Leptin Induces Direct Vasodilation Through Distinct Endothelial Mechanisms

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In this study, we reveal that leptin evokes an acute hypotensive effect in 6-hydroxydopamine sympathectomized rats (response to maximal leptin dose, mean blood pressure: from 92 ± 4 to 78 ± 2 mmHg, $P < 0.01$). This hemodynamic effect is related to a direct action of the hormone on vascular tone, since in aortic and mesenteric rings increasing doses of leptin evoke a dose-dependent vasorelaxation (aorta: from 3 ± 1 to 36 ± 3, $n = 15$; mesenteric: from 6 ± 1 to 30 ± 5, $n = 10$), which is impaired by endothelial denudation. In particular, leptin-evoked vasorelaxation is impaired by nitric oxide synthase inhibition in aorta (Δ% of maximal response: from 36 ± 3 to 3 ± 1, $P < 0.01$) and by endothelium-derived hyperpolarizing factor (EDHF) inhibition in mesenteric arteries (Δ% of maximal response: from 30 ± 5 to 7 ± 2, $P < 0.01$), suggesting that vasorelaxation evoked by leptin is heterogeneous and related to the vascular bed. Finally, the inhibition of nitric oxide synthase by N^G-nitro-L-arginine-methyl ester does not modify blood pressure response to leptin, suggesting a predominant role of the EDHF mechanism in the hypotensive effect of leptin. *Diabetes* 49:293–297, 2000

Obesity is one of the most significant risk factors for vascular disorders such as hypertension and coronary artery disease (1,2). The discovery of the *ob* gene and analysis of its gene product have led to the identification of leptin, a novel adipocyte hormone, involved in the control of body weight (3–5). Several studies have dissected the metabolic effects of leptin and the mechanisms by which these actions are accomplished. A few studies have also examined the impact of leptin on blood pressure homeostasis. In particular, Haynes et al. (6) have demonstrated that acute intravenous leptin infusion is able to markedly increase sympathetic nervous activity without altering arterial blood pressure, suggesting that the lack of pressor effect coupled with sympathetic activation may result in opposite leptin influences. In contrast, chronic leptin administration evokes a sympathetic overactivity and blood pressure increase. These effects are more evident when leptin is infused into a carotid artery (7) or directly into cerebroventricles (8), suggesting that, in these circumstances, the hypertensive leptin influences are not affected by other opposite actions of the hormone.

This conflicting evidence in regard to leptin's effect on blood pressure homeostasis prompts us to better clarify the link between leptin and the primary mechanisms involved in blood pressure control. Although it has been well recognized that leptin promotes an effect on sympathetic nervous activity, the mechanism by which leptin counteracts the effect of sympathetic nervous system activity on blood pressure is still unclear.

On this issue, it has been recently reported that the leptin receptor is expressed in endothelial cells and is functionally active for the leptin-dependent angiogenic activity (9). It is also known that the endothelium has a pivotal role in blood pressure control (10) and, therefore, it may be hypothesized that leptin receptors on endothelium may also be involved in the modulation of vascular tone.

The first purpose of this study was to examine in vivo whether leptin has hemodynamic properties hidden by the concomitant sympathetic activation. For this reason, leptin's effect on blood pressure was tested after the removal of sympathetic nervous influences, obtained by chemical sympathectomy with 6-hydroxydopamine.

The second aim was to explore whether in both conduit and resistance arteries leptin is able to directly influence vascular tone and, eventually, to characterize the mechanisms by which this vascular action is accomplished.

RESEARCH DESIGN AND METHODS

Animals. A total of 60 normotensive Wistar Kyoto rats (Charles River, Como, Italy) were housed in groups of two per cage and received standard chow and water ad libitum. At the time of the study, the rats were 12–14 weeks old. The experimental procedures were performed in accordance with the Italian government's directions concerning the protection of animals used for scientific purposes.

Chemical sympathectomy. Destruction of efferent sympathetic nerve endings was accomplished by administration of 6-hydroxydopamine (Sigma, St. Louis, MO) (11). In particular, rats were injected with 150 mg/kg of 6-hydroxydopamine intraperitoneally and, subsequently, a mini-osmotic pump delivering 1.0 mg/kg of the drug daily was inserted subcutaneously. After 7 days, during the surgical session to attain femoral artery and vein catheterization, a further intravenous injection of 6-hydroxydopamine was administered (150 mg/kg dissolved in 0.2% ascorbic acid). Rats used as controls were treated in a similar way, with substitution of the drug with the vehicle alone (0.2% ascorbic acid). Before the blood pressure–monitoring session described below was started, the effectiveness of sympathectomy was tested by comparing the pressor and heart rate (HR) responses to tyramine (i.e., a drug that releases norepinephrine from sympathetic endings) at 150 μg/kg i.v. in treated versus control rats.

