Endotoxin-induced alteration in the expression of leptin and β3-adrenergic receptor in adipose tissue

DAN E. BERKOWITZ, DANIEL BROWN, KYOUNG MIN LEE, CHARLES EMALA, DAVID PALMER, YING AN, AND MICHAEL BRESLOW
Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

Berkowitz, Dan E., Daniel Brown, Kyoun Min Lee, Charles Emala, David Palmer, Ying An, and Michael Breslow. Endotoxin-induced alteration in the expression of leptin and β3-adrenergic receptor (β3-AR), through a mechanism involving TNF-α. Increasing doses of Escherichia coli endotoxin (lipopolysaccharide, LPS) resulted in dose-dependent elevations of plasma leptin (maximal response ~7-fold, half-maximal effective dose of ~16 µg/100 g body wt) and white fat leptin mRNA in C3H/HeOuJ mice. LPS also produced a large decrease in adipose tissue β3-AR mRNA, and a parallel reduction in β3-agonist-induced activation of adenyl cyclase. Changes in plasma leptin and β3-AR mRNA were preceded by an approximately threefold increase in white fat TNF mRNA. TNF administration resulted in changes similar to those seen with LPS. We conclude that endotoxemia results in an induction of leptin mRNA and a decrease in β3-AR mRNA in adipose tissue, an effect that may be mediated by alterations in TNF-α.

ANOREXIA AND CACHEXIA, syndromes of depressed appetite, weight loss, and wasting, complicate many diseases, including cancer (33), AIDS (10), chronic infection, and other critical illnesses (3). Both decreased caloric intake and an increased metabolic rate have been observed in cachectic patients (8), but little is known regarding the specific mechanisms responsible for altered appetite and metabolism. Substantial animal and human data suggest a possible role of cytokines, such as tumor necrosis factor (TNF) and interleukin (IL)-1 (19); however, the cellular mechanisms by which these factors induce anorexia and cachexia are unknown.

Recent data indicate that cytokines alter the expression of several adipose tissue-specific genes (28). TNF decreases the activity of lipoprotein lipase (11), reduces expression of the glucose transporter GLUT-4 (29), and increases the rate of lipolysis (20). The effect of TNF on GLUT-4 is hypothesized to contribute to development of insulin resistance (15, 16). Related studies suggest a role for endogenous TNF in the regulation of fat cell size, with increases in fat cell lipid content leading to augmented TNF production, which in turn initiates responses to limit adipocyte size (14).

A variety of animal and human studies have suggested a possible etiologic role for TNF-α and other cytokines in cachexia (19, 34). We hypothesize that TNF may contribute to wasting by altering adipocyte gene expression. Two possible candidate genes are those coding for synthesis of leptin and the β3-adrenoceptor (β3-AR). Leptin is a fat cell hormone that regulates both appetite and metabolic activity (for review, see Refs. 2, 27). Recent data suggest that leptin is the principal means by which information regarding the adequacy of peripheral fat stores is communicated to the central nervous system, with decreases in plasma leptin concentration playing a central role in triggering adaptive responses to starvation (1). Moreover, Grunfeld et al. (13) have demonstrated that lipopolysaccharide (LPS) and exogenous TNF both prevent fasting-induced decreases in leptin. A second possible site for cytokine action is the β3-AR, which is important in transducing signals for sympathetically mediated lipolysis in white adipose tissue and thermogenesis in brown adipose tissue (for review, see Ref. 31). Little is known regarding possible effects of cytokines on this receptor system; however, TNF is known to reduce β3-AR signal transduction in select cell lines (5). The importance of the β3-AR system in energy homeostasis is highlighted by studies demonstrating weight gain after targeted disruption of this receptor system (32) and weight loss with administration of exogenous β3-AR agonists (36). The present study examines the effect of LPS on these two adipocyte systems and probes the potential role of TNF in mediating these actions.

