

# The Inhibitory Effect of Leptin on Angiotensin II-Induced Vasoconstriction in Vascular Smooth Muscle Cells Is Mediated via a Nitric Oxide-Dependent Mechanism

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Leptin inhibits the contractile response induced by angiotensin (Ang) II in vascular smooth muscle cells (VSMCs) of the aorta. We studied *in vitro* and *ex vivo* the role of nitric oxide (NO) in the effect of leptin on the Ang II-induced vasoconstriction of the aorta of 10-wk-old Wistar rats. NO and nitric oxide synthase (NOS) activity were assessed by the Griess and <sup>3</sup>H-arginine/citrulline conversion assays, respectively. Stimulation of inducible NOS (iNOS) as well as Janus kinases/signal transducers and activators of transcription (JAK/STAT) and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways were determined by Western blot. The contractile responses to Ang II were evaluated in endothelium-denuded aortic rings using the organ bath system. Changes in intracellular Ca<sup>2+</sup> were measured in VSMCs using fura-2 fluorescence. Leptin significantly ( $P \leq 0.01$ ) stimulated NO release and NOS activity in VSMCs. Leptin's effect on NO was abolished by the NOS inhibitor, N<sup>G</sup>-monomethyl L-arginine, or the

iNOS selective inhibitor L-N<sup>6</sup>-(1-iminoethyl)-lysine. Accordingly, leptin increased iNOS protein expression, with a comparable time course with that of NO production and NOS activity. Leptin also significantly increased STAT3 ( $P \leq 0.01$ ) and Akt ( $P \leq 0.001$ ) phosphorylation. Moreover, either the JAK2 inhibitor, AG490, or the PI3K inhibitor, wortmannin, significantly ( $P \leq 0.05$ ) abrogated the leptin-induced increase in iNOS protein. Finally, both N<sup>G</sup>-monomethyl L-arginine and L-N<sup>6</sup>-(1-iminoethyl)-lysine inhibitors completely blunted ( $P \leq 0.001$ ) the leptin-mediated inhibition of the Ang II-induced VSMC activation and vasoconstriction. These findings suggest that the endothelium-independent depressor action of leptin is mediated by an increase of NO bioavailability in VSMCs. This process requires the up-regulation of iNOS through mechanisms involving JAK2/STAT3 and PI3K/Akt pathways. (*Endocrinology* 148: 324–331, 2007)

**O**BESITY CONSTITUTES A major risk for the development of cardiovascular disorders, such as hypertension and coronary artery disease (1, 2). Several central and peripheral abnormalities that can explain the development or maintenance of high blood pressure in obesity have been identified. Leptin, an adipocyte-derived hormone, is known to participate in the control of body weight, regulating food intake and energy expenditure (3). Furthermore, leptin has been also shown to contribute to blood pressure homeostasis inducing a pressor response attributable to sympathoactivation (4) and a depressor response attributable to the vasodilation of conduit and resistance vessels (5, 6). Our group reported, for the first time, that nitric oxide (NO) represents the key molecule for the depressor response induced by leptin (7). Subsequent studies showed that leptin acts on the endothelium inducing the synthesis of NO through the ac-

tivation of the endothelial NO synthase, hence evoking an endothelium-dependent vasodilation (8–12). However, because leptin receptors are also expressed in the underlying smooth muscle layer, vascular smooth muscle cells (VSMCs) also represent an important target for the vascular actions of leptin (13, 14). In previous studies we reported that leptin acts on VSMCs inhibiting the increase of cytosolic calcium ([Ca<sup>2+</sup>]<sub>i</sub>) induced by angiotensin II (Ang II) and consequently blunting the contractile response of the aorta caused by this potent vasoactive peptide (13, 14). This inhibition of the Ang II-induced vasoconstriction was shown to be leptin receptor mediated because no effects of leptin were found in the aorta and VSMCs obtained from leptin receptor-deficient obese Zucker rats (13). To date, the postreceptor mechanisms responsible for this vascular effect of leptin remain unknown.

NO plays an important role in the maintenance of the vascular tone. In VSMCs, NO is mainly produced by the inducible NO synthase (iNOS), a Ca<sup>2+</sup>-independent enzyme that is regulated at transcriptional and posttranscriptional levels (15). Furthermore, cytokine-induced iNOS up-regulation is mediated, among others, by Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (16–18). On the one hand, the main signaling cascades activated by leptin receptors, which belong to the class I cytokine receptor superfamily, include the JAK/STAT and

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Abbreviations: Ang II, Angiotensin II; [Ca<sup>2+</sup>]<sub>i</sub>, cytosolic calcium; FBS, fetal bovine serum; iNOS, inducible NOS; JAK, Janus kinase; L-NIL, L-N<sup>6</sup>-(1-iminoethyl)-lysine; NMMA, N<sup>G</sup>-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PI3K, phosphoinositide 3-kinase; STAT, signal transducers and activators of transcription; VSMC, vascular smooth muscle cell.

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PI3K/Akt pathways (18). On the other hand, a functional relation between leptin and NO has been established in quite diverse sites and biological actions (19–21). With regard to the cardiovascular sphere in particular, leptin has been shown to increase NO synthase (NOS) activity in endothelial cells (12, 22, 23) and cardiomyocytes (24, 25) through the activation of PI3K/Akt and JAK2/STAT3 pathways, respectively.

Given that many of the physiological and pathophysiological effects of Ang II are opposed by NO (26), it seems plausible that NO operates as the intracellular mediator responsible for the depressor response of leptin in VSMCs. Therefore, the aim of the present study was to examine *ex vivo* and *in vitro* the potential role of NO in the inhibition of leptin on the Ang II-induced contractile response. The effect of leptin on NO production, NOS activity, and iNOS expression was measured directly in VSMCs. To gain further insight into the signaling cascades activated by leptin, the potential involvement of JAK2/STAT3 and PI3K/Akt transduction signals was analyzed using specific pharmacological inhibitors. To further corroborate the participation of NO on the vascular actions of leptin, the effect of the  $N^G$ -monomethyl-L-arginine (NMMA), a nonselective inhibitor of NOS, and L-N<sup>6</sup>-(1-iminoethyl)-lysine (L-NIL), a selective inhibitor of iNOS, on the inhibitory effect of leptin on the Ang II-induced VSMC activation and vasoconstriction of the aorta was analyzed.

