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Ghrelin stimulates angiogenesis in human microvascular endothelial cells: Implications beyond GH release ☆

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Abstract

Ghrelin, a peptide hormone isolated from the stomach, releases growth hormone and stimulates appetite. Ghrelin is also expressed in pancreas, kidneys, cardiovascular system and in endothelial cells. The precise role of ghrelin in endothelial cell functions remains unknown. We examined the expression of ghrelin and its receptor (GHSR1) mRNAs and proteins in human microvascular endothelial cells (HMVEC) and determined whether ghrelin affects in these cells proliferation, migration and *in vitro* angiogenesis; and whether MAPK/ERK2 signaling is important for the latter action. We found that ghrelin and GHSR1 are constitutively expressed in HMVEC. Treatment of HMVEC with exogenous ghrelin significantly increased in these cells proliferation, migration, *in vitro* angiogenesis and ERK2 phosphorylation. MEK/ERK2 inhibitor, PD 98059 abolished ghrelin-induced *in vitro* angiogenesis. This is the first demonstration that ghrelin and its receptor are expressed in human microvascular endothelial cells and that ghrelin stimulates HMVEC proliferation, migration, and angiogenesis through activation of ERK2 signaling.
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Keywords: Ghrelin; GHS-R—growth hormone secretagogue receptor; Angiogenesis; Endothelial cells; Proliferation; Migration; ERK2

Ghrelin is a 28-amino acid peptide hormone recently isolated from rat and human stomach [1,2]. A major focus of research on ghrelin has been on its role in neuroendocrine regulation in the central nervous system [2–4], where through its receptor—growth hormone secretegogue receptor (GHSR)—ghrelin regulates growth hormone (GH) secretion at the hypothalamic and pituitary level [3–6]. Independently of GH-secretagogue actions, ghrelin elicits orexigenic (appetite-stimulating) action by increasing expression and release of neuropeptide Y and agouti-related protein, neuropeptides involved in the central regulation of appetite and food intake [5,6]. Although ghrelin is produced mainly in the stomach by A-like cells in rodents and by P/D_1 cells in humans [1,2,7], its expression has been demonstrated in other organs, such as pancreas, salivary glands, and kidneys [7–11]. Recently, ghrelin and its receptor were also detected in the cardiovascular system, in the heart, vasculature, and endothelial cells of large vessels, indicating that this peptide may play roles in cardiovascular functions including vasodilation, increased contractility and cardioprotection [12–16]. Moreover, exogenous ghrelin inhibits proinflammatory response in human umbilical vein endothelial cells *in vitro* and reverses endothelial dysfunction in patients with metabolic syndrome [17,18]. Also, recent studies demonstrated that ghrelin exerts a potent protective action on the gastric mucosa and accelerates the healing of ischemia/reperfusion-induced gastric lesions [19,20]. Since healing of gastric lesions requires angiogenesis—sprouting new capillary blood vessels [21], this indirectly indicates that ghrelin may stimulate angiogenesis. However, direct evidence for angiogenesis-stimulating action of ghrelin is lacking.

There are only few studies on expression of ghrelin in endothelial cells. Recent study demonstrated expression

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of immunoreactive ghrelin in endothelial cells of human arteries and veins [14]. However, the precise role of ghrelin in endothelial cell function remains unknown. Two studies (from the same group) reported that ghrelin may inhibit bFGF-stimulated angiogenesis [22,23], but these reports are inconclusive and the direct effect of ghrelin on proliferation, migration and angiogenesis in human microvascular endothelial cells (HMVEC) remains unknown.

In this study, we examined expression of ghrelin and its receptor, GHSR1 mRNAs and proteins in HMVEC and determined whether treatment with exogenous ghrelin activates MAPK/ERK2 signaling and affects proliferation, migration, and *in vitro* angiogenesis in HMVEC. Our findings provide the first evidence that ghrelin and GHSR1 are constitutively expressed in HMVEC and that ghrelin stimulates endothelial cell migration, proliferation, and angiogenesis via activation of ERK2 signaling pathway.

Materials and methods

Cell lines and reagents. Human dermal microvascular endothelial cells (HMVEC) were obtained from Cambrex Biosciences (Walkersville, MD). Cells were maintained in culture as an adherent monolayer in endothelial cell growth media (EGM-2 MV; Cambrex Biosciences). Recombinant human ghrelin, anti-ghrelin, and anti-GHSR1 antibodies were purchased from Phoenix Pharmaceutical (Belmont, CA). MEK inhibitor PD98059 was purchased from BIOMOL (Plymouth Meeting, PA).

Ghrelin and GHSR1 mRNA analysis by RT-PCR. Human ghrelin and GHSR1 specific RT-PCR primers (GenBank Accession Nos. NM_016362 and NM_004122) used for analysis of mRNA expression by RT-PCR were purchased from Superarray System (Frederick, MD). Cells were lysed in Trizol Reagent for the RNA isolation. Total RNA from each sample was used in the reverse transcription reaction to synthesize first strand cDNA. Ten microliters of cDNA was used in each PCR. Each cycle set used a denaturing temperature (95 °C) for 30 s, annealing temperature (55 °C) for 90 s, and extension temperature (72 °C) for 30 s for a total of 40 cycles for each primer. PCR fragments were separated on a 2% agarose gel containing ethidium bromide (0.5 μg/ml), visualized and photographed under UV illumination.

