Leptin increases endothelin type A receptor levels in vascular smooth muscle cells

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1Institute of Physiology, 2Department of Surgery, and 3Faculty of Medicine, School of Medicine, National Yang-Ming University; 4Division of Endocrinology and Metabolism, Department of Medicine, and 5Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan

Submitted 15 February 2007; accepted in final form 22 November 2007

Juan C-C, Chuang T-Y, Lien C-C, Lin Y-J, Huang S-W, Kwok CF, Ho L-T. Leptin increases endothelin type A receptor levels in vascular smooth muscle cells. Am J Physiol Endocrinol Metab 294: E481–E487, 2008. First published December 4, 2007; doi:10.1152/ajpendo.00103.2007.—Leptin, one of the adipocyte-secreted peptides, is involved in the control of appetite and body weight. Several studies have demonstrated that plasma leptin levels are elevated in obese subjects and are positively correlated with body weight. The arterial endothelin (ET) system plays an important role in the regulation of vascular tone, and ET-1 overexpression may be involved in the pathogenesis of the hypertension associated with insulin resistance. This study was performed to explore the regulatory effects of leptin on ET receptor expression and ET binding in A10 vascular smooth muscle cells (VSMCs) by use of Northern blotting, immunoblotting, and a 125I-labeled ET-1 binding assay. The effect of leptin on ET receptor-mediated cell proliferation was also tested. The results showed that leptin caused a significant increase in [125I]-ET-1 binding, which was time- and dose-dependent. Immunoblotting showed that expression of the ET type A receptor (ETaR) in leptin (10^{-7}\, M)-treated cells was increased by up to 2.3-fold compared with controls. Levels of ETaR mRNA measured by Northern blotting were also increased by up to 2.2-fold in leptin (10^{-7}\, M)-treated cells. Pretreatment with an ERK inhibitor, PD-98059 (2.5 \times 10^{-5}\, M), blocked the leptin-induced increase in 125I-ET-1 binding. Finally, ET-1 (10^{-7}\, M)-stimulated cell proliferation was enhanced by leptin (10^{-7}\, M) pretreatment, with a maximal increase of twofold compared with controls. In conclusion, leptin increases ETaR expression in VSMCs in a time- and dose-dependent manner. This effect is ERK dependent and is associated with increased ET-1-stimulated cell proliferation. These findings provide support for roles for leptin and the ET system in the pathogenesis of obesity-associated hypertension.

Although it is produced by other tissues, such as stomach, heart, and placenta (19, 23, 24), leptin, the product of the ob gene, is synthesized and secreted mainly by adipose tissue (37). In ob/ob mice, a mutation in the ob gene, which results in a lack of circulating leptin, causes obesity, and the administration of recombinant leptin causes weight loss in these mice (10, 22). Animal studies have shown that administration of leptin reduces food intake (9). The plasma concentration of leptin increases rapidly with overeating (18) and is positively correlated with total body fat mass (6). In addition, a chronic increase in circulating leptin causes a sustained increase in arterial pressure (28), transgenic skinny mice overexpressing leptin have a high blood pressure (2), and a genetic epidemiological study has shown that leptin gene polymorphism is closely associated with a higher incidence of hypertension independent of obesity (29). These studies suggest a direct effect of leptin on blood pressure.

The signal transduction pathways regulated by leptin are diverse and are similar to the cytokine and growth factor receptor-mediated signaling pathways. They include the JAK/STAT pathway, SH2 domain-containing tyrosine phosphatase pathway, mitogen-activated protein kinase (MAPK) pathway, suppressors of cytokine signaling pathway, phosphatidylinositol 3-kinase (PI3K) pathway, protein kinase B (PKB) pathway, protein kinase C (PKC) pathway, cyclic AMP pathway, and nitric oxide pathway (see review, Ref. 28). A recent study has demonstrated that in CHO cells the leptin-leptin receptor interaction induces the synthesis of c-fos and c-jun, the major components of the transcription factor AP-1 (20).

Endothelin-1 (ET-1) is a powerful vasoconstrictive peptide produced by endothelial cells and smooth muscle cells (36). Its vascular actions are mediated by two specific receptors, ETaR and ETbR, located, respectively, primarily on vascular smooth muscle cells (VSMCs) and endothelial cells (ECs). ETaR activation causes long-lasting vasoconstriction by increasing intracellular calcium levels (3), whereas ETbR activation causes vasorelaxation by releasing nitric oxide and prostacyclin (27). In addition, ET-1 may potentiate the pressor effects of other vasoconstrictive substances, such as angiotensin II (38) and norepinephrine and serotonin (37). ET-1 also promotes proliferation of VSMCs and fibroblasts, which may cause vascular wall thickening (5, 15). All of these actions may contribute to the development of hypertension and other cardiovascular diseases.

