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 nmol/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,6,11 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 transferase 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 KpnI and BglII. The plasmids pET[490]Luc and pET[297]Luc were generated through digestion of pET[3604]Luc with exonuclease III (Promega). The plasmid pl[TATA]Luc has been previously described as pHTF(111) and was generously provided by Dr N. Mackman 20 (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 trans-
Cells were labeled with rabbit anti-c-fos and rabbit anti-c-jun (10 µg/mL) (Santa Cruz Biotechnology Inc) for 1 hour, washed with PBS, and incubated with FITC-labeled goat anti-rabbit IgG (Jaxell-Accurate) (8 µg/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 µg each were size-fractionated by 1.2% formaldehyde agarose gel electrophoresis and transferred to Hybond N membranes (Amersham). A 480-bp SacI 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.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts from HUVECs were isolated as previously described. Oligonucleotides containing the ET-1–derived AP-1 sequence (5'-GCTTGGTGTACTAATAACAC-3'; Pharmacia, Freiburg, Germany) were end-labeled with polynucleotide kinase in the presence of [γ-32P]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
Application of Phosphorothioate Oligonucleotides

The sequence of the used human Ob-Rb antisense oligonucleotide is 5'-CAAGCCAGACCGCTCGTG-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' GCAGCCGGAAACAGCGGT-3'. Oligonucleotides were synthesized as phosphorothioate derivates 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 13.1±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-R 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 was measured. As shown in Figure 4B, 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).

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

EMSA using [\(^{32}\)P]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 \(\mu\)g 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.

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-
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 Kd13.3±3.1 nmol/L and Kd21390±198 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...
other cell types. The importance of leptin–Ob-Rb interaction for ET-1 induction became evident in experiments using antisense oligonucleotides against Ob-Rb because we were able to show that these oligonucleotides downregulated Ob-Rb mRNA, and consequently prevent leptin-mediated ET-1 induction.

Recently, Bouloumie et al. demonstrated that engagement of the leptin receptor on HUVECs activates both the MAP kinases Erk1/2 and the JNK/SAP kinases. JNK phosphorylates c-jun leading to its nuclear translocation, homo- or heterodimerization with members of the Jun or the Fos family, and the formation of the transcription factor AP-1. Sequences highly homologous to the octanucleotide consensus AP-1 binding site are present in the 5'-flanking region of the human ET-1 gene. Thus, leptin-induced JNK activation may lead via formation of AP-1 and its binding to the ET-1 promoter to the observed ET-1 production. In the present study, we demonstrate increased nuclear immunofluorescence staining of c-jun and c-fos, the major components of transcriptionally active AP-1, after incubation of HUVECs with leptin. Overexpression of a dominant-negative c-jun point mutant was able to bind other members of the AP-1 family like c-fos but unable to bind DNA, reduced leptin-induced ET-1 promoter activity in transfection studies. Furthermore, using several ET-1 promoter constructs, we showed that stimulation of BAECs with leptin resulted in increased ET-1 promoter activity as long as at least one potential AP-1 binding site was present. These data are in accordance with several other studies demonstrating that the transcription factor AP-1 is not only necessary for the basal transcriptional activity of ET-1, but also for the regulation of ET-1 transcription after stimulation of endothelial cells by cytokines like interleukin-II and thrombin. Furthermore, it is important to mention that the effect on ET-1 expression seen in our study is in the same range described with other stimuli such as interleukin-1, TNF-α, or thrombin, suggesting a pathophysiological significance of such an observed increase in ET-1 after leptin stimulation.

Although the leptin concentrations are in the upper range found in obese subjects, the present study provides further evidence for a direct link between adipocytes and endothelium, which may be of importance in the development of obesity-related diseases like hypertension and atherosclerosis. These symptoms are at least partly caused by endothelial dysfunction, which may also include increased production of the vasoconstrictor and mitogen ET-1.

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References


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