A murine model of obesity implicates the adipokine milieu in the pathogenesis of severe acute pancreatitis

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Obesity is epidemic in the 21st century; currently, over one-third of adult Americans are considered to be clinically obese (28). Fatty infiltration of visceral organs such as the liver, heart, gallbladder, and pancreas leads to a proinflammatory milieu and organ dysfunction (1, 18, 25, 26, 39). Adipose tissue has historically been considered solely as an energy storage depot. Over the past decade, however, the role of adipose tissue as a metabolic organ has been increasingly appreciated. Adipocytes produce a number of pleiotropic molecules collectively called adipokines (38). Adipokines have a wide variety of endocrine, paracrine, and autocrine effects, including regulation of satiety, energy metabolism, immune function, and angiogenesis. Importantly, adipokines are also potent regulators of the inflammatory response. The first discovered and best characterized adipokines are leptin and adiponectin (34, 43). Leptin is produced by adipocytes that are important regulators of the inflammatory response. Adipokines (including leptin and adiponectin) are pleiotropic molecules produced by adipocytes that are important regulators of the inflammatory response. We hypothesized that the altered adipokine milieu observed in obesity contributes to the increased severity of pancreatitis. Lean (C57BL/6J), obese leptin-deficient (LepOdb), and obese hyperleptinemic (LepDb) mice were subjected to AP by six hourly intraperitoneal injections of cerulein (50 μg/kg). Severity of AP was assessed by histology and by measuring pancreatic concentration of the proinflammatory cytokines IL-1β and IL-6, the chemokine MCP-1, and the marker of neutrophil activation MPO. Both congenitally obese strains of mice developed significantly more severe AP than wild-type lean animals. Severity of AP was not solely related to adipose tissue volume; LepOdb mice were heaviest; however, LepDb mice developed the most severe AP both histologically and biochemically. Circulating adiponectin concentrations inversely mirrored the severity of pancreatitis. These data demonstrate that congenitally obese mice develop more severe AP than lean animals when challenged by cerulein hyperstimulation and suggest that alteration of the adipokine milieu exacerbates the severity of AP in obesity.

Adipokine milieu; obesity

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MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee of Indiana University and performed in accordance with protocols outlined in the American Physiological Society’s Guiding Principles in the Care and Use of Animals.

Animals and diet. Female obese Lep\textsuperscript{Ob} (leptin deficient) and Lep\textsuperscript{Db} (hyperleptinemic) and wild-type lean mice (C57BL/6J) were obtained from the Jackson Laboratory (Bar Harbor, ME). Adult 7-wk-old animals were allowed to acclimate for 1 wk in a climate-controlled room with a 12:12-h light-dark cycle. At 8 wk of age, mice were fed a diet consisting of (% calories) 25% fat (soybean oil and corn oil), 55% carbohydrate, and 20% protein (Dyets, Bethlehem, PA). All experiments were performed in animals at 12 wk of age; typical experiments included 10–16 animals in experimental groups.

Induction of AP and tissue preparation. AP was induced by pancreatic hyperstimulation with the cholecystokinin agonist cerulein (Sigma-Aldrich, St. Louis, MO). Sixteen mice of each strain (lean C57BL/6J, Lep\textsuperscript{Ob}, and Lep\textsuperscript{Db}) were administered 50 \( \mu \)g/kg cerulein intraperitoneally hourly for 6 h. Ten mice of each strain served as control, receiving intraperitoneal injection of vehicle (0.5% NH\textsubscript{4} saline) on the same time schedule. Nine hours after the first injection, mice were anesthetized with ketamine (50 mg/kg) and xylazine (15 mg/kg) and underwent intraabdominal necrectomy. Blood was collected at this time by ventricular puncture. A portion of pancreas was preserved in 10% formalin for histological analysis, and the remainder was immediately frozen in liquid nitrogen and preserved at \(-80^\circ\text{C}\) for further analysis. A portion of lung was similarly frozen in liquid nitrogen and preserved at \(-80^\circ\text{C}\). Blood was centrifuged at 15,000 rpm, and serum was stored for subsequent analysis. Chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Microscopy and histological analysis. Formalin-preserved pancreas was embedded in paraffin blocks, sectioned into 5-\(\mu\text{m}\) segments, and stained with hematoxylin and eosin. Sections of pancreas were taken from a standard location, the mid body of the gland. One section from each animal was evaluated. Histological severity of pancreatitis was determined by three separate observers who were unaware of the animal strain or treatment. A total pancreatitis score was determined by a validated method based on the degree of inflammation, vacuolization, and edema (32).

