Ghrelin has novel vascular actions that mimic PI 3-kinase-dependent actions of insulin to stimulate production of NO from endothelial cells

Micaela Iantorno,1 Hui Chen,1 Jeong-a Kim,1 Manfredi Tesauro,2 Davide Lauro,2 Carmine Cardillo,3 and Michael J. Quon1

1Diabetes Unit, National Center for Complementary and Alternative Medicine, National Institutes of Health, Bethesda, Maryland; 2Department of Internal Medicine, University of Rome “Tor Vergata,” Rome; and 3Department of Internal Medicine, Catholic University “Sacro Cuore,” Rome, Italy

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Ghrelin is an orexigenic peptide hormone secreted by the stomach. In patients with metabolic syndrome and low ghrelin levels, intra-arterial ghrelin administration acutely improves their endothelial dysfunction. Therefore, we hypothesized that ghrelin activates endothelial nitric oxide synthase (eNOS) in vascular endothelium, resulting in increased production of nitric oxide (NO) using signaling pathways shared in common with the insulin receptor. Similar to insulin, ghrelin acutely stimulated increased production of NO in bovine aortic endothelial cells (BAEC) in primary culture (assessed using NO-specific fluorescent dye 4,5-diaminofluorescein) in a time- and dose-dependent manner. Production of NO in response to ghrelin (100 nM, 10 min) in human aortic endothelial cells was blocked by pretreatment of cells with Nω-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor), wortmannin [phosphatidylinositol (PI) 3-kinase inhibitor], or (D-Lys3)-GHRP-6 (selective antagonist of ghrelin receptor GHSR-1a), as well as by knockdown of GHSR-1a using small-interfering (si) RNA (but not by mitogen/extracellular signal-regulated kinase inhibitor PD-98059). Moreover, ghrelin stimulated increased phosphorylation of Akt (Ser773) and eNOS (Akt phosphorylation site Ser1179) that was inhibitable by knockdown of GHSR-1a using siRNA or by pretreatment of cells with wortmannin but not with PD-98059. Ghrelin also stimulated phosphorylation of mitogen-activated protein (MAP) kinase in BAEC. However, unlike insulin, ghrelin did not stimulate MAP kinase-dependent secretion of the vasoconstrictor endothelin-1 from BAEC. We conclude that ghrelin has novel vascular actions to acutely stimulate production of NO in endothelium using a signaling pathway that involves GHSR-1a, PI 3-kinase, Akt, and eNOS. Our findings may be relevant to developing novel therapeutic strategies to treat diabetes and related diseases characterized by reciprocal relationships between endothelial dysfunction and insulin resistance.

ghrelin; nitric oxide; endothelium

Ghrelin is a 28-amino-acid peptide hormone secreted predominantly from the X/A-like cells in the oxyntic mucosa of the stomach that has central actions to stimulate growth hormone release, orexigenesis, and regulate metabolic homeostasis of adipose tissue (28, 29, 66, 67). The majority of circulating ghrelin originates from the gastrointestinal tract (29). However, ghrelin and its mRNA have also been detected in the hypothalamus (54) and cardiomyocytes (22). The principal cognate receptor for ghrelin, GHSR-1a, is a seven-transmembrane G protein-coupled receptor that mediates many, if not most, of the biological actions of ghrelin (for reviews, see Ref. 5, 29, and 61). Displaceable binding of labeled ghrelin analogs, as well as GHSR-1a mRNA, is detectable not only in pituitary but in many human peripheral tissues, including vascular endothelium, myocardium, and monocytes (2, 15, 20, 24). Therefore, both in vitro and in vivo evidence that ghrelin has important direct actions in peripheral tissues (independent of growth hormone) to regulate cardiovascular homeostasis (5), inflammation (15, 30), adipocyte function (8, 19, 43), bone formation (18), and gastric motility (33).

