Minireview

Cardiovascular actions of the ghrelin gene-derived peptides and growth hormone-releasing hormone

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Abstract

In 1976, small peptide growth hormone secretagogues (GHSs) were discovered and found to promote growth hormone (GH) release from the pituitary. The GHS receptor (GHS-R) was subsequently cloned, and its endogenous ligand ghrelin was later isolated from the stomach. Ghrelin is a 28-amino acid peptide, whose acylation is essential for binding to GHS-R type 1a and for the endocrine functions, including stimulation of GH secretion and subsequent food intake. Unacylated ghrelin, the other ghrelin form, although devoid of GHS-R binding is an active peptide, sharing many peripheral effects with acylated ghrelin (AG). The ghrelin system is broadly expressed in myocardial tissues, where it exerts different functions. Indeed, ghrelin inhibits cardiomyocyte and endothelial cell apoptosis, and improves left ventricular (LV) function during ischemia–reperfusion (I/R) injury. In rats with heart failure (HF), ghrelin improves LV dysfunction and attenuates the development of cardiac cachexia. Similarly, ghrelin exerts vasodilatory effects in humans, improves cardiac function and decreases systemic vascular resistance in patients with chronic HF. Obestatin is a recently identified ghrelin gene peptide. The physiological role of obestatin and its binding to the putative GPR39 receptor are still unclear, although protective effects have been demonstrated in the pancreas and heart. Similarly to AG, the hypothalamic peptide growth hormone-releasing hormone (GHRH) stimulates GH release from the pituitary, through binding to the GHRH-receptor. Besides its proliferative effects in different cell types, at the cardiovascular level GHRH inhibits cardiomyocyte apoptosis, and reduces infarct size in both isolated rat heart after I/R and *in vivo* after myocardial infarction. Therefore, both ghrelin and GHRH exert cardioprotective effects, which make them candidate targets for therapeutic intervention in cardiovascular dysfunctions.

Keywords: ghrelin, GH secretagogues, growth hormone-releasing hormone, heart

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Introduction

Synthetic growth hormone secretagogues (GHSs) are a family of ligands, including peptidyl and non-peptidyl molecules and small synthetic compounds, with the ability to stimulate growth hormone (GH) secretion from the pituitary.[1] GHSs act mainly through the GHS receptor (GHS-R), a seven-transmembrane-spanning G-protein-coupled receptor that was cloned in 1996.[2]

Ghrelin, a 28-amino acid peptide, was isolated from the human and rat stomach and identified in 1999 as an endogenous ligand for the GHS-R type 1a.[3] The ghrelin peptides were found to circulate in two distinct forms, acylated ghrelin (AG) and unacylated ghrelin (UAG). Ghrelin acylation on the third serine residue, promoted by ghrelin *O*-acyltransferase,[4,5] is essential for GHS-R1a binding and for the main endocrine functions of AG, including stimulation of GH secretion from the pituitary, induction of food intake and regulation of energy homeostasis.[1] Besides its central effects, AG has a broad range of peripheral functions, including roles in circulation, digestion, inflammation, glucose metabolism, and cell proliferation and survival.[1,6,7] Ghrelin is mainly synthesized and secreted by X/A-like cells in the oxyntic glands of the stomach;[8] however, lower ghrelin expression and secretion have been also demonstrated in other tissues, including the gastrointestinal tract, pancreas, brain, pituitary gland, kidney, lung, placenta and the heart.[1,3] Similarly, besides the hypothalamus and pituitary, GHS-R mRNA expression has been shown in the heart and blood vessels, suggesting cardiovascular functions of the ghrelin system.[2,9] Moreover, cultured cardiomyocytes were found to be able to synthesize ghrelin, suggesting that ghrelin may exert a paracrine/autocrine function in cardiac muscle.[10]

UAG, whose concentration in blood is far greater than that of AG, is devoid of GHS-R binding and of the typical AG-induced endocrine effects. However, it shares many of the AG peripheral functions, particularly the cellular effects, likely through a common and as yet unidentified receptor recognized by both AG and UAG.[11–13]

The 23-amino acid peptide obestatin (Ob) is a recently identified product of the ghrelin gene, and like ghrelin is mainly produced in the stomach. Ob was identified as the G-protein coupled receptor 39 (GPR39) ligand and claimed to be a physiological opponent of AG on food intake and gastrointestinal motility.[14–16] However, its physiological role is still unclear, as the anorexigenic effects of Ob as well as its capacity to bind GPR39 have since been questioned.[17–21] Ob exerts both central and peripheral effects, which include inhibition of thirst, modulation of mnemonic functions, regulation of cell proliferation and survival in different cell types, such as pancreatic β-cells and cardiomyocytes.[22–24]

Interestingly, one of the peculiar features of ghrelin and GHSs is the synergistic relationship with the 44-amino acid hypothalamic peptide growth hormone-releasing hormone (GHRH) in GH release.[25,26] However, GHRH and its receptor, isolated in 1982 from human pancreatic neoplasms,[27,28] are entirely distinct from ghrelin and its cognate receptor, GHS-R1a.

