

Cooperation Between Insulin and Leptin in the Modulation of Vascular Tone

Carmine Vecchione, Alessandra Aretini, Angelo Maffei, Gennaro Marino, Giulio Selvetella, Roberta Poulet, Valentina Trimarco, Giacomo Frati, Giuseppe Lembo

Abstract—High levels of insulin and leptin have been reported in human hypertension, suggesting a role for these metabolic hormones in blood pressure homeostasis. These hormones interact on intermediate metabolism, but nothing is known about their interaction at the vascular level. Our data demonstrate that insulin (0.6 nmol/L) is able to enhance vasodilation induced by leptin (10^{-11} to 10^{-6} mol/L; percentage change in maximal vasodilation, $39 \pm 3\%$ vs $26 \pm 2\%$; $n=6$, $P<0.03$) but not by acetylcholine. Moreover, we demonstrate by 4,5-diaminofluorescein (DAF)-2 that insulin potentiates leptin-induced nitric oxide (NO) release. Finally, Western blotting studies show that insulin enhances the leptin-induced phosphorylation of Akt in Ser⁴⁷³ and Thr³⁰⁸ and of endothelial NO synthase in Ser¹¹⁷⁷. In conclusion, our data demonstrate that insulin and leptin cooperate in the modulation of vascular tone through enhancement of endothelial NO release. This phenomenon could have a major impact on the regulation of the cardiovascular system, principally in those clinical conditions characterized by endothelial NO dysfunction and metabolic disorders, such as arterial hypertension. (*Hypertension*. 2003;42:166-170.)

Key Words: insulin ■ leptin ■ endothelium-derived factors ■ nitric oxide synthase ■ phosphorylation ■ vasorelaxation

Insulin and leptin are two metabolic hormones that play key roles in the modulation of intermediate metabolism. Dysregulation of their levels and impairment of their action have been reported in cardiovascular disease such as arterial hypertension.^{1,2} In particular, in humans, hyperinsulinemia and hyperleptinemia have been associated with high blood pressure levels,^{3,4} suggesting that these hormones might be involved in blood pressure homeostasis.

On this issue, it has been observed that insulin and leptin, independent of metabolic action, exert influences on the cardiovascular system. In particular, short-term infusion of both hormones induces contrasting effects on the modulation of vascular tone in vivo because they are able to increase sympathetic nervous activity and exert a direct vasodilator effect.⁵⁻⁷ A correct balance between these actions participates in the healthy control of vascular function.

It has been clearly demonstrated that the endothelium represents the main vascular target of insulin and leptin, because both hormones exert vasorelaxant effects through endothelial nitric oxide (NO) release.^{7,8} In particular, the effect of insulin on endothelial NO is mediated by phosphatidylinositol 3-kinase-dependent Akt activation, which directly phosphorylates endothelial nitric oxide synthase (eNOS) in Ser¹¹⁷⁷.⁹⁻¹¹ In addition, we have also recently demonstrated that leptin's effect on endothelial NO release is mediated by Akt.¹² Thus, insulin and leptin evoke effects on

vascular tone by increasing eNOS activity through an Akt-dependent mechanism. It is well known that insulin and leptin can interact on several target tissues. In particular, leptin enhances the insulin effect on glucose production in the liver, reverses hepatic and skeletal muscle insulin resistance in patients with lipodystrophy, and influences specific intracellular components of the insulin-signaling cascade in hepatic cells.¹³⁻¹⁵

However, all of the aforementioned studies have focused their attention on the interaction between the two hormones at the metabolic level. So far, no data are available on the possible interaction between insulin and leptin at the vascular level. Therefore, the aim of this study was to explore the vascular interplay between these two hormones.

Methods

Animals

The studies were conducted on male Wistar-Kyoto rats (WKY, $n=32$; Charles River, Calco, Italy), aged 12 to 14 weeks. Rats were housed 2 per cage, kept in a temperature-controlled (23°C to 25°C) room with a 12-hour light/dark cycle, and fed standard rodent chow and water ad libitum. The experiments were performed after the rats had been acclimatized to their housing conditions for at least 1 week. On the day of the experiments, the rats were weighed and then decapitated. The thoracic aorta was dissected out from each rat, cleaned of adhering perivascular tissue, and placed in cold Krebs-Henseleit bicarbonate buffer solution of the following composition

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From the Department of Angio-Cardio-Neurology (C.V., A.A., A.M., G.M., G.S., R.P., V.T., G.F., G.L.), IRCCS Neuromed, Pozzilli (IS), and the Department of Experimental Medicine and Pathology (G.L.), La Sapienza University, Rome, Italy.