## Materials and Methods

### Animals

Ten-week-old male normotensive Wistar rats ( $n = 30$ ) (breeding house of the University of Navarra) were used in the present study. Rats were housed in a temperature-, humidity-, and light-controlled room with free access to tap water and fed *ad libitum* with a standard rodent chow (2014S Teklad global 14% protein rodent maintenance diet; Harlan, Barcelona, Spain). All experimental procedures conformed with the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609) and were approved by the Ethical Committee for Animal Experimentation of the University of Navarra (036/03). Rats were killed by decapitation. The thoracic aorta was carefully excised, dissected out, and processed for each study.

### Isolation of VSMCs

Primary VSMCs were obtained from the thoracic aorta by the tissue explants method, as previously described (27). Briefly, the smooth muscle tissue was longitudinally opened and cut in small pieces that were grown in plastic six-well plates and maintained at 37°C in a humidified incubator with an atmosphere of 95% air–5% CO<sub>2</sub>. Tissue explants were cultured in DMEM containing 20% fetal bovine serum (FBS) (Life Technologies, Inc., Gaithersburg, MD) and antibiotic-antimycotic products (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) (Life Technologies). The medium was changed initially after 24 h and then every 2–3 d. After about 8–10 d, when cells had formed a confluent monolayer, they were harvested by addition of 0.05% trypsin, and the culture was continued up to four to six passages using DMEM containing 10% FBS. The previous day of each experiment, media of subconfluent cultures were switched to DMEM containing 0.1% FBS.

### Evaluation of NO production and NOS activity

Quiescent VSMCs were stimulated during 30 min with leptin (10 nmol/liter) (PreproTech EC, Inc., Rocky Hill, NJ) in the presence or absence of NMMA (10 µmol/liter) (Sigma, St. Louis, MO) or L-NIL (10 µmol/liter) (Sigma). The concentration of leptin as well as that of the

pharmacological NOS inhibitors to carry out the experiments was chosen on the basis of prior experiments performed in our laboratory (13, 14, 28). One sample per assay was used to obtain control responses in the presence of solvent. Samples of the culture media were collected at different times (0, 10, 20, and 30 min) for the measurement of nitrates and nitrites, as an index of NO production, with a commercial kit (Cayman Chemical, Ann Arbor, MI) based on the Griess reaction following the manufacturer's protocol. The inter- and intraassay coefficients of variation were 6.5 and 3.3%, respectively. Stimulated cells were harvested and homogenized in a lysis buffer [25 mmol/liter Tris, 1 mmol/liter EDTA, 1 mmol/liter EGTA (pH 7.40)] supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) for the determination of NOS activity. The protein content of the homogenates was determined by the method of Bradford (29). NOS activity was measured by the L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline conversion assay, using a commercial kit (Stratagene, La Jolla, CA). The inter- and intraassay coefficients of variation were 9.1 and 6.3%, respectively. Briefly, samples of 20 µg protein were incubated at room temperature for 1 h in the reaction buffer [25 mmol/liter Tris-HCl (pH 7.40), 3 µmol/liter tetrahydrobiopterin, 1 µmol/liter flavin adenine dinucleotide reduced, 1 µmol/liter flavin mononucleotide reduced, 1 mmol/liter nicotinamide adenine dinucleotide phosphate reduced, 0.6 mmol/liter CaCl<sub>2</sub>] supplemented with L-[<sup>3</sup>H]arginine (1 µCi/µl) (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). L-[<sup>3</sup>H]citrulline was quantified by scintillation. All assays were performed in duplicate.

### Western blot studies

Quiescent VSMCs were stimulated for 30 min with leptin (10 nmol/liter). At different times of the stimulation (0, 2, 5, 10, 20, and 30 min), cells were harvested, homogenized, and protein content measured as previously described (13). In some experiments cells were preincubated for 30 min with the following specific pharmacological inhibitors before leptin treatment: tyrphostin AG490 (10 µmol/liter) (Sigma) for JAK2; wortmannin (10 µmol/liter) (Tocris, Ellisville, MO) for PI3K; actinomycin D (5 µg/ml) (Calbiochem, La Jolla, CA) for transcription; and cycloheximide (10 µg/ml) (Calbiochem) for protein synthesis. Equal amounts of protein (30 µg) were run out in 8% SDS-PAGE, subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA), and blocked in Tris-buffered saline with Tween 20 containing 5% nonfat dry milk for 1 h at room temperature. Blots were then incubated overnight at 4°C with Akt, phospho-(Thr<sup>308</sup>)-Akt (Upstate, Lake Placid, NY), STAT3, phospho-(Tyr<sup>705</sup>)-STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), iNOS (BD Transduction Laboratories, San Jose, CA), or β-actin (Sigma) antibodies. The antigen-antibody complexes were visualized using peroxidase-conjugated antimouse or anti-rabbit IgG antibodies (1:5000) and the enhanced chemiluminescence detection system (Amersham Biosciences). The intensity of the bands was determined by densitometric analysis and normalized with β-actin density values.

### Measurement of intracellular free Ca<sup>2+</sup> concentrations

[Ca<sup>2+</sup>]<sub>i</sub> was measured by fura-2 AM (Molecular Probes, Leiden, The Netherlands) microfluorometry in combination with digital imaging as previously reported (27). Basal and Ang II (100 nmol/liter) (Sigma)-induced changes in the excitation ratio of fura-2 (340/380 nm) were measured after the preincubation with leptin (0.01, 0.1, 1, and 10 nmol/liter) during 10 min in VSMCs. The concentration of Ang II to carry out the experiments was chosen on the basis of previous studies performed in our laboratory (13, 14, 27). In a subset of experiments, cells were pretreated for 10 min with NMMA (10 µmol/liter) or L-NIL (10 µmol/liter) (Sigma). The [Ca<sup>2+</sup>]<sub>i</sub> was calculated as described by Gryniewicz *et al.* (30).