Only later was GHRH identified and characterized from the hypothalamus.[29] GHRH plays a key role in induction of GH synthesis and release from the pituitary and is essential for expansion of the somatotrope lineage during pituitary development.[30] Extrahypothalamic production of GHRH has been implicated in tumorigenesis, suggesting peripheral actions through autocrine and/or paracrine mechanisms.[31] The direct effect of GHRH on tumors was demonstrated in studies using GHRH antagonists, which potently inhibited cancer cell growth independently of the GH/IGF-1 axis, likely through blockade of tumoral GHRH.[32]

The actions of GHRH involve the stimulation of the G protein-coupled receptor GHRH-R, whose expression, besides the pituitary, has been detected in extrapituitary tissues, such as the kidney and placenta, as well as the heart.[33,34] Similarly, GHRH mRNA, apart from the hypothalamus, was found in peripheral tissues including the heart, suggesting cardiovascular effects of the hormone.[34–36]

The present paper will give an overview of the main cardiovascular actions of the ghrelin gene-derived peptides and of GHRH. To date, a number of studies have shown the cardiovascular effects of AG, UAG and GHSs, whereas little is known about the effects of Ob. Moreover, a direct cardioprotective role of GHRH has been recently demonstrated both *in vitro* and *in vivo*, opening new perspectives for pharmacological interventions in cardiac function and heart failure (HF).

Inotropic effects of ghrelin and GHSs

Several i*n vivo* studies have suggested a physiological role of GHSs as inotropic regulators. However, it is difficult to establish whether the enhancement of cardiac output[37,38] observed in these conditions is due to a direct effect on cardiac contractility, rather than on vasodilation and reduced after-load, or secondary to increased secretion of GH. The possibility that GHSs may directly modulate cardiac contractility was suggested by a few *in vivo* studies, showing that intravenous hexarelin injection (a peptidic GHS) is able to increase left ventricular ejection fraction (LVEF) in the absence of decreased peripheral resistance in both healthy volunteers[39] and inpatients with growth hormone deficiency (GHD).[40] More recently, similar increases of LVEF and end-systolic volume in the absence of blood pressure or heart rate alterations have been reported after ghrelin administration to healthy volunteers.[41] However, some of the positive effects on contractility, at least in non-GHD conditions, may be explained by increased GH secretion, which has been demonstrated to have inotropic effects, possibly related to alterations in Ca^{2+} transients.[42]

In vitro studies on the effects induced by different GHSs on isolated papillary muscle contractility were performed in our laboratory to investigate the mechanism responsible for their inotropic actions.[43,44] Hexarelin induced time-dependent effects on rat papillary muscle, i.e. a transient increase followed by a reduction of contractile force. The negative inotropic effect of hexarelin was reduced by blockade of nitric oxide (NO) synthesis, and abrogated by pretreatment with indomethacin or removal of endocardial endothelium. However, hexarelin had no significant effect on either calcium transients or on L-type Ca^{2+} current (ICa), measured in isolated ventricular cells. Taken together, these findings suggest that the effects of hexarelin are mainly due to release of NO and prostacyclin (PGI2) from the endocardial endothelium, rather than due to direct effects on cardiac cells.[44] Additional experiments were performed to compare the effects of hexarelin to those of AG and their endogenous derivatives des-Gln14-ghrelin and UAG on mechanical tension developed by guinea pig papillary muscle and on ICa,L of isolated ventricular cells. In agreement with our previous observations, all the GHSs investigated reduced the tension developed in a dose-dependent manner; moreover, their action depended on NO synthesis as well as endocardial endothelium integrity. The order of potency of the GHSs investigated (UAG > AG = des-Gln14-ghrelin > hexarelin) was consistent with the binding experiments performed on ventricular membranes where either AG or UAG, and hexarelin, recognized a common high-affinity binding site. These results indicated that the cardiotropic action of natural and synthetic ghrelin analogues reflects the interaction with a novel GHS receptor (peculiarly common for AG and UAG), leading to release of cyclooxygenase metabolites from endothelial cells (ECs).[43]

The contractile effects of ghrelin and expression of its receptor GHS-R1a were further investigated in normal and hypertrophic myocardium by Soares *et al.*[45,46] Besides confirming the negative inotropic and lusitropic effects of ghrelin, and the role of cycloxygenase, NO synthase and endocardial endothelium in mediating its effects, they showed that they are independent of the receptor GHS-R1a, since they were not affected by the specific

antagonist D-Lys(3)-GHRP-6. Interestingly, the effects of ghrelin and expression of its receptor are preserved in right ventricular hypertrophy, suggesting that ghrelin may be a new target in the progression to HF.

