Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis

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OBESITY is associated with several chronic disorders such as diabetes, hyperlipidemia, and cardiovascular diseases (6). Although liver disease is not widely appreciated as a complication of alcoholic liver injury, a disorder histologically indistinguishable from NAFLD (1, 47). Because the major source of endotoxin is the gut lumen, increased intestinal permeability has been suggested as an important factor in alcohol-induced liver disease. Indeed, several animal studies have supported the view that chronic ethanol abuse disrupts the intestinal epithelium integrity, leading to increased portal endotoxemia and exposure of the liver to high levels of LPS (20). Current data on intestinal dysfunction in NASH patients are, however, contradictory (4, 55). Obesity, which is often associated with NASH, is now considered a low-grade systemic inflammatory condition. Persistent high circulating levels of inflammatory cytokines, which are often observed in obese patients, may cause impairment of intestinal barrier function by altering structure and localization of tight junctions (TJs) (7, 17). The sealing properties of TJs rely on a cluster of functional proteins that selectively regulate the paracellular transport of ions, solutes, and peptides from the lumen to the intestinal mucosa and bloodstream. Occludin, claudin family members, and junctional adhesion molecule are linked to the actin cytoskeleton by cytoplasmic rafts formed by catenins and zonula occludens-1 in the intestinal mucosa, and higher circulating levels of inflammatory cytokines and portal endotoxemia compared with lean control mice. Moreover, HSCs isolated from ob/ob and db/db mice showed lower intestinal resistance, profoundly modified distribution of occludin and zonula occludens-1 in the intestinal mucosa, and more pronounced lipopolysaccharide-induced proinflammatory and fibrogenic responses than HSCs from lean animals. In conclusion, genetically obese mice display enhanced intestinal permeability leading to increased portal endotoxemia that makes HSCs more sensitive to bacterial endotoxins. We suggest that in metabolic syndrome, patients may likewise have a greater intestinal mucosa permeability and increased lipopolysaccharide levels in portal blood that can contribute to the liver inflammatory damage.

Toll-like receptors; endotoxins; fibrosis; tight junction proteins; hepatic stellate cells

Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 292: G518–G525, 2007. First published October 5, 2006; doi:10.1152/ajpgi.00024.2006.—A small percentage of pathologically obese subjects with fatty livers develop histological signs of necroinflammation and fibrosis, suggesting a variety of cofactors in the pathogenesis of obesity-related liver diseases including nonalcoholic steatohepatitis. Since several observations have linked bacterial endotoxins to liver damage, the aim of this study was to determine the effect of obesity on intestinal mucosal integrity and portal blood endotoxemia in two strains of obese mice: leptin-deficient (ob/ob) and hyperleptinemic (db/db) mice. Murine intestinal mucosal barrier function was assessed using a Ussing chamber, whereas ileum tight junction proteins were analyzed by immunocytochemistry and Western blot analysis. Circulating proinflammatory cytokines and portal blood endotoxin levels were measured by ELISA and the limulus test, respectively. The inflammatory and fibrogenic phenotype of murine hepatic stellate cells (HSCs) was determined by ELISA and quantitative RT-PCR. Ob/ob and db/db mice showed lower intestinal resistance, profoundly modified distribution of occludin and zonula occludens-1 in the intestinal mucosa, and higher circulating levels of inflammatory cytokines and portal endotoxemia compared with lean control mice. Moreover, HSCs isolated from ob/ob and db/db mice showed higher membrane CD14 mRNA levels and more pronounced lipopolysaccharide-induced proinflammatory and fibrogenic responses than HSCs from lean animals. In conclusion, genetically obese mice display enhanced intestinal permeability leading to increased portal endotoxemia that makes HSCs more sensitive to bacterial endotoxins. We suggest that in metabolic syndrome, patients may likewise have a greater intestinal mucosa permeability and increased lipopolysaccharide levels in portal blood that can contribute to the liver inflammatory damage.

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6db/db (db/db) mice, which are functionally deficient for the long-form leptin receptor], we showed that metabolic syndrome is associated with an abnormal distribution of junctional proteins within the intestinal mucosa that causes increased intestinal permeability leading to portal endotoxemia. In addition, HSCs isolated from the livers of ob/ob and db/db mice are more sensitive to LPS, developing a stronger inflammatory and fibrogenic phenotype than HSCs from lean control mice.

MATERIALS AND METHODS

Animals. Male 12-wk-old wild-type C57BL/6j mice, mice genetically deficient in leptin (ob/ob mice), and mice functionally deficient for the long-form leptin receptor (db/db mice) were obtained from Harlan (Oderzo, Italy), housed in groups of 2 mice/cage, kept under controlled temperature and humidity conditions, and received standard chow and tap water ad libitum. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Padua.