### Preparation of isolated endothelium-denuded aortic rings

The thoracic aorta was cleaned and placed into Krebs-Henseleit solution as previously reported (13, 14). The endothelium of the aorta was removed by gently rubbing the intimal surface with the tip of a small steel stick. The aorta was cut into ring segments (2 to 3 mm in length, six per rat) that were placed in a vessel containing 20 ml Krebs-Henseleit solution oxygenated with a 95% O<sub>2</sub>–5% CO<sub>2</sub> mixture and maintained at

37 C in a thermostated bath (LE 13206 Thermostat; Letica Scientific Instruments, Barcelona, Spain). Two stainless steel wires were inserted into the vascular lumen; one was anchored to a stationary support, and the other was connected to a force-displacement isometric transducer (Tri 110; Letica Scientific Instruments). Changes in isometric forces were analyzed and recorded by an Isolated Organs Data Acquisition program (Proto5; Letica Scientific Instruments). The rings were incubated to equilibration at a resting tension of 2.5 g for 60 min, with buffer changes every 15 min during this period. The lack of functional endothelium was confirmed by the complete absence of relaxation evoked by acetylcholine (10  $\mu\text{mol/liter}$ ) (Sigma) in KCl (80 mmol/liter)-precontracted aortic rings. The rings were then washed and stretched, if necessary, until a stable baseline force was obtained. After equilibration, endothelium-denuded aortic rings were exposed to murine leptin (0.01, 0.1, 1, and 10 nmol/liter) for 30 min, and subsequently the response to Ang II (100 nmol/liter) was evaluated. In a second set of experiments, vessels were pretreated during 10 min with NMMA (10  $\mu\text{mol/liter}$ ) or L-NIL (10  $\mu\text{mol/liter}$ ). One sample per experiment was used to obtain control responses (100%) in the presence of the solvent. Contractile responses were evaluated in milligrams of tension and expressed as percentage of contraction of the KCl (80 mmol/liter)-induced response in each experiment.

### Statistical analysis

Data are presented as mean  $\pm$  SEM. The contractile response was obtained from 10 aortic rings isolated from Wistar rats ( $n = 10$ ). As otherwise indicated, 10 determinations were performed in isolated VSMCs obtained from the aorta of Wistar rats ( $n = 5$  per experiment). Statistical differences among mean values were determined using the two-way or one-way ANOVA, followed by Dunnett's *t* test, as appropriate.  $P < 0.05$  was considered statistically significant. Analyses were performed by the SPSS/Windows software (version 11.0.1; SPSS Inc., Chicago, IL).

## Results

### Effect of leptin on NO release and NOS activity

The stimulation with leptin induced a statistically significant increase in both NO production and NOS activity as compared with basal conditions in VSMCs (Fig. 1). Exposure to the nonspecific NOS inhibitor, NMMA, and the selective inhibitor of iNOS, L-NIL, abolished the stimulatory effect of leptin on NO production and NOS activity.

### Effect of leptin on iNOS expression

To establish the potential mechanism of action triggered by leptin to produce NO, the expression of iNOS protein after leptin stimulation was studied by Western blotting. Leptin induced a significant increase in iNOS expression in VSMCs, with a similar time course to that of NO production and NOS activity (Fig. 2A). To discern whether mRNA and/or protein synthesis were necessary for the up-regulation of iNOS induced by leptin, the response of cells after the incubation with the transcription inhibitor actinomycin D, and the protein synthesis inhibitor, cycloheximide, was also analyzed. As shown in Fig. 2B, actinomycin D inhibited by approximately 25% the expression of iNOS induced by leptin. In addition, cycloheximide completely blunted leptin-induced iNOS expression.

### Role of JAK2/STAT3 and PI3K/Akt pathways in leptin-induced iNOS activation

The ability of leptin to trigger JAK2/STAT3 and PI3K/Akt was examined by the degree of phosphorylation/activation of the downstream molecules STAT3 and Akt after leptin treatment. Leptin activated the phosphorylation of STAT3 in

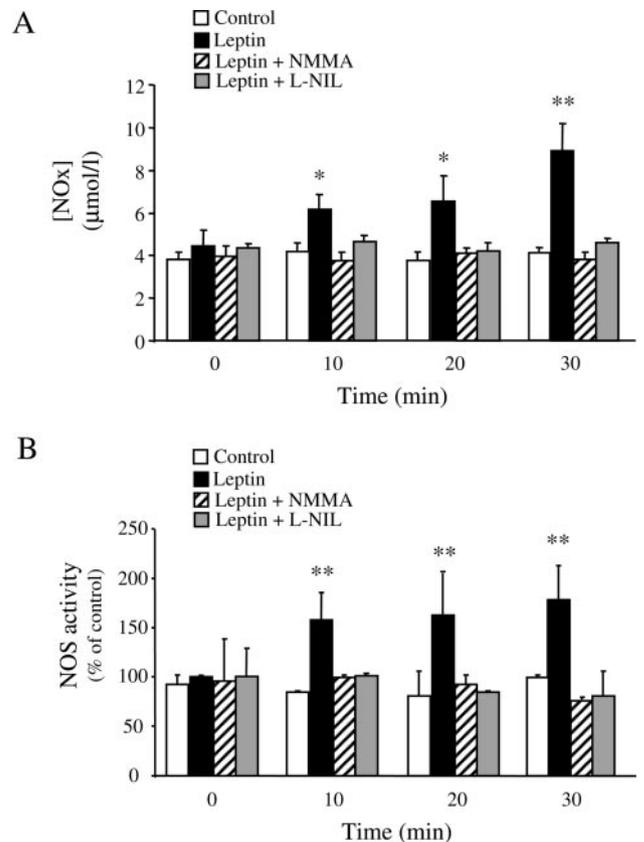


FIG. 1. Effect of leptin on NO production and NOS activity in VSMCs. Bar graphs show the differences in the time course of accumulation of nitrites in the culture media (A) and NOS activity (B) of leptin (10 nmol/liter)-stimulated cells in the presence or absence of the NOS inhibitor, NMMA (10  $\mu\text{mol/liter}$ ), and the selective iNOS inhibitor, L-NIL (10  $\mu\text{mol/liter}$ ). Data are expressed as mean  $\pm$  SEM of 10 assays performed in isolated VSMCs from the aorta of Wistar rats ( $n = 5$ ). Each assay was performed in duplicate. \*,  $P < 0.05$ , \*\*,  $P < 0.01$  vs. unstimulated cells.