Chemical assays. Pancreatic tissue was homogenized in buffer containing 50 mM Tris, 250 mM NaCl, 5 mM EDTA, 1 mM NaF, 20 mM Na\textsubscript{2}PO\textsubscript{4}, 0.02% NaN\textsubscript{3}, proprietary detergent, and protease inhibitor (Sigma) at a volume of 50 \(\mu\text{L}\) per gram tissue. Homogenates were centrifuged at 10,000 rpm at 4°C for 15 min, and protein concentration of the supernatant was assayed (Bio-Rad, Hercules, CA). Pancreatic tissue levels of the cytokines IL-1\(\beta\) and IL-6 and the chemokine monocye chemoattractant protein-1 (MCP-1) were determined with commercially available ELISA kits (R & D Systems, Minneapolis, MN). Pancreatic and lung tissue concentration of myeloperoxidase (MPO) was determined by ELISA (Cell Sciences, Canton, MA). Serum levels of leptin and adiponectin were determined by using ELISA kits from Linco Research (St. Charles, MO). Serum insulin concentration was determined by ELISA (Crystal Chem, Downers Grove, IL). Serum glucose and amylase concentration were determined by colorimetric assay according to the manufacturer’s instructions (Stanbio Laboratory, Boerne, TX).

Statistical analysis. Statistical analyses were performed by use of SigmaStat software (Jandel, San Rafael, CA). Data are expressed as means \pm SE. ANOVA and Tukey’s tests were applied as appropriate. A \(P\) value of <0.05 was accepted as statistically significant.

RESULTS

Animal weight. Twelve-week-old mice were weighed just prior to injection of cerulein or vehicle. Lean mice were significantly lighter than both congenitally obese strains of mice: lean 19.8 \(\pm\) 0.2 g, Lep\textsuperscript{Ob} 52.4 \(\pm\) 0.6 g, Lep\textsuperscript{Db} 43.8 \(\pm\) 0.5 g \((P < 0.001)\). Of the two congenitally obese strains, Lep\textsuperscript{Ob} mice were significantly heavier than Lep\textsuperscript{Db} \((P < 0.001)\).

Histological severity of pancreatitis. Mice of all three strains injected with vehicle (control group) did not manifest any histological changes of AP. Supramaximal stimulation by six hourly injections of 50 \(\mu\text{g}\)/mg ip cerulein resulted in histological changes of AP including edema, intracellular vacuolization, and inflammatory cell infiltrate. Figure 1 shows representative micrographs from each strain. The histological pancreatitis score for all animals is shown in Table 1. After cerulein hyperstimulation, both obese strains developed significantly more severe histological pancreatitis \((P < 0.05)\) than lean animals. Of the obese mice, Lep\textsuperscript{Db} mice developed significantly more severe histological pancreatitis than Lep\textsuperscript{Ob} animals \((P < 0.05)\).

Cytokines and chemokines in AP. Relative to control animals of the same strain, pancreatic tissue levels of the proinflammatory cytokines IL-1\(\beta\) and IL-6 were significantly elevated after induction of AP (Fig. 2). In congenitally obese (Lep\textsuperscript{Ob} and Lep\textsuperscript{Db}) mice subjected to AP, pancreatic IL-1\(\beta\) and IL-6 levels were significantly elevated compared their counterparts in the control group \((IL-1\beta P < 0.01, IL-6 P < 0.001)\). Similar to histological evaluation of AP, tissue levels of both cytokines increased in a stepwise fashion among groups: lean < Lep\textsuperscript{Ob} < Lep\textsuperscript{Db} \((IL-1\beta P < 0.01, IL-6 P < 0.001)\).

Relative to animals in their respective control groups, pancreatic tissue levels of the chemoattractant molecule MCP-1 (a potent attractor/activator of monocytes and macrophages) were significantly increased in all three strains of mice after induction of AP by cerulein hyperstimulation \((P < 0.001)\; (Fig. 3A)\). Within the cerulein group, Lep\textsuperscript{Ob} and Lep\textsuperscript{Db} mice demonstrated significantly elevated levels of MCP-1 relative to lean mice \((P < 0.001)\). Similarly, all three strains of mice demonstrated significant increases in pancreatic levels of MPO (a marker for intrapancreatic neutrophil sequestration/activation) after induction of pancreatitis by cerulein hyperstimulation relative to control animals of the same strain \((P < 0.03)\; (Fig. 3B)\).

