The microvascular endothelial cell in shock
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ADIPONECTIN DEFICIENCY ACCENTUATES SEPSIS MORBIDITY AND MORTALITY ASSOCIATED WITH ENDOTHELIAL DYSFUNCTION

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ABSTRACT

Increasing evidence suggests that the endothelium plays a critical role in sepsis pathophysiology. Adiponectin is an adipocyte-derived anti-inflammatory cytokine that has been shown to attenuate endothelial activation. Previous studies have demonstrated that sepsis is associated with reduced circulating levels of adiponectin. Thus, we hypothesized that sepsis-mediated adiponectin deficiency results in accentuated endothelial activation and secondary multi-organ dysfunction. We show that circulating levels of adiponectin are reduced in endotoxemia, but increased in cecal ligation puncture (CLP).

Quantitative RT-PCR for adiponectin and its receptors revealed no changes respectively significant reduction in gene expression in either model of sepsis, the pattern of response being model and organ specific. Adiponectin deficiency resulted in increased expression of endothelial adhesion and coagulation molecules in the lung, liver and kidney during sepsis, increased macrophage and neutrophil infiltration, and vascular leakage in the liver and kidney during experimental sepsis. This was accompanied by impaired survival following CLP and increased blood levels of interleukin (IL)-6, sVEGFR1, and soluble endothelial adhesion molecules sE-selectin and sICAM-1. Finally, adiponectin deficiency promoted end-organ injury in the liver and kidney while the lungs were not affected. These data suggest a protective role of adiponectin in sepsis and a role in diminishing endothelial dysfunction during sepsis.
INTRODUCTION

Over 750,000 cases of severe sepsis are diagnosed every year in the US. The mortality rate continues to be unacceptably high. Increasing evidence suggests that the endothelium plays an important role in sepsis pathophysiology (reviewed in). In animal and human models of sepsis, endothelial dysfunction is manifested by increased expression of cell adhesion molecules, enhanced production of procoagulants and cytokines, and altered release of nitric oxide. Collectively, these changes lead to increased leukocyte trafficking, fibrin deposition, vasomotor dysfunction, hemostatic imbalance, vascular permeability and inflammation.

Obesity is thought to be an independent risk factor for sepsis morbidity and mortality. Adipose tissue is a highly active endocrine organ. Adipocytes produce a number of adipokines, of which adiponectin is the most abundant. Circulating levels of adiponectin are reduced in patients with obesity, insulin resistance, atherosclerosis and related inflammatory disorders. Adiponectin exerts profound anti-inflammatory and anti-atherosclerotic actions via its receptors, AdipoR1 and AdipoR2, which are expressed in liver and skeletal muscle, endothelial cells, macrophages, and smooth muscle cells in vivo and in vitro. Previous studies have shown that the vasculoprotective effects of adiponectin are mediated by a suppression of endothelial cell activation, including cell adhesion molecule expression and nitric oxide release.

Recent studies have implicated a protective role of adiponectin in sepsis. However, the extent to which adiponectin deficiency is associated with endothelial cell dysfunction in sepsis remains largely unknown. In the present study, we show that mice that are null for adiponectin have increased mortality and morbidity in sepsis, including worsened endothelial cell dysfunction. Our findings suggest that adiponectin may exert a protective role in sepsis at the level of the vasculature.
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Materials and Methods

Sepsis models and tissue sample preparation

Male C57Bl/6 (Charles River), adiponectin knockout mice (adipoq⁻/⁻, C57Bl/6j background) and wild-type littermates at eight weeks of age were used for the study. In the endotoxemia model, mice were injected i.p. with 16 mg/kg lipopolysaccharide (LPS). CLP was performed as previously described\textsuperscript{14, 15}. Sham operation was performed the same way as CLP procedure except the caecal puncture. Blood samples were collected in different groups at 16 h after LPS or CLP. Subsequently, animals were systemically perfused with PBS and organs were harvested and snap-frozen for RNA isolation and histological analysis. In subgroups of LPS-injected and control mice, epididymal white fat was harvested, and primary mouse adipocytes were separated from the stromal vascular fraction as previously described\textsuperscript{16}. The Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee approved all animal studies.