Whether ET-1 plays a pathogenic role in obesity is not clear. However, increased vascular ET-1 mRNA and protein levels are seen in experimental obesity (33). Increased plasma ET-1 levels have also been found in obese normotensive and hypertensive subjects (32). In genetic epidemiological studies, the Lys198Asn polymorphism of the preproET-1 gene was found to be linked to obesity-associated hypertension (4, 32). Thus, variations in the gene encoding the preproET-1 peptide may affect susceptibility to hypertension in obese individuals.

The abovementioned studies indicate that there is an association between obesity and hypertension; however, the under-
lying pathogenic mechanism has not been clarified. The aims of this study were to explore the regulatory effect of leptin on ET receptor expression in VSMCs and its cellular molecular mechanism. We first investigated leptin receptor expression in VSMCs by use of reverse transcriptase-polymerase chain reaction (RT-PCR). We then explored the effects of leptin on ET receptor expression and the underlying mechanisms by means of an 125I-labeled ET-1 binding assay, immunoblotting, and Northern blotting. Finally, we tested the effects of leptin on ET-1-induced cell proliferation in VSMCs.

MATERIALS AND METHODS

Cell culture. The A10 cell line (ATCC, Rockville, MD), a VSMC cell line isolated from rat embryonic aorta, was used in this study. The VSMCs were seeded on 12-well plates or 10-cm Petri dishes (Falcon; Becton-Dickinson) and grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U/ml penicillin and 100 µg/ml streptomycin (all from GIBCO-BRL, Gaithersburg, MD), and 10% fetal bovine serum (BioWest, Nuaillé, France) (complete medium) in humidified air with 5% CO2. The cells were grown to 90% confluence, and the medium was changed every 4 days. Before each experiment, the cells were incubated for 6 h in the absence of serum in DMEM containing 0.1% bovine serum albumin. This study protocol was used in all experiments.

125I-ET-1 total binding. VSMCs in 12-well plates were cultured in the absence or presence of 10-7 M leptin (R&D Systems, Minneapolis, MN) for the indicated time and then were incubated for 1 h at 37°C in binding buffer (Hanks’ buffered saline containing 25 mM HEPES, pH 7.4, 0.2% BSA, and 0.1% glucose) containing 20 pM 125I-ET-1 (Amersham, Aylesbury, UK) plus various concentrations (10-12 to 10-6 M) of unlabeled ET-1 (Peptide Institute, Osaka, Japan). The cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and solubilized by incubation for 30 min at room temperature in 1% Triton X-100, and then the bound 125I-ET-1 was measured on a γ-counter. Nonspecific binding was determined in the presence of 1 µM unlabeled ET-1 and was subtracted from the total binding to give the specific binding. A Scatchard plot was used to determine the number of ET-1 binding sites and the dissociation constant.

Immunoblotting. Whole cell lysates were prepared by sonication of the cells in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 125 µM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate); then samples (100 µg of total protein) in 50 µl of reducing sample buffer were boiled for 3 min and resolved on 10% SDS gels for 90 min at 100 V, and the proteins were transferred to polyvinylidene difluoride membranes at 21 V for 120 min. The membranes were then blocked by incubation at room temperature for 30 min with 5% skimmed milk in PBS and incubated for 24 h at 4°C with primary antibody (Chemicon International, Temecula, CA) followed by incubation for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody (Chemicon International), and then bound antibody was detected using chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK). To detect multiple signals using a single membrane, the membrane was incubated for 20 min at 37°C with stripping buffer (50 mM Tris-HCl, 2% SDS, 0.75% 2-mercaptoethanol) prior to reblotting with a different antibody (13). β-Actin was used as the internal control.

Total RNA extraction. Total RNA was extracted from VSMCs using a Tri Reagent kit (Molecular Research Center, Cincinnati, OH). The integrity of the extracted total RNA was examined by 1% agarose gel electrophoresis and the RNA concentration determined by UV light absorption at 260 nm. RNA samples for RT-PCR were incubated for 30 min at 37°C with RNase-free DNase I and then for 10 min at 100°C to inactivate the DNase I.

