Leptin treatment ameliorates acute lung injury in rats with cerulein-induced acute pancreatitis

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Abstract

AIM: To determine the effect of exogenous leptin on acute lung injury (ALI) in cerulein-induced acute pancreatitis (AP).

METHODS: Forty-eight rats were randomly divided into 3 groups. AP was induced by intraperitoneal (i.p.) injection of cerulein (50 µg/kg) four times, at 1 h intervals. The rats received a single i.p. injection of 10 µg/kg leptin (leptin group) or 2 mL saline (AP group) after cerulein injections. In the sham group, animals were given a single i.p. injection of 2 mL saline. Experimental samples were collected for biochemical and histological evaluations at 24 h and 48 h after the induction of AP or saline administration. Blood samples were obtained for the determination of amylase, lipase, tumor necrosis factor (TNF-α), interleukin (IL)-1β, macrophage inflammatory peptide (MIP)-2 and soluble intercellular adhesion molecule (sICAM)-1 levels, while pancreatic and lung tissues were removed for myeloperoxidase (MPO) activity, nitric oxide (NOx) level, CD40 expression and histological evaluation.

RESULTS: Cerulein injection caused severe AP, confirmed by an increase in serum amylase and lipase levels, histopathological findings of severe AP, and pancreatic MPO activity, compared to the values obtained in the sham group. In the leptin group, serum levels of MIP-2, sICMA-1, TNF-α, and IL-1β, pancreatic MPO activity, CD40 expression in pancreas and lung tissues, and NOx level in the lung tissue were lower compared to those in the AP group. Histologically, pancreatic and lung damage was less severe following leptin administration.

CONCLUSION: Exogenous leptin attenuates inflammatory changes, and reduces pro-inflammatory cytokines, nitric oxide levels, and CD40 expression in cerulein-induced AP and may be protective in AP associated ALI.

INTRODUCTION

Acute pancreatitis (AP) is a non-infectious inflammatory reaction of the pancreas, associated with autodigestion of the organ[1]. Lung injury is the most important manifestation of extra abdominal organ dysfunction in acute pancreatitis[2]. Approximately one-third of patients develop acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) accounts for 60% of all deaths in the first week[3]. Apart from mechanical ventilatory support, few therapies have shown any clinical benefit. New agents such as anti-cytokines and anti-nuclear factor kappa are still being evaluated in experimental models of pancreatitis[1,4]. ALI is characterized by an increase in pulmonary microvascular permeability, with protein-rich transudate spilling into the alveolar spaces, resulting in decreased lung compliance. Such physicochemical alterations are mediated by the local release of cytotoxic and vasoactive substances. Recent studies have shown that cytokines and adhesion molecules such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), macrophage inflammatory peptide-2 (MIP-2), intercellular adhesion molecule-1 (ICAM-1) and CD40 (50-kDa protein expressed on the membranes of B lymphocytes, monocytes, dendritic cells, and biliary epithelial cells) as well as neutrophil activation and adhesion contribute to the development and severity of AP and ALI[2,5-7].

Leptin is a peptide hormone that is produced...
predominantly by white adipose cells\textsuperscript{[8]}. The mature protein, encoded by the obese (\textit{ob}) gene, is localized on human chromosome 7 and mouse chromosome 6\textsuperscript{[9]}. In addition to metabolic and endocrine functions, leptin also plays a regulatory role in hematopoiesis, immunity and inflammation\textsuperscript{[10,11,12]}. Alterations in immune and inflammatory responses are present in leptin or leptin-receptor-deficient animals, as well as during starvation and malnutrition. Both conditions are characterized by low levels of circulating leptin. Leptin is believed to play a role in immune function, as it is a member of the helical cytokine family with a structure resembling the hematopoietic cytokines IL-2, IL-6 and IL-13\textsuperscript{[10]}. The leptin receptors (Ob-R) show amino acid sequence homology to hematopoietic (class 1) cytokine receptors\textsuperscript{[13,14]}. Besides fat tissues, Ob-R is also present in other tissues such as liver, pancreas, lung, and kidney\textsuperscript{[15]}. Leptin exerts proliferative and anti-apoptotic activity in a variety of cell types including T lymphocytes, leukemia cells, and hematopoietic progenitors. It also affects cytokine production, the activation of monocytes/macrophages, wound healing and angiogenesis. Moreover, leptin production increases acutely during infection, inflammation, and pancreatitis\textsuperscript{[16,17]}. Recent studies have demonstrated that AP in rats and humans is accompanied by an increase in plasma levels of leptin\textsuperscript{[15,16]}. Furthermore, the elevation of leptin mRNA expression in the pancreas during AP suggests that the increased level of this hormone in serum originates from the pancreas\textsuperscript{[18]}. Another study showed that exogenous leptin administration was associated with a reduction in the severity of AP, presumably through a decrease in the production of plasma pro-inflammatory cytokines and NO\textsuperscript{[19]}. Whether exogenous leptin has any effect on AP-associated lung injury remains unclear, however, its anti-inflammatory and anti-apoptotic actions would suggest that it may reduce the occurrence of extra-abdominal organ dysfunctions such as ALI. In the present study, we examined the effects of exogenous leptin on lung injury in a cerulein model of AP.

