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

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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 (t-Lys)3-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 (Ser473) 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

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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 as previously described (16, 35). BAEC were serum starved overnight with endothelial basal medium (EBM) before experimental procedures. Human aortic endothelial cells (HAEC) in primary culture (Cambrex) 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 4,5-diaminofluorescein diacetate (DAF-2 DA; Cayman Chemical, Ann Arbor, MI) as described previously (16). Briefly, BAEC or HAEC were grown to 95% confluence in Lab-Tek (Rochester, NY) chamber slides and then serum starved overnight or for 2 h, respectively, in phenol red-free EBM supplemented with L-arginine (100 μM). BAEC or HAEC were loaded with DAF-2 DA (3 μM) for 30 or 10 min, respectively, at 37°C. In some experiments, N^6-nitro-L-arginine methyl ester (L-NAME; 100 μM), wortmannin (100 nM), or PD-98059 (25 μM) was added to the media 30 min before loading with DAF-2 DA. After being loaded with DAF-2 DA, cells were washed three times with EBM at 37°C and kept in the dark. Next, BAEC or HAEC were treated with insulin (100 nM, 5 min) or ghrelin (100 nM, 10 min; human (octanoyl)-ghrelin; Biovision, Mountain View, CA) in the absence or presence of (o-Lys3)-GHRP-6 (10 μM; Phoenix Pharmaceuticals, Belmont, CA). For time-course experiments, cells were treated with ghrelin (100 nM) for 0, 1, 3, 5, or 30 min. For dose-response experiments, cells were treated with ghrelin for 10 min at concentrations of 0, 0.1, 1, 10, 100, or 1,000 nM. In some experiments, HAEC were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol with human GHSR-1a duplex siRNA (catalog no. 005513; Dharmacon) or scrambled siRNA as a control [nontargeting small-interfering (si) RNA pool, catalog no. 001210; Dharmacon]. After treatment with ghrelin or insulin, cells were fixed in 4% paraformaldehyde (vol/vol) for 5 min at 4°C. Fixed cells were visualized using an Olympus IX81 inverted microscope with attached charge-coupled device (CCD) camera (Retiga Exi; Burnaby, BC, Canada) using appropriate filters with a peak excitation wavelength of 480 nm and a peak emission wavelength of 510 nm. Images were captured using IP Labs Software (Scanalytics, Fairfax, VA).

Immunofluorescence microscopy. BAEC were grown to 95% confluence in Lab-Tek chamber slides and serum starved overnight in phenol red-free EBM. Cells were then treated without or with insulin (100 nM, 5 min) or ghrelin (100 nM, 10 min) and subsequently fixed with 4% paraformaldehyde at room temperature for 12 min. Cells were then washed three times with PBS and incubated with 0.5% Triton X-100 in PBS with 10% horse serum for 10 min. Next, cells were washed three times with PBS and then blocked with 2% BSA in PBS for 1 h. This was followed by incubation with primary polyclonal antibodies against phospho-eNOS (S1177; Cell Signalling Technology, Beverly, MA) or eNOS (Transduction Laboratories, Lexington, KY) (1:400 dilution in blocking solution) overnight at 4°C. Cells were then washed three times with PBS followed by incubation with secondary antibodies (1:1,000 dilution) for 1 h at room temperature (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), Akt, phospho-Akt (S473), 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.

Endothelin-1 assay. BAEC were grown in 60-mm dishes and then serum starved overnight. At the beginning of each experiment, media was replaced with fresh EBM. Cells were treated without or with wortmannin (100 nM) or PD-98059 (25 μM) for 1 h and then stimulated without or with insulin (100 nM, 5 min) or ghrelin (100 nM, 10 min). After insulin or ghrelin stimulation, 1 ml of conditioned media was collected from each dish for determination of endothelin (ET)-1 concentrations using an ELISA microplate assay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s instructions.

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 GHSR-1a (but not in cells transfected with scrambled control siRNA; Fig. 3B). Moreover, ghrelin-stimulated phosphorylation of Akt and eNOS was completely inhibited in HAEC transfected with siRNA against human GHSR-1a (but not in cells transfected with scrambled control siRNA; Fig. 4). As expected, expression of GHSR-1a in HAEC was substantially reduced 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...
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. 3](image-url)

**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.

![Fig. 4](image-url)
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-1α. 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 Nω-monomethyl-L-arginine (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 regulation of metabolism, including insulin (35, 73, 74), estrogen (60), leptin (69), adiponectin (7), HDL (34), and dehydroepiandrosterone (DHEA) (16) that acutely activate eNOS in vascular endothelium by a PI 3-kinase-dependent signaling mechanism leading to phosphorylation of eNOS by Akt. Intriguingly, ghrelin and insulin share important central and peripheral biological actions to regulate energy homeostasis through stimulation of food intake, glucose metabolism, and regulation of adipocyte function (11, 13, 40, 43, 44, 56, 62–64, 66, 67, 71, 75). Metabolic and vasodilator actions of insulin are both regulated by highly parallel signaling pathways requiring PI 3-kinase (but not MAP kinase; see Ref. 27). This represents one potential mechanism underlying the ability of vascular actions of insulin to help couple metabolic and hemodynamic homeostasis (27). Thus the findings of our present study raise the possibility that ghrelin may also have functions similar to those of insulin to couple metabolic and cardiovascular physiology.

**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 stimulated 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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REFERENCES


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