Ussing chamber experiments. Five-centimeter-long segments of the small intestine were collected from age-matched wild-type, ob/ob, and db/db mice, rinsed free of intestinal contents, and opened along the mesenteric border. Mucosal preparations were mounted in Ussing chambers connected to a voltage-clamp apparatus (EVC 4009, World Precision Instruments, Sarasota, CA), as described previously (14). After a 30-min equilibration period, the potential difference (PD; in mV) and short-circuit current (Isc, in μA/cm²) were recorded every 15 min for 60 min. The electrical resistance (in Ω·cm²) was calculated using Ohm’s law from the open-circuit PD and Isc. PD and resistance values were corrected for junctional potentials (<0.1 mV) between luminal and serosal solutions and the buffer resistance, respectively. Epithelial permeability to horseradish peroxidase (HRP) was also determined by a colorimetric assay.

Immunofluorescence analysis of occludin and ZO-1. Ob/ob, db/db, and age-matched wild-type lean mice were killed by cervical dislocation, the abdomen was opened, and a segment of the ileum was immediately removed, longitudinally opened, washed with PBS, mounted in embedding medium (Tissue-Tek, Sakura, The Netherlands), and stored at −80°C until use. Cryosections (8 μm) were fixed in acetone at −20°C for 5 min. Nonspecific background was blocked by an incubation with 2% donkey serum in TBS and 0.3% Triton X-100 extraction buffer as described above. Using commercially available ELISA kits, IL-6 (Bender Med-Tecan) at 450 nm. Cytokine levels were expressed as nanograms per milliliter. Sensitivities of the assays were performed in duplicate.

Isolation and culture of murine HSCs. HSCs were isolated from livers of wild-type, ob/ob, and db/db mice as previously described (9). HSC purity, confirmed after the first subculture passage by the morphological light microscopic appearance, staining of fat droplets with oil red O, immunofluorescent staining for α-smooth muscle actin (Sigma) and vitamin A-specific ultraviolet fluorescence, was ≥96%. Any presence of contaminating Kupffer and endothelial cells was ruled out by the absence of nonspecific esterase activity and immunofluorescent staining for factor VIII (Dako, Milan, Italy), respectively.

Subconfluent HSCs were trypsinized, seeded in six-well plates, and grown in complete medium to reach 80% confluence. The FBS concentration was gradually reduced to 1% (vol/vol), and cell monolayers were incubated for 6 days in medium containing LPS (10 ng/ml) from Salmonella enteritidis (Sigma). Media and stimuli were replaced daily. At least one internal control was performed in each experiment and consisted of HSCs incubated with medium supplemented with 1% (vol/vol) FBS.

RNA extraction and quantitative RT-PCR analysis. Total RNA was isolated from cultured HSCs using OMNIzol (Euroclone, Milan, Italy). Contaminating DNA was removed with a DNA-free TM Kit (Ambion, Milan, Italy), and cDNA was synthesized using standard procedures. GAPDH, membrane CD14 (mCD14), IL-6, monocyte chemoattractant protein (MCP)-1, fibroactin, and collagen type I steady-state mRNA-specific transcript levels were quantified using real-time PCR technology (ABI Prism 7700 Sequence Detector, Applied Biosystems), SYBR Green PCR Core Reagent kits (Applied Biosystems), and specific sets of primers (see Table 1).

Cytokine ELISA assays. HSCs were cultured in medium containing 1% (vol/vol) FBS alone or supplemented with LPS, as described above. Using commercially available ELISA kits, IL-6 (Bender Med-Systems, Milan, Italy) and MCP-1 (BioLegend, San Diego, CA) levels were measured in HSC-conditioned medium, whereas IL-1β, TNF-α, INF-γ (Endogen, Woburn, MA), and IL-6 levels were assessed in serum samples collected from ob/ob, db/db, and lean mice. Optical densities were measured using an ELISA plate reader (Spectra I Tecan) at 450 nm. Cytokine levels were expressed as nanograms per milliliter. Sensitivities of the assays ranged from 3 to 12 pg/ml.
Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
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<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
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<td>5’-TCTCCATGTTGTTGAAGACA-3’</td>
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<tr>
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<td>264</td>
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<td>5’-CCGGAGAGGAGACTTCAC-3’</td>
<td>5’-TCCAGATTCCACAGGA-3’</td>
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<tr>
<td>Mouse MCP-1</td>
<td>170</td>
<td>5’-AGTCAGGCGTTGATTACAATGTTG-3’</td>
<td>5’-TATTGGAATATTCAACCCACTICTC-3’</td>
</tr>
<tr>
<td>Mouse fibronectin</td>
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<td>5’-TGTTGATATCGTGTAGTTGGCC-3’</td>
</tr>
<tr>
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<td>5’-AGAAACATCCACCTATCACTCGCAAGA-3’</td>
<td>5’-GTTGATTGTATCGATGACTGTC-3’</td>
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MCP-1, monocyte chemoattractant protein-1.

