Published in final edited form as:
*J Immunol*. 2010 July 1; 185(1): 517–524. doi:10.4049/jimmunol.0903975.

# Leptin Exacerbates Sepsis-Mediated Morbidity and Mortality

**Nathan I. Shapiro**[*,†], **Eliyahu V. Khankin**[*,‡], **Matijs Van Meurs**[*,†], **Shou-Ching Shih**[*,§], **Shulin Lu**[*,†], **Midori Yano**[*,†], **Pedro R. Castro**[*,†], **Eleftheria Maratos-Flier**[¶], **Samir M. Parikh**[*,‖], **S. Ananth Karumanchi**[*,‡,‖], and **Kiichiro Yano**[*,†]

[*]Center for Vascular Biology Research, Harvard Medical School, Boston, MA 02215

[†]Department of Emergency Medicine, Harvard Medical School, Boston, MA 02215

[‡]Division of Molecular and Vascular Medicine, Harvard Medical School, Boston, MA 02215

[§]Department of Pathology, Harvard Medical School, Boston, MA 02215

[¶]Division of Endocrinology, Harvard Medical School, Boston, MA 02215

[‖]Division of Nephrology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

## Abstract

The adipose-derived hormone leptin is well known for its contribution to energy metabolism and satiety signaling in the hypothalamus. Previous studies suggested that obesity is an independent risk factor for sepsis morbidity and mortality, and it is associated with elevated baseline levels of circulating leptin in normal, nonseptic patients. In mouse endotoxemia and cecal ligation puncture models of sepsis, we observed elevated levels of leptin and soluble leptin receptor (sLR). Exogenously administered leptin increased mortality in endotoxemia and cecal ligation puncture models and was associated with increased expression of adhesion and coagulation molecules, macrophage infiltration into the liver and kidney, and endothelial barrier dysfunction. Conversely, longform leptin receptor-deficient mice were protected from sepsis morbidity and mortality and had less endothelial dysfunction. Furthermore, an in vitro study revealed that leptin-induced endothelial dysfunction is likely mediated, at least in part, by monocytes. Moreover, administration of an sLR conferred a survival benefit. Human septic patients have increased circulating sLR concentrations, which were correlated with disease severity indices. Together, these data support a pathogenic role for leptin signaling during sepsis.

There are 750,000 cases of severe sepsis diagnosed every year in the United States, accompanied by unacceptably high mortality (1). Increasing evidence suggests that endothelial cells play an important role in sepsis pathophysiology. Animal and human sepsis studies demonstrated a pathogenic role for endothelial activation/dysfunction, leading to upregulation of endothelial-related adhesion and coagulation molecules (2, 3). This results in



Address correspondence and reprint request to Dr. Kiichiro Yano, Department of Emergency Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, RN-280B, Boston, MA 02215. kyano@bidmc.harvard.edu.

**Disclosures**
The authors have no financial conflicts of interest.

an increase in leukocyte trafficking, coagulation, vascular permeability, and inflammation, characteristic of the sepsis phenotype.

Leptin is an adipocyte-derived circulating hormone/cytokine that regulates adipose tissue mass through hypothalamic effects on satiety and energy expenditure, in addition to playing a role in regulating immune homeostasis (4). Elevated baseline levels of leptin are found in obese patients (5), and obesity seems to be an independent, “dose-dependent” risk factor for sepsis morbidity and mortality (6, 7). The leptin receptor OB-R is a member of the class I cytokine receptor family and exists in at least six isoforms derived from RNA splicing variants known as OB-Ra-f (8, 9). Deficiency of leptin or its long-form receptor gene (OB-Rb) in animals and humans results in increased fat deposition, leading to a series of abnormalities in various physiologic processes, including abnormal angiogenesis (10, 11), hyperlipidemia, hyperglycemia, and increased insulin secretion (4). Deficiency of this receptor impairs innate and adaptive immune responses (12, 13). Prior studies demonstrated that the leptin receptor OB-Rb is expressed in endothelial cells, as well as leukocytes, lymphocytes, monocytes, and macrophages (14–17), and it mediates leptin actions. In contrast, soluble leptin receptor (sLR) (Ob-Re) is expressed by several nonimmune cells and is thought to mediate the transport and degradation of leptin. Although there is accumulating evidence regarding the role of leptin and its receptors in sepsis, very little is known about the specific role of leptin signaling in the endothelial activation/dysfunction that is noted in sepsis.

Prior studies demonstrating a role for leptin in sepsis have yielded conflicting results. Mice deficient in the long-form receptor seem to be unaffected (18) or protected (19) from the deleterious effects of endotoxin. Because leptin is the only known ligand for the receptor, this finding suggests that leptin acts through its long-form receptor during endotoxemia. However, other investigators showed that leptin-deficient mice (ob/ob) are sensitized to endotoxin in such a way that exogenous leptin administration is protective in this ob/ob model (18, 20, 21). These contradictory findings have yet to be resolved and have impaired our understanding of the mechanisms by which obese individuals are more susceptible to adverse outcomes in sepsis (6, 7).

Therefore, the goal of this study was to investigate the role of leptin in sepsis and its effects on endothelial activation/dysfunction in vivo and in vitro. Because the metabolic abnormalities that arise in mice genetically deficient in leptin may independently exacerbate sepsis (18), we compared outcomes between obese long form leptin receptor-deficient (db/db) mice and nonobese wild-type (WT) mice that transiently overexpress sLR (and thereby block intracellular signaling downstream of OB-Rb). Moreover, we tested the role of exogenous leptin administration in a fashion that produces a metabolic profile similar to vehicle-treated WT mice. In this article, we report that leptin and its long-form receptor axis play a pathogenic role in sepsis-associated endothelial dysfunction. We also propose a potential role for antileptin therapy in reducing sepsis morbidity and mortality.

## Materials and Methods

### Sepsis models and tissue sample preparation

Eight-week-old male C57BL/6 mice (purchased from Charles River Laboratories, Wilmington, MA) and male db/db mice (B6.BKS[D]-Lepr$^{db}$/J) and its control (C57BL/6J) (purchased from The Jackson Laboratory, Bar Harbor, ME) were used in this study. Murine endotoxemia models and cecal ligation puncture (CLP) were performed as previously described, with minor modifications, using a single 21-gauge needle puncture technique (22). For tissue sample preparation, animals were perfused with PBS, and organ tissue samples were harvested and snap-frozen for RNA isolation. Another set of organ samples was fixed with buffered formalin for 1 h, washed with PBS for 1 h, and incubated in 20% sucrose overnight at 4°C. The organ samples were embedded in OCT compound for immunohistochemistry. Blood samples were assayed for blood glucose, blood urea nitrogen (BUN), and alanine aminotransferase (ALT). Plasma cytokine levels of mouse leptin, sLR, soluble vascular endothelial growth factor receptor-1 (sVEGFR1), and IL-6 were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN). All animal studies were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

### Exogenous leptin/sLR protein administration

To administer mouse leptin or sLR recombinant protein (R&D Systems), we used osmotic pumps (model 1007D) that are capable of 7-d continuous in situ infusion (ALZET osmotic pumps, Cupertino, CA). In brief, mouse leptin or sLR recombinant protein was reconstituted in PBS containing 5% BSA. Osmotic pumps were primed according to the manufacturer's instructions with mouse leptin (0.5 mg/ml) or sLR protein (Fc chimera; 1 mg/ml). For the endotoxin experiments, leptin pumps or control pumps (5% BSA) or sLR pumps or control pumps (5% BSA) were implanted into the abdominal cavity 3 d prior to LPS administration. For the CLP model, a combination of injection and pump infusion was used. Mice were injected i.p. with 10 mg mouse leptin protein or 20 μg mouse sLR every 12 h for 1 d and then osmotic pumps carrying leptin, sLR, or their controls (5% BSA) were implanted when CLP was performed. We also confirmed that 5% BSA and Fc fragment did not show any difference in sepsis mortality using experimental sepsis models (LPS and CLP) (Supplemental Fig. 4). Supplemental mouse leptin or sLR was injected into the mice using the same regimen for another 2 d after the implantation of osmotic pumps and was titrated to augment circulating leptin levels until pump-derived mouse leptin achieved the targeted 30 ng/ml levels.

### Survival studies

Survival studies were performed using endotoxemia and CLP models. Survival was assessed over 96 h for endotoxin, comparing 16 mg/kg body weight LPS injection to placebo controls. For the CLP, survival was assessed over 96 h after the CLP procedure compared with mice undergoing sham operation.

### Body fat measurement

Mouse body fat mass was analyzed using dual-energy X-ray absorptiometry (Lunar PIXImus2 mouse densitometer; GE Medical Systems, Madison WI), as previously described (23). The machine was calibrated daily using a phantom provided by the manufacturer. The system has an image-acquisition time of 5 min. Fat weight, lean weight, and percentage adiposity (fat weight/[fat weight + lean weight]) were predicted by the measurement.