**MATERIALS AND METHODS**

**Experimental animal models and Study groups**

Forty-eight female Wistar rats weighing 230-260 g were used in the study. The experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, and was approved by the Committee for Research and Animal Ethics of Gazi University. The rats were maintained at 23°C in a 12 h light dark cycle, with free access to water and standard rat chow. Prior to the start of the experiments, the rats were deprived of food while drinking water was available ad libitum. Three experimental groups were established: sham group (\textit{n} = 16), acute pancreatitis (AP) group (\textit{n} = 16) and leptin group (\textit{n} = 16). AP was induced by intraperitoneal (i.p.) injection of cerulein (Sigma-Aldrich Chemical, Steinheim, Germany) diluted in saline and administered at the dose of 10 mg/kg i.p. dissolved in 1 mL of saline followed by cerulein injections. The sham group was injected with 2 mL 0.9% NaCl solution i.p. All groups were randomly subdivided into 24 h and 48 h groups, each group containing 8 rats. Frossard \textit{et al.}\textsuperscript{[3]} have demonstrated that administration of supramaximal dose of cerulein to rats led to AP associated ALI; the severity of ALI was time-dependent, with maximal injury occurring at 24 h and 48 h after the start of cerulein injections. Therefore, in our study, the animals were sacrificed at 24 h and 48 h after the cerulein or saline administration.

Following cessation of treatment, animals were anesthetized by an intramuscular injection of 40 mg/kg ketamine (Ketalar\textsuperscript{®}, Parke Davis, Eczacibasi, Istanbul, Turkey) and 5 mg/kg xylazine (Rompum\textsuperscript{®}, Bayer AG, Leverkusen, Germany). The abdominal and thoracic cavities were opened and blood, pancreatic and lung samples were obtained. The blood samples were stored at -80°C for biochemical analysis, which was run in duplicates. Random cross-sections of the pancreatic head, body and tail, and of the right lung were fixed in a 40 g/L solution of formaldehyde, in 0.1 mol/L phosphate-buffed saline (pH 7.4), and embedded in paraffin. Samples of pancreas and lung tissues were stored at -70°C for subsequent biochemical measurements.

**Serum amylase and lipase measurements**

Serum amylase and lipase were determined with a Beckman Coulter LX-20\textsuperscript{®} System analyzer (Fullerton, CA, USA) using Beckman kits (Fullerton, CA, USA), according to the supplier’s specifications.

**Serum TNF-\textit{\alpha}, IL-1\textit{\beta}, MIP-2 and Soluble ICAM-1 measurements**

Serum levels of TNF-\textit{\alpha}, IL-1\textit{\beta}, MIP-2 (Biosource International, Camarillo, CA, USA) and soluble ICAM-1 (sICAM-1, Quantikine, R&D Systems, MN, USA) were determined by using a commercially available enzyme-linked immunosorbent assay (ELISA). TNF-\textit{\alpha}, IL-1\textit{\beta}, MIP-2 and sICAM-1 levels were determined from a standard curve for the combination of these cytokines. The concentrations were expressed as pg/mL.

**Measurement of tissue myeloperoxidase activity**

Pancreas and lung tissues were homogenized in TRIS buffer. Sequestration of neutrophils within the pancreas and lung was evaluated by quantitation of tissue myeloperoxidase (MPO) activity by the ELISA method (Hbt Hyicult Mouse MPO ELISA kit, Uden, Netherlands).

**Measurement of NOx level**

Total nitrate/nitrite (NOx) concentration was measured in a simple two-step process by using the Cayman Chemical NOx Assay Kit. The first step was the conversion of nitrate to nitrite, utilizing nitrate reductase. The second step was the addition of the Griess reagents, which converted nitrite into a deep-purple azo-compound. Photometric measurement of the absorbance due to this azo-chromophore accurately determined nitrite.
concentration. Concentrations were expressed as nmol/mg protein\(^{[39]}\).

**Histological examination**

Sections of the pancreas and lung (4μm thick) were stained with hematoxylin-eosin (HE) and examined with a light microscope as described previously\(^{[22]}\).