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EDHF, endothelium-derived hyperpolarizing factor; HR, heart rate; L-NAME, N^G-nitro-L-arginine-methyl ester; MBP, mean blood pressure; ODQ, 1H-1,2,4 oxadiazolo[4,3-a]quinoxalin-1-one; PBS, phosphate-buffered saline.

Blood pressure measurement. Two days before testing leptin's effect on blood pressure, all rats were anesthetized as described before, and polyethylene catheters (PE-50; Becton Dickinson, Sparks, MD) were inserted into a femoral artery and vein. Both catheters were filled with heparinized saline solution (100 µU/ml) and exteriorized subcutaneously at the interscapular area. After the surgery, the animals were housed in single cages and allowed to recover.

Direct intrafemoral arterial pressure was measured in conscious, freely moving rats after an overnight fast. At 8:00 A.M., the arterial catheter was connected to a pressure transducer (Statham P23db; Gould, Cleveland, OH) through an extension of polyethylene (PE-50) tubing (Becton Dickinson). After a resting period of at least 30 min, arterial blood pressure was monitored and recorded on a polygraph (Gould, Oxnard, CA) for 15 min after each stimulus.

Each rat, from both the sympathectomized and control groups, received intravenously increasing doses of leptin (7, 70, and 700 µg/kg dissolved in 100 µl of 0.9% phosphate-buffered saline [PBS]). The injection of 100 µl of the vehicle did not alter arterial pressure in either study group (data not shown).

In further groups of six intact and seven sympathectomized animals, the pressor response to leptin was accomplished during the inhibition of endogenous nitric oxide production realized by the administration of N^G-nitro-L-arginine-methyl ester (L-NAME) (15 mg/kg in bolus, followed by 30 mg · kg^{-1} · h^{-1}). Since nitric oxide inhibition per se evokes a marked increase of blood pressure, the hemodynamic variable used to reveal leptin effects in vivo, we decided to clamp blood pressure effects of nitric oxide inhibition with a concomitant infusion of sodium nitroprusside at a dose capable of restoring basal blood pressure levels. This strategy was adopted to rule out the incovenience inherent in testing the leptin effect in animals with high blood pressure levels induced by L-NAME, which may influence cardiovascular responses.

Vascular reactivity on aortic and mesenteric rings. On the day of the experiments, the rats were weighed and then decapitated. The thoracic aorta and the main branch of the mesenteric artery were dissected out from each rat and placed in cold Krebs-Henseleit bicarbonate buffer solution with the following composition (mmol/l): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 5.6. The aorta and mesenteric artery were cleaned of the adhering perivascular tissue and cut into rings 3 mm long. Aortic and mesenteric rings were suspended in isolated tissue baths filled with 20 ml Krebs solution continuously bubbled with a mixture of 5% CO_2 and 95% O_2 (pH 7.37–7.42) at 37°C. One end of the aortic and mesenteric rings was connected to a tissue holder and the other end to an isometric force transducer. The signal was passed to a Gould pressure processor and then acquired in a computerized system by Gould's Data Acquisition and Signal Analysis. The analysis of the generated curves was performed by the View II software (Gould, Oxnard, CA) and the sensitivity of the system was 5 ± 1 mg of tension generated. The rings were equilibrated for 90 min in the unstretched condition, and the buffer was replaced every 20 min. The length of the smooth muscle was increased stepwise in the equilibration period to adjust passive wall tension to 2 g for the aorta and 0.5 g for mesenteric artery. This tension was found optimal for contractions of the aorta and mesenteric artery by testing the vasoconstrictions to norepinephrine (10^{-3} mol/l). Once basal tension was established, the length of the rings was not altered thereafter. Caution was taken to avoid endothelium damage, and the functional integrity of this structure was reflected by the response to 10^{-7} mol/l acetylcholine. Increasing doses of human recombinant leptin (10^{-13} – $5 \cdot 10^{-10}$ mol/l, dissolved in 0.9% PBS) (Calbiochem-Novabiochem, La Jolla, CA) were tested both on untreated aortic and mesenteric rings and on rings preconstricted with phenylephrine (10^{-6} mol/l). In some experiments, the endothelium was mechanically removed. Furthermore, a dose response curve to leptin was tested in the presence of different pharmacological tools such as L-NAME ($3 \cdot 10^{-4}$ mol/l, 30 min), a nitric oxide synthase inhibitor; Brefeldin A ($3.5 \cdot 10^{-5}$ mol/l, 90 min), an endothelium-derived hyperpolarizing factor (EDHF) inhibitor (12); and 1H-1,2,4 oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10^{-6} mol/l, 15 min), an inhibitor of the smooth muscle soluble guanylyl cyclase selectively induced by nitric oxide (13).

