Leptin receptor blockade reduces intrahepatic vascular resistance and portal pressure in an experimental model of rat liver cirrhosis

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Leptin, a hormone of 16 kDa expressed from the ob gen (9), regulates weight, appetite, and body thermogenesis and prevents fat storage in nonadipose tissue (35). Although leptin is produced primarily by adipocytes, expression of leptin and of its receptors has been described in other tissues such as heart, skeletal muscle, reproductive system, immune system, endothelium, and liver (39). Elevated levels of leptin, probably produced by hepatic cells in response to inflammation, have been described in experimental models of fibrosis and cirrhosis and in cirrhotic patients (5, 30, 31, 37).

Leptin has been shown to impair vascular tone and NO bioavailability. Leptin administration to obese rats impairs acetylcholine-mediated coronary vasodilatation, both in vivo and ex vivo (23). Recent studies demonstrate that leptin is able to increase $O_2^-$ production in endothelial cells, leading to a marked decrease in NO levels together with an increase in peroxynitrite accumulation (24). On the other hand, leptin is actively involved in liver fibrogenesis. The absence of leptin or its receptor, as occurs in ob/ob mice or fa/fa rats, is associated with a significant reduction of fibrosis in experimental models of liver injury (2). Moreover, exogenous leptin administration potentiates progression of liver fibrosis (6, 19, 21, 28), a situation that is prevented administering leptin antagonists (7).

Given the above, the present study aimed at investigating whether 1-wk leptin blockade has a role modulating the increased hepatic vascular tone observed in cirrhosis. For this, we tested the effects of a nonselective blockade of leptin receptor using monoclonal antibodies (ObR-Ab) (32) on the hepatic and systemic hemodynamics and tested hepatic NO bioavailability in an experimental model of cirrhosis induced by CCl₄ in rats.

MATERIALS AND METHODS

Animals and induction of cirrhosis. Male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 50–75 g were induced to cirrhosis by inhalation of carbon tetrachloride (CCl₄) three times a week. Phenobarbital (0.3 g/l) was added to the drinking water as previously described (10). When cirrhotic rats developed ascites (after 14–16 wk), administration of CCl₄ and phenobarbital was stopped and treatment was started 1 wk later. Control animals only received phenobarbital. Animals were kept in environmentally controlled animal facilities at the Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

PORTAL HYPERTENSION IS THE main complication of cirrhosis of the liver. It is mainly due to increased intrahepatic resistance, which results from structural changes inherent to progressive fibrosis and dynamic changes due to increased hepatic vascular tone (11). Deficient nitric oxide (NO) bioavailability within the liver circulation, derived both from reduced NO synthesis by endothelial NO synthase (eNOS) (17, 27, 34) and increased vascular tone (11). Deficient nitric oxide (NO) bioavailability within the liver. It is mainly due to increased intrahepatic resistance, which results from structural changes inherent to progressive fibrosis and dynamic changes due to increased hepatic vascular tone (11).

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Characterization of leptin and leptin receptor ObR in control and cirrhotic animals. Leptin levels were analyzed in serum from control and cirrhotic rats (n = 12 per group) by using a commercially available enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI) following manufacturer’s instructions. Leptin receptor protein expression was assessed by Western blot in hepatic homogenates from control and cirrhotic animals (n = 6 per group) as described below. ObR antibody specificity was validated by analyzing leptin receptor expression in liver tissue from db/db mice (Charles River Laboratories).

Leptin receptor antagonist ObR-Ab administration. SH-SY5Y human neuroblastoma cells (kindly provided by Dr. Sanfelici, IIBB/CSIC/IDIBAPS) were used to test the specificity of the Ob receptor (all isoforms) blocker antibody (ObR-Ab, Alpha Diagnostic International, San Antonio, TX). These cells express active ObR that upon leptin administration signals through Signal Transducer Activator of Transcription 3 (STAT3). Cells cultured in DMEM supplemented with retinoic acid were starved for 16 h, incubated with ObR-Ab (200 nM) or its vehicle for 1 h, and afterward stimulated with leptin (100 ng/ml, R&D Systems, Minneapolis, MN), IL-6 (50 ng/ml, R&D Systems) or vehicle for 15 min (1). ObR blockade was defined as a reduction in the ratio of phosphorylated STAT3 (p-STAT3) to total STAT3 determined by Western blot (1, 32).

