Leptin Induces Endothelin-1 in Endothelial Cells In Vitro

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Abstract—Leptin, a protein encoded by the obese gene, is produced by adipocytes and released into the bloodstream. In obese humans, serum leptin levels are increased and correlate with the individual’s body mass index and blood pressure. Elevated serum concentrations of endothelin-1 (ET-1), a potent vasoconstrictor and mitogen, were also observed in obese subjects. The pathomechanisms underlying this ET-1 increase in obesity are poorly understood. In the present study, we investigated the influence of the ob gene product leptin on the expression of ET-1 in human umbilical vein endothelial cells (HUVECs). Binding studies using 125I-radiolabeled leptin revealed high- and low-affinity leptin binding sites on HUVECs (Kd1 = 13.1 ± 3.1 nmol/L and Kd2 = 1390 ± 198 nmol/L, respectively), mediating a time- and dose-dependent increase of ET-1 mRNA expression and protein secretion after incubation of HUVECs with leptin. This leptin-induced ET-1 expression was inhibited by preincubation of HUVECs with 0.75 mmol/L antisense phosphorothioate oligonucleotides directed against the leptin receptor Ob-Rb. Furthermore, after incubation with leptin, increased nuclear staining of c-fos and c-jun, the major components of the transcription factor AP-1, and increased AP-1 DNA binding were observed. Transient transfection studies with ET-1 promoter constructs showed that leptin-induced promoter activity was abolished in the absence of AP-1 binding sites or by cotransfection with a plasmid overexpressing a mutated jun, which is able to bind c-fos but not DNA. Thus, leptin upregulates ET-1 production in HUVECs via a mechanism potentially involving jun binding members of the bZIP family. (Circ Res. 2002;90:711-718.)

Key Words: endothelin • obesity • hypertension • vasculature

Although the association of obesity and hypertension is well established,1 there is, however, scarce information about the underlying pathomechanisms. Recently, the adipocyte gained more attention, serving not only as a passive energy storage pool but also as an important source of endocrine-active peptides, thus leading to a novel concept of adipocyte function.

One of the adipocyte-derived peptides involved in the control of body weight is the recently identified hormone leptin,2 a protein encoded by the obese (ob) gene modulating lipid metabolism,3 hematopoiesis,4 pancreatic β-cell function,5 and angiogenesis.6,7 Mutation of the ob gene causes severe obesity in ob/ob mice, which can be reversed through administration of exogenous leptin.8–10 In contrast, serum leptin levels in obese humans are increased and correlate with their body mass index,11 suggesting that obese subjects are resistant to endogenous leptin.

Several alternate spliced isoforms of the leptin receptor (Ob-R) have been cloned (reviewed in Tartaglia12) containing a single transmembrane domain and an extracellular domain homologous to the class I cytokine receptor family, but differing in the length of their cytoplasmic tail. The receptor with the full-length cytoplasmic domain (Ob-Rb, 302 amino acids) is predominantly expressed in the hypothalamus13 and is the most important isoform capable of signal transduction. Importantly, the Ob-Rb with the long cytoplasmic tail is also expressed on the surface of endothelial cells,6,7 indicating that leptin might transduce Ob-Rb-dependent signals in these cells. Furthermore, recent studies demonstrated that leptin–Ob-R interaction on CHO-cells induces the synthesis of c-fos and c-jun,14 the major components of the transcription factor AP-1, which is also involved in regulating the expression of the vasoconstrictor and mitogen endothelin-1 (ET-1) in endothelial cells.15

Several studies reported increased serum concentrations of ET-1 in obese subjects, suggesting a participation of ET-1 in the pathogenesis of obesity-associated hypertension and atherosclerosis.16,17 However, the mechanism leading to ET-1 induction in these subjects is unknown. In the present study, we describe a direct link between adipocytes and the endothelium and demonstrate that leptin is able to upregulate ET-1 production in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Reagents
Reagents were obtained as follows: human recombinant leptin and tumor necrosis factor-α (TNF-α) were from Sigma (St Louis, Mo).
DMEM, RPMI 1640, HEPES buffer solution, L-glutamine, penicillin-streptomycin mixture, and PBS (pH 7.4) were from BioWhittaker (Walkersville, Md). Fetal calf serum (FCS) was from Boehringer Mannheim (Mannheim, Germany). [32P]-dCTP (3000Ci/mmol at 10Ci/mL) and 125I-Leptin (2000Ci/mmol) were obtained from Amersham (Braunschweig, Germany). Anti-c-Fos (sc-52) and anti-c-Jun (sc-45) antibodies were obtained from Santa Cruz Biotechnology Inc. AGE albumin was prepared as described previously.18