RT-PCR analysis. One microgram of total RNA was reverse transcribed at 42°C for 1 h using poly(T)12–18 primers and SUPER RT reverse-transcriptase (HT Biotechnology, Cambridge, UK) in a 50-µl reaction volume. Five microliters of the reaction mix in a total volume of 50 µl was then used in the PCR under the following conditions: 1 cycle of 95°C × 5 min, 35 cycles of 95°C × 30 s, 55°C × 1 min, 72°C × 1 min, and a final 7-min extension period. The primers used were OB-Rb (leptin long-form receptor) sense primer 5'-AGTGG AAACCT GATGA AGAGC A-3', OB-Rb antisense primer 5'-TTACA CAGTT AAGTC ACAAC TC-3', ETAR sense primer 5'-CAGAT CCACA TTAAG GTGGG-3', ETAR antisense primer 5'-CAATG ACCAC GTGAG AAAGC-3', β-actin sense primer 5'-TGGAG AAGAG TCATG AGCTG CCT-3', and β-actin antisense primer, 5'-GTGCC ACCAC AGACG ACTGT GT-3'. These amplicy fragments of different sizes: 508 bp for OB-Rb cDNA, 412 bp for ETAR cDNA, 323 bp for ETAR cdNA, and 202 bp for β-actin cDNA. Ten micrograms of 18S ribosomal DNA poly(A)+ RNA from the same RT template solution were combined and electrophoresed on a 2% agarose gel. After ethidium bromide staining, the relative levels of OB-Rb and β-actin mRNA in the original total RNA extract were determined.

dDNA probe preparation and Northern blot analysis. The ETAR cDNA probes were prepared from the ETAR PCR products. The PCR products were cloned into the Smal site of the pGEM-3zf(+) vector (Promega, Madison, WI) and their identities confirmed by sequencing. Northern blotting was carried out according to a previously described procedure (16). For hybridization, the inserted ETAR cDNA probes were cut out and radiolaabeled using a random primer labeling system (12) and reagents from Promega. After hybridization, the membranes were washed and autoradiographed using an intensifying screen at −80°C. GAPDH mRNA was used as the internal control. Hybridization signals, as arbitrary densitometric units, were expressed as the ETAR mRNA/GAPDH mRNA ratio for comparison.

Cell proliferation assayed by cell counting and the MITT method. In direct cell counting, cell numbers were quantified using a hemocytometer after trypsinization. In the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells in secondary cultures maintained in DMEM containing 10% FBS were trypsinized and seeded on 96-well plates at ~1 × 104 cells/well in 100 µl of DMEM and 0.5% FBS. After incubation for 24 h at 37°C with leptin and/or ET-1, 15 µl of MTT was added (final concentration 0.5 mg/ml) for 4 h, then 100 µl of DMSO was added to dissolve the formazan crystals formed, and the optical density was measured on an ELISA plate reader using test and reference wavelengths of 570 and 630 nm, respectively. MTT reduction is proportional to the number of viable cells.

Data analysis. Experiments were repeated at least four times and the results expressed as means ± SD of the number of observations. Statistical significance was assessed by one-way analysis of variance or Student’s t-test. A P value of <0.05 was considered statistically significant.

RESULTS

Leptin receptor expression in VSMCs. The RT-PCR results showed that the OB-Rb, the functional form of the OB-R, was expressed in VSMCs and normal rat aorta, as well as in positive control adipocytes (Fig. 1).

Effects of leptin on ET-1 binding to VSMCs. To examine whether leptin had an effect on ET-1 binding to VSMCs, the cells were incubated with 10-7 M leptin for 0–48 h, and then specific ET-1 binding was measured as described in MATERIALS AND METHODS. As shown in Fig. 2A, a significant increase in 125I-ET-1 binding were seen after 12 h of leptin incubation,
maximal stimulation being seen at 24–36 h. These data show that leptin treatment caused a time-dependent increase in ET-1 binding to VSMCs.

VSMCs were then incubated for 24 h with various concentrations of leptin (0–10^{-7} M). Figure 2B shows that incubation with 10^{-10} M leptin resulted in a significant increase in 125I-ET-1 binding compared with the control. This response was dose dependent, with an ED_{50} (half-maximally effective dose) of 1.76 nM. The maximal level of 125I-ET-1 binding, seen in the presence of 10^{-7} M leptin, was ~1.8-fold higher than in the control.

To further explore the effect of leptin on ET receptor binding characteristics, VSMCs were incubated with and without 10^{-7} M leptin for 24 h, and then the 125I-ET-1 binding assay was performed. As shown in Fig. 3, ET-1 binding was significantly increased in the leptin-treated cells compared with the controls. Scatchard analysis (Fig. 3, inset) showed that there was no difference in the dissociation constant for ET-1 binding between the two sets of cells, but the ET-1 binding capacity was significantly increased in the leptin-treated cells compared with the controls (372 ± 21 vs. 177 ± 15 fmol/1.25 × 10^5 cells, P < 0.05).