Correspondence to Giuseppe Lembo, MD, PhD, IRCCS Neuromed, Località Camerelle, 86077 Pozzilli (IS), Italy. E-mail lembo@neuromed.it

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(in mmol/L): NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ · 7 H₂O 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 5.6. All experimental procedures were in accordance with guidelines for research on animals at our institutions.

Vascular Reactivity

Aortic rings were suspended in isolated tissue baths filled with 20 mL Krebs' solution continuously bubbled with a mixture of 5% CO₂ and 95% O₂ (pH 7.37 to 7.42) at 37°C. One end of the aortic ring was connected to a tissue holder and the other, to an isometric force transducer. The signal was passed to a pressure processor (Gould Instruments) and then acquired in a computerized system by Gould's DASA (Data Acquisition and Signal Analysis). Analysis of the generated curves was performed with View II software (Gould Instruments), and the sensitivity of the system was 5 ± 1 mg of tension generated. The rings were equilibrated for 90 minutes in the unstretched condition, and the buffer was replaced every 20 minutes. The length of the smooth muscle was increased stepwise during the equilibration period to adjust passive wall tension to 2.0 g. 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 acetylcholine (10⁻⁶ mol/L, Sigma). The maximal contraction evoked by phenylephrine was considered as the baseline when subsequent vasorelaxations were evoked. Vasorelaxation was expressed as percentage reduction in contraction (the maximal vasorelaxation attained with papaverine being 100% vasorelaxation).

On aortic rings precontracted with phenylephrine (10⁻⁶ mol/L, Sigma), we evaluated the vascular effect evoked by increasing doses of leptin (10⁻¹¹ to 10⁻⁷ mol/L, Calbiochem). Furthermore, dose-response curves to leptin and to acetylcholine (10⁻⁹ to 10⁻⁵ mol/L) were tested after preincubation with insulin (0.6 nmol/L, 20 minutes; Lilly). Finally, the insulin effect on leptin vasorelaxation was tested in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 300 μmol/L, 10 minutes; Sigma), an eNOS inhibitor.

Evaluation of NO Production by DAF-2

Aortic rings were incubated for 2 hours in the dark in aerated (95% O₂, 5% CO₂) Krebs' buffer containing 4,5-diaminofluorescein (DAF)-2 diacetate (10 μmol/L, Alexis). Leptin (10 nmol/L) was administered during the last 30 minutes of DAF-2 incubation, alone, and after 20 minutes of exposure to insulin (0.6 nmol/L). Some experiments were also performed in the presence of L-NAME (300 μmol/L).

Vascular rings were removed and frozen at -20°C. They were cut into 10-μm-thick sections in a cryostat (Jung CM3000, Leica). Sections were placed onto microscope slides without any mounting medium or coverslip. Specimens were observed under a fluorescence microscope (Axiophot2, Zeiss) at 200× magnification. Twenty-four-bit color pictures were taken at 3 different wavelengths (corresponding to green, red, and blue fluorescence) with a digital camera system coupled to imaging software (Spot, Diagnostic Instruments) under constant exposure time, gain, and offset. To account for fluorescence decay, all images were taken in the first 30 seconds of light exposure. The images were then merged so that the 515-nm emitted fluorescence due to DAF appeared green above a purple all-wavelength emitted autofluorescence background derived from the fibers of the vessel. Green fluorescence intensity was quantified with the imaging software.

