available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/peptides

Ghrelin induces proliferation in human aortic endothelial cells via ERK1/2 and PI3K/Akt activation

Fabio Rossi, Antonella Castelli, Maria J. Bianco, Cora Bertone, Marina Brama, Vittorio Santiemma *

Dipartimento di Fisiopatologia Medica, Sapienza Università di Roma, Rome, Italy

ARTICLE INFO

Article history:
Received 17 April 2008
Received in revised form
3 July 2008
Accepted 7 July 2008
Published on line 17 July 2008

Keywords:
Ghrelin
Endothelial cell
Aorta
Proliferation

ABSTRACT

The direct ghrelin (Ghr) involvement in cardiovascular (CV) system homeostasis has been suggested by the expression of its receptor in CV tissues and by evidence that ghrelin mediates CV activities in animals and in humans. Moreover, low Ghr plasma levels have been reported in pathological conditions characterized by high cardiovascular risk. In the present study, we investigated Ghr effect on proliferation of human aortic endothelial cell (HAEC) and involved transduction pathways. Our results indicate that ghrelin elicited proliferation in a dose-dependent manner (EC_{50} about of 5 nmol/L) in cultured HAEC, and that this effect was inhibited by the receptor antagonist (D-Lys3)-GHRP-6. Western blot experiments documented an activation of external receptor activated kinases (ERK1/2) and Akt in a dose-dependent fashion, as well as involvement of the cAMP pathway in ERK1/2 phosphorylation. Experiments conducted with appropriate pharmacological inhibitors to investigate Ghr-induced HAEC proliferation confirmed the involvement of ERK1/2 and I3P/Akt pathways, as well as the role of AMP cyclase/PKA pathway in ERK1/2 phosphorylation. Our results indicate that Ghr promotes HAEC proliferation, and thus may be a protective factor against vascular damage. The low ghrelin serum levels reported in insulin-resistant states may not be able to effectively counteract endothelial cell injury.

1. Introduction

Ghrelin (Ghr), an acyl-peptide consisting of 28 amino acids esterified with octanoic acid on Ser3, is the natural ligand of growth hormone secretagogue receptor (GHSR). Ghrelin secretion is pulsatile, and it is related to feeding behavior [31]. Ghr plasma levels are in the nanomolar range in humans [6]. The largest amount of circulating ghrelin is produced by X/A-like cells in the oxyntic mucosa of the stomach [18]. However, ghrelin and its mRNA were also detected in cardiomyocytes [14], and immunoreactive ghrelin was reported in endothelial cells [17].

Direct Ghr involvement in cardiovascular system homeostasis has been supported by the expression of its receptor in heart and vessels and by evidence that ghrelin mediates cardiovascular activities in animals and in humans. Ghrelin administration in man-in normal subjects and in patients with chronic heart failure – significantly decreases systemic vascular resistance, and it increases cardiac index and stroke volume index [22,11]. In addition, low Ghr plasma levels have been reported in pathological conditions characterized by high cardiovascular risk, such as uncomplicated obesity, insulin-resistant obesity, type 2 diabetes mellitus, and hypertension [28,21,24].

* *Corresponding author at*: Dipartimento di Fisiopatologia Medica, Policlinico Umberto I, Sapienza Universita' di Roma, Viale del Policlinico 00161 Roma, Italy Tel.: +39 06 49970707/4; fax: +39 06 49970704.
E-mail address: vittorio.santiemma@uniroma1.it (V. Santiemma).
0196-9781/$ – see front matter
doi:10.1016/j.peptides.2008.07.002

The characterized receptor of ghrelin is the G-protein-coupled receptor GHSR-1a, that mediates many of Ghr biological actions [7,6,5,29]. GHSR-1a mRNA has been detected in human brain and peripheral tissues [13,12], including cardiovascular tissues such as myocardium [3] and vascular endothelium [12]. The density of ghrelin receptors in the vascular system is up-regulated with atherosclerosis [16]. Other binding sites, distinct from GHSR-1a and not yet characterized, have been identified in a large number of tissues, including heart [19].

In a previous study we demonstrated that Ghr binds to GHS-R 1a and inhibits angiotensin II-induced migration in human aortic endothelial cells [25]. Conflicting data were reported about ghrelin effect on cell proliferation. In thyroid and breast cell lines, Ghr inhibits FCS-induced proliferation [20], in other cell types, such us human hepatoma (HepG2) prostate, adrenal, pancreatic, cardiac, adipose cells and pituitary, ghrelin stimulates proliferation [19,15,10].