Effects of leptin on ETAR mRNA and protein levels in VSMCs. The major ET receptor subtype in vascular muscle is the ET_{A}R (3). To confirm this, we examined using RT-PCR whether the ET_{B}R was expressed in VSMCs and found that ET_{B}R expression was undetectable in control VSMCs or in VSMCs treated with 10^{-7} M leptin (Fig. 4). We then investigated the effect of leptin on ET_{A}R expression in VSMCs by use of Western and Northern blots. Figure 5A shows Western blots of extracts of cells incubated for 24 h with 10^{-9} to 10^{-7} M leptin, in which the size of the band in both the control and leptin-treated cells was consistent with the reported molecular weight of the ET_{A}R (11). Densitometry showed a dose-dependent increase in ET_{A}R levels, the levels in 10^{-7} M leptin being 2.3-fold higher than in the control. Northern blotting showed a dose-dependent increase in ETAR mRNA levels in VSMCs incubated with leptin for 24 h, levels in 10^{-7} M leptin being 2.2-fold higher than in controls (Fig. 5B).

Effect of inhibitors of PKC, MAPK, adenylyl cyclase, or PI 3-kinase on leptin-stimulated ET-1 binding to VSMCs. To gain insight into the signaling pathway involved, VSMCs were
preincubated for 1 h in the presence or absence of various inhibitors acting on different signaling pathways, and then they were incubated for a further 24 h with $10^{-7}$ M leptin during the continued presence or absence of these inhibitors before the ET-1 binding assay was performed; the agents used were the PKC inhibitor H7 ($6 \times 10^{-6}$ M); the MAPK inhibitors PD-098059 (PD, an ERK pathway inhibitor, $2.5 \times 10^{-5}$ M) and SB-203580 (SB, a p38 pathway inhibitor, $2 \times 10^{-5}$ M); the adenyl cyclase inhibitor SQ-22536 (SQ, $10^{-6}$ M), and the PI 3-kinase inhibitor wortmannin (W, $10^{-7}$ M). As shown in Fig. 6, pretreatment with the ERK inhibitor PD-098059 completely blocked leptin-stimulated ET-1 binding ($P < 0.05$), whereas the other inhibitors had no effect, showing that ERK activation was necessary for leptin-stimulated ET-1 binding to VSMCs.

**Effects of leptin on MAPK phosphorylation.** As shown in Fig. 7A, exposure of VSMCs to $10^{-7}$ M leptin led to phosphorylation of ERK1 and ERK2 within 5 min, maximal phosphorylation of both being seen in 10 min and the signal gradually decaying and being lost by 30 min. Pretreatment of VSMCs with the ERK inhibitor PD-098059 ($2.5 \times 10^{-5}$ M) completely blocked leptin-induced ERK1 and ERK2 phosphorylation (Fig. 7B). Taken together with the data shown in Fig. 6, these findings show that leptin increases ET-1 binding in VSMCs via ERK activation.

**Effects of leptin on ET-1-stimulated cell proliferation.** To determine whether this increase in ETAR expression was associated with any biological effect, ET-1-stimulated cell proliferation was measured after 24 h of pretreatment with or without $10^{-7}$ M leptin. Cell counting (Fig. 8A) showed that $10^{-7}$ M ET-1 alone slightly increased cell numbers 1.3-fold compared with the controls ($P < 0.05$), whereas 24 h of pretreatment with $10^{-7}$ M leptin, followed by addition of $10^{-7}$ M ET-1, caused cell numbers to increase to twice those seen in controls ($P < 0.05$) and 1.5-fold compared with cells treated with ET-1 alone ($P < 0.05$). Both assays showed that leptin alone has little effect on cell proliferation.
We then used the ETAR antagonist BQ-610 and the ETBR antagonist BQ-788 to confirm that the ETAR was involved in the effect of leptin on ET-1-stimulated proliferation in VSMCs. As shown in Fig. 9, BQ-610 completely prevented the effect of leptin on proliferation, but BQ-788 had no effect, demonstrating that the effect of leptin on ET-1-stimulated cell proliferation was mediated through upregulation of the ETAR. Furthermore, pretreatment of VSMCs with the ERK inhibitor PD-98059 completely blocked the effect of leptin on ET-1-stimulated proliferation in VSMCs (Fig. 9), showing that ERK activation was
necessary for leptin-enhanced ET-1-stimulated cell proliferation in VSMCs.