a time-dependent manner (Fig. 3A), whereas a maximal phosphorylation of Akt was observed after 10 min of leptin stimulation with attenuation of the phosphorylation thereafter (Fig. 3B). Because the induction of iNOS can be mediated via JAK2/STAT3 and PI3K/Akt (15–17), we further investigated the involvement of these transduction signals in the up-regulation of iNOS induced by leptin. Specific cell-permeable chemical inhibitors were added to the culture media before the addition of leptin. The inhibition of the upstream molecules JAK2 and PI3K with AG490 and wortmannin, respectively, significantly reduced leptin-induced iNOS expression (Fig. 3C). Neither wortmannin nor tyrosine kinase inhibitor AG490 modified basal iNOS protein levels (data not shown).

### Effect of leptin on the Ang II-induced increase of $[Ca^{2+}]_i$ in VSMCs in the presence of NOS inhibitors

Responses to Ang II (100 nmol/liter) were assessed in quiescent VSMCs from Wistar rats in the absence or presence of different concentrations of leptin. Ang II (100 nmol/liter) induced a maximal increase of  $[Ca^{2+}]_i$  after 2–6 sec of the stimulation, and baseline or plateau  $[Ca^{2+}]_i$  values were

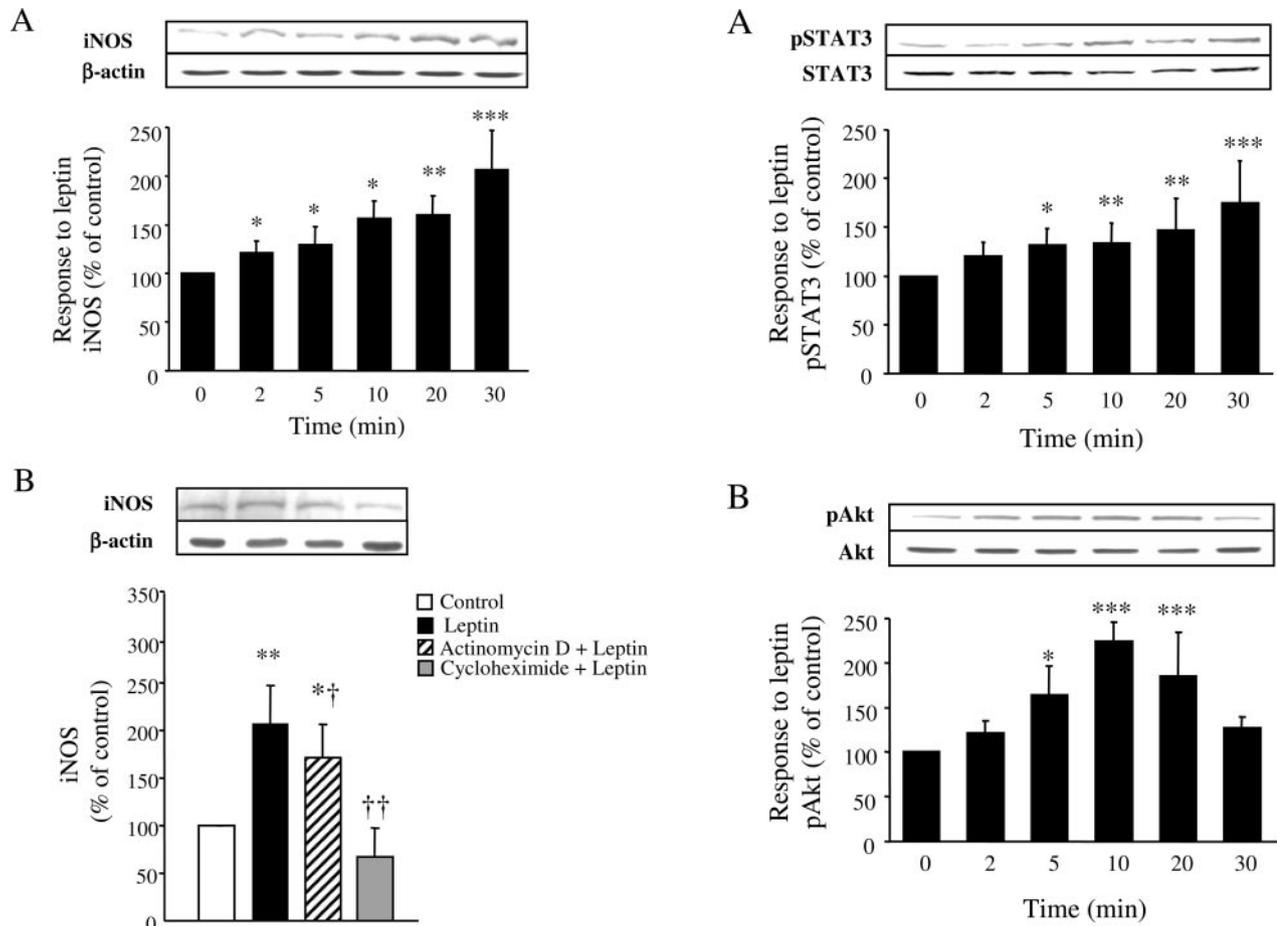


FIG. 2. Effect of leptin on iNOS expression in VSMCs. A, Histograms illustrate the differences in the time course of iNOS expression in VSMCs stimulated with leptin (10 nmol/liter). B, Effect of the mRNA synthesis inhibitor, actinomycin D (5  $\mu$ g/ml), and the protein synthesis inhibitor cycloheximide (10  $\mu$ g/ml) on the up-regulation of iNOS evoked by leptin. Representative blots of 10 replicates are shown on the top of the histograms. Each bar represents mean  $\pm$  SEM for 10 determinations performed in isolated VSMCs from the aorta of Wistar rats ( $n = 5$ ). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  vs. unstimulated cells; †,  $P < 0.05$ , ††,  $P < 0.01$  vs. leptin-stimulated cells in the absence of inhibitors.

achieved within the following 30–40 sec (Fig. 4). Leptin reduced in a concentration-dependent manner the peak of  $[Ca^{2+}]_i$  after Ang II (100 nmol/liter) stimulation (Fig. 4A). The adipokine also accelerated the recovery rate to basal  $[Ca^{2+}]_i$  values (Fig. 4, B and C). Pretreatment with either the NOS inhibitor, NMMA (Fig. 4B), or the selective iNOS inhibitor, L-NIL (Fig. 4C), blunted the depressor action of leptin (10 nmol/liter) on Ang II (100 nmol/liter)-induced VSMC activation. NOS inhibitors did not modify the Ang II-induced peak of  $[Ca^{2+}]_i$  or the return to basal calcium levels.

