

Multiple Cytokines and Acute Inflammation Raise Mouse Leptin Levels: Potential Role in Inflammatory Anorexia

Pasha Sarraf,* Robert C. Frederich,‡ Ewa M. Turner,* Grace Ma,*
Nora T. Jaskowiak,* Dennis J. Rivet III,* Jeffrey S. Flier,‡
Bradford B. Lowell,‡ Douglas L. Fraker,* and H. Richard Alexander*

From *The Surgical Metabolism Section, Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and ‡Division of Endocrinology, Beth Israel Hospital, Boston, Massachusetts 02215

Summary

Several inflammatory cytokines, most notably tumor necrosis factor (TNF) and IL-1, induce anorexia and loss of lean body mass, common manifestations of acute and chronic inflammatory conditions. In C57BL/6 female mice, the administration of TNF, IL-1, and, to a lesser extent, leukemia inhibitory factor (LIF), produced a prompt and dose-dependent increase in serum leptin levels and leptin mRNA expression in fat. IL-10, IL-4, ciliary neurotrophic factor, and IL-2, cytokines not known to induce anorexia or decrease food intake, had no effect on leptin gene expression or serum leptin levels. After administration of *Escherichia coli* lipopolysaccharide (LPS), leptin gene expression and leptin levels were increased. These findings suggest that leptin levels may be one mechanism by which anorexia is induced during acute inflammatory conditions.

Anorexia and loss of lean body mass are hallmark manifestations of acute or chronic disease including infection or cancer. The role of TNF, IL-1, IL-6, and leukemia inhibitory factor (LIF) as endogenous mediators of the host response to infection or malignancy has been extensively studied (1–8). Chronic administration of TNF or LIF to mice (6, 9, 10), or IL-1 to rats (2, 11) results in significant anorexia and weight loss. Mice engrafted with Chinese hamster ovary (CHO) cells genetically engineered to produce TNF, IL-6, or LIF demonstrate profound wasting comparable to chronic starvation or cancer cachexia (12–14). Alternatively, anti-TNF antibodies and a receptor antagonist against IL-1 ameliorate the manifestations of cancer cachexia in mice (15, 16). Also, cachectic patients with HIV-related lymphoma treated with anti-IL-6 antibodies demonstrate weight gain without change in tumor burden (17).

Though evidence has mounted that inflammatory diseases mediate energy and weight dysregulation through their associated cytokines, the mechanisms are unknown. Many recent studies have suggested that the *ob* gene product, leptin, may play a central role in energy regulation (18, 19). This protein is expressed specifically in white adipose tissue (18), is strongly correlated with total body fat mass (20, 21), and when given to lean or *ob/ob* mice reduces food intake and in the *ob/ob* mice increases basal metabolic rate leading to weight loss (22–24). Therefore, we investigated the hypothesis that inflammatory cytokines might elevate leptin, a potential explanation for their anorectic effects.

Materials and Methods

Animals. Female C57BL/6 mice (10–12 wk/18–22 g) were housed (5/cage) and raised on open formula rat/mouse ration (Zeigler, Gardners, PA) and water ad libitum, with a 12 h light–dark cycle beginning at 6:30 a.m. All experiments were conducted in compliance with the Animal Care and Use Committee of the National Institute of Health. In the diurnal variation experiment animals had free access to food. In all other experiments, food was withdrawn 2 h before onset of the dark cycle. In the refeeding experiment only, food was reintroduced after 7–7.5 h of fasting. Animals were killed at indicated timepoints for retroperitoneal fat and serum harvest.

Reagents. The following cytokines were obtained: mTNF- α (Genentech, South San Francisco, CA), hIL-1 β (Biologic Response Modifiers Program [BRMP], Frederick, MD), hIL-2 (Cetus, Chiron Corp., Emeryville, CA), mIL-10 (BRMP, Frederick, MD), mIL-4 (BRMP, Frederick, MD), mLIF (Genentech, South San Francisco, CA), mCNTF (R&D Systems, Minneapolis, MN), and mIL-6 (BRMP, Frederick, MD). *E. coli* lipopolysaccharide (serotype 0127:B8) was purchased from Sigma (St. Louis, MO).

Cytokine Response Studies. After a 7-h fast, mice were given an i.p. injection of 0.2 ml of a control carrier solution of PBS (Biofluids, Rockville, MD) with 0.5% endotoxin-free fatty acid-poor BSA (Calbiochem-Novabiochem, La Jolla, CA) or LPS, or one of the indicated cytokines.