Intravenous infusion of ghrelin acutely lowers blood pressure in both humans (38, 39) and rats (58). Nevertheless, from these in vivo interventions, it is not clear if the hypotensive actions of ghrelin are the result of its direct effects on hemodynamic homeostasis or resultant elevations in circulating growth hormone or other secondary factors. One well-established major mechanism for lowering blood pressure involves decreasing peripheral vascular resistance (55). However, in rats, the hypotensive effects of ghrelin appear to be independent of vasodilator actions of nitric oxide (NO; see Ref. 58). Furthermore, intra-arterial administration of ghrelin in humans in vivo may have acute vasodilator actions to increase forearm blood flow in an NO-independent fashion (41), and ghrelin treatment of human internal mammary arteries ex vivo appears to cause acute vasodilation in an endothelium-independent manner (70). On the other hand, in patients with the metabolic syndrome who have lower circulating ghrelin levels than in healthy subjects, intra-arterial ghrelin infusion acutely improves their endothelial dysfunction by increasing bioavailability of NO (65). Moreover, a number of both central (52, 59) and ex vivo peripheral actions (25, 51) of ghrelin are NO dependent. Therefore, the precise mechanisms by which ghrelin exerts its peripheral and/or central hemodynamic actions have not been fully elucidated. In particular, the roles of vascular endothelium and NO in mediating the cardiovascular actions of ghrelin remain unresolved. Intriguingly, ghrelin and insulin share some common central and peripheral actions to regulate energy homeostasis, including the stimulation of food intake and glucose metabolism (11, 44, 56). These actions of insulin to regulate energy homeostasis are phosphatidylinositol (PI) 3-kinase dependent (40, 44, 56). Vasodilator actions of insulin to stimulate production of NO in vascular endothelium that help to couple metabolic and hemodynamic homeostasis

Address for reprint requests and other correspondence: M. J. Quon, NC- CAM, NIH, 10 Center Dr., Bldg. 10, Rm. 6C-205, Bethesda, MD 20892-1632 (e-mail: quonm@nih.gov).

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are also PI 3-kinase dependent (26, 27). Consequently, in the present study, we hypothesized that ghrelin acutely activates endothelial nitric oxide synthase (eNOS) in vascular endothelium, resulting in increased production of NO using PI 3-kinase-dependent signaling pathways shared in common with insulin. The results of our study may be relevant to understanding molecular mechanisms underlying direct cardiovascular actions of ghrelin.

**MATERIALS AND METHODS**

**Cell culture.** Bovine aortic endothelial cells (BAEC) in primary culture (Cell Applications, San Diego, CA) were grown in endothelial growth medium-microvascular (EGM-MV; Cambrex, Walkersville, MD) and used between passages 3 and 5. BAEC were serum starved overnight in EGM-2 MV and used between passages 3 and 5. HAEC were grown to 95% confluence in Lab-Tek (Rochester, NY) and used between passages 3 and 5 as previously described (16, 17). Cytokine-depleted human aortic endothelial cells (HAEC) in primary culture (Cambrex) were grown in growth medium-microvascular (EGM-2 MV) before experimental procedures. Human aortic endothelial cells (HAEC) were grown in EGM-2 MV and used between passages 3 and 5. HAEC were serum starved for 2 h with EBM before experimental procedures.

**Evaluation of NO production in fixed cells.** Production of NO in BAEC or HAEC was assessed using the NO-specific fluorescent dye (Alexa Fluor 568-conjugated goat anti-rabbit IgG or Alexa Fluor 488-conjugated goat anti-mouse IgG; Molecular Probes, Eugene, OR). Red or green immunofluorescence in the cells was evaluated using an Olympus IX81 microscope with appropriate filters. Images were captured using an attached CCD camera in conjunction with IP Labs Software.