### Histological analyses

For macrophage detection in situ, immunohistochemistry for CD11b (macrophage-1 Ag [Mac-1]) was performed on 5-μm snap-frozen liver or kidney sections using rat monoclonal anti-mouse CD11b Ab (BD Biosciences, San Jose, CA). The avidin-biotin method and 3-amino-9-ethylcarbazol chromogen were used to amplify and visualize the signal (24). Hematoxylin was used for nuclear stains.

### Cell culture studies

HUVECs were cultured in microvascular endothelial growth medium-2 media (Lonza, Walkersville, MD). A human monocyte cell line (THP-1; American Type Culture Collection, Manassas, VA) was maintained in 10% FBS RPMI 1640 media (Invitrogen, Carlsbad, CA). One million THP-1 cells (or no cells) were incubated in 0.5% FBS RPMI 1640 media with 1 μg/ml mouse leptin recombinant protein (R&D Systems), 1 μg/ml LPS, leptin plus LPS (both 1 μg/ml), and control (1 μg/ml BSA in PBS) for 24 h. In the meantime, HUVECs were preincubated with serum-starvation medium (0.5% FBS endothelial basal medium-2) for 20 h. Conditioned media from monocytes with a series of treatments and incubated culture media were added to serum-starved HUVECs and cultured for 4 h. In all experiments, cells were incubated at 37°C with 5% $CO_2$.

### Real-time quantitative PCR

Tissue and cultured-cell RNA were isolated and purified as previously described, and cDNA preparation and real-time PCR were performed as previously described (2, 25). Primer sequences for targeted molecules are shown in Supplemental Table I.

### Human studies

Human samples were prospectively collected from patients presenting to the emergency department at the Beth Israel Deaconess Medical Center. The inclusion criteria were age ≥18 y and suspected infection, as determined by the treating clinician. Blood samples were collected in EDTA tubes and centrifuged, and plasma was frozen at −80°C within 30 min of acquisition. Human leptin and sLR levels were assayed using commercially available kits (R&D Systems). The APACHE-II score was calculated, and serum lactate levels were measured upon presentation; these are two well-established markers of illness severity in sepsis (26, 27). Descriptive statistics were used to describe the population, and the Spearman correlation coefficient was used to describe the relationship among leptin, sLR, and body mass index (BMI). Lactate levels were normalized using a log transformation. Linear regression, controlling for confounders, was used to assess the relationship among leptin, sLR, and the illness severity markers, such as lactate and APACHE-II score. This study was

approved by the Beth Israel Deaconess Medical Center Committee for Clinical Investigations.

### Statistical analyses for animal studies

A two-way ANOVA followed by the Fisher exact test was used to compare morbidity markers, including cytokine levels, vascular leakage, BUN levels, ALT levels, and gene expression. We compared the following groups: control versus leptin treated and control versus sLR treated, each with or without sepsis (LPS or CLP). The Wilcoxon log-rank test was used to assess statistical significance for the survival studies; $p < 0.05$ was considered significant.

## Results

### Plasma leptin levels are elevated in sepsis and contribute to mortality

LPS administration in mice resulted in increased circulating leptin levels compared with PBS-injected controls (28.3 ± 8.4 ng/ml versus 2.1 ± 0.8 ng/ml, respectively; $p < 0.01$). Circulating leptin levels were also increased in mice subjected to CLP compared with sham-operated mice (24.8 ± 1.0 ng/ml and 1.0 ± 0.5 ng/ml, respectively; $p < 0.01$) (Fig. 1*A*). Time-course experiments using endotoxemia models revealed a time-dependent increase in plasma leptin levels (Fig. 1*B*).

To investigate the role of leptin in sepsis morbidity and mortality, we administered exogenous leptin protein or 5% BSA in PBS (control) using osmotic pumps. In the exogenously augmented leptin group, circulating leptin levels of ~30 ng/ml were targeted and were achieved at 2 d after leptin pump administration (Fig. 2*A*). These levels were maintained for a week (data not shown). The administration of exogenous leptin in lean mice was associated with significantly lower survival rates in the endotoxemia model (0 and 37.5%, leptin treated versus control, respectively; $p < 0.01$) (Fig. 1*C*) and the CLP model (0 and 37.5%, leptin treated versus control, respectively; $p < 0.01$) (Fig. 1*D*).

To assess the role of the long-form leptin receptor during sepsis, we repeated the endotoxemia and CLP sepsis experiments using db/db mice, which lack a functional long-form receptor. Receptor-deficient mice showed improved survival during endotoxemia (50 and 12.5%, db/db mice versus WT control mice, respectively; $p < 0.05$) (Fig. 1*E*) and CLP induced-sepsis (62.5 and 25%, db/db mice versus WT control mice, respectively; $p < 0.04$) (Fig. 1*F*). Exogenously administered leptin in normal mice accentuated sepsis mortality, and leptin receptor-deficient mice were protected during sepsis. Together, these findings support a pathogenic role for leptin and its long-form receptor axis in sepsis.

### Exogenous leptin results in further induction of soluble VEGFR1 and IL-6 during endotoxemia, and its induction is attenuated in db/db mice

Given the well-studied role of altered energy metabolism in sepsis, we investigated the metabolic consequences of exogenous leptin administration. Blood glucose measurement revealed a significant reduction in blood glucose levels in leptin-treated mice at day 1 (89.8 ± 17.3 mg/dl) compared with control mice (125 ± 18.3 mg/dl) (Fig. 2*B*); however, this

difference disappeared after day 1, and there was no difference in the glucose levels between the leptin- and control-treated groups on subsequent days (Fig. 2*B*). LPS administration (on day 4) resulted in a significant decrease in blood glucose levels (hypoglycemia). However, the glucose levels were similar in the leptin- and control-treated groups (Fig. 2*B*) during endotoxemia. Fat that stores energy for the body is broken down to release glycerol and free fatty acids when necessary. The glycerol can be converted to glucose by the liver; thus, fat is used a source of glucose. Similarly, there was no difference in fat weight (Supplemental Fig. 1*C*), lean weight (Supplemental Fig. 1*B*), or percentage adiposity (Fig. 2*C*), suggesting that there is no change in the source of glucose with or without endotoxemia. Lack of alterations in glucose metabolism provides evidence that we were able to examine the effects of leptin, independent of its effects on the hypothalamic axis.

We and other investigators previously showed that circulating sVEGFR1 and IL-6 reflect the severity of inflammation in experimental and human sepsis (28, 29). ELISA assays for sVEGFR1 and IL-6 revealed no baseline difference in circulating levels between leptin- and control-treated mice. However, in endotoxemia, exogenously administered leptin resulted in a 2.2-fold increase in sVEGFR1 levels (Fig. 2*D*) and a 3-fold increase in IL-6 levels (Fig. 2*E*). Next, we repeated endotoxin experiments using db/db mice. Although circulating leptin levels were similarly elevated during endotoxemia in WT and db/db mice (Fig. 2*F*), endotoxemic db/db mice showed 2.2-fold lower levels of circulating sVEGFR1 (Fig. 2*G*) and 9.8-fold lower levels of circulating IL-6 (Fig. 2*H*). These results showed that exogenous leptin exacerbates inflammation without inducing major alterations in energy metabolism, effects that are reversed in the absence of a long-form leptin receptor.

## Exogenous leptin results in upregulation of inflammatory mediators during endotoxemia, whereas leptin receptor deficiency attenuates inflammatory mediator expression

Having found that alterations in leptin and its long-form receptor axis concordantly affects sVEGFR1 levels, and because previous reports demonstrated an expression of leptin long-form receptor on endothelial cells, we hypothesized that leptin signaling may have broader actions in mediating the vascular endothelial dysfunction that underlies sepsis. To test this concept, we measured the expression of a panel of vascular-related genes in organ beds commonly affected by sepsis (i.e., liver and kidney). During endotoxemia, we detected an upregulation of proinflammatory and procoagulant molecules in the liver and kidneys of leptin-treated mice compared with control mice, whereas there was a decrease in proinflammatory and procoagulation molecule expression in db/db mice (also compared with controls) (Fig. 3).

There was no difference in baseline gene expression between control and leptin- treated mice without LPS administration. Compared with control-treated animals, endotoxemic leptin-treated mice revealed significant vascular bed-specific alterations in the expression of adhesion and coagulation molecules (Fig. 3*A*–*X*). Exogenously administered leptin significantly increased the expression of ICAM-1 (6.3-fold), VCAM-1 (2.6-fold), E-selectin (4.6-fold), P-selectin (3.6-fold), and cyclooxygenase-2 (COX-2) (10.0-fold) and decreased plasminogen activator inhibitor-1 (2.1-fold) in the liver during exdotoxemia. It increased the expression of ICAM-1 (3.6-fold), VCAM-1 (1.8-fold), E-selectin (1.4-fold), P-selectin (2.7-

fold), and COX-2 (1.8-fold) in the kidney during endotoxemia. Conversely, deficiency of a long-form leptin receptor (Fig. 3*M–X*) resulted in a significant decrease in expression of VCAM-1 (1.4-fold), E-selectin (7.8-fold), P-selectin (2.3-fold), and COX-2 (6.4-fold) in the liver during exdotoxemia and in a significant decrease in the expression of ICAM-1 (5.1-fold) and COX-2 (4.1-fold) in the kidney during exdotoxemia. Together, these data suggest that changes in leptin signaling directly affect the vasculature in sepsis. These data also support a pathogenic role for leptin signaling in mediating endothelial dysfunction during experimental sepsis.