#### Effect of leptin on Ang II-induced contractile response in the presence of NOS inhibitors

The vascular response to Ang II (100 nmol/liter) was also evaluated in endothelium-denuded aortic rings after the preincubation with different concentrations of leptin for 30 min.

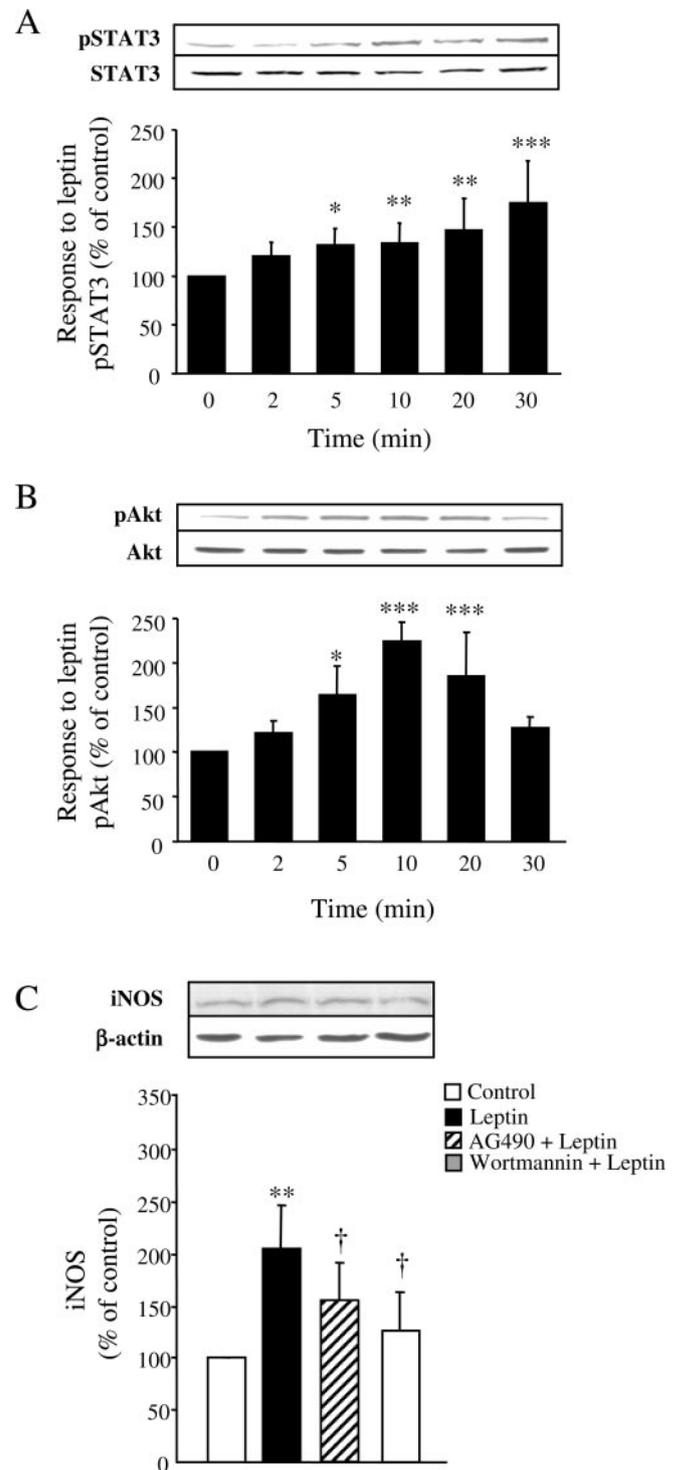


FIG. 3. Western blot analysis showing the effect of leptin (10 nmol/liter) on STAT3 (A) and Akt (B) phosphorylation in VSMCs. C, Effect of the JAK2 inhibitor AG490 (10  $\mu$ mol/liter) and the PI3K inhibitor wortmannin (10  $\mu$ mol/liter) on the iNOS expression induced by leptin in VSMCs. Representative blots of 10 replicates are shown on the top of the histograms. Each bar represents mean  $\pm$  SEM for 10 determinations performed in isolated VSMCs from the aorta of Wistar rats ( $n = 5$ ). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  vs. unstimulated cells; †,  $P < 0.05$ , ††,  $P < 0.01$  vs. leptin-stimulated cells in the absence of inhibitors.