**Immunoblotting.** BAEC were grown in 60-mm dishes, serum-starved overnight, and then treated with either insulin (100 nM, 5 min) or ghrelin (100 nM, 10 min). In some experiments, wortmannin (100 nM) or PD-98059 (25 μM) was added to cells 1 h before treatment with insulin or ghrelin. Cell lysates were prepared using 300 μl of lysis buffer (100 mM NaCl, 20 mM HEPES, pH 7.9, 1% Triton X-100, 1 mM Na3VO4, 4 mM sodium pyrophosphate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and complete protease inhibitor cocktail; Roche Applied Sciences, Indianapolis, IN). Samples (45 μg total protein) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using antibodies against eNOS (Transduction Laboratories), phospho-eNOS (S1177; Cell Signaling Technology), Akt, phospho-Akt (Ser473), p44/42 mitogen-activated protein (MAP) kinase, phospho-p44/42 MAP kinase (Thr202/Tyr204; Cell Signaling Technology), GHSR-1a (Alpha Diagnostics International), or β-actin (Sigma) according to standard methods.

**Statistics.** Paired Student’s t-tests were used where appropriate. P values <0.05 were considered to represent statistical significance.

**RESULTS**

**Ghrelin acutely stimulates production of NO in vascular endothelial cells.** To evaluate whether ghrelin mimics vascular actions of insulin to acutely stimulate production of NO from vascular endothelium, we treated HAEC in primary culture with ghrelin (100 nM, 10 min) without or with pretreatment of cells with l-NAME [nitric oxide synthase (NOS) inhibitor] or wortmannin (PI 3-kinase inhibitor). In HAEC loaded with the NO-specific fluorescent dye DAF-2, we observed a significant increase in green fluorescence (indicative of NO production) in response to ghrelin treatment that was comparable to that observed with insulin treatment (Fig. 1). Both insulin- and ghrelin-stimulated production of NO in HAEC was completely blocked by pretreatment of cells with l-NAME or wortmannin (Fig. 1). In similar experiments conducted in BAEC, we observed a dose- and time-dependent increase in NO production in response to ghrelin treatment (Fig. 2). Taken together, these results suggest that ghrelin mimics the PI 3-kinase-dependent vasodilator actions of insulin to stimulate production of NO from vascular endothelium.

**Ghrelin-stimulated production of NO in vascular endothelial cells requires GHSR-1α and involves Akt and eNOS, but not MAP kinase.** We next explored potential signaling mechanisms mediating ghrelin-stimulated production of NO from vascular endothelium. Many, if not most, biological actions of ghrelin are initiated by binding of ghrelin to its cognate cell surface receptor GHSR-1α (21, 28, 29, 61). Therefore, we evaluated the role of GHSR-1α in ghrelin-stimulated produc-
tion of NO by treating HAEC with either ghrelin or insulin in the absence or presence of (D-Lys3)-GHRP-6, a selective antagonist of GHSR-1a. Production of NO in response to ghrelin (but not insulin) was substantially and significantly inhibited by (D-Lys3)-GHRP-6 (Fig. 3A). To more specifically address the role of GHSR-1a, we also examined HAEC transfected with siRNA designed to specifically reduce expression of human GHSR-1a. Production of NO in response to ghrelin (but not insulin) was substantially and significantly inhibited in cells transfected with siRNA against human GHSR-1a (but not in cells transfected with scrambled control siRNA; Fig. 4).

Insulin-stimulated production of NO requires PI 3-kinase-dependent activation of Akt that then directly phosphorylates and activates eNOS (35, 73, 74). Therefore, we used phosphospecific antibodies to evaluate the ability of ghrelin to acutely stimulate phosphorylation of Akt and eNOS in BAEC. In cell lysates prepared from BAEC treated with either insulin or ghrelin, we observed a significant increase in phosphorylation of Akt at Ser473 and eNOS at Ser1179 (Fig. 5, A and B, lanes 1–3). This response to insulin or ghrelin was blocked by pretreatment of cells with wortmannin (Fig. 5, A and B, lanes 4–5) but not PD-98059 (Fig. 5, A and B, lanes 6–7). Moreover, in intact cells (BAEC), both insulin and ghrelin acutely stimulated phosphorylation of eNOS at its Akt phosphorylation site Ser1179 without substantially altering the expression of eNOS.
These results are consistent with our findings that ghrelin-stimulated production of NO in vascular endothelial cells is PI 3-kinase dependent (Fig. 1). Moreover, these results suggest that postreceptor signaling mechanisms required for ghrelin-stimulated production of NO in vascular endothelium are shared in common with pathways regulating vasodilator actions of insulin.