## Exogenous leptin promotes inflammation, barrier dysfunction, and organ dysfunction during endotoxemia

Given the molecular correlates of leptin-augmented septic inflammation that we observed, we next asked whether physiologic parameters are impaired in leptin-treated endotoxemic mice. First, we performed immunohistochemistry for Mac-1 (CD11b), a macrophage surface marker, using liver and kidney tissue sections. Histological analyses revealed very few or no Mac-$1^+$ cells in nonendotoxemic mice. However, LPS administration resulted in a significant increase in Mac-$1^+$ cells in the liver and kidney in control-treated mice during endotoxemia. This was further increased by exogenous leptin treatment, as evidenced by the higher levels of Mac-$1^+$ cells in leptin-treated endotoxic mice compared with control-treated endotoxic mice (Fig. 4*A–D*, kidney; 4*E–H*, liver). These results were confirmed by Mac-1 quantitative real-time PCR on the kidney (Fig. 4*I*) and liver (Fig. 4*J*). Real-time PCR also showed greater TNF-α mRNA expression in the kidney (Fig. 4*K*) and liver (Fig. 4*L*) of endotoxemic leptin-treated mice, suggesting a leptin-mediated inflammatory response. Moreover, the BUN level, a measure of kidney damage, was increased during endotoxemia, with a further increase in leptin-treated mice (125 ± 8.50 mg/dl) versus control-treated mice (65.0 ± 13.8 mg/dl; $p < 0.01$) (Fig. 4*M*). Endotoxemic leptin-treated mice also showed higher ALT levels compared with control-treated mice (Fig. 4*N*).

Finally, leptin-treated mice (compared with control-pump mice) with LPS administration developed greater vascular leakage in the kidney, small intestine, and spleen (Fig. 5). These findings suggest that elevated leptin during sepsis may exacerbate inflammation and organ dysfunction in an organ-specific fashion.

## Protective role of circulating sLR in experimental sepsis

Our initial studies revealed elevated circulating levels of sLR in WT animals subjected to endotoxemia (7.7 ± 0.8 ng/ml versus 0.3 ± 0.1 ng/ml; $p < 0.01$) or CLP (7.03 ± 0.6 ng/ml versus 1.55 ± 0.5 ng/ml; $p < 0.01$) compared with their relevant controls (Fig. 6*A*). Having studied other biological conditions in which a soluble receptor fragment inhibits ligand signaling (2, 30), we hypothesized that sLR elevation may be protecting animals from the injurious effects of increased leptin during sepsis. To test our hypothesis, we performed survival studies using exogenous sLR treatment delivered by i.p. osmotic pumps (sLR levels ~40 ng/ml were achieved). There was no major difference in body weight or in blood glucose levels when comparing control and sLR-treated mice pre- and post-LPS administration (data not shown). sLR treatment resulted in improved survival compared with control treatment during endotoxemia (87.5 versus 37.5%; $p < 0.05$) (Fig. 6*B*) and CLP

(75.0 versus 25.0%; $p < 0.05$) (Fig. 6*C*). Improved survival was associated with a significant 5.0-fold reduction in circulating leptin levels ($p < 0.001$) (Fig. 6*D*), a 3.4-fold decrease in sVEGFR1 ($p < 0.05$) (Fig. 6*E*), and a 2.3-fold decrease in IL-6 ($p < 0.05$) (Fig. 6*F*) during endotoxemia. The mouse Leptin ELISA kit (R&D Systems) that we used detects mostly free leptin but not total leptin (bound version) (Supplemental Fig. 3*A*). The real-time PCR for leptin using white fat tissue (epididymal fat) from mice treated with sLR showed that there was no difference in leptin mRNA expression levels between sLR-treated mice and controls during endotoxemia (Supplemental Fig. 3*B*). Therefore, a reduced level of circulating leptin is likely due to binding of sLR to leptin protein, not to the inhibition of leptin expression. These data demonstrated that systemic blockade of leptin by sLR conferred a survival benefit.

### Leptin-induced endothelial activation is mediated by monocytes

To investigate the mechanisms underlying leptin-induced endothelial dysfunction in sepsis, HUVECs were cultured with conditioned media (CM) from monocytes treated with leptin only, LPS only, and leptin plus LPS. Each was combined with 0.5% FBS RPMI 1640 media (without any cells). There was a significant induction of ICAM-1, VCAM-1, E-selectin, and tissue factor gene expression by LPS alone with media (Fig. 7). CM from monocytes cultured with LPS showed further upregulation of these molecules. CM from monocytes cultured with LPS plus leptin caused a further induction of ICAM-1 (1.9-fold), VCAM-1 (1.9-fold), and E-selectin (2.2-fold), but not tissue factor, compared with CM from LPS-treated monocytes. Leptin alone, with or without monocytes, did not induce adhesion molecule expression in HUVECs. There was no such synergistic effect found between LPS and leptin when HUVECs were cultured with CM from macrophages treated with both (data not shown). These data suggested that leptin-induced endothelial dysfunction is mediated, at least in part, by monocytes.

### Plasma levels of human sLR show an association with morbidity markers in sepsis

Based on the findings of the pathogenic role for leptin and sLR in experimental sepsis, we looked for an association among circulating free leptin levels, sLR levels, and morbidity markers in early-stage septic patients. We studied 146 human subjects presenting to the emergency department with clinical signs of an infection. The mean age was 58.5 ± 20.2 y, 49% were male, the mean APACHE-II score was 12.1 ± 8.5, and the mean lactate level was 2.0 ± 1.7 mg/dl. For biomarkers, the mean leptin level was 19.2 ± 29.0 ng/ml, the mean leptin receptor level was 23.3 ± 11.3 ng/ml, and the mean IL-6 level was 754 ± 2036 pg/ml. Leptin levels were inversely correlated with leptin receptor levels (Spearman correlation coefficient $r = -0.31$; $p < 0.001$), leptin was positively correlated with BMI ($r = +0.49$; $p < 0.001$), whereas leptin receptor levels were negatively correlated with BMI ($r = -0.20$; $p < 0.011$). In a multivariate model controlling for age, BMI, gender, and diabetes, leptin was not associated with lactate ($p = 0.71$), whereas a change of 1 SD in leptin receptor level was associated with a change in lactate of 1.2 mg/dl ($p < 0.001$; β for log lactate 0.015 with SE 0.004; $R^2$ for model = 0.18). Similarly, controlling for the same covariates, leptin was not associated with APACHE- II score ($p = 0.17$), whereas a change of 1 SD in leptin receptor was associated with a change in APACHE-II score of 1.7 points ($p < 0.001$; β for APACHE-II 0.15 with SE 0.046; $R^2$ for model = 0.42). Finally, leptin was significantly associated with

IL-6 ($p < 0.05$; β coefficient 0.013 with SE 0.0068; $R^2$ for model = 0.10), such that a change of 1 SD in leptin level was associated with a change in IL-6 level. These results showed an association between leptin and inflammatory response (measured by IL-6), as well as an association with the sLR response and illness severity, as determined by lactate levels and APACHE-II score.

## Discussion

In this study, we observed that levels of leptin and sLR increased in two different mouse models of sepsis. Our subsequent experiments provide compelling data that excess leptin potentiates the adverse molecular and physiological effects of sepsis associated with pronounced endothelial dysfunction. Moreover, we showed that, in two different models of inhibited leptin signaling by sLR (subtracting leptin) and leptin receptor deficiency (interfering leptin signaling), the effects of LPS or CLP are markedly attenuated. These findings are the basis of our conclusion that excess leptin signaling in sepsis is deleterious to the host.

Our findings are contradictory to previous publications that described a protective role for leptin in sepsis (18, 20, 21). Compared with these previous studies using leptin-deficient mice or leptin receptor-deficient mice in models of sepsis, all of our animal experiments were specifically designed to minimize the metabolic effects of altered leptin signaling to enable us to focus on its vascular inflammatory effects. Faggioni et al. (18) showed that LPS injection resulted in increased lethality in leptin-deficient ob/ob mice. Forty hours prior to LPS administration (5 mg/kg), ob/ob mice were treated with leptin, resulting in an increased survival during endotoxemia. These leptin-deficient mice have several severe metabolic defects, including higher basal glucose levels and severe adiposity. The results of Wang et al. (20) showed that ob/ob mice had impaired kidney function after the administration of 0.3 mg/kg LPS compared with their WT littermates. Leptin replacement for 10 days improved serum creatinine and mean arterial pressure at 16 h after LPS injection. Because leptin administration in ob/ob mice reverses their metabolic derangements, it is unclear from these two studies whether leptin per se is salutary or whether restoration of normal metabolism confounded the interpretation of the protective effects of leptin in endotoxemia in both studies. Impaired glucose regulation was shown to influence sepsis mortality (31). In a study by Faggioni et al. (21), they used animals fasted before leptin administration. The fasting resulted in decreased leptin levels associated with decreased glucose levels in those mice. Thus, in previous studies that described a protective role for leptin during endotoxemia using ob/ob or fasted WT mice, the mice showed metabolic abnormalities before leptin treatment, and the treatment improved metabolic abnormalities before LPS administration. Conversely, in our studies, mice were not fasted, and exogenous leptin-treated lean mice did not show any major metabolic alterations. These methodologic differences can explain the discrepancy between our findings and those in previous studies.

