

# Natural (ghrelin) and synthetic (hexarelin) GH secretagogues stimulate H9c2 cardiomyocyte cell proliferation

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## Abstract

Recent experimental data demonstrate cardiovascular effects of the GH secretagogues (GHSs) hexarelin and ghrelin, the proposed natural ligand for the GHS receptor. Moreover, specific cardiac binding sites for GHSs have been suggested. The aim of the present study was to investigate if the natural ligand ghrelin and synthetic GHS peptide hexarelin and analogues have direct effects on the cardiomyocyte cell line, H9c2. Hexarelin stimulated thymidine incorporation in a dose-dependent manner with significant responses at 3  $\mu$ M ( $147 \pm 3\%$  of control,  $P < 0.01$ ) and elicited maximal effects at concentrations around 30  $\mu$ M. This activity was seen already after 12 h of incubation with a maximal effect after 18 h ( $176 \pm 9\%$  of control,  $P < 0.01$ ). Ghrelin also had a significant stimulatory effect on thymidine incorporation ( $129 \pm 2\%$  of control at 3  $\mu$ M and 18 h,  $P < 0.05$ ). The stimulatory effect on thymidine incorporation of hexarelin, Tyr-Ala-hexarelin, EP80317 and ghrelin was specific and no stimulatory

effect was observed with the truncated GH-releasing peptide EP51389 or the non-peptidyl GHS MK-0677. In competitive binding studies, <sup>125</sup>I-labeled Tyr-Ala-hexarelin was used as radioligand and competition curves showed displacement with hexarelin, Tyr-Ala-hexarelin, EP80317 and ghrelin, whereas MK-0677 and EP51389 produced very little displacement at 1  $\mu$ M concentration, adding further support for an alternative subtype binding site in the heart compared with the pituitary. In conclusion, we have demonstrated a dose-dependent and specific stimulation of cardiomyocyte thymidine incorporation by natural and synthetic GHS analogues, suggesting increased cell proliferation and binding of GHS to H9c2 cardiomyocyte cell membranes. These findings support potential peripheral effects of GHS on the cardiovascular system independent of an increased GH secretion.

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## Introduction

The growing family of synthetic growth hormone (GH) secretagogues (GHSs) (Camanni *et al.* 1998) consists of peptides and non-peptides structurally derived from met-enkephalin and synthesized by Bowers and collaborators in the early 1980s (Momany *et al.* 1981, Bowers 1998). Since the peptidyl GHSs have very low oral bioavailability and short half-lives, several small non-peptidyl molecules have been designed which are less susceptible to degradation and have higher bioavailability. The spiroindolin derivative MK-0677 is a small non-peptidyl GHS with excellent oral bioavailability (Smith *et al.* 1997). Using MK-0677 as a ligand, a receptor in membranes isolated from pituitary and hypothalamic tissue was identified and it was suggested to be a G-protein coupled receptor (Pong

*et al.* 1996). Subsequently, the receptor was cloned from porcine, human and rat pituitary tissue (Howard *et al.* 1996, McKee *et al.* 1997a) and revealed no similarities to any other known G-protein coupled receptor. The receptor showed 96% homology between rat and human, and studies on the pufferfish have shown that it has been conserved for at least 400 million years (Palyha *et al.* 2000). Recent studies have demonstrated other subtypes of the receptor, which are expressed also in peripheral tissues (McKee *et al.* 1997b, Tan *et al.* 1998). Specific binding sites for GHSs have been found in thyroid (Cassoni *et al.* 2000) and cardiac (Ong *et al.* 1998a, Bodart *et al.* 1999, 2002, Papotti *et al.* 2000) tissue.