To further investigate the signaling system underlying the direct effects of GHSs on cardiomyocytes, the effects of ghrelin and hexarelin were recently studied on isolated ventricular myocyte shortening, Ca(2+) transients and ICa,L.[47] Both GHSs produced a positive inotropic effect and increased the amplitude of Ca(2+) transients and ICa,L, which were abrogated by blockade of GHS-R1a or protein kinase C (PKC). Although partially conflicting, taken together, these data suggest that on isolated cardiac cells (i.e. in the absence of ECs) GHSs increase the ICa,L, through GHS-R1a receptor and PKC signaling cascade, thus exerting a direct positive inotropic effect. In the case of papillary muscle, which is composed of multiple cell types, the negative inotropic effect may occur through indirect action via the endothelium, with a possible release of NO and PGI2 from these cells. It is possible that *in vivo* experiments with observations of the integrated vascular response could help to shed some light on the vasodilatory effects of ghrelin.

Vasodilation and endothelial function

In vivo studies

Several clinical and experimental studies have demonstrated that GHSs exert an important cardiovascular action as vasodilatory agents. It has been shown that a single injection of AG caused a significant decrease in blood pressure in healthy volunteers.[48] A modest but significant decrease in systolic blood pressure was also observed in hypophysectomized (hx) rats treated with AG for two weeks (unpublished observations from Isgaard laboratory). However, the vasodilatory effect of ghrelin and GHSs is not entirely consistent in the literature, as described in other studies.[39–41] This may possibly be due, at least in part, to differences in administration modes and dosing, or may be related to differences in animal species.

For instance, it has recently been shown that intracoronary infusion of AG decreases coronary blood flow without affecting the other hemodynamic parameters or plasma levels of GH in the pig.[49] By using pharmacological blocking agents, these authors showed that the coronary vasoconstriction induced by ghrelin was independent from muscarinic and alpha-adrenergic receptor stimulation. Instead, it probably involved the inhibition of a vasodilatory beta2-adrenergic receptor-mediated effect related to the release of NO.

The cardiovascular actions of endogenous AG have been recently demonstrated in conscious rats treated with its receptor antagonist [D-Lys-3]-GHRP-6.[50] Mean arterial pressure and heart rate were dose-dependently elevated after application of [D-Lys-3]-GHRP-6. Sympathetic blockade of both alpha-adrenoreceptors and beta1-adrenoreceptors abolished these hemodynamic alterations, suggesting that the increases of arterial pressure and heart rate induced by injection of ghrelin antagonist are, at least in part, due to the activation of the sympathetic nervous system. These results indicated that the use of the ghrelin antagonist system as a therapeutic target for reduction in food intake might lead to serious side-effects such as elevated blood pressure, particularly since many patients will already have high blood pressure as part of their metabolic syndrome.

The hemodynamic effects of ghrelin and Ob have been recently compared in spontaneously hypertensive rats.[51] In addition to confirming the hypotensive action of AG, which reduced mean arterial pressure and heart rate without altering the baroreflex sensitivity, these authors showed that Ob did not affect mean blood pressure and the other hemodynamic parameters.

Several possible mechanisms for the vasodilatory effect of ghrelin have been suggested. Besides the direct effect of AG observed in several *in vitro* studies (see the following section), another interesting possible mechanism reported is a central effect, involving decreased sympathetic nervous activity and arterial pressure, observed after unilateral microinjection of AG into the nucleus of the solitary tract of rats.[52] These findings are in line with a previous report describing decreased mean arterial pressure and renal sympathetic nerve activity by ghrelin in rabbits, thus suggesting that central ghrelin participates in the regulations of the sympathetic nerve activity to the kidney and the baroreceptor reflex.[53]

While intravenous administration of ghrelin receptors agonists (non-peptide ghrelin receptor agonists, GSK894490 and CP464709, and two peptide receptor agonists, AG and UAG) caused biphasic changes in blood pressure, a brief drop followed by a blood pressure increase, their direct application to the spinal cord caused only a blood pressure increase.[54] The maximum effects occurred when the agonist was applied at spinal cord levels T9 to T12. By using polymerase chain reaction and *in situ* hybridization, ghrelin receptor gene expression was detected and localized into spinal cord neurons, including autonomic preganglionic neurons of the intermediolateral cell columns at all levels from T3 to S2. In conclusion, the partially conflicting results obtained in studies on the vasomotor effect of ghrelin depend on the different responses evoked by stimulation of ghrelin receptors, according to their localization. While infusion of AG into the nucleus of the solitary tract decreases mean arterial blood pressure and sympathetic nerve activity, the stimulation of receptors present on sympathetic preganglionic neurons increases blood pressure; on the other hand, receptors present on blood vessels are responsible for the observed decreases in blood pressure.