It is noteworthy that we found differences in leptin, sVEGFR1, and IL-6 levels among the mice strains used in our studies. C57BL/6j mice that were used as controls for db/db mice were more sensitive to endotoxin compared with C57BL/6 mice, leading to higher levels of

plasma leptin, sVEGFR1, and IL-6. This observation is in line with previous publications that described variations in endotoxin sensitivity among different mouse strains (32, 33).

We also found that administration of sLR conferred a survival benefit in both murine sepsis models. This experiment used an acute subtractive approach (i.e., blocking endogenous leptin) to bolster the conclusion (that leptin is injurious in sepsis) from our initial acute additive experiments of administering extra leptin. In fact, our results take the subtractive approach one step further to show that genetic “blockade” in leptin signaling, via the db/db mouse, similarly confers protection in sepsis across a range of relevant outcomes. This last finding is in agreement with a previous study showing that db/db mice were comparatively resistant to endotoxin (32). Therefore, three different sets of experiments in two different models of sepsis all favor the same conclusion that excess leptin worsens outcomes in this disease.

The role of leptin signaling in the septic vasculature is a relatively novel area of study. It was also reported that HUVECs express a long-form leptin receptor. Interestingly, however, in the HUVEC experiments, we could not detect any direct effects of leptin on endothelial activation. Leptin-induced endothelial activation was indirectly mediated by monocytes. Our findings are in accordance with previous studies in which adipocyte-derived leptin was demonstrated to interact with monocytes, which, in turn, secreted an array of cytokines, such as TNF-α, which are known to induce barrier dysfunction and inflammation in endothelial cells (33–36). It was reported that leptin also induces IL-10 production in human monocytes (37, 38) and that IL-10 induces E-selectin expression in small- and large-vessel endothelial cells (39). Further investigation is required to elucidate a detailed mechanism underlying leptin-mediated endothelial activation.

The translational relevance of our findings obviously bears most on the case of severe obesity, a condition in humans that is associated with increased sepsis-induced morbidity and mortality (6, 7) and is associated with elevated levels of circulating leptin (5). Although the nonhypothalamic signaling role of excess leptin in severe obesity is not completely understood (it is generally agreed that there is hypothalamic leptin resistance in severe obesity), our results suggest that elevated leptin may result in marked endothelial cell activation, leading to a more severe sepsis phenotype in obese patients. Finally, it must be acknowledged that the predisposition to adverse outcomes in sepsis experienced by obese individuals may be associated with excess leptin, but it is caused by other substances elaborated from the adipose tissue, from associated metabolic derangements, or other leptin-independent factors.

The elevation of sLR in mouse models of sepsis also has an added clinical implication and raises the question of whether this may be a biomarker for the disease. Although other investigators found elevated leptin in sepsis (40, 41), we did not find elevated levels of leptin in septic patients. One explanation for this discrepancy is methodologic differences: previous studies used human leptin RIA that detects mostly total leptin not free leptin. Because of its biological relevance, we used a human leptin ELISA kit that primarily detects free leptin (Supplemental Fig. 2). Previous studies measured total leptin levels containing, in part, the bound version of leptin that lacks biological relevance. Therefore, an association

between elevated levels of total leptin and better sepsis outcome does not guarantee a protective role of leptin. We advocate that sLR levels have the most biologic relevance and should be measured.

Our correlation study using human septic plasma revealed that sLR levels were associated with APACHE-II score and correlated with lactate levels. We also found increased levels of sLR in experimental sepsis models. Conversely, Anderson et al. (42) recently demonstrated that LPS administration resulted in elevated levels of leptin, not sLR, in humans. This may be explained by the dosage of LPS. We injected 16 mg/kg of LPS to mice i.p., but they injected 3 ng/kg of LPS to humans i.v., suggesting that the LPS dose may be too low to induce sLR response. Further investigation is required to elucidate the role of sLR in sepsis. Our mechanistic experiments suggest that sLR in sepsis should be adaptive and represents a component of the compensatory anti-inflammatory mechanism; therefore, one prediction is that survivors of severe sepsis have higher sLR values compared with nonsurvivors. Conversely, higher leptin levels may indicate the presence of a more severe insult with a larger compensatory response. In addition to a biomarker role, our experiments suggest that inhibition of leptin signaling could be of therapeutic benefit in sepsis. These concepts merit further investigation.

In summary, our data strongly suggest a causal link between sepsis and increased circulating leptin. Leptin may play a critical role in the pathogenesis of sepsis-associated multiorgan dysfunction. Further studies are required to determine mechanisms underlying leptin-mediated endothelial dysfunction, tissue heterogeneity in leptin responses, and the diagnostic and therapeutic potential of leptin family molecules in human sepsis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Dr. William C. Aird, Dr. Evans Rosen, and Dr. Richard Wolfe for critical discussions on this project. We also express thanks to Dan Li for technical support on a quantitative real-time PCR.

This work was supported in part by National Institutes of Health Grants R01HL091757, P50GM076659, and R01HL093234-01 (to N.I.S.). S.A.K. is an investigator at the Howard Hughes Medical Institute.

## Abbreviations used in this paper

| | |
|---|---|
| **ALT** | alanine aminotransferase |
| **BMI** | body mass index |
| **BUN** | blood urea nitrogen |
| **CLP** | cecal ligation puncture |
| **CM** | conditioned media |
| **COX-2** | cyclooxygenase-2 |

| | |
|---|---|
| **CTL** | control |
| **db/db** | long form leptin receptor-deficient |
| **LEP** | leptin |
| **Mac-1** | macrophage-1 Ag |
| **sLR** | soluble leptin receptor |
| **sVEGFR1** | soluble vascular endothelial growth factor receptor-1 |
| **WT** | wild-type. |

## References


1. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit. Care Med. 2001; 29:1303–1310. [PubMed: 11445675]
2. Yano K, Liaw PC, Mullington JM, Shih SC, Okada H, Bodyak N, Kang PM, Toltl L, Belikoff B, Buras J, et al. Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality. J. Exp. Med. 2006; 203:1447–1458. [PubMed: 16702604]
3. Reinhart K, Bayer O, Brunkhorst F, Meisner M. Markers of endothelial damage in organ dysfunction and sepsis. Crit. Care Med. 2002; 30(Suppl.)(5):S302–S312. [PubMed: 12004252]
4. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature. 1998; 395:763–770. [PubMed: 9796811]
5. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat. Med. 1995; 1:1155–1161. [PubMed: 7584987]
6. Bercault N, Boulain T, Kuteifan K, Wolf M, Runge I, Fleury JC. Obesity-related excess mortality rate in an adult intensive care unit: A risk-adjusted matched cohort study. Crit. Care Med. 2004; 32:998–1003. [PubMed: 15071392]
7. El-Solh A, Sikka P, Bozkanat E, Jaafar W, Davies J. Morbid obesity in the medical ICU. Chest. 2001; 120:1989–1997. [PubMed: 11742933]
8. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, et al. Identification and expression cloning of a leptin receptor, OB-R. Cell. 1995; 83:1263–1271. [PubMed: 8548812]
9. Tartaglia LA. The leptin receptor. J. Biol. Chem. 1997; 272:6093–6096. [PubMed: 9102398]
10. Sierra-Honigmann MR, Nath AK, Murakami C, García-Cardeña G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR. Biological action of leptin as an angiogenic factor. Science. 1998; 281:1683–1686. [PubMed: 9733517]
11. Park HY, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY, Kim HS. Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro. Exp. Mol. Med. 2001; 33:95–102. [PubMed: 11460888]
12. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature. 1998; 394:897–901. [PubMed: 9732873]
13. Sánchez-Margalet V, Martín-Romero C, Santos-Alvarez J, Goberna R, Najib S, Gonzalez-Yanes C. Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action. Clin. Exp. Immunol. 2003; 133:11–19. [PubMed: 12823272]
14. Zhao Y, Sun R, You L, Gao C, Tian Z. Expression of leptin receptors and response to leptin stimulation of human natural killer cell lines. Biochem. Biophys. Res. Commun. 2003; 300:247–252. [PubMed: 12504075]