The promoterless plasmid pG12-basic, the β-galactosidase control plasmid pSV-β-Gal, and the chloramphenicol transference control vector pCAT-control were obtained from Promega. The mutated Jun plasmid pDB7, a derivate of the point mutant Mut14,19 was obtained from Dr D. Bohmann (EMBL, Heidelberg, Germany). The human endothelin-1 cDNA was a present of Dr T. Yanagisawa (University of Texas Southwestern Medical Center, Dallas, Tex). For functional promoter studies, the 3604-bp long 5'-flanking region of the human ET-1 gene was inserted into the multiple cloning site of the plasmid pG12-basic after digestion with Kpn and BglII. The plasmids pET490Luc and pET297Luc were generated through digestion of pET3604Luc with exonuclease III (Promega). The plasmid pl[TATA]Luc has been previously described as pHTF(111) and was generously provided by Dr N. Mackman (The Scripps Research Institute, La Jolla, Calif). All plasmids were verified by sequencing.

Cell Culture
Primary cultures of HUVECs were established according to Jaffe et al21 with minor modifications.22 Bovine aortic endothelial cells (BAECs) were cultured in DMEM supplemented with 10% FCS as previously described.23 All experiments were performed with BAECs of passage 4 to 6 and primary cultures of HUVECs, which had been confluent for 3 days.

Leptin-Receptor Analysis
Leptin-receptor analysis were performed as described by Golden et al.24 Briefly, plasma membranes of HUVECs or BAECs were incubated with 0.5 nmol/L 125I-leptin and varying concentrations of unlabeled leptin (from 0.5 to 200 nmol/L) in Ringer-HEPES buffer+0.1% BSA overnight at 4°C. Furthermore, the contents of each incubation tube were combined with 4 mL ice-cold PBS (pH 7.4) and filtered immediately by rapid vacuum filtration through preconditioned (blocking with ice-cold PBS+5% dry milk powder at 4°C overnight) Whatman GF-D filters. After the initial filtration, the incubation tube was washed once with 4 mL ice-cold PBS, and this solution was used to wash the filter. Filters were washed 8 more times with ice-cold PBS. Radioactivity remaining on filters was determined by gamma counting. Number of binding sites and their dissociation constants were calculated by means of Scatchard transformation. The experiments were repeated 3 times, each with HUVECs obtained from different isolations.

Immunofluorescence Studies
HUVECs were isolated as described, seeded in chamber slides, and grown to confluence. Cells were fixed with 1% paraformaldehyde.
for 1 hour and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Cells were labeled with rabbit anti-\(\text{c-fos}\) and rabbit anti-\(\text{c-jun}\) (10 \(\mu\)g/mL) (Santa Cruz Biotechnology Inc) for 1 hour, washed with PBS, and incubated with FITC-labeled goat anti-rabbit IgG (Jaxell-Accurate) (8 \(\mu\)g/mL) for 1 hour. Bound antibody was detected using a Zeiss Axiophot microscope equipped for epiluminescence.

**Determination of Endothelin-1**

Endothelin-1 contents of cell culture supernatants were determined by ELISA (Biomedica). Coefficient of variation was estimated from repeated measurements on various days of reference samples and were similar to those given by the manufacturer.