In addition, adequate hepatic leptin receptor blockade was analyzed by administering ObR-Ab (4 or 8 μg/kg body wt) or its vehicle 2 h before administering leptin (1 μg/g body wt) to control rats, and evaluating p-STAT3/STAT3 in liver samples.

Once ObR-Ab efficacy was confirmed, ObR-Ab (8 μg/kg, n = 12) or IgG (8 μg/kg, n = 12) was administered to cirrhotic rats via the tail vein every other day for 1 wk. Drug administration and subsequent experiments were performed blindly. In cirrhotic animals, hepatic ObR blockade was further validated by analyzing IL-6 mRNA expression (26).

Splanchnic and systemic in vivo hemodynamic studies. Two hours after the last dose of ObR-Ab or vehicle, rats were anesthetized with ketamine (100 mg/kg ip, Imalgene, Barcelona, Spain) and midazolam (5 mg/kg ip, Laboratorio Reig Jofre, Barcelona, Spain). A tracheotomy and cannulation with PE-240 catheter (Portex, Kent, UK) was performed to maintain adequate ventilation during the anesthesia. Then PE-50 catheters were introduced into femoral artery and ileocolic vein to continuously measure mean arterial pressure and portal pressure, respectively. A perivascular nonconstrictive ultrasonic flow probe (T206, Transonic Systems, Ithaca, NY) placed around the portal vein, as close as possible to the liver, measured the portal vein blood flow. Blood pressures and flows were registered on a multichannel computer-based recorder (Powerlab 4SP, ADInstruments; Mountain View, CA). Temperature was monitored and maintained at 37 ± 0.5°C during all procedure, and after 20 min of stabilization hemodynamic values were taken. At the end of the in vivo hemodynamic study, blood samples from cava vein were obtained and stored for further determinations. Intrahepatic vascular resistance was calculated as portal pressure/portal vein blood flow.

Nitric oxide bioavailability. NO bioavailability in livers from rats treated with ObR-Ab or vehicle was assessed by analyzing its surrogate markers cyclic guanosine monophosphate (cGMP) and phosphorylated vasodilator-stimulated phosphoprotein (p-VASP). cGMP was determined by enzyme immunoassay (Cayman Chemical) (14). p-VASP was determined by Western blot.

Nitric oxide signaling pathway characterization. Activity of hepatic NO synthase was evaluated through three different approaches. The phosphorylated form of eNOS at Ser1176 (active) was analyzed and referred to total eNOS protein expression by Western blot. NO synthase activity was also analyzed by determining the conversion of 14C-labeled L-arginine to 14C-labeled L-citrulline, according to a previously reported method (29), and expressed as picomoles per minute per gram protein. Additionally, eNOS dimer (“coupled” active form) and monomer (“uncoupled” inactive form) were determined using a nonreducing SDS-PAGE Western blot.

Evaluation of O2− and nitrotyrosine. Hepatic O2− levels were quantified using a commercially available assay (Sigma, Tres Cantos, Madrid, Spain) with minor modifications. Briefly, liver were homogenized in buffer containing 20 mM HEPES, 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose. After centrifugation at 1,500 g for 5 min at 4°C, the supernatant was recovered and incubated with WST-1 during 20 min at 37°C. O2−-derived absorbance at 440 nm was proportional to the amount of O2− radical. Positive (exogenous O2− generating enzyme) and negative (samples with high antioxidant capacity) internal controls were included. Nitrotyrosine content, as secondary marker of the O2−-mediated NO scavenging to form peroxynitrite, was analyzed in liver sections (10 μm) previously fixed in 10% formalin and embedded in paraffin, by use of a polyclonal rabbit anti-nitrotyrosine antibody (Millipore, Billerica, MA) as previously described (25).

Liver fibrosis evaluation. Paraffin-embedded liver slides were stained with Sirius red to detect fibrous tissue components. The degree of fibrosis was assessed by image analysis techniques with the freeware NIH Image J 1.38 (National Institutes of Health) as previously described (15). The results were expressed as a fibrosis ratio (%), calculated as the ratio of the Sirius red-positive area to the total area examined from 10 independent images for each animal. Additionally, α-smooth muscle actin (α-SMA) protein expression and procollagen I mRNA expression were analyzed by Western blot and quantitative real-time PCR, respectively.

