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Cardiovascular Research 69 (2006) 227 – 235

Cardiovascular Research

www.elsevier.com/locate/cardiores

Functional and immunocytochemical evidence for a role of ghrelin and des-octanoyl ghrelin in the regulation of vascular tone in man

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Received 21 July 2005; received in revised form 22 August 2005; accepted 1 September 2005
Available online 12 October 2005
Time for primary review 28 days

Abstract

Objective: Ghrelin is the recently identified endogenous ligand for the ghrelin receptor GHS-R1a and known to regulate growth hormone secretion and appetite. Ghrelin also is a potent vasodilator and improves cardiac performance after systemic administration. Generally, the octanoyl modification on Ser^3 has been considered essential for biological activity. Recently however, cardiovascular actions of des-octanoyl ghrelin have been reported in rodents. Our aim was to investigate if ghrelin and ghrelin receptor protein are expressed within the human vasculature, to determine if des-octanoyl ghrelin, like ghrelin, is a vasodilator in human artery and to test the acute effect of ghrelin peptides on cardiac contractility.

Methods: Distribution of ghrelin and ghrelin receptor was determined using standard immunocytochemistry and confocal microscopy. Ghrelin peptides were tested for vasodilator actions in human isolated arteries and their effect on cardiac contractility was investigated in human isolated paced atria.

Results: Immunoreactive ghrelin was detected in endothelial cells of human arteries and veins where it localized to intracellular vesicles but not to the Weibel–Palade bodies of the regulated pathway, suggesting constitutive ghrelin production. Specific antisera detected ghrelin receptor on vascular smooth muscle cells and cardiomyocytes. Ghrelin ($pD_2 = 8.60 \pm 0.1$, $E_{max} = 55.8 \pm 8.9$, mean ± standard error of the mean) and des-octanoyl ghrelin ($pD_2 = 8.8 \pm 0.2$, $E_{max} = 54.7 \pm 5.3$) showed comparable ($P > 0.05$) endothelium independent vasodilator potency and efficacy in reversing endothelin-1 induced constriction in human artery. Neither ghrelin nor des-octanoyl ghrelin had effects on contractile force in paced atria.

Conclusions: We show widespread expression of ghrelin and its cognate receptor in the human cardiovascular system with functional evidence suggesting a role for both ghrelin and the more abundant endogenous form des-octanoyl ghrelin in the paracrine regulation of vascular tone in man.

Keywords: Hypertension; G protein; Receptor; Myocytes; Vasodilation

1. Introduction

Ghrelin, the endogenous ligand for the human G protein-coupled growth hormone secretagogue receptor (GHS-R1a, recently designated as ghrelin receptor [1]), is a gastric peptide, cleaved from a 117 amino acid precursor with a post-translational octanoyl modification that is unique among peptide hormones [2].

A major focus of ghrelin research is its role in neuroendocrine regulation in the central nervous system (CNS). Ghrelin and ghrelin receptors are present in discrete regions of the brain where ghrelin regulates growth hormone (GH) secretion at the hypothalamic and pituitary level by triggering secretion of growth hormone releasing hormone from hypothalamic neurons and by direct stimulation of GH

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doi:10.1016/j.cardiores.2005.09.001

release from the adenohypophysis [2,3]. Independently of GH-secretagogue actions, ghrelin elicits orexigenic effects by increasing expression and release of neuropeptide Y and agouti-related protein, two key neuropeptides involved in the central regulation of appetite and food intake. Conversely, ghrelin inhibits the action of proopiomelanocortin, a physiological antagonist of feeding behaviour in the hypothalamus [4].

In agreement with widespread expression of ghrelin mRNA in peripheral tissues [5], the novel ghrelin receptor system has also been implicated in non-CNS actions including control of gastric motility and acid secretion [6], energy homeostasis [4] and reproductive function [7]. Ghrelin shows pro- and anti-proliferative effects on tumour cells [8,9], modulates T-cell biology [10] and possesses regulatory properties in the cardiovascular system, both in the heart [11,12] and vasculature [12–18]. [^{125}I-His^{9}]-ghrelin-binding has previously been demonstrated in human heart and blood vessels using radioligand binding [19] and ghrelin was shown to possess potent endothelium independent vasodilator effects in human isolated artery [15]. In vivo studies showed a significant drop in blood pressure accompanied by increased cardiac output after ghrelin infusion in healthy volunteers [14,18,20], and both in vitro and in vivo endothelium independent vasodilator effects for ghrelin have been reported in other species [15,17,20]. Nevertheless, meal related changes in circulating ghrelin levels, mainly derived from gastric endocrine cells [21,22], do not cause apparent changes in cardiovascular parameters [4,21,23,24]. Since the initial characterization of the ghrelin receptor, the octanoyl group esterified onto Ser^{3} of the peptide has been considered essential for biological activity [2,25,26]. However, peripheral effects of des-octanoyl ghrelin were recently reported in animals [27–32], which may have major physiological implications as more than 90% of circulating peptide lacks the octanoyl modification [2].

We hypothesize that ghrelin is a ubiquitously expressed endothelium derived peptide in the human cardiovascular system, which if released locally can interact with specific receptors present on the underlying smooth muscle cells to cause vasodilatation. We further propose that in humans, des-octanoyl ghrelin may have peripheral functional activity comparable to ghrelin, in contrast to the CNS where the former peptide is reported to be inactive [2]. To test this hypothesis, our aims were firstly to determine the cellular localization of the ghrelin receptor and its cognate ligand in human large and small arteries and veins from representative vascular beds. We visualized immunoreactive peptide to intracellular vesicles within endothelial cells to seek evidence that the peptide might be synthesized by either the secretory or constitutive pathway [33]. Finally we compared the vascular and cardiac actions of ghrelin and des-octanoyl ghrelin on human isolated arteries and paced atrial appendage in vitro.