Recently, an endogenous ligand for the GHS receptor (GHS-R) was identified in rat stomach and designated ghrelin (Kojima *et al.* 1999). This ligand was found to have

no structural homology with any of the synthetic GHSs and the existence of an *n*-octanoyl group at the Ser3 residue seemed to be necessary for the GH-releasing activity. The plasma concentration of active *n*-octanoylated ghrelin is around 4 pM and the total amount (both active and inactive form) is around 200 pM in rats (Hosoda *et al.* 2000b). The circulating level of total ghrelin is also around 200 pM in the human (Ariyasu *et al.* 2001). Ghrelin has been shown to have a very short half-life (Tschöp *et al.* 2000), suggesting the importance of rapid changes of the plasma concentration.

The most prominent and well-characterized effect of GHSs is the ability to release GH both *in vivo* and *in vitro* and it has been suggested that they act at both the pituitary and the hypothalamic level (Bowers *et al.* 1984, Dickson *et al.* 1993, Shimon *et al.* 1998). More recently, the GHSs have also been proposed to have peripheral effects. Hexarelin, which is a 2-methyl D-Trp derivative of GH-releasing peptide-6 (GHRP-6) (His-D-Trp-Ala-Trp-D-Phe-Lys-NH<sub>2</sub>) (Deghenghi *et al.* 1994, Ghigo *et al.* 1994), has been shown to improve cardiac function in rats after experimental myocardial infarction (Tivesten *et al.* 2000) and to be protective against postischemic dysfunction of perfused hearts isolated from GH-deficient (De Gennaro Colonna *et al.* 1997), senescent (Rossoni *et al.* 1998) and hypophysectomized (Locatelli *et al.* 1999) rats. Moreover, when hexarelin was injected i.v. in a single dose to hypopituitary adult patients, a significant increase in left ventricular ejection fraction was observed, suggesting a GH-independent inotropic effect (Bisi *et al.* 1999). Ghrelin has been shown to acutely decrease systemic vascular resistance in GH-deficient rats, suggesting that ghrelin also has a GH/insulin-like growth factor (IGF-I)-independent vasodilatory effect (Nagaya *et al.* 2001b).

Specific binding to rat cardiac membranes has been suggested using an iodinated and photoactivatable derivative of hexarelin, <sup>125</sup>I-labeled Tyr-Bpa-Ala-hexarelin (Ong *et al.* 1998a, Bodart *et al.* 1999). Papotti *et al.* (2000) have also shown specific binding to human cardiac tissue using iodinated Tyr-Ala-hexarelin. Binding of hexarelin to H9c2 cells has previously been shown (Filigheddu *et al.* 2001); however, to the best of our knowledge no studies demonstrating direct effects of GHS and also binding of ghrelin to cardiomyocytes have yet been published.

The H9c2 cardiomyocyte cell line (Kimes & Brandt 1976) is derived from embryonic rat ventricle and shows both cardiac and skeletal muscle cell properties (Kimes & Brandt 1976, Hescheler *et al.* 1991). It has been used in several studies to investigate different cellular mechanisms in cardiomyocytes (Sipido & Marban 1991, Wu *et al.* 1996, Chen *et al.* 2000) and has been shown to be responsive to IGF-I (Chen *et al.* 1995, Bahr *et al.* 1997).

The aim of the present study was to investigate possible direct effects of hexarelin and ghrelin on H9c2 cells. Moreover, iodinated Tyr-Ala-hexarelin was used to show specific binding to a GHS-R in H9c2 cells.

