0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(9):3555–3560

doi: 10.1210/en.2002-220075

Leptin Inhibits Angiotensin II-Induced Intracellular Calcium Increase and Vasoconstriction in the Rat Aorta

ANA FORTUÑO, AMAIA RODRíGUEZ, JAVIER GÓMEZ-AMBROSI, PAULA MUÑIZ, JAVIER SALVADOR, JAVIER DíEZ, AND GEMA FRÜHBECK

Division of Cardiovascular Pathophysiology (A.F., P.M., J.D.) and Metabolic Research Laboratory (A.R., J.G.-A., G.F.), University of Navarra School of Medicine, and Departments of Endocrinology (J.S., G.F.) and Cardiology and Cardiovascular Surgery (J.D.), Clínica Universitaria de Navarra, 31008 Pamplona, Spain

Besides its role in body weight control leptin may also act as a vasoactive hormone. This study was designed to investigate whether leptin modifies angiotensin II (ANG II)-induced vascular responses. The expression of functional leptin receptors (OB-Rb) was detected in vascular smooth muscle cells (VSMCs) from adult Wistar rats by RT-PCR. Immunocytochemistry and Western blot analysis further showed the expression of OB-R protein in VSMCs. The ANG II (10^{-7} mol/liter)-induced increase in intracellular Ca^{2+} was blocked ($P < 0.01$) by leptin (10^{-8} mol/liter). Moreover, in calcium-free buffer leptin was able to inhibit 65% of the ANG II-induced calcium release from intracellular stores. In endothelium-denuded aortic rings from adult Wistar rats no effect of leptin on basal tension was observed. However, the ANG II-induced isometric contraction was reduced ($P < 0.05$) by leptin (10^{-8} mol/liter). The experiments were also performed in age- and sex-matched Zucker rats, in which no effect of leptin on ANG II-induced calcium increase and vasoconstriction was observed. It is concluded that leptin blocks the vasoconstrictor action of ANG II and inhibits the ANG II-induced increase in intracellular Ca^{2+} in VSMCs through OB-Rb. These findings provide new insight into the physiological effects of leptin on blood pressure regulation. (*Endocrinology* 143: 3555–3560, 2002)

OBESITY IS an important risk factor for the development of vascular disorders such as hypertension (1, 2). The *ob* gene product, leptin (3), is a peptidic hormone produced mainly by adipose tissue that acts as a signaling mechanism to regulate body fat content (4) through binding to leptin receptors located in hypothalamic nuclei (5). In recent years the potential participation of leptin on blood pressure homeostasis has been investigated. Chronic high dose administration of leptin has been reported to increase arterial pressure and heart rate in conscious rats by peripherally or centrally mediated mechanisms (6, 7). The presence of functional leptin receptors (OB-Rb) in endothelial cells provides a new target for leptin action, namely the vascular wall. Leptin induces direct vasodilation through distinct endothelial mechanisms (8) that oppose the sympathetic-mediated pressor response (9). Besides, our group had previously shown that leptin induces the release of nitric oxide (NO) at the same time as it triggers sympathoexcitation (10). Thus, leptin seems to exert dual effects on the regulation of vascular tone.

The vasoactive peptide angiotensin II (ANG II) is an important factor regulating blood pressure (11). Alterations in the synthesis of and responsiveness to ANG II have been implicated in the pathophysiology of arterial hypertension (12). In addition, Barton *et al.* (13) found that obesity is associated with activation of the renin-angiotensin system in nonadipose tissue. Besides, an adipose tissue-specific increase in the production of angiotensinogen during the onset of obesity has recently been reported (14). According to previous studies, the link between angiotensinogen production and the control of adipose mass involves ANG II, which has been implicated in the differentiation of adipose precursors by paracrine/autocrine mechanisms (15). Therefore, the present study explored the possibility that leptin might interfere with the vascular responses induced by ANG II through OB-Rb. To test this hypothesis, the *in vitro* effects of leptin on both ANG II-induced increases in the intracellular free calcium concentration ($[Ca^{2+}]_i$) transients in vascular smooth muscle cells (VSMCs) and isometric contraction of endothelium-denuded and intact aortic rings from Wistar rats as well as leptin receptor-deficient obese Zucker rats were assessed. In addition, the expression of OB-Rb in VSMCs from Wistar rats was investigated.

Abbreviations: ANG II, Angiotensin II; $[Ca^{2+}]_i$, intracellular free calcium concentration; NA, noradrenaline; NO, nitric oxide; VSMC, vascular smooth muscle cell.

Materials and Methods

Animals

Ten-week-old male Wistar rats (breading house of University of Navarra) and obese Zucker *fa/fa* rats (Harlan, Barcelona, Spain) were used in the study. Rats were maintained under controlled conditions of room temperature (20 ± 2 C), relative humidity (50 ± 10%), ventilation (at least 15 complete changes of air/h), and artificial light-dark cycle (lights on from 0800–2000 h). Animals had free access to tap water and were fed *ad libitum* an isoenergetic (13.39 MJ/kg), isoproteic (14%) rodent maintenance diet containing 0.13% sodium (2014S Teklad Global 14% Protein Rodent Maintenance Diet, Harlan, Barcelona, Spain). The investigation was performed according to the European Community Guidelines for animal ethical care and use of laboratory animals (Directive 86/609) and was approved by the local ethical committee (011/99; 001/01). Rats were killed by decapitation. The thoracic aorta was carefully excised, dissected out, and processed for each study.