Western Blot Analysis

Aortas were kept in Krebs' solution at 37°C in an incubator (95% O₂, 5% CO₂) for 1 hour before stimulation with insulin (0.6 nmol/L, 20 minutes) and/or leptin (10 nmol/L, 10 minutes). Then the aortas were frozen in LN₂ and homogenized in the following lysis buffer: 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% sodium deoxycholate, 10 mmol/L NaF, 1 mmol/L Na₃VO₄, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 nmol/L okadaic acid, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin. Insoluble material was removed by centrifugation at

14 000 rpm in a Microfuge for 15 minutes at 4°C. An aliquot of supernatant was used for Bradford protein determination. Lysates (35 μg) were subjected to Western blot analysis with 8% sodium dodecyl sulfate polyacrylamide gels and run on a Maxi-gel apparatus (Bio-Rad). Gels were electroblotted onto nitrocellulose membranes (Bio-Rad) for 1 hour with use of a semi-dry electroblotting system (Bio-Rad Transblot system), and the filters were blocked for 1 hour at room temperature in a buffer of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk. Blots were then incubated overnight at 4°C with anti-phospho(Ser⁴⁷³) Akt (1:500, Cell Signaling), phospho(Thr³⁰⁸) Akt (4 μg/mL, Upstate Biotechnology), or phospho(Ser¹¹⁷⁷) eNOS (Cell Signaling) antibodies. Blots were washed 3 times with 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20 and incubated for 1 hour with peroxidase-coupled anti-rabbit (Amersham) secondary antibodies. Finally, protein detection was performed with a commercially available kit (ECL, Amersham). To detect protein levels, the membranes were stripped and reblotted with anti-Akt (Cell Signaling) or eNOS (Transduction Laboratories) antibodies. Detection was performed as described earlier. The intensity of the bands was quantified by scanning densitometry with NIH Image 1.61 software.

Analysis of Akt phosphorylation was also performed on human aortic endothelial cells (BioWhittaker). Cells were placed in essential growth medium (10 ng/mL human endothelial growth factor, 1 mg/mL hydrocortisone, 50 mg/mL gentamicin, 50 μg/mL amphotericin-B, and 3 mg/mL bovine brain extract) containing 10% fetal bovine serum on 60-mm plates and were studied before cell confluence at passages 4 to 6. Plates were washed with phosphate-buffered saline and starved for 24 hours in endothelial basal medium containing 1% fetal bovine serum. Then, Akt phosphorylation in response to insulin and/or leptin was assessed as described previously.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed by 2-way ANOVA followed by the Bonferroni test.

Results

Vascular Reactivity Studies

Insulin significantly enhanced the leptin-induced, dose-dependent vasorelaxation (Figure 1A), but it did not modify that evoked by acetylcholine (Figure 1B). In the presence of L-NAME, leptin vasorelaxation was impaired (percentage of maximal vasorelaxation: 2 ± 1% vs 25 ± 3%; *P* < 0.01), and insulin did not further influence it (data not shown).

DAF-2 Studies

As shown in Figure 2, we directly assessed NO production by DAF-2 and observed that leptin evoked, in WKY aortas, an increase in DAF-2 fluorescence compared with control. Furthermore, insulin did not modify basal DAF-2 fluorescence, but it markedly potentiated that evoked by leptin. In the presence of L-NAME, insulin and leptin did not evoke any effects.

Western Blot Analysis

In aortic rings, insulin or leptin exposure was able to evoke Akt phosphorylation in Ser⁴⁷³ (Figure 3) and Thr³⁰⁸ (data not shown) compared with control. Exposure to both hormones further enhanced Akt phosphorylation compared with leptin and insulin alone. In addition, leptin induced eNOS Ser¹¹⁷⁷ phosphorylation compared with control, whereas insulin did not significantly influence it. The same insulin levels were able to greatly potentiate eNOS Ser¹¹⁷⁷ phosphorylation evoked by leptin (Figure 4).

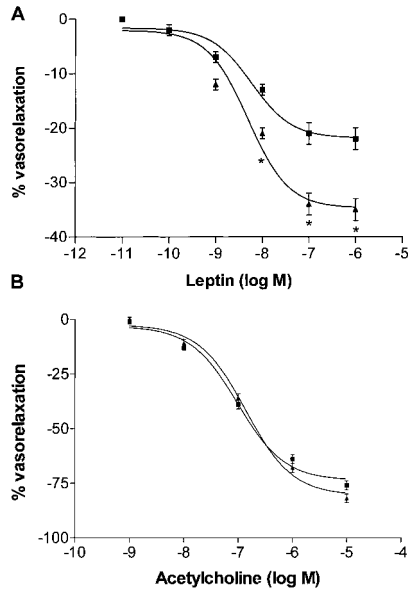


Figure 1. Vasular responses of aortic rings from WKY rats (n=12), precontracted with phenylephrine, to increasing doses of leptin (A) and acetylcholine (B), either alone (squares) or after preincubation (triangles) with 0.6 nmol/L insulin. *P<0.01 vs leptin alone.