**Immunohistochemical assessment of CD40**

Specimens were fixed in a 40g/L solution of formaldehyde in 0.1 mol/L phosphate-buffed saline (pH 7.4), and embedded in paraffin wax from which four-μm-thick sections were taken on a slide coated with poly-L-lysine in order to be de-waxed for immunohistochemical assessment. The specimens were treated with 3% hydrogen peroxidase, following 10 min of endogenous peroxidase blockage. Antigen retrieval was performed with EDTA. Following the processing of the samples with a streptavidin biotin peroxidase complex, 3-amino-9-ethylcarbazole was applied as a chromogen, and hematoxylin was used for counterstaining. CD40 is expressed on the surface of B lymphocytes. Tonsil tissue was used as a positive control since the tonsils are lymphoid organs where B lymphocytes are commonly present in lymphoid folicles. CD40 staining was graded from 0 to 4; the number of cells which revealed CD40-positive staining obtained from five × 400 magnified fields was divided by the total cell number. According to these observations, less than 50% of the stained cell population was considered focally stained, and more than 50% of the stained cell population was considered diffusely stained. Grade 0: no staining; grade 1: slight cytoplasmic or membranous staining in <50% of the cell population (focal); grade 2: slight cytoplasmic or membranous staining in >50% of the cell population (diffuse); grade 3: strong cytoplasmic or membranous staining in <50% of the cell population (focal); grade 4: strong cytoplasmic or membranous staining in >50% of the cell population (diffuse).

**Statistical analysis**

All values were expressed as mean±SE and the results were compared by analysis of variance (ANOVA) with post hoc analysis using the Bonferroni test. The log rank test was used for survival analysis. \(P < 0.05\) was considered statistically significant. Statistical evaluation was carried out using the SPSS 12.0 software (SPSS, Chicago, IL, USA).

**RESULTS**

Kaplan-Meier survival curves (Figure 1) showed absence of mortality after the sham operation (100% survival), while the survival rate was reduced to 62.5% in the AP group \((P < 0.001)\) vs sham). Treatment with leptin was associated with significant improvement in the survival rate (87.5%) throughout the 48 h observation period \((P < 0.05)\).

**Effects of exogenous leptin on pancreatic injury in AP**

Supramaximal doses of cerulein injections resulted in severe AP in the animals. This was confirmed by an increase in serum amylase and lipase levels \((P < 0.001)\), histopathological findings of severe AP \((P < 0.05, Table 3, Figures 2C and D)\), and pancreatic MPO activity, as a measurement of neutrophil infiltration \((P = 0.004, Tables 1 and 2)\) in the cerulein injected rats compared to saline administered rats. Serum amylase levels and pancreatic MPO activity in rats treated with leptin were markedly lower at 24 h compared to cerulein-only injected rats \((P < 0.001, Tables 1 and 2)\). However, there was no significant difference in serum lipase levels between the AP group and the leptin treated group (Tables 1 and 2). Histopathological examination of the pancreas confirmed the beneficial effect of exogenous leptin treatment on AP (Table 3). In the pancreatic tissue from the AP group,
Effects of exogenous leptin on ALI associated with AP

Tissue MPO activities and NOx levels in lung tissues increased significantly in cerulein administered rats compared with rats that received saline, indicating that increased neutrophil infiltration and microvascular permeability were the result of pancreatitis. Treatment with leptin decreased lung MPO activities and tended to decrease at 48 h. sICAM levels remained high during the 48 h study period. Treatment with leptin decreased TNF-α, IL-1β, MIP-2, and sICAM levels in cerulein administered rats (P < 0.001, Tables 1 and 2). Serum cytokines and sICAM levels were markedly higher in both AP and leptin groups compared to the sham group (P < 0.001, Figure 4A and B). In rats treated with leptin, there was a significant decrease in the lung CD40 expression (P < 0.001, Table 5).

Effects of exogenous leptin on cytokines in AP

Serum cytokines and sICAM levels were markedly higher in both AP and leptin groups compared to the sham group (P < 0.001, Tables 1 and 2). Serum TNF-α, IL-1β and MIP-2 levels increased at 24 h after cerulein injection and tended to decrease at 48 h. sICAM levels remained high during the 48 h study period. Treatment with leptin decreased TNF-α, IL-1β, MIP-2, and sICAM levels in cerulein administered rats (P < 0.001, Tables 1 and 2).
decrease in cytokines and sICAM levels in the leptin group may contribute to the attenuation of lung injury associated with cerulean-induced AP.