## Materials and Methods

### Peptides

Hexarelin, recombinant human GH and IGF-I (lot 1009, 4100 U/mg) were from Pharmacia & Upjohn (Stockholm, Sweden) and the GHS, Tyr-Ala-hexarelin, EP51389, EP80317 and MK-0677 were all provided by Euro-peptides (Argenteuil, France). The rat ghrelin came from Neosystem (Strasbourg, France). The GHS EP80317 (Haic-D-2-Me-Trp-D-Lys-Trp-D-Phe-Lys-NH<sub>2</sub>) is a hexapeptide with a primary structure similar to the hexapeptide hexarelin (His-D-2-Me-Trp-Ala-Trp-D-Phe-Lys-NH<sub>2</sub>), although EP80317 is characterized by a D-Lys in the third position, which has been previously suggested to be of importance for blunting of the GH-releasing property (Momany *et al.* 1981). Somatostatin-14 (SRIF-14) was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). The iodination of Tyr-Ala-hexarelin with <sup>125</sup>I (specific activity 2200 Ci/mmol) was performed by EuroDiagnostica AB (Malmö, Sweden), using the chloramine-T method followed by purification on a  $\mu$ -Bondapak C<sub>18</sub> column (Sweden Waters AB, Söllerntuna, Sweden).

### Cell culture

H9c2 cardiac muscle cell line was obtained from American Type Culture Collection (Manassas, VA, USA) at passage 14 and cultured in DMEM complete culture medium (DMEM/F12 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.5 mg/l Fungizone and 50 mg/l gentamicin). Experiments were performed at passage 19–25. The stock of cells used for experiments were sub-cultured 1:5 twice a week. Cells used for thymidine incorporation were seeded in triplicates in six-well plates at  $4 \times 10^3$  cells/cm<sup>2</sup>, cultured for 2 days, washed twice with 2 ml DMEM and starved for 24 h in DMEM starvation medium (DMEM/F12 supplemented with 0.5% BSA, 2 mM L-glutamine, 0.5 mg/l Fungizone and 50 mg/l gentamicin). After the starvation the cells were washed once with DMEM and used for GHS stimulation. DMEM/F12, L-glutamine, Fungizone, Hepes and Dulbecco's PBS were all from Gibco BRL and FCS was from Harlan Seralab Ltd (Loughborough, UK). BSA came from Sigma Chemical Company (St Louis, MO, USA). Gentamicin was purchased from Seromed (Berlin, Germany). The cell culture bottles and plates came from Costar (High Wycombe, Bucks, UK).

### Thymidine incorporation

The cells were incubated for 18 h at 37 °C with various concentrations of GHS, or for various lengths of time with 10  $\mu$ M GHS in starvation medium. As vehicle control, wells with only starvation medium were used. During the

last 60 min, 1  $\mu\text{Ci}$  [methyl- $^3\text{H}$ ]thymidine (Amersham Pharmacia Biotech) per ml was added. The cells were washed three times with 3 ml ice-cold Dulbecco's PBS (containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and solubilized with 500  $\mu\text{l}$  10% SDS at room temperature. DNA was precipitated with 500  $\mu\text{l}$  ice-cold 20% trichloroacetic acid (TCA) and collected on Whatman GF/C glass fiber filters. The precipitate was washed twice with ice-cold 10% TCA and twice with ice-cold 95% ethanol. The radioactivity associated with the filters was quantified using liquid scintillation (Beta counter; Beckman LS6500, High Wycombe, Bucks, UK). The c.p.m. data from each time point were corrected against the vehicle control, which was set to 100% thymidine incorporation. The results for the different GHSs in various concentrations are presented as percent of control. This is done separately for each thymidine incorporation experiment performed.

#### Membrane preparation

The membrane preparation and binding protocol used was a slight modification of the one used by Muccioli *et al.* (1998). H9c2 cells were cultured in 162  $\text{cm}^2$  flasks and harvested at confluency. The cells were washed once in PBS (without  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$ ) and scraped off with a rubber policeman in 0.34 M sucrose. They were homogenized with a Dounce homogenizer in 50 mM Tris-HCl buffer also containing 2.5 mM EGTA, 0.34 M sucrose and protease inhibitors, pH 7.4. After homogenization they were centrifuged at 30 000  $g$  for 30 min at +4 °C. The pellet was resuspended and homogenized in ice-cold resuspension buffer (50 mM Tris-HCl, 2.5 mM EGTA, 0.003% bacitracin). The protein concentration was determined using a Bio Rad DC Protein Assay kit.