In vitro studies

Several *in vitro* studies were performed to investigate the mechanisms responsible for the hypotensive effect of GHSs. Wiley and Davenport[55] first demonstrated that ghrelin exerts an antagonistic action against the long-lasting vasoconstrictor effect of endothelin-1 in isolated human mammary arteries. As it is also able to exert its vasodilatory effects in endothelium-denuded preparations, it has been suggested that ghrelin is an effective endothelium-independent vasodilator.[55] Studies on aortic ring preparations from GH-deficient rats treated with ghrelin

showed an increased maximal acetylcholine-induced vasorelaxation. This vasodilatory effect was accompanied by an increase in endothelial nitric oxide synthase (eNOS) expression.[56] Moreover, the effect of ghrelin could be inhibited by *N*(G)-nitro-L-arginine methyl ester, a non-selective NOS inhibitor, adding further support for a NO-dependent pathway. However, the same research group has also reported possible vasodilatory effects independent of NO mechanisms.[57]

In contrast with the coronary vasoconstrictor effect described by Pemberton *et al.*[58] in isolated perfused rat hearts, due to PKC and L-type Ca(2+) channel activation, we observed that ghrelin (50 nmol/L) significantly reduced the vasoconstrictor effect exerted by endothelin-1 in guinea-pig isolated hearts.[59] To investigate the mechanism responsible for the vasodilatatory effect of ghrelin, NO production was studied in cultured bovine aortic endothelial (BAE-1) cells by using NO fluorimetric confocal imaging; on these cells, ghrelin induced an increase in NO production, which was blocked by the phosphoinositide-3 kinase (PI3K) inhibitor Wortmannin.[59] Our results were confirmed on BAE-1 and in human aortic endothelial cells by Iantorno *et al.*[60] In addition, they showed that the stimulatory action of ghrelin could be blocked by pretreatment of cells with L-nitro-arginine methyl ester (L-NAME) or (D-Lys3)-GHRP-6 (selective antagonist of ghrelin receptor GHSR-1a), as well as by knock-down of GHSR-1a. Moreover, ghrelin increased the level of phosphorylation of Akt (Ser473) and eNOS (Akt phosphorylation site Ser1179).

The ability of ghrelin to activate NO synthase in ECs was confirmed by Xu *et al.*,[61] both in cultured ECs and in intact vessels. Ghrelin induced phosphorylation of eNOS on an activation site and production of NO in human umbilical vein ECs and bovine aortic ECs. The eNOS phosphorylation was also observed in mouse aortas perfused *ex vivo* with ghrelin and in aortic tissues isolated from mice injected with ghrelin. Activation of eNOS and NO production induced by ghrelin depend on a ghrelin receptor/Gq protein/calcium-dependent pathway, leading to stimulation of AMP-activated protein kinase (AMPK) and Akt activation. It has been suggested that calmodulin-dependent kinase is a potential convergence point for the regulation of Akt and AMPK activation in ghrelin signaling.

Taken together, these results suggest a physiological role for ghrelin as modulator of vasomotor tone in several districts, including coronary vessels. By stimulating NO production and release from the endothelium using a signaling pathway that involves GHSR-1a, PI3K/Akt and eNOS, ghrelin may antagonize the effect of vasoconstrictor agents. Furthermore, since eNOS activation is critical for ghrelin inhibition of vascular inflammation, these data highlight the therapeutic potential for ghrelin to correct endothelial dysfunction associated with atherosclerotic vascular diseases and metabolic syndrome. Indeed, a recent clinical observation evaluating forearm blood flow responses suggests that short-term infusion of ghrelin to patients with the metabolic syndrome can normalize the endothelin-1/NO balance, possibly through the stimulation of NO production.[62] Interestingly, single nucleotide polymorphisms of the ghrelin gene were found to be associated with hypertension in a study of more than 1100 hypertensive subjects and 1400 control subjects.[63]

Cardioprotective effects against ischemia and reperfusion and antiapoptotic effects