**DISCUSSION**

To our knowledge, the present study is the first to demonstrate that exogenous leptin administration significantly reduces ALI in rats with cerulein induced AP. The exogenous leptin treatment reduced cerulein induced pancreatitis, and pancreatitis-associated lung injury, with reduction in the serum concentrations of TNF-α, IL-1β, MIP-2 and sICAM-1, decrease in the pancreatic and pulmonary neutrophil activation, and in MPO activity, and NOx, and CD40 levels. In addition, the exogenous leptin treatment significantly reduced mortality rates in rats with AP induced by supramaximal doses of cerulein.

Histological findings of ALI were more prominent after injection of supramaximal doses of cerulein compared to those seen in the sham group at 24 h and 48 h; maximum improvements in histological features of ALI were observed at 48 h after leptin administration. During AP, lung injury was associated with accumulation of neutrophils in the interstitial and alveolar spaces, a common finding in both clinical and experimental studies [1,2]. It has been shown that reduction in circulating serum neutrophils, and strategies which interfere with neutrophil recruitment, such as ICAM-1 blocking antibodies are accompanied with decrease in neutrophil infiltration and in pancreatic and distant organ damage in AP[3,23]. In our study, induction of AP was associated with a significant increase in pancreatic and lung MPO activity, indicating that neutrophils were sequestered in both organs. Exogenous leptin treatment markedly decreased the MPO activity. Jaworek et al[15] have shown that exogenous leptin treatment reduced neutrophil infiltration. However, an exact explanation of the effects of leptin on neutrophil activation remains unknown. ICAM-1 and MIP-2 play an important role in the activation and the adhesion of neutrophils [23,24]. A better understanding of the effects of leptin on these factors may help in defining their effects on neutrophil activation.

ICAM-1 is an inducible protein expressed on the surface of endothelial cells. ICAM-1 plays an important role in neutrophil adhesion and neutrophil mediated lung injury during AP. Serum ICAM-1 concentrations peak at 24 h after cerulein administration[23]. In the present study, exogenous leptin significantly reduced serum ICAM-1 concentrations compared with the sham group. These findings suggest that neutrophil activation in the lung may be ameliorated by leptin administration.

| Group          | Pancreas   | Lung      |
|----------------|------------|-----------|
|                | 24 h       | 48 h      | 24 h     | 48 h     |
| Sham group     | 0          | 0         | 0        | 0        |
| AP group       | 2.6 ± 0.8' | 3.4 ± 0.4' | 2.6 ± 0.8' | 3.1 ± 0.6' |
| Leptin group   | 1.5 ± 0.6  | 2.1 ± 0.3  | 1.5 ± 0.6  | 1.9 ± 0.5  |

*P < 0.05 vs the leptin group.

![Sham Group-24 h](A) ![Sham Group-48 h](B)

![AP Group-24 h](C) ![AP Group-48 h](D)

![Leptin Group-24 h](E) ![Leptin Group-48 h](F)

**Figure 3** Immunohistochemical localization of CD40 expression in the pancreas in the sham group (A, B), AP group (C, D) and leptin group (E, F). (Divisions A, C, E on the figure represent pulmonary sections at 24 h and B, D, F represent pulmonary sections at 48 h).

**Figure 4** Immunohistochemical localization of CD40 expression in lung tissues. There was no staining in the sham group (A, B), whereas marked staining was observed in the AP group (C, D). CD40 staining was significantly reduced in leptin-treated rats (E, F). (Divisions A, C, E on the figure represent pulmonary sections at 24 h and B, D, F represent pulmonary sections at 48 h).
During AP, MIP-2 is involved in neutrophil activation and sequestration in the pancreas and lungs. MIP-2 is a potent rodent chemokine, homologous to GRO-β, which binds to the C-X-C chemokine receptor-2. We found that cerulein induced AP was associated with a significant increase in serum MIP-2 concentrations, and that leptin treatment substantially decreased the MIP-2 concentration. The effect of leptin administration on serum MIP-2 concentration is not clearly established. To the best of our knowledge, this is the first study demonstrating the effect of leptin on serum MIP-2 concentration. A decrease in the concentration of MIP-2 may therefore play an important role in reducing neutrophil adhesion and sequestration. Moreover, Ob-R is present in the pancreas and lungs. Therefore, leptin may also reduce pancreatic neutrophil activation by affecting its lung receptors. Several in vitro studies have shown that endogenous leptin increases neutrophil activation. However, administration of a higher dose of exogenous leptin compared to the baseline circulating level may result in neutrophil inhibition.

