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High-Dose Leptin Activates Human Leukocytes Via Receptor Expression on Monocytes

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Leptin is capable of modulating the immune response. Proinflammatory cytokines induce leptin production, and we now demonstrate that leptin can directly activate the inflammatory response. RNA expression for the leptin receptor (Ob-R) was detectable in human PBMCs. Ob-R expression was examined at the protein level by whole blood flow cytometry using an anti-human Ob-R mAb 9F8. The percentage of cells expressing leptin receptor was 25 ± 5% for monocytes, 12 ± 4% for neutrophils, and 5 ± 1% for lymphocytes (only B lymphocytes). Incubation of resting PBMCs with leptin induced rapid expression of TNF-α and IL-6 mRNA and a dose-dependent production of TNF-α and IL-6 by monocytes. Incubation of resting PBMCs with high-dose leptin (250 ng/ml, 3–5 days) induced proliferation of resting cultured PBMCs and their secretion of TNF-α/H9251 and IL-6/H9251 (8), and leptin levels rise after surgical stress (9). Leptin receptors are expressed on human umbilical cord leukocytes (10), and leptin activates umbilical cord monocytes, polymorphonuclear cells, and lymphocytes (11). Rodents with genetic abnormalities in leptin and leptin receptor expression have deficits in macrophage phagocytosis and endotoxin-mediated induction of TNF-α and IL-6 (12).  

High-dose leptin is now being used as a potential therapy for obesity (13), and high levels of leptin are found in extreme obesity due to leptin resistance and also in renal failure (14, 15). Injection of leptin elicits a mild localized inflammation (16), but the physiological basis for this reaction has not been established. To date, studies have focused on the effects of physiological levels (as determined by circulating plasma levels) of leptin on activated lymphocytes. In this study, we confirm the specificity of a mAb for the leptin receptor and demonstrate that monocytes are the predominant cells expressing Ob-R in the peripheral blood. Our results demonstrate that high-dose leptin induces the production of proinflammatory cytokines in resting PBMCs, which is compatible with the observed expression of Ob-R on peripheral blood monocytes.  

Materials and Methods  
All the materials were purchased from Sigma-Aldrich (Poole, U.K.) unless otherwise stated.  

Blood samples  
Peripheral blood (50 ml) was collected into heparinized tubes from healthy adult volunteers (aged 20–45 years).  

Measurement of endotoxin activity in recombinant human leptin and blocking of endotoxin activity  
Endotoxin activity in recombinant human leptin was measured using a QCL-1000 pyrogen testing kit (BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions. The potential influence of endotoxin contamination on observed effects was excluded by the addition of poly-myxin-B in control experiments (17). This was added at the same time as endotoxin and leptin.
Leptin functional assay

Biological activity of recombinant leptin was tested in transcription assays performed in human epithelial kidney (HEK293) cells transiently transfected with the leptin receptor long form and a reporter construct containing a Stat3-binding element fused to a minimal thymidine kinase promoter and luciferase. Luciferase activity was measured as previously reported (18).

RT-PCR

For the analysis of leptin receptor and cytokine gene expression, total RNA was extracted from 4 × 10^7 PBMCs using TRIzol reagent (Life Technologies, Paisley, U.K.). Total RNA (1 μg) was reverse-transcribed and PCR amplification was performed using previously published primers for Ob-Ra and Ob-Rb (4). For cytokine gene analysis, mRNA was extracted at the indicated time points, cDNA was prepared, and PCR amplifications were performed using previously characterized primers for IL-6, TNF-α, IFN-γ, and GAPDH (19).

Anti-leptin receptor mAb

A mAb (9F8) raised against the recombinant human leptin receptor extracellular domain (20) was used for the study. Specificity of mAb 9F8 was shown by the lack of cross-reactivity with extracellular domains from other closely related receptors of the same class I cytokine receptor superfamily (growth hormone and prolactin receptors).

Transfection of HEK293 cells with Ob-R

The leptin receptor expression plasmid was a kind gift from A. Welcher (Amgen, Thousand Oaks, CA, CA). HEK293 cells were transfected with 9 μg of leptin receptor cDNA using a calcium phosphate transfection kit (Life Technologies). Receptor expression was confirmed by assessing the binding of ^125I-labeled leptin (Biogenes, Poole, U.K.).

Validation of 9F8 by flow cytometric analysis of transfected and nontransfected HEK293 cells

Transfected and nontransfected HEK293 cells were suspended in PBS/BSA (washing buffer) at a density of 1 × 10^7 cells/ml. Cells (10^6; 100 μl) were transferred to 12 × 75 mm polycarbonate tubes (BD Biosciences, Oxford, U.K.) and incubated with 5 μg of an anti-leptin receptor Ab (9F8) or isotype-matched negative control Ab (R&D Systems, Abingdon, U.K.) for 30 min on ice. Primary Ab binding was detected by incubation with biotinylated goat anti-mouse IgG polyclonal Ab (1 μg; Calbiochem, Nottingham, U.K.), followed by incubation with streptavidin-R-PE (SAV-R-PE) (R&D Systems) for 30 min on ice. Cells were washed before flow cytometric analysis. The specificity of 9F8 was tested by preincubation with a recombinant form of the extracellular domain of the human leptin receptor rhOb-R (R&D Systems).

