Leptin Effect on Endothelial Nitric Oxide Is Mediated Through Akt–Endothelial Nitric Oxide Synthase Phosphorylation Pathway

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Recent evidence suggests that besides its action on the central nervous system, leptin can modulate vascular tone through local mechanisms involving nitric oxide (NO) release. In this study, using a fluorescent probe for direct determination of NO, we demonstrated both in endothelial cells and in vessels that leptin is able to stimulate NO release. The effect of leptin on NO is abolished by erbstatin A, a Ca^{2+}-independent tyrosine kinase inhibitor, whereas it is not influenced by calcium removal or by other protein phosphorylation inhibitors, such as genistein (an ATP-dependent tyrosine-kinase inhibitor) or wortmannin and LY294002 (two different phosphatidylinositol [PI] 3-kinase inhibitors). Accordingly, leptin-induced vasorelaxation in aortic rings was abolished only by erbstatin A. Furthermore, immunoblotting studies revealed that leptin evokes Akt phosphorylation, with a comparable time course in both endothelial cells and vessels. Also in this experimental system, the effect of leptin was abolished by erbstatin A and not by other inhibitors. Finally, a considerable increase in endothelial NO synthase (eNOS) phosphorylation in Ser^{1177} was found when vessels were treated with leptin. In conclusion, leptin induces NO production by activating a PI 3-kinase–independent Akt-eNOS phosphorylation pathway. *Diabetes* 51:168–173, 2002

Obesity, defined as an increased mass of adipose tissue, is a multifactorial disease and is the consequence of relative inefficiency in self-adapting energy intake to energy requirements in predisposed individuals (1). This pathological condition is associated with a high incidence of hypertension and cardiovascular complications (2,3), which have in turn been linked to both the activation of the sympathetic nervous system (4) and the impairment of endothelial function (5), two pivotal phenotypical traits of obese subjects. Leptin, a protein released by adipose tissue, is strongly implicated in the development of obesity (6,7). In fact, both leptin deficiency and defects in the leptin receptor are sufficient to produce obesity, even if the more common causes of obesity are certainly not caused by genetic defects of leptin signaling but depend instead on overfeeding, with a remarkable increase in leptin plasma levels. Actually, leptin has been regarded as a hormonal signal linking adipose tissue status with a number of key central nervous system circuits to decrease appetite and increase energy expenditure. Thus, normal leptin production and action are critical for maintaining energy balance, and this effect is realized through an increase of sympathetic nervous activity (6). In particular, intravenous leptin infusion increases norepinephrine turnover and sympathetic nerve activity to thermogenic brown adipose tissue but, at the same time, also increases sympathetic nerve activity to tissues not usually considered thermogenic, including the kidney, hindlimb, and adrenal gland (8). This accessory effect of leptin could perturb cardiovascular homeostasis if not adequately counterbalanced. On this issue, our laboratories and others have demonstrated that leptin receptors are also present on endothelium and that increasing doses of the hormone are able to exert a clear vasorelaxant response, which may contribute to counterbalance the effects of leptin-induced sympathetic overactivity (9,10).

Recent studies have inferred that nitric oxide (NO) could be one of the endothelial mediators of the actions of leptin on vascular tissues (9). On this issue, it is noteworthy to emphasize the strong impact of endothelial NO dysfunction on cardiovascular homeostasis, as clearly evidenced by both human and animal studies (5,11,12). Consequently, in a chronic condition of hyperleptinemia, typical of obesity, the sympathetic overactivity evoked by the hormone could be inadequately balanced by a compromised endothelial NO function, thus increasing the cardiovascular risk observed in obesity. Moreover, recent studies have suggested that NO can also be a mediator in the glucose uptake induced by leptin, as well as by other metabolic factors, and that the endothelial production may play a dominant role in NO-modulated glucose metabolism (13–15). Therefore, a better definition of the interaction between leptin and endothelial NO is needed to improve our knowledge of the mechanisms connecting metabolic and cardiovascular function.

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DAF-2, 4,5-diaminofluorescein; DAF-2DA, DAF-2 diacetate; EGM, endothelial growth medium; eNOS, endothelial nitric oxide synthase; FAU, fluorescence arbitrary units; FBS, fetal bovine serum; HAEC, human aortic endothelial cell; L-NAME, L-nitroarginine-methyl-ester; NO, nitric oxide; PBS, phosphate-buffered saline; PI, phosphatidylinositol; TBST, Tris-buffered saline with Tween.