Immunohistochemistry. Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and killed by the placement of a 22-gauge butterfly catheter in the left ventricle and in situ constant pressure fixation (100 mmHg) with 4% paraformaldehyde in 0.1 mol/l PBS, pH 7.3. Aorta, carotid, and mesenteric arteries were harvested, embedded in paraffin, sectioned (5 µm), and placed on glass slides. Sections were treated with 5% pronase in PBS and then incubated with leptin receptor antiserum (Santa Cruz, Heidelberg, Germany). The avidin-biotin peroxidase complex method (Vector, Burlingame, CA) was used to label the primary antibody. The primary antiserum was raised in goats against a peptide corresponding to amino acids 874–894 of the leptin receptor. This peptide is common to both the long and short isoforms of the leptin receptor. Control experiments were accomplished using the above-described method omitting the primary antibody. Preabsorption controls were also performed using the recombinant protein (50 µmol/l) incubated with the primary antiserum for 2 h at room temperature.

Statistical analysis. Data are expressed as means ± SE. Statistical differences were calculated with analysis of variance followed by Bonferroni test for intergroup comparisons, or unpaired Student's *t* test when only two groups were compared. A threshold value of $P < 0.05$ was taken as significant.

RESULTS

Blood pressure studies. The effectiveness of sympathectomy was tested by intravenous injection of tyramine. In particular, tyramine increased mean blood pressure (MBP) (from 102 ± 4 to 158 ± 8 mmHg, $n = 5$, $P < 0.01$) and HR (from 288 ± 12 to 348 ± 7 beats/min, $P < 0.01$) in control rats, whereas it did not evoke any significant response in animals treated with 6-hydroxydopamine (MBP: from 93 ± 4 to 94 ± 5 mmHg, NS; HR: from 275 ± 10 to 280 ± 12 beats/min, NS, $n = 7$). As shown in Fig. 1, in control rats intravenous injection of increasing doses of leptin did not change blood pressure, even if a significant increase in HR (from 310 ± 10 to 344 ± 12 beats/min, $n = 7$, $P < 0.01$) was recorded. In contrast, increasing doses of leptin injection evoked a blood pressure decrease in animals treated with 6-hydroxydopamine, without altering HR (from 280 ± 11 to 282 ± 10 beats/min, NS, $n = 7$) (Fig. 1).

In additional series of experiments, L-NAME produced a marked increase of blood pressure both in intact (MBP: from 97 ± 4 to 131 ± 4 mmHg, $P < 0.01$) and sympathectomized rats (MBP: from 90 ± 3 to 127 ± 4 mmHg, $P < 0.01$), whereas the HR decreased in intact animals (from 305 ± 17 to 252 ± 15 beats/min, $P < 0.01$) and remained substantially unmodified in sympathectomized animals (HR: from 287 ± 12 to 285 ± 15 beats/min, NS). The concomitant infusion of sodium nitroprusside restored basal blood pressure in both study groups and abolished the reflex increase of heart rate in intact rats, whereas it did not influence heart rate in sympathectomized rats. In these latter experimental conditions, leptin did not influence blood pressure (response to maximal leptin dose, MBP: 98 ± 5 vs. 95 ± 4 mmHg, NS) but increased HR (response to maximal leptin dose: from 305 ± 12 to 338 ± 11 beats/min, $P < 0.01$) in intact animals. More important, in sympathectomized rats, the adipocyte hormone induced a significant blood pressure decrease (response to maximal leptin dose, MBP: from 88 ± 4 to 65 ± 4 mmHg, $P < 0.01$) without altering HR (response to maximal leptin dose: 292 ± 5 vs. 290 ± 4 beats/min, NS).