METHODS

Animal protocols. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee and are accepted by the American Association for Accreditation in Laboratory Animal Care. Animals were housed at 25°C, five to a cage, with free access to standard rodent diet and water. For all experiments, 8-wk-old C3H/HeOuJ mice were used. In preliminary experiments, we found that high-dose LPS produces marked anorexia, reducing food intake from 3.1 to 0 g/day. Therefore, to avoid confounding effects of varying food intake on leptin expression, all animals were fasted after LPS (or saline) administration. To determine the effect of increasing doses of LPS on the expression of leptin and β3-AR mRNA, animals (n = 5/group) received intraperitoneal endotoxin at one of the following doses: 0, 0.5, 5.0, 50, or 500 µg/100 g. For this dose-response study, animals were killed 16 h after LPS administration. After the animals were killed, blood was obtained for measurement of leptin concentration, and epididymal white fat was removed and rapidly frozen in liquid nitrogen for quantification of β3-AR, leptin, and TNF mRNA concentrations and adenyl cyclase responses to β3-agonist stimulation.

To determine the time course of LPS-induced changes in adipose tissue mRNA levels and plasma leptin, animals...
received LPS (500 mg/100 g) and were killed 0, 2, 4, 8, and 16 h later (n = 5/group). Plasma leptin concentrations and white fat leptin, β3-AR, and TNF mRNA concentrations were measured.

To evaluate whether observed effects of LPS on leptin and β3-AR mRNA expression may have occurred secondary to TNF induction, white fat TNF mRNA was measured in LPS-treated animals. In separate experiments, four animals received exogenous TNF (17 mg/100 g ip), and plasma leptin and fat cell leptin and β3-mRNA were measured 16 h later. As in the LPS protocols, food was withheld for the 16-h exposure period.

RT-PCR. For RT-PCR of TNF-α mRNA, 1 µg of total RNA was reverse transcribed with Moloney murine leukemia virus RT (GIBCO BRL, Gaithersburg, MD) for 30 min at 37°C in a solution containing 500 nM oligo(dT), 0.2 mM of each dNTP, 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol. RNA template was digested with RNase H. cDNA was denatured for 5 min at 94°C and submitted to 30 cycles of amplification in a solution containing 0.5 U of Taq polymerase (Perkin Elmer) and 1 mM sense and antisense oligonucleotides. For TNF, the amplified fragment corresponds to nucleotides 946-1738 of the mouse genomic sequence (23). This sequence spans an intron (intron 2); we are thus able to determine that the amplified fragment does not arise from genomic contamina tion. The sense and antisense primers were 1) GGC CAG ACC CTC ACA CTC AG and 2) CTT GGG GCA GGG GCT CTT GA. To determine the linearity of amplification for quantitation, PCR was performed for 25–30 cycles and found to be linear over this range. For further PCR reactions, 27 cycles were chosen. The PCR cloning of the leptin and β3-AR RNase protection assay (RPA) probes is described below.

Mouse leptin and β3-AR cRNA probe synthesis. Fragments of the leptin and β3-AR genes were amplified by using RT-PCR with specific primers containing EcoRI/BamH I primer linkers. These DNA fragments were cloned into the transcription vector, pGEM-4Z. The leptin PCR product is a 1,090–1,347 bp of the mouse leptin sequence (23). The PCR product used for probe is a 257 bp fragment corresponding to nucleotides 1,000–1,347 bp of the mouse leptin sequence (23). Both probes were sequenced in their entirety before use. Mouse white fat cDNA was used as template for the PCR reactions (GIBCO BRL). Radiolabeled cRNA probes for use in the RPA were synthesized from cloned cDNA fragments using [α-32P]CTP (NEN) and T7 polymerase (Ambion, Austin, TX) according to their published protocol.