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In this study, we have looked for direct evidence of leptin action on endothelial NO by using a 4,5-diaminofluorescein (DAF-2) fluorescent probe, which allows a quantitative determination of NO production (16,17). Furthermore, we have dissected the signal transduction pathways linking leptin to endothelial NO release, combining both pharmacological and molecular studies.

RESEARCH DESIGN AND METHODS

The studies were conducted in two different experimental systems: endothelial cells and aortic rings. Human aortic endothelial cells (HAECs) (Biowhittaker, Belgium) were placed in endothelial growth medium (EGM) (10 ng/ml h-endothelial growth factor, 1 mg/ml hydrocortisone, 50 mg/ml gentamicin, 50 μg/ml amphotericin-B, 3 mg/ml bovine brain extract) containing 10% fetal bovine serum (FBS) and then transferred to 60-mm plates and studied before cell confluence passages 4–6. Plates were washed with phosphate-buffered saline (PBS) and starved for 24 h in EGM containing 0.5% FBS.

Aortic rings were isolated from 65 Wystar Kyoto rats (WKY; Charles River Laboratory) at the age of 12–14 weeks. The animals were previously housed at two per cage, kept in a temperature-controlled room (between 23°C and 25°C) with a 12-h light/dark cycle, and provided food and water ad libitum. The experiments were performed after the rats were acclimatized to our housing conditions for at least 1 week. On the day of experiments, the rats were weighed and then decapitated. The thoracic aorta was dissected and placed in cold Krebs-Henseleit bicarbonate buffer solution with the following composition (in mmol/l): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO4{\cdot}7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 5.6. The vessels were cleaned of the adhering perivascular tissue and cut into rings 3-mm long. All experimental procedures were in accordance with the guidelines for research in animals at our institution.

Evaluation of NO production by DAF-2. Aortic rings were incubated for 2 h in the darkness in aerated (95% O_2, 5% CO_2) Krebs buffer containing DAF-2 diacetate (DAF-2DA 10 μmol/l; Alexis). Endothelial cells were incubated for 1 h in the darkness in red phenol-free Dulbecco's modified Eagle's medium containing DAF-2DA on multiwell slides. In both systems, leptin (10 ng/ml; Calbiochem) was administered in the last 30 min of DAF-2 incubation, either alone or after 30 min exposure to L-nitroarginine-methyl-ester (L-NAME, 300 μmol/l; Sigma), an inhibitor of NO synthase.

In aortas, the leptin effect was also evaluated by incubating the rings in calcium-free medium with 1 mmol/l EGTA; acetylcholine (1 μmol/l) was used as a control. Moreover, the effect of the hormone on aortic rings was also evaluated after incubation with genistein (10 μmol/l, 30 min; Calbiochem), an ATP-dependent tyrosine-kinase inhibitor; wortmannin (0.1 μmol/l, 30 min; Calbiochem) and LY294002 (30 μmol/l, 10 min; Calbiochem), two different phosphatidylinositol (PI)-3 kinase inhibitors; and erbstatin A (3 μmol/l, 30 min; Calbiochem), a Ca^{2+}-independent tyrosine kinase inhibitor.

Vascular rings were rapidly removed and frozen at −20°C. At 1–3 days after inclusion, they were cut in 10 μm-thick sections in a Jung CM3000 cryostat (Leica, Nussloch, Germany). Sections were placed onto microscope slides without any mounting medium or coverslip. Endothelial cells were fixed in 4% paraformaldehyde and mounted in 80% glycerol in PBS.