2. Methods

2.1. Tissue collection

The investigation conformed to the 'Declaration of Helsinki'. Human left ventricular and atrial myocardium and coronary artery were from heart transplant patients. Left internal mammary artery (LIMA), saphenous vein and atrial appendage were from coronary artery bypass graft surgery patients. Samples of kidney, lung and adrenal gland were from histologically normal regions obtained following excision of tumors. For immunohistochemistry tissue was snap frozen in liquid nitrogen and stored at −70 °C, for in vitro pharmacology tissue was collected in Krebs' solution (4 °C) and transported to the laboratory. Human umbilical vein endothelial cells (HUVECs) were cultured as described previously [34], grown to subconfluency in 24 well plates and stored at −70 °C (used at passage 2–3).

2.2. Immunocytochemistry

Immunocytochemistry was carried out as described previously [34]. Briefly, fixed cryostat tissue sections, blocked with non-immunized swine serum, were incubated with rabbit-anti-human ghrelin antiserum (1:300 dilution, 48 h, 4 °C, Phoenix Pharmaceuticals, Belmont, CA, USA) or rabbit-anti-human ghrelin receptor antiserum (1:500 dilution, 96 h, 4 °C, Phoenix Pharmaceuticals). In adjacent sections, primary antisera were omitted or primary rabbit anti-ghrelin antiserum was pre-absorbed with ghrelin peptide (1 μM, Peptide Institute, Osaka, Japan) as negative controls. Staining was visualized using the peroxidase anti-peroxidase method. In sections of rat brain used as a positive control, ghrelin receptor-like immunoreactivity (LI) was detected in hypothalamic neurons as previously described [35] (rat and human GHS-R1a show 96% amino acid sequence homology [36]) (Fig. 2f).

2.3. Fluorescent double staining and confocal microscopy

Fluorescent double staining was carried out as described previously [34]. Fixed sections or HUVECs in 24 well plates were blocked with goat serum, followed by incubation with rabbit-anti-human ghrelin antiserum (1:100 dilution) and mouse-anti-van Willebrand factor (vWF) monoclonal antibody (1:50 dilution) or mouse-anti-calnexin antibody (1:200)(24 h, 4 °C). Adjacent sections or wells were incubated with rabbit-anti-human ghrelin receptor antiserum (1:100 dilution) and mouse-anti-vWF (1:50 dilution), mouse-anti-smooth muscle α-actin monoclonal antibody (1:100 dilution) or mouse-anti-calnexin antibody (1:200)(24 h, 4 °C). Specific staining was visualized with fluorescent secondary antibodies and sections were mounted using medium containing 4′,6′-diamino-2-phenylindole hydrochloride (DAPI, Vector Laboratories, Burlingame, CA, USA) as a marker of cell nuclei. Confocal imaging

was performed using a confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany). Deconvolution was carried out using Huygens maximum likelihood estimation image restoration (Scientific Volume Imaging BV, The Netherlands).

2.4. In vitro pharmacology

2.4.1. Reversal of ET-1 constriction in LIMA

Endothelium denuded rings (4 mm) of LIMA were mounted in 5 ml baths containing oxygenated Krebs' solution (mM: NaCl, 90; $NaHCO_3$, 45; KCl, 5; $MgSO_4 \cdot 7H_2O$, 0.5; $Na_2HPO_4 \cdot 2H_2O$, 1; $CaCl_2$, 2.25; fumaric acid, 5; glutamic acid, 5; glucose, 10; sodium pyruvate, 5; pH 7.4; gassed with 95% O_2/5% CO_2 at 37 °C) for the measurement of isometric force development and experiments were carried out as described previously [15]. Optimum resting tension was determined by repeated application of 0.1 M KCl at increasing levels of basal tension until no further increase in developed isometric force was obtained. Successful removal of functional endothelium was confirmed by showing the absence of a vasodilator response to 1 μM acetylcholine after pre-constriction with 1 μM phenylephrine. Vessels were washed with fresh Krebs' containing G-nitro-L-arginine-methyl ester (L-NAME, 300 μM) and indomethacin (5 μM) (Sigma-Aldrich, Poole, UK) to exclude the possibility of endothelium dependent vasodilatation and after a 1 h equilibration period artery rings were pre-constricted with ET-1 (10 nM). Cumulative concentration-response curves to ghrelin and des-octanoyl ghrelin (Phoenix Pharmaceuticals) (0.1–300 nM) were constructed. Experiments were terminated by addition of atrial natriuretic peptide (ANP, 10 nM) followed by *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10 μM) (Sigma-Aldrich, Poole, UK) to determine maximal receptor mediated and receptor-independent vasodilatation. Adjacent ET-1 pre-constricted rings, with no ghrelin peptides added, served as time-matched controls. Vasodilator responses were expressed as percentage reversal of the ET-1 constriction and the negative logarithm of the EC_{50} value (pD_2) and the maximum response (E_{max}) were determined using iterative curve fitting software (FigP, Biosoft, Cambs, UK).

2.4.2. Ghrelin actions in human paced atria

Human atrial appendages were cut into 3–4 mm strips parallel to atrial trabecular muscle bundles and set up in 10 ml organ baths containing oxygenated Krebs' solution for isometric tension recordings as described before [37]. After a 1 h equilibration period, atrial strips were paced using field stimulation (1 Hz square-wave pulse of 5 ms duration, voltage just above threshold (<4.0 V) and set to optimum resting tension by increasing basal tension until no further increase in developed tension was observed. Tissue strips were adjusted to 50% of optimum resting tension and left to equilibrate for 30 min. Cumulative concentration-response curves were constructed to ghrelin and des-octanoyl ghrelin (0.1–300 nM). Experiments were terminated by adding isoprenaline (0.2 mM, Sigma-Aldrich) followed by $CaCl_2$ (6.7 mM) to determine maximal β_1-adrenoceptor-mediated and external Ca^{2+}-mediated inotropic responses.