15. Sánchez-Margalet V, Martín-Romero C, González-Yanes C, Goberna R, Rodríguez-Baño J, Muniain MA. Leptin receptor (Ob-R) expression is induced in peripheral blood mononuclear cells by in vitro activation and in vivo in HIV-infected patients. Clin. Exp. Immunol. 2002; 129:119–124. [PubMed: 12100031]
16. Fujita Y, Murakami M, Ogawa Y, Masuzaki H, Tanaka M, Ozaki S, Nakao K, Mimori T. Leptin inhibits stress-induced apoptosis of T lymphocytes. Clin. Exp. Immunol. 2002; 128:21–26. [PubMed: 11982586]
17. Caldefie-Chezet F, Poulin A, Tridon A, Sion B, Vasson MP. Leptin: a potential regulator of polymorphonuclear neutrophil bactericidal action? J. Leukoc. Biol. 2001; 69:414–418. [PubMed: 11261788]
18. Faggioni R, Fantuzzi G, Gabay C, Moser A, Dinarello CA, Feingold KR, Grunfeld C. Leptin deficiency enhances sensitivity to endotoxin-induced lethality. Am. J. Physiol. 1999; 276:R136–R142. [PubMed: 9887187]
19. Madiehe AM, Mitchell TD, Harris RB. Hyperleptinemia and reduced TNF-alpha secretion cause resistance of db/db mice to endotoxin. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2003; 284:R763–R770. [PubMed: 12571077]
20. Wang W, Poole B, Mitra A, Falk S, Fantuzzi G, Lucia S, Schrier R. Role of leptin deficiency in early acute renal failure during endotoxemia in ob/ob mice. J. Am. Soc. Nephrol. 2004; 15:645–649. [PubMed: 14978166]
21. Faggioni R, Moser A, Feingold KR, Grunfeld C. Reduced leptin levels in starvation increase susceptibility to endotoxic shock. Am. J. Pathol. 2000; 156:1781–1787. [PubMed: 10793089]
22. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. Nat. Protoc. 2009; 4:31–36. [PubMed: 19131954]
23. Segal-Lieberman G, Trombly DJ, Juthani V, Wang X, Maratos-Flier E. NPY ablation in C57BL/6 mice leads to mild obesity and to an impaired refeeding response to fasting. Am. J. Physiol. Endocrinol. Metab. 2003; 284:E1131–E1139. [PubMed: 12582011]
24. Yano K, Brown LF, Detmar M. Control of hair growth and follicle size by VEGF-mediated angiogenesis. J. Clin. Invest. 2001; 107:409–417. [PubMed: 11181640]
25. Yano K, Okada Y, Beldi G, Shih SC, Bodyak N, Okada H, Kang PM, Luscinskas W, Robson SC, Carmeliet P, et al. Elevated levels of placental growth factor represent an adaptive host response in sepsis. J. Exp. Med. 2008; 205:2623–2631. [PubMed: 18852292]
26. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. Crit. Care Med. 1985; 13:818–829. [PubMed: 3928249]
27. Shapiro NI, Howell MD, Talmor D, Nathanson LA, Lisbon A, Wolfe RE, Weiss JW. Serum lactate as a predictor of mortality in emergency department patients with infection. Ann. Emerg. Med. 2005; 45:524–528. [PubMed: 15855951]
28. Hack CE, De Groot ER, Felt-Bersma RJ, Nuijens JH, Strack Van Schijndel RJ, Eerenberg-Belmer AJ, Thijs LG, Aarden LA. Increased plasma levels of interleukin-6 in sepsis. Blood. 1989; 74:1704–1710. [PubMed: 2790194]
29. Shapiro NI, Yano K, Okada H, Fischer C, Howell M, Spokes KC, Ngo L, Angus DC, Aird WC. A prospective, observational study of soluble FLT-1 and vascular endothelial growth factor in sepsis. Shock. 2008; 29:452–457. [PubMed: 18598002]
30. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J. Clin. Invest. 2003; 111:649–658. [PubMed: 12618519]
31. van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaers D, Ferdinande P, Lauwers P, Bouillon R. Intensive insulin therapy in the critically ill patients. N. Engl. J. Med. 2001; 345:1359–1367. [PubMed: 11794168]
32. Faggioni R, Feingold KR, Grunfeld C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. FASEB J. 2001; 15:2565–2571. [PubMed: 11726531]
33. Kuldo JM, Ogawara KI, Werner N, Asgeirsdóttir SA, Kamps JA, Kok RJ, Molema G. Molecular pathways of endothelial cell activation for (targeted) pharmacological intervention of chronic inflammatory diseases. Curr. Vasc. Pharmacol. 2005; 3:11–39. [PubMed: 15638780]

34. Zarkesh-Esfahani H, Pockley G, Metcalfe RA, Bidlingmaier M, Wu Z, Ajami A, Weetman AP, Strasburger CJ, Ross RJ. High-dose leptin activates human leukocytes via receptor expression on monocytes. J. Immunol. 2001; 167:4593–4599. [PubMed: 11591788]
35. Santos-Alvarez J, Goberna R, Sánchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. Cell. Immunol. 1999; 194:6–11. [PubMed: 10357875]
36. Zarkesh-Esfahani H, Pockley AG, Wu Z, Hellewell PG, Weetman AP, Ross RJ. Leptin indirectly activates human neutrophils via induction of TNF-alpha. J. Immunol. 2004; 172:1809–1814. [PubMed: 14734764]
37. Chatzantoni K, Papathanassopoulos P, Gourzoulidou E, Mouzaki A. Leptin and its soluble receptor in plasma of patients suffering from remitting-relapsing multiple sclerosis (MS) In vitro effects of leptin on type-1 and type-2 cytokine secretion by peripheral blood mononuclear cells, T-cells and monocytes of MS patients. J. Autoimmun. 2004; 23:169–177. [PubMed: 15324935]
38. Mouzaki A, Panagoulias I, Dervilli Z, Zolota V, Spadidea P, Rodi M, Panitsas FP, Lagadinou E, de Lastic AL, Georgakopoulos T. Expression patterns of leptin receptor (OB-R) isoforms and direct in vitro effects of recombinant leptin on OB-R, leptin expression and cytokine secretion by human hematopoietic malignant cells. Cytokine. 2009; 48:203–211. [PubMed: 19665393]
39. Vora M, Romero LI, Karasek MA. Interleukin-10 induces E-selectin on small and large blood vessel endothelial cells. J. Exp. Med. 1996; 184:821–829. [PubMed: 9064342]
40. Bornstein SR, Licinio J, Tauchnitz R, Engelmann L, Negrão AB, Gold P, Chrousos GP. Plasma leptin levels are increased in survivors of acute sepsis: associated loss of diurnal rhythm, in cortisol and leptin secretion. J. Clin. Endocrinol. Metab. 1998; 83:280–283. [PubMed: 9435456]
41. Arnalich F, López J, Codoceo R, Jim nez M, Madero R, Montiel C. Relationship of plasma leptin to plasma cytokines and human survivalin sepsis and septic shock. J. Infect. Dis. 1999; 180:908–911. [PubMed: 10438392]
42. Anderson PD, Mehta NN, Wolfe ML, Hinkle CC, Pruscino L, Comiskey LL, Tabita-Martinez J, Sellers KF, Rickels MR, Ahima RS, Reilly MP. Innate immunity modulates adipokines in humans. J. Clin. Endocrinol. Metab. 2007; 92:2272–2279. [PubMed: 17374708]