In cerulein-treated animals, both obese strains of mice had significantly more MPO than lean mice \((P < 0.01)\).

Adipokines in AP. As anticipated, serum levels of leptin were low in lean mice, extremely low in Lep\textsuperscript{Ob} mice, and markedly elevated (8-fold higher than lean) in Lep\textsuperscript{Db} mice \((P < 0.001)\; (Fig. 4A)\). Induction of pancreatitis did not significantly change circulating leptin levels in lean or Lep\textsuperscript{Ob} animals; however, Lep\textsuperscript{Db} mice subjected to AP had significantly higher serum leptin compared with Lep\textsuperscript{Db} in the control group \((P < 0.001)\). Within the control group, both obese strains of mice were observed to have significantly lower serum adiponectin vs. lean mice \((P < 0.02)\; (Fig. 4B)\). After induction of pancreatitis, serum adiponectin levels decreased significantly in each strain relative to their respective control \((P < 0.01)\). Within the pancreatitis group, serum adiponectin was significantly lower in Lep\textsuperscript{Db} mice compared with both lean and Lep\textsuperscript{Ob} mice \((P < 0.02)\). Interestingly, the pattern of serum adiponectin levels inversely mirrored the severity of pancreatitis (as measured both by histology and pancreatic tissue concentration of proinflammatory cytokine upregulation).

Serum biochemical parameters. Measurements of serum amylase, glucose, and insulin are shown in Table 2. Serum amylase was significantly elevated in all three strains of mice.
after induction of pancreatitis ($P < 0.05$). Within the pancreatitis group, no significant differences were observed among lean, Lep$^{Ob}$, or Lep$^{Db}$ mice. Serum glucose was significantly higher at rest (control group) in both obese strains of animals compared with lean mice ($P < 0.05$); Lep$^{Db}$ mice had significantly higher serum glucose than Lep$^{Ob}$ mice under these conditions ($P < 0.002$). Induction of AP had no effect on serum glucose in lean animals but caused a significant decrease in serum glucose in both obese strains of mice ($P < 0.01$). Under these conditions, serum glucose was significantly lower in Lep$^{Db}$ compared with Lep$^{Ob}$ mice ($P < 0.05$). In the control group, serum insulin was significantly elevated in both obese strains compared with lean mice. Interestingly, induction of AP significantly decreased serum insulin in both obese strains (relative to their in-strain control); Lep$^{Db}$ mice had significantly lower serum insulin concentrations than Lep$^{Ob}$ mice after induction of pancreatitis ($P < 0.01$).

**Lung MPO.** Induction of AP resulted in elevation of MPO levels in the lung of animals in all three strains, consistent with systemic inflammatory response (Fig. 5). These values were significantly less than the MPO elevation observed in the pancreatic parenchyma. Consistent with the histology and pancreatic cytokine data, lung MPO levels were highest in the Lep$^{Db}$ mice ($P < 0.05$).

**DISCUSSION**

The most important finding of this study was that congenitally obese mice (Lep$^{Ob}$ and Lep$^{Db}$) sustained significantly worse AP than wild-type lean (C57BL/6J) animals when subjected to cerulein hyperstimulation. This observation mimics the clinical situation seen in humans, where obesity is well established as an independent risk factor for development of severe AP (8, 23, 31, 37). Both obesity and AP represent...
substantial clinical concerns; despite the clear impact of obesity on severity of pancreatitis, a remarkable paucity of experiments have evaluated this association. In fact, only two studies have been published on pancreatitis using obese animal models (35, 36). In the first study, genetically obese (Zucker fa/fa) rats and rats with diet-induced obesity were shown to have significantly increased mortality and severity of pancreatitis relative to lean wild-type control animals (35). In follow-up experiments from the same investigators, rats with diet-induced obesity had no difference in histological pancreatitis but increased early mortality relative to control animals (36). These data were somewhat confounded, however, by the concurrent administration of endotoxin (36).

In the present study, both obese strains of mice (LepOb and LepDb) manifest more severe pancreatitis by histological analysis, increased pancreatic tissue levels of the proinflammatory cytokines IL-1β and IL-6, increased pancreatic tissue levels of the chemokine MCP-1, and the marker of neutrophil activation MPO. Taken together, these data suggest that the increased severity of AP seen in obesity is directly related to increased inflammatory cell upregulation, activation, and infiltration into the pancreatic parenchyma. LepOb mice were significantly more obese than LepDb animals; however, the LepDb mice developed the most severe pancreatitis (both histologically and biochemically). This observation suggests that the volume of adipose tissue per se is not in itself solely responsible for the increased severity of pancreatitis in obesity and that other factors such as the adipokine milieu are important modulators of the inflammatory response. It was therefore quite intriguing to observe that across all three strains of animals studied serum levels of the potent anti-inflammatory adipokine adiponectin inversely mirrored the severity of AP.