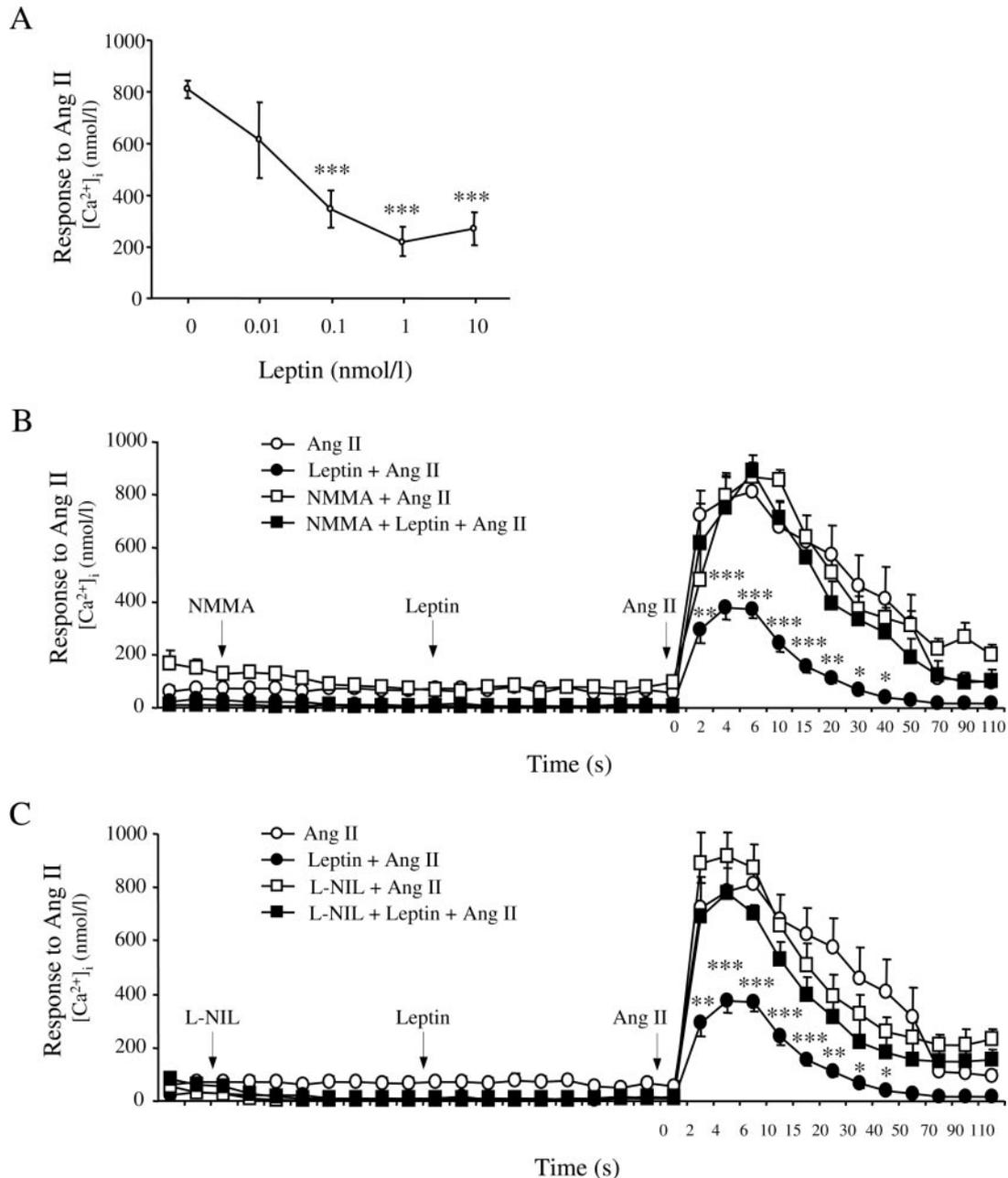


FIG. 4. Impact of NOS inhibitors on the depressor action of leptin on Ang II-induced increase in  $[Ca^{2+}]_i$  of VSMCs. *A*, Line graphs show the Ang II (100 nmol/liter)-induced changes in  $[Ca^{2+}]_i$  in VSMCs from Wistar rats in the absence or after preincubation with different concentrations of leptin. Line graphs show the differences in the time course of Ang II-induced calcium mobilization in the absence or presence of leptin (10 nmol/liter), the NOS inhibitor NMMA (10  $\mu$ mol/liter) (B), or the selective iNOS inhibitor L-NIL (10  $\mu$ mol/liter) (C). Maximal stimulated  $[Ca^{2+}]_i$  occurred at 2–6 sec, and the recovery to basal concentrations was measured thereafter. Values are expressed as mean  $\pm$  SEM of independent experiments performed in 10–15 cells/group isolated from the aorta of Wistar rats ( $n = 4$ ). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  vs. Ang II control response in the absence of inhibitors.

Leptin inhibited in a concentration-dependent manner the Ang II (100 nmol/liter)-induced vasoconstriction in Wistar rats (Fig. 5A). In addition, preincubation of endothelium-denuded aortic rings with leptin (10 nmol/liter) for 30 min induced a significant ( $P = 0.030$ ) decrease in the basal wall tension ( $-17 \pm 2$  vs.  $21 \pm 4$  mg).

The effect of leptin (10 nmol/liter) on the Ang II-induced vascular response was reexamined in the presence of the

NOS inhibitors NMMA and L-NIL (Fig. 5, B and C). Both NOS inhibitors completely abolished the inhibitory effect of leptin on the Ang II-mediated vasoconstriction. Moreover, the presence of NMMA or L-NIL blunted the decrease of the passive vasomotor tone induced by leptin ( $16 \pm 3$  and  $16 \pm 5$  vs.  $21 \pm 4$  mg, respectively). The passive wall tension of endothelium-denuded aortic rings was not affected by the presence of NOS inhibitors.