Ghrelin stimulates phosphorylation of MAP kinase but not secretion of ET-1 from vascular endothelial cells. In addition to its NO-dependent vasodilator actions, insulin also has opposing vasoconstrictor actions mediated by MAP kinase-dependent secretion of ET-1 from vascular endothelium (6, 45). To determine if ghrelin also mimics vasoconstrictor actions of insulin, we evaluated the ability of ghrelin to acutely stimulate phosphorylation of MAP kinase and secretion of ET-1 from BAEC. In cell lysates prepared from BAEC treated with either insulin or ghrelin, we observed a significant increase in phosphorylation of MAP kinase at Thr202/Tyr204 (Fig. 6A, lanes 1–3). This response to insulin or ghrelin was blocked by pretreatment of cells with PD-98059 (Fig. 6A, lanes 6–7) but not wortmannin (Fig. 6A, lanes 4–5). The blockade of MAP kinase signaling by PD-98059 pretreatment did not inhibit insulin- or ghrelin-stimulated production of NO in HAEC (Fig. 6B). Interestingly, unlike insulin, ghrelin treatment of BAEC was unable to significantly increase the secretion of ET-1 into conditioned media (Fig. 6C). As we previously demonstrated (45), insulin-stimulated secretion of ET-1 was inhibitable by pretreatment of BAEC with PD-98059 but not wortmannin (Fig. 6C). Thus, although ghrelin stimulates phosphorylation of both PI 3-kinase- and MAP kinase-dependent pathways in vascular endothelium, our data suggest that ghrelin only mimics NO-dependent vasodilator actions of insulin but not ET-1-dependent vasoconstrictor actions of insulin.

**DISCUSSION**

Ghrelin is a peptide hormone secreted by the stomach that was recently cloned and identified as the endogenous ligand for the former orphan receptor GHSR-1a (28, 29, 61). The principal physiological function of GHSR-1a was previously thought to be stimulation of growth hormone release from the pituitary (61). However, the fact that GHSR-1a mRNA and protein is expressed in many cell types and tissues, including cardiomyocytes, myocardium, vascular endothelium, and monocytes, raises the possibility that ghrelin may also have important physiological actions in peripheral tissues that are growth hormone independent (2, 5, 15, 20, 24). Indeed, results from ghrelin null mice and GHSR-1a null mice have implicated ghrelin and ghrelin receptor action as essential regulators of energy homeostasis that are likely to be at least partially independent of growth hormone action since none of these mice have abnormalities in growth (13, 62–64, 71, 75). On the other hand, a recently discovered naturally occurring human mutation in the human ghrelin receptor gene results in short
stature (42). The cardiovascular phenotypes of ghrelin null mice, ghrelin receptor null mice, and humans with ghrelin receptor mutations have not been reported to date. However, with respect to cardiovascular physiology and pathophysiology, ghrelin has been suggested to have direct vasodilator, cardiotropic, cardioprotective, and anti-inflammatory actions (for review, see Ref. 5). Indeed, we recently demonstrated that, in patients with the metabolic syndrome (and low circulating ghrelin levels), intra-arterial ghrelin infusion acutely improves their endothelial dysfunction by increasing bioavailability of NO (65). Although some biological actions of ghrelin seem to be NO dependent (25, 51, 52, 59), other studies suggest that vasodilator actions of ghrelin may be NO independent (41, 58, 70). Therefore, to gain further insight into molecular mechanisms underlying direct vasodilator actions of ghrelin, we evaluated the ability of ghrelin to directly stimulate production of NO from vascular endothelial cells in primary culture.