As shown in Figure 5, in endothelial cells, insulin induced only slight Akt phosphorylation. Leptin induced Akt phosphorylation to a degree comparable to that observed in whole vessels. The combination of the two hormones induced a significant increase in Akt phosphorylation compared with leptin and insulin alone.

Discussion

Our results demonstrate that insulin and leptin cooperate in the modulation of vascular tone through enhancement of endothelial NO release. In our study, the vascular interaction between insulin and leptin has been demonstrated on several grounds. In particular, by using different methodological approaches, we have observed that cooperation between the two hormones is revealed at functional, biochemical, and

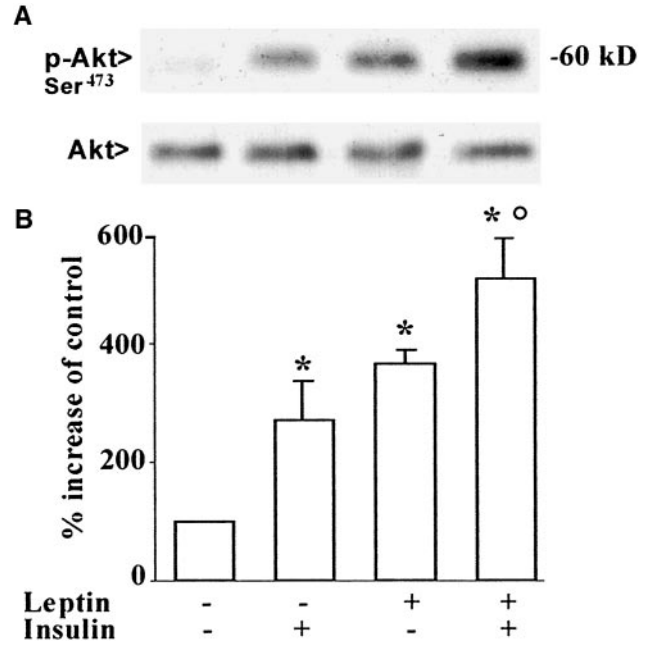


Figure 3. Akt phosphorylation in Ser⁴⁷³ on aortic vessels from WKY (n=8), under control conditions and after stimulation with 10 nmol/L leptin, 0.6 nmol/L insulin, or the combination of both. A, Representative Western blot. B, Quantification of Akt phosphorylation. *P<0.01 vs control, °P<0.01 vs leptin or insulin alone.

molecular levels. Insulin is able to enhance leptin-evoked vasodilatation but does not modify that evoked by acetylcholine, thus suggesting that insulin's effect on leptin vasorelaxation is not due to a general enhancement of endothelial function but depends on a selective interaction between the two hormones.

Our data provide the first evidence of direct cross-talk between leptin and insulin at the vascular level, showing the strong functional relevance of the combination of both hormones on vascular tone. This finding is in agreement with previous observations on metabolic target tissues, which have reported that leptin and insulin signaling can be interconnected.¹³⁻¹⁵

