Role and Regulation of Adipokines during Zymosan-Induced Peritoneal Inflammation in Mice

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Adipokines, cytokines mainly produced by adipocytes, are active participants in the regulation of inflammation. Administration of zymosan (ZY) was used to investigate the regulation and role of adipokines during peritonitis in mice. Injection of ZY led to a significant increase in leptin levels in both serum and peritoneal lavage fluid, whereas a differential trend in local vs. systemic levels was observed for both resistin and adiponectin. The role of leptin in ZY-induced peritonitis was investigated using leptin-deficient ob/ob mice, with and without reconstitution with exogenous leptin. Leptin deficiency was associated with delayed resolution of peritoneal inflammation induced by ZY, because ob/ob mice had a more pronounced cellular infiltrate in the peritoneum as well as higher and prolonged local and systemic levels of IL-6, TNFα, IL-10, and chemokine (C-X-C motif) ligand 2 compared with wild-type mice. Reconstitution with exogenous leptin exacerbated the inflammatory infiltrate and systemic IL-6 levels in ob/ob mice while inhibiting production of TNFα, IL-10, and chemokine (C-X-C motif) ligand 2. In contrast with the important role of leptin in regulating each aspect of ZY-induced peritonitis, adiponectin deficiency was associated only with a decreased inflammatory infiltrate, without affecting cytokine levels. These findings point to a complex role for adipokines in ZY-induced peritonitis and further emphasize the interplay between obesity and inflammation. (Endocrinology 149: 4080–4085, 2008)

A DIPOSE TISSUE IS not simply an inert storage depot for lipids but is also important in the integration of endocrine, metabolic, and inflammatory signals (1–3). Adipokines, such as leptin, adiponectin (APN) and resistin are active modulators of inflammation (4–9). In particular, leptin is a cytokine-like hormone important in the regulation of energy-demanding physiological processes, such as hemoapoiesis, angiogenesis, wound healing, and the immune and inflammatory response (1, 10–18). During infection and inflammation, the increase in leptin production has strongly suggested that leptin is part of the cytokine cascade that modulates the immune response (19, 20). Leptin protects T cells from apoptosis and modulates T-cell proliferation and activation, macrophage phagocytosis, and expression of adhesion molecules (21). However, both pro- and antiinflammatory effects have been described for leptin. In models of T-cell-mediated inflammation, such as experimental allergic encephalomyelitis, collagen-induced arthritis, and autoimmune hepatitis, leptin-deficient ob/ob mice are protected (22, 23). In contrast, in models in which inflammation is independent of adaptive immunity, such as zymosan (ZY)-induced arthritis or administration of endotoxin [lipopolysaccharide (LPS)], leptin appears to exert antiinflammatory properties (12, 24).

The role of APN in regulating inflammatory responses also appears to be tissue and context specific (25–27). The biology of APN has mostly been investigated in the context of insulin sensitivity and atherosclerosis (28–30). A strong epidemiological relationship between low circulating APN and diabetes, metabolic syndrome, and cardiovascular disease has been reported (1). In accordance, several antiinflammatory effects have been described for APN, including inhibition of TNFα production and activity, inhibition of nuclear factor-κB activation, and induction of antiinflammatory cytokines as well as down-regulation of adhesion molecules (17, 31–37). Based on these observations and on the protective role of APN in cardiovascular disease, this adipokine is generally considered as an antiinflammatory molecule. However, several reports demonstrated that APN can have proinflammatory properties (38–42).

To better understand the regulation of adipokines and the role these proteins play in inflammation, we challenged mice with ZY, a cell wall product of the yeast Saccharomyces cerevisiae (43). ZY is a potent stimulator of innate immunity, activating macrophages mostly through Toll-like receptor 2 (TLR2), although other receptors are also involved (44–50). We first evaluated the regulation of adipokines during ZY-induced peritonitis. Second, to better understand the role played by leptin and APN in ZY-induced peritonitis, we investigated the effect of ZY-induced inflammation in leptin-deficient ob/ob mice as well as APN knockout (KO) mice.