**RNA Isolation, Northern Hybridization, and Reverse Transcription-Polymerase Chain Reaction**

Total RNA of HUVECs was prepared using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. For Northern analysis, RNA samples of 20 \(\mu\)g each were size-fractionated by 1.2% formaldehyde agarose gel electrophoresis and transferred to Hybond N membranes (Amersham). A 480-bp \(\text{Sac}\) I insert of the ET-1 cDNA was labeled with \(\alpha\)-\(^{32}\)P\)-dCTP by the random prime technique and used as an ET-1–specific probe. After washing, filters were exposed at \(-70^\circ\)C to Kodak XAR-5 films using intensifying screens. To show same amount of RNA loading in each lane, filters were additionally hybridized with GAPDH. The relative density of the signals was determined by laser densitometry.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts from HUVECs were isolated as previously described. Oligonucleotides containing the ET-1–derived AP-1 sequence \(5’\-\text{GCCTGTTGGTACATAAACAC-3’}\); Pharmacia, Freiburg, Germany) were end-labeled with polynucleotide kinase in the presence of \(\gamma\)-\(^{32}\)P\)-ATP and assayed for transcription factor binding activity. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled consensus oligonucleotides.

**Transient Transfection of BAECs**

Logarithmically growing BAECs were transfected as described by Bierhaus et al with minor modifications. Briefly, plasmid DNA used in transfection studies was isolated by CsCl equilibrium density gradient centrifugation.

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**Figure 3.** Leptin enhances ET-1 mRNA expression in HUVECs. A, Dose dependence (left): HUVECs were incubated with increasing concentrations of leptin for 6 hours. Total mRNA was isolated as described in the Materials and Methods section. Northern blot analysis was performed using a 480-bp \(\text{Sac}\) I insert of the ET-1 cDNA (ET-1; top) and with GAPDH (bottom). B, Time dependence (right): HUVECs were incubated with 500 ng/mL leptin for different time periods. Shown are the ET-1 (top) and the GAPDH signals (bottom). In the lower part, scanning densitometry quantification (normalized to the concentration of GAPDH mRNA) are given.
centrifugation. Cells growing in DMEM containing 10% FCS were transfected by the calcium phosphate method using the ProFection Mammalian Transfection System (Promega) and were exposed to the phosphorothioate oligonucleotides in culture medium at a concentration of 0.75 μmol/L asOb-Rb (lanes 4). Phosphorothioate oligonucleotides in culture medium decreases at time periods longer than 36 hours.

**Application of Phosphorothioate Oligonucleotides**

The sequence of the used human Ob-Rb antisense oligonucleotide is 5'-CAAGCCAGACCGGCTCGTGTG-3'. It comprises the region +3 bp to +21 bp of the human published DNA sequence for Ob-R.

The used sense Ob-Rb oligonucleotide sequences are complementary to the described sequences. The sequence of the scrambled sequence oligonucleotide was 5'-GCAGGCGGAACACCGGTT-3'. Oligonucleotides were synthesized as phosphorothioate derivatives on a Gene Assembler Plus by Pharmacia. Oligonucleotides were added to cell culture medium at a concentration of 0.75 μmol/L and applied 48 hours before harvest or incubation with leptin, respectively. Furthermore, oligonucleotides were renewed every 24 hours, as stability of oligonucleotides in culture medium decreases at time periods longer than 36 hours.

**Statistical Analysis**

For statistical analysis, we used a SPSS 9.0 software package (SPSS Inc.). Mean values were compared by 1-way ANOVA or Student’s t test where appropriate. Unless otherwise indicated mean values and standard error of the mean are given.

**Results**

**Binding of Leptin to Human Umbilical Vein Endothelial Cells**

For determination of specific binding of leptin to HUVECs, Scatchard analysis was performed. To avoid rapid incorporation, isolated plasma membranes from HUVECs were incubated with 125I-leptin. Scatchard analysis of the obtained data indicate the presence of 2 independent leptin binding sites (Figure 1): a high-affinity binding site with a dissociation constant (Kd1) of 131±3.1 nmol/L (estimate±SD) and a second, low-affinity binding site with an estimated dissociation constant (Kd2) of 1390±198 nmol/L. Similar experiments with isolated plasma membranes from BAECS showed that these cells have 2 leptin binding sites with a Kd1 of 6.6±0.7 nmol/L and a Kd2 of 104.9±8.8 nmol/L (data not shown).

**Influence of Leptin on ET-1 Secretion by HUVECs**

To study a potential effect of leptin on endothelial ET-1 production, HUVECs were subjected to various concentrations of human recombinant leptin for several time periods. As shown in Figures 2A and 2B, a dose- and time-dependent increase of ET-1 in cell culture supernatants was detected. Consistently, Northern blot analysis showed that incubation of HUVECs with leptin led to an increase of ET-1 mRNA in a time- and dose-dependent manner (Figures 3A and 3B).