Experiments performed on different *in vivo* and *in vitro* models by several research groups demonstrated that GHSs may protect the heart against dysfunctions induced by ischemia and reperfusion (I/R). De Gennaro Colonna *et al.*[64] first showed that a long-lasting treatment with GH or hexarelin in GH-deficient rats may ameliorate the recovery of left ventricular end diastolic pressure (LVEDP) and contractility of isolated *ex vivo* coronary perfused hearts undergoing I/R, in comparison to control hearts from GH-deficient rats. The protective effects of GH or hexarelin were confirmed in a subsequent study, performed on *ex vivo* perfused hearts from hypophysectomized rats.[65] It is noteworthy that in both these studies, performed in two different models of GH-deficient rats, the cardioprotective effects of hexarelin were independent of increased GH secretion, suggesting a direct action on cardiac receptors. Myocardial stunning is a clinical entity, which can be defined as transient myocardial ischemia involving both systolic and diastolic function. The possible protective effects of GH and GHSs on myocardial stunning, i.e. the transient, reversible cardiac dysfunction induced by a short period of ischemia followed by reperfusion, were studied *ex vivo* in hearts isolated from rabbits pretreated with GH or GHRP-2 for two weeks.[66] When the hearts were subjected to 15 min I followed by 80 min R, those pretreated with GHRP-2 showed a significant improvement of diastolic function compared with non-ischemic hearts, while pretreatment with GH was ineffective. However, neither GH nor GHRP-2 had significant effects on systolic function.

Several *in vitro* studies were performed to investigate the mechanisms responsible for the protective effect of GHSs against I/R. First of all, experiments were performed to verify whether acute administration of GHSs may exert a protective effect. To this purpose, the possible protective effect of ghrelin and the synthetic secretagogues hexarelin and MK-0677 were tested on isolated perfused rat hearts subjected to I/R.[67] While hexarelin and AG significantly reduced infarct size, MK-0677 and UAG were ineffective. The PKC inhibitor chelerythrine reduced the protection provided by hexarelin. Further evidence for a protective action of ghrelin against cardiac I/R was provided by the study by Chang *et al.*,[68] which showed that administration of ghrelin during R resulted in an improvement in coronary flow, heart rate, left ventricular systolic pressure and LVEDP, enhanced rates of left ventricular contraction and relaxation, and reduced myocardial release of lactate dehydrogenase and myoglobin, indicating protection against cardiomyocyte injury. Interestingly, the receptor-binding assay demonstrated that the maximum binding capacity of ghrelin to sarcolemmal membranes was significantly increased after I and was further increased after I/R. Taken together, these data demonstrate that ghrelin and hexarelin exert specific

cardioprotective effects against myocardial I/R injury, which are independent of GH secretion and likely involve binding to cardiovascular receptors, a process that is upregulated during I/R.

In vitro experiments underlining the protective effect of GHSs against myocardial I/R dysfunction encouraged further studies to verify if these hormones maintain these properties *in vivo* and to identify their possible clinical correlates. Indeed, a significant decrease in ghrelin plasma levels has been observed in patients with myocardial infarction (MI).[69]

The effect of ghrelin administration on left ventricular (LV) remodeling following MI has been recently investigated by Soeki *et al.*[70] These authors showed that LV enlargement induced by MI was significantly attenuated in rats treated with ghrelin for two weeks from the day after MI operation. This effect was accompanied by a decrease in LV end-diastolic pressure and an increase in the peak rate of the rise and fall of LV pressure. The improvement of cardiac function was related to the suppression of the increase in heart rate and plasma norepinephrine concentration induced by MI, which ultimately exacerbates chronic cardiac dysfunction. Interestingly, similar protective effects (i.e. reduction in the increased sympathetic nerve activity and mortality rate) were observed also after early ghrelin treatment (1 min, or 2 h, after the infarct) by Schwenke *et al.*[71] Although further investigations are needed to establish whether the early beneficial effects of ghrelin also have long-term benefits for improving cardiac function, taken together, these data suggest the potential usefulness of this peptide as a new cardioprotective hormone early after MI.

Several studies were devoted to investigating the mechanism responsible for the protective effects of ghrelin against cardiac dysfunction and cell death after I/R. On the basis of these results, it is possible to hypothesize that this is due to several factors, such as direct protective, antiapoptotic and anti-inflammatory effects.

In vitro experiments showed that both AG and UAG inhibit apoptosis of primary adult and H9c2 cardiomyocytes and ECs.[72] The protective effect of these peptides depends on the activation of extracellular signal-regulated kinase (ERK) 1/2 and PI3K/Akt. Interestingly, the fact that both AG and UAG recognize common high-affinity binding sites on H9c2 cardiomyocytes, which do not express GHSR-1a, suggests the presence of a novel, yet to be identified cardiac receptor, distinct from GHSR-1a. Several reports further confirmed the antiapoptotic effect of ghrelin, independently of the method used to induce cell damage, also in different cell types through similar signaling pathways.[13] Iglesias *et al.*[10] showed that ghrelin protects against the apoptosis-inducer cytosine arabinoside (AraC) in mouse adult cardiomyocyte cell line HL-1. Moreover, it has been recently shown that similarly to H9c2 cells, in HL-1 cardiomyocytes and primary cultured-mouse and rat cardiomyocytes, UAG recognizes specific binding sites and both AG and UAG exert antiapoptotic effects. Interestingly, the same study showed that AG and UAG had opposing metabolic effects. Indeed, AG inhibited insulin-induced glucose uptake and had no effect on fatty acid uptake, whereas UAG reversed the AG inhibitory effect on glucose uptake and increased fatty acid uptake and glucose transporter-4 translocation to the cell membrane.[73]

A beneficial effect of this peptide was also reported in a model of chronic HF in the rat,[74] in which the suppression of cardiomyocyte apoptosis was accompanied by improvement of LV function and heart remodeling in chronic heart failure (CHF).