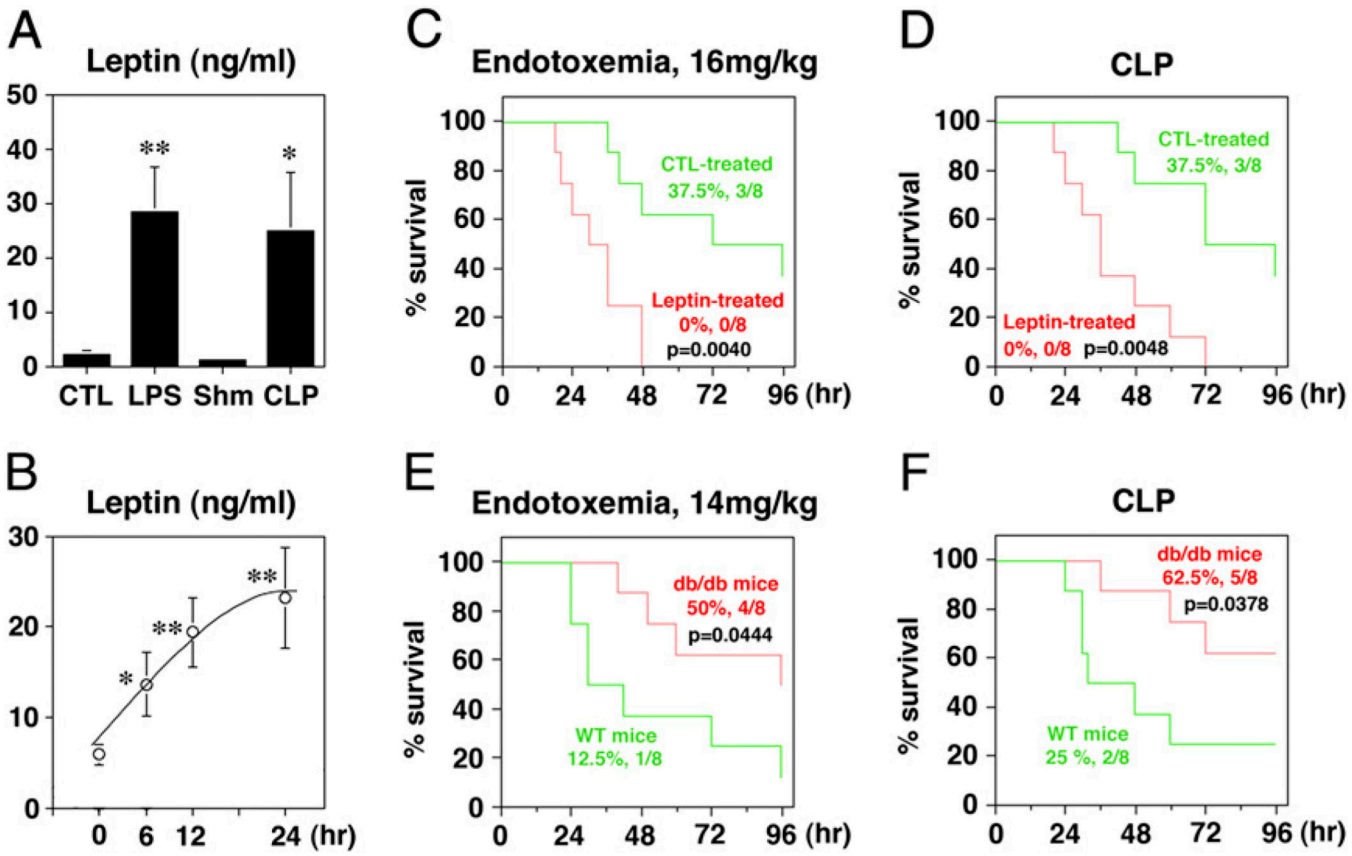


**Figure 1.**

Circulating levels of leptin in mouse models of sepsis and survival studies with exogenous leptin administration and in db/db mice. The levels of leptin in the endotoxemia and CLP models (*A*) and the level of leptin in LPS-injected mice at the time points indicated (*B*). Survival studies on exogenous leptin-treated WT mice during endotoxemia (*C*) and CLP (*D*). Survival studies of db/db mice during endotoxemia (*E*) and CLP (*F*). All data are mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.001$.

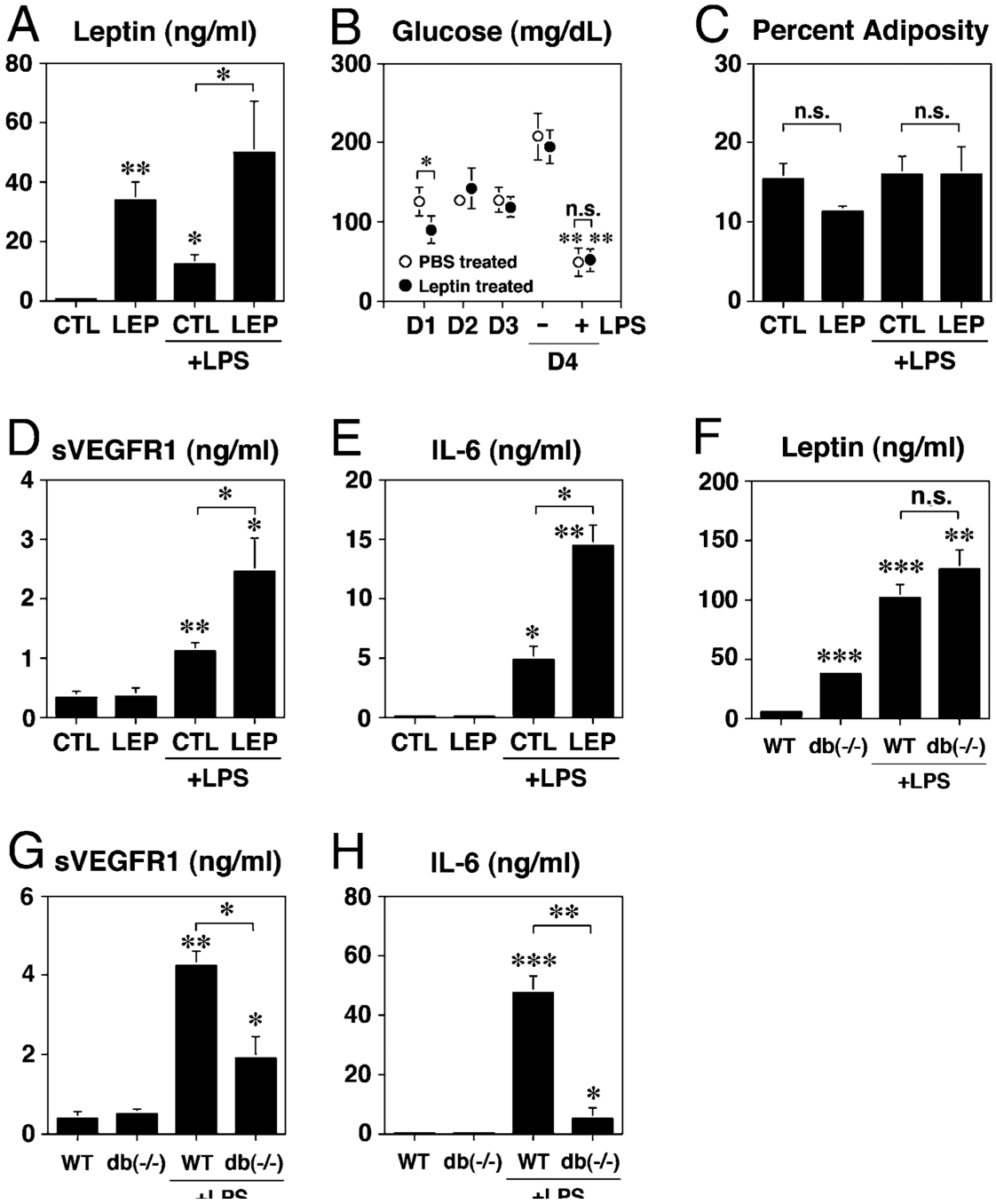


**Figure 2.**

Effects of exogenous leptin or a deficiency in the long-form leptin receptor during experimental sepsis. Three days prior to LPS administration, mice were implanted i.p. with control (CTL) pumps or leptin (LEP) pumps. Blood samples were taken at 24 h after LPS administration and assessed for plasma levels of leptin (*A*), blood glucose levels (*B*), percentage adiposity (*C*), sVEGFR1 (*D*), and IL-6 (*E*). Blood and tissue samples of db/db and WT mice were taken 24 h after LPS administration (16 mg/kg) and assayed for leptin (*F*), sVEGFR1 (*G*), and IL-6 (*H*). Data in *A*–*F* are mean ± SD of three independent experiments. **p* = 0.05; ***p* = 0.001; ****p* = 0.0001.