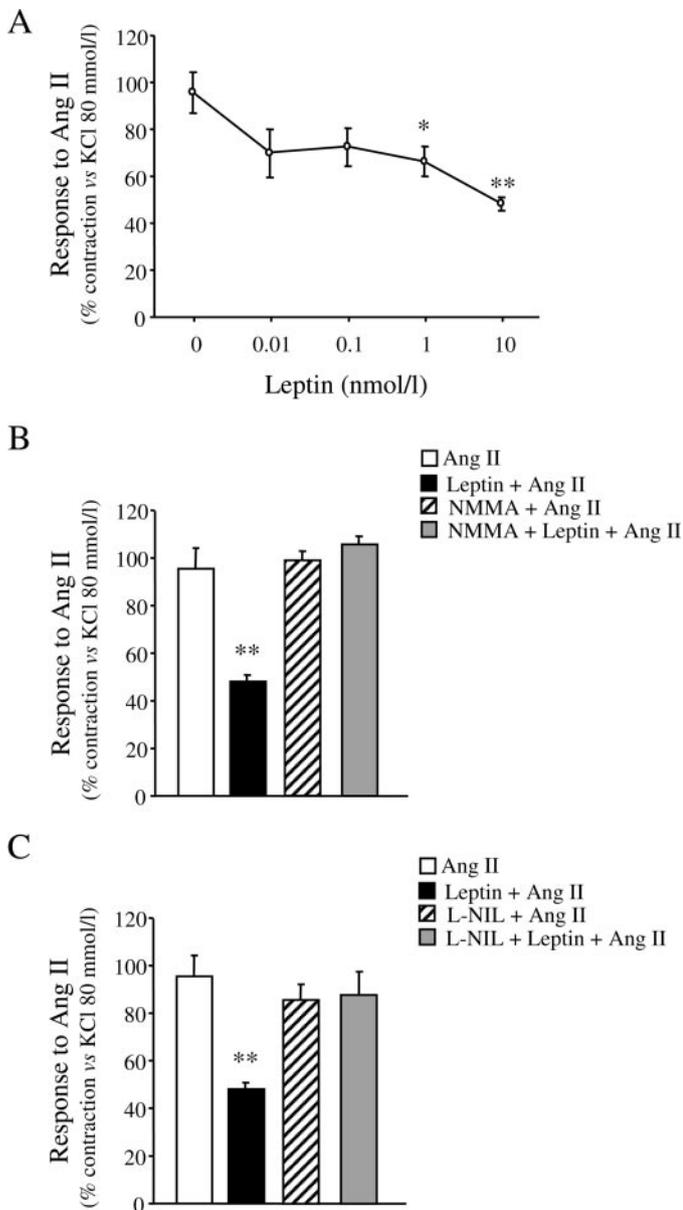


FIG. 5. Impact of NOS inhibitors on the depressor action of leptin on Ang II-induced contractile response. A, Line graphs show the inhibition exerted by leptin on the Ang II (100 nmol/liter)-induced vasoconstriction of endothelium-denuded aortic rings. Both NMMA (10  $\mu$ mol/liter) (B) and L-NIL (10  $\mu$ mol/liter) (C) blunted the depressor action of leptin (10 nmol/liter) on the Ang II (100 nmol/liter)-induced response in endothelium-denuded vessels. Data are expressed as mean  $\pm$  SEM of the contractile response of 10 aortic preparations obtained from Wistar rats ( $n = 10$ ). \*,  $P < 0.05$ , \*\*,  $P < 0.01$  vs. Ang II control response in the absence of inhibitors.

### Discussion

Several *ex vivo* studies have reported that leptin exerts a direct vasodilation through different endothelial mechanisms, depending on the specific vascular bed at which it acts. In the aorta and coronary arteries, leptin reportedly induces vasodilation via NO (8, 9, 31), whereas the relaxation induced by the hormone in mesenteric arteries is mediated by the endothelium-derived hyperpolarizing factor (8, 32). Moreover, leptin treatment *in vivo* has been shown to reverse

the endothelial dysfunction of leptin-deficient obese (*ob/ob*) mice by increasing NO bioavailability in vessels (10). However, previous data suggest that the smooth muscle layer also constitutes an important target for the vasorelaxant actions of leptin (13). The present study has further explored the complex mechanisms by which leptin exerts its depressor action operating directly on VSMCs.

Our data show, for the first time, that leptin induces the synthesis of NO in VSMCs through the activation of iNOS with leptin-induced NO production and NOS activity in VSMCs being completely prevented by an iNOS inhibitor. Furthermore, leptin increased the synthesis of iNOS protein mainly at a posttranscriptional level because the inhibition of RNA transcription reduced only 25% of the leptin-induced iNOS expression, whereas the inhibition of the translation totally blunted the leptin-induced iNOS up-regulation. The ability of leptin to up-regulate the iNOS isoform has been shown in other cell types such as chondrocytes, glioma cells, macrophages, or cells from the gastric mucosa (33–36).

To gain further insight into the mechanisms by which leptin promotes iNOS up-regulation, we evaluated the signaling cascades potentially involved. In VSMCs, JAK2 kinases induce the tyrosine phosphorylation and nuclear translocation of the STAT3 transcription factor, which binds to its response element in the iNOS gene promoter, inducing iNOS transcription (15, 17). On the other hand, the phosphorylation of Akt has been shown to induce a concomitant activation of the transcription factor nuclear factor- $\kappa$ B, a pivotal regulator of iNOS expression (37). Recent studies reported that the activation of JAK2/STAT3 and PI3K/Akt transduction signals is related to the induction of iNOS by leptin in rat cardiomyocytes and human chondrocytes (25, 33). Our findings show that leptin treatment induced the phosphorylation/activation of the STAT3 and Akt downstream molecules. A similar pattern of phosphorylation after acute stimulation with leptin has been found for STAT3 in rat VSMCs (25, 38) and Akt in isolated aortic rings (12). Furthermore, the inhibition of the JAK2 and PI3K upstream molecules decreased the expression of iNOS evoked by leptin in VSMCs. Taken together, we conclude that activation of the JAK2/STAT3 and PI3K/Akt pathways induced by leptin constitutes an early step for the up-regulation of iNOS, which is in accordance with studies performed by other authors (25, 33).

The vasoconstrictive action of Ang II is based on its ability to elevate the  $[Ca^{2+}]_i$  in the smooth muscle layer (39). The intracellular mechanisms triggered by Ang II to increase the  $[Ca^{2+}]_i$  in VSMCs after binding the Ang II receptor type 1 include the rapid release of calcium sequestered in the sarcoplasmic reticulum and the additional increase of the calcium influx from the extracellular compartment. Our group previously tested the effect of leptin on the contractile response of Ang II and other vasoconstrictors, such as noradrenaline and endothelin-1 (13, 14, 40). The Ang II-induced vasoconstriction was inhibited by leptin in the aorta, whereas no effects of leptin were found on the noradrenaline- and endothelin-1-induced contraction (13, 40). Leptin attenuates the Ang II-induced contraction by blocking the release of  $Ca^{2+}$  sequestered in the intracellular stores, the major mechanism used by Ang II to increase the  $[Ca^{2+}]_i$  (39). Therefore, the lack of effect of leptin on the contraction of noradrenaline

and endothelin-1 may be ascribed to the fact that these vasoconstrictors exert their contractile properties mainly via a  $\text{Ca}^{2+}$  influx from the extracellular compartment.