#### Receptor binding assay

Tyr-Ala-hexarelin have been reported to have the same GH-releasing activity as hexarelin in rats (Ong *et al.* 1998a) and  $^{125}\text{I}$ -labeled Tyr-Ala-hexarelin has been used in other studies to show GHS-R binding (Muccioli *et al.* 1998, Cassoni *et al.* 2000, Papotti *et al.* 2000). To show binding-site specificity, different GHSs, natural (ghrelin) and synthetic (hexarelin, Tyr-Ala-hexarelin, as well as two different hexarelin analogues, EP80317 and EP51389, MK-0677) and the neuropeptide SRIF-14 were used as competitors in a displacement assay. The concentration of the competitors ranged from 1 nM to 10  $\mu\text{M}$ . Non-specific binding was calculated as binding which was not displaced by 1  $\mu\text{M}$  unlabeled Tyr-Ala-hexarelin. The data are presented as a percent of specific binding in the absence of unlabeled competitor.

Approximately 100  $\mu\text{g}$  of membrane protein were incubated in triplicate at 0 °C for 60 min together with 1.5 nM radioligand and different concentrations of competitor in assay buffer (50 mM Tris-HCl, 2.5 mM EGTA,

0.1% BSA, 0.003% bacitracin). The final volume was 0.5 ml. The binding reaction was terminated by adding 4 ml assay buffer followed by filtration over Whatman GF/B filters presoaked for 60 min in 0.5% polyethyleneimine to minimize non-specific binding of the radioligand to the filters. Filters were rinsed three times with assay buffer and the bound radioactivity measured in an LKB 1260 gamma counter (Wallac, Turku, Finland). The dissociation constant ( $K_d$ ) and the number of binding sites ( $B_{\text{max}}$ ) were calculated from a saturation curve (Fig. 4) using non-linear regression and the GraphPad Prism 3 program (GraphPad, San Diego, CA, USA). The radioligand competition curves were also analyzed with the GraphPad program and  $\text{IC}_{50}$  values for the various GHSs were calculated.

#### RT-PCR for GHS-R1a

Total RNA extraction from frozen rat hypothalamus and from the H9c2 cell line was performed using TRIzol (Life Technologies, S Giuliano Milanese, Italy) according to the modified single-step RNA isolation method of Chomzynski & Sacchi (1987). The reverse transcriptase reaction was performed using 3  $\mu\text{g}$  total RNA with Moloney murine leukemia virus reverse transcriptase (PE Applied Biosystems, Foster City, CA, USA) under the conditions recommended by the supplier. The forward and reverse primer sequences (TIB Molbiol, Genova, Italy) for rat GHS-R1a were 5'-GTCGAGCGCTAC TTCGC-3' and 5'-GTACTGGCTGATCTGAGC-3' respectively (Yokote *et al.* 1998). The GHS-R primers were located on different exons. The primers for  $\beta$ -actin were 5'-GGTCATCTTCTCGCGGTTGGCCTTGG GGT-3' (forward) and 5'-CCCCAGGCACCAGGGCG TGAT-3' (reverse). After an initial denaturation step (94 °C for 10 min), 32 cycles of PCR were carried out in a 50  $\mu\text{l}$  volume with AmpliTaq Gold Polymerase (PE Applied Biosystems) in a GeneAmp PCR System 9600 (PE Applied Biosystems) under the following conditions: one cycle, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min for elongation. The PCR products (492 bp for GHS-R1a and 230 bp for  $\beta$ -actin) were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining.