Cell isolation and culture

Primary VSMCs were obtained from the thoracic aorta of Wistar and Zucker rats and cultured by the tissue explants method in accordance

with previously published procedures (16). The culture was continued up to eight passages using 5% fetal bovine serum with an antibiotic-antimicotic product (Life Technologies, Inc., Gaithersburg, MD; 10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B as Fungizone in 0.85% saline). Immunocytochemical characterization of isolated VSMCs was performed on primary cultures, using specific antibodies against smooth muscle-specific α-actin (DAKO Corp., High Wycombe, UK). VSMCs were differently processed depending on the study protocol.

Detection of leptin receptors in VSMCs

Total RNA was extracted in TRIzol reagent (Life Technologies, Inc.) from a 75-mm^2 flask of VSMCs, according to the manufacturer's instructions. The yield and quality of the RNA were assessed by measuring absorbance at 260 and 280 nm and by electrophoresis on 1.5% agarose gels. One microgram of RNA was used to synthesize first strand cDNA after treatment for 60 min at 37 C with 10 U ribonuclease-free deoxyribonuclease I (Roche Molecular Biochemicals, Mannheim, Germany). The RT reaction was carried out in a volume of 20 μl containing 50 mmol/liter Tris-HCl (pH 8.3), 75 mmol/liter KCl, 3 mmol/liter $MgCl_2$, 10 mmol/liter dithiothreitol, 100 ng random hexamers (Roche Molecular Biochemicals), 1 mmol/liter of each deoxy-NTP (Bioline, London, UK), 20 U ribonuclease inhibitor (Promega Corp., Madison, WI), and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and incubated at 37 C for 60 min. The enzyme was inactivated by heating at 95 C for 5 min. Four microliters from the RT reaction were amplified in a 50-μl reaction mixture containing 40 ng of each primer, 16 mmol/liter $(NH_4)_2SO_4$, 67 mmol/liter Tris-HCl (pH 8.8), 2 mmol/liter $MgCl_2$, 0.1% Tween 20, 0.2 mmol/liter of each deoxy-NTP, and 1 U BIOTAQ polymerase (Bioline). Primers used to amplify rat OB-Ra and OB-Rb cDNA were a common forward primer (5′-ACACTGTTAATTTCACACCAGAG-3′) used in combination with a specific reverse primer either for the short (a) form (5′-AGTCATTCAAACCATAGTTTAGG-3′) or the long (b) form (5′-TTCCAAAAGCTCATCCAACCC-3′). As a control, mock amplifications were carried out in the presence of RNA template and *Taq* polymerase, but in the absence of reverse transcriptase. As a control for RNA quality and quantity, β-actin mRNA was amplified from all RNA samples using primers 5′-TCTACAATGAGCTGCGTGTG-3′ and 5′-GGTCAGGATCTTCATGAGGT-3′, based upon the sequence of rat β-actin. cDNA was amplified for 40 (OB-Ra), 45 (OB-Rb), and 35 cycles (β-actin) using the following parameters: 94 C for 30 sec, 55 C (OB-Ra and OB-Rb) and 59 C (β-actin) for 30 sec, and 72 C for 30 sec, with a final extension step at 72 C for 7 min. Amplifications were carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. Images were captured with the Gel Doc 1000 UV fluorescent gel documentation system and Molecular Analyst 1.4.1 software (Bio-Rad Laboratories, Inc., Hercules, CA).

VSMCs from the aortas of Wistar rats were homogenized in ice-cold lysis buffer containing sacarose (0.32 mol/liter) and protease inhibitor cocktail (Roche). The protein content of the crude preparations was measured by the Bradford method (17), using lyophilized BSA (Bio-Rad Laboratories, Inc.) as standard. Equal amounts of proteins (30 μg) were run out in 8–12% SDS-PAGE, subsequently transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) by electrotransfer, and blocked in 4% Tris-buffered saline-Tween 20 for 60 min at room temperature under agitation. The membranes were incubated overnight at 4 C under agitation with an N-terminal polyclonal antibody against OB-R (1:500) in 4% Tris-buffered saline-Tween 20. The antigen-antibody complexes were visualized using peroxidase-conjugated antigoat IgG antibody (1:10,000) and the enhanced chemiluminescence ECL detection system (Amersham Pharmacia Biotech).

The presence of OB-R immunoreactivity on intact VSMCs was studied by immunocytochemistry. Cells were grown on four-well plates, fixed with 4% paraformaldehyde, and washed with cold PBS. Fixed cells were treated with 5% dimethylsulfoxide and 0.25% Triton X-100 solution. After blocking with 4% fetal bovine serum for 1 h, cells were incubated overnight at 4 C with rat polyclonal antiserum specific for the N-terminal region of OB-R (1:100). After washing three times, cells were incubated with biotinylated goat antirat IgG antibody (1:100) in PBS for 1 h, washed, and reacted with avidin-fluorescein isothiocyanate conjugate (1:100; Sigma, St. Louis, MO) for 30 min. Finally, VSMCs were mounted in synthetic resin with 4′,6-diamidino-2-phenylindole. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Measurement of $[Ca^{2+}]_i$ in VSMCs

Measurement of $[Ca^{2+}]_i$ was performed as previously described (16). Briefly, nonconfluent cultures of VSMCs grown on round 22-mm coverslips were incubated in a humidified incubator for 45 min with fura-2/AM (4–7 μmol/liter) dissolved in dimethylsulfoxide with 0.02% pluronic acid. Fura-2-loaded VSMCs were placed in a thermostatically perfusion chamber on top of the stage of an inverted microscope equipped for epifluorescence (Diaphot, Nikon, Badhoevedorp, The Netherlands) and attached to an image analysis system (Magiscal Applied Imaging, Newcastle upon Tyne, UK). Upon binding Ca^{2+}, fura-2 exhibits an absorption shift from 340 to 380 nm that can be monitored by measuring the emission at 510 nm. The $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* (18).