We investigated the ghrelin effect on human aortic endothelial cell (HAEC) proliferation and the involved transduction pathways.

2. Materials and methods

Ghrelin and (D-Lys3)-GHRP-6 were purchased from Bachem AG (Italy). PD 98059 and wortmannin (specificity: IC_{50} = 50 nM and 300 nM, respectively, for PI3 and MAPK activation induced by PDGF) were obtained by SIGMA (Milan, Italy). SQ22.536 and KT5720 were purchased from Calbiochem (Milan, Italy). HAEC, endothelial cell basal medium (ECBM) and medium supplements were obtained by BioWhittaker (Europe). Primary antibody for ERK1/2, p-ERK1/2 (phosphorylated Ser 473), Akt and p-Akt, and secondary antibody HRP conjugated were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

2.1. HAEC culture

After thawing according to the method suggested by Bio Whittaker, cryopreserved cells were placed at density of 2500 cells/cm^2 in 75 mL plastic flask (Falcon) with ECBM supplemented with 1 μg/mL hEGF (human recombinant endothelial growth factor), 0.1% hydrocortisone, 50 mg/mL gentamicin, 50 μg/mL amphotericin-B and 10% fetal calf serum, and then maintained at 37 °C in 5% CO_2 atmosphere. The medium was changed every 48 h. Cells were subcultured at 70–80% confluence. All experiments were performed at cell passage 4–5.

2.2. Western blotting

After stimulation, subconfluent HAECs were washed twice with cold PBS on ice bed. Then, they were scraped and lysed in RIPA buffer for 1 h at 4 °C, and lysate was centrifuged at 22,000 rpm for 12 min. The solubilized proteins were loaded on a 10% SDS gel, and, after running, the gel was blotted on a nitrocellulose paper. Aspecific binding on nitrocellulose paper was saturated with free fatty milk for 18 h at 4 °C for non-phosphorylated proteins or 3% bovine serum albumin for phosphorylated proteins. After incubation with primary and secondary HRP conjugated antibody, the proteins were detected with ECL Western blotting detection systems.

2.3. Cell growth analysis

To evaluate proliferation, subconfluent HAECs were subcultured in 24-well plates (Falcon Plastic, Los Angeles, CA) at a density of 20×10^3 cells/well and cultured overnight in M199 containing 10% FCS at 37 °C. Then, cells were made quiescent by incubation for 24 h in serum-free medium. Adherent cells were treated with graded concentration of ghrelin or with drug solvent only. After 24 h cells were rinsed with PBS and removed by trypsin/EDTA treatment. Then, they were suspended in PBS for counting in a cell counter chamber. Cell viability was determined by trypan blue dye exclusion. Antagonists and inhibitors were added to cell culture 20 min before ghrelin treatment.

The cellular growth was also evaluated by using the sulforhodamine B based In Vitro Toxicology Assay Kit (TOX 6 assay kit Sigma–Aldrich, Milano, Italy). The sulforhodamine B method is simple, accurate, and yields reproducible results. It was applied as modified by Papazisis KT [23]. In brief, cells were fixed using 50% TCA, then the plates were layered with sulforhodamine B solution. The incorporated dye was then solubilized in sulforhodamine B assay solubilization solution (10 mM Tris), and absorbance was spectrophotometrically measured at wavelength of 530 nm using an Ultraspec 2100pro spectrophotometer (Amersham biosciences, Milano, Italy). As in all the cellular growth experiments the data obtained with sulforhodamine B were strictly correlated with the number of cells detected in the cell counter chamber ($r = 0.87$), the results presented are those with sulforhodamine B.

Mitogen-activated protein kinase (MAPK) and tyrosine kinase involvement in the mitogenic effect of Ghr were determined by adding the MAPK inhibitor PD 98059 (30 μM) and PI3K inhibitor wortmannin (100 nM). SQ 22.536 (10 μM), a specific inhibitor of AMP cyclase, and KT5720 (1 μM), inhibitor of cAMP-dependent protein kinase (PKA) were used to investigate the involvement of cAMP.