RNA quantification by RPA. RNA was extracted from fat tissue by the method of Chomczynski using RNAzol (Tel-test, Friendswood, TX) and quantitated spectrophotometrically at 260/280 nm. RPAs were performed as previously described (21). In brief, 20 µg of total RNA were coprecipitated with ~105 counts/min of the cRNA probe and then dissolved in 30 µl of hybridization buffer (80% formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, and 1 mM EDTA). The solution was then heated for 5 min at 85°C, followed by an overnight incubation at 55°C. RNase digestion was accomplished by using 300 µl of RNase solution (RNase A (40 µg/ml, Sigma) and RNase T1 (800 U/ml, Gibco BRL) in 300 mM sodium acetate and 5 mM EDTA) at 30°C for 60 min and was terminated by addition of 10 ml of 20% SDS and 2.5 ml of proteinase K (15 mg/ml) for 30 min at 37°C. After phenol-chloroform extraction, RNA was precipitated with 20 µg of tRNA as a carrier. After resuspension in 4 ml of loading buffer (80% formamide, 20 mM EDTA (pH 8.0), 0.1% bromophenol blue, and 0.1% xylene cyanole), the samples were separated by electrophoresis on a denaturing 8 M urea-6% acrylamide gel, followed by drying and exposure to XAR-5 (Kodak) film for 12–48 h at ~70°C. Proteored fragments were quantified after exposure of Phospho mager screens by image-analysis software (Imagequant, Molecular Dynamics, Sunnyvale, CA). Because it has been shown that LPS and cytokines increase levels of mRNA for actin and cyclophillin (13), two mRNAs commonly used for normalizing data, equal amounts of total RNA were coprecipitated in our RPA protocol.

Measurement of plasma leptin. Mouse plasma leptin concentrations were measured by radioimmunoassay (Linco Research, St. Charles, MO). Samples were incubated overnight with mouse leptin antibody, after which [125I]-labeled leptin was added and allowed to equilibrate for 18 h. Precipitated antibody was separated by centrifugation and counted in a Minaxi 5000 gamma counter, and leptin concentrations were derived by interpolation from a standard curve. The sensitivity of this assay is 0.2 ng/ml, and intra-assay variability is <3%.

Adenyl cyclase activity. Frozen adipose tissue was homogenized in 2 ml of cold buffer [0.1 M HEPES (pH 7.4), 25 mg/ml aprotinin, and 25 mg/ml leupeptin] in ice using a Tissumizer high-speed cutting blade (Tekmar, Cincinnati, OH). The homogenate was used immediately for protein determination and adenylyl cyclase assays. Adenyl cyclase activity was determined as previously described with minor modifications (4). Briefly, adipocyte membranes (60 mg) were incubated for 10 min at 30°C in 100 ml of buffer [50 mM HEPES (pH 8.0), 50 mM NaCl, 0.4 mM EDTA, 0.25 mg/ml BSA, 5 mM MgCl2, 1 mM [γ-32P]ATP (0.1-0.3 mCi/mmol), 1 mM cAMP, 7 mM creatine phosphate, and 50 mM creatine kinase]. Adenyl cyclase activity was determined in the basal (unstimulated) state and in response to GTP (10 mM), GTP plus isoproterenol (100 mM), GTP plus the β3-AR-selective agonist CL-316243 (1 mM-100 mM), forskolin (10 mM), NaF-AlCl3 (10 mM NaF + 100 mM AlCl3), and MnCl2 (20 mM). Preliminary experiments confirmed the linearity of adenylyl cyclase activity at the protein concentrations and incubation times used. cAMP was isolated by sequential column chromatography through Dowex and alumina (24), with column recoveries of 75–90%. Protein was assayed with the Pierce Chemical (Rockford, IL) bicinchoninic acid (BCA) protein assay reagent, consisting of BCA and copper sulfate solutions (3). BSA was used as a standard (26).