Vascular reactivity studies. Increasing doses of recombinant leptin induced a slight decrease of passive wall tension (data not shown) in both conduit and resistance vessels. Leptin evoked a clear dose-dependent relaxation both in aortic (Fig. 2) and mesenteric rings (Fig. 3) preconstricted with phenylephrine (10^{-6} mol/l). The leptin-mediated vasorelaxation was impaired by the endothelium denudation both in conduit and resistance arteries (Figs. 2 and 3). However, whereas in aortic rings leptin-induced vasorelaxation was abolished by L-NAME or ODQ (Fig. 2), in mesenteric rings the dose-dependent vasorelaxation evoked by the adipocyte hormone was counteracted by Brefeldin A (Sigma-Aldrich S.R.L., Milan, Italy) and was not influenced by L-NAME (Fig. 3).

Immunohistochemical analysis. As shown in the aorta (Fig. 4), a dense immunostaining for leptin receptor was present on the endothelium, whereas no labeling was exhibited by the media. In the preabsorption controls, there was no labeling in the vessels. Similar results were obtained in carotid and mesenteric arteries (data not shown).

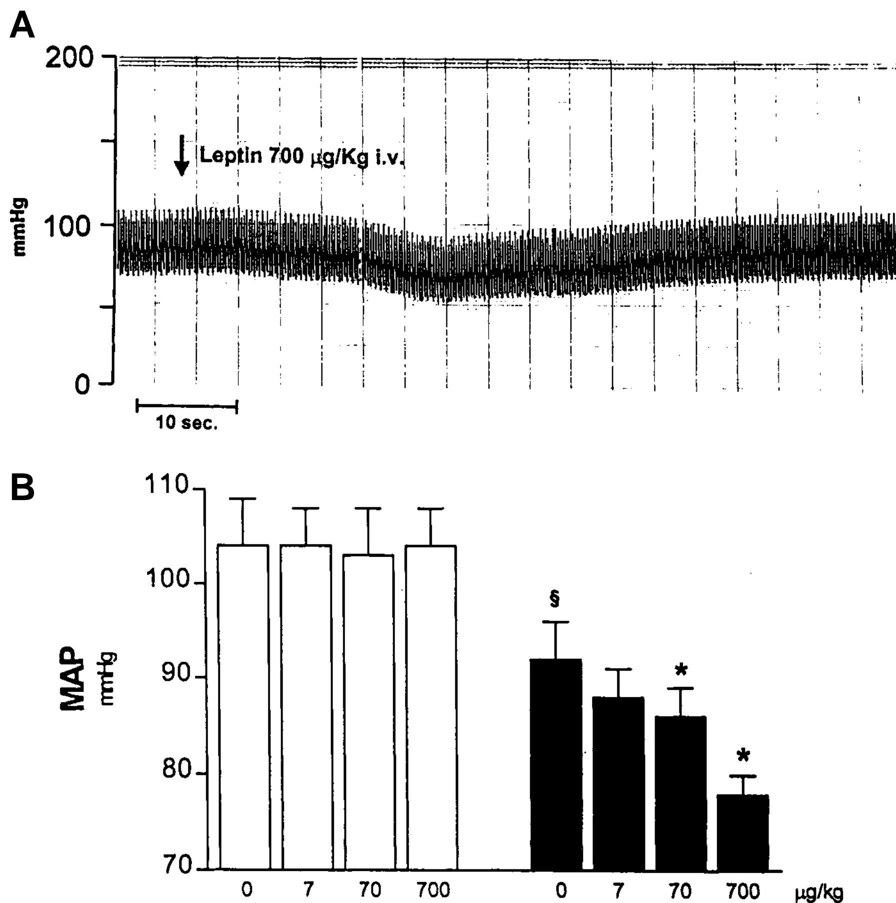

FIG. 1. A: Effect of intravenous injection of the highest leptin dose on arterial blood pressure in a rat sympathectomized with 6-hydroxydopamine. B: Effect of increasing doses of leptin on MPB in sympathectomized (■; n = 7) and in control (□; n = 5) rats. §P < 0.01 as compared with control rats; *P < 0.01 as compared with 0 (vehicle).

DISCUSSION

This study demonstrates that leptin evokes a hypotensive effect when sympathetic nervous system influences are abolished. Such hemodynamic action of leptin fits with the direct endothelial-mediated vasorelaxation evoked by the hormone on both conduit and resistance arteries, through nitric oxide or EDHF mechanisms, respectively.