Measurement of mouse cytokines, soluble endothelial adhesion molecules, blood urea nitrogen levels and alanine aminotransferase levels

To harvest plasma or serum samples from mice, blood samples were collected by heart puncture into heparinized and non-treated tubes respectively, centrifuged, and the supernatant was stored until use at -80°C. To obtain serum, blood samples were coagulated overnight at 4°C before centrifugation. Plasma levels of mouse adiponectin, leptin, soluble vascular growth factor receptor-1 (sVEGFR1, also known as Flt-1), interleukin-6 (IL-6), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1) and sE-selectin were measured by commercially available ELISA kits (R&D systems, Minneapolis, USA) according to the manufacturer’s instructions. Blood urea nitrogen (BUN) and alanine aminotransferase (ALT) were measured as previously described\textsuperscript{17, 18}.

Tissue RNA isolation and quantitative RT-PCR analysis

Tissue RNA was isolated and purified cDNA was prepared for real-time PCR was performed as previously described\textsuperscript{15, 19, 20}.
Permeability assay

Sixteen hours after the CLP procedure, mice were anesthetized and injected i.v. with 200 μl Evans blue dye (1% in saline). Forty minutes later, mice were systemically perfused via heart puncture with PBS containing 2 mM EDTA for 5 minutes. Organs (lung, liver, and kidney) were harvested and minced, and then incubated in formamide for 3 days to extract Evans blue dye. Absorbances were measured at 620 nm.

Survival studies

Survival studies were performed in the CLP model. Both male adiponectin knockout mice and age-matched wild-type littermates were used for the study. Survival was assessed from 0 up to 96 h after CLP operation.

Statistical analysis

A one way analysis of variance followed by a Bonferroni correction was used to compare cytokine levels, vascular leakage, ALT, BUN levels and gene expression. The Wilcoxon log-rank test was used for the survival studies. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered significant when p < 0.05.

RESULTS

Plasma adiponectin levels are reduced in mouse models of sepsis

To investigate the effect of sepsis on circulating levels of adiponectin, mice were subjected to CLP or endotoxemia. Compared with unperturbed control mice, CLP resulted in reduced levels of adiponectin (6.9 ± 0.86 μg/ml vs. 4.8 ± 0.37 μg/ml) (figure 4.1A). Surprisingly, however, sham-operation was associated with a further reduction in plasma levels (3.6 ± 0.26 μg/ml). Compared with non-injected controls, mice with endotoxemia (LPS 16 mg/kg i.p.) revealed significantly lower plasma adiponectin levels (8.2 ± 1.5 μg/ml vs. 5.9 ± 0.56 μg/ml) (figure 4.1B).
mRNA levels of adiponectin and its receptors are altered in an organ-specific manner in mouse models of sepsis

We next performed real-time PCR analysis of adiponectin and its receptors, adiponectin receptor-1 and adiponectin receptor-2, using total RNA from various organs of CLP- or LPS-treated mice and their respective controls. The highest baseline expression of adiponectin mRNA was detected in epididymal adipose tissue (451.2 ± 34.6 mRNA copies/10^6 18S copies) followed by skeletal muscle (39.3 ± 10.5 mRNA copies/10^6 18S copies) (figure 4.2A). Low mRNA levels were found in other organs. In mice subjected to CLP, adiponectin mRNA expression was significantly decreased in lung (5.9-fold), kidney (4.7-fold) and epididymal fat tissue (1.8-fold), as compared with non-treated mice (figure 4.2A). Similar changes were observed in sham-operated mice. There were no detectable changes in adiponectin receptor1 mRNA levels in any of the organs tested (figure 4.2B). However, adiponectin receptor-2 revealed reduced mRNA expression in small intestine (2.5-fold) and epididymal fat tissue (2.8-fold) in CLP treated mice, compared with non-treated control and sham-operated mice, respectively (figure 4.2C).

In endotoxemia, adiponectin mRNA levels were significantly reduced in skeletal muscle (3.2-fold) and epididymal fat tissue (2.4-fold) (figure 4.2D). Adiponectin receptor1 mRNA expression was significantly decreased in brain (2.4-fold), lung (1.4-fold), skeletal muscle (1.3-fold) and fat tissue (2.0-fold) (figure 4.2F). Adiponectin receptor2 mRNA expression was significantly downregulated in lung (1.3-fold), liver (2.1-fold), kidney (1.4-fold), small intestine (3.8-fold), skeletal muscle (1.9-fold) and adipose tissue (12-fold) (figure 4.2G).
Thus, sepsis results in organ-specific changes in mRNA expression of adiponectin and its receptors.

To identify the cell type(s) that express adiponectin, adipocytes and stromal vascular cells (SVC) were separated from LPS-treated or non-treated mouse epididymal fat pads as previously described\textsuperscript{16}. Under normal conditions, adiponectin was preferentially expressed in the adipocyte fraction (figure 4.2E). Adiponectin in the adipocyte fraction but not the SVC fraction was significantly reduced in endotoxemia (figure 4.2E).