Data analysis. ANOVA techniques were used to evaluate effects of both varying doses of LPS and exposure times on plasma leptin and fat cell mRNA levels. Dunnnett’s test was used for pairwise comparisons. Nonlinear curve fit analysis was used to establish dose-response relationships. Effects of TNF on leptin and fat cell mRNA were evaluated by use of independent t-tests, with Bonferroni correction for multiple comparisons. RNA data are expressed as counts and are expressed as means ± SE. Effects of LPS on basal and stimulated adenylyl cyclase activity were analyzed by paired, two-tailed Student’s t- test. Dose-response curves of CL-316243-stimulated adenylyl cyclase activity in LPS and control animals were determined by use of nonlinear curve-fit analysis and were compared by Friedman nonparametric repeated measures test, with Dunn’s multiple posttest used for comparisons between groups. Data were considered to be significantly different at P < 0.05.

RESULTS

Escherichia coli endotoxin produced dose-dependent increases in plasma leptin concentrations (Fig. 1), with a half-maximal effective dose of ~16 µg/100 g. At the
Adenyl cyclase activity was not altered (85 ± 66 nmoles cAMP·mg protein·min−1) by LPS (Fig. 4), and plasma leptin concentrations were approximately seven times higher than baseline concentrations in saline-treated controls (13.9 ± 6.0 pmol cAMP·mg protein−1·min−1). There was no effect of LPS on cyclase activation by G protein activators (GTP and NaF) or by direct activators of adenyl cyclase (forskolin and Mn).

Studies examining effects of LPS on adipocyte TNF mRNA expression demonstrated increased expression of this cytokine [26.6 ± 1.1 vs. 88.4 ± 8.1 (arbitrary units × 10³), n = 5, P < 0.01]. When TNF was administered to mice, leptin and β3-AR leptin mRNA expression changed in a manner similar to that seen with LPS (Fig. 4), and plasma leptin concentrations increased ~10-fold (1.63 ± 0.1 vs. 16.8 ± 0.6, n = 4, P < 0.01). To further probe the relationship between TNF and adipocyte gene transcription, the time course of LPS-induced alterations in fat cell TNF, β3-AR mRNA, and plasma leptin was determined (Fig. 5). TNF induction was observed 2 h post-LPS and appeared to be maximal at this time (Fig. 5A). β3-AR mRNA was decreased at 4 h, with the maximal decrease seen at 8 and 16 h (Fig. 5B). Plasma leptin concentrations were increased in two of five animals at 8 h and in five of five animals at 16 h (Fig. 5C).

**DISCUSSION**

The present study demonstrates that endotoxin administration results in dramatic changes in the expression of TNF, leptin, and β3-AR in white fat. LPS increases leptin production in a dose-dependent manner and, at higher doses, produces plasma levels significantly above those seen in untreated mice. LPS also reduces adipose tissue β3-AR mRNA levels and results in reduced β3-AR agonist-induced adenyl cyclase activation. Changes in fat cell leptin and β3-AR mRNA concentrations are preceded by increases in adipose tissue TNF mRNA. Moreover, administration of exogenous TNF is sufficient to produce the changes in leptin and β3-AR mRNA produced by endotoxin. These data suggest that LPS alters the synthesis of these fat cell proteins by stimulation of TNF.

Other investigators have shown that endotoxin increases leptin production. Grunfeld et al. (13) demonstrated anorexia and increased leptin mRNA in hamsters treated with endotoxin, IL-1, and TNF. Using Western blot analysis, they demonstrated that leptin levels in fasted, LPS-treated animals were similar to those seen in control fed animals. The authors hypothesized that cytokines block the normal inhibition of leptin synthesis that occurs with fasting. In contrast, we observed marked elevations of plasma leptin concentrations in LPS-treated mice. Levels in our animals were far greater than those seen in fed animals. These data suggest pathological overexpression of leptin rather than simply interference with the signaling mechanism responsible for fasting-induced inhibition of leptin syn-