ELISA measurement of ghrelin concentration. Ghrelin levels in culture supernatants and in HMVEC lysate were determined using human Bioassay ELISA kit from the US Biological (Swampscott, MA), according to the manufacturer's protocol. Cells were grown in 6-well plate for 48 h, and the culture supernatant and cell lysate were collected. Fifty microliters of either: culture supernatant, cell lysate or recombinant human ghrelin protein at different concentrations, and 25 μl of ghrelin polyclonal antibody and 25 μl biotinylated ghrelin standard peptide were added to each well. After 2 h incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system. The reactions were stopped by addition of 100 μl of 0.18 N H_2SO_4 and absorbance was determined using an ELISA microtiter plate reader (DYNEX Technologies, Inc. Chantilly, Virginia) at 450 nm. A curve of the absorbance vs the concentration of ghrelin in the standard wells was plotted. We determined ghrelin concentration in the samples by comparing the absorbance of the samples to the standard curve.

GHSR1 protein expression by Western blot analysis. Cell lysates were subjected to SDS–PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibody against GHSR1 followed by peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using ECL detection system (Amersham, Arlington Heights, IL), similarly as in our previous study [24].

Immunocytochemical (ICC) staining for ghrelin and GHSR1. Ghrelin protein expression and localization in HMVEC were analyzed by ICC staining. Briefly, endothelial cells were plated on 24-well coverslips coated with rat type I collagen gel, cultured for 48 h and used for immunostaining. Samples were washed twice with PBS and fixed in 4% paraformaldehyde (Sigma–Aldrich) in PBS for 10 min and permeabilized with acetone at −20 °C for 5 min. After washing twice with PBS, cells were incubated with a blocking solution (DAKO, Carpentaria, CA) for 7 min at room temperature. Excess blocking solution was drained and samples were incubated with either anti-ghrelin or anti-GHSR1 antibodies for 2 h at room temperature. The samples were then rinsed with PBS and incubated with Alexa 488-conjugated secondary antibody (Molecular Probes, Eugene, CA). Cells were then washed, mounted using anti-fade mounting media (Molecular Probes, Eugene, CA) and examined under a Nikon epifluorescence microscope. As a negative control we performed staining using all reagents except the primary antibody.

Effect of ghrelin on endothelial cell proliferation. Endothelial cell proliferation was determined using bromodeoxyuridine (BrdU) ELISA (Chemicon, Temecula, CA) and BrdU staining. Briefly, endothelial cells were seeded into 96-well culture plates in triplicate and allowed to adhere overnight. The cultures were then washed and refed with medium alone (control) or medium containing different concentrations of ghrelin. Following 24 h incubation, cell proliferation was determined by BrdU ELISA following the product instruction. For the BrdU staining, cells were plated on 24-well coverslips coated with rat collagen gel and following treatment with ghrelin for 24 h, cells were incubated with BrdU at final concentration at 10 μM for 2 h prior to experiment termination. Then, after fixation and treatment with 2 N HCl at 37 °C for 1 h, anti-BrdU antibody (Chemicon, Temecula, CA) was added and cells were then rinsed with PBS and incubated with Alexa 488-conjugated secondary antibody and evaluated under a Nikon epifluorescence microscope.

Effect of ghrelin on endothelial cell migration. Endothelial cell migration in response to ghrelin was determined utilizing method described in our previous study for assessment of cell migration (25). Transwell 6.5 mm chambers (Corning Costar, Cambridge, MA) with polycarbonate membrane containing 8.0-μm pores were coated with matrigel. Endothelial cells (2×10^4) were plated onto transwell chambers in triplicate and incubated in medium (serum free) alone or medium containing different concentrations of ghrelin and incubated at 37 °C in CO_2 for 2 h. MTT [3-(14, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] was added and cells were incubated for additional 2 h. Cells from the top of the transwell chambers were removed using a cotton swab (residual cells). Cotton swabs containing residual cells and transwell chamber (migrated) cells were placed in 24-well plates containing 400 μl of DMSO. Following 1 h of gentle shaking, 100-μl samples were removed and absorbency was determined at 570 nm using an ELISA plate reader. The migratory activity was calculated as a percent migration $= A/[A + B] \times 100$, where A is the absorbance of migrated cells and B is the absorbance of residual cells.

Effect of ghrelin on in vitro angiogenesis—capillary-like tube formation on matrigel. An *in vitro* capillary tube formation assay was performed as described in our previous studies [24,25]. Briefly, 2×10^4 HMVEC were plated on a Matrigel (BD Biosciences, Mountain View, CA) coated 48-well plate with either medium alone (control) or medium containing ghrelin 0.1, 1, and 10 nM. After 6 h, the plates were examined for capillary tube formation under an inverted Nikon microscope and photographed. Each assay was done in triplicate and each experiment was repeated three times. The total length of capillary tube in control and each treatment group was measured in five random fields on each coded samples by two independent observers. The differences in tube formation were examined and quantified using the image analytical software—Metamorph vision 7.0 (Universal Imaging Corporation, West Chester, PA).

Effect of ghrelin on ERK2 phosphorylation in HMVEC and effect of MEK/ERK2 inhibitor on ghrelin-induced angiogenesis. To determine whether MAPK/ERK2 mediates signaling involved in ghrelin-induced angiogenesis, we examined in HMVEC ERK2 phosphorylation in response to ghrelin treatment using the same methods as in our previous study [24]. HMVEC monolayers were pretreated with vehicle or MEK/ERK2 inhibitor PD98059 (10 μM) for 30 min and then were treated with either medium only or ghrelin 1 nM for 30 min. Cells were lysed and isolated protein subjected to Western blotting using total Erk and p-Erk antibodies, identically as in our previous study [24]. To further determine