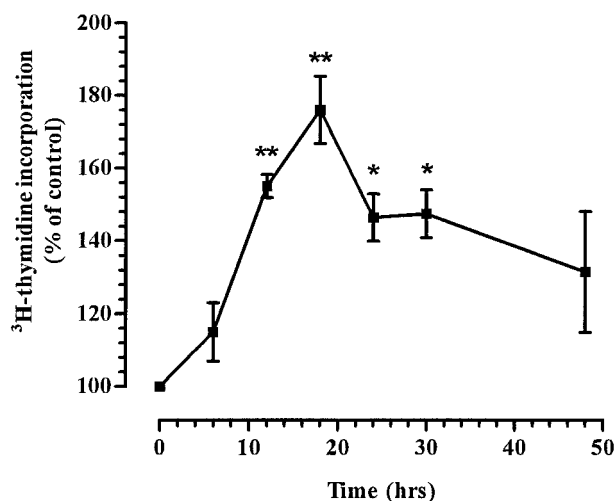
#### Statistical analysis

Data are presented as means  $\pm$  S.E.M. unless otherwise stated. The statistical significance was tested using Student's *t*-test.

## Results

#### Stimulatory effects of hexarelin and ghrelin on H9c2 cells

A time course study of hexarelin (10  $\mu\text{M}$ ) showed significant effects on thymidine incorporation already after 12 h



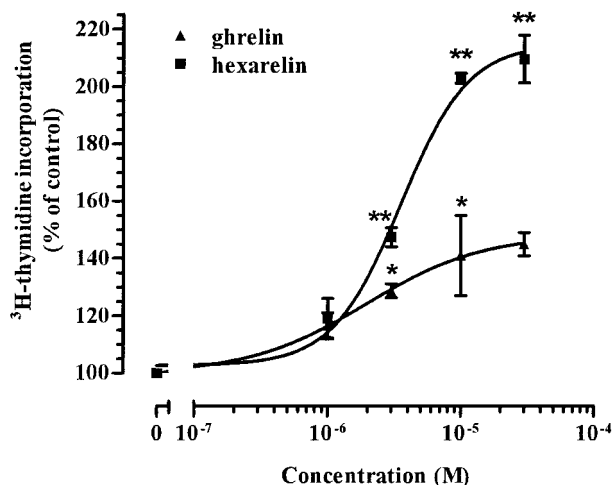
**Figure 1** Time-course effect of hexarelin on [ $^3\text{H}$ ]thymidine incorporation in H9c2 cells. Cells were incubated with  $10\ \mu\text{M}$  hexarelin. After the indicated length of time the cells were harvested and the radioactivity was measured. The data were corrected against the basal vehicle background level and the c.p.m. from each time point is shown as a percent of vehicle control  $\pm$  S.E.M. and represents results from three separate experiments in triplicate. \*\* $P < 0.01$ , \* $P < 0.05$  vs vehicle control.

( $155 \pm 3\%$  of control,  $P < 0.01$ ) and maximal effects after 18 h ( $176 \pm 9\%$  of control,  $P < 0.01$ ); thereafter it decreased (Fig. 1). The stimulatory effect on thymidine incorporation was dose-dependent for both hexarelin and ghrelin with significant responses at  $3\ \mu\text{M}$  ( $147 \pm 3\%$  of control ( $P < 0.01$ ) and  $129 \pm 2\%$  of control ( $P < 0.05$ ) respectively). Maximal effects with hexarelin were obtained using concentrations around  $30\ \mu\text{M}$  ( $210 \pm 8\%$  of control,  $P < 0.01$ ) (Fig. 2).