![Fig. 1. Plasma leptin concentrations in fasted control animals and in mice receiving exogenous endotoxin (lipopolysaccharide, LPS; 0.5, 5, 50, or 500 µg). Plasma leptin concentration was measured 16 h after LPS administration. *P < 0.01; n = 5.](http://ajpendo.physiology.org/)

![Fig. 2. Expression of leptin and β3-adrenergic receptor (β3-AR) mRNA in endotoxin-treated (500 mg/100 g) and control mice. A: a, autoradiogram of RNAse protection assay (RPA) demonstrating increased expression of leptin; Lep PF, leptin protected fragment. b, Volume integration and quantitation of leptin mRNA from phosphorimager of RPA gel. mRNA is expressed as arbitrary units or counts. *P < 0.01; n = 4. B: a, autoradiogram of RPA demonstrating decreased expression of β3-AR mRNA; P, probe; PF, protected fragment. b, Volume integration and quantitation of β3-AR mRNA from phosphorimager of RPA gel. mRNA is expressed as arbitrary units or counts. *P < 0.01; n = 5.](http://ajpendo.physiology.org/)
thesis. Kirchgessner et al. (17) present data both in vivo (mouse model) and in vitro (3T3-L1 adipocytes in culture) to suggest a mechanism by which TNF-α acts directly on adipocytes to release preformed pools of leptin, since TNF-α stimulation is insensitive to cyclohexamide, a protein synthesis inhibitor, while being significantly inhibited by the secretion inhibitor brefeldin A. Although this mechanism may be important in the rapid regulation of leptin release, this does not explain the significant rise in the steady-state concentration of leptin mRNA that we and other investigators have demonstrated with LPS and TNF-α. Thus both transcriptional and posttranslational mechanisms may be important in the leptin response to TNF-α.

Endotoxin and TNF have not previously been reported to affect β3-AR mRNA levels or β3-AR function. Our results indicate a dramatic reduction in mRNA levels, reflecting either a reduction in mRNA synthesis or an accelerated breakdown of this message. Accompanying the reduced β3-AR mRNA expression was a reduced adenyl cyclase response to β3-AR agonist stimulation. Observation of normal responses to direct activators of G proteins and adenyl cyclase suggests a specific effect on β3-ARs and not their signal transduction pathways. Although the magnitude of the reduction in adenyl cyclase response was smaller than the decrease in mRNA concentration, cyclase studies were performed 16 h after LPS administration. Because there is a significant lag between changes in β3-AR mRNA expression and changes in β3-AR cyclase-mediated activity (7), inadequate time may have elapsed to manifest the full consequences of reduced receptor synthesis.

The observed changes in leptin and β3-AR mRNA after LPS administration were preceded by augmented adipose tissue TNF mRNA production. Adipose tissue

- **Fig. 3.** Adenylyl cyclase activity in adipose tissue membranes of control and LPS-pretreated mice (n = 9). A: adenylyl cyclase activity was measured under basal (unstimulated) conditions and in response to GTP (10 mM), GTP + isoproterenol (100 mM), and NaF-ACl3 (10–100 mM) for 10 min at 30°C. Isoproterenol activity was decreased in LPS animals. *P < 0.05. B: adenylyl cyclase activity was measured in response to GTP (10 mM) + increasing concentrations of selective β3-AR agonist CL-316243. Adenylyl cyclase activity in response to CL-316243 was significantly decreased in adipose tissue membranes of LPS-pretreated mice. *P < 0.05.