Evaluation of hepatic function and injury. Albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured in serum by standard techniques.

Western blot analysis. Protein expression of ObR, STAT3, p-STAT3, eNOS, p-eNOS, p-VASP, and α-SMA was assessed by Western blot in livers from cirrhotic rats treated with ObR-Ab or IgG. Briefly, livers were collected, snap frozen in liquid N2, and homogenized in Triton-lysis buffer as previously described (13). Aliquots from each sample containing equal amounts of protein (40–100 μg) were run on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. To evaluate the eNOS dimer-to-monomer ratio, low-voltage and low-temperature SDS-PAGE was run with nonheated tissue homogenates. After the transfer, the blots were subsequently blocked for 1 h with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk and were subsequently incubated with a primary antibody recognizing ObR (Alpha Diagnostic International), STAT3 (Cell Signaling Technology, Beverly, MA), p-STAT3 (Cell Signaling), eNOS (BD Transduction Laboratories, Lexington, KY), p-eNOS (Cell Signaling), p-VASP (Calbiochem, Darmstadt, Germany), or α-SMA (Sigma) overnight at 4°C. Then membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature.

Protein expression was determined by densitometric analysis using the Science Lab Image Gauge (Fuji Photo Film GMBH, Düsseldorf, Germany). After stripping, blots were assayed for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) expression as standardization of sample loading. Quantitative densitometric values of all proteins were normalized to GAPDH.

Quantitative real-time TaqMan PCR. Hepatic RNA was isolated, and real-time TaqMan PCR was performed as previously described (15).

Statistical analysis. Statistical analysis was performed with the SPSS 18.0 for Windows statistical package (IBM, Armonk, NY). All results are expressed as means ± SE. Comparisons between groups were performed with the Mann-Whitney test for unpaired data. Differences were considered significant at a P value < 0.05.

RESULTS

Levels of leptin and its receptor in cirrhotic animals. Leptin circulating levels were significantly higher in serum from cirrhotic animals compared with controls (Fig. 1A). Similarly,
leptin receptor (ObR) expression in livers from cirrhotic animals was significantly higher than in control animals (Fig. 1B). As internal negative control, no ObR expression was detected in liver homogenates from db/db mice (Fig. 1B).

Effectiveness of ObR-Ab blocking leptin receptor. As previously described (1), leptin produced a significant upregulation in p-STAT3 in SH-SY5Y cells, which was blunted by pretreating cells with ObR-Ab (Fig. 1C). Contrarily, STAT3 activation induced by IL-6 was not affected by ObR-Ab (Fig. 1C), thus validating its specificity for ObR.

Similarly, after leptin administration to control animals, the hepatic expression of p-STAT3 was significantly lower in rats pretreated with ObR-Ab than in those treated with its vehicle, confirming an adequate leptin receptor blockade by ObR-Ab (Fig. 1D). Considering that the greater inhibition of p-STAT3 expression by ObR-Ab was achieved with the 8 μg/kg dose, this concentration was chosen for all subsequent experiments. In cirrhotic rats the ratio p-STAT3/STAT3, as well as the hepatic expression of IL-6, were significantly lower in the group treated with ObR-Ab compared with those treated with IgG, confirming an efficient blockade of the leptin receptor also in cirrhotic animals (Fig. 1, E and F).

Hemodynamic effects of ObR-Ab administration in cirrhotic rats. Cirrhotic rats receiving ObR-Ab exhibited significantly lower portal pressure than those receiving IgG-vehicle (mean decrease −19%; P = 0.02; Fig. 2) with no significant differ-