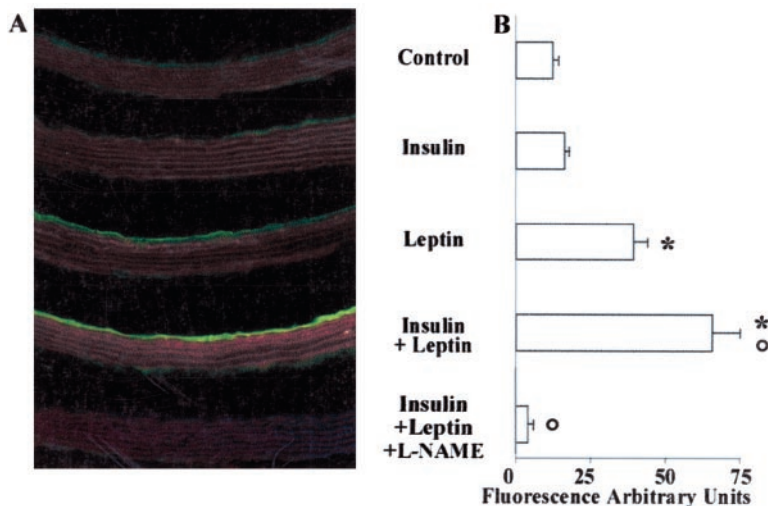


Figure 2. NO production in aortic rings from WKY (n=5), as assessed by DAF-2 fluorescence (green), under basal conditions, after stimulation with 10 nmol/L leptin or 0.6 nmol/L insulin, and after concomitant stimulation with leptin and insulin, alone or after preincubation with 0.3 mmol/L L-NAME. A, Representative high-power photomicrograph. B, Quantification of DAF-2 fluorescence. *P<0.01 vs control, °P<0.01 vs leptin or insulin alone.

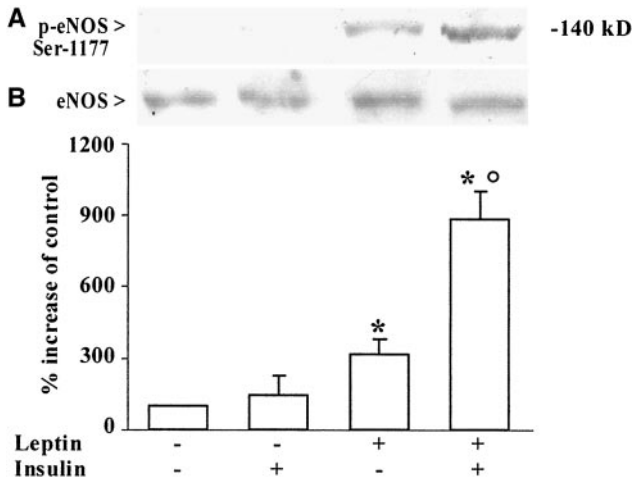


Figure 4. eNOS phosphorylation in Ser¹¹⁷⁷ on aortic vessels from WKY (n=6), under control conditions, after stimulation with 10 nmol/L leptin or 0.6 nmol/L insulin, or after concomitant stimulation with leptin and insulin. A, Representative Western blot. B, Quantification of eNOS phosphorylation. *P<0.01 vs control, °P<0.01 vs leptin or insulin alone.

The dose of insulin used in our study was in the physiologic range, corresponding to the levels of the hormone that are reached in the postprandial state. Previous studies have revealed that these insulin levels do not evoke direct vasodilation, induced only by pharmacologic doses of the hormone,^{16,17} but are able to blunt the vasoconstriction evoked by several agonists.^{18,19} However, L-NAME, an NOS inhibitor, abolishes both pharmacologic and physiologic vasorelaxant effects of insulin, thus indicating that mechanisms related to NO are involved in the vasorelaxant effects of the hormone.^{17,20,21} Nevertheless, although high levels of insulin clearly induce endothelial NO release, this effect is not detectable at physiologic levels of the hormone. Thus, we hypothesized that the potentiation of leptin vasodilation evoked by physiologic insulin levels could depend on sensitization of leptin intracellular signaling converging on NO release.

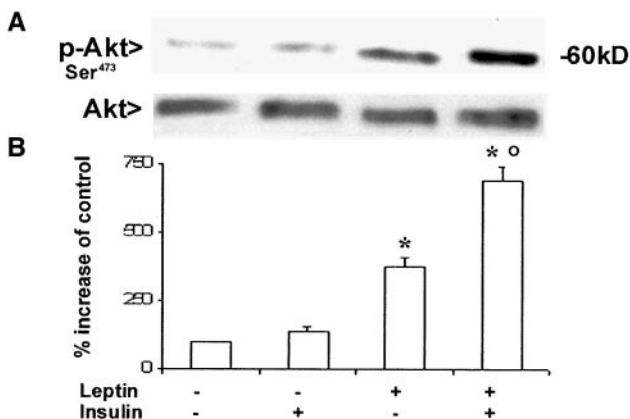


Figure 5. Akt phosphorylation in Ser⁴⁷³ on endothelial cells (n=4), under control conditions and after stimulation with 10 nmol/L leptin, 0.6 nmol/L insulin, or the combination of both. A, Representative Western blot. B, Quantification of Akt phosphorylation. *P<0.01 vs control, °P<0.01 vs leptin or insulin alone.