Specimens were observed under an Axiophot2 fluorescence microscope (Zeiss, Jena, Germany) equipped with a fluorescein isothiocyanate filter (excitation 450–490 nm, emission 515–560 nm). The magnification used was 20× for vessels, 40× for cells. We obtained and analyzed 24-bit color pictures using a digital camera system coupled to imaging software (Spot; Diagnostic Instruments, Sterling Heights, MI) under constant exposure time, gain, and offset. To account for fluorescence decay, all images were taken in the first 30 s of light exposure. The fluorescence intensity of 15–20 cells per experiment, or of four to six sections per ring (selectively on the endothelium), was measured and expressed as fluorescence arbitrary units (FAU) ranging from 0 (absolute black) to 255 (absolute green), using the above-mentioned software. This method had been validated in a preliminary set of experiments by using known agonists and antagonists of NO synthase, as well as possible contaminants, and it had been shown to have good sensitivity and specificity.

Vascular reactivity. Aortic rings were suspended in isolated tissue baths filled with 20 ml Krebs' solution continuously bubbled with a mixture of 5% CO_2/95% O_2 (pH 7.37–7.42) 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 Gould Instruments pressure processor and then acquired in a computerized system by data acquisition and signal analysis software (DASA; Gould Instruments). Analysis of the generated curves was performed using View II software (Gould Instruments), 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 2.0 g. This tension was found to be optimal by testing the contractions to norepinephrine (10^{-3} mol/l). Once basal tension was established, the length of the rings was not altered any further. 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 for subsequent evoked vasorelaxations. Vasorelaxation was expressed as the percent reduction in contraction (the maximal vasorelaxation attained with papaverine being 100% vasorelaxation).

Increasing doses of leptin (0.01–10 ng/ml) were tested on aortic rings preconstricted with phenylephrine (10^{-6} mol/l; Sigma). Furthermore, dose-response curves to leptin were tested in the presence of L-NAME, genistein, wortmannin, LY294002, and erbstatin A, as above. Finally, a dose-response curve to bradykinin (10^{-8} to 10^{-5} mol/l) was performed to compare the effects elicited by leptin with those of a more classical physiological endothelial NO synthase (eNOS) agonist.

Analysis of Akt and eNOS phosphorylation. Leptin (10 ng/ml) was administered for 2, 5, 10, 15, and 30 min in the presence of calcium, in calcium-free medium with the addition of 1 mmol/l EGTA and, finally, in the presence of genistein, wortmannin, LY294002, or erbstatin A, as above. Subsequently, HAECs and aortas were lysated on ice in the following lysis buffer: 50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% Na-deossicolate, 1% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Insoluble material was removed by centrifugation at 14,000 rpm in microfuge for 30 min at 4°C. An aliquot of supernatant was used for Bradford protein determination.

Lysates from cell cultures and blood vessels were subjected to Western blotting analyses using 8% SDS polyacrylamide gel and run on a maxigel apparatus (Bio Rad, Milan, Italy). Gels were electroblotted on polyvinylidine fluoride membrane (Bio Rad) for 1 h using a semidry electroblotting system (Transblot system; Bio Rad), and filters were blocked for 1 h at room temperature in Tris-buffered saline with Tween (TBST) (20 mmol/l Tris-HCl, 150 mmol/l NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. Blots were then incubated overnight at 4°C with phospho-(Ser^{1177}) eNOS (Cell Signaling, Beverly, MA) and phospho-(Thr^{308}) Akt (4 μg/ml) antibodies (Upstate Biotechnology, Lake Placid, NY). Blots were washed three times with TBST buffer and incubated for 1 h with peroxidase-coupled anti-sheep (Sigma) and anti-rabbit (Amersham) secondary antibodies diluted 1:5,000 in TBST. Proteins were revealed by an enhanced chemiluminescence kit (Amersham). The intensity of the bands was quantified by scanning densitometry using the National Institutes of Health Image 1.61 software.

Statistical analysis. Statistical analysis was performed either by Student's t test or by two-way analysis of variances followed by Bonferroni test, as appropriate.

RESULTS

Evaluation of NO production by DAF-2. As shown in Figs. 1 and 2, leptin induced an increase in DAF-2 fluorescence as compared with basal condition, both in cells (69 ± 4 vs. 29 ± 2 FAU; $P < 0.01$) and in vessels (86 ± 7 vs. 38 ± 1 FAU; $P < 0.01$). Endothelium removal abolished DAF-2 fluorescence, as did L-NAME exposure (cells 30 ± 2 vs. 69 ± 4, vessels 40 ± 2 vs. 86 ± 7; $P < 0.01$). Furthermore, leptin-induced DAF-2 fluorescence was not affected by a lack of calcium (83 ± 3 vs. 86 ± 7; P = NS), in contrast to that observed with acetylcholine (41 ± 3 vs. 94 ± 4; $P < 0.01$).