![Fig. 1. Characterization of leptin, leptin receptor, and leptin receptor blocking antibody. A: levels of leptin in serum from control and cirrhotic animals (n = 12 per group). B: representative Western blots and densitometric quantification of leptin receptor (ObR), and housekeeping protein GAPDH in livers from control and cirrhotic rats (n = 6 per group) #P < 0.05 vs. control. Inset: representative ObR and GAPDH immunoblots in wild-type (wt) and db/db mice. C: Western blots of phosphorylated-STAT3 (active form; p-STAT3), total STAT3, and GAPDH in SH-SY5Y cells incubated with leptin, IL-6, or vehicle, in the presence of ObR-Ab or its vehicle (representative images from 3 independent experiments). D: blots of depicted proteins and densitometric quantification determined in livers from control rats pretreated with ObR-Ab and afterward treated with a single dose of leptin (n = 3 per group). E: hepatic IL-6 mRNA expression in cirrhotic rats receiving ObR-Ab or its vehicle IgG (n = 8 per group). F: representative Western blots and quantification of depicted proteins in livers from cirrhotic rats treated with ObR-Ab or its vehicle IgG (n = 12 per group). Values represent means ± SE. *P < 0.05 vs. IgG.]

![Fig. 2. Effects of leptin receptor blockade on hepatic and splanchnic hemodynamics. Portal pressure (PP), portal blood flow (PBF), and intrahepatic vascular resistance (IVR) measured in CCl4-cirrhotic rats treated with ObR-Ab (8 μg/kg body wt) or its vehicle IgG every other day during 1 wk (n = 12 per group). Values represent means ± SE.]

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ences in portal blood flow. As a consequence, portal vascular resistance was lower in cirrhotic animals receiving ObR-Ab than in those treated with IgG; however, this difference did not reach statistical significance ($P = 0.1$; Fig. 2). ObR-Ab administration did not significantly modify mean arterial pressure ($102 \pm 7$ mmHg in IgG vs. $97 \pm 9$ mmHg in ObR-Ab group).

Effects of ObR-Ab administration on the nitric oxide pathway in the cirrhotic liver. To understand the molecular mechanisms partly responsible for the decrease in portal pressure, we characterized hepatic NO bioavailability, eNOS expression and activity, oxidative stress, and protein nitrotyrosination. Figure 3 shows that cirrhotic animals treated with ObR-Ab exhibited a significant increase in hepatic NO bioavailability, as indicated by the measurement of its secondary messenger cGMP, and by increased expression of p-VASP, a surrogate of NO activity, compared with cirrhotic animals receiving IgG. These observations were not associated with significant changes in eNOS or p-eNOS, in eNOS coupling status, or in eNOS activity (Fig. 4), altogether discarding a possible increase in NO biosynthesis by eNOS due to leptin receptor blockade. Contrarily, oxidative stress quantification evidenced that liver tissue from ObR-Ab-treated animals exhibited a significant reduction in $O_2^{-}$ levels that was associated with a significant reduction in protein nitrotyrosination, as shown by immunohistochemistry (Fig. 5).

Leptin receptor blockade effects on liver fibrosis. Liver fibrosis levels were characterized by Sirius red staining followed by computational analysis, and by α-SMA and procollagen I quantification. No differences in percentage of fibrotic...
tissue areas or in α-SMA protein expression were observed when comparing cirrhotic animals that received ObR-Ab or IgG; however, livers from animals receiving ObR-Ab exhibited reduced levels of procollagen I mRNA expression (Fig. 6).

Hepatic function and liver injury in response to ObR-Ab administration. There were no significant differences in the values of AST (157 ± 69 in IgG vs. 180 ± 88 U/l in ObR-Ab group), ALT (73 ± 19 in IgG vs. 80 ± 18 U/l in ObR-Ab group), and albumin (25 ± 4 in IgG vs. 24 ± 3 g/l in ObR-Ab group) between cirrhotic rats receiving IgG and those who received ObR-Ab.

DISCUSSION

Leptin, a hormone encoded by the Ob gene, has six recognized receptor isoforms (Ob-Ra-Ob-Rf) (36), but only the long receptor (Ob-Rb) mediates the biological actions of leptin through the activation of the JAK/STAT pathway (9, 36). Specifically, in the liver, Ikejima et al. (20) showed the presence of Ob-Rb and Ob-Ra receptors. Subsequently, another study confirmed that sinusoidal endothelial cells also express the gene for leptin (33). In patients with cirrhosis, regardless of its etiology, high serum levels of leptin have been described (18, 30, 39); moreover, a role for leptin in hepatic fibrosis has been hypothesized (2, 6, 21).