Since leptin is a pleiotropic hormone, it is expected that its effects at supraphysiological dose may be similar to the effects in normal doses. In contrast, Konturek et al and Warzecha et al indicated that high doses of exogenous leptin had greater effect, and markedly attenuated pancreatic damage in both cerulein-induced and ischemia/reperfusion-induced pancreatitis models. We found similar effects with exogenous leptin in this study, where high doses of leptin exhibited the protective effects in AP and in AP associated lung injury. Nevertheless, these dichotomous effects arising from differing doses of leptin remain poorly understood and require further investigation.

TNF-α, and IL-1β are derived predominantly from activated macrophages and act via specific cell membrane-bound receptors in inflammatory situations such as AP and AP associated ALI. The severity of pancreatitis has been shown to correlate with TNF-α and IL-1β levels. After the onset of pancreatitis, cytokine production from lung parenchyma increases significantly, and large quantities of chemokines, including TNF-α and IL-1β are released from macrophages via the p38 mitogen activated protein-kinase pathway. Specific treatments that target the reduction of TNF-α and IL-1β levels reduce the severity of ALI in AP. Konturek et al have shown that exogenous leptin treatment reduced plasma TNF-α level and pancreatic IL-4 expression in rats with AP. In another study, Warzecha et al concluded that leptin treatment reduced plasma IL-1β levels. Ob-R is a member of the class I cytokine receptor superfamily. These receptors are membrane-spanning glycoproteins. Leptin is recognized as a pro-inflammatory hormone, and it shares structural similarities to cytokines IL-6, IL-15 and granulocyte colony-stimulating factor. However, our understanding of the role of leptin in inflammation is incomplete. Endogenous leptin protects against TNF-mediated toxicity. Ob/ob mice and db/db mice, as well as mice treated with leptin-receptor antagonist displayed increased sensitivity to the lethal effects of TNF. The addition of exogenous leptin protected against TNF-mediated toxicity in ob/ob mice, but did not increase the protective effect of endogenous leptin in wild-type mice. Our study showed that AP was accompanied with increased plasma levels of TNF-α and IL-1β compared to the sham group, and exogenous leptin reduced these cytokine levels at 24 h and 48 h following cerulein injection. Overstimulation of Ob-R might also result in a decrease in cytokine levels. Taken together, exogenous leptin treatment appears to play an important role in the reduction of pancreatic damage during AP, through a reduction in the pro-inflammatory cytokines.

Leider et al have shown that the expression of iNOS correlated with apoptotic changes in the lung, suggesting that NO overproduction is an important factor in the development of a systemic inflammatory reaction in response to severe pancreatitis. In the present study, the NOx concentration in lung tissues was considerably higher in rats with cerulein-induced AP, compared with controls. However, a significant decrease in the NOx level was observed with leptin treatment. Leptin was able to reduce ALI by decreasing the NOx level, which resulted in the reduction of inflammation and neutrophil activation.

CD40, a protein member of the TNF receptor superfamily, is expressed on the membrane of a variety of cells, including B lymphocytes, monocytes, biliary and acinar cells. CD40 binds to its ligand CD40L, a membrane glycoprotein, and mediates major immunoregulatory signals involved in auto-immune disease, inflammatory bowel disease and acute experimental pancreatitis. Daoussis et al have demonstrated that the CD40 ligation on antigen-presenting cells is associated with the following: (1) enhanced cell survival; (2) secretion of cytokines (TNF-α, IL-1β, IL-6, MIP); (3) enhanced monocyte activity, and (4) NO synthesis. In a recent study, Frossard et al concluded that elevation of serum CD40 levels is a prognostic factor for AP. In our study, pancreatic and pulmonary CD40 staining level in the AP group was significantly higher than the sham group, and exogenous leptin treatment reduced both pancreatic and pulmonary CD40 staining. Exogenous leptin may contribute to the reduction in MPO activity by decreasing lung CD40 levels. However, whether lung CD40 levels are suppressed by leptin is unclear. Leptin directly reduces pancreatic CD40 levels and inhibits monocyte activation, which plays an important role in acute lung injury.

In conclusion, supramaximal doses of exogenous leptin treatment significantly abrogated ALI in cerulein-induced AP. Exogenous leptin markedly reduced both neutrophil activation and the levels of soluble pro-inflammatory factors such as cytokines, chemokines, NOx and CD40 that are known to be involved in the development of ALI. However, the pathophysiological role of exogenous leptin in AP and pancreatitis-associated lung injury requires further investigation.

ACKNOWLEDGMENTS

This study was presented at the 41st Congress of The European Society for Surgical Research, in Rostock, Germany, 17-21 May 2006. The authors would like to thank Syn WK. MD for excellent technical assistance.

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