Finally, to determine the role of tyrosine kinases in modulating leptin-evoked NO production, the effect of the hormone was also evaluated after incubation with different tyrosine kinase inhibitors. In particular, the increase in DAF-2 fluorescence evoked by leptin was abolished by erbstatin A, whereas it was not modified by wortmannin, LY294002, or genistein (Fig. 3).

Studies on vascular reactivity. As shown in Fig. 4, increasing doses of leptin evoked a dose-dependent vasodilation in aortic rings that was abolished by L-NAME and erbstatin A but was not modified by the other kinase inhibitors. The maximal vasodilatation elicited by leptin

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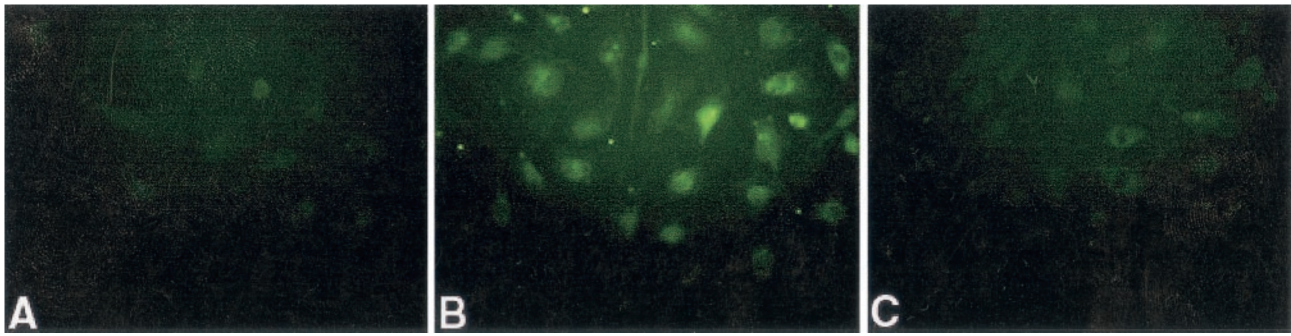

FIG. 1. NO production in endothelial cells under basal conditions (*A*) and after the application of leptin, either alone (*B*) or after preincubation with the NOS inhibitor L-NAME (*C*). The high-power micrographs are representative of six experiments.

was comparable to that obtained with the more classical agonist bradykinin (Δ% maximal vasodilatation 25.3 ± 1.8 vs. 26.3 ± 3.2; P = NS).

Analysis of Akt and eNOS phosphorylation. Leptin induced time-dependent Akt phosphorylation in threonine^{308} (with a maximum observed after 10 min) in both endothelial cells (Fig. 5*A*) and vascular tissue (Fig. 5*B*). According to the previous data, calcium removal did not affect leptin-evoked Akt phosphorylation in both endothelial cells and vascular tissue (data not shown). More important, Fig. 6 shows that erbstatin A—but not genistein, LY294002, or wortmannin—impaired the threonine^{308}–Akt phosphorylation induced by leptin. The protein kinase inhibitors alone were ineffective on Akt phosphorylation. Insulin was used as a positive control to monitor the effectiveness of the PI 3-kinase inhibitors in our experimental system. Finally, leptin was able to evoke eNOS phosphorylation in serine^{1177} in vascular tissue (Fig. 7).

DISCUSSION

Our data demonstrate that leptin is able to induce NO production through a PI 3-kinase–independent Akt-eNOS phosphorylation pathway. So far, it is well documented that leptin has a receptor system on vascular tissues (10,18), but there is only indirect evidence that leptin can