#### Specificity of the stimulatory effect

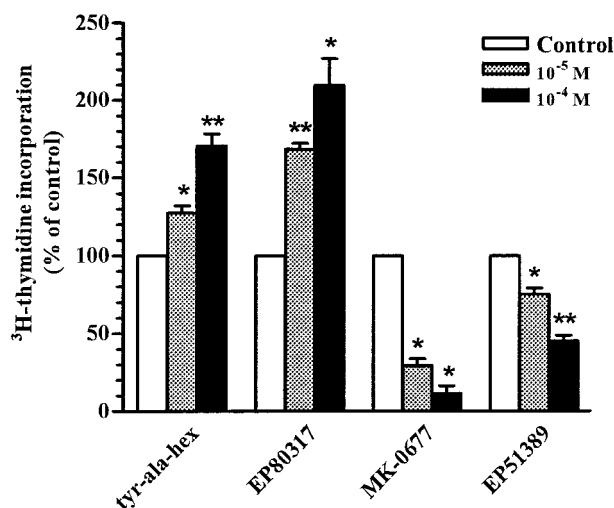
To study the specificity of GHSs on stimulating H9c2 cells, we also incubated cells with EP51389, a truncated GHRP derivative with strong GH-releasing activity (Bodart *et al.* 1999, Locatelli *et al.* 1999), EP80317, a GHRP antagonist which does not release GH but has a strong affinity to the pituitary/hypothalamus GHS-R (Muccioli *et al.* 2001) and MK-0677, a non-peptidyl GHS (Smith *et al.* 1997).

We found an effect on thymidine incorporation in H9c2 cells with hexarelin and ghrelin (Fig. 2). Moreover, both Tyr-Ala-hexarelin and EP80317 also had a stimulatory effect on thymidine incorporation but neither the small non-peptidyl GHS, MK-0677, nor the truncated GHRP derivative, EP51389, showed that stimulatory effect. In fact both MK-0677 and EP51389 had a negative impact on thymidine incorporation, which resulted in thymidine incorporation being lower than in the control (Fig. 3). When hexarelin and MK-0677 were combined (1+1) at



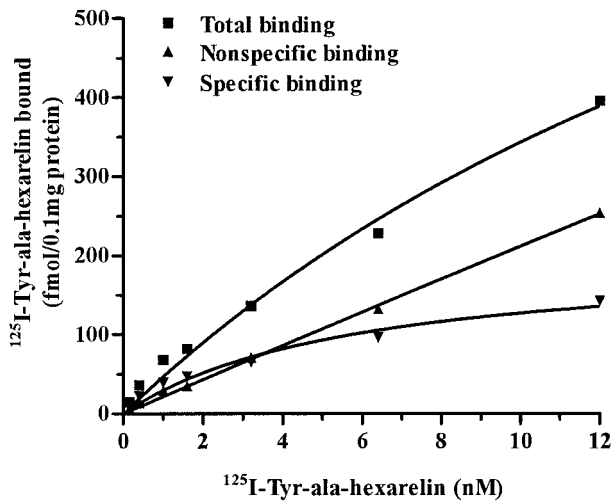
**Figure 2** Dose-response effect of hexarelin and rat ghrelin on [ $^3\text{H}$ ]thymidine incorporation in H9c2 cells. Cells were incubated with indicated concentrations of GHSs. After 18 h the cells were harvested and the radioactivity was measured. The c.p.m. data, which are corrected against the vehicle control, are presented as percent of controls  $\pm$  S.E.M. and represent results from three separate experiments in triplicate, except for the highest ghrelin concentration ( $30\ \mu\text{M}$ ), which represents three wells from one experiment. Data were fitted by non-linear regression analysis. \*\* $P < 0.01$ , \* $P < 0.05$  vs vehicle control.

$10\ \mu\text{M}$  the stimulatory effect of hexarelin on thymidine incorporation was completely attenuated and was even lower than for the vehicle control. The tendency was the same when EP51389 was combined with hexarelin, but



**Figure 3** Specificity of the GHS effect on H9c2 cells. Cells were incubated with two different concentrations (10 and  $100\ \mu\text{M}$ ) of each GHS. After 18 h the cells were harvested and the radioactivity was measured. The c.p.m. data, which are corrected against the vehicle control, are presented as percent of controls  $\pm$  S.E.M. and represent results from three separate experiments in triplicate. \*\* $P < 0.01$ , \* $P < 0.05$  vs vehicle control.





