Hyperleptinemia and reduced TNF-α secretion cause resistance of db/db mice to endotoxin

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Madiehe, Abram M., Tiffany D. Mitchell, and Ruth B. S. Harris. Hyperleptinemia and reduced TNF-α secretion cause resistance of db/db mice to endotoxin. Am J Physiol Regul Integr Comp Physiol 284: R763–R770, 2003; 10.1152/ajpregu.00610.2002.—Leptin deficiency in ob/ob mice increases susceptibility to endotoxic shock, whereas leptin pretreatment protects them against LPS-induced lethality. Lack of the long-form leptin receptor (Ob-Rb) in db/db mice causes resistance. We tested the effects of LPS in C57BL/6J db/db/J (BL/3J) mice, which express only the circulating leptin receptors, compared with C57BL/6J db/db (BL/6J) mice, which express all short-form and circulating isoforms of the leptin receptor. Intraperitoneal injections of LPS significantly decreased rectal temperature and increased leptin, corticosterone, and free TNF-α in fed and fasted BL/3J and BL/6J mice. TNF-α was increased three- and fourfold in BL/3J and BL/6J, respectively. LPS (100 μg) caused 50% mortality of fasted BL/6J mice but caused no mortality in fasted BL/3J mice. Pretreatment of fasted BL/3J mice with 30 μg leptin prevented the drop in rectal temperature, blunted the increase in corticosterone, but had no effect on TNF-α induced by 100 μg LPS. Taken together, these data provide evidence that fasted BL/3J mice are more resistant than BL/6J mice to LPS toxicity, presumably due to the absence of leptin receptors in BL/3J mice. This resistance may be due to high levels of free leptin cross-reacting with other cytokine receptors.

loss of body weight and lack of appetite are serious complications associated with acute and chronic infections due to increased levels of inflammatory mediators and cytokines needed for killing pathogens (24, 25). A severe inflammatory response can, therefore, cause deleterious changes in nutrition that adversely affect growth, reproduction, and the immune system (3). LPS, a gram-negative bacterial endotoxin, has been extensively used for experimental induction of an inflammatory response; its administration results in anorexia, fever, and increased cytokine production in rodents (27, 33). The anorexia and host responses to LPS infection are mediated by IL-1β and TNF-α (7). The involvement of leptin in the immune response to LPS has been demonstrated by hypersensitivity of ob/ob mice to endotoxin administration. Leptin-deficient (ob/ob) mice are hypersensitive to the lethal effects of LPS and TNF-α, but C57BL/6J db/db mice are resistant to LPS-induced anorexia and lethality (10, 12, 36). Leptin administration to ob/ob mice blunts the increased sensitivity to LPS and TNF-α (36).

Flier (16) hypothesized that leptin signals a shift between adequate and inadequate stores of energy, whereby a fall in leptin may lead to a series of physiological and metabolic adaptations that improve survival during times of energy insufficiency. One potential adaptation could be in the interaction between leptin and the immune system. Malnutrition is known to compromise immune function by reducing resistance to infection (31). The susceptibility to LPS-induced lethality is increased in fasted, but not fed, wild-type mice (13). Leptin protected against LPS-induced lethality in ob/ob mice but did not mediate the anorexia of inflammation in starved wild-type mice (12). Leptin has been shown to directly stimulate phagocytic activity of macrophages (18, 28) and to enhance endotoxin-induced production of TNF-α, IL-6, and IL-12 (28). Furthermore, leptin prevents the increased susceptibility to endotoxic shock in mice that have been fasted for 48 h (11) and reverses starvation-induced suppression of cellular immune responses (29). The latter suggests that leptin may provide a critical link between malnutrition and immunosuppression (11, 31).

Leptin mRNA expression and circulating protein concentrations are stimulated within hours of LPS administration in fasted animals (14, 19, 35). TNF-α and IL-1β have been shown to be involved in LPS-induced leptin expression and secretion (9, 15). It was initially thought that cytokine-induced hyperleptinemia might be involved in the anorexia that accompanies infection. However, leptin was recently shown not to be essential in LPS-induced anorexia (12) but was suggested to participate in the host response to inflammation by modulating the immune and cytokine response after LPS treatment (10). Furthermore, administration of exogenous leptin did not affect either proinflammatory or anti-inflammatory cytokine responses during LPS-induced acute inflammation (6).