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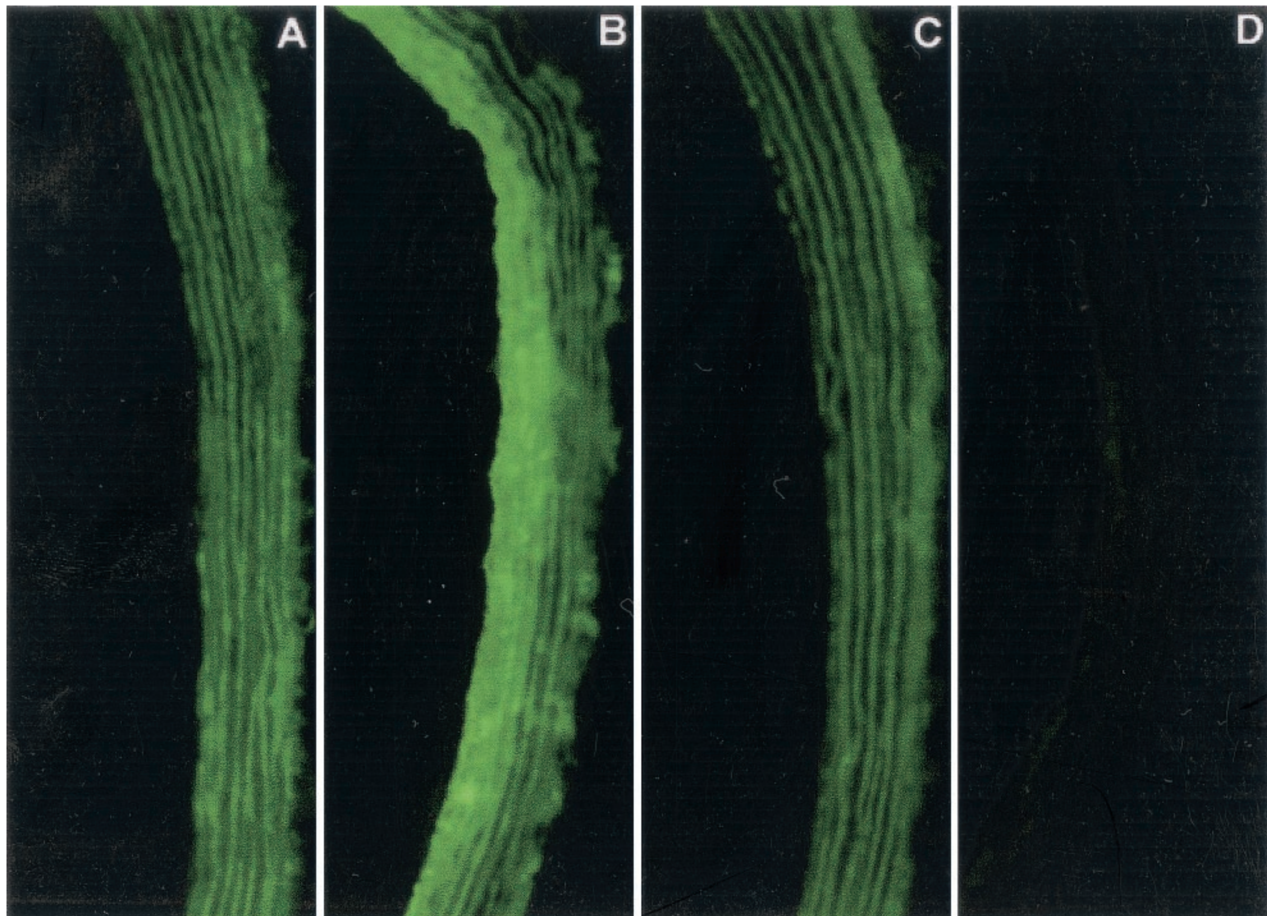

FIG. 2. NO production in aortic rings under basal conditions (*A*) and after the application of leptin, either alone (*B*) or after preincubation with the NOS inhibitor L-NAME (*C*) or after endothelium removal (*D*). The high-power micrographs are representative of eight experiments.

