Growth hormone-releasing hormone promotes survival of cardiac myocytes in vitro and protects against ischaemia-reperfusion injury in rat heart

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Abstract

Aims The hypothalamic neuropeptide growth hormone-releasing hormone (GHRH) stimulates GH synthesis and release in the pituitary. GHRH also exerts proliferative effects in extrapituitary cells, whereas GHRH antagonists have been shown to suppress cancer cell proliferation. We investigated GHRH effects on cardiac myocyte cell survival and the underlying signalling mechanisms.

Methods and results Reverse transcriptase–polymerase chain reaction analysis showed GHRH receptor (GHRH-R) mRNA in adult rat ventricular myocytes (ARVMs) and in rat heart H9c2 cells. In ARVMs, GHRH prevented cell death and caspase-3 activation induced by serum starvation and by the β-adrenergic receptor agonist isoproterenol. The GHRH-R antagonist JV-1-36 abolished GHRH survival action under both experimental conditions. GHRH-induced cardiac cell protection required extracellular signal-regulated kinase (ERK)1/2 and phosphoinositide-3 kinase (PI3K)/Akt activation and adenylyl cyclase/cAMP/protein kinase A signalling. Isoproterenol strongly upregulated the mRNA and protein of the pro-apoptotic inducible cAMP early repressor, whereas GHRH completely blocked this effect. Similar to ARVMs, in H9c2 cardiac cells, GHRH inhibited serum starvation- and isoproterenol-induced cell death and apoptosis through the same signalling pathways. Finally, GHRH improved left ventricular recovery during reperfusion and reduced infarct size in Langendorff-perfused rat hearts, subjected to ischaemia–reperfusion (I/R) injury. These effects involved PI3K/Akt signalling and were inhibited by JV-1-36.

Conclusion Our findings suggest that GHRH promotes cardiac myocyte survival through multiple signalling mechanisms and protects against I/R injury in isolated rat heart, indicating a novel cardioprotective role of this hormone.

1. Introduction

Many pathological states of the heart are associated with cardiac myocyte cell death and apoptosis, including myocardial infarction and heart failure.1 β-Adrenergic receptors (β-ARs) belong to the family of seven transmembrane receptors and mediate the regulatory effects on cardiac function of the catecholamine neurotransmitter epinephrine and norepinephrine.2 Their chronic stimulation has been shown to cause cardiac myocyte apoptosis in vitro and in vivo.3 Moreover, cardiac myocytes undergo apoptosis in response to stimuli related to ischaemia, such as serum or nutrient deprivation.4,5 Thus, the control of myocyte loss through the suppression of cell death pathways represents a logical strategy to prevent myocardial failure. Growth hormone-releasing hormone (GHRH) is a 44-amino acid peptide,

structurally related to the family of ‘brain-gut peptides’, including glucagon, glucagon-like peptide 1, vasoactive intestinal peptide, and secretin.6,7 The role of hypothalamic GHRH in the regulation of GH synthesis and release from the anterior pituitary is well known,8 as well as its effect on somatotrope cell proliferation during development.9 GHRH actions involve the stimulation of its receptor (GHRH-R), a G protein-coupled receptor that activates at least two transduction pathways, the adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) via the Gsα subunit,10 and the Ras/MAPK pathway through the βγ subunits.11

GHRH-R is primarily expressed in the pituitary but gene expression has also been detected in extrapituitary tissues, such as kidney and placenta.6 Similarly, GHRH mRNA, besides the hypothalamus, was demonstrated in peripheral tissues, including the heart.6,12,13

Extrahypothalamic production of GHRH has been implicated in tumorigenesis, suggesting peripheral action of the hormone through autocrine and/or paracrine mechanisms.6,14,15 GHRH-direct action in tumours was demonstrated in studies using GHRH antagonists, which potently inhibited cancer cell growth independently of GH/insulin-like growth factor axis, likely through blockade of tumoral GHRH. Moreover, the presence of either splice variants (SVs) of GHRH-R or pituitary-type GHRH-R has been demonstrated in human cancers, suggesting that they would mediate the effects of tumoral GHRH and GHRH antagonists.7 Besides direct and indirect actions in tumour cells, GHRH has been described to stimulate steroidogenesis and to regulate immune cells function.6

Neurohormones, such as corticotropin-releasing hormone-related peptides, were recently reported to exert positive cardiovascular effects through GPCRs-mediated mechanisms.16 Moreover, we and others have shown cardioprotective actions of the GH-releasing peptide ghrelin and its analogues.17,18 On the basis of these evidence, we hypothesized that a neurohormone with GH-releasing properties such as GHRH would elicit biological responses in the heart. We investigated whether GHRH would inhibit serum starvation- and β-AR-induced cell death in adult rat ventricular myocytes (ARVMs) and in H9c2 cardiac cells and determined the underlying signalling mechanisms. In addition, we assessed the cardioprotective role of GHRH during ischaemia–reperfusion (I/R) injury in the isolated rat heart.

2. Methods

2.1 Reagents

Rat GHRH (GRF, 1-44), PD98059, LY294002, MDL-12330A, isobutylmethylxanthine (IBMX), MTT, MEM, isoproterenol, Hoechst 33258, Mammalian Cell Lysis Kit were from Sigma-Aldrich (Milan, Italy). KT-5720 was from Biomol Research Laboratory Inc. (DBA, Italy). Cell culture reagents were from GIBCO BRL Invitrogen (Milan, Italy). JV-1-36 was from Bachem (Germany). P-ERK1/2, P-Akt (Ser473), phosphorylated cAMP response element-binding protein (P-CREB) (Ser133), and CREB antibodies were from Cell Signaling Technology (Euroclone, Milan, Italy); anti-CREM was from Abcam, UK. Total antibodies were from Santa Cruz Biotechnology (DBA, Milan, Italy). Reverse transcriptase–polymerase chain reaction (RT–PCR) and real-time PCR reagents were from Applied Biosystem (Italy). Primers were from TibMolBiol (Genoa, Italy).