\textbf{Figure 4.2.} mRNA levels of adiponectin, adiponectin receptor 1 and - 2 during endotoxemia and CLP in various organs and in endotoxemic primary adipocytes. Quantitative RT-PCR analyses (mRNA copy number per 106 copies of 18S) of adiponectin (A, D), adiponectin receptor 1 (B, F), and Adiponectin receptor 2 (C, G) gene expression in multiple organs in CLP and endotoxemia models, respectively. White, grey and black bars represent non-treated, sham-operated and CLP treated mice, respectively (A-C). White and black bars represent non-treated and LPS-treated mice, respectively (D, F, G). Adiponectin mRNA expression in isolated adipocytes and stromal vascular cell fraction (SVC) isolated from epididymal fat tissue, in the absence or presence of LPS for 24 hrs (E). Data are expressed as mean ± SEM (n=6), * are statistical significant with P <0.05. B: brain, Lu: lung, H: heart, Li: liver, K: kidney, SI: small intestine, M: skeletal muscle, and F: epididymal fat pad.
Adipoq<sup>−/−</sup> mice have increased mortality in CLP

To determine whether adiponectin plays a protective role in sepsis, we carried out a survival study of adipoq<sup>−/−</sup> mice subjected to CLP. As shown in figure 4.3 none of the adiponectin deficient mice survived the observation period of 96 h after CLP, compared to 35.7% survival in the wild-type littermates (p=0.0080) (figure 4.3). Thus, adiponectin has a protective effect in sepsis.

Adipoq<sup>−/−</sup> mice have increased circulating levels of inflammatory markers and endothelial adhesion cell adhesion molecules in CLP

To investigate the role of adiponectin in inflammation and endothelial activation, we measured circulating levels of established sepsis biomarkers (IL-6, sVEGFR1 and leptin) as well as the soluble forms of endothelial adhesion molecules (sICAM-1, sVCAM-1 and sE-selectin). When subjected to CLP, plasma sVEGFR1 (1.9-fold) and IL-6 (7.3-fold) were significantly elevated in adipoq<sup>−/−</sup> vs. adipoq<sup>+/+</sup> mice, whereas leptin levels were induced to comparable levels in knockout and wild-type mice (figure 4.4A-C). In response to CLP, adipoq<sup>−/−</sup> mice also demonstrated higher circulating levels of sICAM-1 (figure 4.4D) and sE-selectin (figure 4.4F), but not sVCAM-1 (figure 4.4E) compared with wild-type control (adipoq<sup>+/+</sup>) animals. In Sham-operated animals (adipoq<sup>−/−</sup> or adipoq<sup>+/+</sup>) no differences could be observed in any of the above markers.

![Figure 4.3. Impaired survival in adiponectin deficient mice during polymicrobial sepsis. Survival studies on adiponectin KO (n=12) and wildtype (n=14) mice after CLP-induced polymicrobial sepsis.](image-url)
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Adipoq<sup>-/-</sup> mice have organ-specific changes in mRNA expression of endothelial activation markers in CLP

To determine the extent to which adiponectin deficiency influences endothelial cell activation, we employed real-time PCR to measure mRNA expression of a panel of vascular-related genes in organs commonly affected by sepsis, namely the liver, kidney and lung. At 16 h following CLP, adipoq<sup>-/-</sup> mice demonstrated significantly higher mRNA expression (compared with adipoq<sup>+/+</sup> mice) of ICAM-1 in the liver, kidney and lung (3.9-fold, 3.5-fold and 1.6-fold, respectively) (figure 4.5A, G, M), E-selectin in the liver, and leptin in the liver, kidney and lung (3.9-fold, 3.5-fold and 1.6-fold, respectively) (figure 4.5B, C, D).

Figure 4.4. Effects of adiponectin deficiency on plasma levels of sVEGFR1, IL-6, leptin, sICAM-1, sVCAM-1 and sE-selectin during CLP-induced microbial sepsis.

Twenty-four hours prior to plasma preparation, mice were treated with CLP or sham. Plasma levels of sVEGFR1 (A), IL-6 (B) and leptin (C), sICAM-1 (D), sVCAM-1 (E) and sE-selectin (F) are shown. Data are expressed as mean ± SEM (n=4) of three independent experiments; * are statistical significant with P <0.05. nd: non detectable levels.
Figure 4.5. Effects of adiponectin deficiency on mRNA expression of endothelial cell adhesion and coagulation related molecules during CLP-induced microbial sepsis.