To examine whether the known long receptor isoform for leptin is expressed on endothelial cells and involved in leptin-mediated ET-1 induction, the following experiments were carried out: in semiquantitative RT-PCR, we were able to demonstrate that antisense phosphorothioate oligonucleotides against Ob-Rb (asOb-Rb) led to a downregulation of Ob-Rb transcripts in HUVECs compared with untreated cells or cells incubated with sense Ob-Rb oligonucleotides (Figure 4A). Furthermore, after 48 hours of incubation with 0.75 μmol/L phosphorothioate oligonucleotides, HUVECs were incubated with leptin for further 48 hours and ET-1 content of cell culture supernatants were measured with ELISA. Leptin-mediated ET-1 induction could be reduced significantly by asOb-Rb, whereas sOb-Rb and scrambled sequence oligonucleotides had no significant effect. The results represent the mean±SEM of 3 independent experiments.
leptin binding to and signaling via Ob-Rb. In contrast, sense Ob-Rb oligonucleotides and also scrambled sequence oligonucleotides had no significant effect on leptin-mediated ET-1 induction.

Induction of c-fos and c-jun in Leptin-Stimulated HUVECs

Immunofluorescence imaging of c-fos and c-jun was performed after 1 and 6 hours of incubation with 500 ng/mL leptin. In unstimulated HUVECs, no specific c-fos and c-jun staining occurred (Figure 5A and 5D), whereas cytoplasmic c-fos expression was detectable after 1 hour exposure to leptin (Figure 5B), and a strong nuclear staining was observed after 6 hours (Figure 5C). In contrast, a distinct nuclear c-jun staining was evident after 1 hour incubation with leptin (Figure 5E), which changed to a perinuclear pattern after 6 hours (Figure 5F).

Figure 5. The effect of leptin on c-fos and c-jun. HUVECs were untreated (A and D) or treated with 500 ng/mL leptin for different time periods (B and E for 1 hour; C and F for 6 hours), fixed in 1% paraformaldehyde, and stained with polyclonal antibodies to c-fos (B and C) and c-jun (E and F). It is apparent from these photomicrographs that leptin caused an increase in nuclear staining intensity for c-fos at 6 hours and for c-jun already after 1 hour. Magnification is 400×. One representative experiment out of 3 different experiments is shown.

Participation of the Transcription Factor AP-1 in ET-1 Induction

EMSA using [32P]AP-1 oligonucleotides complimentary to the AP-1 binding site at position −109 of the human ET-1 promoter. Nuclear extracts were prepared from HUVECs induced for various time periods with leptin (500 ng/mL). A total of 10 μg nuclear extract was included in each binding reaction and DNA protein complexes were analyzed on 4% native polyacrylamide gels. EMSA detected AP-1 binding to the binding site at position −109 of the human ET-1 promoter. The leptin-inducible AP-1 complex is indicated with an arrow. Specificity of binding was ascertained by competing with a 160-fold molar excess of cold AP-1 consensus oligonucleotides included in the binding reaction. A representative autoradiograph is shown.

Figure 6. Leptin induces binding of AP-1 to the AP-1 binding site at position −109 of the human ET-1 promoter. Nuclear extracts were prepared from HUVECs induced for various time periods with leptin (500 ng/mL). A total of 10 μg nuclear extract was included in each binding reaction and DNA protein complexes were analyzed on 4% native polyacrylamide gels. EMSA detected AP-1 binding to the binding site at position −109 of the human ET-1 promoter. The leptin-inducible AP-1 complex is indicated with an arrow. Specificity of binding was ascertained by competing with a 160-fold molar excess of cold AP-1 consensus oligonucleotides included in the binding reaction. A representative autoradiograph is shown.

Figure 7A. Participation of the Transcription Factor AP-1 in ET-1 Induction

EMSA using [32P]AP-1 oligonucleotides complimentary to the AP-1 binding site at position −109 of the human ET-1 promoter showed leptin-inducible binding of protein complexes (Figure 6). The stimulatory effect was detectable over a time period of at least 24 hours. Treatment of nuclear extracts with excess of cold AP-1 resulted in the suppression of leptin effect.