Although leptin induces a sympathetic nervous system activation, as evidenced by the increase in HR and regional sympathetic nervous activity (6), any blood pressure change is detectable during the exposure to the hormone, suggesting that the sympathetic-mediated pressor effect may be offset by other leptin actions. In this study, we provide evidence that a leptin hypotensive effect is disclosed when sympathetic control is removed by chemical sympathectomy. Thus, it is possible to speculate that the influence of leptin on blood pressure homeostasis is the result of a balance between the pressor action through sympathetic activation and the hypotensive effect of the hormone. However, in particular circumstances, the increase of sympathetic nervous activity induced by leptin might overcome its opposite hypotensive effect,

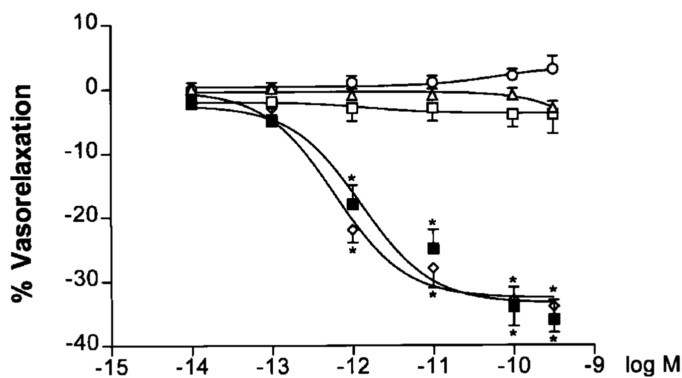

FIG. 2. Vascular response of aortic rings precontracted with phenylephrine to increasing doses of leptin (■, n = 15) in endothelium-denudated vessels (□; n = 5) after exposure to L-NAME (△; n = 5), ODQ (○; n = 5), or brefeldin A (◇; n = 5). *P < 0.01 as compared with control.

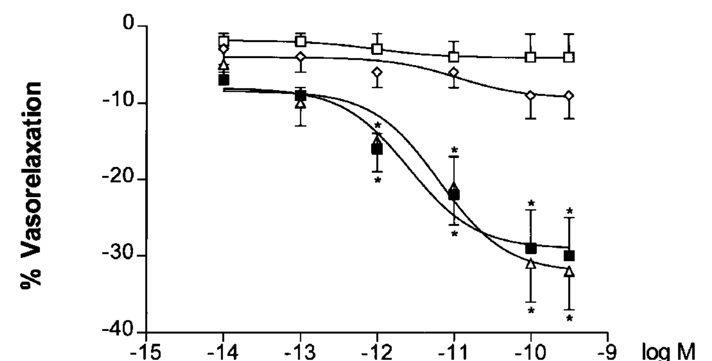

FIG. 3. Vascular response of mesenteric rings precontracted with phenylephrine to increasing doses of leptin (■; n = 10), in endothelium-denudated vessels (□; n = 5) after exposure to L-NAME (△; n = 5) or brefeldin A (◇; n = 5). *P < 0.01 as compared with control.

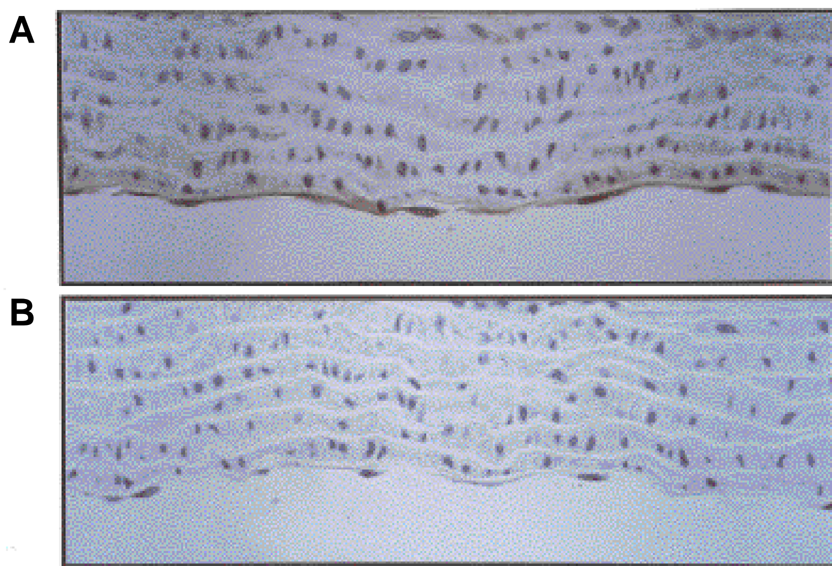

FIG. 4. A: Leptin-receptor immunoreactivity labeling of the aorta. B: Experimental control omitting primary antibody. Original magnification ×40.

resulting in an increase of arterial blood pressure. In this regard, there is evidence that the acute intracerebroventricular infusion of leptin evokes a sympathetic overactivity and a concomitant increase of blood pressure (8), indicating that in these experimental conditions, with a minimal spillover of the adipocyte hormone in the systemic circulation, the sympathetic-mediated pressor effect of leptin is not counteracted by the hypotensive action of the hormone. This suggests that the hypotensive effect of leptin has to be ascribed to peripheral actions of the hormone.