It has been reported that increased levels of endogenous ghrelin during the progression of doxorubicin-induced cardiomyopathy and HF might provide a compensatory self-protective effect.[74] The pathways responsible for these protective effects were investigated *in vitro* on primary cultured cardiomyocytes challenged with doxorubicin in the presence or absence of ghrelin or a tumor necrosis factor-α (TNF-α) antagonist.[75] Ghrelin not only reduced apoptosis and markers of oxidative stress but also increased antioxidative enzyme activity and retained mitochondrial membrane potential and energy metabolism. Moreover, ghrelin increased mitochondrial antiapoptosis-related gene protein expression, reduced cytoplasmic cytochrome C (Cyt C) release and strengthened the activation of NF-κB. Since all these effects were abrogated by the TNF-α antagonist, the authors suggested that the antioxidative and antiapoptotic effects of ghrelin are due to its ability to upregulate TNF-α and involve the TNF-α/NF-κB activation pathways.[75] On the other hand, ghrelin was found to improve cardiac performance in rats with CHF, to reduce cardiomyocyte apoptosis and to decrease inflammatory cytokines, such as TNF-α-and NF-κB.[76] Proinflammatory and proapoptotic cytokines, such as TNF-α, are upregulated in patients with congestive HF, particularly in those with cardiac cachexia, and have been implicated in the pathophysiology of this disease.[77] Moreover, ghrelin has been shown to inhibit TNF-α-induced cytokine release and NF-κB activation in human ECs.[78] Accordingly, ghrelin has been previously found to counteract cytokine-induced apoptosis and to reduce inflammation both *in vitro* in different cell types and *in vivo*.[13,79,80]

Endoplasmic reticulum dysfunction and the consequent Ca^{2+} overload are among the key intracellular mechanisms involved in heart reperfusion injury. The possibility that ghrelin may reduce endoplasmic reticulum stress has been recently investigated on the isolated rat heart undergoing I/R.[81] Preadministration of ghrelin *in vivo* greatly ameliorated the damaged heart function, attenuated myocardial injury and apoptosis, and decreased the expression of endoplasmic reticulum stress markers. Moreover, ghrelin directly inhibited the myocardial endoplasmic reticulum stress response induced *in vitro* by tunicamycin or dithiothreitol in rat cardiac tissue. These findings support the hypothesis that ghrelin could protect the heart against I/R injury, at least in part, through inhibiting myocardial endoplasmic reticulum stress.

The number of clinical trials with GHSs and ghrelin in conditions of impaired cardiac function is very limited so far. Acute administration of hexarelin increased LVEF in patients with ischemic but not dilated cardiomyopathy.[82] Moreover, in anesthetized patients undergoing bypass surgery, short-term hexarelin infusion was found to increase

LVEF, cardiac output and mean arterial pressure without any changes in peripheral resistance.[83]

A more recent clinical trial with short-term administration of ghrelin to patients with CHF also demonstrated beneficial effects.[48] Twelve patients with CHF received a single intravenous infusion of human ghrelin or placebo. Ghrelin significantly increased cardiac index, stroke volume index and decreased systemic vascular resistance within 60 min. To our knowledge, only one study with a longer duration of treatment of patients with congestive HF has been published so far.[84] In this open study, 10 patients with CHF of heterogeneous etiology were treated for three weeks with intravenous administration of ghrelin. Ghrelin significantly increased LVEF, LV mass and decreased LV end-systolic volume. Moreover, ghrelin also increased peak workload and peak oxygen consumption during exercise and there was a reported decrease in plasma norepinephrine. No serious adverse events were observed.