2.4. Statistical analysis

Data are presented as means ± standard error of mean (S.E.M.). Statistical analysis was performed by using the Student's paired *t*-test for cell proliferation experiments. One-way ANOVA with post-hoc Dunnet test was used with the densitometric analyses of Western blot experiments. $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Ghrelin effect on HAEC proliferation

In cultured HAEC, increasing concentrations (0.01–100 nmol/L) of ghrelin elicited proliferation in a classic dose-dependent manner, showing a EC_{50} of 5 nmol/L (Fig. 1). It was a receptor-mediated event, as suggested by the curve morphology and

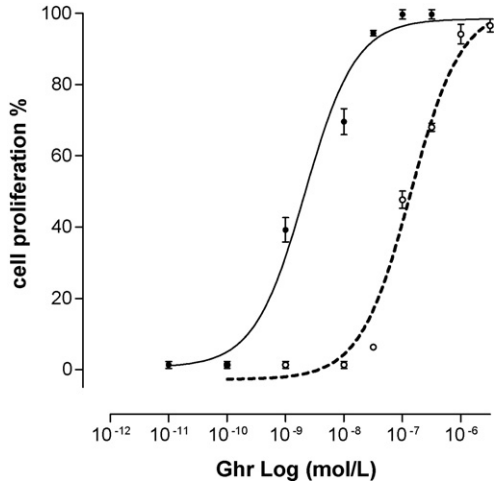


Fig. 1 – Dose–response curve of HAEC proliferation induced by increasing doses of ghrelin (closed circle). A single dose (100 nM) of (D-Lys^3)-GHRP-6 inhibited Ghr effect, producing a significant rightward parallel shift in the dose–response curve without affecting maximal response (open circle). The results are expressed as percentage of proliferation. Each point is expressed as the mean of three separate experiments run in triplicate. Bars show S.E.M.

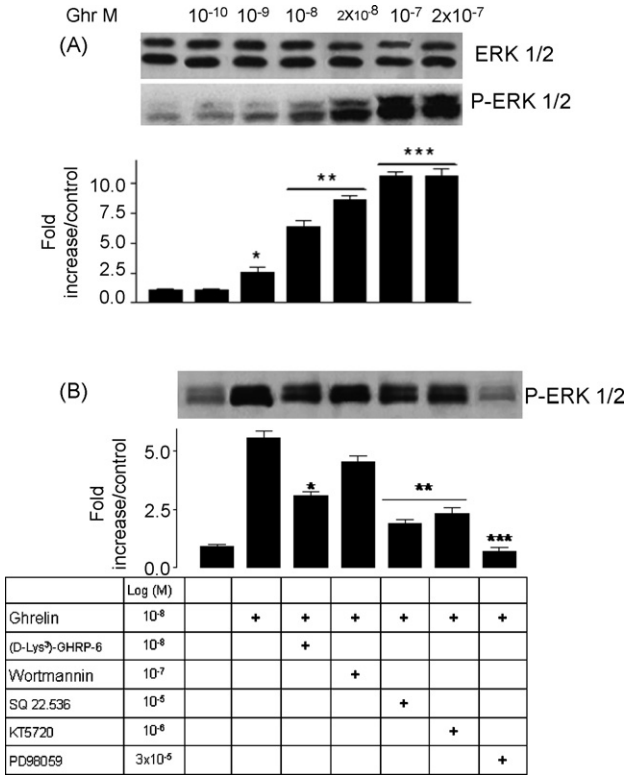


Fig. 2 – (A) Increasing Ghr doses enhanced ERK1/2 phosphorylation in dose-dependent fashion. Results are representative of those observed in three independent experiments. Bar graphs are representative of densitometric analyses of five separate experiments expressed as the fold increase from the control (untreated cells) (means ± S.E.) (*P < 0.05, **P < 0.01, ***P < 0.005 versus control). (B) Effect of (D-Lys^3)-GHRP-6 and of pharmacological inhibition of P I3 kinase, AMP cyclase, PKA and ERK1/2 on Ghr-induced ERK activation. Bar graphs are representative of densitometric analyses of five separate experiments expressed as the fold increase from the control (means ± S.E.) (*P < 0.05, **P < 0.01, ***P < 0.005 versus ghrelin alone). One-way ANOVA with post-hoc Dunnet test was used for the statistical analisys of data. The relative level of ERK activation was determined by densitometric scanning of the phospho-ERK bands and normalized to the control (untreated cells) signal.

confirmed by experiments with (D-Lys^3)-GHRP-6, a specific ghrelin antagonist. (D-Lys^3)-GHRP-6, at the dose of 100 nmol/L, inhibited the ghrelin effect, producing a significant rightward parallel shift in the dose–response curve without affecting maximal response (Fig. 1). Performing experiments with increasing doses of (D-Lys^3)-GHRP-6 and ghrelin, a pA_2 value of 7.91 (7.99–7.83, 95% confidence limits) and a slope of 1.03 (0.82–1.24, 95% confidence limits) were obtained by Schild analysis of data, showing a typical receptor-antagonism.