Leptin plays an important role not only in the regulation of appetite, body weight, and energy expenditure but also in the immune response (8). Leptin performs...
these functions by binding to its receptor, Ob-R (37). There are five isoforms of the receptor (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re) produced by alternative mRNA splicing (26). Spontaneous mutations in the leptin receptor lead to the development of obesity in mice (26). A mutation in C57BL/6J db/db (BL/6J) mice produces short-form receptors (Ob-Ra, Ob-Rc, Ob-Rd) and the circulating receptor (Ob-Re) but no long-form receptor (Ob-Rb). A mutation in C57BL/6J db/db (BL/6J) mice produces only the truncated Ob-Re (26). Both genotypes are hyperleptinemic but do not respond to leptin by reducing food intake or body weight due to lack of functional Ob-Rb (37). These experiments were, therefore, carried out to test the hypothesis that mice that lack membrane-bound Ob-R will, like ob/ob mice, be hypersensitive to LPS due to the absence of leptin signaling.

METHODS

Animal housing. Mice were obtained from a colony maintained at the University of Georgia. They were housed individually in shoebox cages with free access to rodent chow (Purina Mills, St. Louis, MO) and water in a room maintained at 75°F, with a 12:12-h light-dark cycle starting from 7 AM, and were handled daily during the 1-wk acclimation period for measurement of body weights. Experimental protocols used were approved by the Institutional Animal Care and Use Committee of the University of Georgia and were conducted in conformity with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society (2).

Experiment 1: effects of LPS on fed BL/3J db/db mice. The objective of this study was to determine whether the absence of leptin signaling in BL/3J db/db mice causes them to be hypersensitive to LPS similar to ob/ob mice.

On the day of experiment, starting at 7:30 AM, 12 11-wk-old female BL/3J mice were divided into two weight-matched groups (6 mice/group). Rectal temperature of each mouse was recorded, and a 100-μl blood sample was collected by tail bleeding at 0 h. One group received intraperitoneal injections of 0.1 ml saline, and the other group was injected with 100 μl of 0.2% LPS in 0.1 ml saline (low-dose LPS). This low dose was chosen based on the lethal effects of LPS in ob/ob mice (10). Fifty-microliter blood samples were collected by tail bleeding 1, 3, and 6 h after injection. Rectal temperatures were recorded 6 h after injection. All mice were decapitated after 6 h.

Experiment 2: effects of LPS on fed BL/6J db/db mice. Because our hypothesis was that BL/3J mice would be as susceptible to LPS toxicity as ob/ob mice, the objective of this experiment was to use BL/6J mice as controls for resistance to high doses of LPS.

Nine female BL/6J mice, aged 11 wk, were housed and handled as described above. On the day of experiment, starting at 7:30 AM, rectal temperature of each mouse was recorded, and a 100-μl blood sample was collected by tail bleeding. Mice were allowed free access to chow and water during the experiment. Each animal received an intraperitoneal injection of either 0.1 ml saline (4 mice/group) or 50 μg LPS (5 mice/group) in 0.1 ml saline. Fed BL/6J mice are resistant to low doses of LPS (12), therefore a dose of 50 μg LPS/mouse was used. Experimental design was the same as for experiment 1.

Experiment 3: effects of LPS and LPS plus leptin on fasted BL/3J db/db mice. Leptin has been shown to protect fasted wild-type mice from LPS-induced lethality (13). Experiment 1 showed that the low dose LPS was not toxic to fed BL/3J mice. Therefore, the objective of this study was to test whether high doses of LPS were lethal in fasted BL/3J mice and whether pretreatment with leptin protected fasted BL/3J mice against LPS.