Statistical analysis. Data are expressed as means ± SE. Comparisons were drawn between groups for parametric data using Student’s t-test. Results were considered statistically significant when P < 0.05.

RESULTS

Lower intestinal resistance and higher portal endotoxemia and systemic proinflammatory cytokine levels in obese mice. As shown in Fig. 1, intestinal specimens collected from either ob/ob and db/db mice revealed disrupted mucosal barrier function. Indeed, a significant decrease in small intestinal transepithelial resistance (Fig. 1A) and higher epithelial permeability to HRP (Fig. 1B) were observed in ob/ob and db/db mice compared with lean controls. No differences in electrophysiological parameters were reported in intestinal specimens collected from ob/ob and db/db mice. We then investigated the effect of the increased intestinal permeability on portal blood endotoxemia. As shown in Fig. 2A, we found higher endotoxin levels in the portal blood of both strains of obese mice than in age-matched wild-type lean animals. LPS levels in the portal blood of ob/ob and db/db mice were 95 ± 16 and 184.6 ± 14.7 pg/ml, respectively, significantly higher than in lean controls (51 ± 9 pg/ml). Greater intestinal permeability is commonly reported in metabolic disorders, but it has also been described in systemic inflammatory conditions (7). As shown in Fig. 2B, circulating levels of IL-1β, IL-6, INF-γ, and TNF-α were significantly higher in obese than in lean mice. As expected, obese mice also had significantly higher blood glucose levels than lean controls (15.82 ± 1.2 mmol/l in ob/ob mice and 16.4 ± 2.6 mmol/l in db/db mice compared with 10.88 ± 0.8 mmol/l in lean controls, P < 0.05).

Intestinal TJ protein delocalization in obese mice strains. Since the altered paracellular permeability involves a disrupted intestinal barrier, we examined the organization and distribution of TJ proteins by immunohistochemistry and Western blot analysis. As shown in Fig. 3, the immunofluorescence assay performed on intestinal sections of wild-type C57BL/6J mice demonstrated an intact network of occludin and ZO-1 with TJ proteins predominantly localized along the apical cellular border. Instead, immunocytochemistry performed on intestinal cryosections obtained from ob/ob mice revealed a substantial decrease in occludin’s staining and discontinuous signals for ZO-1, suggesting a dramatic redistribution of both proteins in the intestinal epithelium of ob/ob mice (Fig. 3). Similar results were obtained with intestinal sections from db/db mice (data not shown).

Since TJs are structurally characterized as soluble or insoluble proteins by their partition into Triton X-100 buffer (33), we analyzed the expression levels and solubility profiles of occludin and ZO-1 in obese and wild-type lean mice. As shown in Fig. 4A, a broad band between 58 and 71 kDa of junction-associated occludin (65 kDa) was present in both soluble and insoluble protein fractions in lean mice. In ob/ob and db/db mice, however, the protein amount in the fractions was remarkably decreased compared with wild-type lean animals. Moreover, in genetically obese mice, the occludin-associated protein pattern was totally changed, since the lowest-molecular-mass proteins (corresponding to the intramembrane fragment) were barely detectable (21, 22). In specimens collected from C57BL/6J wild-type mice, ZO-1 was exclusively associated with the Triton X-100-insoluble fraction (Fig. 4B), whereas in ob/ob and db/db mice, ZO-1 clearly partitioned also in the soluble fraction. These findings suggest a shift of intestinal junctional protein from the cytoskeleton, causing a decrease in paracellular sealing.

Enhanced sensitivity of HSCs from obese mice to endotoxin. Because Yang and coworkers (57) reported greater susceptibility of obese mice to LPS-mediated liver injury, we evaluated whether persistent portal endotoxemia might increase the sen-
sitivity of HSCs to bacterial cell-wall products, thus favoring the development of an activated phenotype. For this purpose, HSCs were isolated from leptin-deficient ob/ob mice and db/db long-isofom leptin receptor-defective mice. As shown in Fig. 5, after 6 days of exposure to low LPS concentrations (10 ng/ml), HSCs from genetically obese mice developed a more evident proinflammatory and profibrogenic phenotype than HSCs isolated from lean controls. Indeed, LPS exposure significantly increased IL-6 and MCP-1 steady-state mRNA-specific transcript levels by 4.6- and 3.1-fold in ob/ob mice and 4.3- and 9.8-fold in db/db mice, respectively. Similarly, fibronectin and collagen type I mRNA upregulation was significantly more pronounced in ob/ob- and db/db-derived HSCs than in cells from lean control mice (Fig. 5A).