Comparative study of lean (C57BL/6J) and these two discrete strains of congenitally obese mice (both on a C57BL/6J background) affords a unique opportunity to evaluate the roles of both increased adiposity and the inflammatory modulating adipokines leptin and adiponectin in the pathogenesis of pancreatitis. Both LepOb and LepDb mice are congenitally obese; however, they manifest this phenotype via different mechanisms. LepOb mice have a spontaneous mutation of the ob (leptin) gene and produce virtually no leptin (43). LepDb mice, on the other hand, have a spontaneous mutation of the leptin receptor and consequently have dramatically elevated circulating levels of leptin (34). The LepDb mouse more closely capitates the human situation, in which leptin resistance leads to hyperleptinemia. Thus, whereas both mice manifest the

Fig. 2. Pancreatic tissue concentration of proinflammatory cytokines IL-1β and IL-6. Mice received either vehicle (Control) or cerulein (50 μg/kg body wt × 6 hourly injections; Cerulein). Nine hours after the first injection, animals were euthanized, and pancreatic tissue levels of IL-1β (A) and IL-6 (B) were determined as described in the text. Data are means ± SE for 5 animals in each control strain and 10 animals in each cerulein strain.

Fig. 3. Pancreatic tissue concentration of chemokines monocyte chemoattractant protein-1 (MCP-1) and MPO. Mice received either vehicle (Control) or cerulein (50 μg/kg body wt × 6 hourly injections; Cerulein). Nine hours after the first injection, animals were euthanized, and pancreatic tissue levels of MCP-1 (A) and MPO (B) were determined as described in the text. Data are means ± SE for 5 animals in each control strain and 10 animals in each cerulein strain.
The discovery of leptin in 1994 and adiponectin shortly thereafter have been followed by an explosion in the study of adipokine biology (34, 38, 43). Both of these molecules are produced predominantly by adipocytes, but their impact on inflammation is dramatically different. In humans, circulating leptin concentration increases directly with increasing obesity, and leptin has been shown in many diverse systems to act predominantly as a proinflammatory mediator (10, 33). The proinflammatory effects of leptin are thought to be mediated mostly by mechanisms regulating differential cytokine production and leukocyte activity (21, 22). Adiponectin, on the other hand, paradoxically decreases as adipose tissue mass increases (2). In the context of obesity, the function of adiponectin as a strong anti-inflammatory molecule has been well established (11). Adiponectin exerts its anti-inflammatory effects through several distinct mechanisms: 1) downregulation of chemokines and adhesion molecules (15, 29); 2) alteration of macrophage and lymphocyte function (19, 30, 41); 3) suppression of proinflammatory cytokine production (24); and 4) upregulation of anti-inflammatory cytokine production (41).

Seven published studies have evaluated the role of leptin in lean animal models of AP. Konturek et al. (17) showed that circulating leptin levels were increased in lean rats after induction of AP by cerulein hyperstimulation, a finding that has been substantiated by two other groups (16, 42). Somewhat paradoxically, the administration of leptin (again in lean animals) has been shown to attenuate the severity of AP (13, 40). In the present experiments, circulating leptin was increased after induction of AP only in the obese LepDb mice. It is not surprising that there was no change in circulating leptin in LepOb mice, who are unable to produce significant amounts of leptin. The observation that AP did not change circulating leptin levels in lean animals may be related to our use of a murine model (as opposed to rat models used in other reports) or use of a lower dose of cerulein than that reported in prior studies. The present experiments are the first to our knowledge that evaluate the role of adiponectin in experimental AP.