2.5. Statistical analysis

E_{max} and pD_2 for ghrelin and des-octanoyl ghrelin were expressed as mean ± standard error of the mean (SEM) and compared using Student's paired two-tailed *t*-test. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Immunocytochemistry

3.1.1. Ghrelin peptide

In human atrial and ventricular myocardium ($n = 13$) ghrelin-LI was detected in endothelial cells lining small intramyocardial blood vessels (Fig. 1a,f), small coronary arteries and endocardial endothelial cells lining the recesses of the atrial chamber (Fig. 1b). In atrial and ventricular cardiomyocytes, vascular smooth muscle cells and coronary adipocytes ghrelin-LI was absent or below the level of detection. In lung ($n = 4$), ghrelin-LI was restricted to endothelial cells of small pulmonary vessels, with no ghrelin-LI detected in vascular smooth muscle of pulmonary vessels, pulmonary epithelium or connective tissue. In kidney ($n = 4$) ghrelin-LI was present in endothelial cells lining small intrarenal vessels, but not in vascular smooth muscle cells, glomeruli, renal tubular epithelial cells or connective tissue (data not shown). In human large conduit vessels ($n = 4–6$) we observed ghrelin-LI in endothelial cells lining saphenous vein (Fig. 1c), coronary artery (Fig. 1d) and LIMA (Fig. 1e) but not in vascular smooth muscle cells forming the media. Staining was absent in sections where the primary antiserum was omitted and in sections incubated with ghrelin pre-absorbed antiserum (Fig. 1g).

3.1.2. Ghrelin receptor

Ghrelin receptor-LI was present in human small intramyocardial (Fig. 2a,c), pulmonary, renal and adrenal blood vessels, but absent or below the level of detection in epithelial cells, nerves or connective tissue. Ghrelin receptor-LI was detected in endothelial cells lining saphenous vein (Fig. 2d), LIMA (Fig. 2e) and coronary artery. Ghrelin receptor-LI was also expressed in the tunica media of saphenous vein (Fig. 2d), LIMA (Fig. 2e) and coronary artery. Low levels of ghrelin receptor-LI were observed in both the atrial and ventricular myocardium. Staining was absent in sections where the primary antiserum was omitted as a negative control (Fig. 2b,g).

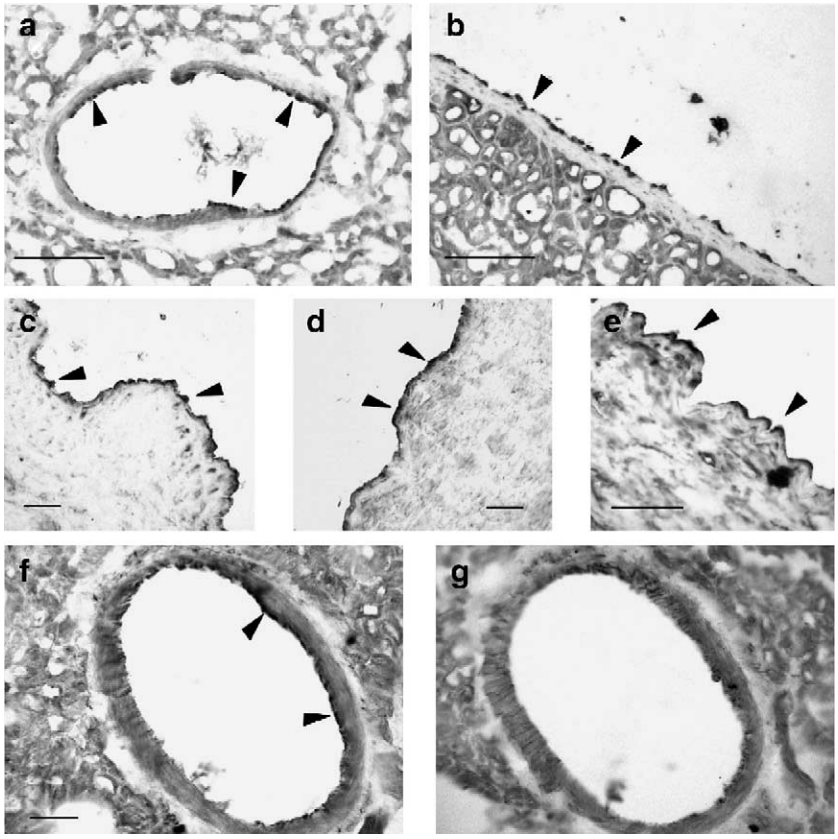

Fig. 1. Ghrelin-LI in human tissues: Representative photomicrographs show ghrelin-LI in endothelial cells from intramyocardial vessels in the ventricle (a) and in endocardial endothelial cells lining atrial recesses (b) (indicated bay arrowheads). Ghrelin-LI was also detected in the endothelial layer of saphenous vein (c), coronary artery (d) and internal mammary artery (e). Photomicrograph (f) shows ghrelin-LI in endothelial cells of intramyocardial blood vessels. In an adjacent section (g) incubated with primary antiserum pre-absorbed with ghrelin no ghrelin-LI was detected. Scale bar=50 μm.