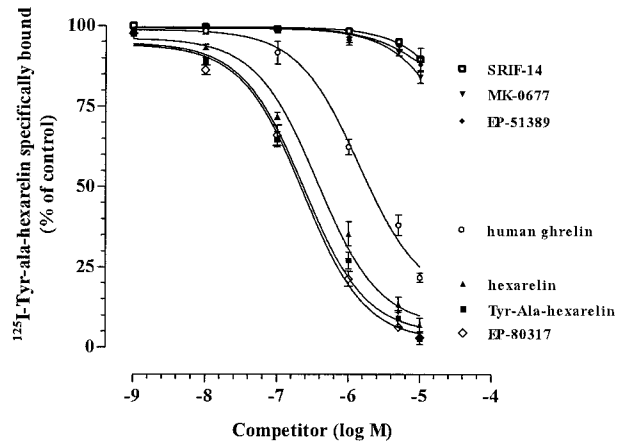
**Figure 4** Saturation curve of <sup>125</sup>I-labeled Tyr-Ala-hexarelin binding to H9c2 cell membranes. A fixed amount of membrane protein (100 µg/tube) was incubated with increasing concentrations of radiolabeled Tyr-Ala-hexarelin alone (total binding) or plus 10 µM of unlabeled Tyr-Ala-hexarelin to define non-specific binding. Specific binding values were obtained by subtracting non-specific binding from total binding. Data in the Figure are from one representative experiment.

here 10 µM GHS did not manage to show significant inhibition of the hexarelin effect on the thymidine incorporation (data not shown).

As positive control, we also performed experiments with IGF-I incubation with H9c2 cells in a concentration of 10 µM, showing a stimulation of thymidine incorporation of 147 ± 7% of control (*P* < 0.001). GH was not able to stimulate thymidine incorporation in the H9c2 cells (data not shown).

*Receptor-binding studies*

H9c2 cell membranes were used for the binding studies and the specific binding of <sup>125</sup>I-labeled Tyr-Ala-hexarelin was calculated as the difference between binding in the absence (total binding) and in the presence of 10 µM unlabeled Tyr-Ala-hexarelin (non-specific binding). Experiments using increasing concentrations of <sup>125</sup>I-labeled Tyr-Ala-hexarelin revealed the presence of a saturable specific binding associated with a high non-specific binding that linearly increased as a function of the radioligand concentrations (Fig. 4). Scatchard analysis of the specific binding data (Figure not shown) demonstrated the existence of a single class of binding sites with a *K<sub>d</sub>* of 7.5 ± 1.0 nM and a *B<sub>max</sub>* of 2023 ± 168 fmol/mg of protein (means ± S.E.M. of three separate experiments). The specificity of <sup>125</sup>I-labeled Tyr-Ala-hexarelin binding to H9c2 membranes was established by determining the ability of different compounds to compete with the radioligand for binding sites. Based on saturation binding



**Figure 5** Competition curves using <sup>125</sup>I-labeled Tyr-Ala-hexarelin as radioligand and several different unlabeled substances as competitors. The ordinate represents specific binding as percentage of control (i.e. specific binding in the absence of unlabeled competitor). Data in the Figure are from one representative experiment.

data, competition was performed at a radioligand concentration (1.5 nM) where the specific binding value (about 58% of total radioactivity bound) was greater than that of non-specific binding. Figure 5 shows that Tyr-Ala-hexarelin, hexarelin, EP80317 and human ghrelin displaced the radioligand Tyr-Ala-hexarelin from binding sites in a dose-dependent manner. The *IC<sub>50</sub>* values (means ± S.E.M. of three separate experiments, all expressed as µM) were 0.25 ± 0.04 for Tyr-Ala-hexarelin, 0.39 ± 0.07 for hexarelin, 0.2 ± 0.05 for EP80317 and 1.4 ± 0.19 for ghrelin. In contrast, no or little competition was observed in the presence of other competitors such as MK-0677, EP51389 or SRIF-14.