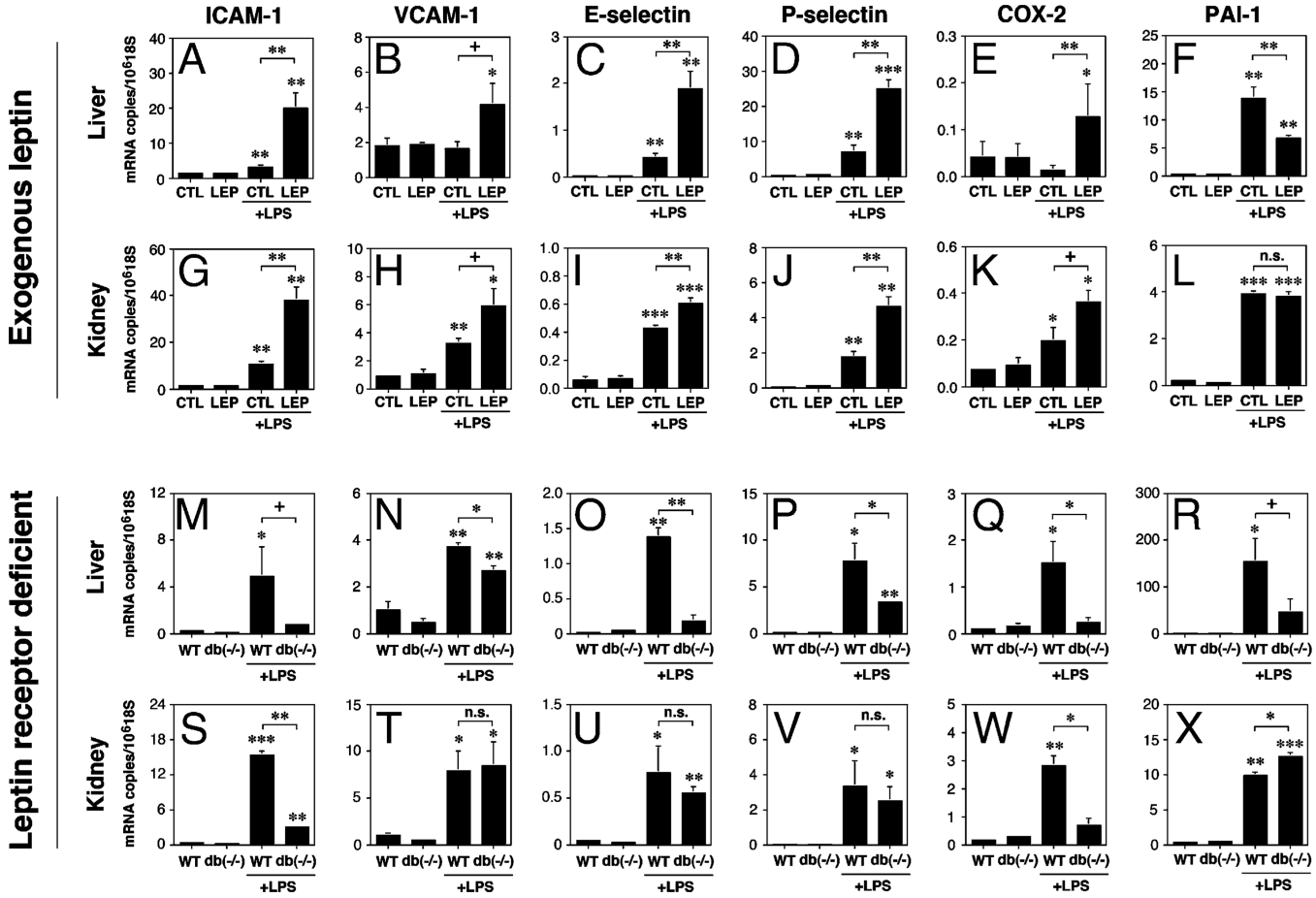


**Figure 3.**

Effects of exogenous leptin on tissue mRNA levels of inflammatory and coagulation molecules in endotoxemic mice. Control-treated (5% BSA in PBS) mice, leptin-treated (LEP) mice, db/db mice, and age-matched littermates (WT) were injected i.p. or not with 16 mg/kg LPS. The results of quantitative real-time PCR analyses (mRNA copy number per $10^6$ copies of 18S) of ICAM-1, VCAM-1, E-selectin, P-selectin, COX-2, and plasminogen activator inhibitor-1 in the liver (*A–F*) and kidney (*G–L*) of leptin- and control-treated mice and in the liver (*M–R*) and kidney (*S–X*) of leptin receptor-deficient mice and their littermates at 24 h after LPS administration. All data are mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

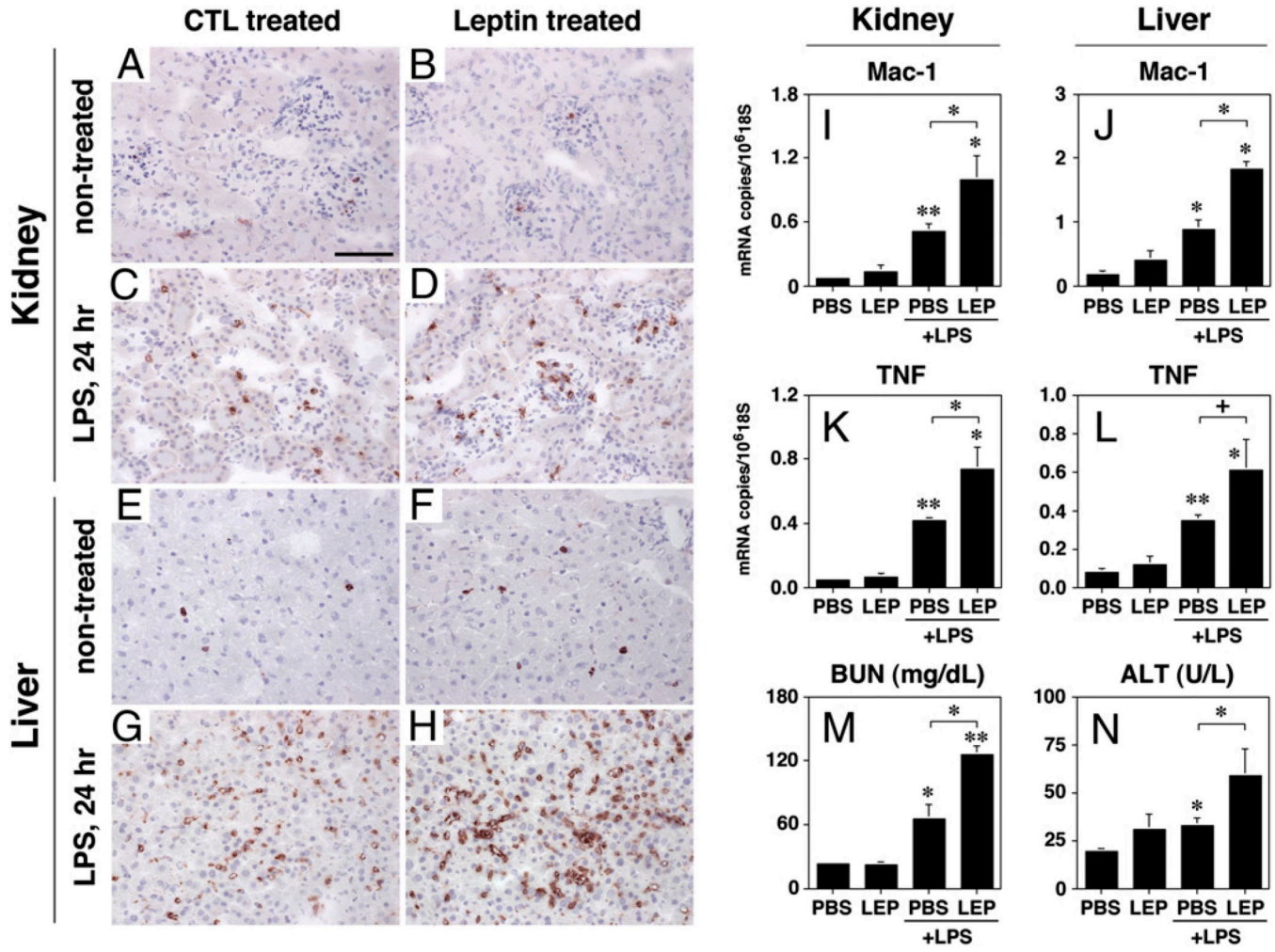


**Figure 4.**

Effects of exogenous leptin on macrophage infiltration, organ function, and vascular leakage during endotoxemia. LEP- and CTL-treated mice were subjected to endotoxemia. Blood samples were taken at 24 h and assayed for serum ALT (*N*) and BUN (*M*). The liver and kidney were harvested at 24 h and stained for Mac-1 (Mac-1, red; Hematoxylin, blue) (kidney, *A–D*; liver, *E–H*) and used for quantitative real-time PCR analyses (mRNA copy number per $10^6$ copies of 18S) of Mac-1 and TNF (*I–L*). All data were expressed as mean ± S.D. of three independent experiments. $^{+}p < 0.1$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared with PBS-treated controls. Scale bar in *A* (50 μm) also applies to *B–H* (original magnification ×630).