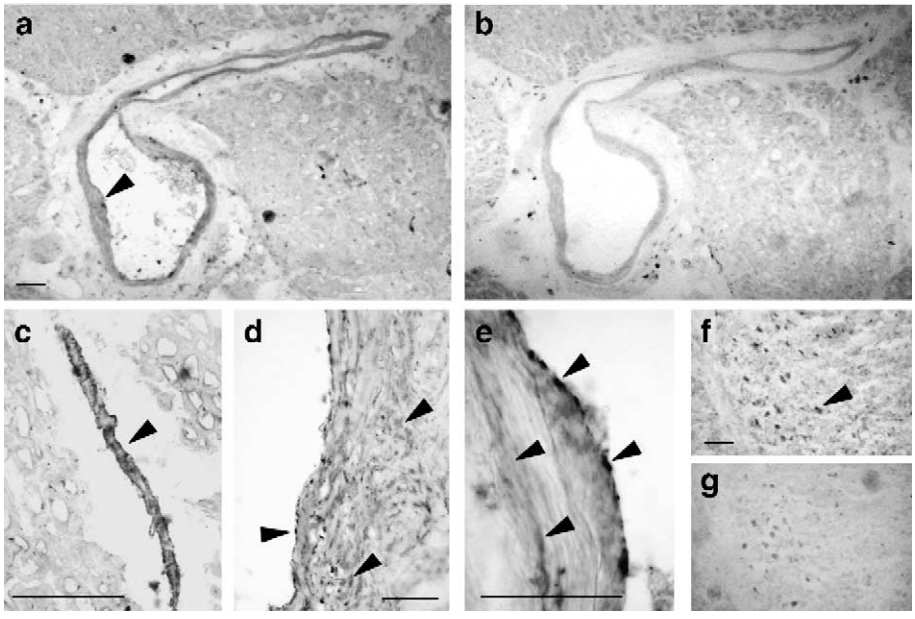

Fig. 2. Ghrelin receptor-LI in human tissues: Ghrelin receptor-LI was present in small resistance vessels of all tissues examined (indicated by arrowheads). Representative photomicrographs show ghrelin receptor-LI in vessels from the left ventricle (a, c). In human large conduit vessels ghrelin receptor-LI was expressed in endothelial cells lining large conduit vessels such as the saphenous vein (d) and internal mammary artery (e). Lower levels of ghrelin receptor-LI were detected in the tunica media of large conduit vessels including saphenous vein (d) and internal mammary artery (e). Photomicrograph (f) shows ghrelin receptor-LI in hypothalamic neurons in rat brain sections used as positive controls. In tissue sections where the primary antibody was omitted no ghrelin receptor-LI was detected (b, g). Scale bar=100 μm.

3.2. Fluorescent double staining and confocal microscopy

3.2.1. Ghrelin peptide

Confocal laser scanning microscopy detected ghrelin-LI and vWF-LI in the cytoplasm of endothelial cells from human large conduit, coronary and intramyocardial vessels. Within the endothelial cell cytoplasm ghrelin-LI was present in sub-cellular compartments of 1–5 μm in diameter (Fig. 3a–d). To further investigate the sub-cellular distribution of ghrelin-LI, staining was carried out in HUVECs: Ghrelin-LI localized to vesicle-like structures (diameter < 1 μm) within the cytoplasm and to structures of ~5 μm diameter localized close to the cell nucleus (Fig. 4a,c). Ghrelin-LI in vesicle-like structures partly colocalized with the endoplasmic reticulum marker calnexin (Fig. 4f). Ghrelin-LI did not colocalize with vWF-LI in rod-like structures of the endothelial cell cytoplasm (Fig. 4c).

3.2.2. Ghrelin receptor

In human myocardium, confocal microscopy detected intense staining for ghrelin receptor-LI on the cell surface of atrial and ventricular cardiomyocytes showing a transversal striated pattern (Fig. 3e–h). Intense staining for ghrelin receptor-LI was present in vascular smooth muscle of intramyocardial blood vessels (Fig. 3i–p), peripheral small resistance and large conduit vessels including LIMA (Fig.