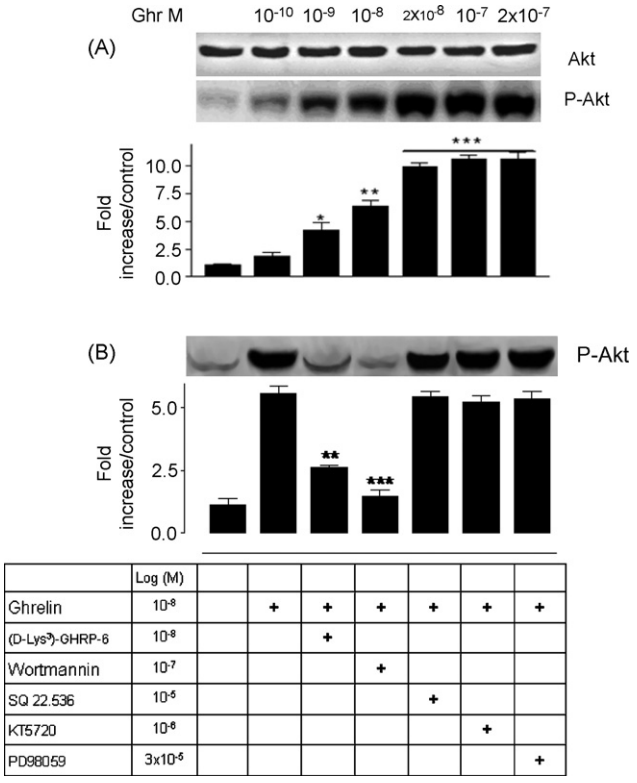
3.2. *ERK1/2 and PI3K/Akt activation*

To evaluate the Ghr effect on ERK1/2 and Akt, Western blotting experiments were performed. Ghrelin stimulation of quiescent HAEC induced phosphorylation of both kinases in a dose-dependent fashion compatible with the results of proliferation experiments (Figs. 2A and 3A). The addition of (D-Lys^3)-GHPR-6 markedly blunted the phosphorylation of both kinases induced by Ghr (Figs. 2B and 3B). The effect of MAPK and Akt pathway inhibition by SQ 22.536, KT5720, wortmannin and PD 98059 was then tested. As shown in Fig. 2B, the specific ERK1/2 inhibitor PD98059 completely abolished ERK phosphorylation, but activation was also blunted by cAMP pathway inhibition by SQ22.536, an AMP cyclase inhibitor, and KT5720P, a PKA inhibitor. As far as Akt was concerned, PI3K inhibition by wortmannin very strongly reduced Akt phosphorylation, whereas cAMP pathway inhibition did not influence it (Fig. 3B).The involvement of PI3K/Akt and the ERK1/2 pathways in HAEC proliferation induced by Ghr was investigated by using wortmannin and PD98059. Both compounds were able to inhibit ghrelin-induced proliferation (Fig. 4). Inhibition of cAMP formation by SQ 22.536 and of cAMP-dependent protein kinase A (PKA) activation by KT5720 were also able to blunt the ghrelin effect (Fig. 4).

4. Discussion

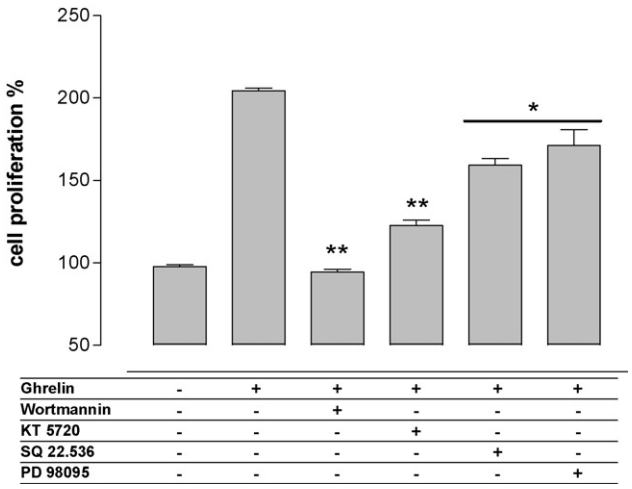
In the present study, we document that ghrelin exerts a direct effect on human aortic endothelial cell proliferation, which is mediated by PI3K-dependent Akt activation and ERK1/2 activation.