Recently, ghrelin and GHS-R1a expression have been investigated in different myocardial areas of patients with CHF as compared with heart-healthy subjects, to better define the role of the ghrelin signaling system in the networks regulating cardiac function and its potential as a target for diagnosis and/or treatment of CHF. Ghrelin expression was decreased in the atrium and ventricles of CHF hearts whereas GHS-R1a expression was increased. These findings suggested a possible association between myocardial expression of ghrelin and GHSR-1a and myocardial function. Ghrelin reduction in CHF hearts may reflect maladaptive processes, whereas GHS-R1a increase may represent a compensatory mechanism.[85]

End-stage CHF is sometimes associated with cachexia which is a severe catabolic state characterized by weight loss, muscle wasting and poor prognosis. Thus it is tempting to speculate that ghrelin and GHSs may have multiple beneficial effects on this patient population by exerting both positive cardiovascular and orexigenic actions. To date there are no long-term clinical trials substantiating this hypothesis, although preliminary data suggest that administration of ghrelin increases muscle strength and lean body mass in patients with CHF.[84]

Cardiovascular effects of Ob

Recently identified as a novel ghrelin gene peptide,[15] Ob is also likely to play a significant role in cardiac function in humans, both under physiological and pathological conditions. Indeed, saliva Ob levels were found to be slightly higher in overweight patients with ischemic heart disease compared with healthy controls[86] and significantly decreased in serum of subjects with type 2 diabetes mellitus[87] and obesity,[88] two pathological conditions that are associated with a number of clinical manifestations involving the cardiovascular system. Moreover, it has been recently shown that Ob levels are increased in the presence of altered ghrelin to Ob ratio in CHF patients with cachexia[89] as well as in spontaneously hypertensive rats[90] (see further details in the following sections). However, to date, the physiological role of Ob is still largely unknown and its cardiovascular effects are yet to be determined.

We have recently demonstrated that Ob prevents apoptosis both in rodent β-cells and human pancreatic islets by binding to specific receptors and activating PI3K/Akt and ERK1/2.[22] Both PI3K/Akt and ERK1/2 have been demonstrated to protect against apoptosis in several cell types and together with PKC, have also been proposed as integral components of antiapoptotic cascades involved in myocardial protection in the setting of I/R, as well as myocardial preconditioning.[91] Bearing in mind the beneficial cardiotropic effects of ghrelin and its analogues, in particular the improvement of myocardial function and the reduction of infarct size after I/R,[68] and in light of the fact that Ob originates from the same pre-prohormone, and that circulating levels of the 29–94-amino acid proghrelin fragment (C-ghrelin) from which Ob is cleaved[92] are modulated in cardiovascular diseases,[69] it seems logical to hypothesize that Ob could also have a role in protecting myocardial function.

The possible protective effects of Ob on acute I/R injury in isolated rat heart and its effects on apoptosis induced in cultured rat cardiomyocytes by an experimental model of I/R were recently investigated.[24] We observed that pretreatment of isolated rat hearts with Ob reduced infarct size and contractile dysfunction induced by I/R. In addition, Ob exerted an antiapoptotic effect on rat H9c2 cells or isolated ventricular myocytes subjected to I/R. In both cases, the cardioprotective effect was due to activation of PKC, PI3K or ERK1/2 pathways. In keeping with these functional findings, radioreceptor binding results revealed the presence of specific high-affinity Ob binding sites, mainly localized on membranes of ventricular myocardium and cardiomyocytes. Although it has been suggested that Ob opposes several actions of ghrelin,[15,93] the protective effect of Ob observed was fully comparable to that afforded by ghrelin, at least in our experimental models.[24] Up to now, this is the first demonstration that, by acting on specific receptors, Ob is able to activate several survival kinases and to protect cardiac cells against myocardial injury and apoptosis induced by I/R.

Cardioprotective actions of GHRH

Until recently, interest in the cardiovascular effects of GHRH had been very scant. Only one study, published in 1988, reported a positive inotropic effect of GHRH in guinea-pig papillary muscle.[94] Since then, the studies on the extrapituitary functions of the hormone were mainly focused on its tumorigenic effects and on the potential antitumor actions of GHRH antagonists.[32] We recently hypothesized that a neurohormone with GH-releasing properties such as GHRH would elicit cardioprotective responses. Indeed, GHRH was found to prevent serum starvation- and isoproterenol-induced apoptosis in adult rat ventricular myocytes and in the cardiac cell line H9c2. Both cell types expressed GHRH-R, and the GHRH-R antagonist JV-1-36 inhibited the GHRH antiapoptotic effect, suggesting receptor-mediated effects of GHRH. GHRH-induced cardiac cell protection required ERK1/2 and PI3K/Akt activation and adenylyl cyclase/cAMP/protein kinase A signaling. Interestingly, GHRH prevented isoproterenol-induced up-regulation of the proapoptotic protein inducible cAMP early repressor, further supporting its cardioprotective