2.2 Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH; Publication No. 85-23, revised 1996), in accordance with Italian law (DL-116, 27 January 1992). Experiments were performed on adult female CD®IGS rats from Charles River (body weight 200–300 g). Rats were anaesthetized by i.p. injection of Nembutal (1 mg/g) and killed by stunning and cervical dislocation.

2.3 Isolation of adult rat ventricular myocytes

ARVMs were obtained from young adult (4–6 months) rats by enzymatic dissociation, as described.19 For Ca2+ measurements and immunofluorescence experiments, ∼10 000 cardiomyocytes were plated on laminin-treated dishes and incubated in M1018 medium (SIGMA) plus 10 mmol/L butanedionemonoxime, 100 U/mL penicillin, 100 μg/mL streptomycin, and ITS (insulin, transferrin, selenium, 1:1000). Cardiomyocytes were placed in a 37°C, 5% CO2 incubator until adhesion (2 h).

2.4 Cell lines

The embryonic rat heart-derived cell line H9c2 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in 100 mm dishes at 37° C with 5% CO2 in DMEM with 10% FBS, 4 mM glutamine, 1% penicillin–streptomycin and grown to subconfluence prior to experiments.

2.5 Isolated heart preparation

Hearts were excised under anaesthesia and perfused as described previously.20 Hearts were allowed to stabilize for 30 min; at this time, baseline parameters were recorded. Then, hearts were randomly assigned to one of the treatment groups described in what follows and subjected to 40 min of global, no-flow ischaemia followed by 120 min reperfusion. Control group hearts (group 1: n = 5) were perfused with physiological solution for additional 20 min before ischaemia. Group 2 hearts were treated with GHRH (50 nmol/L; n = 5) for 20 min before ischaemia; groups 3 and 4 with GHRH (50 nmol/L), in the presence of LY294002 (10 μmol/L) or JV-1-36 (10 nmol/L) (n = 4 in both cases). Myocardial injury was assessed as described.20

2.6 Cell survival

Cell survival was assessed by MTT assay (Sigma-Aldrich) as described.21

2.7 Hoechst staining

Morphological changes in the nuclei of apoptotic cells were detected by Hoechst 33258 as described.22

2.8 Caspase-3 activity

Caspase-3 activity was assessed in cell lysates, as reported previously.22

2.9 cAMP

H9c2 cells and ARVMs were starved for 4 h and incubated in the presence of the phosphodiesterase inhibitor IBMX (100 and 50 μM, respectively). Intracellular cAMP concentration was measured, as described previously.22

2.10 Ca2+ transients

Ca2+ transient measurements in electric field-stimulated cardiomyocytes were performed as described previously.19

2.11 Western blotting

Immunoblot analysis was performed as described.22 Forty micrograms of proteins for P-ERK, P-Akt, P-CREB, and CREM were resolved in 12% SDS–PAGE and incubated with the specific antibody. Nuclear extracts (50 μg) were used for inducible cAMP early repressor (ICER) detection.23,24 Blots were reprobed with antibodies against ERK1/2, Akt, CREB, or β-actin for normalization.

2.12 Reverse transcriptase–polymerase chain reaction

Total RNA extraction from rat pituitary, H9c2 cells and rat cardiac myocytes and reversed transcription to cDNA from 3 μg RNA, was performed as described.21 The primer sequences and amplification conditions are described in Supplementary material online. The PCR products were 199 bp for GHRH-R, 609 bp for GHRH, and 201 bp for 18S rRNA. Rat pituitary was used as positive control; negative control consisted of buffer alone with no RNA. 18S rRNA amplification served as internal control.

2.13 Real-time polymerase chain reaction

ICER mRNA was assessed by real-time PCR on ABI-Prism 7300 (Applied Biosystem), as described.22 Rat ICER (Rn00565271_m1) and 18s rRNA (Hs99999901_s1) assays were from Applied Biosystem (Milan, Italy). Results were normalized to 18s rRNA, and relative quantification was performed using the comparative Ct (2-ΔΔCt) method. mRNA levels were expressed as fold induction over control.

2.14 Immunofluorescence

Cardiomyocytes were fixed for 30 min (4% paraformaldehyde in 0.1 mol/L PBS, pH 7.3). Cells were incubated with 0.3% Triton and 1% serum albumin and stained with the primary antibody 24 h at 4°C. Receptor detection was performed with polyclonal antibody against hGHRH-R (Abcam, 1:100). Slides were incubated for 1 h with the secondary antibody (Cy3 Sigma, 1:1000), then mounted with DABCO, and observed 24 h later under confocal microscope, as described in Supplementary material online.

2.15 Statistical analysis

Results are expressed as means ± SE. Statistical analysis were performed using Student's t-test or ANOVA on ranks (Kruskal–Wallis one-way analysis of variance). Significance was established when $P < 0.05$.

3. Results

3.1 Expression of GHRH receptor in adult rat ventricular myocytes and H9c2 cardiac cells

RT–PCR analysis showed full-length GHRH-R mRNA expression in both ARVMs and H9c2 rat cardiac cells (Figure 1A). The primers used for GHRH-R25 potentially amplified both the short and the long isoforms of rat GHRH-R mRNA described in rat pituitary.10

Figure 1

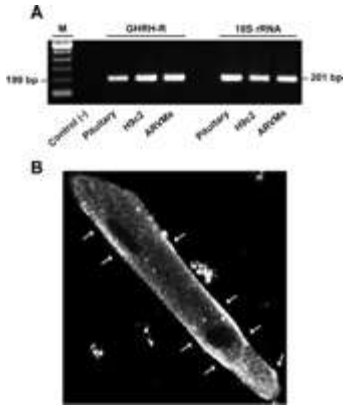

Figure 1

(A) Growth hormone-releasing hormone receptor (GHRH-R) mRNA expression determined by reverse transcriptase–polymerase chain reaction in H9c2 cells and adult rat ventricular myocytes (ARVM) cultured in the presence of serum. M, 100 bp ladder. Buffer alone was used as negative control (−), and rat pituitary as positive control. 18S rRNA was used as control gene. (B) Confocal microscopy image (magnification: ×100) showing growth hormone-releasing hormone receptor immunofluorescence staining on cardiac myocyte membrane (indicated by arrows).