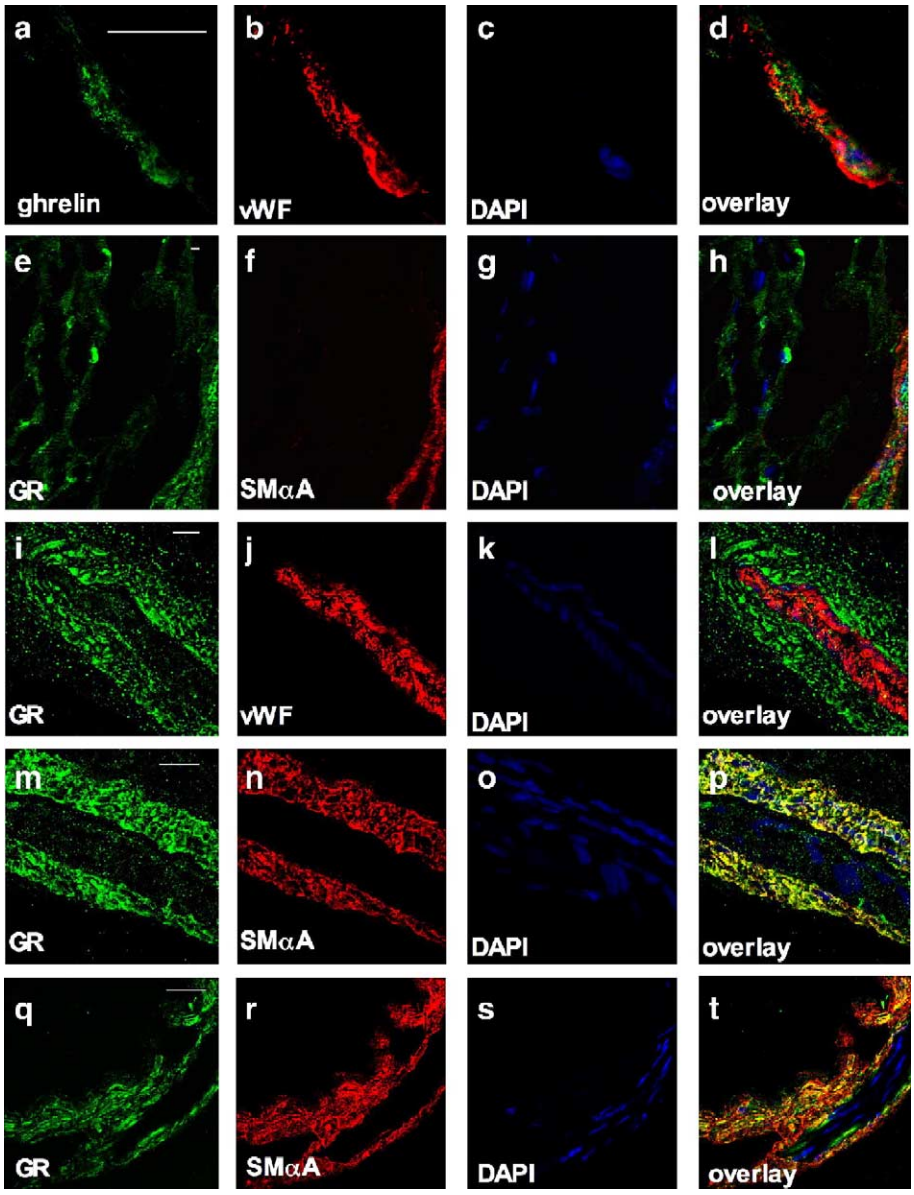

Fig. 3. Representative photomicrographs show confocal images of fluorescent double staining for ghrelin and vWF (a–d), ghrelin receptor (GR) and the smooth muscle marker α-actin (SMαA) (e–h, m–p and q–t) and ghrelin receptor and the endothelial marker vWF (i–l). DAPI is used as a marker for the cell nucleus. The first row shows single optical sections, rows 2–5 show maximum intensity overlays of optical sections acquired over a range of ~20 μm. Ghrelin-LI is present in coronary artery endothelial cells (a–d). Ghrelin receptor-LI was detected in cardiomyocytes (e–h), coronary artery endothelial cells (i–l) and vascular smooth muscle cells of coronary artery (m–p) and internal mammary artery (q–t). Scale bar = 10 μm.

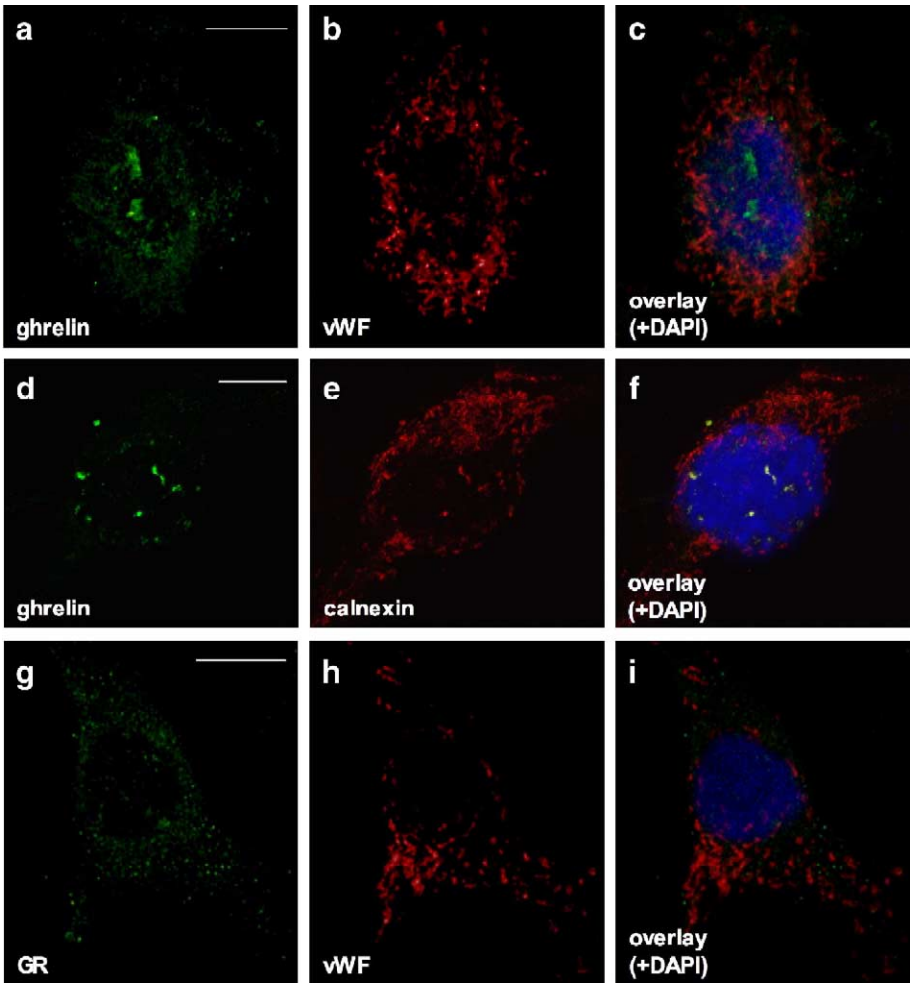

Fig. 4. Representative photomicrographs showing deconvolved confocal images of fluorescent double staining for ghrelin (a–c, d–f) or ghrelin receptor (GR) (g–i) and the endoplasmic reticulum marker calnexin (d–f) or endothelial cell marker vWF (a–c, g–i) in cultured HUVECs. Yellow colour in the right column (overlay) indicates spatial colocalization of the target antigens visualised as red and green fluorescence in individual channels. Scale bar=10 μm.