Materials and Methods

Mice

Care of mice followed institutional guidelines under protocols approved by the University of Illinois at Chicago. All mice used in these experiments had a C57BL6 background. Adiponectin KO mice were generated as previously described (51). Mice heterozygous for APN
were mated and 6–to 8-wk-old littersmates used for each experiment. Mice were genotyped by PCR of tail DNA, and APN deficiency was confirmed by measuring serum APN using a specific ELISA (R&D Systems, Inc., Minneapolis, MN). Age-matched female leptin-deficient (C57BL/6) ob/ob mice, their lean littermates (+/−), and C57BL/6j mice were obtained from The Jackson Laboratory (Bar Harbor, ME). A group of ob/ob mice received ip injections of recombinant murine leptin (R&D Systems) for 5 d (1 mg/kg) before ZY administration.

**Induction and evaluation of peritonitis**

Mice were injected ip with 100 mg/kg of a sterile suspension of ZY (Sigma Chemical Co., St. Louis, MO) in saline. The dose of ZY was chosen in accord with previously published data (52, 53). Control mice received an ip injection of saline. All injections were performed between 0900 and 1100 h. Approximately 0.5 ml blood was collected from the retroorbital plexus under isoflurane anesthesia at different time points after ZY injection, and serum was prepared. Mice were euthanized by cervical dislocation immediately after bleeding, and peritoneal lavage fluid (PLF) was collected, as follows. Mice were instilled with 5 ml ice-cold sterile RPMI in the peritoneal cavity, followed by gentle manual massage and removal of 3 ml fluid. The PLF was centrifuged, supernatants were collected for cytokine measurement, and the cells were resuspended in 1 ml RPMI and counted on a hemocytometer.

**Cytokine and adipokine measurement**

Chemokine (C-X-C motif ligand 2 (CXCL2), leptin, APN, and resistin levels were measured using ELISA kits from R&D Systems. TNFα, IL-6, and IL-10 levels were measured using ELISA kits from e-Bioscience (San Diego, CA). The distribution of APN multimeric forms was examined in serum and PLF after fractionation as previously described (20).

**Statistical analysis**

Data are expressed as mean ± SEM. The statistical significance of differences between treatment and control groups was determined by factorial ANOVA. Statistical analyses were performed using the XLStat software (Addinsoft, Brooklyn, NY).

**Results**

**Effect of ZY administration on adipokine levels**

To investigate the effect of ZY-induced peritoneal inflammation on systemic and local adipokine levels, wild-type (WT) mice received an ip injection of ZY or vehicle. Basal levels of leptin, resistin, and APN were 20-, 7-, and 1000-fold higher in serum than in PLF, respectively (Fig. 1, A–C). Administration of ZY induced a significant increase in both circulating and PLF leptin at 6 h, with levels returning to baseline by 24 h (Fig. 1A). In contrast, a differential trend in local vs. systemic levels was observed for both resistin and APN. As shown in Fig. 1B, although serum resistin levels were not significantly altered at any time point after ZY administration, PLF resistin showed a 2-fold increase at 2 h followed by a 3-fold peak at 6 h, returning to baseline levels at 24 h. Finally, whereas a statistically significant reduction in serum APN levels was observed at 2 and 6 h after ZY (58 and 52% reduction, respectively), PLF APN levels did not change significantly after ZY injection (Fig. 1C). Fractionation of serum and PLF obtained from vehicle- and ZY-injected mice demonstrated that the ratio of high to middle-low molecular weight (MW) APN was not significantly altered by ZY administration but did not differ between serum and PLF (data not shown).

**Role of leptin in ZY-induced inflammation**

To investigate whether leptin and/or obesity plays a role in modulating inflammation after administration of ZY, the response of ob/ob mice, with or without leptin replacement, was compared with that of WT animals. Leptin replacement in ob/ob mice led to serum leptin levels that were comparable to those observed in WT mice (serum leptin was 3.3 ± 1.1 vs. 2.1 ± 0.3 ng/ml in WT vs. leptin-injected ob/ob mice, respectively; data are mean ± SEM; n = 3). However, this short course of leptin replacement did not induce a significant body weight loss in ob/ob mice (body weight was 47.4 ± 0.4 vs. 47.4 ± 0.7 g in ob/ob vs. leptin-replaced ob/ob mice, respectively; data are mean ± SEM; n = 5). Thus, the group of leptin-replaced ob/ob mice was obese but had leptin levels comparable to those of WT mice. This group was used to differentiate between a direct effect of leptin vs. the effect of obesity on ZY-induced inflammation.