GHRH-R protein expression in ARVMs was assessed by immunofluorescence staining. Cardiomyocytes presented a clear fluorescence signal for GHRH-R, localized in proximity to the membrane surface (Figure 1B).

3.2 GHRH prevents serum starvation- and ISO-induced cell death, and caspase-3 activation in adult rat ventricular myocytes

Both serum deprivation and the β-AR agonist ISO induce cell death and apoptosis in cultured cardiac myocytes.3,4,26 Therefore, GHRH-protective effect was evaluated in ARVMs cultured in serum-deprived medium, either alone or with ISO. As expected, cell survival decreased in serum-free medium, with respect to serum-treated cells (Figure 2A) and was further reduced by ISO in serum-starved conditions (Figure 2B). GHRH increased cell survival under serum deprivation; moreover, it markedly counteracted ISO-induced cell death and restored survival up to rates that exceeded those of serum-free medium alone. In both experimental conditions, the best protective effect was observed at 0.5 μmol/L (Figure 2A and B).

Figure 2

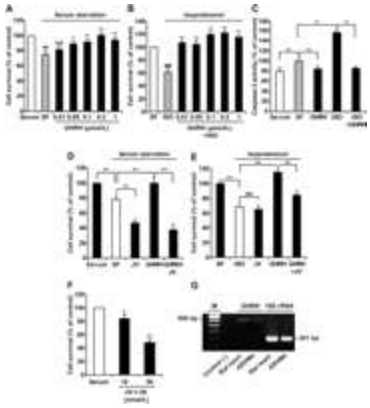

Figure 2

Growth hormone-releasing hormone (GHRH)-induced survival and inhibitory action of the growth hormone-releasing hormone antagonist JV-1-36 in adult rat ventricular myocytes (ARVMs). The cells were cultured in the presence of serum or in serum-free medium (SF) for 48 h, in either presence or absence of ISO (10 μmol/L) and growth hormone-releasing hormone, that was added 40 min prior to ISO. (A) Survival effect of growth hormone-releasing hormone, at the indicated concentrations, assessed by MTT under serum starvation. (B) Survival effect of growth hormone-releasing hormone in ISO-treated cells. (A and B) Data are expressed as per cent of control (serum and SF, respectively) and are the mean ± SE of five replicates (n = 3) [##P < 0.01 vs. serum (A) or vs. SF (B); *P < 0.05, **P < 0.01 vs. SF (A) or vs. ISO (B); ns, not significant). (C) Caspase-3 activation, expressed as percentage of control (SF). Growth hormone-releasing hormone was used at 0.5 μmol/L. Values are the means ± SE of three independent experiments (**P < 0.01). (D and E) Cell survival assessed in the presence of JV-1-36 (50 nmol/L) and growth hormone-releasing hormone (0.5 μmol/L), under serum starvation or treatment with ISO, respectively. (F) Effect of JV-1-36, at the indicated concentrations, on survival of adult rat ventricular myocytes cultured in the presence of serum (1%). For D–F, results are means ± SE of three independent experiments, each performed in five replicates (*P < 0.05; **P < 0.01). (G) Growth hormone-releasing hormone mRNA assessed by reverse transcriptase–polymerase chain reaction in adult rat ventricular myocytes cultured in the presence of serum. M, 100 bp ladder; buffer alone was used as negative control (−). 18S rRNA was used as control gene.

The activity of the apoptotic marker caspase-326 increased in serum-free medium and, particularly, in the presence of ISO. GHRH prevented caspase-3 activation under both experimental conditions (Figure 2C).

These results suggest that GHRH protects ARVMs against cell death and caspase-3 activation induced by serum starvation and β-AR stimulation.

3.3 GHRH antagonist JV-1-36 inhibits GHRH survival effect in adult rat ventricular myocytes

The human GHRH analogue JV-1-36 possesses strong antagonistic activity, both in vitro and in vivo.7,27 Moreover, the human GHRH-R gene is highly homologous to its rat counterpart,10 and JV-1-36 was found to be active in rat GH3 cells transfected with human GHRH-R.27 To assess the involvement of GHRH-R in GHRH survival effect, ARVMs were cultured in the presence of JV-1-36, either alone or with GHRH, under both serum starvation and ISO. JV-1-36 blocked GHRH-induced cell survival in serum-free conditions (Figure 2D) and inhibited GHRH-protective effect upon β-AR stimulation (Figure 2E). Interestingly, in serum-starved cells alone, but not with ISO alone, JV-1-36 strongly reduced ARVM survival (Figure 2D and E, respectively). This effect was lost at lower concentrations and reduced at higher concentrations of the antagonist (data not shown). JV-1-36 per se decreased cardiac myocyte survival in the presence of serum (Figure 2F).