Because leptin induces ET-1 expression and the ET-1 promoter harbors 3 potential binding sites for the transcription factor AP-1,15 we suspected that these binding sites may be involved in leptin-induced ET-1 upregulation. Therefore, we performed transient transfection studies with different promoter constructs to investigate whether the number of these AP-1 sites influences ET-1 promoter activity (Figure 7A).

As shown in Figure 7B, leptin-stimulated BAECs that were transiently transfected with a plasmid containing the full-
Figure 7A shows a diagram of the ET-1 promoter activity. A, Top, Organization of the ET-1 promoter, which was cloned in front of the reporter gene luciferase. Bottom, ET-1 luciferase reporter constructs used in this study. Numbers indicate the distance in base pairs upstream from the transcription start site. B, BAECs were transiently transfected with several ET-1 promoter constructs as indicated for 8 hours before known stimulators of ET-1 transcription (TNF-α and AGE-albumin) or leptin (500 ng/mL) was added for 42 hours. Cotransfection with a plasmid overexpressing mutated jun (0.8 μg/mL medium) inhibits leptin-mediated ET-1 induction. After harvest, luciferase activity was determined in the cell lysates and normalized for transfection efficiency to the amount of β-galactosidase activity expressed by the control plasmid pSV-β-Gal. Luciferase activity of unstimulated cells is taken for 100%. The results represent the mean±SEM of at least 3 experiments that were performed in triplicate. *P<0.05 compared with unstimulated BAECs; #P<0.05 compared with leptin-stimulated BAECs.

Discussion

It is well known that obesity is related to hypertension, but the underlying mechanism is not completely understood. There is increasing evidence that adipocytes not only function as energy storage pools but also as immunologically and endocrinologically active cells, because they are able to secrete, eg, TNF-α, plasminogen activator inhibitor-1, and leptin. Recently, it could be demonstrated that intravenous infusion of leptin for 12 days increased arterial pressure in Sprague-Dawley rats, and that long-term overexpression of leptin in the liver of transgenic skinny mice was accompanied by blood pressure elevation compared with nontransgenic littermates. Additionally, Agata et al demonstrated a significant positive correlation between mean blood pressure and leptin serum levels in lean subjects with essential hypertension. Thus, hyperleptinemia itself seems to play an important role in the development of hypertension independent of obesity.

There is increasing evidence that the endothelium may be a target for leptin action. Thus, endothelium-derived vasoactive substances like ET-1 may play a role in hyperleptinemia-related hypertension. ET-1 levels are elevated in hypertensive patients, and there is evidence for its role in the pathogenesis of hypertension as endothelin antagonists are able to lower blood pressure in animal models. Furthermore, Ferri et al were able to demonstrate that ET-1 levels of obese men were significantly higher compared with nonobese men. The authors concluded that obesity is responsible for the observed increase in circulating ET-1 levels. Therefore, hyperleptinemia and, as shown in the present study, subsequent induction of ET-1 may contribute to increased blood pressure found in obese subjects, although it has to be stated that this may be just one possible mechanism.

Leptin-induced ET-1 secretion of HUVECs may also contribute to the high cardiovascular risk observed in obese subjects. Various studies indicate that ET-1 is able to promote several steps in the development of atherosclerosis, eg, fibroblast and smooth muscle cell proliferation. Indeed, increased plasma ET-1 levels have been reported in patients with atherosclerotic lesions. Furthermore, the high abundance of ET-1 immunoreactivity in atherosclerotic tissue as demonstrated by several groups is consistent with the long-lasting increase in leptin-mediated AP-1 binding and subsequent increased rate of ET-1 transcription as shown in our study.

Our findings that HUVECs express high- and low-affinity binding sites for leptin with a Kd=1.5±1.4 nmol/L and Kd=1.9±1.8 nmol/L, respectively, are in accordance with data published by Golden et al. Additionally, using RT-PCR, we were able to demonstrate mRNA expression of the leptin receptor with the long cytoplasmic tail in HUVECs as published previously by 2 other groups. There are no data available about the fate of the leptin receptor expressed on HUVECs after engagement, but it seems to be permissible to assume an internalization of the receptor as shown with...
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References


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