Detection of Ob-R on peripheral blood leukocytes by whole blood flow cytometry

Heparinized whole blood (150 μl) and 10 μl of goat serum (Serotec) were incubated for 15 min on ice, after which 1 μg of 9F8 or isotype-matched negative control Ab were added. Cells were washed in PBS containing 7.5% v/v goat serum, incubated with 0.2 μg of biotinylated goat anti-mouse IgG, and finally incubated with 10 μl of the SAV-R-PE conjugate. Incubations were conducted for 30 min on ice and cells were washed twice with washing buffer. Erythrocytes were lysed with 2 ml of Erythrolyse (Serotec, Oxford, U.K.) for 30 min on ice. Cells were washed before flow cytometric analysis. The percentage of 9F8 was tested by preincubation with a recombinant form of the extracellular domain of the human leptin receptor rhOb-R (R&D Systems).

Results

Ob-R gene expression of PBMCs

PCR products for both short (Ob-Ra) and long (Ob-Rb) isoforms of leptin receptor were detectable in human PBMCs (Fig. 1).

Validation of 9F8 for detection of Ob-R expression by flow cytometry

HEK293 cells were transfected with Ob-R and expression of leptin receptor was confirmed by incubation with ^125I-labeled leptin in the presence or absence of unlabeled leptin. Ob-R-transfected HEK293 cells had high levels of leptin-specific binding (8 ± 1%).
but nontransfected cells also showed a low level of specific binding (0.7 ± 0.1%). Ob-R-transfected cells were used in flow cytometry to test the anti-leptin receptor Ab (9F8). Transfected and nontransfected HEK293 cells were incubated with either 9F8 or isotype-matched control Ab, and Ab binding was detected by flow cytometry. Binding of 9F8 to transfected cells was apparent, but 9F8 also bound to nontransfected cells, confirming that HEK293 cells express low levels of the leptin receptor (Fig. 2). The specificity of 9F8 for the leptin receptor was further tested by preincubation of Ab with recombinant human (rh)Ob-R, which completely abolished binding of 9F8 Ab to HEK293 cells (data not shown).

Leptin receptor expression on peripheral blood leukocytes

Whole blood flow cytometric analysis of samples from 10 subjects using 9F8 revealed that 25 ± 5% of monocytes, 12 ± 4% of polymorphonuclear cells, and 5 ± 1% of lymphocytes express the Ob-R (Fig. 3). Two-color flow cytometry revealed that lymphocyte expression was primarily related to CD20+ cells (B lymphocytes,
50 ± 6%) with T lymphocytes showing no expression. Activation of PBMCs by incubation with endotoxin (10 ng/ml) for 2 h resulted in no change in Ob-R expression on monocytes, and incubation up to 3 days with endotoxin, PHA, or MLR did not alter Ob-R expression on lymphocyte populations.

Leptin bioassay

The biological activity of the recombinant leptin was tested in a function assay using a Stat3-luciferase reporter (Fig. 4a). Leptin showed a dose response between 1 and 100 ng/ml.

Effect of leptin on intracellular cytokine production by peripheral blood monocytes

Whole blood was incubated with leptin in doses of 10–1000 ng/ml and production of IL-6 and TNF-α by CD14⁺ monocytes was determined using flow cytometry. Leptin at doses of 50–1000 ng/ml induced the production of both IL-6 and TNF-α (p < 0.01; Fig. 4b). The observed effects were not due to endotoxin contamination of the leptin preparation, as levels of endotoxin (<0.00036 ng/ml) were below those that we have previously shown to be the minimum dose (0.01 ng/ml) required to induce IL-6 (21). The possibility of endotoxin contamination was further excluded by the demonstration that polymyxin-B, an endotoxin inhibitor (17), completely inhibited the induction of TNF-α by 0.1 ng/ml endotoxin, yet it had no effect on leptin-induced TNF-α production (Fig. 5).

