

Obesity Alters Cytokine Gene Expression and Promotes Liver Injury in Rats With Acute Pancreatitis

Ralf Segersvärd¹, Jon A. Tsai¹, Margery K. Herrington² and Feng Wang¹

Objective: Obesity is a negative prognostic factor in patients with critical illnesses such as acute pancreatitis (AP). The outcome of AP is determined by the severity of systemic inflammation and organ dysfunction. In a previous study, we found that AP caused more deaths in obese rats than in lean rats. In the present study, we examined whether the effect of obesity on rats with AP is associated with distinct alterations in inflammatory cytokine expression in organs involved in AP.

Methods and procedures: AP was induced in lean and obese Zucker rats by pancreatic infusion of taurocholic acid. All survivors were killed 8 h later. Gene transcripts for two proinflammatory cytokines (interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α)) and two anti-inflammatory cytokines (IL-10 and pancreatitis-associated protein (PAP)) were determined in the pancreas, liver, and lungs by quantitative real-time polymerase chain reaction. The severity of AP was assessed by means of histology and serology.

Results: Obese AP rats had higher TNF- α mRNA in all organs examined, lower IL-10 and IL-6 mRNA in the pancreas, and lower PAP mRNA in the liver, compared to lean AP rats. Lean and obese AP rats had similar pancreatic lesions as assessed by histology. However, steatohepatitis and increased serum alanine amino transferase levels, which are indications of hepatic injury, were present in obese but not lean AP rats.

Discussion: Our findings suggest that altered inflammatory cytokine expression and increased vulnerability in the liver underlie the detrimental influence of obesity on AP.

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INTRODUCTION

Obesity is one of today's most visible public health problems (1). Obesity has recently been recognized as a negative prognostic factor in a number of critical illnesses including trauma, burns, and acute pancreatitis (2–4), and is now considered an independent prognostic factor for mortality in intensive care unit patients (5). A recent meta-analysis showed that the association of acute pancreatitis (AP) with obesity increases the morbidity and mortality of the disease (4). Although the mechanisms behind this are unclear, it has been proposed that the poorer outcome from AP in obese patients is related to alterations in immune system reactions in response to the insult of AP (6).

AP is an inflammatory disease of the pancreas that may affect remote organs such as the liver and lungs through the induction of a systemic inflammatory response syndrome and promote the development of distant organ failure (7,8). The ultimate outcome in AP is believed to depend on this systemic illness (9). The pathophysiological mechanisms behind the

systemic inflammatory response syndrome and organ failure in AP include complex, interrelated processes. In a pancreas with a local inflammatory response following injury, activated macrophages not only play a key role in releasing proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), but also induce a simultaneous expression of anti-inflammatory cytokines such as IL-10 and pancreatitis-associated protein (PAP) that downregulate proinflammatory cytokines (10,11). If the local inflammatory reaction is not contained, it is disseminated and gets amplified by circulating immune cells and the endothelium. Inflammatory cascades are induced in remote organs, with endothelial activation and massive polymorphonuclear leukocyte (PMNL) infiltration. Decreased tissue oxygenation and PMNL-induced tissue injury are believed to contribute to the development of organ failure (12). Importantly, not only the level of cytokines but also the balance between the pro- and anti-inflammatory responses determine the magnitude of the systemic inflammatory response syndrome (13,14).

¹Division of Surgery, Department of Clinical Science, Intervention and Technology, Karolinska University Hospital Huddinge, Stockholm, Sweden; ²Department of Biology, Adams State College, Alamosa, Colorado, USA. Correspondence: Ralf Segersvärd (ralf.segersvard@ki.se)

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We have previously observed decreased survival in genetically obese *fa/fa* Zucker rats in an experimental model of severe AP, even though the degree of pancreatic injury was similar in lean and obese rats. This led us to hypothesize that obesity alters the inflammatory response in rats with AP (15). To test this hypothesis, we undertook the present study using the same AP model.

METHODS AND PROCEDURES

Animals

Ten-week-old male obese (*fa/fa*) and lean (*FA/?*) Zucker rats (Zuc-Lepr^{fa}; Charles River, Uppsala, Sweden) were purchased at least 1 week before the experiment. The animals were kept on a 12-h dark–light cycle at 21 °C with free access to water and standard chow. The research protocol (S50/01) was approved by the Animal Ethics Committee at the Karolinska Institutet, Stockholm, Sweden.