Although this hypotensive effect is mainly detectable when leptin is infused at supraphysiological range, a tendency to decrease blood pressure is noticed also at a lower dose. Furthermore, the hemodynamic effect of leptin may have a different threshold concentration when superimposed with other factors, such as hypertension or obesity.

To identify whether this latter effect of the adipocyte hormone may be related to a direct action on vascular tone, we analyzed the effects of increasing doses of leptin on conduit and resistance vessels. In particular, in an elementary model of vascular function, such as vascular rings, leptin induces a clear vasorelaxation both in aortic and mesenteric arteries, which is abolished by endothelial denudation, suggesting that the integrity of endothelium is critical for leptin vascular action. Moreover, evidence that leptin receptors are only on the endothelium of the vessels further strengthens the concept that the direct leptin vasorelaxation is entirely dependent on endothelial mechanisms.

Our study has also explored the intimate mechanisms by which leptin exerts its vasorelaxant action. In particular, the leptin-evoked vasorelaxation on aorta is abolished by L-NAME or ODQ, which specifically inhibits endothelial nitric oxide function at two distinct sites. In contrast, the vasorelaxant effect of leptin on mesenteric arteries is counteracted by Brefeldin A, an EDHF inhibitor, and is not influenced by endothelial nitric oxide inhibition, indicating that the mechanisms involved in the vasorelaxation evoked by leptin are heterogeneous and related to vascular bed.

Finally, our data indicate that during inhibition of endothelial nitric oxide production, leptin does not increase blood pressure levels in intact rats and is still able to induce a hypotensive response in sympathectomized animals, suggesting that the vasorelaxant effect of leptin on conduit vessels through the nitric oxide mechanism has only a marginal role in the hypotensive action of the hormone. On the other hand, the observation that leptin vasorelaxation on mesenteric arteries involves EDHF release allows the hypothesis that the acute hypotensive effect evoked by the hormone depends on its action on resistance vessels, which have a main role in the blood pressure homeostasis as compared to conduit vessels (15).

Last year, Frühbeck (16) showed the hypotensive effect of a single-bolus injection of leptin in anesthetized rats treated with the ganglion-blocking agent chlorisondamine. These findings are in agreement with our observations, even if a main methodological difference with our study has to be pointed out. Actually, Frühbeck monitored the leptin effect on blood pressure 90 min after the injection of the hormone, whereas we evaluated the hemodynamic consequences in conscious rats just after the infusion of leptin. In particular, we observed that the hypotensive response to a single-bolus injection of leptin has a short time course, as shown in Fig. 1A. In contrast, Frühbeck evidenced a late hypotensive component evoked by leptin, ascribed to a nitric oxide mechanism, omitting the description of the early effects of the hormone on blood pressure. These results, taken together with our observations, suggest that the late hypotensive effect of leptin may be dependent on a mechanism involving mainly conduit arteries, in which leptin exerts its vasorelaxant effect through a nitric oxide mechanism. On the other hand, we cannot entirely exclude the possibility that the hypotensive effect evoked by leptin may be exerted through other vascular mechanisms.

In conclusion, leptin's effect on blood pressure homeostasis results from the balance of a direct vasorelaxant action realized by endothelial mechanisms and a concomitant sympathetic nervous system activation. It is noteworthy to emphasize that the hemodynamic effect induced by leptin resembles some of the actions evoked by insulin (17,18). In particular, both hormones have the ability to stimulate the sympathetic nervous system and concomitantly to evoke a vasorelaxant effect that blunts the impact of the sympathetic nervous system on blood pressure homeostasis. Since several

studies have clearly demonstrated that leptin is able to modulate insulin-regulated metabolic responses (19), it will be interesting to explore whether the interaction between the two hormones is evident also at the cardiovascular level. Moreover, it has to be considered that even though there is an increased prevalence of hypertension among overweight individuals, the pathophysiological mechanisms underlying this common association are still unknown. Thus, defects in leptin vascular action may contribute to the inadequate increase of peripheral vascular resistance, a pivotal phenotypical trait of hypertension associated with obesity.

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