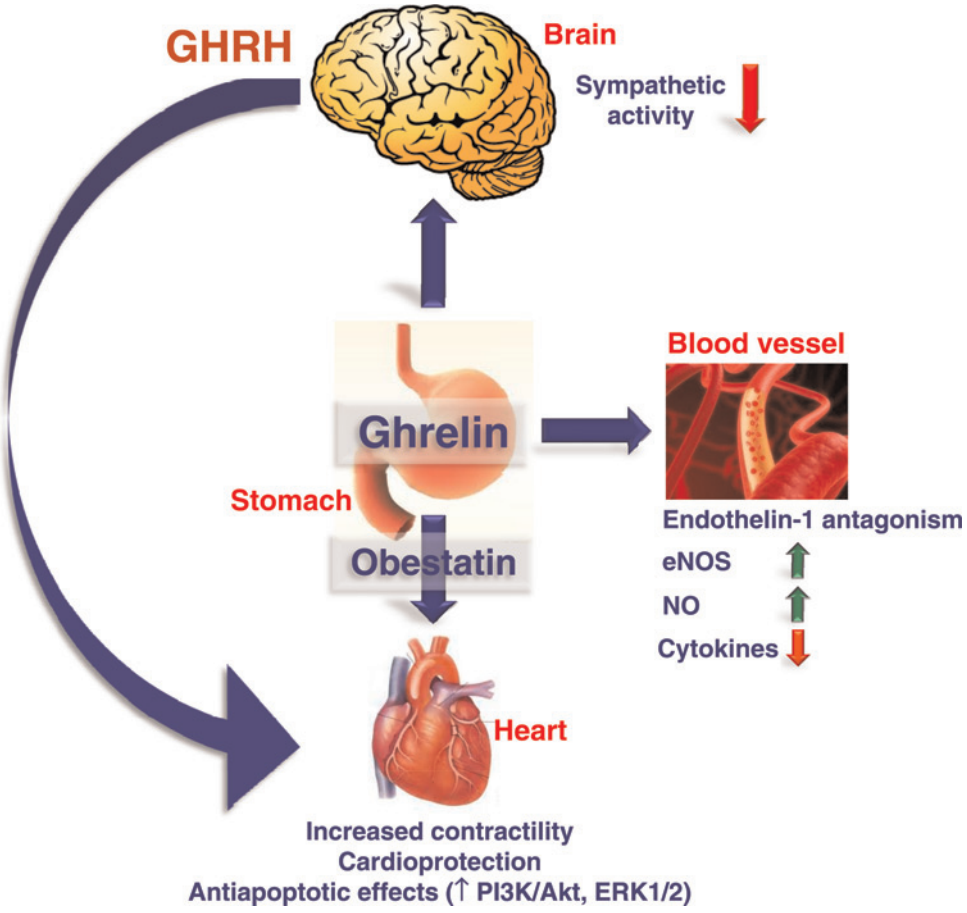

Figure 1 Schematic illustration of possible interactions between growth hormone-releasing hormone (GHRH), ghrelin, the central nervous system (CNS) and the cardiovascular system. Exogenous growth hormone secretagogue (GHS) or ghrelin may interact directly with blood vessels and antagonize the effect of endothelin-1, increase eNOS expression and the production of NO. An alternative pathway may be through the CNS leading to decreased sympathetic nervous activity. Additional vasoactive effects include inhibition of cytokine release from the blood vessels. Both GHRH and ghrelin have been found to exert cardioprotective and antiapoptotic effects, and ghrelin also increases contractility. eNOS, endothelial nitric oxyde synthase; NO, nitric oxide; ERK1/2, extracellular signal-regulated kinase; PI3K, phosphoinositide-3 kinase

role. In isolated rat hearts subjected to I/R injury, pretreatment with GHRH enhanced LVDP recovery and strongly reduced the development of diastolic contracture, as well as infarct size following reperfusion. These effects were likely triggered by GHRH-R and dependent on PI3K/Akt signaling, as suggested by their inhibition in the presence of JV-1-36 and LY294002.[35]

More recently, Kanashiro-Takeuchi *et al.* showed that a GHRH agonist (GHRH-A) has a cardioprotective role *in vivo* after acute MI. Animals receiving GHRH-A had improved cardiac structure and function and reduced infarct size. In addition, cardiac fibrosis was markedly reduced by GHRH-A, which also increased Bcl-2 antiapoptotic protein in cardiomyocytes of the infarct zone. These effects were found to be direct, and did not involve the GH/IGF-1 axis, because the circulating levels of these hormones were not increased by GHRH-A treatment.[36]

Conclusions

It is becoming more and more evident that GHSs, besides their main stimulatory effect on GH release, exert important cardiovascular actions (as summarized in Figure 1). Interestingly, in addition to ghrelin, Ob, the new peptide of the ghrelin gene family also exerts cardioprotective actions. Moreover, a new player, GHRH, is now demonstrating its relevant role in cardiac function and protection. Although further studies are required to highlight the mechanisms underlying the effects of these peptides, as well as their cardiovascular actions in different animal models, the findings reported so far suggest potential therapeutic applications of the ghrelin gene products and GHRH in cardiovascular dysfunctions.

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