**Table 2. Serum biochemical parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lean</th>
<th>LepOb</th>
<th>LepDb</th>
<th>Lean</th>
<th>LepOb</th>
<th>LepDb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase, U/l</td>
<td>1,331 ± 212</td>
<td>3,028 ± 206</td>
<td>2,408 ± 164</td>
<td>13,527 ± 851*</td>
<td>12,938 ± 851*</td>
<td>11,979 ± 576*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>261 ± 17</td>
<td>439 ± 56†</td>
<td>637 ± 69†</td>
<td>249 ± 17</td>
<td>232 ± 20*</td>
<td>344 ± 19†‡</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>3.2 ± 0.8</td>
<td>15.6 ± 0.6†</td>
<td>14.8 ± 1.4†</td>
<td>1.1 ± 0.02</td>
<td>5.1 ± 0.8†</td>
<td>8.9 ± 1.3†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = at least 9 in each control group and at least 14 in each cerulein strain. *P < 0.05 vs. control; †P < 0.05 vs. lean within control group (control/cerulein); ‡P < 0.05 vs. LepOb within group (control/cerulein).
real, the mechanisms modulating increased severity of AP in obesity are likely complex and multifactorial. The fact that adipokines are potent modulators of inflammation and that the adipokine milieu is markedly altered in the setting of obesity suggests that these molecules may play a role. Indeed, our finding that serum adiponectin concentration inversely mirrored the severity of AP suggests that downregulation of this anti-inflammatory molecule in the setting of obesity may be more important than the influence of the proinflammatory adipokine leptin.

Our finding that induction of pancreatitis increases pulmonary MPO concentration is consistent with many prior reports in small animal models of cerulein-induced pancreatitis (5, 20, 27) and indicative of a systemic inflammatory response associated with the genesis of pancreatitis. The fact that pulmonary MPO concentration was significantly elevated only in the obese LepDb animals may be related either to the increased severity of the local (pancreatic) insult or alternatively to the entire organism being “primed” to perpetuate a systemic inflammatory response (i.e., by the presence of a significantly altered adipokine milieu: hyperleptinemia and/or hypoadiponectinemia).

The finding of increased pancreatic MPO concentration in obese mice suggests a greater degree of neutrophil infiltration and/or activation in these animals relative to lean, wild-type mice. Although we also observed a greater degree of leukocyte infiltration in the obese mice histologically (Table 1), the role played by macrophages in this process is less obvious from our data. It is intriguing to speculate that specific leukocyte subtypes (i.e., neutrophil vs. monocyte/macrophage) may play differential roles in the pathogenesis of AP specifically in the context of obesity. We are not yet, however, confident of our ability to accurately and reliably quantify these relative subclasses of leukocytes. Further experiments using immunohistochemical staining and flow cytometry techniques may be helpful in this regard.

Serum amylase elevation is generally accepted as a marker of AP (12, 44). In the present experiments, serum amylase was found to be elevated in all three strains of mice after induction of AP. No differences in the degree of hyperamylasemia were observed among these three strains. Although some basic investigators have used the degree of amylase elevation as a surrogate for degree of pancreatic inflammation, this marker is widely variable (5, 6). Indeed, no clinical study has ever been able to correlate the degree of hyperamylasemia with the severity or duration of AP (44).

As expected, both strains of obese mice demonstrated the metabolic consequences of obesity (hyperinsulinemia and hyperglycemia) relative to lean animals. Interestingly, induction of AP led to a significant decrease in serum insulin and glucose in both obese strains of mice. Serum insulin and glucose levels were unchanged after induction of AP in lean mice. This finding may be related to increased insulin sensitivity in the face of a systemic inflammatory event; however, the reason why it would manifest differently in obese vs. lean mice is not clear.

In summary, these experiments demonstrated that congenitally obese mice developed more severe AP than lean wild-type animals when challenged by cerulein hyperstimulation. The severity of pancreatitis was not solely related to volume of adipose tissue. Thus the observation that pancreatitis severity was inversely mirrored by circulating concentrations of the anti-inflammatory adipokine adiponectin is particularly intriguing. Further dissection of the adipokine milieu’s impact on the development of severe AP of obesity is clearly of high importance for two reasons. First, the prevalence of obesity in the United States (and the world) has reached epidemic proportions, and the incidence shows no sign of slowing. Second, despite over a century of research and literally billions of dollars invested in clinical therapeutic trials, the precise mechanisms involved in the pathogenesis of AP remain elusive, and currently no specific medical therapy for AP exists beyond general support. Interrogating the mechanisms by which AP develops from novel angles (such as obesity and the influence of specific adipokines like adiponectin) offers the potential for unique observations that may ultimately lead to the development of directed therapeutic agents. It is exciting to speculate that manipulation of the adipokine milieu has the potential to impact the severity of AP. Thus continued investigation along these lines is most certainly warranted.

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GRANTS

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REFERENCES