A significantly higher number of resident cells were present in the peritoneum of vehicle-injected ob/ob compared with WT mouse; reconstitution with recombinant leptin led to a further increase in the number of peritoneal cells (Fig. 2A). Administration of ZY induced a significantly more pronounced infiltrate in ob/ob compared with WT mice at both 6 and 24 h, with a further increase observed in the group of ob/ob that had received exogenous leptin (Fig. 2A).

Administration of ZY induced significantly higher levels of IL-6 in both serum (Fig. 2B) and PLF (Fig. 2C) in ob/ob compared with WT mice. Although the systemic response
was enhanced at both 6 and 24 h, PLF IL-6 levels were significantly higher in ob/ob compared with WT mice only at the later time point (Fig. 2, B and C). Administration of exogenous leptin to ob/ob mice further increased the systemic IL-6 response compared with non-leptin-injected ob/ob mice without significantly altering PLF IL-6 levels (Fig. 2, B and C). Thus, administration of leptin enhanced the systemic IL-6 response of ob/ob mice although not significantly altering local IL-6 levels.

In contrast with its potentiating effects on IL-6 production, leptin reconstitution of ob/ob mice reduced production of TNFα, IL-10, and CXCL2. In fact, a significantly enhanced and prolonged local response to ZY was observed for each of the three cytokines in ob/ob compared with WT mice, with leptin administration significantly dampening the heightened cytokine response of ob/ob mice (Fig. 2, D–F). A similar trend was observed when serum cytokine levels were measured (not shown).

Role of leptin in ZY-induced modulation of adipokine levels

The effect of leptin on regulation of APN and resistin levels after ZY administration was investigated using ob/ob and leptin-reconstituted ob/ob mice. Leptin administration to ob/ob mice normalized basal serum APN levels (Fig. 3A). Although administration of ZY significantly reduced circulating APN in WT mice, as already shown above in Fig. 1C, no significant change in serum APN was observed in ob/ob or leptin-replaced ob/ob mice receiving ZY. No significant changes in PLF APN levels in response to ZY were observed in any of the three groups (data not shown).

Although ZY did not significantly alter systemic resistin levels in any of the mice studied (data not shown), the kinetics of the PLF resistin response of ob/ob and leptin-replaced ob/ob mice was significantly prolonged compared with that of WT mice, which in contrast fully recovered by 24 h (Fig. 3B).

Role of APN in ZY-induced inflammation

To investigate a potential role for APN in ZY-induced inflammation, the response of WT and APN KO mice was compared. Although a significantly reduced inflammatory infiltrate was observed at 6 and 24 h after ZY in the peritoneal

Fig. 2. Effect of ZY in ob/ob mice. WT (■), ob/ob (□) and leptin-injected ob/ob (■) mice were injected with ZY or vehicle. Cellular infiltrate (A) as well as serum IL-6 (B) and PLF IL-6 (C), TNFα (D), IL-10 (E), and CXCL2 (F) were evaluated at the indicated time points. The inset in B shows IL-6 levels at 24 h after ZY. Data are mean ± SEM of four to five mice per group. * P < 0.05; ** P < 0.01; *** P < 0.001 vs. WT. ♦, P < 0.01; †††, P < 0.001 vs. ob/ob.

Fig. 3. APN (A) levels in serum and resistin (B) levels in PLF after ZY. WT (■), ob/ob (□), and leptin-injected ob/ob (■) mice were injected ip with ZY or vehicle. Circulating APN (A) as well as PLF resistin (B) were evaluated at the indicated time points. Data are mean ± SEM of four to five mice per group. * P < 0.05; *** P < 0.001 vs. WT. ○, P < 0.05 vs. ob/ob.
cavity of APN KO compared with WT mice (Fig. 4), serum and PLF cytokines, leptin, and resistin levels were not significantly different between WT and APN KO mice at any of the time points analyzed (data not shown).