In addition, we detected GHRH mRNA in both rat heart and ARVMs (Figure 2G), suggesting autocrine/paracrine survival action of endogenous GHRH, that may be inhibited by GHRH antagonists, as described previously for other cell models.7

3.4 ERK1/2 and PI3K/Akt are involved in GHRH survival effects in adult rat ventricular myocytes

ERK1/2 and PI3K/Akt play essential roles in cardiac myocyte cell survival.4,5,28 In ARVMs, GHRH increased the phosphorylation of ERK1/2 at 5 min, with a subsequent decrease at 15 min and a strong reduction at 30 min (Figure 3A). Akt was strongly activated by GHRH at 5 min, further increased at 15 min, and decreased to basal level at 30 min (Figure 3B). ERK1/2 and PI3K/Akt-specific inhibitors, PD98059 and LY294002, respectively, blocked the survival action of GHRH under both serum deprivation and ISO. No effect was observed using these compounds alone (Figure 3C and D).

Figure 3

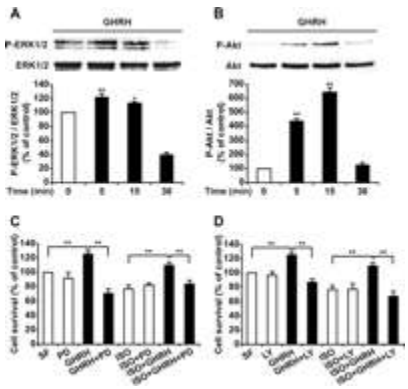

Figure 3

ERK1/2 and Akt signalling in growth hormone-releasing hormone (GHRH)-induced adult rat ventricular myocyte (ARVM) survival. ERK1/2 (A) and Akt (B) phosphorylation assessed by western blot on lysates from cells stimulated, for the indicated times, with 0.5 µmol/L growth hormone-releasing hormone in serum-free medium (upper panels). Equal protein loading was determined by reprobing with antibodies to the respective total proteins (lower panels). Blots are representative of three independent

experiments. Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as per cent of basal (*P < 0.05, **P < 0.01). (C and D) Cell survival (48 h) in serum-free medium (SF) or with ISO (10 µmol/L), in the presence of growth hormone-releasing hormone, with or without either PD98059 (10 µmol/L) or LY294002 (5 µmol/L). Data are from three independent experiments (*P < 0.05; **P < 0.01).

These results suggest that GHRH prevents ARVM cell death through ERK1/2 and Akt signalling.

3.5 GHRH signals through cAMP pathway and counteracts ISO-induced inducible cAMP early repressor increase in adult rat ventricular myocytes

Because in pituitary somatotropes GHRH-R is coupled to AC,6 we evaluated GHRH effect on intracellular cAMP levels in ARVMs. In the presence of GHRH, cAMP strongly increased at 5 min to 15 min, decreasing thereafter although remaining higher than control up to 90 min (Figure 4A). AC and PKA-specific inhibitors (MDL12330A and KT5720, respectively) blocked GHRH survival effect in either serum starvation or ISO (Figure 4B and C). GHRH even induced phosphorylation, on serine 133, of the transcription factor CREB, at 5 min (Figure 4D).

Figure 4

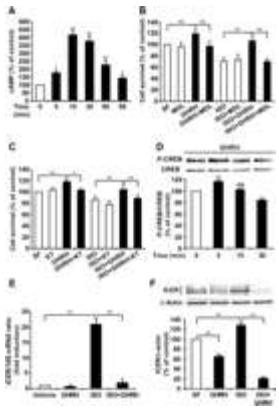

Figure 4

cAMP/protein kinase A signalling in growth hormone-releasing hormone (GHRH)-induced adult rat ventricular myocyte (ARVM) survival. (A) cAMP levels in serum-starved cells incubated, for the indicated times, with 0.5 µmol/L growth hormone-releasing hormone in the presence of isobutylmethylxanthine (100 µmol/L). Results, expressed as per cent of control (time: 0 min), are means ± SE experiments performed in triplicate (n = 3), (*P < 0.05, **P < 0.01). (B and C) Survival of adult rat ventricular myocytes cultured in serum-free medium (SF) alone or with ISO (10 µmol/L), either with or without growth hormone-releasing hormone (0.5 µmol/L), MDL12330A (3 nmol/L), and KT-5720 (0.5 µmol/L), which were added 30 min prior to growth hormone-releasing hormone. Data are from three experiments (*P < 0.05, **P < 0.01). (D) cAMP response element-binding

protein (CREB) phosphorylation on serine 133 (upper panel) evaluated by western blot on lysates from adult rat ventricular myocytes incubated with growth hormone-releasing hormone (0.5 μmol/L) for the indicated times. The same membrane was reprobed with cAMP response element-binding protein antibody, for normalization (lower panel). Shown are representative blots from three separate experiments. Histogram shows phosphorylated cAMP response element-binding protein (P-CREB) normalized to total cAMP response element-binding protein and reported as per cent of basal (**P < 0.01; ns, not significant). (E) Inducible cAMP early repressor (ICER) mRNA assessed by real-time PCR. Adult rat ventricular myocytes were incubated for 48 h in serum-free medium alone (vehicle) or in either presence or absence of growth hormone-releasing hormone (0.5 μmol/L) and ISO (10 μmol/L). Results, normalized to 18S rRNA transcript, are expressed as fold increase over vehicle and are means ± SE of three experiments (**P < 0.01). (F) Inducible cAMP early repressor protein assessed by western blot from nuclear extracts. Membranes were reprobed with antibodies to β-actin (lower panel). Graph represents the densitometric analysis of inducible cAMP early repressor normalized to β-actin and reported as per cent of basal (**P < 0.01).

In the present study, we show that ISO and GHRH exert opposite action on cardiac myocyte survival; however, ISO, like GHRH, is known to signal through the cAMP/PKA pathway.3 To identify differences between ISO- and GHRH-induced signalling, we investigated the effect of the two compounds on the expression of ICER, a positive mediator of cardiac myocyte apoptosis in response to β-AR stimulation.24 Figure 4E shows that, as expected, ISO strongly increased ICER mRNA. In contrast, although having no effect alone, GHRH completely reversed ISO-induced ICER upregulation. Immunoblot analysis of ARVM nuclear fraction evidenced that GHRH even reduced ICER protein under serum starvation and completely blocked ISO-induced ICER increase (Figure 4F).