Effect of leptin on proinflammatory cytokine mRNA expression

PBMCs were incubated in the presence or absence of leptin for between 1 and 24 h and cytokine gene expression was determined by RT-PCR using cytokine-specific primers. Leptin induced IL-6 and TNF-α gene expression within 1 h (Fig. 6), whereas it had no effect on induction of IL-4. The IL-4 levels in samples from both resting and leptin-activated PBMCs were below the detection limit of the assay, although PHA-stimulated PBMCs produced high levels of IL-4 (122 ± 14 pg/ml). Leptin also significantly increased the proliferation of PBMCs (PBMCs only, 923 ± 178; PBMCs plus leptin, 1253 ± 254 cpm [³H]thymidine uptake, p < 0.01). When coincubated with endotoxin, leptin augmented
the cytokine response to endotoxin with a pattern similar to the induction of cytokines by leptin alone. Endotoxin alone at the dose of 0.01 ng/ml did not induce IFN-γ (Fig. 7).

**Effect of leptin on PBMCs activated by MLR**

Leptin augmented the production of TNF-α, IL-6, and IFN-γ, but not IL-4 from MLR-activated PBMCs in a similar pattern to that seen in nonactivated PBMCs (Fig. 8). In addition, the MLR-induced proliferation was augmented by leptin (PBMCs only, 923 ± 178; MLR only, 3867 ± 372; MLR plus leptin, 6350 ± 584 cpm [3H]thymidine uptake, p < 0.001).

**MAPK inhibitors and leptin activation of PBMCs**

PBMCs were preincubated with two mitogen-activated protein kinase (MAPK) inhibitors, PD98059 (inhibits ERK) and SB203580 (inhibits p38), and then exposed to either endotoxin or leptin. The p38 inhibitor, SB203580, consistently inhibited both the endotoxin and leptin induction of TNF-α by ~30%, whereas PD98059 had no effect (Fig. 9). The biological activity of the ERK inhibitor PD98059 had previously been demonstrated in our functional bioassay.

**Discussion**

We have examined the possibility that high-dose leptin can induce proinflammatory cytokines in resting PBMCs and explored the mechanism by which this could occur. Our results confirm that human circulating leukocytes express mRNA for both the long and short forms of the leptin receptor (5). We carefully validated the specificity of a mouse monoclonal anti-human Ob-R Ab and used this to examine Ob-R expression in human PBMCs. Ob-R expression was predominantly found on monocytes with low levels on neutrophils. Only a very small proportion of resting lymphocytes expressed Ob-R, and these were exclusively B cells. Compatible with monocyte expression of Ob-R we observed our demonstration that in vitro leptin rapidly induces the production of TNF-α and IL-6, this
occurs in nonactivated as well as activated PBMCs, and the effect of leptin was seen to be additive to the actions of endotoxin.

The expression of Ob-R on human umbilical cord blood leukocytes has been reported using flow cytometry (10). In this study the proportion of monocytes expressing the Ob-R was comparable to that reported in this work (23 ± 4%); however, the proportion of lymphocytes expressing the receptor was greater (16 ± 4%) (10). The difference in results may be relate to the different source of leukocytes. In our data, activation of monocytes and lymphocytes did not alter Ob-R expression.

Leptin treatment of activated PBMCs (MLR), in an experimental design similar to that reported in this study, has been shown to increase Th1 (IFN-γ) and suppress Th2 (IL-4) cytokine production (5). In the previous study, IL-6 and TNF-α were not measured and there was no effect of leptin on resting PBMCs. Our finding that leptin can activate resting PBMCs may relate to the higher dose of leptin used and confirms a study demonstrating that leptin can activate and stimulate the proliferation of resting human PBMCs (22). Leptin has been shown to have a direct effect on activated T lymphocytes (5), suggesting that leptin directly acts on T lymphocytes. We found no evidence of Ob-R on resting T lymphocytes by whole blood flow cytometry, and our data suggest that the predominant action on resting PBMCs is through activation of monocytes.

Our RT-PCR experiments demonstrate that there is an acute stimulation of cytokines by leptin, and the longer incubation studies confirm cytokine production by nonactivated PBMCs. Leptin treatment also enhanced the cytokine response to endotoxin and the MLR. Results from rodent studies confirm our observations in the human. In mice, thioglycollate-elicited peritoneal macrophages produce no detectable TNF-α or IL-6, whereas leptin pretreatment augments proinflammatory cytokine release from endotoxin-stimulated macrophages (12). In the RAW murine macrophage cell, leptin at a high dose of 1 μg/ml activates resting cells to produce significant levels of IL-1R antagonist after 24 h and also augmented the response to endotoxin in an additive manner (23). Intracerebroventricular and i.p. injection of leptin into rats induces the production of IL-1β and increases body temperature (24).

Contamination of any component of the cell incubation by endotoxin could activate cells and induce the production of inflammatory cytokines. This seems unlikely in our experiments because endotoxin levels in the leptin preparation were well below that which is required to activate PBMCs (21). In addition, polymyxin-B (an inhibitor of endotoxin) treatment inhibited the response to endotoxin but had no effect on leptin. The profile of cytokine induction by leptin was different from that of endotoxin, with leptin inducing significant levels of IFN-γ, an effect not seen with low levels of endotoxin.