Discussion

The aim of the present study was to investigate the role of leptin and APN in regulating inflammation during ZY-induced peritonitis. We demonstrate that adipokines are differentially modulated after administration of ZY and that although leptin plays a major role in regulating the inflammatory response to ZY, APN deficiency has only a minor effect. Although leptin and resistin levels were in the same order of magnitude in serum and PLF on untreated lean mice, APN’s concentration was approximately 1000-fold lower in PLF compared with serum. This major difference between serum and PLF APN levels is similar to that previously reported for other body fluids, such as bronchoalveolar lavage and cerebrospinal fluid (54, 55). Mechanisms including active exclusion, increased degradation, and selective transport of low vs. high MW APN have been proposed to explain the low levels of APN present in cerebrospinal fluid (56, 57). Whether these mechanisms are responsible for maintaining low levels of APN in PLF remains to be investigated, although our data obtained from fractionation studies indicate that the distribution of middle/low vs. high MW APN did not significantly differ between serum and PLF.

In agreement with previous data demonstrating that acute inflammation reduces circulating APN levels (58, 59), we found a significant reduction in serum APN during ZY-induced peritonitis. Interestingly, although inflammation had a profound effect on systemic APN, no significant changes were observed in the amount of APN present in PLF after ZY injection. Lack of modulation at the local level was unique to APN, because each of the other adipokines and cytokines measured showed more pronounced changes locally than systemically in response to ZY. These results, together with data indicating a role for leptin and obesity in regulating APN levels during inflammation (see Fig. 3), point to a complex regulation of APN production and compartmentalization in the course of inflammatory responses.

The role and regulation of APN during inflammation is multifaceted and highly dependent upon the stimuli and tissues involved (60). During ZY-induced peritonitis, endogenous APN did not play a crucial role in modulating the inflammatory response. In fact, when compared with WT mice, APN KO mice had a minor reduction in cellular infiltrate, with no significant changes in terms of cytokine or adipokine levels. These data are in agreement with previous results obtained by our group using the models of inflammation induced by injection of LPS or concanavalin A and demonstrating negligible effects of APN deficiency in the regulation of cytokine production (61).

In contrast with the apparently minor role of APN in the inflammatory response to ZY, leptin significantly contributed to modulation of cellular infiltrate and cytokine production. An overall increase in the inflammatory response to ZY was observed in ob/ob compared with WT mice, including a more pronounced cellular infiltrate in the peritoneal cavity as well as higher local and systemic levels of each of the cytokines measured. Furthermore, whereas in WT mice cytokine levels returned to baseline values by 24 h after ZY, a more prolonged cytokine response, particularly at the local level, was observed in ob/ob mice, indicating failure to effectively resolve the inflammatory response, in agreement with previous data obtained using the model of ZY-induced arthritis (24). Delayed resolution of peritoneal inflammation induced by ZY was, however, not secondary to reduced IL-10 production, as previously suggested for the increased sensitivity of ob/ob mice to LPS (12), because levels of this antiinflammatory cytokine were highly elevated in ob/ob mice injected with ZY.

Whereas leptin deficiency was associated with generalized worsening of the inflammatory response, a selective effect of reconstitution with exogenous leptin was observed. Administration of leptin further increased the already elevated inflammatory infiltrate and systemic IL-6 levels in ob/ob mice. Because the schedule of leptin reconstitution used in these experiments did not induce significant weight loss, exacerbation of these parameters by leptin suggests that obesity, rather than leptin deficiency, is responsible for the differences between WT and ob/ob mice. In contrast, production of TNFα, IL-10, and CXCL2 was significantly reduced by leptin, indicating that lack of leptin is directly responsible for the increased production of these mediators in ob/ob mice. Levels of resistin, which is mainly produced by adipocytes in mice and by macrophages in humans, are usually increased during active inflammation (62). Although ZY did not significantly alter systemic resistin levels in mice, local levels of this cytokine followed kinetics similar to that of the other cytokines measured. In particular, an overlapping pattern of local response was observed for resistin and IL-6, the kinetics of production of both being prolonged in ob/ob mice regardless of administration of exogenous leptin, suggesting similar mechanisms of regulation for these two cytokines.

In conclusion, our results further extend understanding of the complex interactions among adipokines, adipose tissue, and inflammation. We demonstrate that inflammation is an important modulator of adipokine levels in mice, while at the same time adipose tissue-derived products exert selective and potent effects on various parameters of the inflammatory response.

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