- **Fig. 4.** Expression of leptin and β3-AR mRNA in tumor necrosis factor-α (TNF-α)-treated and control mice. Autoradiogram (top) of RPA demonstrating expression of leptin (Lep) and β3-AR mRNA. P, probe; PF, protected fragment. A: volume integration and quantitation of leptin mRNA. B: volume integration and quantitation of β3-AR mRNA from phosphoimage of RPA gel. mRNA is expressed as arbitrary units or counts. *P < 0.01; n = 4–5.
TNF mRNA was maximally increased at 2 h, whereas changes in $\beta_3$-AR mRNA were not seen until 4 h (maximal at 8 h), and plasma leptin levels did not increase until 8–16 h after LPS. This temporal sequence suggests that TNF plays a critical intermediary role in LPS-induced changes in leptin and $\beta_3$-AR. This hypothesis is supported by the observation that exogenous TNF produced changes in adipocyte gene expression identical to those seen with LPS. Although the observed temporal sequences are consistent with adipose tissue TNF being principally responsible for affecting leptin and $\beta_3$-AR synthesis, we cannot exclude the participation of other cytokines in modulating adipocyte mRNA synthesis, especially in light of reports demonstrating leptin stimulation by exogenous IL-1 (25). It is also possible that circulating cytokines, rather than locally produced TNF, are responsible for the observed effects of LPS. Systemic cytokines could act directly on the adipocyte, or indirectly, via diverse effects on hormonal secretion and autonomic nervous system activity (30). Finally, we do not know the identity of the cell type responsible for TNF production in adipose tissue (adipocyte vs. stromal cell vs. resident macrophage).

The clinical relevance of the observed changes in fat cell protein synthesis is unknown at this time, but we speculate that the rise in plasma leptin levels with LPS and TNF administration may contribute to sepsis-induced weight loss. Pathological production of TNF occurs in many animal models of cachexia and in some patients with cancer cachexia. Moreover, administration of exogenous TNF causes weight loss; however, little is known about the mechanisms by which TNF alters appetite and metabolism. Given the central role that leptin plays in regulating both appetite and metabolic rate, it is attractive to postulate that TNF-induced hyperleptinemia suppresses appetite and prevents the usual compensatory responses to starvation. However, recent data from LPS-treated, leptin-deficient mice suggest that leptin is not essential for LPS-induced appetite suppression (6). TNF-induced hyperleptinemia may still contribute to weight loss in some cachectic states by preventing the normal adaptive responses to starvation. Available data suggest that a fall in plasma leptin concentration is the trigger responsible for neuroendocrine changes that reduce metabolic rate and terminate nonessential functions, such as reproduction, during starvation (1, 9). Cytokine-mediated increases in leptin would appear to interfere with this signaling and prevent initiation of adaptive responses. Leptin is not elevated in many wasting states (i.e., AIDS; Refs. 12, 35), and additional studies are required to determine whether hyperleptinemia contributes to weight loss in states with elevated cytokines.

The physiological relevance of reduced $\beta_3$-AR expression in LPS-treated animals is unclear. $\beta_3$-ARs are important in sympathetically mediated lipolysis and thermogenesis. $\beta_3$-AR knockout mice are prone to excess weight gain (22), presumably as a result of reduced energy expenditure and impaired fat utilization. LPS administration causes a marked increase in sympathetic nervous system activity. Increased sympathetic traffic to adipose tissue augments lipolysis and nonshivering thermogenesis. Whether the reductions in $\beta_3$-AR expression and agonist-induced adenylyl cyclase production that we observed act to attenuate sympathetic effects in fat is not known.

The present study examines only short-term effects of LPS administration; it is not known whether long-standing infections are characterized by chronically elevated leptin levels. Equally important, it is not known whether cytokines increase leptin levels in humans. Finally, although leptin and $\beta_3$-AR play an
important role in metabolic regulation, the contribution of cytokine-mediated changes in the expression of these fat cell proteins to metabolic abnormalities in sepsis and other states with elevated cytokines remains unknown.

D. Brown is a Foundation for Anesthesia Education and Research/Marion Roussel Anesthesiology Research Fellow. Address for reprint requests: D. E. Berkowitz, Dept. of Anesthesiology and Critical Care Medicine, Tower 711, The Johns Hopkins Hospital, 600 N. Wolfe St., Baltimore, MD 21287-8711.

Received 27 August 1997; accepted in final form 4 February 1998.

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