3q–t). Lower levels of ghrelin receptor-LI were present in vWF-positive vascular endothelial cells (Fig. 3i–p). In HUVECs ghrelin receptor-LI localized to vesicle-like structures (<1 μm) (Fig. 4g,i) and partly colocalized with calnexin-LI. Ghrelin-LI did not colocalize with vWF-LI in rod-like structures of the endothelial cell cytoplasm (Fig. 4i).

3.3. In vitro pharmacology

3.3.1. Reversal of ET-1 constriction in LIMA

Ghrelin reversed ET-1-induced vasoconstriction with a $pD_2 = 8.6 \pm 0.1$ and an $E_{max} = 55.8 \pm 8.9\%$ in mammary artery from five patients (one female, four males, mean age 67±5 years) (Fig. 5a). In adjacent vessels rings obtained from the same patient, des-octanoyl ghrelin reversed ET-1 induced vasoconstriction with a comparable pD_2 (8.8±0.2, $n=5$, $P=0.5$ vs. ghrelin) and E_{max} (54.7±5.3%, $n=5$, $P=0.9$ vs. ghrelin) (Fig. 5b). There was no significant difference ($P>0.05$) between basal resting tension (11.5±1.9 vs. 14.8±2.6 mN/mm), isometric force developed to 0.1 M KCl (14.0±1.8 vs. 11.3±2.5 mN/mm), isometric force developed to 10 nM ET-1 (9.3±1.5 vs. 7.0+1.1 mN/mm) and percentage reversal of ET-1 pre-constriction by 10 nM ANP (111.9±11.1 vs. 112.8±15.2%) and 10 μM SNAP (150.4±14.9 vs. 171.5±29.1%) in vessel rings treated with ghrelin or des-octanoyl ghrelin.

3.3.2. Ghrelin actions in human paced atria

In adjacent human isolated paced atria strips from six patients (six males, mean age 63±6 years), mean contractile force at optimum resting tension (7.4±1.9 mN) was increased to 12.5±3.4 mN in the presence of 0.2 mM isoprenaline. In the same tissues no response was observed to ghrelin or des-octanoyl ghrelin at concentrations up to 300 nM.

4. Discussion

In this study we show for the first time expression of ghrelin in endothelial cells of human arteries and veins and

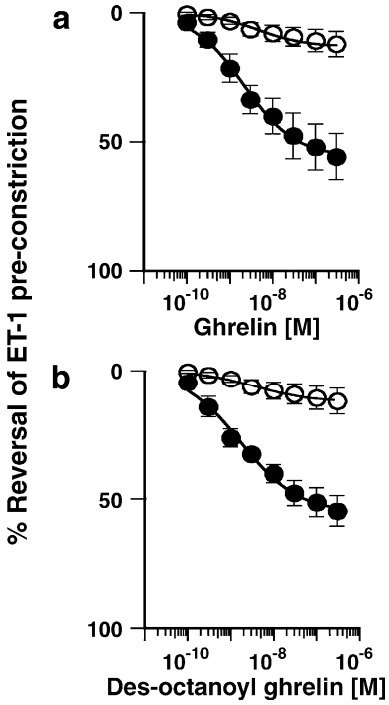

Fig. 5. Mean cumulative concentration-response curves for ghrelin ($n=5$, a) and des-octanoyl ghrelin ($n=5$, b) reversing ET-1 pre-constriction (●). Open circles (○) are time-matched controls. Results are expressed as percentage reversal of ET-1 pre-constriction (mean±SEM).

in secretory vesicles of cultured endothelial cells using immunocytochemistry. We report ghrelin receptor staining in human endothelial cells, vascular smooth muscle cells and cardiomyocytes and demonstrate vasoactive properties for des-octanoyl ghrelin in human isolated artery. Ghrelin and des-octanoyl ghrelin show comparable endothelium independent vasodilator potency and efficacy. Neither ghrelin nor des-octanoyl ghrelin had any effect on contractile force in human atria.

Our results showing ghrelin-LI in vascular and endocardial endothelial cells are consistent with the expression of ghrelin mRNA in HUVECs [38] and the detection of small amounts of ghrelin-LI in homogenates of rat and mouse heart and rat aorta using specific ghrelin radioimmunoassays (RIA) [39,40]. Ubiquitous expression of ghrelin peptide in endothelial cells may explain the widespread distribution of low levels of ghrelin mRNA reported in all human tissues examined [5]. Immunocytochemical localization of ghrelin receptors in human cardiomyocytes, vascular smooth muscle cells and endothelial cells from different vascular beds substantiates reports showing low levels of ghrelin receptor mRNA in peripheral human tissues with highest levels detected in myocardium [41,42] and corroborates observation of [^{125}I-His^{9}]-ghrelin binding in human heart and blood vessels [19]. The discovery of equipotent endothelium independent vasodilator actions for ghrelin and des-octanoyl ghrelin in isolated human artery is in agreement with previously reported vasodilator effects for ghrelin [15]. This provides evidence for biological activity of both octanoylated and non-octanoylated ghrelin in the human cardiovascular system which so far has only been reported in animals [27,28,43].

