups, respectively. Images were taken at ×20 magnification. B: Coll1α immunoreactivity as shown by immunohistochemistry in liver slices from the DIO (i), DIO + BDCM (ii), ob/ob (iii), ob/ob + leptin (iv), p47phox KO (v), miR21 KO (vi), Hu CTRL (vii), and Hu NASH (viii) groups, respectively. Images were taken at ×20 magnification. C: morphometric analysis of the immunoreactivity of A. D: morphometric analysis of the immunoreactivity of B. *P < 0.05 and #P < 0.01 are considered statistically significant.
binding of nuclear transcription factor NF-κB to its promoter (37). In the present study, we show that NF-κB binding activity to the DNA was significantly higher in the DIO + BDCM group (NASH mouse model) and human NASH livers (Fig. 3, A and B). The activation NF-κB was dependent on leptin and NADPH oxidase activation because leptin KO (ob/ob) mice and p47phox KO mice had significantly lower NF-κB activation (Fig. 3A). Human livers had a significant increase in NF-κB activation, an index of oxidative stress and inflammatory signaling pathway (Fig. 3B). Coupled with the NF-κB activation, our studies also showed that the miR21 levels in the NASH livers were significantly elevated (Fig. 3, C and D). The levels of miR21 were dependent on the NADPH oxidase subunit p47phox and leptin. The results correlate well with observations reported previously about NF-κB-mediated miR21 induction in vitro through a binding of NF-κB to the miR21 promoter region (32). However, the above phenomenon could not be established in the in vivo models of NASH because of procedural complications involving transfection in primary cells from the NASH mice at this point. Thus it can be justified to assume that NF-κB translocation in the NASH livers might bind to miR21 promoter, causing its induction, apart from the other functions that it is predicted to perform in NASH (Fig. 3, C and D).

A recent review by Noetel et al. (25) describes the current knowledge of the role of miR21 in liver fibrosis. Inflammatory microenvironments in the NASH livers are predicted to promote TGF-β signaling, which in turn can stimulate SMAD2/3 phosphorylation and their association with SMAD4. Furthermore, the above events can upregulate miR21, which by suppressing SMAD7 can further add to the increased TGF-β-mediated profibrogenic responses, events that are known in other fibrotic mechanisms in the lung and kidney (6, 17). We are not aware of any reports that establish a direct role of NADPH oxidase activation-induced miR21 in TGF-β signaling and subsequent liver fibrosis. To prove the role of miR21-induced SMAD7 repression and SMAD2/3-SMAD4 interactions, we used miR21 KO mice in parallel to p47phox and leptin KO (ob/ob) mice. Results showed that miR21 KO mice that show significantly lower inflammation in similarly used NASH models (Pourhoseini S, Seth RK, Das S, Dattaroy D, Kadiiska M, Nagarkatti M, Michelotti GA, Diehl AM, and Chatterjee S, unpublished data) had higher SMAD7 levels when treated identically with high-fat diet and hepatotoxin challenge compared with the DIO + BDCM group (Fig. 4, B and D). Interestingly, SMAD7 proteins were significantly decreased in human NASH livers compared with controls (Fig. 4, B and D). Leptin KO (ob/ob) and p47phox KO mice had a small increase in protein levels compared with the DIO + BDCM group at 4 wk, but the reported increase was not statistically significant (Fig. 4D). This might be due to the genetic makeup of these mice and a very low level of inflammation that triggers SMAD7 protein expression. Also, it is
worth reporting the levels of TGF-β in leptin, p47phox, and miR21 KO mice (Fig. 5, A and B). Results showed that these KO mice had significantly decreased TGF-β levels compared with the DIO + BDCM group (Fig. 5). The results also establish that TGF-β levels are significantly regulated upstream of miR21 induction, and leptin, NADPH oxidase, and miR21 are linked to cause an upregulation of TGF-β.

TGF-β signaling is crucial for fibrogenesis in NASH. SMAD2/3 association with SMAD7 in the nucleus is a crucial event in this process (22). This important mechanistic association and its regulation by miR21 have never been shown in liver fibrosis in vivo or in human NASH livers. We show by immunofluorescence imaging ex vivo the colocalization of SMAD2/3-SMAD4 in the nuclei of NASH livers (Fig. 6). Murine and human NASH livers had a significant increase in the number of colocalization events compared with corresponding controls, whereas leptin, p47phox, and miR21 KO mice had significantly fewer colocalization events compared with NASH groups (Fig. 6C). The results described in this study assume great significance because we show that NADPH oxidase through miR21 not only repressed SMAD7 as a profibrogenic mechanism, but also contributed partly to enhance SMAD2/3-SMAD4 association in the nucleus, a key event in liver fibrogenesis. The leptin-mediated NADPH oxidase induction of miR21 also played a significant role in the downstream events of TGF-β signaling pathway by regulating the levels of CTGF and the intracellular form of FN (EDAFN) (Fig. 7).

Finally, we studied the implications of the leptin-mediated NADPH oxidase activation and subsequent miR21 upregulation in stellate cell activation, collagen levels, and fibrosis, key events in NASH pathogenesis. Predictably, our results showed a direct involvement of leptin-mediated NADPH oxidase and a miR21 role in fibrogenesis because leptin KO (ob/ob), p47phox KO, or miR21 KO mice had significantly decreased levels of α-SMA, Col1α, and picrosirius red staining compared with mouse and human NASH models (Figs. 8 and 9). Importantly, NASH pathogenesis for proving stellate cell activation and fibrosis in both preclinical and clinical settings relies on the levels of α-SMA, Col1α, and picrosirius red staining.

In summary, we show that leptin-mediated NADPH oxidase upregulated the levels of miR21 through NF-kB activation predictively by binding to the miR21 promoter in vivo, observations that have been reported previously. The resultant upregulation of miR21 caused fibrogenesis in NASH by SMAD2/3-SMAD4 association in the nucleus and SMAD7 repression. Our results will help advance the current knowledge about the mechanisms of NADPH oxidase-mediated fibrogenesis in NASH and will stimulate newer studies that can help develop therapeutic approaches for this clinically silent disease.

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DISCLOSURES

No conflicts of interest financial or otherwise are declared by the authors.

Fig. 10. Schematic representation of the role of leptin-induced miR21 in modulating TGF-β pathway in NASH fibrogenesis. SBE, SMAD binding element.
AUTHOR CONTRIBUTIONS


REFERENCES