Leptin/Serum Levels. Leptin levels were measured by RIA as described (20, 21) using the procedure as directed by Linco (St. Charles, MO), except that all reagents were used at one-half recommended volume.

Reverse Transcriptase-PCR. Total RNA was extracted from frozen fat tissue samples by the guanidinium–thiocyanate/CsCl

method. An *ob* gene cDNA probe for Northern blotting was generated as follows. First strand cDNA was synthesized from 3 μg of total RNA derived from an untreated control group, using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and oligo(dT) priming according to the directions of the manufacturer. The reaction was carried out at 40°C for 1 h in a final volume of 60 μl PCR mix. Amplification primers were synthesized using the mouse *ob* gene sequence: sense, 5'-AATGTGCTGCAGACCCCTGTG-3', and antisense, 5'-CATTCAGGGCTAACATCCAAC-3'. Hot-start PCR was performed using the protocol for Ampliwax in the following reaction: 2.5 μl PCR buffer (10 \times), 10.5 μl sterile water, 5 μl of each primer (4 μM), 2 μl dNTP mix (10 mM each dNTP) were mixed in a PCR microfuge tube, followed by a single Ampliwax PCR Gem, heated to 80°C, and allowed to cool at room temperature. On the solidified wax layer, 7.5 μl PCR buffer (10 \times), 56.5 μl sterile water, 10 μl cDNA template, and 1 μl Taq polymerase (5 U/ μl) were mixed and heated at 95°C for 5 min, followed by 37 cycles using a 1-min denaturation step at 94°C, a 1-min annealing step at 60°C, and a 2-min extension step at 72°C. An additional 8-min extension step at 72°C was added after 37 cycles. PCR reactions were performed in a thermocycler (Perkin-Elmer Cetus model 480, Norwalk, CT). A single predicted 500-bp PCR product was obtained as resolved on a 2% agarose gel. The product was cloned with the Invitrogen T/A cloning kit (San Diego, CA), produced in quantity, purified, random prime labeled with P32, and used as a cDNA probe for our Northern blots. A quantity of the product was subjected to restriction enzyme analysis for confirmation of specificity.

Northern Blot Analysis. Total RNA was extracted from frozen fat tissue pooled from each experimental group as described above. Equal amounts of total RNA (25 μg /lane) were subjected to gel electrophoresis using 1% agarose gels containing 0.6 M formaldehyde. RNA was blotted onto Duralon-UV (Stratagene, Inc., La Jolla, CA) nylon membranes and ultraviolet cross-linked. Hybridization and autoradiography were performed using standard techniques. Equal loading of RNA was confirmed by ethidium bromide staining of the agarose gel or hybridization of nylon membranes with a chicken β -actin cDNA probe (Oncor, Gaithersburg, MD). Each lane represents RNA from tissue pooled from five or six mice. Each experiment was done between three to six times with consistent results.

Statistical Methods. Individual comparisons were evaluated with Student's *t* test using the Statview™ program.

Results

We first examined the characteristics of leptin gene expression and serum levels under simple physiological manipulations, including circadian variation in freely feeding mice and the response to acute starvation and refeeding. As previously demonstrated at the mRNA level (25), leptin levels under conditions of ad libitum feeding were lowest in the middle of light cycle and highest in the middle of the dark cycle (Fig. 1), consistent with the well-established diurnal but primarily nocturnal food intake behavior of rodents (26). When animals were fasted beginning 2 h before the night cycle, the nocturnal rise was abolished. When 7-h fasted animals were refed, there was an exuberant increase in leptin gene expression and serum levels higher than freely fed controls and were manifest as early as 3 h after feeding (Fig. 1).

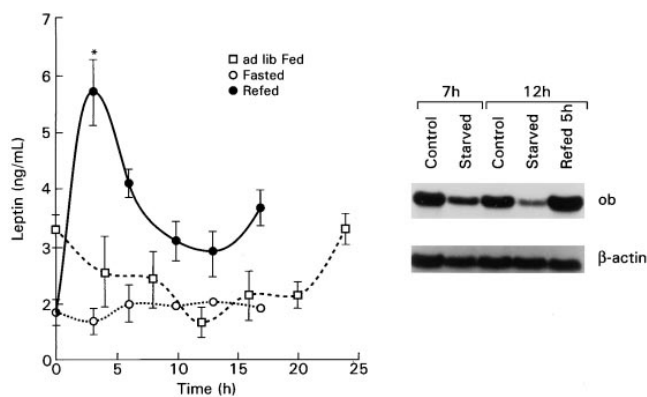


Figure 1. Leptin levels in freely feeding mice at intervals throughout a 24-h period and after short-term fasting and refeeding. The initial point represents midnight, 5.5 h after beginning the dark cycle in the ad libitum fed diurnal experiment, 7 h after the commencement of the fast in the fasting and refeeding experiments, and the beginning of refeeding in the latter experiment. Each point represents the mean \pm SEM of 6–8 individually measured mice. Northern blot shows *ob* gene expression in adipose tissue from freely fed mice (control), decreased expression after a 7- or 12-h fast (starved), and increased 5 h after refeeding groups of mice starved for 7 h (refed 5 h).