The canonical ERK MAP kinase cascade is stimulated upon the binding of extracellular growth factors, such as EGF and PDGF, to their respective transmembrane receptor tyrosine kinases, including Akt. The autophosphorylation of the



	Log (M)							
Ghrelin	10^{-8}		+	+	+	+	+	+
(D-Lys^3)-GHRP-6	10^{-8}			+				
Wortmannin	10^{-7}				+			
SQ 22.536	10^{-5}					+		
KT5720	10^{-6}						+	
PD98059	$3x10^{-5}$							+

Fig. 3 – (A) Increasing Ghr doses induced Akt phosphorylation in dose-dependent fashion. Results are representative of those observed in three independent experiments. Bar graphs are representative of densitometric analyses of five separate experiments expressed as the fold increase from the control (means ± S.E.) (*P < 0.05, **P < 0.01, ***P < 0.005 versus untreated cell). (B) Effect of (D-Lys^3)-GHRP-6 and of pharmacological inhibition of PI3 kinase, AMP cyclase, PKA and ERK1/2 on Ghr-induced Akt activation. Bar graphs are representative of densitometric analyses of five separate experiments expressed as the fold increase from the control (means ± S.E.) (P < 0.01, ***P < 0.005 versus ghrelin alone). One-way ANOVA with post-hoc Dunnet test was used for the statistical analisys of data. The relative level of Akt activation was determined by densitometric scanning of the phospho-Akt bands and normalized to the control (untreated cells) signal.**



Ghrelin	-	+	+	+	+	+
Wortmannin	-	-	+	-	-	-
KT 5720	-	-	-	+	-	-
SQ 22.536	-	-	-	-	+	-
PD 98095	-	-	-	-	-	+

Fig. 4 – Effect of pharmacological inhibition of ghrelin-induced proliferation in HAEC. The ghrelin effect was strongly inhibited by P I3 kinase inhibition with wortmannin and ERK1/2 inhibition elicited by PD98095. Also cAMP inhibition by SQ 22.536 and PKA inhibition by KT5720 significantly blunted ghrelin effect. The results are expressed as percentage of proliferation. Each histogram represents the mean ± S.E.M. of three separate experiments run in triplicate. *indicates significant difference (P < 0.05), ** (P < 0.01) versus ghrelin-treated samples.

cytoplasmic tails of the receptor on tyrosine leads to recruitment and activation of the kinases Raf, MEK, and ERK. Also Ghr has been reported to activate the ERK1/2 via a G(i)-dependent pathway that involves PI3K/Akt [4]. The activation of this pathway mediates the Ghr inhibitory effect on endothelial cell apoptosis [2].

The involvement of both MAPK and PI3K pathways does not exclude the possibility that also a receptor type other than a G-protein-coupled receptor is involved in signal transduction. In a recent paper, activation of ERK and PI3K/Akt pathways was reported to be mediated not only by acylated Ghr via GHS-R1a, but also by desacylated Ghr via other unidentified receptor, unable to bind (D-Lys^3)-GHRP-6 [8]. We have already reported [25], and confirm in the present paper, that Ghr binds (D-Lys^3)-GHRP-6-responsive GHS-R1a in HAEC. However, investigation of possible actions of desacyl-Ghr in HAEC is in progress in our lab.

Our Western blot results document that SQ 22.536, a specific AMP cyclase inhibitor, and KT5720, a specific inhibitor of cAMP-dependent PKA, were able to markedly blunt ERK1/2 activation, suggesting that the cAMP pathway activates ERK1/2 in HAEC. In addition, pharmacological inhibition of either ERK1/2 and Akt or AMP cyclase and PKA effectively inhibited ghrelin-induced proliferation.

This is consistent with the reported capability of cAMP to modulate cell proliferation by regulating the extracellular signal-regulated kinase [30]. cAMP-mediated ERK activation leads to the activation of transcription factor Elk-1 [34]. This action is independent of Ras and requires the expression of the B-Raf isoform of serine/threonine kinase Raf [32]. In endothelial cells, the major Raf isoform expressed is B-Raf [33].