Ghrelin-stimulated production of NO in vascular endothelial cells requires GHSR-1a and involves Akt and eNOS, but not MAP kinase.

Fig. 3. Ghrelin-stimulated production of NO in HAEC is mediated by its cognate receptor GHSR-1a. A: cells were serum starved and loaded with DAF-2 DA as described in MATERIALS AND METHODS before treatment with either insulin (100 nM, 5 min) or ghrelin (100 nM, 10 min) in the absence or presence of (D-Lys³)-GHRP-6 (10 μM), a selective antagonist of GHSR-1a. After treatment, cells were fixed in 4% paraformaldehyde for 5 min at 4°C and then viewed using an epifluorescent microscope. Emission of green light (510 nm) from cells excited by light at 480 nm is indicative of NO production (top). Bottom: phase-contrast view of cells corresponding to images in top. A representative experiment is shown for experiments that were repeated independently 3 times. B: HAEC seeded in Lab-Tek chamber slides were transfected with scrambled control small-interfering (si) RNA (100 nmol) or GHSR-1a siRNA (100 nmol) as described in MATERIALS AND METHODS. After transfection (48 h), cells were treated as described in A and then viewed using an epifluorescent microscope. A representative experiment is shown for experiments that were repeated independently 3 times.

Fig. 4. Ghrelin-stimulated phosphorylation of endothelial nitric oxide synthase (eNOS) and Akt in HAEC is mediated by its cognate receptor GHSR-1a. HAEC were transfected with scrambled control siRNA (100 nmol) or GHSR-1a siRNA (100 nmol) as described in MATERIALS AND METHODS. After transfection (48 h), cells were serum starved for 8 h and then treated without or with ghrelin (100 nM, 10 min). Cell lysates were then subjected to immunoblotting as described in MATERIALS AND METHODS. Representative immunoblots obtained from gels run in parallel and immunoblotted with anti-phospho-Akt (S473), anti-phospho-eNOS (S1177), anti-GHSR-1a, and anti-β-actin antibodies are shown.
PI 3-kinase (but not MAP kinase). Moreover, we found that ghrelin-stimulated phosphorylation of Akt and eNOS at its Akt phosphorylation site also required expression of GHSR-1a. Interestingly, pretreatment of cells with the PI 3-kinase inhibitor wortmannin completely inhibited ghrelin-stimulated phosphorylation of Akt and production of NO in endothelial cells without completely inhibiting phosphorylation of eNOS. This raises the possibility that there may not be a direct correspondence between eNOS phosphorylation and NO production. Alternatively, this may reflect a difference in sensitivity between our phosphospecific antibodies and our ability to detect production of NO using the NO-specific fluorescent dye DAF-2. Because PI 3-kinase-dependent phosphorylation of Akt and eNOS is known to increase production of NO in vascular endothelium (14), our data strongly suggest that, in vascular endothelial cells, ghrelin binds to its cognate receptor (GHSR-1a), resulting in activation of PI 3-kinase that then stimulates phosphorylation and activation of Akt that, in turn,
phosphorylates and activates eNOS, resulting in increased production of NO. It is possible that ghrelin is also stimulating production of NO in endothelial cells through activation of other receptors known to bind ghrelin (e.g., CD36; see Refs. 1, 3, and 4). However, this possibility seems unlikely since we were able to completely inhibit ghrelin-stimulated production of NO by reducing expression of GHSR-1a. Our results are concordant with previous studies demonstrating that ghrelin stimulates phosphorylation of Akt in cultured H9c2 cardiomyocytes (2) and HAEC (53; although GHSR-1a mRNA is undetectable in H9c2 cells by RT-PCR). More importantly, the results of our present study are fully consistent with our previous report demonstrating NO-dependent vasodilator actions of ghrelin in humans (65). A previous study by Okumura et al. (41) concluded that vasodilation of the brachial artery in response to intra-arterial infusion of ghrelin is not NO dependent. However, conclusions from this plethysmographic study in humans were based on experiments where the NOS inhibitor L-NMMA was infused for only 5 min (by contrast with the 15-min infusion of L-NMMA in our previous study; see Ref. 65). Thus eNOS activity may not have been completely inhibited in the experiments of Okumura et al.