We herein show that cirrhotic animals exhibit elevated levels of leptin and hepatic expression of its receptor compared with control rats, mirroring what has been described in human cirrhosis. In addition, the present study demonstrates for the first time that the blockade of the receptor of leptin in cirrhotic rats is associated with a significant decrease in portal pressure.
In fact, portal pressure in ObR-Ab-treated animals was 19% lower than in rats treated with IgG. This reduction in portal pressure was not related to significant changes in portal blood flow, suggesting that it was due to a reduction in intrahepatic vascular resistance.

As discussed below, the mechanism by which leptin-receptor blockade decreases portal pressure appears to be related to a reduced hepatic vascular tone: the so-called dynamic component of the increased resistance to portal flow through the cirrhotic liver. Low hepatic NO bioavailability, due to reduced eNOS activity and increased NO scavenging by an excess of radical oxygen species, has been described as an essential mechanism contributing to increase hepatic vascular tone in cirrhosis (3, 22). The results of our study suggest that increased levels of leptin may contribute to increased portal pressure precisely by influencing these pathophysiological alterations. Indeed, herein we demonstrate that ObR-Ab administration to cirrhotic rats produced a significant increase in hepatic NO bioavailability, as evidenced by the significantly increased levels of hepatic cGMP and VASP phosphorylation. Subsequently, we ascertained the underlying mechanisms of NO increment by analyzing eNOS expression and activity, and O2- -mediated NO scavenging. Leptin receptor blockade was not associated with changes in eNOS protein expression or in eNOS activity, demonstrated by absence of changes in phosphorylated eNOS and in eNOS monomer/dimer expression. On the other hand, leptin receptor blockade resulted in a significant amelioration of oxidative stress and protein nitrotyrosination, which is a surrogate marker of scavenging of NO by O2- , producing peroxynitrite. Altogether these results suggest that the increment in hepatic NO bioavailability observed in rats treated with the leptin receptor-blocking antibody resulted from a reduction in O2- -levels, and therefore in NO scavenging, with no increase in eNOS activity. Previous studies suggested that leptin-derived endothelial O2- -mainly originates from NADPH oxidase and uncoupled eNOS (32). Our results showing no improvement in eNOS coupling status after leptin-receptor blockade, together with a previous report demonstrating a lack of role for NADPH oxidase in the pathogenesis of the increased hepatic vascular tone in cirrhosis (12), suggest that leptin may activate another prooxidant mechanism in cirrhotic hepatic sinusoidal cells, most likely by further decreasing hepatic superoxide dismutase activity. Although the present study mainly focused on characterizing the increment in hepatic NO bioavailability to explain the beneficial effects of ObR blockade, we cannot discard that the inhibition of other leptin-dependent pathways would also contribute to ameliorate portal hypertension in cirrhosis. As example, it is conceivable that leptin receptor blockade may downregulate VEGF-mediated angiogenesis (4), which we and others have demonstrated actively contributes to portal hypertension pathophysiology (8). Nevertheless future studies are required to validate this hypothesis. It is worthy to note that ObR-Ab administration had no deleterious effects on mean arterial pressure or in liver blood test.

As previously mentioned, elevated leptin levels in serum from cirrhotic patients have been considered as a mediator of enhanced liver fibrosis (21, 39). In the present study, no differences in hepatic fibrous tissue content or in hepatic stellate cells activation marker α-SMA were observed when comparing rats treated with ObR-Ab or its vehicle, indicating no net effects on liver fibrosis after 1-wk administration. Our results indicate that portal pressure reduction due to ObR-Ab administration is derived from an improvement in NO bioavailability rather than from a reduction in fibrosis. However, our data demonstrating reduced levels of procollagen I mRNA expression in livers from animals receiving ObR-Ab suggest that using higher doses or longer periods of treatment with ObR-Ab may have additional effects reducing liver fibrosis.

In conclusion, our study shows for the first time that leptin might be involved in the pathogenesis of portal hypertension in cirrhosis. Blockade of hepatic leptin receptors results in a decrease in portal pressure through a reduction in intrahepatic vascular resistance without modifying systemic hemodynamics. These data suggest that leptin can be a potential target in the treatment of portal hypertension.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