The intracellular signaling cascade underlying the depressor action of leptin on Ang II-induced vasoconstriction has not been completely disentangled. The potential role of NO, a key modulator of the vascular tone, on this effect of leptin has been addressed by several groups. On the one hand, NO has been shown to induce a direct vasodilation through mechanisms that include the activation of the guanylyl cyclase that converts GTP into cGMP in VSMCs (41). On the other hand, NO has been observed to reduce the vasoconstriction induced by Ang II by activating calcium clearing mechanisms in VSMCs, such as the sarcoplasmic  $\text{Ca}^{2+}$ -AT-Pase and  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, and reducing intracellular calcium transients, decreasing phospholipase C and inositol 1,4,5-trisphosphate receptor activity (26). Interestingly, our data indicate that leptin induced a decrease of the passive wall tension of the endothelium-denuded aorta that was inhibited in the presence of NOS inhibitors. Moreover, under NOS activity blockade, the inhibition of leptin on the Ang II-induced increase of  $[\text{Ca}^{2+}]_i$  in VSMCs and the vasoconstriction of endothelium-denuded aortic rings was completely blunted. Thus, our data provide evidence that the depressor action of leptin in the smooth muscle layer of the aorta produces a direct vasorelaxant effect as well as a reduction of the vasoconstrictor potential of Ang II through NO-dependent mechanisms. Similar findings of hypotensive effects of leptin via NO have been reported in rat myocardium (24), kidneys (42), and endothelium of conduit vessels (aorta) (8, 9) and resistance vessels (mesenteric and coronary arteries) (8, 9, 43). Taken together, these data support the notion that NO represents a key mediator of the cardiovascular effects of leptin (5).

The effect of the inhibition of the upstream molecules JAK2 and PI3K was not directly tested in the vascular preparations. Nonetheless, our data show that iNOS activation is necessary for the depressor action of leptin on the Ang II-induced vasoconstriction in isolated aortic rings. Interestingly, we found that leptin-stimulated up-regulation of iNOS was effectively blocked by pharmacological inhibitors of JAK2 (AG490) and PI3K (wortmannin), which is in accordance with previously published studies performed in isolated myocytes (25, 33). Thus, our findings suggest that the JAK2/STAT3 and PI3K/Akt pathways are required for this vascular effect of leptin, although the participation of other transduction signals involved in the up-regulation of iNOS may not be ruled out.

It has been recently reported that oxidative stress further participates in the Ang II-induced vasoconstriction (44). Ang II induces the generation of reactive oxygen species, which scavenge NO to reduce NO bioavailability and generate the potent oxidative peroxynitrite radical, known to play relevant roles in vascular pathophysiology (44). Our findings support that leptin counteracts the Ang II-induced vasoconstriction by increasing NO bioavailability in the vascular wall of normotensive rats. However, in spontaneously hypertensive rats, leptin resistance has been observed at the VSMC level in relation to a loss of antagonism of the Ang II-induced contractile response (14). Taken together, under

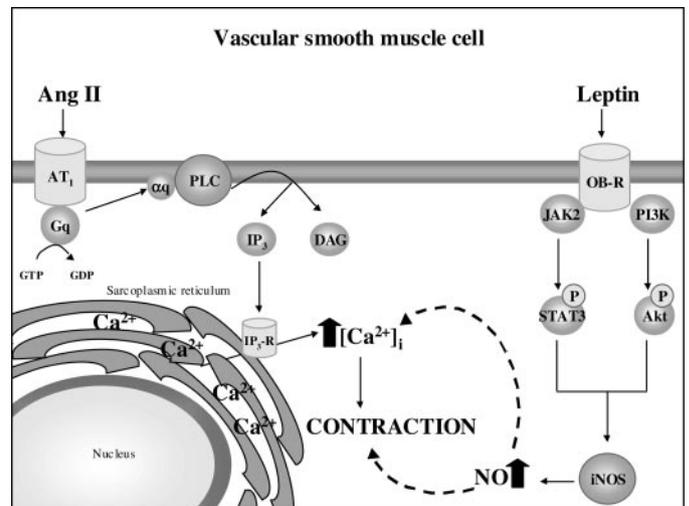


FIG. 6. Schematic diagram of the effect of leptin on Ang II-induced vasoconstriction. In a normotensive state, leptin attenuates the Ang II-induced increase of  $[\text{Ca}^{2+}]_i$  and the subsequent contractile response of VSMCs by increasing NO bioavailability in the vascular wall. However, in the setting of hypertension, leptin's counterregulatory action is overridden by the effects of Ang II, despite a clear hyperleptinemia. *Striped arrows* indicate inhibition; *solid arrows* indicate stimulation. AT<sub>1</sub>, Angiotensin II type 1 receptor; PLC, phospholipase C; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; IP<sub>3</sub>-R, inositol 1,4,5-trisphosphate receptor; OB-R, leptin receptor; P, phosphorylated residue; Gq, guanine nucleotide binding protein (9);  $\alpha$ ,  $\alpha$ -subunit of Gq protein.

physiological conditions, NO produced by leptin may account for the decrease of Ang II-induced vasoconstriction to maintain the vascular tone (Fig. 6). However, obesity-associated hypertension is characterized by hyperleptinemia, leptin resistance, and stimulation of the renin-angiotensin system (45–47). Thus, in the context of obesity-associated hypertension, hyperleptinemia may constitute a compensatory mechanism for vascular leptin resistance due to an exacerbated response to Ang II that decreases NO bioavailability and hence attenuates the vascular effects of leptin.

In conclusion, our results provide evidence that the depressor action of leptin on the Ang II-induced contractile response is mediated by NO-dependent mechanisms. Thus, leptin exerts a vasodilator effect on vascular beds by increasing NO release not only at the endothelial level but also in the smooth muscle layer. This statement is supported by the findings reported herein: 1) leptin stimulates the JAK2/STAT3 and PI3K/Akt pathways, enhancing iNOS expression and activity; 2) this effect takes place mainly at a posttranscriptional level; and 3) the subsequent NO production blunts the Ang II-induced VSMCs activation and vasoconstriction.

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