Preparation of isolated aortic rings

The thoracic aorta was removed immediately after sacrifice, cleaned, and placed into Krebs-Heinseleit solution as previously described (16). The endothelium of the aorta was removed by gently rubbing the intimal surface with the tip of a small steel stick. In some experiments the endothelium of the aorta was preserved to analyze the effect of leptin on intact aortic rings. Both intact and endothelium-denuded rings were incubated for 60 min to equilibrate at a resting tension of 2.5 g, with buffer changes every 15 min to reach equilibrium conditions. The lack of a functional endothelium was confirmed by demonstrating the complete absence of relaxation induced by acetylcholine (10^{-5} mol/liter) in 80 mM KCl-precontracted aortic rings. The rings were then washed and stretched if necessary until a stable baseline force was obtained. The contraction with KCl was also used to test the contractile response of the tissue.

Study of vasoactive responses

Cumulative concentration-response curves to 10^{-9}–10^{-6} mol/liter ANG II were obtained to determine the concentration needed for submaximal effects and the pD_2 value. In a second group of experiments, VSMCs or aortic rings were previously incubated with different concentrations of leptin (10^{-10}–10^{-7} mol/liter) before testing the ANG II-induced response. To study the specificity of the inhibitory effect of leptin on ANG II-mediated actions the response to another vasoactive substance was further tested. As leptin has been shown to induce a sympathoexcitation (1, 7, 19–21), and the sympathetic nerve activity represents one of the most important factors controlling ongoing vasoconstriction, the effect of leptin on the noradrenaline (NA; 10^{-7} mol/liter)-induced contractile response was examined. One sample per experiment was used to obtain control responses (100%) in the presence of the solvent. Repetitive use of experimental products on the same aortic ring preparations was avoided to stay clear of possible desensitization.

Statistical analysis

Values are given as the mean ± SEM. Statistical differences between mean values were determined using one-way ANOVA, followed by Dunnett's *t* test. When the number of determinations were less than 10, statistical analysis was performed using the nonparametric Kruskal-Wallis test. $P < 0.05$ was considered statistically significant. Concentration-response curves were fitted by nonlinear regression, the concentration giving 50% of the maximal response (EC_{50}) was determined, and the pD_2 was calculated as $-\log EC_{50}$ (mol/liter).

Results

Expression of leptin receptors in VSMCs

RT-PCR was used to detect mRNAs of the short (OB-Ra) and full-length (OB-Rb) isoforms in RNA isolated from confluent cultures of VSMCs from Wistar rats (Fig. 1A). The