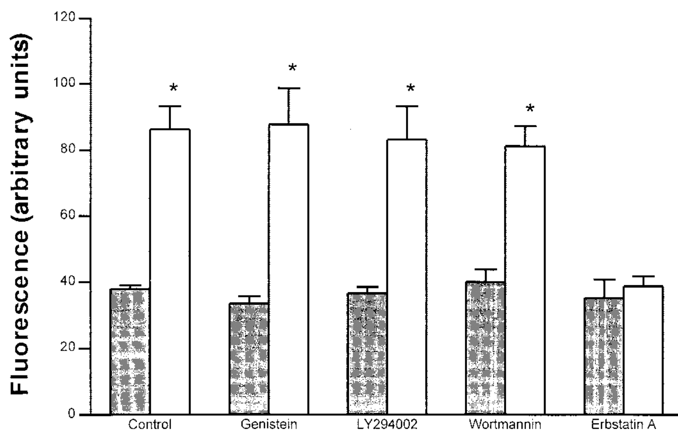

FIG. 3. DAF-2 fluorescence in the endothelium of aortic rings after application of leptin, either alone or after preincubation with the protein kinases inhibitors genistein, erbstatin A, wortmannin, or LY294002. Full bars represent basal conditions, empty bars represent leptin-treated vessels. *P < 0.01 as compared with basal, n = 7.

activate NO production in this compartment (9,10,19). In particular, the interaction between leptin and NO has been mainly derived either by analysis of leptin hypotensive effects in the absence of simultaneous sympathetic nervous system activation or by analysis of leptin vasorelaxant effects on isolated vessels. In the present study, using a technique that allows a direct measurement of NO production through a novel fluorescent probe, we have clearly observed that leptin evokes an unquestionable increase in fluorescence in vascular tissues, and the concomitant exposure to an NO inhibitor completely prevents the effect of the hormone. Thus, it is now clear that, besides other possible targets of leptin vascular action, the hormone is able to directly activate NO production in the vessels. Such an effect is entirely dependent on the presence of the endothelium. In fact, endothelium removal prevents the vasorelaxation and the increase of fluorescence evoked by the hormone, demonstrating that the production of NO induced by leptin is of endothelial origin. This latter evidence fits with our observations and other previous studies showing that vascular leptin receptors are present only on endothelium. Moreover, it should be emphasized that leptin-evoked vasodilatation was similar to that observed with the more classical eNOS agonist bradykinin, suggesting a similar physiological relevance.

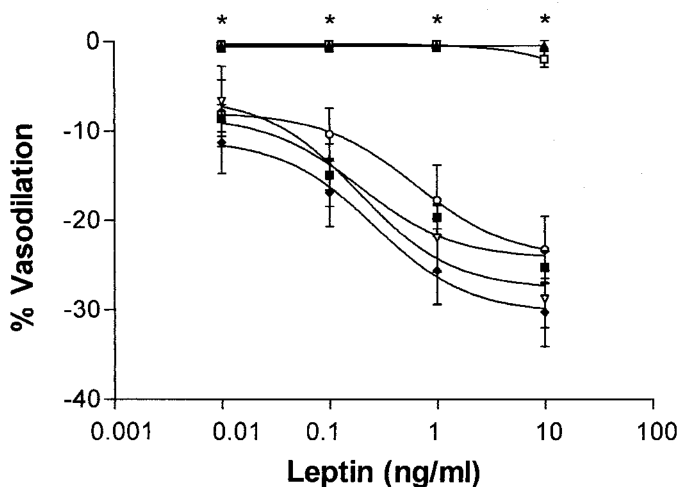

FIG. 4. Vascular response of aortic rings precontracted with phenylephrine to increasing doses of leptin, either alone (■) or after preincubation with the protein kinase inhibitors genistein (◆), wortmannin (△), LY294002 (○) or Erbstatin A (▲), or with the NOS inhibitor L-NAME (□). *P < 0.01 as compared with control, n = 24.

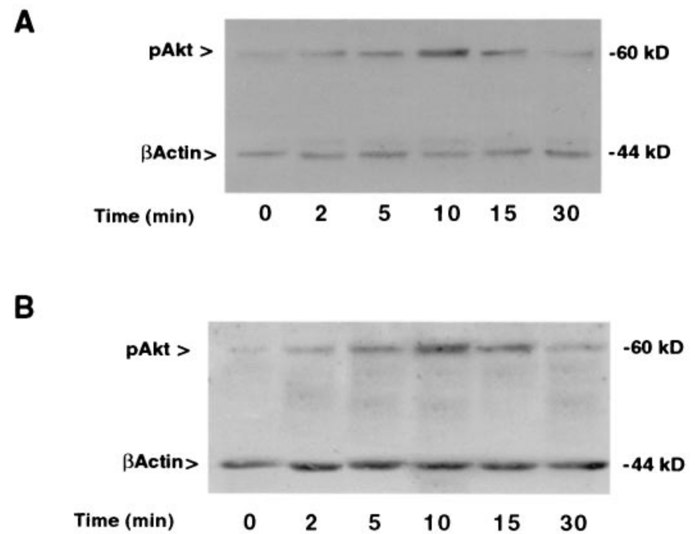

FIG. 5. Representative Western blot showing Akt phosphorylation after acute stimulation with leptin, both in endothelial cells (A) (n = 6) and in aortic tissue (B) (n = 5).

