Cooperation Between Insulin and Leptin in the Modulation of Vascular Tone

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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^{-8} mol/L; percentage change in maximal vasodilation, 39±3% vs 26±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 Ser^{473} and Thr^{308} and of endothelial NO synthase in Ser^{1177}. 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. In particular, in humans, hyperinsulinemia and hyperleptinemia have been associated with high blood pressure levels, 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. 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. 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 Ser^{1177}. In addition, we have also recently demonstrated that leptin’s effect on endothelial NO release is mediated by Akt. 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.

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:

- Sodium chloride 118.0 mmol/L
- Sodium bicarbonate 25.0 mmol/L
- Potassium chloride 4.7 mmol/L
- Calcium chloride 2.5 mmol/L
- Magnesium chloride 1.2 mmol/L
- Glucose 11.0 mmol/L
- Sodium pyruvate 11.0 mmol/L
- Sodium lactate 5.0 mmol/L
- Ethylenediaminetetraacetic acid (EDTA) 0.7 mmol/L

The pH was adjusted to 7.4 with sodium hydroxide, and the solution was gassed with a mixture of 95% oxygen and 5% carbon dioxide.

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administered during the last 30 minutes of DAF-2 incubation, alone,
response curves to leptin and to acetylcholine (10^(-9) to 10^(-6) M) and/or leptin (10 nmol/L, 10 minutes). Then the aortas were
sliced into 10-μm-thick sections in a cryostat (Jung CM3000, Leica). The images were then merged so that the 515-nm emitted fluores-
cence background derived from the fibers of the vessel could be greatly potentiated. 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 Mini-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 Trans blot 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-phosphoSer(1177) Akt (1:500, Cell Signaling), phosphoThr(308) Akt (4 μg/mL, Upstate Biotechnology), or phosphoSer(1177) 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 antibod-
ies. Finally, protein detection was performed with a commercially available kit (ECL, Amersham). To detect protein levels, the mem-
branes were stripped and reblinked with anti-Akt (Cell Signaling) or eNOS (Transduction Laboratories) antibodies. Detection was per-
formed as described earlier. The intensity of the bands was quanti-
fied 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**

Aortas were kept in Krebs’ solution at 37°C in an incubator (95% O_2, 5% CO_2) for 1 hour before stimulation with insulin (0.6 mmol/L, 20 minutes) and/or leptin (10 nmol/L, 10 minutes). Then the aortas were frozen in LN_2 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 Na_3VO_4, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L okadaic acid, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin. Insoluble material was removed by centrifugation at
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 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 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 1. Vascular 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.

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, 0.6 nmol/L insulin, or the combination of both. 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 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.
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.

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.5,13 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 Seo177,10,11 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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References

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