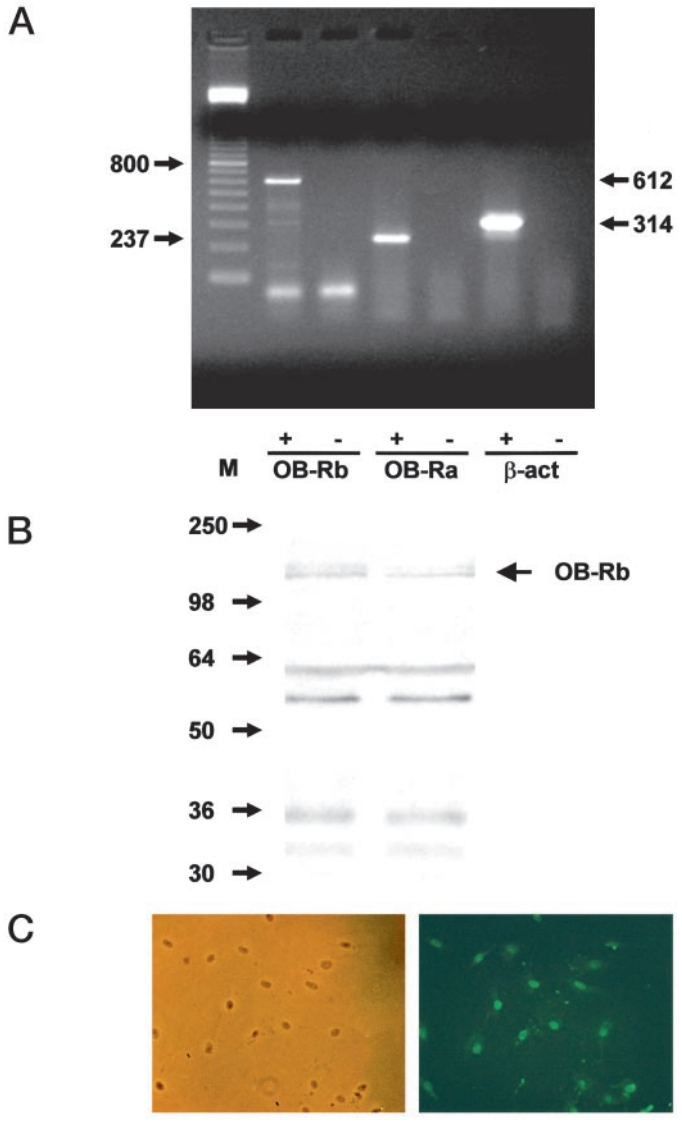

FIG. 1. A, mRNA expression of the long (OB-Rb) and short (OB-Ra) leptin receptor mRNA isoforms in VSMCs. DNA was amplified from first strand cDNA synthesized from total RNA in presence (+) or absence (−) of reverse transcriptase. The lengths of the RT-PCR products correspond to the sizes predicted from the nucleotide sequences encoding OB-Rb (612 bp), OB-Ra (237 bp), and β-actin (314 bp). B, Western blot analysis of leptin receptor (OB-R) in VSMCs of Wistar rats is shown using duplicate sample preparations and incubated with a polyclonal antibody against OB-R. A mayor band of 120–130 kDa was detected in all experiments performed (n = 12). Sizes corresponding to molecular weight markers are indicated. C, Immunocytochemical identification of OB-R in cultured VSMCs from rat aorta. In the *left panel*, VSMCs are visualized with an optic microscope. In the *right panel*, the same microscopic field is visualized with a fluorescence filter for fluorescein isothiocyanate. Most cells are immunopositive, with a whole cell pattern of staining. Representative pictures from six experiments are shown. Original magnification, ×200.

receptor splice variants OB-Ra and OB-Rb were expressed in VSMCs, as demonstrated by PCR products of 237 and 612 bp, respectively. The easy detection of OB-R expression confirms the vascular smooth muscle as a potential target for leptin action.

The presence of the OB-R protein in VSMCs from Wistar rats was evaluated by Western blot and immunocytochemical analysis. As shown in Fig. 1B, a 130-kDa band was found in all experiments performed (n = 12). Due to the N-terminal nature of the polyclonal antibody used against OB-R, four additional bands of lower molecular weights were also observed. No immunoreactivity was found without the primary antibody (data not shown). The immunocytochemical analysis of the cellular distribution of OB-R indicates that all VSMCs were immunopositive, exhibiting a whole-cell staining pattern (Fig. 1C).

Effects of leptin on ANG II-induced $[Ca^{2+}]_i$ responses in VSMCs

In the presence of 1.3 mmol/liter Ca^{2+} in the bathing medium, ANG II increased $[Ca^{2+}]_i$ in VSMCs from Wistar rats in a concentration-dependent manner ($pD_2 = 8.6 \pm 0.6$). A concentration of 10^{-7} mol/liter ANG II, inducing a response of 728.2 ± 51.1 nmol/liter (basal $[Ca^{2+}]_i$, 64.8 ± 9.3 nmol/liter), was chosen for subsequent experiments.

Although no basal effects of leptin on $[Ca^{2+}]_i$ in VSMCs were observed (data not shown), all leptin concentrations tested inhibited ($P < 0.01$) ANG II-induced $[Ca^{2+}]_i$ stimulation in VSMCs from Wistar rats (Fig. 2A). The inhibitory

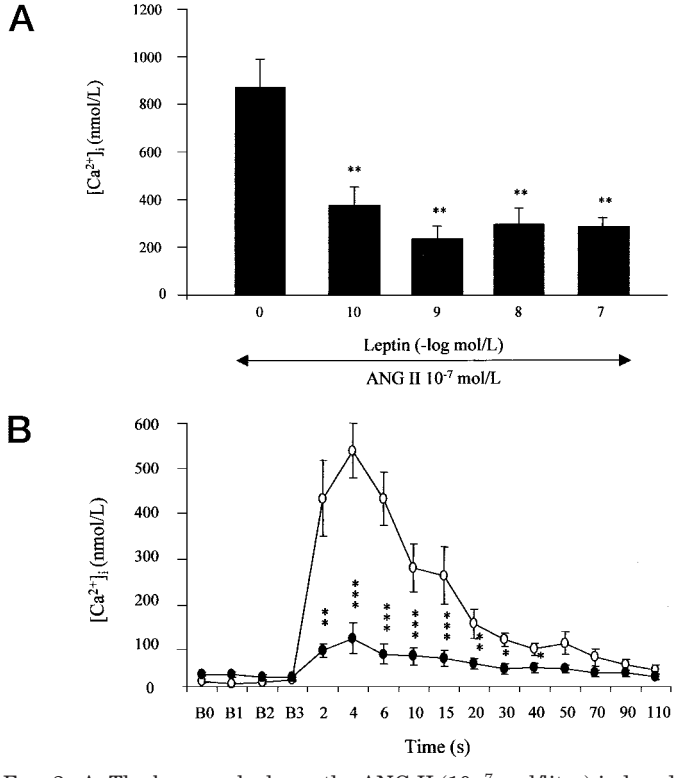

FIG. 2. A, The bar graph shows the ANG II (10^{-7} mol/liter)-induced changes in $[Ca^{2+}]_i$ in absence of or after incubation for 30 min of VSMCs from adult Wistar rats with different concentrations of leptin. B, Representative line graphs showing the differences in the time course of the ANG II-induced $[Ca^{2+}]_i$ increase in the absence (○) and presence (●) of 10^{-8} mol/liter leptin. Maximal stimulated $[Ca^{2+}]_i$ occurred at 1–3 sec, and the recovery time to basal concentrations was measured thereafter. Values are the mean ± SEM from 10–15 determinations/concentration. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* curve obtained in the absence of leptin).

effect was independent of cell passage. VSMCs exposed to ANG II showed a rapid rise in $[Ca^{2+}]_i$ that reached a maximum in 10 sec, declined rapidly within 30–40 sec, and then gradually decreased in the next 1–2 min to near-resting values (Fig. 2B). In the presence of leptin (10^{-8} mol/liter) the maximum value reached was inhibited by approximately 75% (Fig. 2B).