Once we established a clear interaction between leptin and endothelial NO production, we tried to better specify the molecular features of this interaction. It is now commonly accepted that endothelial NO synthase can be activated in a Ca^{2+}-dependent and -independent manner (20). Interestingly, in contrast to that observed with acetylcholine (a stimulus commonly used to test endothelial NO release), leptin activates NO formation through a Ca^{2+}-independent mechanism. In fact, the absence of Ca^{2+} is able to prevent acetylcholine-induced fluorescence, whereas it does not influence the effect of leptin on NO production. So far, only a few stimuli able to affect endothelial NO formation have been characterized on the basis of their Ca^{2+} dependence, in part because, until some years ago, endothelial NO synthase was classified only as a Ca^{2+}-dependent enzyme (21). Our technical approach with DAF-2 is in agreement with a recent study demonstrating the role of calcium in insulin action on NO release (22).

Recently, it has been demonstrated that a serine/threonine kinase Akt (also known as protein kinase B or Rac kinase), which influences various cellular processes, including glucose metabolism and cell survival, is also an important regulator of endothelial NO production (23,24), activating eNOS through phosphorylation on serine^{1177}. However, there is conflicting evidence on the possibility that leptin may affect Akt phosphorylation and activity in several tissues (25–27). Actually, the data of the present study clearly indicate that leptin is able to enhance Akt phosphorylation in a time-dependent manner in both endothelial cells and isolated vessels. More importantly, we demonstrate that leptin is able to induce eNOS phosphorylation on serine^{1177}, the phosphorylation site specific to the Akt-induced activation of the enzyme. These findings

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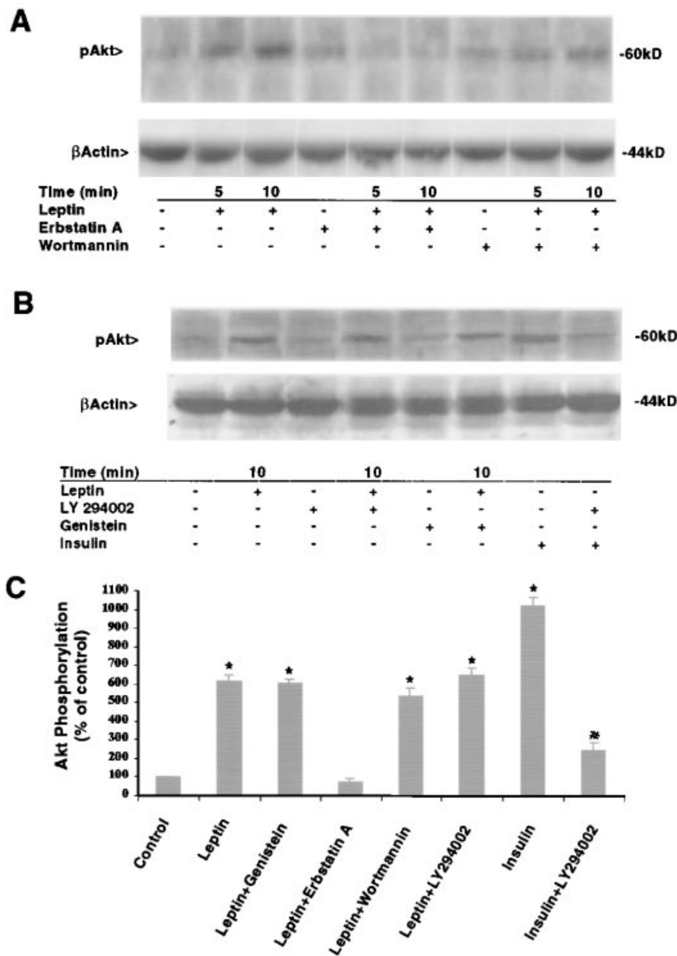