In addition, LPS exposure induced the release of higher levels of IL-6 (12.2 ± 0.87 ng/ml for ob/ob-derived HSCs and 7.98 ± 0.88 ng/ml for db/db-derived HSCs; Fig. 5B) and MCP-1 (53.1 ± 3.4 ng/ml for ob/ob-derived HSCs and 54.9 ± 5.3 ng/ml for db/db-derived HSCs; Fig. 5C) in HSCs isolated from obese mice compared with HSCs isolated from lean mice.
NASH is a multifactorial disorder mainly affecting overweight/obese subjects with associated metabolic syndrome. Obesity is considered a mild systemic inflammatory condition and is thought to cause serious complications, including liver disease, because 73% of patients with cryptogenic cirrhosis are obese (10, 11). Fatty livers are less tolerant of various stressors, including fatty acid metabolites, endotoxins, and inflammatory cytokines, but the factor(s) triggering liver damage and fibrosis are still not clear (44, 57). Although it is an intriguing hypothesis, the potential role of intestinal endotoxins in liver damage is based only on indirect evidence (47, 55). In this study, we report innovative observations linking obesity with endotoxin-mediated liver damage. We found that the mucosal barrier function was significantly impaired in two different animal models of obesity, due to an abnormal distribution of TJ proteins, thus favoring endotoxin leakage into the portal blood. Liver exposure to gut-derived endotoxin contributes to the establishment of the activated phenotype in HSCs. In this study, we used two animal strains, ob/ob and db/db mice, which have been extensively used as models of obesity (3). Both animal strains present a defect in leptin activity: ob/ob mice express a truncated inactive form of leptin, whereas db/db mice express a signaling-incompetent long isoform of the hormone (15). Although the molecular mechanisms causing leptin deficiency differ between ob/ob and db/db mice, these animals show a similar phenotype in regard to hyperphagia, hypometabolism, and obesity (30), but also some relevant differences (24). Thus, since leptin also modulates an inflammatory process, ob/ob mice are less prone to develop inflammatory and fibrogenetic diseases (38), whereas db/db mice develop inflammation and liver fibrosis following...

Fig. 4. Redistribution of occludin and ZO-1 in small intestine specimens of ob/ob and db/db mice. Mucosal intestinal specimens collected from ob/ob, db/db, and age-matched lean control (w/t) mice were lysed in 1% Triton X-100 extraction buffer. Forty micrograms of the total protein fraction (total f.), Triton X-100-insoluble fraction (insol. f.), and Triton X-100-soluble fraction (sol. f.) were fractionated by SDS-PAGE and immunoblotted for occludin (A) or ZO-1 (B). Immunocomplexes were visualized by ECL. Western blotting detection reagents. Results are representative of at least 3 independent experiments.

Fig. 5. Increased sensitivity to bacteria endotoxins of hepatic stellate cells (HSCs) isolated from ob/ob and db/db mice. HSCs were isolated from w/t, ob/ob, and db/db mice and incubated with medium alone (control) or supplemented with 10 ng/ml LPS for 6 days. A: total RNA was isolated from HSCs, and quantitative RT-PCR analysis was performed to quantify IL-6, monocyte chemoattractant protein (MCP)-1, fibronectin, collagen type I, and membrane (m)CD14 mRNA transcript levels. Data are given as ratios of stimulated versus respective control nonstimulated HSCs; °P < 0.05 vs. HSCs isolated from w/t mice. B and C: IL-6 and MCP-1 were measured by ELISA in the conditioned medium. °P < 0.05 vs. respective control nonstimulated HSCs; °P < 0.02 vs. LPS-stimulated HSCs isolated from w/t mice.
exposure to a proper stimulus through a short-receptor isoform present in peripheral tissues (48). Nevertheless, we observed that circulating proinflammatory cytokine levels and changes in intestinal mucosa resistance were comparable between ob/ob and db/db mice regardless of the associated metabolic syndrome. In this respect, in ob/ob and db/db mice, the intestinal abnormalities seemed to correlate to the obese phenotype rather than to the loss of leptin function or its circulating level. Indeed, adipocytes spontaneously produce and release into the circulation inflammatory cytokines, and obesity is now recognized as a mild systemic inflammatory condition (18, 23).