These results suggest the involvement of cAMP/PKA signalling and ICER in GHRH-induced ARVMs survival.

3.6 Intracellular Ca2+regulation in adult rat ventricular myocytes

GHRH and ISO behaved differently with respect to intracellular Ca2+ regulation, despite the common increase in cAMP levels, as GHRH did not modify basal Ca2+ transients amplitude (0.4 ± 1.5% vs. control for 0.5 μmol/L GHRH; 57 ± 11% vs. control for 1 μmol/L ISO) (P < 0.005, n = 9).

3.7 GHRH prevents cell death and apoptosis in H9c2 cells

GHRH survival action was next investigated in H9c2 rat cardiac cells that express both β1- and β2-ARs and possess biochemical and electrophysiological characteristics of adult cardiac myocytes.29 As for ARVMs, GHRH reduced serum starvation- and ISO-induced cell death, at all concentrations tested (Figure 5A). Furthermore, GHRH prevented apoptosis by reducing the incidence of fragmented and/or pycnotic apoptotic nuclei (Figure 5B), activated ERK1/2 and PI3K/Akt pathways (Figure 5C and D), and increased

cAMP levels (Figure 5E). The time points of ERK1/2 and Akt phosphorylation, and cAMP increase were similar to those observed in ARVMs. GHRH antiapoptotic effects were prevented by the blockade of ERK1/2 and PI3K/Akt signalling (with PD98059 and LY294002, respectively) and by inhibition of AC and PKA (with MDL12330A and KT5720, respectively) (see Supplementary material online, Figure S1A–D). No effect was observed with these inhibitors alone. GHRH antiapoptotic action was also blocked by JV-1-36, which even increased apoptosis alone under serum starvation (see Supplementary material online, Figure S1E). JV-1-36 pro-apoptotic effect was also observed in the presence of serum alone (data not shown). Notably, siRNA targeted to GHRH, which strongly reduced GHRH mRNA (see Supplementary material online, Figure S1F), decreased H9c2 cell survival in either presence or absence of serum (see Supplementary material online, Figure S1G), suggesting autocrine/paracrine survival action of endogenous GHRH.

Figure 5

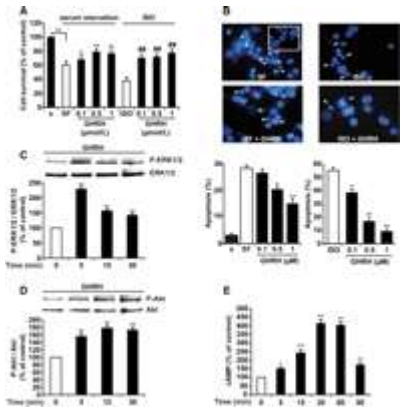

Figure 5

Growth hormone-releasing hormone (GHRH) prevents H9c2 cell death and apoptosis. Cells were cultured in the presence of serum (s) or in serum-free medium (SF) for 48 h, with or without ISO (100 µmol/L) and growth hormone-releasing hormone, which was added 40 min prior to ISO. (A) Survival effect of growth hormone-releasing hormone, at the indicated concentrations. Data, expressed as per cent of control (s, serum), are means ± SE of five replicates (n = 3) (*P < 0.05, **P < 0.01 vs. SF; ##P < 0.01 vs. ISO). (B) Apoptosis assessed by Hoechst 33258 nuclear immunofluorescence staining (magnification: ×100) in cells cultured in serum-free medium either alone (left panels) or in the presence of ISO (right panels), with or without growth hormone-releasing hormone. Inset: cells cultured with serum; arrows, apoptotic cells. Apoptosis was measured by counting fragmented/condensed Hoechst-stained nuclei (graphs). Values are expressed as per cent of apoptotic cells and are means ± SE of duplicate determinations (500 cells each, n = 3); *P < 0.05, **P < 0.01. ERK1/2 (C) and Akt (D) phosphorylation assessed by western blot on H9c2 cells stimulated, for the indicated times, with 0.5 µmol/L growth hormone-releasing hormone (upper panels). Blots were reprobed with antibodies to total proteins (lower panels). Graphs show densitometric analysis of phosphorylated proteins normalized to total proteins and reported as per cent of basal (**P < 0.01) (n = 3). (E) Growth hormone-releasing hormone-induced cAMP increase. Serum-starved cells were cultured for the indicated times with growth hormone-releasing hormone (0.5 µmol/L), in the presence of isobutylmethylxanthine

(100 µmol/L), which was added 30 min before stimulation. (*P < 0.05, **P < 0.01 vs. basal time point) (n = 3).

3.8 GHRH improves contractile recovery and reduces infarct size in isolated rat hearts subjected to ischaemia-reperfusion