Twenty-seven 11-wk-old female BL/3J mice were housed and handled for 1 wk as described above. On the day of experiment, a baseline 100-μl blood sample was collected by tail bleeding, starting at 7:30 AM, and mice were fasted for 24 h. The next day, a 100-μl blood sample was collected by tail bleeding before injections (0 h). One group of six mice received an intraperitoneal injection of 30 μg leptin in 0.1 ml PBS and were tail bled 1 h after leptin injection. Mice were then injected with either saline (8 mice/group) or 50 μg (8 mice/group) or 100 μg (5 mice/group) LPS in 0.1 ml saline. The leptin-pretreated group was injected with 100 μg LPS. Blood samples (50 μl) were collected by tail bledding at 1, 3, and 6 h postinjection. Rectal temperatures were recorded at 6 h, and mice were killed by decapitation 24 h after injection.

Experiment 4: effects of LPS on fasted BL/6J db/db mice. Sixteen female BL/6J db/db mice, aged 11 wk, were housed and handled as described above. On the day of experiment, a baseline 100-μl blood sample was collected by tail bleeding, starting at 7:30 AM, and mice were fasted for 24 h. The next day, a 100-μl blood sample was collected by tail bleeding at 0 h. Mice were injected with saline (4 mice/group), 50 μg LPS (6 mice/group), or 100 μg LPS (6 mice/group) in 0.1 ml saline. Because leptin pretreatment has been shown to confer resistance to LPS in BL/6J mice, it was omitted in this experiment. Experimental design was the same as for experiment 3.

Blood and tissue samples. Trunk blood was collected for measurement of serum leptin, corticosterone, and TNF-α concentrations. Serum was prepared by centrifugation and stored at −80°C until analysis for leptin, corticosterone, and TNF-α, as described below. Spleen, liver, inguinal, retroperitoneal, and uterine fat pads were dissected and weighed.

Hormone analysis. For each hormone assay, samples from all experiments were analyzed together in duplicate to avoid interassay variations. Corticosterone was measured by RIA (rat RIA kit: ICN Diagnostics, Costa Mesa, CA) in serum from the 0-, 1-, and 6-h time points. Free TNF-α was measured by ELISA (murine TNF-α quantikine kit; R&D Systems, Minneapolis, MN) in both 0- and 1-h serum samples, diluted 1:25 in saline. Leptin was measured by RIA (murine leptin RIA kit, Linco Research, St. Charles, MO) in the 0-, 1-, 3-, 6-, and 24-h samples with serum from LPS- and leptin-injected mice diluted 1:100 in assay buffer, and the serum from saline-treated mice diluted 1:20.

RESULTS

Experiment 1. Treatment of fed BL/3J mice with 10 μg LPS had no effect on serum leptin levels 1 h postinjection (Fig. 1); however, at 3 and 6 h, leptin levels were significantly higher in LPS-injected mice compared with saline controls. LPS did not affect corticosterone levels 1 h postinjection, but caused a significant increase in corticosterone 6 h postinjection. Serum TNF-α was higher in LPS- than saline-injected mice 1 h postinjection (Fig. 1). Treatment of fed BL/3J mice with 10 μg LPS caused a significant decrease in rectal temperature compared with saline controls (−2.7 ± 0.4 vs. −0.6 ± 0.2°C, P < 0.01).

Experiment 2. LPS treatment did not affect serum leptin levels in fed BL/6J mice 1 h postinjection, but caused a 1.5- and 4-fold increase in serum leptin levels
at 3 and 6 h, respectively (Fig. 2). LPS treatment did not affect corticosterone levels at 1 h postinjection, but significantly increased corticosterone levels after 6 h (Fig. 2, P < 0.05). Serum TNF-α was significantly increased by LPS treatment 1 h postinjection (Fig. 2, P < 0.05). Treatment of mice with 50 μg LPS caused a significant decrease in rectal temperature (−2.0 ± 0.1 °C vs. −0.2 ± 0.2°C, P < 0.005).

Experiment 3. Fasting decreased body weight of saline- and LPS-treated BL/3J mice, and treatment with 50 or 100 μg LPS after fasting did not cause further weight loss (Fig. 3). Pretreatment of BL/3J mice with 30 μg leptin, 1 h before injection of 100 μg LPS, partially prevented the loss of body weight (Fig. 3, P < 0.05). There were no differences in rectal temperatures of fasted BL/3J mice at 0 h, but temperature was significantly lower in LPS than control animals 6 h after injection (Fig. 3, P < 0.0001). Leptin partially prevented the hypothermia caused by LPS treatment (Fig. 3, P < 0.0001).