The sealing properties of the intestinal mucosa require an efficient and anatomically intact epithelium as well as efficient apical cell junctional complexes (37). TJ function is strictly related to the expression, localization, and integrity of a variety of proteins in the lateral membrane; thus, cytokines and bacterial toxins can modify intestinal mucosal barrier function, affecting the expression and/or distribution of specific proteins (19, 26). Therefore, in patients with inflammatory bowel diseases, increased TNF-α affects TJ functionality and alters adhesion molecule expression, influencing mucosal barrier efficacy (50). In obese subjects, soluble factors arising directly from fat tissue or produced as a consequence of the associated metabolic syndrome, like hyperinsulinemia, might directly affect TJ function (17, 36, 49, 54). Thus, both obese mice strains, ob/ob and db/db, show increased circulating levels of inflammatory cytokines such as INF-γ and TNF-α, which are able to induce a redistribution of several junctional proteins (2), and IL-1β, which is capable of decreasing TJ ion selectivity in epithelial and endothelial cell monolayers (16, 40). The leakage of intestinal-derived endotoxins into the portal blood, due to the direct effect of ethanol on the intestinal mucosa (after oral ethanol feeding), is a well-established mechanism of alcohol-induced liver damage (1, 20). In the absence of ethanol-mediated mucosal damage, portal endotoxemia in leptin-deficient ob/ob mice as well as in functionally leptin-resistant db/db mice reached abnormally high values, supporting the existence of impaired mucosal barrier function in the presence of obesity. Indeed, when we performed Ussing chamber experiments, we found significantly decreased electrophysiological parameters and a concomitant increase in HRP fluxes, suggesting that altered paracellular permeability is involved in the disruption of intestinal barrier function in genetically obese mice (Figs. 1 and 2). We observed, by confocal microscopy and Western blot analysis, a striking reorganization of occludin and ZO-1 in the ileum of ob/ob and db/db mice, suggesting the rupture of TJ links with the cytoskeleton, a condition known to compromise the sealing properties of TJs (33). Therefore, the substantial changes in expression/distribution of two key TJ proteins in obese mice may explain the enhanced paracellular permeability observed in the intestinal mucosa that can lead to an abnormal leakage of bacterial endotoxins into the portal blood and LPS exposure of hepatic cellular populations.

HSC activation is generally secondary to liver damage and due to the disruption of the normal extracellular matrix and to the release of proinflammatory cytokines from Kupffer cells, hepatocytes, and infiltrating inflammatory cells. Recent evidence, however, has shown that HSCs can directly respond to LPS via a specific receptor complex and contribute to establish and/or enhance endotoxin-mediated liver injury (9, 43). Indeed, our data demonstrated an increase in liver susceptibility to LPS-mediated damage in obese mice that might depend to some degree on enhanced HSC reactivity to LPS (57). Obese mice-derived HSCs seemed to lack the LPS tolerance that develops in a variety of cell types after LPS stimulation to inhibit inflammatory cytokine production to prevent autotoxic effects by inflammatory mediator overproduction (43). On the other hand, HSCs from ob/ob and db/db mice showed enhanced responsiveness to inflammatory mediators similar to what is observed in myofibroblasts, macrophages, and endothelial cells purified from tissues affected by chronic inflammatory disorders and soaked in a milieu rich in inflammatory cytokines (5, 34, 35). Thus, both ob/ob- and db/db-derived HSCs exposed to endotoxin developed an inflammatory and fibrogenic phenotype, whereas this response was observed in the livers of db/db mice but not in ob/ob mice following CCl4 treatment, supporting a role for leptin as a proinflammatory factor required to amplify inflammatory reactions in vivo (28). Thus, proinflammatory cytokines increase fibronectin mRNA levels and protein deposition through a NF-kB-dependent pathway in HSCs isolated from leptin-deficient mice (29).

In conclusion, we demonstrated disrupted intestinal barrier function in two strains of genetically obese mice leading to a consistent leakage of bacterial endotoxins into the portal blood circulation. Several factors may contribute to the enhanced intestinal mucosal permeability in obese subjects, such as hyperinsulinemia and high circulating levels of inflammatory cytokines, exposing the fatty liver (already predisposed to free radical-mediated damage), to a high load of gut-derived bacterial endotoxins (42, 57). The persistent exposure to LPS and LPS-induced soluble proinflammatory mediators may activate HSCs, which develop to a fibroinflammatory phenotype, amplifying the liver damage. These effects could be further enhanced in patients carrying specific polymorphisms in genes coding for inflammatory cytokines and/or receptors for bacteria-derived endotoxins (8, 13, 51, 41, 53).

REFERENCES