PKA can activate ERKs by additional mechanisms that enhance ERK signaling. PKA phosphorylation of a common kinase interaction motif (KIM), within a family of ERK-directed phosphotyrosine phosphatases (PTPases), results in the release of bound ERK and subsequent increase in ERK activity [5,27].

In HAEC, we already documented that ghrelin activates the cAMP/PKA pathway [25] and inhibits Ang II-induced migration. With the present paper, another effect of cAMP/PKA activation, namely HAEC proliferation, is added.

Conflicting data have been reported about ghrelin effect on cell proliferation. A large number of endocrine and nonendocrine neoplastic cells express Ghr and Ghr-receptor,

suggesting that the hormone is likely to play an important autocrine/paracrine role in neoplastic conditions. In thyroid and breast cell lines, Ghr inhibits FCS-induced proliferation [15], whereas in other cell types, ghrelin stimulates proliferation [19,10,15]. Regarding the Ghr effect on angiogenesis, previous studies indicate that ghrelin inhibits basic FGF-induced formation of capillary-like structures in HUVEC, and angiogenic response to FGF-2 in the chick embryo chorioallantoic membrane *in vivo* assay [1]. In rat brain microvascular endothelial cells as well, ghrelin inhibits cellular proliferation [9], whereas, more recently, Ghr was reported to stimulate human microvascular endothelial cells [20]. Other studies documented that Ghr prevents death of porcine aortic endothelial cell and improves endothelial dysfunction in patients with metabolic syndrome [2]. However, no studies were conducted to investigate the role of ghrelin in proliferation of human aortic endothelial cells nor the signaling pathways involved.

Correctly regulated endothelial cell proliferation is essential to maintain vessel anti-thrombogenic properties, and to avoid smooth muscle cell proliferation, both important components of atherogenesis. In this context, the Ghr proliferation promoting effect in HAEC reported in the present paper and Ghr inhibition of Angiotensin II-induced proliferation and contraction on vascular smooth muscle cells [26], suggest that Ghr is involved in vascular wall homeostasis, and that low ghrelin serum levels may contribute to the pro-atherosclerotic state present in pathological conditions characterized by insulin-resistance.

REFERENCES

[1] Baiguera S, Conconi MT, Guidolin D, Mazzocchi G, Malendowicz LK, Parnigotto PP, et al. Ghrelin inhibits in vitro angiogenic activity of rat brain microvascular endothelial cells. Int J Mol Med 2004;4:849–54.

[2] Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, et al. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/Akt. J Cell Biol 2002;159:1029–37.

[3] Bodart V, Bouchard JF, McNicoll N, Escher E, Carriere P, Ghigo E, et al. Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. Circ Res 1999;85:796–808.

[4] Camiña JP, Lodeiro M, Ischenko O, Martini AC, Casanueva FF. Stimulation by ghrelin of p42/p44 mitogen-activated protein kinase through the GHS-R1a receptor: role of G-proteins and beta-arrestins. J Cell Physiol 2007;213:187–200.

[5] Cao JM, Ong H, Chen C. Effects of ghrelin and synthetic GH secretagogues on the cardiovascular system. Trends Endocrinol Metab 2006;17:13–8.

[6] Casanueva FF, Diéguez C. Ghrelin: the link connecting growth with metabolism and energy homeostasis. Rev Endocr Metabol Disord 2002;3:325–38.

[7] Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, et al. Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. J Clin Endocrinol Metab 2001;86:1738–45.

[8] Chung H, Seo S, Moon M, Park S. Phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase-3{beta} and ERK1/2 pathways mediate protective effects of acylated and unacylated ghrelin against oxygen-glucose deprivation-induced apoptosis in primary rat cortical neuronal cells. J Endocrinol)2008;(Jun 9) [Epub ahead of print].

[9] Conconi MT, Nico B, Guidolin D, Baiguera S, Spinazzi R, Rebuffat P, et al. Ghrelin inhibits FGF-2 mediated angiogenesis in vitro and in vivo. Peptides 2004;25:2179–85.

[10] Duxbury MS, Waseem T, Ito H, Robinson MK, Zinner MJ, Ashley SW, et al. Ghrelin promotes pancreatic adenocarcinoma cellular proliferation and invasiveness. Biochem Biophys Res Commun 2003;309:464–8.