In Ca^{2+}-free medium the ANG II (10^{-7} mol/liter) response was 574.3 ± 96.6 nmol/liter, representing 71 ± 12% of the ANG II response in the presence of extracellular calcium. Leptin (10^{-8} mol/liter) inhibited ($P < 0.01$) by 65% the ANG II (10^{-7} mol/liter)-induced increase in $[Ca^{2+}]_i$ in VSMCs from Wistar rats (Fig. 3A). However, the same leptin concentration was not able to inhibit ANG II (10^{-7} mol/liter)-induced $[Ca^{2+}]_i$ stimulation in VSMCs obtained from Zucker rats (Fig. 3B).

Effects of leptin on the response induced by vasoactive agonists in aortic rings

ANG II elicited a concentration-dependent increase in the vascular tone of aortic rings of Wistar rats ($pD_2 = 8.3 \pm 0.6$). On the basis of this concentration-response curve, a concentration of 10^{-7} mol/liter was chosen to carry out the next experiments. No basal effect of leptin on endothelium-denuded aortic rings from Wistar rats was observed (data not shown). However, the ANG II (10^{-7} mol/liter)-induced vasoconstriction was inhibited ($P < 0.05$) by leptin at 10^{-8} mol/liter (Fig. 4A). No inhibitory effect of leptin was observed on ANG II-induced vasoconstriction in endothelium-denuded aortic rings from Zucker rats (Fig. 4B).

To further analyze the physiological significance of leptin on ANG II-induced vasoconstriction, the effects of leptin were examined in intact vessels. In intact aortic rings, leptin (10^{-8} mol/liter) produced a significant ($P = 0.029$) decrease in passive wall tension (−23.1 ± 2.4 *vs.* −12.5 ± 2.7 mg) that was not observed in endothelium-denuded rings. Contrary to what was observed in endothelium-denuded preparations, no effect of leptin on ANG II-induced vasoconstriction was evident in intact aortic rings (Fig. 4A).

Finally, to test the specificity of the inhibitory effect of leptin on ANG II, the response to a well known vasoactive substance such as NA was also studied. As expected in both intact and endothelium-denuded aortic rings, NA (10^{-7} mol/liter) produced a statistically significant ($P < 0.001$) vasoconstriction. As shown in Fig. 5, no significant effect of leptin (10^{-8} mol/liter) on the NA-induced contractile response was observed in either intact or endothelium-denuded aortic rings.

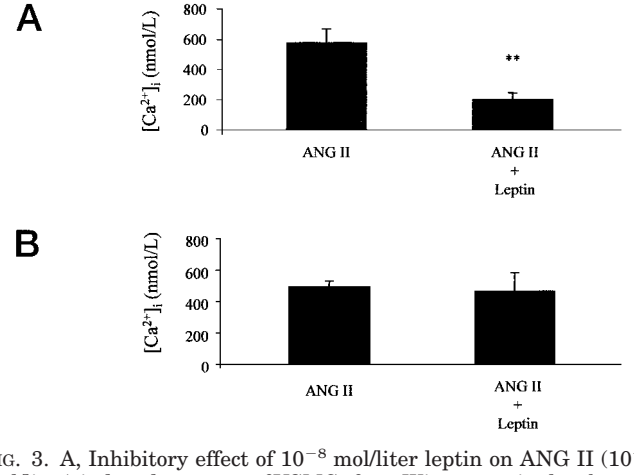

FIG. 3. A, Inhibitory effect of 10^{-8} mol/liter leptin on ANG II (10^{-7} mol/liter)-induced response of VSMCs from Wistar rats in the absence of calcium in extracellular medium. B, Effect of 10^{-8} mol/liter leptin on ANG II-induced response of VSMCs obtained from obese Zucker rats. Values are the mean ± SEM from seven to nine determinations. **, $P < 0.01$ *vs.* control response to ANG II.

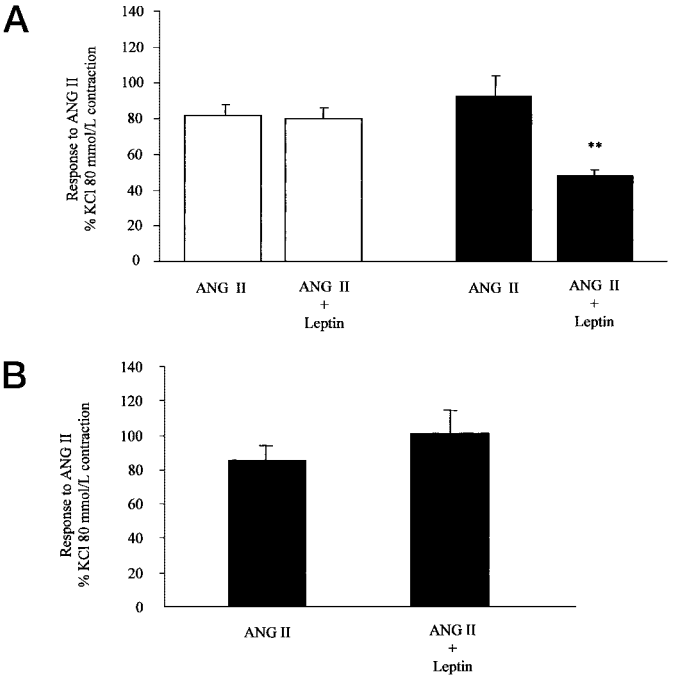

FIG. 4. A, Response to ANG II (10^{-7} mol/liter)-induced vasoconstriction of intact (□) and endothelium-denuded (■) aortic rings in the absence of or after preincubation for 30 min with leptin (10^{-8} mol/liter). B, Histograms showing the ANG II (10^{-7} mol/liter)-induced changes in tension of endothelium-denuded aortic rings from adult obese Zucker rats in the absence or presence of leptin (10^{-8} mol/liter). Values are the mean ± SEM from at least 10 determinations/concentration. **, $P < 0.01$ *vs.* control response.