After a 7-h evening fast, mice were treated with a single intraperitoneal injection of LPS, or multiple cytokines at doses that have an anorectic effect (Ma, G. and H.R. Alexander, manuscript in preparation). Fig. 2 shows that leptin levels are significantly increased by LPS, TNF, IL-1, and LIF in a dose-related manner. LPS and TNF increased leptin levels nearly five fold to levels greater than that seen in animals acutely fasted and refed. IL-1 and LIF increased fasting leptin by approximately twofold, a level similar to that observed in fed animals. IL-6 had a trend to increase leptin, which did not reach significance. LPS administration at sublethal (1 and 10 mg/kg) and lethal doses (20 and 30 mg/kg) produced a

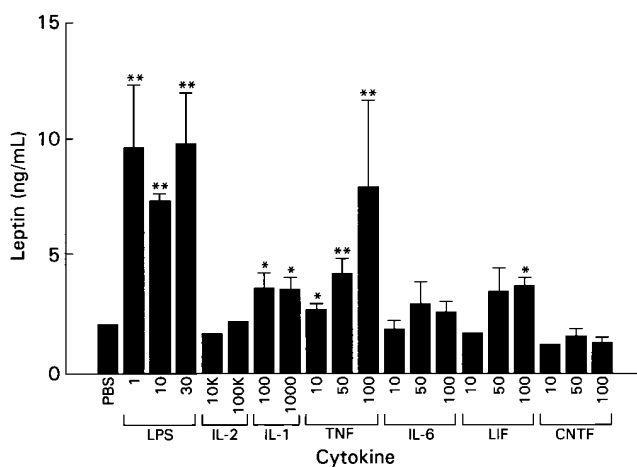


Figure 2. Leptin levels after i.p. administration of various cytokines and LPS. Mice were injected with LPS (mg/kg) or recombinant cytokine in the doses shown (IL-2, IL-1: U/mouse; TNF, IL-6, LIF, CNTF: $\mu\text{g}/\text{kg}$) or PBS after a 7-h fast and sera and adipose tissue harvested 5 h later. Further details are in Materials and Methods. Each bar represents the mean \pm SEM serum leptin level of 6–7 mice. Significance compared with the PBS-treated animals is indicated as * $P < 0.05$, ** $P < 0.01$.

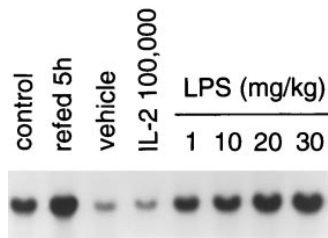


Figure 3. Effect of LPS on *ob* gene expression. After a 7-h fast mice were injected i.p. with LPS at the doses indicated and adipose tissue harvested 5 h later as described. *ob* gene expression is low in fasted animals treated with PBS and increased in a dose-dependent manner after LPS.

dose-dependent increase in *ob* gene expression in fasted mice (Fig. 3). In this model, 20–30 mg/kg of LPS resulted in a 30–40% lethality by 72 h after administration (data not shown). These effects were specific, because IL-10 and IL-4, which generally exhibit anti-inflammatory characteristics, as well as IL-2 and ciliary neurotrophic factor (CNTF), had no effect on leptin mRNA expression in retroperitoneal fat (data not shown) or serum leptin levels (see Fig. 2).

We next examined the time course of effects of TNF and IL-1 on leptin levels (Fig. 4). After a 7-h fast, mice were injected with 100 μ g/kg of TNF, or 1,000 U of IL-1. Expression of the leptin gene in retroperitoneal fat increased within 2 h after injection and was maximal 6–8 h after TNF administration (data not shown), while leptin serum levels reached a maximal three-fold elevation by 7 h, and subsequently returned toward baseline at 18 h. IL-1 induced a slower increase in leptin to a maximal twofold increase by 10 h, which persisted for at least another 8 h.