The protective effect against serum starvation- and β-AR-induced cell death in ARVMs and H9c2 cells led us to test whether GHRH displays its beneficial effect on myocardial function, in rat hearts subjected to I/R injury. Cardiac weight (894 ± 32 mg), cardiac to body weight ratio (2.9 ± 0.2 mg/g), and baseline functional parameters did not differ significantly among the different groups (Figure 6). Group 1 hearts showed stable cardiac performance during the period (20 min) in which they were perfused with Tyrode solution alone before ischaemia. GHRH had minimal effects on left ventricular diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), and maximum rate of rise of LVDP (+dP/dtmax) in group 2 hearts. The rise of LVEDP caused by ischaemia (about 10 mmHg over baseline value) was comparable in both experimental groups. Reperfusion caused a rapid, marked worsening of diastolic contracture developed during ischaemia in control hearts, which reached a first peak at 15–20 min (about 20 mmHg over pre-ischaemic values), then tended to further increase to 25 mmHg over baseline values at the end of reperfusion (Figure 6). Both LVDP and +dP/dtmax showed a marked rise at the beginning of reperfusion, followed by a slow decline to finally reach 25–30% of baseline values at the end of reperfusion. The increase of LVEDP, the fall of LVDP, and +dP/dtmax during reperfusion were significantly reduced in GHRH-treated hearts in comparison to control hearts. In group 1 hearts, the infarct size measured at the end of reperfusion was 49.9 ± 3.2% of the area at risk. GHRH exerted a significant cardioprotective effect in group 2 hearts, in which the infarct size was reduced to 17.9 ± 1.2% of the area at risk (P < 0.001 vs. control hearts). Both LY294002 and JV-1-36 completely blocked the protective effect of GHRH on myocardial function (Figure 6) and on infarct size (respectively, 53.3 ± 5.1 and 52.8 ± 7.4% of the area at risk; P < 0.001 vs. group 2 hearts) in the hearts of groups 3 and 4. Preliminary experiments showed that LY294002 or JV-1-36 did not significantly alter per se myocardial performance in control conditions or after I/R.

Figure 6

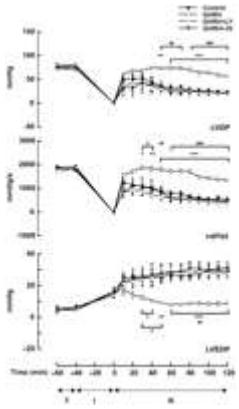

Figure 6

Growth hormone-releasing hormone (GHRH) improves the recovery of left ventricular end-diastolic pressure (LVEDP, bottom trace), maximum rate of rise of developed

pressure (+dP/dtmax, middle trace), and left ventricular developed pressure (LVDP, upper trace) during reperfusion in Langendorff-perfused rat hearts subjected to 40 min of ischaemia (I), followed by 120 min of reperfusion (R). Control hearts (group 1; n = 5) were perfused with physiological solution alone before I and during R. Group 2 (n = 5) hearts were treated with growth hormone-releasing hormone (50 nmol/L; n = 5) for 20 min (T) before ischaemia. Group 3 and 4 hearts were treated with growth hormone-releasing hormone (50 nmol/L) in the presence of LY294002 (10 μmol/L) or JV-1-36 (10 nmol/L), respectively (n = 4 in both cases). Baseline values were left ventricular diastolic pressure: 5.6 ± 1.0 and 4.9 ± 1.4 mmHg; left ventricular developed pressure: 76.6 ± 3.3 and 80.3 ± 4.1 mmHg; +dP/dtmax: 1902 ± 84 and 1918 ± 72 mmHg/s; CF: 7.7 ± 2.2 and 7.9 ± 2.6 mL/min/g (groups 1 and 2, respectively). Baseline values for groups 3 and 4 hearts were superimposable to those of groups 1 and 2. Values are expressed as means ± SE. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, group 1 vs. group 2; #P < 0.05, ##P < 0.01, ###P < 0.001, group 3 or 4 vs. group 2.

4. Discussion

This study shows that GHRH protects ARVMs and H9c2 cardiac cells against serum starvation- and ISO-induced cell death. GHRH survival effects involve cAMP/PKA, ERK1/2, and PI3K/Akt signalling and are inhibited by the GHRH antagonist JV-1-36. In addition to its effects in vitro, GHRH improves left ventricular recovery and reduces infarct size in isolated rat heart subjected to I/R injury, through PI3K/Akt- and GHRH-R-mediated mechanisms.

Cardiac myocyte cell death occurs in failing hearts and contributes to progressive myocardial dysfunction.1 Both serum deprivation and β-AR stimulation have been described to reduce cardiac cell survival.3,4,26 Here, we show that GHRH increased cell survival in ARVMs cultured in the absence of serum and completely restored myocyte viability upon treatment with ISO. GHRH also inhibited the activity of the apoptotic molecule caspase-3, whose overexpression in mice has been shown to increase infarct size and depress cardiac function.30

At variance with human cancers and mouse tissues where SVs of GHRH-R have been described,7,12 only pituitary isoforms of the receptor were demonstrated in rat.10 Accordingly, in both ARVMs and H9c2 cells, we found pituitary GHRH-R mRNA expression. GHRH-R protein was also present on ARVM membranes, suggesting receptor-mediated effects of GHRH. Indeed, GHRH-induced protection against serum starvation and ISO was reduced by its antagonist JV-1-36, which likely interfered with GHRH binding to its receptor.27 JV-1-36 decreased ARVM viability even in the absence of GHRH, in either presence or absence of serum but not upon treatment with ISO, where cell survival was likely too low to be further reduced. The antiproliferative actions of GHRH antagonists in cancer cells were shown to be partly due to blockade of the autocrine/paracrine proliferative effects of locally produced GHRH.7 Therefore, on the basis of our results obtained with JV-1-36 and of GHRH mRNA expression in rat heart,13 ARVMs, and H9c2 cells, it is conceivable that endogenous GHRH may exert autocrine/paracrine survival action in cardiac myocytes. This hypothesis is, at least partly, sustained by our results showing that in H9c2 cells transfected with siGHRH,

where GHRH mRNA expression was inhibited, cell survival was reduced in both presence and absence of serum. In addition, direct pro-apoptotic effects of GHRH antagonists have been proposed in other cell types,7 implying that multiple mechanisms in the inhibitory actions of GHRH antagonists, all related to GHRH-R or SVs.

ERK1/2 and PI3K/Akt play important roles in cardiac myocyte survival and function.4,5,28 Accordingly, GHRH promoted ERK1/2 and Akt phosphorylation in ARVMs, and specific inhibition of these pathways blocked the survival action of the hormone. Although GHRH was previously shown to stimulate the MAPK pathway,11 to our knowledge, this is the first data showing GHRH-induced Akt activation.