Acute experimental pancreatitis

Necrotizing AP was induced by ductal infusion of sodium taurocholate (Sigma Chemicals, St. Louis, MO) into the pancreas (15). Lean ($n = 10$) and obese rats ($n = 9$) were premedicated with midazolam (1.0 mg/kg, subcutaneously) and anesthetized by a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After a midline laparotomy, a polyethylene catheter (PE 10) was inserted transduodenally 5 mm into the common biliopancreatic duct. The duct was occluded by vascular clamps placed at the hilum of the liver and at the intraduodenal part of the duct. Sodium taurocholate solution (3.5%, 0.4 ml) was infused into the pancreas in 1 min, using an infusion pump (Harvard Apparatus, Milford, MA). After the abdominal wall was closed, the animals received a subcutaneous injection of saline (1 ml/100 g) and buprenorphine (0.05 mg/kg, Schering-Plough, Stockholm, Sweden). The animals were allowed to recover under heat lamps. Food and water were provided *ad libitum*. Seven obese rats and seven lean rats were left untreated as two control groups.

The surviving rats in the AP and control groups were killed 8 h after the induction of AP in the AP groups, a time corresponding to the point at which survival curves in obese and lean AP rats started to separate in a previous study (15). If an animal died before this time point, the time of death was recorded, the pancreas was harvested for histology, and the animal was used for analysis of the survival rate. Surviving animals were anesthetized with inhaled isoflurane (~3%; Abbot Scandinavia AB, Solna, Sweden). Tissue samples were taken from the pancreas, liver, and lungs. Blood was collected for serum analysis when the animals were killed by cardiac puncture and exsanguination.

Quantitative real-time polymerase chain reaction gene expression analysis

Frozen pancreatic, liver, and lung tissues were ground in liquid nitrogen. Total RNA was extracted, treated with DNase (Ambion, Austin, TX), and separated by gel electrophoresis. Degraded RNA samples were excluded from further processing. cDNA was synthesized using 1 µg of total RNA and random primers (Promega, Madison, WI). The cDNA was subjected to quantitative real-time polymerase chain reaction analysis using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The transcripts of TNF- α , IL-6, IL-10, and PAP were targeted using primer/probe mixes (20 \times) pre-designed by Applied Biosystems. The IDs of these mixes were Rn00562055_m1, Rn00561420_m1, Rn00563409_m1, and Rn00583920_m1, respectively. The 18S primers and probe (TaqMan Ribosomal RNA Control Reagents) were used at concentrations of 100 and 400 nmol/l, respectively. The real-time amplification began with 50 °C for 2 min and 95 °C for 10 min, and then had 45 cycles of 15 s at 95 °C and 1 min at 60 °C. All reactions were run in duplicate with 18S as the internal control. Samples with abnormal real-time curves were excluded from further

analysis. For each sample, cytokine gene expression was normalized to the internal control (18S). The expression in lean control rats was regarded as the baseline to which data in the other three groups were compared.

Histological examination

Samples from the pancreatic head and the liver were fixed in 4% formalin and embedded in paraffin. The tissues were sectioned (5 µm) and stained with hematoxylin and eosin. An investigator who was unaware of the nature of the intervention scored the amount of acinar and fat necrosis in the pancreatic sections (maximum score 14) according to the Spormann criteria (16). Liver sections were examined for signs of steatohepatitis and necrosis.

Other assays

The left epididymal fat pad was resected and weighed when rats were killed. The weight of the epididymal fat pad expressed as a percentage of body weight was used as an index of intra-abdominal fat. Hepatic fat content was determined by using an isopropanol/heptan/1 N sulphuric acid solution (40/10/1) to extract fat from a known amount of homogenized liver. The decrease in sample weight was used to calculate the percent fat of liver wet weight. Serum amylase was measured using a quantitative colorimetric assay (Phadebas Amylase Test; Pharmacia & Upjohn, Sweden). Serum triglycerides, alanine amino transferase, creatinine, and glucose were measured using an automatic analyzer (Modular system; Roche Centralized Diagnostics, Stockholm, Sweden). Serum insulin was measured by radioimmunoassay (Linco Research, St. Charles, MO).