We performed a dose response for the effect of leptin on TNF-α and IL-6 production. The biological activity of the recombinant leptin was tested in a functional bioassay measuring leptin signaling. This confirmed the biological activity of leptin at doses as low as 1 ng/ml. Higher doses of leptin were required to induce cytokine production (50–1000 ng/ml) than are usually found in venous blood under physiological conditions (1–100 ng/ml). Our repeated experiments on PBMCs were performed with a leptin dose of 250 ng/ml. This dose was chosen because it was clear from our dose response studies that this dose of leptin could activate PBMCs. Similar higher serum leptin levels are found in some nonphysiological conditions, such as in children with chronic renal failure (leptin levels up to 400 ng/ml are reported in Ref. 15) and following leptin treatment (25). Treatment of a patient with genetic leptin deficiency with low levels of leptin (0.028 mg/kg of lean body mass) increased the serum leptin up to 107 ng/ml (26). In obese but otherwise healthy subjects treated with leptin (1 mg/kg/day), serum leptin levels up to 736 ng/ml have been reported (25). Thus the high leptin doses we used in our study were similar to those found in some nonphysiological conditions and may lead to activation of PBMCs.

The high dose response of leptin in our study may reflect the insensitivity of our method for measuring cytokine production from incubated PBMCs, although in activated PBMCs a lower dose of leptin was reported as effective (5). Another possibility is that incubation of PBMCs with leptin over time may result in degradation of leptin and/or loss of cytokines. The higher dose of leptin may be relevant, because leptin levels could vary between tissues and leptin levels in fat tissue may be greater than those found in serum. There are other possibilities for the high dose-dependent activation of PBMCs by leptin. It is recognized that short forms of the leptin receptor can activate the MAPK signaling pathway, and it may be that either the dose response of these receptors is different from that of the long form of the receptor (27) or that leptin is activating another receptor. Recombinant leptin is known to have a lower potency than native leptin, which may be due to a different pattern of glycosylation (28).

The class I cytokine receptors are known to act through the Janus kinase/STAT and MAPK pathways (29). Both long and short leptin receptor isoforms can activate the MAPK pathway (30). The major MAPK pathways are: ERK, c-Jun NH2-terminal kinase, and p38 MAPK. A wide variety of inflammatory mediators, such as cytokines, activate p38 MAPK in several cell types. In neutrophils, TNF-α activates p38 MAPK specifically, whereas others, such as GM-CSF, ILMP, IL-8, and PMA preferentially phosphorylate ERK 1/2 (31). To determine which MAPK signaling pathway is recruited by leptin to produce TNF-α in monocytes, PD98059 and SB203580 were used to block ERK and p38 respectively. PD98059 did not show any effect on either endotoxin-stimulated or leptin-activated monocytes, despite its inhibitory effects on leptin induction of serum response element transfected cells (data not shown). SB203580 reduced the percentage of activated monocytes by 30% in leptin-activated cells, showing involvement of p38 MAPK signaling in leptin induction of TNF-α in monocytes. Currently there is no specific inhibitor of the Janus kinase/STAT signaling pathway, so we were unable to examine the potential role of this pathway in PBMCs.

The physiological significance of leptin-induced proinflammatory cytokine production requires investigation. Leptin-deficient or -resistant rodents are more susceptible to the toxic effects of TNF (32). Although one might therefore expect that leptin would reduce levels of TNF, the reverse appears to be true. It is possible that leptin induces a negative regulator of TNF, and that the rise in TNF levels occurs in response to partial TNF resistance and is therefore compensatory. It may be that the local induction of proinflammatory cytokines provides a pathway for some metabolic actions of leptin. The production of IL-6 in the hypothalamus is known to activate the hypothalamo-pituitary axis (33), and TNF alone can produce an anorectic action (34). Another possibility is that the high leptin levels seen in obesity could be responsible for some of the adverse effects of central obesity. The local activation of TNF could play a role in atherogenesis or insulin resistance (35). Our data suggest that leptin directly activates monocytes and that the activation of T lymphocytes may be in response to the release of inflammatory cytokines. To date, the presence of Ob-R on human B lymphocytes has not been demonstrated. It has been proposed that leptin treatment may augment the immune response in malnourished individuals (6).
The fact that leptin can increase the response to endotoxin and promote proinflammatory cytokine release from apparently non-activated PBMCs should be further investigated in humans. Particular care should be taken in human therapeutic trials that leptin therapy does not activate an unwanted inflammatory response, and leptin-treated patients may be more susceptible to an inflammatory response following infection.

In conclusion, human blood leukocytes express leptin receptor, and the predominant leptin receptor-expressing cell type is the monocyte. In vitro, leptin modulates the immune response, inducing the production of inflammatory cytokines, and has an additive action on cytokine production from PBMCs exposed to endotoxin.

Acknowledgments

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References