The results of quantitative RT PCR analyses (mRNA copy number per 10^6 copies of 18S) of ICAM-1 (A,G,M), VCAM-1 (B,H,N), E-selectin (C,I,O), P-selectin (D,J,P), COX-2 (E,K,Q), and plasminogen activator inhibitor-1 (F,L,R) in the liver (A–F) and kidney (G–L) and lung (M–R) of Sham operated (Sham) respectively CLP (CLP) operated wild type (WT) and adiponectin knock out (KO) mice. All data are mean ± SEM (n≥3). * are statistical significant with P <0.05.
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In response to CLP, adipoq\(^{-/-}\) mice have increased infiltration of leukocytes and barrier dysfunction in the kidney and liver

We next wished to determine whether the increased expression of endothelial cell adhesion molecules in adiponectin-null mice is associated with tissue influx of macrophages and neutrophils. To that end, we carried out immunohistochemistry using antibodies against Mac-1 (a marker of monocytes/macrophages) and Ly6G (a marker of neutrophils). As shown in figure 4.6A-D, CLP resulted in elevated Mac-1 and Ly6G staining in the kidney and liver of knockout mice compared with wild-type mice. CLP-mediated influx of macrophages in adipoq\(^{-/-}\) mice occurred primarily in the glomerular and peritubular regions of the kidney and the sinusoids of the liver.

Vascular leakage is another hallmark of sepsis. To determine the effect of adiponectin deficiency on barrier function in sepsis, we injected Evans Blue in mice subjected to CLP and quantitated extravasation of the dye in liver, kidney and lung. Compared with wild-type controls, adiponectin knockout resulted in increased CLP-mediated vascular permeability in the liver (1.5-fold) (figure 4.6E) and kidney (1.9-fold) (figure 4.6F), but not the lung (figure 4.6G).

Adipoq\(^{-/-}\) mice have worsened renal and liver function in CLP

We next investigated the effects of adiponectin deficiency on liver and kidney function during sepsis. Adiponectin deficiency resulted in increased levels of both blood urea nitrogen (BUN) (2.2-fold) (figure 4.7A) and alanine aminotransferase (ALT) (3.1-fold)
Figure 4.6. Effects of adiponectin deficiency on polymorphonuclear neutrophils and macrophage infiltration, and vascular barrier function in kidney and liver in polymicrobial sepsis.

Immunohistochemical detection of macrophage influx in mouse kidneys (A) and liver (C), of Sham mouse tissue, respectively CLP exposed mice as assessed by Mac-1 in mouse (Mac-1, red; Hematoxylin blue). Immunohistochemical detection of neutrophil influx as assessed by Ly6G in mouse kidneys (B) and liver (D), of sham-operated mouse tissue, respectively CLP exposed mice (Ly6G: red, Hematoxylin: blue). Twenty-four hours prior to i.v. injection of Evans blue dye, mice were treated with CLP. Quantitative data of Evans blue extravasation in the liver (E), kidney (F) and lung (G) is shown. Data is normalised for the OD 620nm in control organs and expressed as mean ± SEM (n=3) of two independent experiments. Scale bar in panel A applies to other panels B, C and D (scale bar= 50 μm).
Adiponectin in sepsis compared to sham-operated mice. Adiponectin deficiency resulted in further induction of both BUN (2.3-fold) (figure 4.7A) and ALT (2.1-fold) (figure 4.7B) levels during sepsis, as compared to CLP in wild-type mice.

DISCUSSION

Adiponectin, a cytokine that is specifically expressed in adipocytes, has anti-inflammatory and vasculoprotective properties. Previous studies have shown that adiponectin levels are reduced in mice subjected to CLP or endotoxemia. Moreover, a small study in humans demonstrated an association between low levels of adiponectin and septic shock. Collectively, these published findings suggest that adiponectin may have a protective role in sepsis. Consistent with this hypothesis, a recent report showed that adiponectin-null mice have increased mortality following CLP.

The goal of the present study was to extend these findings and determine the effect of adiponectin deficiency on endothelial cell activation and dysfunction. Our data confirm that adiponectin levels are indeed reduced in response to endotoxemia or CLP, compared with non-injected and sham-operated controls, respectively. Also consistent with previous reports, we found that adiponectin deficiency is associated with reduced survival in CLP. Finally, we demonstrated that adiponectin deficiency is indeed associated with increased endothelial dysfunction in sepsis.