Tissue myeloperoxidase (MPO) activity was used as an index of PMNL infiltration. Tissue samples were homogenized in ice-cold 20 mmol/l phosphate-buffered saline and then centrifuged. The pellets were dissolved in 0.5% hexadecyl trimethyl ammonium bromide in 50 mmol/l phosphate-buffered saline (pH 6.0) and then sonicated. Following three cycles of freezing and thawing, the preparation was incubated for 2 h at 60 °C and then centrifuged at 10,000g for 10 min. Supernatants were collected and the protein concentration was measured. In a 96-well plate, supernatants containing 15 µg of protein were incubated in triplicate with 100 µl of 3,3',5,5'-tetramethylbenzidine (Vector Laboratories, Burlingame, CA) for 3 min. The reaction was stopped by the addition of 100 µl sulphuric acid (1N), and absorbance was read at 450 nm. MPO activity was expressed as absorbance/milligram protein.

Statistics

Data are means \pm s.e.m. Sigmaplot software was used for all calculations (Jandel, Sausalito, CA). Histological data were analyzed by the Wilcoxon/Kruskal Wallis test. Survival data were analyzed by Fisher's exact test. The rest of the data were analyzed by two-way ANOVA with the Student–Newman–Keul post-test for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Lean and obese rats and their survival in AP

The obese control rats had significantly greater body weights (obese 368 ± 14 g, lean 306 ± 12 g; $P < 0.05$) and more intra-abdominal fat (epididymal fat pad percentage of body weight: obese 2.0 ± 0.2 , lean 0.7 ± 0.1 ; $P < 0.001$) than the lean control rats. As shown in **Table 1**, obese control rats had higher serum levels of triglycerides and insulin and had more hepatic fat than the lean control rats. No deaths occurred in the two control groups. Two lean AP rats and four obese AP rats died before the end of the experiment. The difference in survival rates between the lean and obese AP rats (80 and 56%, respectively) was not significant ($P = 0.3$).

Table 1 Serological parameters and liver fat

	Lean Zucker rats		Obese Zucker rats	
	Controls (n = 7)	AP (n = 8)	Controls (n = 7)	AP (n = 5)
Amylase (kU/l)	7.3 ± 3.2	34.6 ± 3.5*	8.8 ± 4.1	28.1 ± 3.5**
ALT (μkat/l)	1.3 ± 1.1	2.7 ± 1.1	2.6 ± 1.1	8.4 ± 1.3**,* [†]
Creatinine (μmol/l)	23 ± 10	79 ± 10*	24 ± 10	83 ± 12*
Insulin (ng/ml)	2.1 ± 0.1	1.5 ± 0.1 [†]	6.2 ± 0.6 [‡]	3.1 ± 0.5*
Glucose (mmol/l)	11.4 ± 1.2	12.3 ± 1.2	14.0 ± 1.2	7.9 ± 1.5**,* [§]
Triglycerides (mmol/l)	1.8 ± 0.2	1.0 ± 0.3**	4.5 ± 0.9 [‡]	1.5 ± 0.4* [¶]
Liver fat (% of LW)	3.1 ± 0.1	3.2 ± 0.2	4.6 ± 0.2 [‡]	5.6 ± 0.3**,* [§]

ALT, alanine amino transferase; AP, acute pancreatitis; LW, liver weight.
[†]*P* < 0.05, ***P* < 0.01, and **P* < 0.001 vs. corresponding control; [§]*P* < 0.05, ****P* < 0.01 and [¶]*P* < 0.001 vs. lean AP; [‡]*P* < 0.001 vs. lean control.

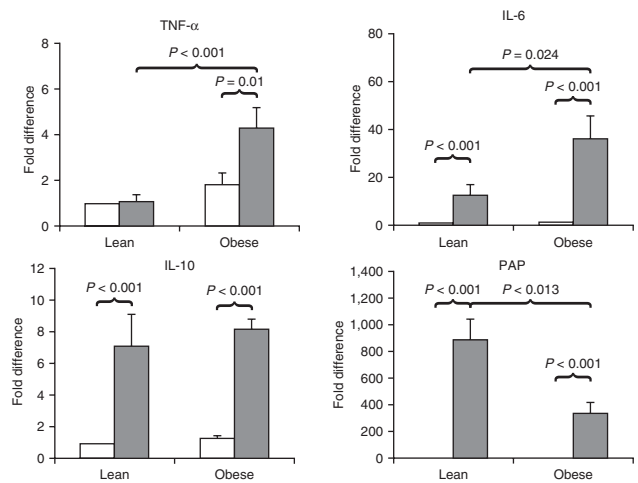


Figure 2 Hepatic mRNA expression of proinflammatory tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and anti-inflammatory IL-10 and pancreatitis-associated protein (PAP) in lean and obese Zucker rats that were either untreated (open square) or had acute pancreatitis (closed square). Data are fold differences in relation to lean control value.