With regard to cAMP, contrasting evidence have been reported. cAMP may either increase cardiac myocyte survival and function, also through ERK signalling,31,32 or induce apoptosis.3,26 Here, GHRH increased intracellular cAMP in ARVMs; moreover, AC and PKA inhibitors prevented GHRH survival action, suggesting cAMP/PKA-mediated mechanisms. In somatotropes, GHRH-induced GH secretion is known to involve cAMP/PKA and phosphorylation of CREB,9 a transcription factor that also acts as antiapoptotic target in different cell types, including cardiac myocytes, regulating Bcl-2 expression via PI3K and MAPK signalling.33,34 Interestingly, we found that in ARVMs, GHRH promoted CREB phosphorylation at serine 133.

The pro-apoptotic protein ICER was found to be elevated in failing human hearts and in cardiac myocytes undergoing apoptosis induced by ISO.24 ICER overexpression stimulates cardiac myocyte apoptosis, in part through inhibition of CREB-mediated transcription and downregulation of Bcl-2 antiapoptotic protein.24 In the present study, ISO strongly upregulated ICER mRNA and protein in ARVMs, as expected. Notably, GHRH, besides counteracting ISO-induced cell death, completely prevented ICER increase. Thus, although both ISO3 and GHRH signal through cAMP, their opposite effect on ARVM survival may be explained by their diverse influence on ICER expression. Interestingly, ICER removal from the cells has been linked to MAPK activity, which phosphorylates and targets ICER to ubiquitin-mediated degradation.35 Therefore, among other possible mechanisms, GHRH may downregulate ICER through ERK1/2 phosphorylation. On the basis of evidence that cAMP increase, due to hormonal activation of Gαs-coupled receptors, promotes ERK phosphorylation in different cells,36 it is not unlikely that in ARVMs, GHRH activates ERK through cAMP induction.

β-AR stimulation causes cardiac myocyte cell death through cAMP-dependent increase in Ca2+ entry,3 which in turn may activate apoptotic mechanisms.1 Despite the induction of cAMP, GHRH was ineffective on basal calcium transients amplitude, suggesting different cAMP compartimentalization mechanisms. This finding is indirectly supported by previous data showing that GHRH antagonists induce cancer cell apoptosis through Ca2+-dependent pathways, whereas GHRH has no effect on Ca2+ influx,37 in contrast with somatotropes, where GHRH-induced Ca2+ increase is essential for GH release.6

GHRH effects were also investigated in the heart-derived cell line H9c2, which possesses many of the characteristics of adult cardiac myocytes.29 Similar to ARVMs, GHRH prevented H9c2 cell death and apoptosis induced by both serum starvation and ISO, through cAMP/PKA, ERK1/2, and PI3K/Akt signalling. Again, GHRH-induced protection was inhibited by JV-1-36, suggesting GHRH-R-mediated signalling.

GHRH cardioprotection was further demonstrated in isolated rat hearts subjected to I/R injury, where 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 signalling, as suggested by their inhibition in the presence of JV-1-36 and LY294002, and are in line with the results obtained in cardiac cells.

In conclusion, this study shows that GHRH promotes cardiac myocyte cell survival through complex mechanisms and affords protection against I/R injury in isolated rat heart. Besides the hypothalamus, GHRH mRNA is present in peripheral tissues, including the heart, where it is diffusely expressed in myocardial cells.6,12,13 However, beyond the effects described in tumours, GHRH autocrine/paracrine effects at the cardiac level have never been shown before. Furthermore, although GHRH was previously reported to exert positive inotropic effect in guinea-pig papillary muscle,38 this is the first study to show the direct cardiovascular effects of the hormone. Thus, GHRH may represent a promising target for pharmacological therapies aimed at promoting cardiac cell survival during heart failure.

1.

van Empel VP, Bertrand AT, Hofstra L, Crijns HJ, Doevendans PA, De Windt LJ . Myocyte apoptosis in heart failure. Cardiovasc Res 2005;67:21-29.

2.

Rockman HA, Koch WJ, Lefkowitz RJ . Seven-transmembrane-spanning receptors and heart function. Nature 2002;415:206-212

3.

Singh K, Xiao L, Remondino A, Sawyer DB, Colucci WS . Adrenergic regulation of cardiac myocyte apoptosis. J Cell Physiol 2001;189:257-265.

4.

Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K . Akt promotes survival of cardiomyocytes in vitro and protects against ischemia–reperfusion injury in mouse heart. Circulation 2000;101:660-667.

5.

Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JH, Chien KR . Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. J Biol Chem 1997;272:5783-5791.

6.

Lin-Su K, Wajnrajch MP . Growth hormone releasing hormone (GHRH) and the GHRH receptor. Rev Endocr Metab Disord 2002;3:313-323.

7.

Schally AV, Varga JL, Engel JB . Antagonists of growth-hormone-releasing hormone: an emerging new therapy for cancer. Nat Clin Pract Endocrinol Metab 2008;4:33-43.

8.

Guillemin R, Brazeau P, Bohlen P, Esch F, Ling N, Wehrenberg WB . Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. Science 1982;218:585-587.

9.

Frohman LA, Kineman RD . Growth hormone-releasing hormone and pituitary development, hyperplasia and tumorigenesis. Trends Endocrinol Metab 2002;13:299-303.

10.

Mayo KE . Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone. Mol Endocrinol 1992;6:1734-1744.

11.

Pombo CM, Zalvide J, Gaylinn BD, Dieguez C . Growth hormone-releasing hormone stimulates mitogen-activated protein kinase. Endocrinology 2000;141:2113-2119.

12.

Christodoulou C, Schally AV, Chatzistamou I, Kondi-Pafiti A, Lamnissou K, Kouloheri S, et al . Expression of growth hormone-releasing hormone (GHRH) and splice variant of GHRH receptors in normal mouse tissues. Regul Pept 2006;136:105-108.