In contrast to the lower levels of plasma adiponectin in sepsis, mRNA levels adiponectin...
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were comparable in tissues examined from CLP and sham-operated controls. There are several possible explanations for this discrepancy. First, transcriptional levels may be significantly reduced in a non-sampled tissue, such as subcutaneous or mesenteric fat. Second, post-transcriptional modifications, including hydroxylation and glycosylation of the adiponectin transcript may affect mRNA stability and translation efficiency\textsuperscript{25, 26}. Finally, it is possible that sepsis inhibits the release of adiponectin into the circulation.

Recent studies have implicated a role for adiponectin in attenuating endothelial cell activation and dysfunction. For example, under \textit{in vitro} conditions, adiponectin has been shown to stimulate the production of nitric oxide\textsuperscript{27}. Adiponectin also inhibits tumor necrosis factor (TNF-\textgreek{a})-induced expression of cell adhesion molecules in endothelial cells\textsuperscript{28}. Mice that are null for adiponectin demonstrate impaired endothelium-dependent vasorelaxation, increased endothelial expression of E-selectin and VCAM-1, increased leukocyte rolling and adhesion in the microcirculation, and reduced production of endothelial nitric oxide\textsuperscript{29}. In a mouse thioglycollate-induced inflammation model, thioglycollate challenge resulted in increased expression of VCAM-1 and ICAM-1 in the aortas of adipq\textsuperscript{-/-} compared with adipq\textsuperscript{+/+} mice\textsuperscript{24}. This effect was reversed with systemic administration of recombinant adiponectin. Our data demonstrating that CLP induces circulating levels of endothelial cell adhesion molecules and mRNA expression of endothelial activation markers (and secondary leukocyte infiltration) provides further evidence for a vasculoprotective role of adiponectin, specifically in the setting of sepsis.

In contrast to the increased basal expression of endothelial VCAM-1 in adipq\textsuperscript{-/-} mice\textsuperscript{29} and the super-induction of VCAM-1 with thioglycollate challenge in these animals\textsuperscript{24}, CLP did not result in increased plasma levels of sVCAM-1 or VCAM-1 mRNA expression in septic or non-septic adipq\textsuperscript{-/-} mice. The differences in results may relate to differences in the model employed (basal expression vs. thioglycollate challenge vs. CLP). Alternately, it is possible that VCAM-1 mRNA and protein expression is induced in untested vascular beds, and/or that protein expression is induced at a post-transcriptional level.

Adiponectin has been shown to protect cultured endothelial cells from TNF-\textgreek{a}-mediated barrier dysfunction associated with increased actin stress fibers, intercellular gap formation and \textgreek{b}-tubulin disassembly\textsuperscript{30}. Our data show that adiponectin deficiency exacerbates CLP-mediated vascular permeability in the liver and kidney. It is possible that this effect is simply a marker of sepsis morbidity. However the observation that
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the lung is spared argues against this possibility, and makes it more likely that the loss of barrier function in the knockout mice is directly related to the loss of adiponectin signaling at the level of the endothelium.

CLP in adipoq\(^{-/-}\) resulted in higher BUN compared with adipoq\(^{+/+}\) controls. Given that the knockout mice also demonstrated increased adhesion molecule expression and leukocyte infiltration as well as enhanced barrier dysfunction in the kidney, it is possible that impaired adiponectin signaling in renal endothelium is responsible for this effect. Similarly, the increased ALT in septic knockout animals corresponds to changes in cell adhesion molecule expression, leukocyte infiltration and vascular permeability in that organ, and is thus consistent with a loss of impaired adiponectin signaling in the liver.

In summary, our data strongly suggest a mechanistic link between adiponectin and sepsis. Adiponectin deficiency exacerbates a spectrum of sepsis-induced vascular phenotypes, which in turn, may contribute to multi-organ dysfunction and death in this disease. Further studies are required to determine mechanisms underlying acquired adiponectin deficiency, as occurs in obesity, and to connect the adiponectin-deficient state to the organ-specific endothelial dysfunction we have reported here. Finally, the prognostic value of measuring circulating adiponectin and the therapeutic potential of adiponectin in patients with sepsis syndrome remain to be determined.

Acknowledgements

This research was supported by institutional funding of Emergency Medicine in Beth Israel Deaconess Medical Center, and by The Dutch Kidney Foundation (KSBP09.0013). We thank Dan Li for technical support and Shou-Ching Shih for valuable input related quantitative real-time PCR.
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