DISCUSSION

Clinical observations have shown that obesity is highly related to hypertension (26), but the underlying pathogenic mechanism is not completely understood. Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ. Adipocytes not only function as energy storage pools but also secrete several factors, such as leptin (17). Mean blood pressure and plasma leptin levels show a significant positive correlation in hypertensive subjects, suggesting that hyperleptinemia itself may play an important role in the development of hypertension (1). Intravenous infusion of leptin for 12 days increases arterial pressure in rats (28). It has therefore been hypothesized that leptin plays a pivotal mediator role in the development of obesity-associated hypertension.

In the present study, we found that the OB-Rb, a functional form of the OB-R with a cytoplasmic domain that transduces leptin signals (28), was expressed in VSMCs, but we did not examine the expression of other OB-Rs. However, Oda et al. (21) have shown that rat aortic VSMCs express a 130-kDa short form of OB-R, and Murakami et al. (20) demonstrated that a short form of the OB-R, OB-Ra, is capable of mediating signal transduction. The OB-R subtypes mediating leptin-upregulated ETAR expression in VSMCs should therefore be determined. In addition, we found that 24-h treatment with leptin did not stimulate proliferation of VSMCs (Fig. 8). However, Oda et al. found that treatment of VSMCs with leptin for 3 days resulted in an increase of ~20% in cell numbers (21). One possible explanation for the difference is the different incubation periods used.

The major finding of the present study was that leptin, acting via the OB-R, upregulated ETAR expression in VSMCs in a time- and dose-dependent manner and that this occurred by activation of the ERK pathway. In addition, ET-1-stimulated cell proliferation was enhanced by leptin, and this effect was prevented by pretreatment with an ETAR antagonist or an ERK inhibitor. Our report provides further support for the above-mentioned hypothesis and suggests that at least part of the effect of leptin on blood pressure regulation may be associated with the increase in ETAR levels. Furthermore, the endothelium may be another target for leptin. Leptin has been shown to induce ET-1 production in endothelial cells (25). This result and our own findings suggest that leptin could activate the vascular ET system, causing vascular contraction and vascular wall thickening and subsequent hypertension. These mechanisms may contribute to the elevated blood pressure seen in obese subjects or subject with hyperleptinemia.

A recent study demonstrated that basal levels of ET-1 production are essential for maintaining normal cardiac function and cardiomyocyte survival in response to age and hemodynamic stress (41). However, high levels of ET-1 have been shown to directly stimulate cardiomyocyte hypertrophy (30) and contractility (14). In addition to regulating the functions of endothelial cells and smooth muscle cells, leptin regulates the function of cardiomyocytes by stimulating ET-1 secretion. For example, it suppresses cardiac contractile function in cardiomyocytes through the ET-1 receptor and NADPH oxidase-mediated pathway (7). In cultured neonatal rat cardiomyocytes, it stimulates the production of ET-1 and reactive oxygen species and subsequently induces hypertrophy of cardiomyocytes, the latter effect also being ETAR mediated (35); however, its effect on ETAR expression was not explored in this study. In cultured rat portal veins, stretching induces secretion of leptin, which causes vascular hypertrophy, but not hyperplasia, in the preparation, and this effect is blocked by an ETAR antagonist or an ERK inhibitor (39). However, the use of a whole tissue preparation may limit the interpretation of some of the data in the aforementioned study. For example, the denuded portal vein is composed of several types of cells, and it is therefore not easy to exclude an effect of non-VSMCs on leptin action in a whole tissue assay system. In our study, we demonstrated that leptin caused an increase in ETAR mRNA and protein levels in a pure VSMC culture system and that it enhanced ET-1-stimulated VSMC proliferation, but not cell hypertrophy. Even though there are some differences in experimental designs, all these studies suggest that activation of the ET system, including ET-1 and ETAR overexpression, plays a crucial role in leptin regulation of cardiovascular function, especially in the obese.

ET-1 stimulates leptin production and secretion in murine adipocytes (34) and circulating ET-1 levels are significantly higher in obese patients than in controls (8). In obese individuals, the cardiovascular system is highly infiltrated with adipose tissue. In such a microenvironment, elevated ET-1 levels enhance leptin production and the elevated levels of cellular or plasma leptin stimulate vascular ET-1 and ETAR expression, creating a vicious circle between these two hormones, with vascular hypertrophy and hypertension being the final result. Our findings provide further support for roles for leptin and ET-1 in the cross talk between the cardiovascular and metabolic systems. Our data also suggest that the metabolic system may affect cardiovascular function and, consequently, blood pressure homeostasis via adipokines, such as leptin.

GRANTS

These studies were supported by research grants from the Tsou’s Foundation. (CSTVGH92-12) and from the Ministry of Education, Aim for the Top University Plan.

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