13.

Matsubara S, Sato M, Mizobuchi M, Niimi M, Takahara J . Differential gene expression of growth hormone (GH)-releasing hormone (GRH) and GRH receptor in various rat tissues. Endocrinology 1995;136:4147-4150.

14.

Kiaris H, Schally AV, Kalofoutis A . Extrapituitary effects of the growth hormone-releasing hormone. Vitam Horm 2005;70:1-24.

15.

Siriwardana G, Bradford A, Coy D, Zeitler P . Autocrine/paracrine regulation of breast cancer cell proliferation by growth hormone releasing hormone via Ras, Raf, and mitogen-activated protein kinase. Mol Endocrinol 2006;20:2010-2019.

16.

Fekete EM, Zorrilla EP . Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. Front Neuroendocrinol 2007;28:1-27.

17.

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;159:1029-1037.

18.

Isgaard J, Barlind A, Johansson I . Cardiovascular effects of ghrelin and growth hormone secretagogues. Cardiovasc Hematol Disord Drug Targets 2008;8:133-137.

19.

Cerra MC, Gallo MP, Angelone T, Quintieri AM, Pulera E, Filice E, et al . The homologous rat chromogranin A1–64 (rCGA1–64) modulates myocardial and coronary function in rat heart to counteract adrenergic stimulation indirectly via endothelium-derived nitric oxide. FASEB J 2008;22:3992-4004.

20.

Penna C, Alloatti G, Cappello S, Gattullo D, Berta G, Mognetti B, et al . Platelet-activating factor induces cardioprotection in isolated rat heart akin to ischemic preconditioning: role of phosphoinositide 3-kinase and protein kinase C activation. Am J Physiol Heart Circ Physiol 2005;288:H2512-H2520

21.

Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, et al . Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3′,5′-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-kinase/Akt signaling. Endocrinology 2007;148:512-529.

22.

Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, et al . Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. Diabetes 2008;57:967-979.

23.

Morisco C, Seta K, Hardt SE, Lee Y, Vatner SF, Sadoshima J . Glycogen synthase kinase 3beta regulates GATA4 in cardiac myocytes. J Biol Chem 2001;276:28586-28597.

24.

Tomita H, Nazmy M, Kajimoto K, Yehia G, Molina CA, Sadoshima J . Inducible cAMP early repressor (ICER) is a negative-feedback regulator of cardiac hypertrophy and an important mediator of cardiac myocyte apoptosis in response to beta-adrenergic receptor stimulation. Circ Res 2003;93:12-22.

25.

Obal F Jr, Fang J, Taishi P, Kacsoh B, Gardi J, Krueger JM . Deficiency of growth hormone-releasing hormone signaling is associated with sleep alterations in the dwarf rat. J Neurosci 2001;21:2912-2918.

26.

Fan GC, Chu G, Mitton B, Song Q, Yuan Q, Kranias EG . Small heat-shock protein Hsp20 phosphorylation inhibits beta-agonist-induced cardiac apoptosis. Circ Res 2004;94:1474-1482.

27.

Kovacs M, Schally AV, Lee EJ, Busto R, Armatis P, Groot K, et al . Inhibitory effects of antagonistic analogs of GHRH on GH3 pituitary cells overexpressing the human GHRH receptor. J Endocrinol 2002;175:425-434.

28.

Matsui T, Rosenzweig A . Convergent signal transduction pathways controlling cardiomyocyte survival and function: the role of PI 3-kinase and Akt. J Mol Cell Cardiol 2005;38:63-71.

29.

Dangel V, Giray J, Ratge D, Wisser H . Regulation of beta-adrenoceptor density and mRNA levels in the rat heart cell-line H9c2. Biochem J 1996;317 (Pt 3):925-931

30.

Condorelli G, Roncarati R, Ross J Jr, Pisani A, Stassi G, Todaro M, et al . Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function. Proc Natl Acad Sci USA 2001;98:9977-9982.

31.

Henaff M, Hatem SN, Mercadier JJ . Low catecholamine concentrations protect adult rat ventricular myocytes against apoptosis through cAMP-dependent extracellular signal-regulated kinase activation. Mol Pharmacol 2000;58:1546-1553.

32.

Takahashi T, Tang T, Lai NC, Roth DM, Rebolledo B, Saito M, et al . Increased cardiac adenylyl cyclase expression is associated with increased survival after myocardial infarction. Circulation 2006;114:388-396.

33.

Mehrhof FB, Muller FU, Bergmann MW, Li P, Wang Y, Schmitz W, et al . In cardiomyocyte hypoxia, insulin-like growth factor-I-induced antiapoptotic signaling requires phosphatidylinositol-3-OH-kinase-dependent and mitogen-activated protein kinase-dependent activation of the transcription factor cAMP response element-binding protein. Circulation 2001;104:2088-2094

34.

Du K, Montminy M . CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 1998;273:32377-32379.

35.

Yehia G, Schlotter F, Razavi R, Alessandrini A, Molina CA . Mitogen-activated protein kinase phosphorylates and targets inducible cAMP early repressor to ubiquitin-mediated destruction. J Biol Chem 2001;276:35272-35279

36.

Stork PJ, Schmitt JM . Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 2002;12:258-266

37.

Rekasi Z, Czompoly T, Schally AV, Boldizsar F, Varga JL, Zarandi M, et al . Antagonist of growth hormone-releasing hormone induces apoptosis in LNCaP human prostate cancer cells through a Ca2+-dependent pathway. Proc Natl Acad Sci USA 2005;102:3435-3440.

38.

Hasegawa J, Saitoh M, Hirai S, Kinugawa T, Kotake H, Mashiba H . Positive inotropic effect of growth hormone releasing factor in the guinea-pig cardiac muscles in vitro. Horm Metab Res 1988;20:130-131