Discussion

The time course and dose-dependent effects of the inflammatory cytokines TNF and IL-1 on leptin gene expression suggest that their anorectic effects may be mediated in part by regulation of leptin gene expression. The finding that IL-6 and LIF are not as potent as TNF or LPS in inducing leptin levels or gene expression is not surprising. Although IL-6 and LIF have pleiotropic inflammatory properties, have been detected in various acute and chronic diseases (2, 10), and have been shown to induce tissue wasting in hosts bearing implantable tumors secreting either cytokine (13, 14), the evidence that IL-6 or LIF induce anorexia or mediate cachexia in various disease states is not consistent (27–29). CNTF, IL-2, IL-4 and IL-10, the latter two being primarily counterinflammatory cytokines, have not been shown to have anorectic effects in mice (30–33) and had no effect on leptin levels or gene expression.

The full syndrome of leptin insufficiency is seen in *ob/ob* mice that lack functional leptin. These mice have increased food-seeking behavior, insulin resistance, hypothermia, decreased sympathetic drive, elevated corticosterone, and infertility, all of which tend to normalize with exogenous leptin (21–23, 34, 35). Acute food restriction in mice, associated with low leptin levels, is characterized by the same abnormalities. In an acutely infected mouse, these abnormalities (except infertility) might be anticipated to impair survival in the face of bacterial infection. In particular, lep-

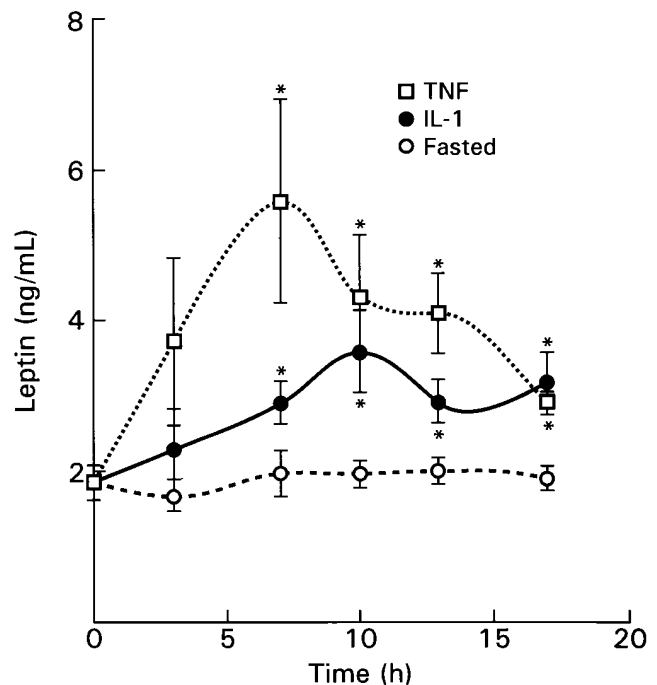


Figure 4. Kinetics of leptin in sera from mice injected with 100 μ g/kg of TNF or 1,000 U of IL-1, after a 7-h fast. All animals were food-deprived during the experimental period. Each point represents mean \pm SEM leptin level of 5–6 mice, except for the 13-h points, which represent 10–14 mice. Compared with fasted controls, significant differences are indicated as * P < 0.05.

tin support of brown fat, basal metabolism, and prevention of hypothermia may be important in preserving the adaptive febrile response. We conjecture that cytokine stimulation of leptin to levels at or above seen in the fed state may have developed to prevent the abnormalities of leptin deficiency during infection, regardless of the food intake.

The leptin receptor, closely related to gp-130 (heterodimer of IL-6 and LIF), is a member of the class I cytokine group (36). Thus, it seems quite likely that the leptin system is ancestrally related to the cytokines. In this context, it is perhaps not surprising that multiple cytokines influence leptin levels, as we have demonstrated. The possibility of the reverse, leptin influence on other cytokines, is plausible based on the wide distribution of some forms of the leptin receptor (36), including primitive hematopoietic stem cells and lymphohematopoietic cell lines (37), and deserves investigation.

In summary, we have demonstrated that multiple cytokines documented to induce anorexia increase leptin, a protein demonstrated to produce anorexia and confirm similar findings with LPS (38). These results strongly advance the hypothesis that cytokine induction of leptin may play a significant role in the anorexia and cachexia of inflammatory diseases such as infections, collagen vascular disease, and cancer. If the cytokine–leptin hypothesis is supported by further studies, it opens a novel approach to combating this significant comorbidity of many common diseases.

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Address correspondence to H. Richard Alexander, Jr., Head, Surgical Metabolism Section, Surgery Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 2B17, Bethesda, MD 20892-1502.

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