There were no significant differences in serum leptin levels at 0 h (Fig. 4), but LPS injection increased serum leptin levels over time (time, P < 0.0001; treatment, P < 0.0001; interaction, P < 0.00001). LPS treatment caused a fivefold increase in serum corticosterone at 3 and 6 h, but the response was not dose dependent. At 24 h postinjection, LPS injection showed significant, dose-dependent increases in serum corticosterone. Pretreatment of fasted BL/3J mice with 30 μg leptin caused an eightfold increase in serum leptin levels 1 h after leptin injection, and LPS treatment did not cause any further increase in serum leptin levels. Leptin pretreatment inhibited the LPS-induced increase in serum leptin levels 24 h postinjection compared with 100 μg LPS-treated mice (P < 0.001). There were no significant

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Fig. 2. Effect of LPS on serum cytokine and corticosterone levels of fed female BL/6J mice in experiment 1. A: serum leptin levels measured before (0 h) and 1, 3, and 6 h after injection with saline (n = 6) or 10 μg LPS (n = 6). B: corticosterone levels measured before (0 h) and 1 and 6 h after injection. C: serum TNF-α levels measured before (0 h) and 1 h after injection. Data are means ± SE. Values that do not share a common superscript letter are significantly different at P < 0.05.
differences in basal serum corticosterone levels between groups at 0 h (Fig. 4). Treatment of fasted BL/3J mice with 50 and 100 μg LPS increased corticosterone levels at 1 and 6 h postinjection compared with saline (time, \( P < 0.005 \); treatment, \( P = 0.07 \); interaction, \( P < 0.005 \)). Pretreatment with leptin prevented the LPS-induced increase in serum corticosterone. There were no differences in serum TNF-α levels measured before injections (Fig. 4), but LPS caused a significant dose-dependent increase in serum TNF-α levels (\( P < 0.001 \)) 1 h after injection. Leptin pretreatment had no effect on LPS-induced TNF-α levels in BL/3J mice.

Treatment of fed BL/3J mice with 10 μg LPS caused a significant increase in spleen weights compared with saline-treated mice (Table 1, \( P < 0.01 \)). Spleen weights were also significantly increased in fasted BL/3J mice treated with 50 and 100 μg LPS compared with saline (\( P < 0.01 \)). Leptin pretreatment did not affect the LPS-induced increase in spleen weight. LPS did not affect liver weights in fed mice; however, fasted BL/3J mice injected with 100 μg LPS had significantly smaller livers than saline- and 50 μg LPS-treated mice (\( P < 0.05 \)). Leptin pretreatment prevented the loss of liver weight caused by 100 μg LPS. LPS had no effect on fat pad weights of fed or fasted BL/3J mice.

**Experiment 4.** Fasting for 24 h decreased body weight of saline- and LPS-injected BL/6J mice (Fig. 5). Treatment of mice with 50 or 100 μg LPS after fasting caused a significant dose-dependent weight loss compared with saline (50 μg, \( P < 0.05 \); 100 μg, \( P < 0.001 \)) and three of the six mice injected with 100 μg LPS died within less than 24 h of injection. There were no differences in rectal temperatures at 0 h, but LPS caused
injected with saline (H11005n experiment 4 Fig. 5. Effect of LPS on body weight and change in rectal temperature, a substantial, dose-dependent decrease in rectal temperature (50 μg, P < 0.01; 100 μg, P < 0.001).

There were no significant differences in serum leptin levels at 0 h, but LPS caused a dose-dependent increase in leptin over time (Fig. 6; time, P < 0.0001; treatment, P < 0.0001; interaction, P < 0.00001). There were no significant differences in basal serum corticosterone levels, but LPS caused a significant dose-dependent increase in corticosterone levels 1 and 6 h postinjection (time, P < 0.001; treatment, P = 0.05; interaction, P < 0.005). LPS treatment caused a significant dose-dependent increase in serum TNF-α levels 1 h after injection (50 μg, P < 0.05; 100 μg, P < 0.005).