Ubiquitous expression of ghrelin-LI in human endothelial cells supports a role for ghrelin as a paracrine cardiovascular mediator acting on ghrelin receptors present on vascular smooth muscle cells, endothelial cells and cardiomyocytes. Such a paracrine circuit may explain how a gastric peptide hormone with plasma levels fluctuating according to nutritional status [4] could be involved in the continuous regulation of blood pressure and cardiac performance. In the cytoplasm of endothelial cells ghrelin is present in secretory vesicles and shows partial colocalization with the endoplasmic reticulum marker calnexin. Ghrelin does however not colocalize with vWF positive Weibel–Palade bodies of the inducible pathway of peptide release, suggesting a constitutive pathway for ghrelin synthesis [33]. Constitutive ghrelin synthesis could be modulated at the transcriptional level by changes in circulating ghrelin levels that act via endothelial ghrelin receptors to form a negative feedback loop. We detected ghrelin receptors on cardiomyocytes (Fig. 4a–d) but did not observe inotropic actions of ghrelin in vitro. However, ghrelin shows long-term effects on cardiac performance [44] that may be mediated by ghrelin activating receptors that we have demonstrated on atrial and ventricular cardiomyocytes. An alternative explanation for the positive long-term effects of ghrelin on cardiac performance would be a reduction in cardiac afterload resulting from ghrelin-induced hypotensive actions.

In humans, des-octanoyl ghrelin is the predominant circulating form of the peptide [31,39,45] which, until recently, was considered biologically inactive [2,46]. However, increasing evidence suggests biological actions for des-octanoyl ghrelin in the cardiovascular system [27,28,43]. In agreement we report equally potent and efficacious endothelium independent vasodilator actions for ghrelin and des-octanoyl ghrelin in human arteries, comparable to those observed for other established vasodilator peptides (Table 1). The ghrelin receptor-LI we observed in human large conduit vessels, including internal mammary arteries, suggests that the vasodilator responses to both peptides are mediated via

Table 1
Potency and efficacy of vasodilator peptides

Agonist	Potency (pD_2)	Efficacy (E_{max}, % reversal of ET-1 pre-constriction)
CGRP	8.08±0.17	76±15
Adrenomedullin	7.63±0.28	58±7.3
Ghrelin*	8.60±0.15*	56±8.9*
Des-octanoyl ghrelin*	8.81±0.22*	55±5.3*
CNP	8.51±0.29	53±6.3
Amylin	7.86±0.30	41±5.4
ANP	7.75±0.14	106±2.0

Vasodilator properties of ghrelin and des-octanoyl ghrelin in human left internal mammary artery compared to other established vasodilator peptides. Our findings are indicated by asterisks (*). Comparative data is taken from Wiley and Davenport [15].

the ghrelin receptor or a structurally related subtype. Neither ghrelin nor des-octanoyl ghrelin induced inotropic actions in isolated human paced atria, indicating that potential drug candidates acting on ghrelin receptors to lower blood pressure would benefit from a reduced risk of causing cardiac arrhythmia [47].

The vasoactive properties of ghrelin peptides and the tissue distribution of ghrelin receptor and ligand reported in this paper suggest a role as an emerging paracrine cardiovascular transmitter system mediating direct cardiovascular actions distinct from GH-dependent cardiovascular ghrelin effects [13]. The discovery of vascular responses to des-octanoyl ghrelin gives new perspective to the relative physiological importance of the ghrelin peptides in cardiovascular regulation, as total circulating ghrelin levels (octanoyl and des-octanoyl) are more than 10 times higher compared to levels of octanoylated peptide alone [31,32,39]. Des-octanoyl ghrelin does not show central effects on orexigenic pathways and GH release [2,48] and the ghrelin receptor, as a class 1 GPCR, is potentially tractable as a target for small molecule agonists. Thus the hypotensive actions of des-octanoyl ghrelin may indicate the possibility to design drugs with selective vascular activity avoiding unwanted effects on the CNS ghrelin receptor. It is interesting to speculate whether the cardiovascular transmitter ghrelin could provide a link between obesity and major cardiovascular co-morbidities such as hypertension, atherosclerosis and heart disease. Obese individuals, similar to carriers of a rare ghrelin gene mutation, show decreased ghrelin plasma levels and are hypertensive [23,49,50]. Endothelial dysfunction, often seen in the obesity associated metabolic syndrome, could result in impaired paracrine endothelial ghrelin synthesis [51] leading to an additional reduction in the hypotensive ghrelin actions. We have demonstrated that ghrelin potently reverses ET-1 induced vasoconstriction, a peptide that is up regulated in atherosclerosis, suggesting that the ghrelin system is a promising candidate for cardiovascular drug discovery.

Acknowledgements

This work was supported by the British Heart foundation and the Cambridge European and Isaac Newton Trusts. We would like to thank Dr Jun Wang (Department of Medicine, University of Cambridge) for culturing HUVECs and Mrs. Rhoda Kuc for technical advice on immunocytochemistry. We would also like to thank Jean Chadderton, the consultant and theatre staff of Papworth Hospital/UK for tissue collection.

References

[1] Foord SM, Bonner TI, Neubig RR, Rosser EM, Pin JP, Davenport AP, et al. International Union of Pharmacology XLVI G protein-coupled receptor list. Pharmacol Rev 2005;57:279–88.

[2] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–60.

[3] Tannenbaum GS, Lapointe M, Beaudet A, Howard AD. Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. Endocrinology 1998;139:4420–3.

[4] Cummings DE, Foster-Schubert KE, Overduin J. Ghrelin and energy balance: focus on current controversies. Curr Drug Targets 2005; 6:153–69.

[5] 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.

[6] Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, et al. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun 2000;276:905–8.

[7] Barreiro ML, Tena-Sempere M. Ghrelin and reproduction: a novel signal linking energy status and fertility? Mol Cell Endocrinol 2004; 226:1–9.

[8] Volante M, Allia E, Fulcheri E, Cassoni P, Ghigo E, Muccioli G, et al. Ghrelin in fetal thyroid and follicular tumors and cell lines: expression and effects on tumor growth. Am J Pathol 2003;162:645–54.

[9] 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.