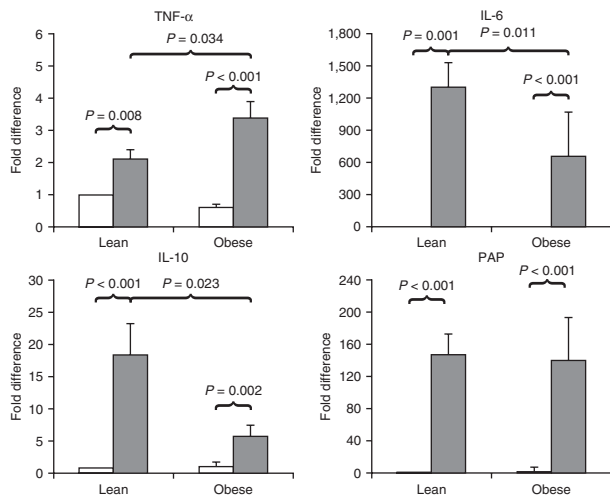


Figure 1 Pancreatic mRNA expression of proinflammatory tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and anti-inflammatory IL-10 and pancreatitis-associated protein (PAP) in lean and obese Zucker rats that were either untreated (open square) or had acute pancreatitis (closed square). Data are fold differences in relation to lean control value.

Inflammatory cytokine expression

In the pancreas, no significant differences were found in any gene expression levels between lean and obese control rats (Figure 1). The transcripts of all genes examined in lean and obese AP rats were significantly higher than in the corresponding control rats. In addition, obese AP rats showed an increase in TNF-α expression and decreases in IL-6 and IL-10 expression, compared to lean AP rats.

In the liver, no significant differences were found between lean and obese control rats for any of the gene expression levels (Figure 2). IL-6, IL-10, and PAP mRNA levels were significantly higher in both lean and obese AP rats, and TNF-α

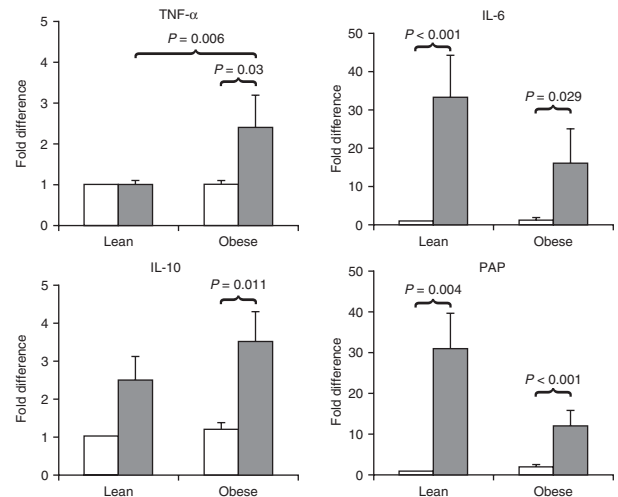


Figure 3 Pulmonary mRNA expression of proinflammatory tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and anti-inflammatory IL-10 and pancreatitis-associated protein (PAP) in lean and obese Zucker rats that were either untreated (open square) or had acute pancreatitis (closed square). Data are fold differences in relation to lean control value.

mRNA was increased in obese AP rats compared to levels in the corresponding control rats. When the two AP groups were compared, obese AP rats had significantly higher TNF-α and IL-6 expression and lower PAP expression.

In the lungs, no significant differences were found between lean and obese control rats in any target genes examined (Figure 3). IL-6 and PAP expression was significantly increased in both lean and obese AP rats compared to the respective control groups. TNF-α and IL-10 mRNA levels in obese AP rats were higher than in the control obese rats. Obese AP rats also had higher TNF-α expression than lean AP rats.