Actually, our results also demonstrate that insulin exposure is able to further increase leptin-evoked Akt phosphorylation in the vessels. This phenomenon has been observed in 2 different Akt residues, such as Ser⁴⁷³ and Thr³⁰⁸, both of which have been demonstrated to induce activation of the catalytic properties of Akt.²² On this issue, a previous observation has shown in hepatic cells that the effects of insulin and leptin on Akt are not additive.¹³ However, those investigators used a dose of insulin that was ≈200-fold higher than that used in our study, which determined that massive Akt activation could not be further increased by concomitant leptin administration.

It is well recognized that Akt is a common molecular target of insulin and leptin. In particular, insulin rapidly activates Akt in skeletal muscle and endothelial cells.^{11,23} Similarly, leptin activates Akt in hepatocytes and vessels.^{9,13} Akt, besides its role in glycogen synthesis,¹³ cell growth,²⁴ and survival,²⁵ can modulate endothelial NO production because it is able to stimulate eNOS activity by phosphorylating the enzyme on Ser¹¹⁷⁷.^{8–11} On this issue, the results of the present study show that insulin enhances leptin-evoked phosphorylation of eNOS in Ser¹¹⁷⁷, thus leading to increased enzymatic activity. This latter concept is sustained by our studies with DAF-2, which directly assessed the production of NO in the vessels and demonstrated higher endothelial NO production with concomitant exposure to insulin and leptin.

It is known that the role of NO on the effects of insulin decreases with diminishing vessel size.²⁶ In our study, we used a conduit vessel (rat aorta), which represents a good model to evaluate the effect of insulin on vascular NO release. In smaller vessels, it has been observed that insulin relaxation is also mediated by other mechanisms, such as hyperpolarizing factors (EDHF).²⁶ Interestingly, we have recently also demonstrated that the action of leptin on small vessels is dependent on EDHF.⁷ Further studies are needed to verify whether the vascular cooperation between the two hormones observed on NO could be extended also to EDHF and, therefore, to smaller vessels.

In our article, we have shown a functional interaction between insulin and leptin in vessels that induced NO release. Furthermore, the combination of the two hormones led to hyperactivation of Akt and eNOS, which could represent the molecular effectors of the interplay between the intracellular signaling of the two hormones at the endothelial level. In our study, however, we do not have a direct demonstration that the observed molecular mechanisms are effectively involved in the vasorelaxant effects. However, a greater eNOS phosphorylation in Ser¹¹⁷⁷ has been clearly related to increased NO vasorelaxation in intact blood vessels,²⁷ and it has been also demonstrated that Akt can selectively phosphorylate eNOS in Ser¹¹⁷⁷,^{10,11} thus inducing NO production. Therefore, we inferred a cause-effect relation between the concomitant increase in both AKT/eNOS phosphorylations and vasorelaxation in response to insulin and leptin.

Evidence emerging from our molecular studies in vessels is that the effect of potentiation on Akt phosphorylation induced by insulin and leptin is not as marked as that observed on eNOS phosphorylation. It is important to note that our results on Akt in whole vessels could reflect an effect of the

hormones on both the endothelium and smooth muscle, whereas only endothelial Akt phosphorylates eNOS in Ser¹¹⁷⁷.^{10,11} Actually, when we restricted our analysis to endothelial cells, we were able to reveal a marked insulin potentiation of leptin-evoked Akt phosphorylation similar to that observed on eNOS phosphorylation and NO production. Moreover, our data indicating Akt as a target of the convergence between insulin and leptin signaling pathways allow us to also hypothesize that the interaction between insulin and leptin could influence other Akt-dependent vascular effects, such as angiogenesis, cell growth, and survival. This is a largely unexplored area, and further data on this issue would better characterize the interaction between insulin and leptin at the vascular level.

In conclusion, our data demonstrate that insulin and leptin cooperate in the modulation of vascular tone through enhancement of endothelial NO release. This phenomenon could have a major impact on the regulation of the cardiovascular system, principally in those clinical conditions characterized by endothelial NO dysfunction and metabolic disorders, such as arterial hypertension.

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