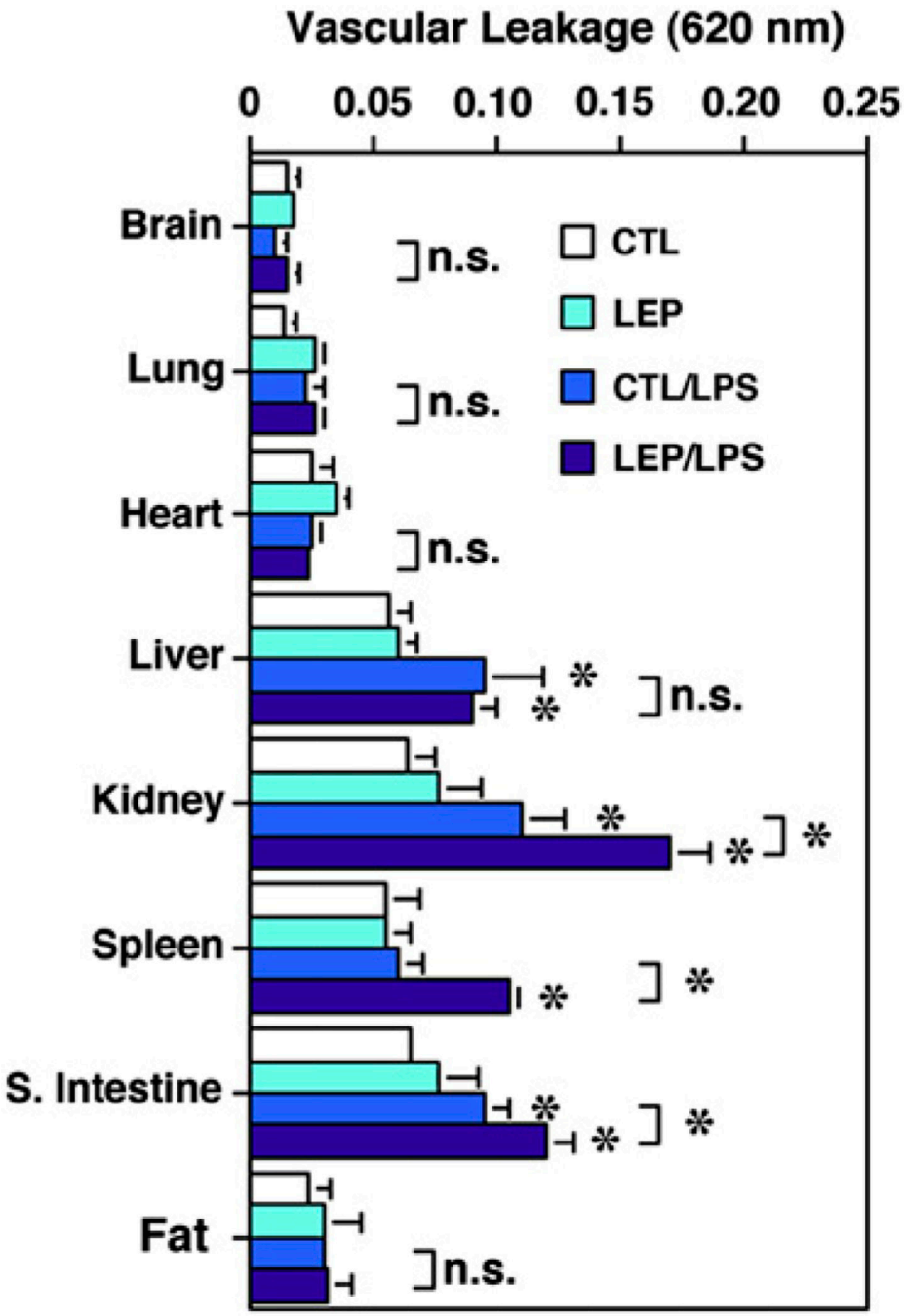


**Figure 5.**

Effects of exogenous leptin on endotoxemia-mediated barrier dysfunction. Mice were injected i.p. with 16 mg/kg LPS (or not injected). Twenty-four hours later, animals were injected with 0.1 ml of 1% Evans blue dye i.v. After 40 min, mice were perfused, and the organs were harvested and incubated in formamide for 3 d. Shown is the quantification of Evans blue extravasation (OD = 620 nm). All data are mean ± SD of three independent experiments. *$p < 0.05$, compared with PBS-treated controls.

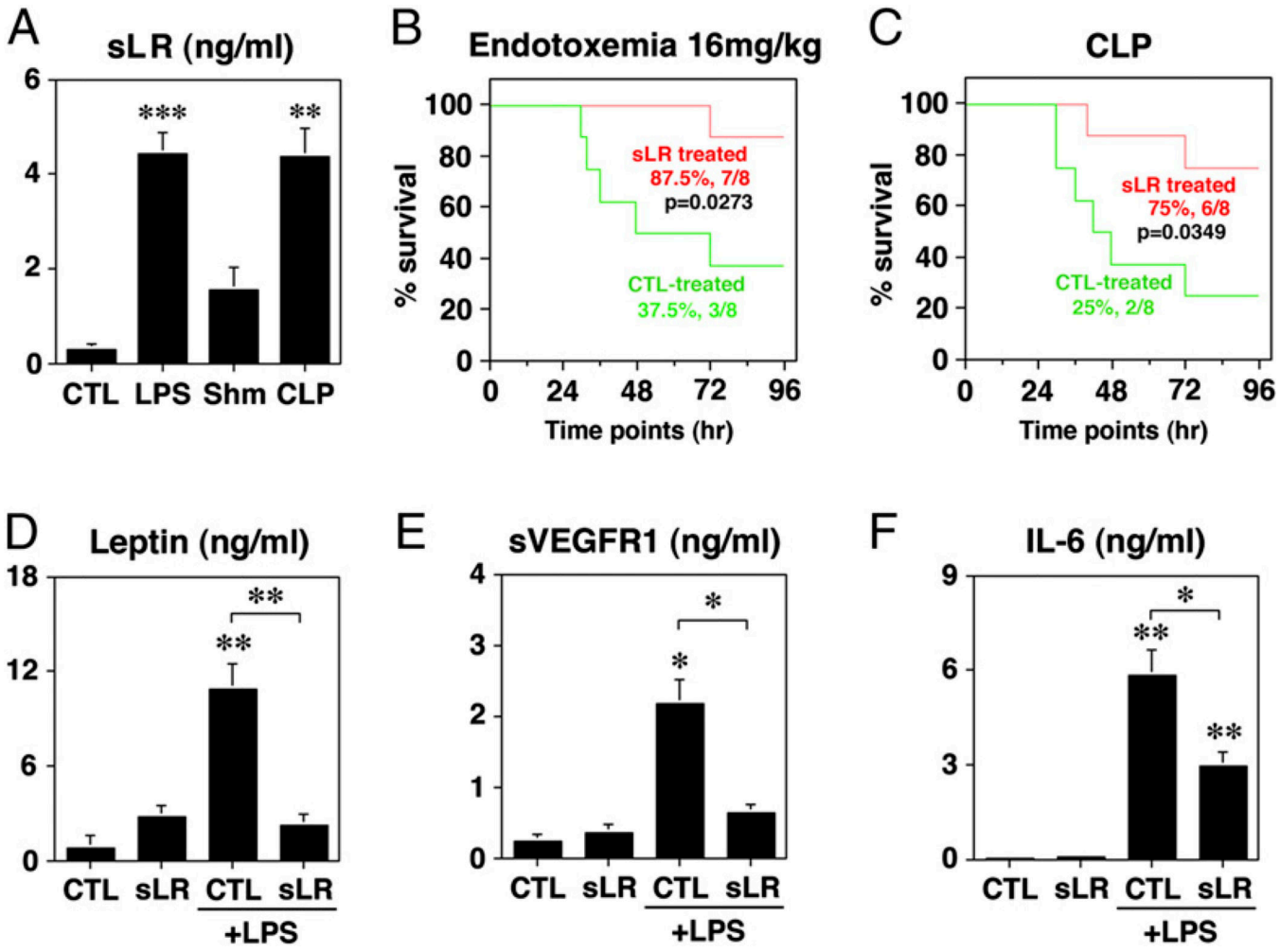


**Figure 6.**

Elevated levels of sLR during experimental sepsis and the effects of sLR on sepsis morbidity and mortality. The levels of sLR in endotoxemia and CLP models (*A*) and survival studies in exogenous sLR-treated mice during endotoxemia (*B*) and CLP (*C*). Blood and tissue samples of exogenous sLR-treated mice and control mice were taken at 24 h after LPS administration (16 mg/kg) and assayed for leptin (*D*), sVEGFR1 (*E*), and IL-6 (*F*). Data in *A* and *D–F* are mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

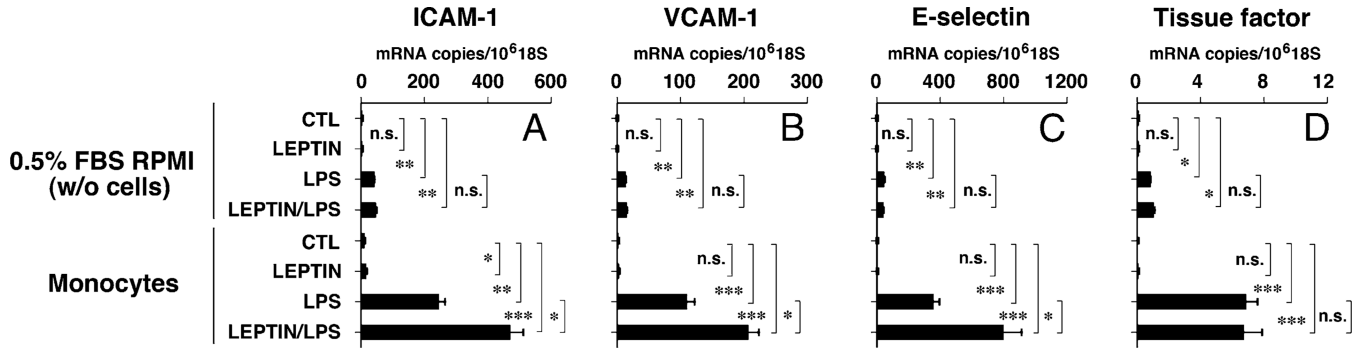


**Figure 7.**

Increased gene expression of ICAM-1, VCAM-1, and E-selectin in HUVECs induced by CM from monocytes treated with leptin, LPS, or both. Monocytes were cultured with leptin, LPS, or both for 24 h. The CM from monocytes were added to serum-starved HUVECs and incubated for 4 h. ICAM-1 (*A*), VCAM-1 (*B*), E-selectin (*C*), and tissue factor (*D*) were quantified by real-time PCR. Data in *A–C* are mean ± SD. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.