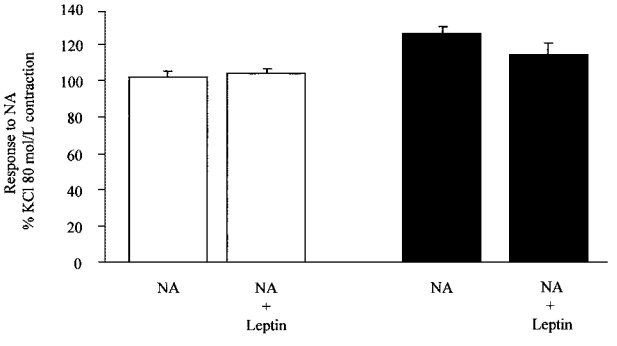

FIG. 5. Response to NA (10^{-7} mol/liter)-induced vasoconstriction of intact (□) and endothelium-denuded aortic rings (■) in the absence of or after preincubation for 30 min with leptin (10^{-8} mol/liter). Values are the mean ± SEM from at least 10 determinations/concentration.

Discussion

In addition to its ability to stimulate sympathetic mechanisms that result in vasoconstriction (1, 7, 19–21), several vascular actions of leptin implying vasodilation have been described. Frühbeck (10) demonstrated in rats with autonomic blockade that leptin infusion induced a systemic vasodilation that was associated with an increase in serum NO_3-NO_2 concentrations. Interestingly, leptin-induced vasodilation was reversed by N^{ω}-nitro-L-arginine methyl ester administration. On the other hand, leptin has been shown to induce a clear vasodilation in both aortic and mesenteric arteries, which is abolished by endothelial denudation, suggesting that endothelium integrity is critical for leptin's vascular relaxing action (8). Furthermore, functional leptin receptors have been identified in endothelial cells (22–24). Therefore, leptin seems to be able to stimulate endothelial NO-mediated vasodilation, although this fact has been questioned by Mitchell *et al.* (25) using the same physiological leptin concentrations as other research groups. Further support of the NO-mediated leptin actions has been provided by Vecchione *et al.* (26), showing that the effect of leptin on endothelial NO is mediated through the Akt-endothelial NOS phosphorylation pathway. The vasodilator effect has been shown to be dependent on the presence of the endothelium. Moreover, findings reported in the present study suggest that VSMCs may represent a target for the vasodilation actions of leptin by opposing ANG II-induced effects leading to vasoconstriction.

The present study confirms that functional leptin receptors are expressed in VSMCs of Wistar rats. The band detected with a molecular mass of approximately 120–130 kDa probably corresponds to OB-Rb, which has been previously described in other animal and human cell types (27–29). This finding supports recent evidence of the expression of functional leptin receptors in calcifying vascular cells (30) and aortic smooth muscle cells (31). Together these results support the hypothesis that leptin plays a relevant role in modulating vasoregulatory function directly through OB-Rb on the vascular wall. Due to the fact that the antibody used to detect OB-R expression in Western blot analysis is raised against the N-terminal region, common to all five isoforms of the receptor, four additional bands of lower molecular weights, probably representing the different receptor isoforms, were also identified in VSMCs (32). The possibility that OB-Rb is functional in VSMCs is supported by other findings reported herein: 1) physiological concentrations of leptin block the ANG II-induced rapid increase in $[Ca^{2+}]_i$ in cultured VSMCs of the aorta from Wistar rats; 2) leptin inhibits the ANG II-induced fast contraction in endothelium-denuded aortic rings from Wistar rats; and 3) no effects of leptin were found in the aorta and VSMCs obtained from Zucker rats with a mutation in the leptin receptor gene that results in the loss of its functional activity.

Central to the direct vasoconstrictor action of ANG II on smooth muscle is its capacity to increase $[Ca^{2+}]_i$. After the interaction with the AT_1 receptor, ANG II mobilizes Ca^{2+} by releasing Ca^{2+} sequestered intracellularly and by increasing Ca^{2+} influx from the extracellular compartment. The former event may occur via phosphoinositide turnover, and the latter in association with voltage-sensitive Ca^{2+} channels (11, 33). As a consequence, $[Ca^{2+}]_i$ increases and VSMC contraction ensue. Our finding that leptin retains the ability to inhibit ANG II-mediated stimulation of VSMC $[Ca^{2+}]_i$ in Ca^{2+}-free medium strongly suggests that the hormone blocks the ANG II-induced release of Ca^{2+} from the intracellular stores. Takekoshi *et al.* (34, 35) reported recently that leptin induces increases in cAMP levels in cultured porcine adrenal medullary chromaffin cells. As cAMP facilitates Ca^{2+} sequestration by the sarcoplasmic reticulum (36), we speculate that cAMP may mediate the ability of leptin to inhibit ANG II-induced stimulation of intracellular Ca^{2+} stores in VSMCs. Further studies are required to elucidate the ability of leptin to stimulate cAMP in these cells.