Treatment of fed or fasted BL/6J mice with LPS significantly increased spleen weights compared with saline-treated mice (Table 2, P < 0.05). There was no effect of LPS on liver or fat pad weight of fed BL/6J mice but there was a significant reduction in liver and fat depot weights in fasted mice.

**DISCUSSION**

The results of this study show that fed and fasted BL/3J mice are resistant to LPS and that leptin inhibits LPS-induced corticosterone secretion in the absence of membrane-bound leptin receptors. We had hypothesized that because BL/3J db/db mice lack membrane-bound leptin receptors, they would be as sensitive to LPS as leptin-deficient ob/ob mice. The heightened sensitivity of ob/ob mice to LPS has been attributed to the absence of leptin necessary for signaling, and LPS resistance in BL/6J db/db mice has been attributed to the absence of Ob-Rα (10, 12). This study shows that the absence of leptin signaling in BL/3J mice did not cause increased susceptibility to high doses of LPS, which suggests that LPS resistance of BL/3J mice is due to the absence of membrane-bound receptors and the increased circulating levels of leptin rather than the absence of leptin signaling. This observation is supported by other studies showing that pretreatment of ob/ob mice with leptin does not exacerbate the anorexic effects of LPS, but rather protects against LPS-induced toxicity (13). Likewise, pretreatment of starved wild-type mice with leptin protects them against the lethal effects of TNF-α (36). The resistance to LPS may also be due to increased free leptin in BL/3J mice who might have lost the functionality of the Ob-Re due to a premature stop codon that produces a truncated receptor (20). This truncated receptor may have lost the tertiary structure required for leptin binding.

LPS caused robust increases in serum leptin in fed and fasted BL/6J and BL/3J mice, and pretreatment of BL/3J mice with leptin inhibited the increase in serum leptin levels caused by 100 μg LPS. These results are consistent with other studies showing that LPS increases serum leptin levels (4, 19, 35). Berkowitz et al.
Table 2. Organ weights of fed and fasted BL/6J db/db mice treated with LPS

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<td>Saline 50 µLPS</td>
<td>Saline 50 µLPS</td>
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<tr>
<td>Spleen, mg</td>
<td>62.0 ± 14*</td>
<td>77.4 ± 3†</td>
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<td>Liver, g</td>
<td>2.83 ± 0.2‡</td>
<td>2.71 ± 0.6*</td>
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<tr>
<td>Inguinal fat, g</td>
<td>5.81 ± 1.2*</td>
<td>5.56 ± 0.4*</td>
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<td>Uterine fat, g</td>
<td>5.21 ± 0.7*</td>
<td>5.29 ± 0.5*</td>
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<td>Retropitoneal fat, g</td>
<td>0.87 ± 0.2*</td>
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Data are means ± SE for groups of 3–6 mice. Values that do not share a common symbol are significantly different at P < 0.05 as determined by 2-way analysis of variance and Duncan's multiple range post hoc test.

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and increased serum leptin levels in fasted hamsters. Moreover, an increase in leptin expression 5 h after an LPS injection has been reported in mice that had been fasted for 7 h before LPS treatment (35). This increase in leptin secretion by LPS was abolished when animals were fed (19, 35).

The LPS-induced stimulation of leptin secretion is not a direct effect of LPS on adipose tissue, because the response can be initiated by central administration of LPS (15), and also because LPS does not stimulate leptin secretion from primary adipocytes. In contrast, TNF-α causes a dose-dependent increase in leptin secretion (15). This indicates that LPS induces synthesis of the proinflammatory cytokine TNF-α, which subsequently increases leptin secretion. Our studies are consistent with a report that leptin administration protects mice from becoming hypersensitive to TNF-α or to endotoxic shock (13, 36). The protective effect of leptin was thought to be limited to animals that were hypoleptinemic, such as ob/ob mice or mice starved for 48 h (13). In this study, we found that, even in mice deficient in membrane-bound Ob-R, exogenous leptin blunts the LPS-induced rise in corticosterone with no effect on the levels of the proinflammatory cytokine TNF-α. The hyperleptinemia present in BL/6J mice may also provide protection against high LPS doses that are lethal to fasted wild-type and ob/ob mice and BL/6J mice. We hypothesize that, in the absence of membrane-bound leptin receptors, especially Ob-Rb, the excess leptin may cross-react with other class I cytokine receptors to modulate the anti-inflammatory response. Furthermore, the presence of high leptin levels may also induce secretion of anti-inflammatory cytokines, such as the endogenous inhibitor of IL-1, the IL-1 receptor antagonist.