[11] Enomoto M, Nagaya N, Uematsu M, Okumura H, Nakagawa E, Ono F, et al. Cardiovascular and hormonal effects of subcutaneous administration of ghrelin, a novel growth hormone-releasing peptide, in healthy humans. Clin Sci 2003;105:431–5.

[12] Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, et al. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 2002;87:2988.

[13] Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, et al. Van der Ploeg LH, Howard AD. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Mol Brain Res 1997;48:23–9.

[14] Iglesias MJ, Pineiro R, Blanco M, Gallego R, Dieguez C, Gualillo O, et al. Growth hormone releasing peptide (ghrelin) is synthesized and secreted by cardiomyocytes. Cardiovasc Res 2004;62:481–8.

[15] Jeffery PL, Herington AC, Chopin LK. Expression and action of the growth hormone releasing peptide ghrelin and its receptor in prostate cancer cell lines. J Endocrinol 2002;172:R7–11.

[16] Katugampola SD, Pallikaros Z, Davenport AP. [125I-His(9)]-ghrelin, a novel radioligand for localizing GHS orphan receptors in human and rat tissue: up-regulation of receptors with atherosclerosis. Br J Pharmacol 2001;134:143–9.

[17] Kleinz MJ, Maguire JJ, Skepper JN, Davenport AP. Functional and immunocytochemical evidence for a role of ghrelin and des-octanoyl ghrelin in the regulation of vascular tone in man. Cardiovasc Res 2006;69:227–35.

[18] Kojima M, Kangawa K. Drug insight: the functions of ghrelin and its potential as a multitherapeutic hormone. Nat Clin Pract Endocrinol Metab 2006;2:80–8.

[19] Korbonits M. Ghrelin—a hormone with multiple functions. Front Neuroendocrinol 2004;25:27–68.

[20] Lia A, Cheng G, Zhu GH, Tarnawski AS. Ghrelin stimulates angiogenesis in human microvascular endothelial cells: implications beyond GH release. Biochem Biophys Res Commun 2007;9:238–43.

[21] McLaughlin T, Abbasi F, Lamendola C, Frayo RS, Cummings DE. Plasma ghrelin concentrations are decreased in insulin-resistant obese adults relative to equally obese insulin-sensitive controls. J Clin Endocrinol Metab 2004;89:1630–5.

[22] Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, et al. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol 2001;280:R1483–7.

[23] Papazisis KT, Geromichalos GD, Dimitriadis KA, Kortsaris AH. Optimization of the sulforhodamine B colorimetric assay. J Immunol Methods 1997;208(Oct 27 (2)):151–8.

[24] Poykko SM, Kellokoski E, Horkko S, Kauma H, Kesaniemi YA, Ukkola O. Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. Diabetes 2003;52:2546–53.

[25] Rossi F, Bertone C, Petricca S, Santiemma V. Ghrelin inhibits angiotensin II-induced migration of human aortic endothelial cells. Atherosclerosis 2006;192:291–7.

[26] Rossi F, Castelli A, Bianco MJ, Bertone C, Brama M, Santiemma V. Ghrelin inhibits contraction and proliferation of human aortic smooth muscle cells by cAMP/PKA pathway activation, doi:10.1016/j.atherosclerosis.2008.06.015.
[27] Saxena M, Williams S, Taskén K, Mustelin T. Crosstalk between cAMPdependent kinase and MAP kinase through a protein tyrosine phosphatase. Nat Cell Biol 1999;1:305–11.
[28] Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, et al. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 2002;87:240–4.
[29] Smith RG. Development of growth hormone secretagogues. Endocr Rev 2005;26:346–60.
[30] Stork PJS, Schmitt JM. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 2002;12:258–66.
[31] Tolle V, Bassant MH, Zizzari P, Poindessous-Jazat F, Tomasetto C, Epelbaum J, et al. Ultradian rhythmicity of Ghrelin secretion in relation with GH, feeding behavior, and sleep-wake patterns in rats. Endocrinology 2002;143:1353–61.
[32] Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 1997;89:73–82.
[33] Wojnowski L, Zimmer AM, Beck TW, Hahn H, Bernal R, Rapp UR, et al. Endothelial apoptosis in Braf-deficient mice. Nat Genet 1997;16:293–7.
[34] Young SW, Dickens M, Tavaré JM. Differentiation of PC12 cells in response to a cAMP analogue is accompanied by sustained activation of mitogenactivated protein kinase. Comparison with the effects of insulin, growth factors and phorbol esters. FEBS Lett 1994;338:212–21.