To improve our understanding of the physiological significance of leptin on ANG II-induced vasoconstriction, the effects were tested not only in denuded aortic rings, but also in intact vessels. Contrary to what was observed in endothelium-denuded preparations, in intact aortic rings no effect of leptin on ANG II-induced vasoconstriction was observed. Several plausible explanations may underlie this finding. Leptin may simultaneously induce the release of other vasoactive substances, with either a direct or indirect effect on the vascular tone of the ring. It is well known that the vasoconstrictor effect elicited by ANG II has a 2-fold component, a direct and an indirect effect, depending on the release of further endothelium-derived factors with a vasoconstrictor effect such as endothelin-1 (37). In addition, the incubation period used in our experiments was 30 min, and during this period the endothelium was exposed to the plentiful release of vasoactive factors such as NO and endothelin-1, among others. In fact, recombinant leptin has been shown to induce a slight decrease in passive wall tension (8). We have confirmed this finding in our study, showing that in intact aortic rings leptin produced a significant decrease in passive wall tension that was not observed in endothelium-denuded rings. The different molecular weights of ANG II and leptin (1 *vs.* 16 kDa, respectively) may also be responsible for the different transport possibilities and, consequently, the different abilities to reach the layer of VSMCs. Interestingly, it has been recently reported that leptin released by the fat depot produces a dose-dependent increase in plasma NO_3-NO_2 concentrations (38). In this sense, it may be speculated that the effects of leptin on the control of vascular tone may arise from two different sources. On the one hand, leptin present in the vascular lumen and, on the other hand, leptin released from perivascular adipocytes may participate in regulation of contraction of the vascular wall.

The lack of effect of leptin on vascular preparations from Zucker rats provides a potential mechanistic explanation for obesity-associated hypertension, *i.e.* the loss of antagonism of ANG II-mediated vasoconstriction. Two arguments add support to this possibility: 1) obesity-associated hypertension is characterized by both leptin resistance (19) and stimulation of the renin-angiotensin system (13); and 2) exaggerated vasoconstriction (39) and prolonged VSMC $[Ca^{2+}]_i$ exposure (40) to ANG II have been reported in Zucker rats.

In conclusion, our results provide additional evidence that OB-Rb are expressed in VSMCs from the aorta of Wistar rats and, therefore, represent a target for leptin action. Our find-

ings indicate that leptin inhibits ANG II-mediated vasoconstriction by blocking the release of Ca^{2+} from VSMC intracellular stores. The lack of effect of leptin on ANG II-mediated effects of vascular preparations obtained from leptin receptor-deficient Zucker rats suggests that OB-Rb are functional in the vascular wall of Wistar rats. Further studies are required to elucidate the pathophysiological relevance of this new vascular action of leptin in human obesity-associated arterial hypertension.

Acknowledgments

Received January 22, 2002. Accepted May 12, 2002.

Address all correspondence and requests for reprints to: Dr. G. Frühbeck, Department of Endocrinology, Clínica Universitaria de Navarra, 31008 Pamplona, Spain. E-mail: gfruhbeck@unav.es.

This work was supported by grants from the Spanish Instituto Carlos III-Fondo de Investigación Sanitaria (FIS 00/1005).