The postreceptor signaling pathway used by ghrelin to stimulate production of NO in vascular endothelium is partially overlapping with that of insulin, which involves insulin receptor/insulin receptor substrate-1/PI 3-kinase/phosphoinositide-dependent kinase-1/Akt/eNOS (27, 35, 37, 73, 74; Fig. 7). Thus ghrelin joins the growing list of hormones involved with NO production and ET-1 secretion from vascular endothelial cells. Therefore, ghrelin joins the growing list of hormones involved with NO production and ET-1 secretion from vascular endothelial cells.

Ghrelin stimulates phosphorylation of MAP kinase but not secretion of ET-1 from vascular endothelial cells. In the present study, we observed that ghrelin, like insulin, acutely stimulates phosphorylation of MAP kinase in vascular endothelial cells. This is consistent with the activation of MAP kinase in vascular endothelial cells in response to insulin that we previously reported (16, 36, 45). In human osteoblasts (12) and some prostate cancer cell lines (72), previous studies have reported that ghrelin stimulates phosphorylation of MAP kinase. Interestingly, by contrast with insulin (16, 45, 46), we found that ghrelin did not stimulate the secretion of the potent vasoconstrictor ET-1 from endothelial cells. Thus ghrelin is unlike DHEA, which mimics insulin action with respect to both NO production and ET-1 secretion (16).

Insulin resistance in diabetes and obesity is characterized by pathway-selective impairment in PI 3-kinase-dependent insulin signaling, but not MAP kinase-dependent insulin signaling, in human skeletal muscle in vivo (10) and rat vasculature ex vivo (23). Thus, in insulin-resistant states, compensatory hyperinsulinemia that serves to maintain euglycemia results in an imbalance between opposing PI 3-kinase-dependent vasodilator actions of insulin and opposing MAP kinase-dependent vasoconstrictor actions of insulin that predisposes to endothelial dysfunction and hypertension (26, 27, 36, 45, 46). We found that ghrelin only mimics the PI 3-kinase-dependent vasodilator actions of insulin but not the opposing MAP kinase-dependent vasoconstrictor actions of insulin (Fig. 7). Therefore, ghrelin may have novel therapeutic potential for both metabolic and cardiovascular diseases characterized by reciprocal relationships between endothelial dysfunction and insulin resistance. Indeed, circulating ghrelin levels are abnormally low in insulin-resistant conditions, including diabetes, obesity, metabolic syndrome, hypertension, coronary heart disease, and atherosclerosis (48–50, 57, 68). Moreover, polymorphisms in the human ghrelin gene are associated with diabetes, impaired glucose tolerance, and hypertension (31, 32, 47). Taken together with the findings in our present study, these observations may help to explain the beneficial effects of intra-arterial ghrelin infusion to improve endothelial dysfunction that we previously reported in patients with the metabolic syndrome (65). Interestingly, lifestyle interventions, including exercise (17), and/or therapeutic interventions that result in weight loss (9) increase plasma ghrelin levels. Thus ghrelin-stimulated production of NO from vascular endothelium may contribute to the beneficial metabolic and cardiovascular outcomes resulting from the successful implementation of these strategies.

In conclusion, we report, for the first time, novel vascular actions of ghrelin to directly stimulate production of NO from vascular endothelial cells using PI 3-kinase-dependent signaling pathways that mimic those of insulin. These findings may
explain, in part, the molecular mechanisms underlying some of the beneficial cardiovascular actions of ghrelin. In addition, our results suggest that novel or existing therapeutic strategies to increase circulating ghrelin levels or ghrelin action may be beneficial for metabolic and cardiovascular diseases characterized by reciprocal relationships between insulin resistance and endothelial dysfunction.

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