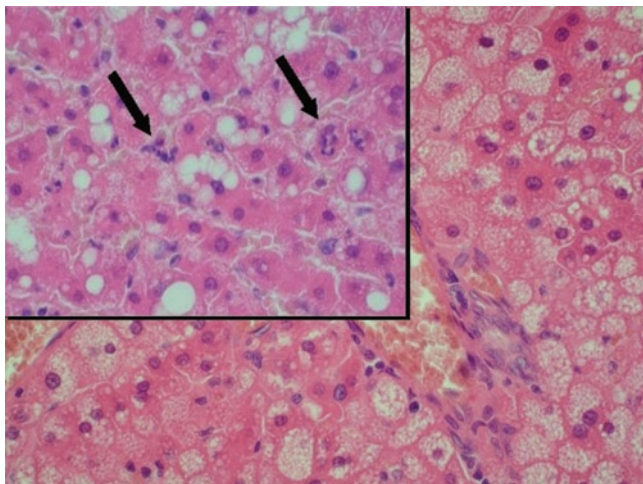


Figure 4 Representative photomicrograph of the liver in obese rats with acute pancreatitis showing extensive microvesicular steatosis. Insert shows focal areas of polymorphonuclear leukocyte infiltration and cytologic ballooning (arrows), indicating steatohepatitis (original magnification $\times 400$).

Histology

Pancreata in the two AP groups showed necrotizing pancreatitis, but the difference in score between the two groups was not significant (lean AP 9.5 ± 1.1 , obese AP 9.7 ± 1.4 , $P = 0.9$). The livers of obese AP rats exhibited focal areas of tissue neutrophil infiltration and cytologic ballooning, indicating steatohepatitis. Extensive macro- and microvesicular hepatic steatosis and scattered sinusoidal PMNLs were also observed (**Figure 4**). The only pathological changes seen in the livers of lean AP rats were scattered PMNLs in the sinusoids.

Other assays

In all the organs studied, MPO activity (absorbance/milligram) was significantly increased in AP rats compared to the corresponding control animals. Pancreatic MPO: lean AP 45 ± 14 vs. lean control 0.9 ± 0.1 , $P < 0.001$; obese AP 32 ± 8 vs. obese control 1.1 ± 0.2 , $P < 0.001$. Liver MPO: lean AP 72 ± 22 vs. lean control 2.1 ± 0.8 , $P < 0.001$; obese AP 55 ± 21 vs. obese control 0.9 ± 0.1 , $P < 0.001$. Lung MPO: lean AP 88 ± 4 vs. control 34 ± 5 , $P < 0.001$; obese AP 81 ± 5 vs. control 33 ± 4 , $P < 0.001$. MPO activity was not significantly different between the AP groups in any of the organs.

As shown in **Table 1**, serum amylase and creatinine levels increased significantly in both obese and lean AP rats compared to the respective control groups. No significant differences were found in the amylase or creatinine levels between the two AP groups. Serum alanine amino transferase levels increased significantly in obese AP but not lean AP rats, compared to corresponding controls. The mean alanine amino transferase level in obese AP rats was significantly higher than in the lean AP animals. Insulin levels decreased in both lean and obese AP rats. Obese AP rats showed decreased glucose levels compared to control rats. The mean glucose level in obese AP rats was also significantly lower than in the lean AP animals. Serum triglycerides decreased significantly in both obese and lean

rats compared to corresponding controls. The mean triglyceride values in obese AP rats were significantly higher than in the lean AP animals. Liver fat content was significantly higher in obese AP but not lean AP rats, compared to the corresponding controls. The mean liver fat content in obese AP rats was also significantly higher than in the lean AP animals.

DISCUSSION

The genetically obese fa/fa Zucker rat suffers from the syndrome of obesity, i.e., insulin resistance, hyperinsulinemia, dyslipidemia, and hepatic steatosis (17), which resembles the metabolic changes seen in human obesity (18). Therefore, induction of AP in obese Zucker rats is a good model to mimic the association between AP and obesity in humans. We have previously observed that AP caused more death in obese rats than in lean rats (15). The present study used this model to investigate a possible mechanism for the impact of obesity on AP.