Pretreatment with leptin did not influence LPS-induced serum TNF-α levels in fasted BL/3J mice. This is consistent with, and could potentially explain, the resistance to LPS observed in fed BL/6J db/db mice compared with fasted lean and leptin-deficient ob/ob mice (12). Leptin has been shown to inhibit the activation of the hypothalamic-pituitary-adrenal (HPA) axis by exerting a direct, dose-dependent inhibition of stimulated cortisol secretion by normal human and rat adrenal cells in vitro (17) and by lowering ACTH secretion (1). Inasmuch as no effect of leptin was observed on adrenal cells obtained from db/db mice, these effects of leptin were suggested to be mediated by...
the Ob-Rb, which is expressed in the adrenal gland (1, 17). In contrast to the lack of activity in vitro, this study shows that the inhibition of corticosterone secretion occurs in the absence of membrane-bound leptin receptor, suggesting that it is not an Ob-Rb-dependent process in vivo, or that the process is not exclusively dependent on Ob-Rb.

In this study, LPS increased corticosterone levels compared with saline controls in BL/3J and BL/6J mice, and exogenous leptin administration significantly blunted the LPS-induced rise in serum corticosterone in fasted BL/3J mice, consistent with glucocorticoids having anti-inflammatory functions in response to LPS injection (20). The synthesis of glucocorticoids and proinflammatory cytokines is connected by feedback loops through the HPA axis (5). That is, corticosterone downregulates the synthesis and activities of proinflammatory cytokines, such as TNF-α, whereas proinflammatory cytokines, especially TNF-α and IL-1β, stimulate release of ACTH from the pituitary gland to increase corticosterone levels (5, 36). Glucocorticoids have also been shown to increase leptin gene expression and secretion, and removal of adrenal steroids decrease serum leptin levels (30). Adrenalectomy also increases the sensitivity to LPS lethality, an effect that is prevented by pretreatment with dexamethasone (32), potentially by increasing serum leptin levels. The inhibition of LPS-induced corticosterone release by leptin treatment is consistent with reports that leptin inhibits stress-induced release of glucocorticoids in food-deprived and immobilized mice (1, 21). Moreover, the rise in serum leptin in pretreated mice could potentially downregulate corticosterone levels in an attempt to decrease the corticosterone-induced rise in leptin.

LPS injection caused three- to fourfold increases in TNF-α secretion of fasted BL/3J and BL/6J mice, respectively, and exogenous leptin administration had no effect on LPS-induced TNF-α levels in fasted BL/3J mice. LPS treatment in vivo increases serum TNF-α levels, which subsequently increase leptin secretion (9, 15). Leptin has recently been demonstrated to correct LPS-induced anorexia in leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice (12).

In conclusion, this study provides evidence that hyperleptinemia present in BL/3J mice, combined with reduced LPS-induced serum TNF-α secretion, increases resistance to LPS-induced toxicity.

Perspectives

Obese humans and genetically obese animals have decreased T cell function and increased leptin levels, but the precise role of leptin on immune function in obesity is still unclear. The link between inflammation, obesity, and diabetes is beginning to unravel, with evidence of this relationship coming from observations that various factors (TNF-α, IL-2, -4, -6, interferon-γ) are modulated similarly in these human conditions. TNF-α and leptin are overexpressed in white adipose tissue of obese rodents and humans (23) and TNF-α plays an important role in the obesity-linked insulin resistance (22). Data presented in this study using Ob-R mutant mice indicate that leptin administration, even in the absence of Ob-Rb, can offer protection against inflammation. Considering that obesity and diabetes are low-grade inflammatory diseases, leptin may be used in certain situations to reduce the inflammatory response.

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