[10] Xia Q, Pang W, Pan H, Zheng Y, Kang JS, Zhu SG. Effects of ghrelin on the proliferation and secretion of splenic T lymphocytes in mice. Regul Pept 2004;122:173–8.

[11] 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: 796–802.

[12] Nagaya N, Miyatake K, Uematsu M, Oya H, Shimizu W, Hosoda H, et al. Hemodynamic, renal, and hormonal effects of ghrelin infusion in patients with chronic heart failure. J Clin Endocrinol Metab 2001; 86:5854–9.

[13] Napoli R, Guardasole V, Matarazzo M, Palmieri EA, Oliviero U, Fazio S, et al. Growth hormone corrects vascular dysfunction in patients with chronic heart failure. J Am Coll Cardiol 2002;39:90–5.

[14] Okumura H, Nagaya N, Enomoto M, Nakagawa E, Oya H, Kangawa K. Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. J Cardiovasc Pharmacol 2002;39:779–83.

[15] Wiley KE, Davenport AP. Comparison of vasodilators in human internal mammary artery: ghrelin is a potent physiological antagonist of endothelin-1. Br J Pharmacol 2002;136:1146–52.

[16] Ishizaki S, Murase T, Sugimura Y, Kakiya S, Yokoi H, Tachikawa K, et al. Role of ghrelin in the regulation of vasopressin release in conscious rats. Endocrinology 2002;143:1589–93.

[17] Shinde UA, Desai KM, Yu C, Gopalakrishnan V. Nitric oxide synthase inhibition exaggerates the hypotensive response to ghrelin: role of calcium-activated potassium channels. J Hypertens 2005;23: 779–84.

[18] Wynne K, Giannitsopoulou K, Small CJ, Patterson M, Frost G, Ghatei MA, et al. Subcutaneous ghrelin enhances acute food intake in malnourished patients who receive maintenance peritoneal dialysis: a randomized, placebo-controlled trial. J Am Soc Nephrol 2005 (July);16(7):2111–8.

[19] 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.

[20] 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.

[21] Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, et al. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 2001;86:4753–8.
[22] Krsek M, Rosicka M, Haluzik M, Svobodova J, Kotrlikova E, Justova V, et al. Plasma ghrelin levels in patients with short bowel syndrome. Endocr Res 2002;28:27–33.
[23] 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.
[24] Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407:908–13.
[25] Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, et al. Structure–activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem Biophys Res Commun 2001;287:142–6.
[26] Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, et al. Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem 2000;43:4370–6.
[27] Bedendi I, Alloatti G, Marcantoni A, Malan D, Catapano F, Ghe C, et al. Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. Eur J Pharmacol 2003; 476:87–95.
[28] Tsubota Y, Owada-Makabe K, Yukawa K, Maeda M. Hypotensive effect of des-acyl ghrelin at nucleus tractus solitarii of rat. Neuroreport 2005;16:163–6.
[29] Muccioli G, Pons N, Ghe C, Catapano F, Granata R, Ghigo E. Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. Eur J Pharmacol 2004;498:27–35.
[30] Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, et al. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of GHS-R1a. Endocrinology 2003;9:9.
[31] Murashita M, Kusumi I, Inoue T, Takahashi Y, Hosoda H, Kangawa K, et al. Olanzapine increases plasma ghrelin level in patients with schizophrenia. Psychoneuroendocrinology 2005;30:106–10.
[32] Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, et al. Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. J Clin Endocrinol Metab 2005;90:2920–6.
[33] Mayadas T, Wagner DD, Simpson PJ. von Willebrand factor biosynthesis and partitioning between constitutive and regulated pathways of secretion after thrombin stimulation. Blood 1989;73: 706–11.
[34] Kleinz MJ, Skepper JN, Davenport AP. Immunocytochemical localisation of the apelin receptor, APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells. Regul Pept 2005;126: 233–40.
[35] Mondal MS, Date Y, Yamaguchi H, Toshinai K, Tsuruta T, Kangawa K, et al. Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. Regul Pept 2005;126:55–9.
[36] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped, AST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[37] Sandilands AJ, O'Shaughnessy KM, Brown MJ. Greater inotropic and cyclic AMP responses evoked by noradrenaline through Arg389 beta 1-adrenoceptors versus Gly389 beta 1-adrenoceptors in isolated human atrial myocardium. Br J Pharmacol 2003;138:386–92.
[38] 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.
[39] Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 2000;279:909–13.
[40] Pemberton CJ, Tokola H, Bagi Z, Koller A, Pontinen J, Ola A, et al. Ghrelin induces vasoconstriction in the rat coronary vasculature without altering cardiac peptide secretion. Am J Physiol Heart Circ Physiol 2004;287:H1522–9.
[41] Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, et al. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 1997;48:23–9.
[42] 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.
[43] 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.
[44] Nagaya N, Uematsu M, Kojima M, Ikeda Y, Yoshihara F, Shimizu W, et al. Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. Circulation 2001;104:1430–5.
[45] Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, et al. Active ghrelin levels and active/total ghrelin ratio in cancer-induced cachexia. J Clin Endocrinol Metab 2005 (May);90(5): 2920–6.
[46] Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R. Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest 2001;24:RC7–9.
[47] Greenberg B, Borghi C, Perrone S. Pharmacotherapeutic approaches for decompensated heart failure: a role for the calcium sensitiser, levosimendan? Eur J Heart Fail 2003;5:13–21.
[48] Muccioli G, Tschop M, Papotti M, Deghenghi R, Heiman M, Ghigo E. Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. Eur J Pharmacol 2002;440:235–54.
[49] 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.
[50] Formiguera X, Canton A. Obesity: epidemiology and clinical aspects. Best Pract Res Clin Gastroenterol 2004;18:1125–46.
[51] Widlansky ME, Gokce N, Keaney Jr JF, Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol 2003; 42:1149–60.