In the current study, the severity of pancreatic injury and the degree of PMNL infiltration were not influenced by obesity, which is consistent with our previous observations (15,19). Cytokine expression was altered in both lean and obese AP rats compared to the control groups. In addition, the profiles of cytokine expression were different between lean and obese AP rats. In the pancreas of obese AP rats, TNF- α gene expression was increased and IL-10 and IL-6 expression was decreased compared to the expression seen in the lean AP rats. IL-10 is known to downregulate TNF- α expression (10). Thus, the blunted IL-10 response may contribute to the exaggerated TNF- α response seen in obese rats. Since the pancreata of lean and obese AP rats displayed similar degrees of leukocyte infiltration and morphological pathology, the different profiles of cytokine expression appear to result from a difference in the functioning of infiltrating immune cells in the pancreas of obese and lean AP rats.

The Kupffer cells in the liver are primarily responsible for hepatic expression of inflammatory mediators during AP (20). Because the size of the Kupffer cell population is not significantly different in obese and lean Zucker rats (21), the increased expression of TNF- α and IL-6 in the liver of obese rats might be due to a change in the functioning of Kupffer cells. Previous studies have not looked at hepatic PAP expression during AP. In the current study, both AP groups showed increased levels of PAP mRNA, but the levels in the lean AP rats were significantly higher than those in the obese AP rats. The blunted PAP expression in the obese AP rats may be of particular interest because PAP has been shown to inhibit TNF- α and IL-6 mRNA expression in macrophages (22). Therefore, in obese AP rats, the smaller increase in hepatic PAP expression may contribute to the increased TNF- α and IL-6 expression in the same organ.

Because hepatic injury is of prognostic value in human AP (23,24), the liver injury observed in the obese AP rats in the present study is an important finding. Both AP groups had decreased insulin levels consistent with previous observations (25). Interestingly, only the obese AP rats had significantly

decreased serum glucose levels. The decrease in serum triglyceride levels in AP rats is consistent with previous observations (19,26) and suggests increased fat utilization. However, the hepatic fat accumulation in the obese AP rats implies that fatty acid delivery exceeded the capacity for fatty acid oxidation or export. The observed microvesiculosis might possibly indicate impairment of mitochondrial β -oxidation, and hence inadequate hepatocyte energy production (27). This would be consistent with a previous study in which AP reduced hepatic energy production (28). Because hepatic glucose production is an energy-consuming process, hepatocyte energy depletion in obese AP rats may explain both the increased injury and the observed hypoglycemia. Indeed, it has been suggested that severe hypoglycemia contributes to shock and death in rodents after necrotizing AP (29).

TNF- α is known to induce hepatocellular injury (30), and inhibition of TNF- α expression has been shown to ameliorate pancreatitis-induced hepatocellular injury in rats (31). The higher expression of TNF- α in obese AP rats may, therefore, be deleterious. It has been recently hypothesized that PAP protects hepatocytes from TNF- α -induced apoptosis (32). Therefore the attenuated hepatic PAP expression seen in obese AP rats may further contribute to TNF- α hepatotoxicity. Previous studies have demonstrated that the fatty liver has reduced tolerance due to the insult of endotoxin, ischemia/reperfusion injury and hepatic resection (33–35). Our data add AP to the list of insults that the fatty liver does not tolerate well.

Lung injury in the present model of AP is mild and transient (36) and small differences could therefore be difficult to detect using conventional methodology, such as MPO activity. The more-sensitive quantitative real-time polymerase chain reaction method used in the present study showed that obese AP rats have significantly higher TNF- α gene expression compared to lean AP rats, suggesting an augmented proinflammatory response in obese rats. Functional heterogeneity of tissue macrophages in different organs, which has been previously observed (37), may have contributed to the variations in the cytokine expression profiles in the pancreas, liver, and lung in the current study.

Obesity is currently regarded as a low-grade chronic proinflammatory condition (38). Whether this aspect of obesity has any effects on acute pathological conditions is not known. The current study, however, found that genetically obese rats had increased systemic inflammation that was characterized by higher TNF- α expression in multiple organs. Because blockade of TNF- α is known to improve survival in experimental AP (39), this observation may help explain the increased mortality in obese AP rats observed at a later time point in our previous study (15). Our new findings support the hypothesis that the increased severity seen in obese patients with AP may be due to an amplified immune response to the local pancreatic injury (6). The current study suggests that altered inflammatory cytokine expression and increased vulnerability in the liver underlie the detrimental influence of obesity on AP.

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DISCLOSURE

The authors declared no conflict of interest.

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