FIG. 6. Representative Western blots showing the effect on Akt phosphorylation of leptin alone and after erbstatin A, wortmannin, LY 294002, and genistein (*A* and *B*). Insulin was used as a positive control. *C*: The quantitation of the blots, considering 10-min exposure to leptin (means + SE; n = 4–7). *P < 0.01 vs. control, †P < 0.01 vs. insulin.

suggest that the Ca^{2+}-independent effect of leptin on endothelial NO production involves Akt phosphorylation and, consequently, the activation of endothelial NO synthase. Our results are in keeping with previous observations demonstrating that stimuli promoting NO release independently from elevations in intracellular calcium raise Akt phosphorylation, thus permitting the activation of eNOS at resting levels of calcium (24,28).

Finally, to determine which pathway accounted for leptin-evoked Akt phosphorylation and NO release in both endothelial cells and isolated vessels, a number of pharmacological agents have been used. The formation of NO, evaluated as fluorescence and vasorelaxation, and the Akt phosphorylation induced by leptin are insensitive to both ATP-dependent tyrosine kinase inhibitors, such as genistein, and PI 3-kinase inhibitors, such as wortmannin and LY294002. In contrast, the exposure of endothelial cells and isolated vessels to a Ca^{2+}-independent tyrosine kinase inhibitor, such as erbstatin A, is able to completely prevent both Akt phosphorylation and the consequent release of NO. Taken together, our data suggest that NO production evoked by leptin is realized through an intracellular calcium-independent signaling pathway converging on Akt phosphorylation, which does not involve the

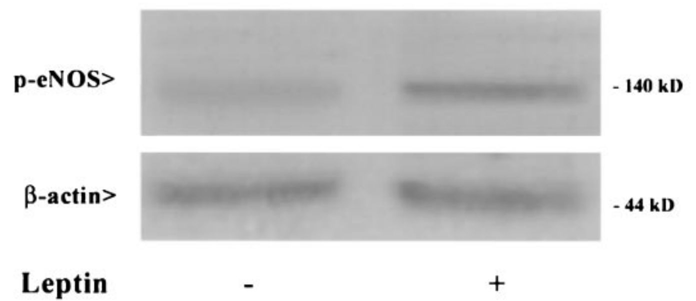

FIG. 7. Representative Western blot, showing eNOS phosphorylation on serine[1177] after 10 min of stimulation with leptin (n = 4).

activation of PI 3-kinase but instead requires tyrosine phosphorylation that is sensitive to erbstatin A but not genistein. This latter finding, that two diverse tyrosine kinase inhibitors realize different effects, could seem conflicting, but several reports have described a similar lack of effect of genistein on NO production realized by application of other stimuli, suggesting that Akt phosphorylation and the consequent NO release is regulated by one or more tyrosine kinases with differential sensitivity to the inhibitors used (29,30). It is noteworthy to emphasize that the inhibitory effect of erbstatin A cannot be ascribed to a direct interference with eNOS tyrosine phosphorylation, because a preliminary event in the eNOS activation cascade, such as Akt phosphorylation, is also inhibited by erbstatin A. This evidence indicates that some tyrosine-phosphorylated protein may regulate Akt phosphorylation and then eNOS activation. On this issue, there is growing evidence that several stimuli, such as insulin or vascular endothelial growth factor, can lead to increased NO formation through an involvement of Akt phosphorylation, depending on the activation of the PI 3-kinase/Akt pathway (31,32). In our study, two independent PI 3-kinase inhibitors have been ineffective to perturb Akt phosphorylation by leptin. However, there is recent evidence in other cellular systems that phosphoinositide-dependent protein kinase 1, the kinase involved in Akt threonine phosphorylation, can also be activated in a PI 3-kinase–independent manner (33,34). Thus, Akt phosphorylation induced by leptin may be ascribed to this alternative pathway.

In conclusion, our study defines for the first time the interaction at the molecular level between leptin and vascular endothelial NO synthesis, shedding further light on the complex action of metabolic factors on vascular function.

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