References

1. **Mark AL, Correia M, Morgan DA, Shaffer RA, Haynes WG** 1999 State-of-the-art-lecture: obesity-induced hypertension: new concepts from the emerging biology of obesity. Hypertension 33:537–541
2. **Hall JE, Brands MW, Dixon WN, Smith Jr MJ** 1993 Obesity-induced hypertension: renal function and systemic hemodynamics. Hypertension 22:292–299
3. **Ahima RS, Flier JS** 2000 Leptin. Annu Rev Physiol 62:413–437
4. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse *obese* gene and its human homologue. Nature 372:425–432
5. **Campfield LA, Smith FJ, Brun P** 1996 The OB protein (leptin): a link between adipose tissue mass and central neural networks. Horm Metab Res 28:619–632
6. **Shek EW, Brands MW, Hall JE** 1998 Chronic leptin infusion increases arterial pressure. Hypertension 31:409–414
7. **Correia ML, Morgan DA, Sivitz WI, Mark AL, Haynes WG** 2001 Leptin acts in the central nervous system to produce dose-dependent changes in arterial pressure. Hypertension 37:936–942
8. **Lembo G, Vecchione C, Fratta L, Marino G, Trimarco V, d'Amati G, Trimarco B** 2000 Leptin induces direct vasodilation through distinct endothelial mechanisms. Diabetes 49:293–297
9. **Hall JE, Brands MW, Hildebrandt DA, Kuo J, Fitzgerald S** 2000 Role of sympathetic nervous system and neuropeptides in obesity hypertension. Braz J Med Biol Res 33:605–611
10. **Frühbeck G** 1999 Pivotal role of nitric oxide in the control of blood pressure after leptin administration. Diabetes 48:903–908
11. **Peach MJ** 1997 Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev 57:313–370
12. **Dzau VJ** 1994 Cell biology and genetics of angiotensin in cardiovascular disease. J Hypertens 12:S3–S10
13. **Barton M, Carmona R, Morawietz H, d'Uscio LV, Goettsch W, Hillen H, Haudenschild CC, Krieger JE, Münter K, Lattmann T, Lüscher TF, Shaw S** 2000 Obesity is associated with tissue-specific activation of renal angiotensin-converting enzyme in vivo: evidence for a regulatory role of endothelin. Hypertension 35:329–336
14. **Hainault I, Nebout G, Turban S, Ardouin B, Ferré P, Quignard-Boulangé A** 2002 Adipose tissue-specific increase in angiotensinogen expression and secretion in the obese (*fa/fa*) Zucker rat. Am J Physiol 282:E59–E66
15. **Darimont C, Vassaux G, Ailhaud G, Negrel R** 1994 Differentiation of preadipose cells: role of prostacyclin upon stimulation of adipose cells by angiotensin-II. Endocrinology 135:2030–2036
16. **Fortuño A, Muñiz P, Ravassa S, Rodriguez JA, Fortuño MA, Zalba G, Díez J** 1999 Torasemide inhibits angiotensin II-induced vasoconstriction and intracellular calcium increase in the aorta of spontaneously hypertensive rats. Hypertension 34:138–143
17. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
18. **Grynkiewicz G, Poenie M, Tsien RY** 1982 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
19. **Dunbar JC, Hu Y, Lu H** 1997 Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. Diabetes 46:2040–2043
20. **Haynes WG, Sivitz WI, Morgan DA, Walsh SA, Mark AL** 1997 Sympathetic and cardiorenal actions of leptin. Hypertension 30:619–623
21. **Hall JE, Hildebrandt DA, Kuo J** 2001 Obesity hypertension: role of leptin and sympathetic nervous system. Am J Hypertens 14:103S–115S
22. **Sierra-Honigmann MR, Nath AK, Murakami C, García-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR** 1998 Biological action of leptin as an angiogenic factor. Science 281:1683–1686
23. **Bouloumie A, Drexler HCA, Lafontan M, Busse R** 1999 Leptin, the product of Ob gene promotes angiogenesis. Circ Res 83:1059–1066
24. **Park H-Y, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY, Kim H-S** 2001 Potential role of leptin in angiogenesis: leptin induces endothelial proliferation and expression of matrix matalloproteinases in vivo and in vitro. Exp Mol Med 33:95–102
25. **Mitchell JL, Morgan DA, Correia MLG, Mark AL, Sivitz WI, Haynes WG** 2001 Does leptin stimulate nitric oxide to oppose the effects of sympathetic activation? Hypertension 38:1081–1086
26. **Vecchione C, Maffei A, Colella S, Aretini A, Poulet R, Frati G, Gentile MT, Fratta L, Trimarco V, Trimarco B, Lembo G** 2002 Leptin effect on endothelial nitric oxide is mediated through Akt-endothelial nitric oxide synthase phosphorylation pathway. Diabetes 51:168–173
27. **Magni P, Vettor R, Pagano C, Calcagno A, Beretta E, Messi E, Zanisi M, Martini L, Motta M** 1999 Expression of a leptin receptor in immortalized gonadotropin-releasing hormone-secreting neurons. Endocrinology 140:1581–1585
28. **Sobhani I, Bado A, Vissuzaine C, Buyse M, Kermorgant S, Laigneau J-P, Attoub S, Lehy T, Henin D, Mignon M, Lewin MJM** 2000 Leptin secretion and leptin receptor in the human stomach. Gut 47:178–183
29. **Sone M, Nagata H, Takekoshi S, Osamura RY** 2001 Expression and localization of leptin receptor in the normal rat pituitary gland. Cell Tissue Res 305:351–356
30. **Parhami F, Tintut Y, Ballard A, Fogelman AM, Demer LL** 2001 Leptin enhances the calcification of vascular cells. Artery wall as a target of leptin. Circ Res 88:954–960
31. **Oda A, Taniguchi T, Yokoyama M** 2001 Leptin stimulates rat aortic smooth muscle cell proliferation and migration. Kobe J Med Sci 47:141–150
32. **Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM** 1996 Abnormal splicing of the leptin receptor in diabetic mice. Nature 15; 379:632–635
33. **Smith JB** 1986 Angiotensin-receptor signaling in cultured vascular smooth muscle cells. Am J Physiol 250:F759–F769
34. **Takekoshi K, Motooka M, Isobe K, Nomura F, Nanmoku T, Ishii K, Nakai T** 1999 Leptin directly stimulates catecholamine secretion and synthesis in cultured porcine adrenal medullary chromaffin cells. Biochem Biophys Res Commun 261:426–431
35. **Takekoshi K, Ishii K, Nanmoku T, Shibuya S, Kawakami Y, Isobe K, Nakai T** 2001 Leptin stimulates catecholamine synthesis in a PKC-dependent manner in cultured porcine adrenal medullary chromaffin cells. Endocrinology 142: 4861–4871
36. **Webb RC, Bohr D** 1981 Regulation of vascular tone, molecular mechanisms. Prog Cardiovasc Dis 21:213–242
37. **Rossi GP, Sacchetto A, Cesari M, Pessina AC** 1999 Interactions between endothelin-1 and the renin-angiotensin-aldosterone system. Cardiovasc Res 43:300–307
38. **Mastronardi CA, McCann SM** 2002 Resting and circadian release of nitric oxide is controlled by leptin in male rats. Proc Natl Acad Sci USA 99:5721–5726
39. **Zemel MB, Reddy S, Shehin S** 1990 Vascular reactivity in Zucker obese rats: role of insulin resistance. J Vasc Med Biol 2:81–85
40. **Abel MA, Zemel MB** 1993 Impaired recovery of vascular smooth muscle intracellular calcium following agonist stimulation in insulin resistant (Zucker obese) rats. Am J Hypertens 6:500–504