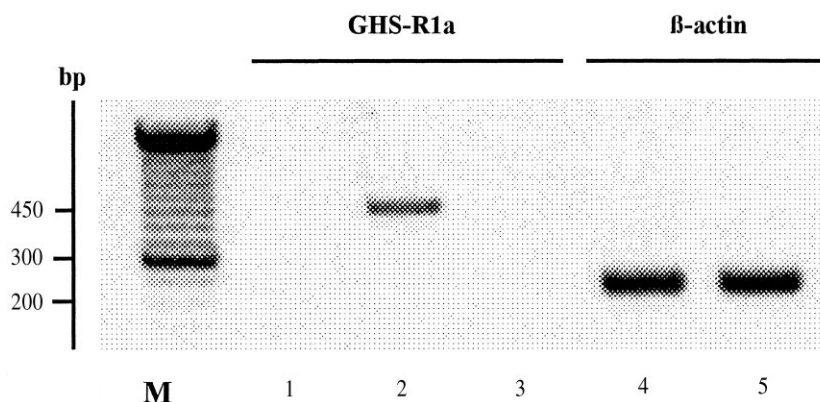
*GHS-R expression*

To study possible expression of the GHS-R, amplification of GHS-R1a mRNA by RT-PCR was performed in the H9c2 cells (Fig. 6). Rat hypothalamus was used as positive control showing a 492 bp target band. Buffer alone was used as negative control. As can be seen in Fig. 6, H9c2 cells do not express detected levels of GHS-R1a mRNA.

**Discussion**

The main findings of the present study were that hexarelin shows binding to H9c2 cardiomyocytes and stimulates thymidine incorporation in a dose-dependent and specific manner. A stimulatory effect on cell proliferation was also observed after incubation with ghrelin, the proposed endogenous ligand for the GHS-R.

To the best of our knowledge this is the first study showing direct stimulatory effects of synthetic GHSs and



**Figure 6** Amplification of GHS-R1a mRNA by RT-PCR in H9c2 rat heart derived cell line. H9c2 cells were cultured in medium (DMEM) containing 10% fetal bovine serum for 24 h. Three micrograms of isolated total RNA were reverse transcribed and one-quarter of the total cDNA was used for PCR with specific GHS-R1a and  $\beta$ -actin primers. The PCR products were separated by agarose gel (1.5%) electrophoresis and visualized with ethidium bromide. The amplified products for GHS-R1a and for  $\beta$ -actin were 492 and 230 bp respectively. M, 50 bp ladder. Buffer alone was used as negative control (lane 1) and rat hypothalamus as positive control (lane 2). As shown in lane 3, H9c2 cells do not express detected levels of GHS-R1a mRNA.  $\beta$ -Actin was used as an internal control for equivalent amounts of amplified samples (lane 4 for hypothalamus and 5 for H9c2). Shown is a representative RT-PCR from three separate experiments performed on three different RNA samples.

ghrelin on cardiomyocyte-like cells. Direct and GH-independent effects of GHSs on the cardiovascular system have previously been suggested and they include improvement of systolic function in rats after experimental infarction (Tivesten *et al.* 2000), cardioprotection against postischemic dysfunction in perfused rat hearts (De Gennaro Colonna *et al.* 1997, Rossoni *et al.* 1998) and increase of left ventricular ejection fraction in hypopituitary patients (Bisi *et al.* 1999). In a recent study Nagaya *et al.* (2001b) reported data of a decrease in systemic vascular resistance in GH-deficient rat treated with a single injection of ghrelins, suggesting that ghrelin has GH/IGF-1-independent vasodilatory effects.

However, at present no conclusive data demonstrating cardiac effects of GHS on the cellular level have been reported. In the present study, a dose-dependent effect of hexarelin and ghrelin was observed with a significant effect at 3  $\mu$ M and a maximal effect for hexarelin at concentrations around 30  $\mu$ M. This is comparable to previously reported concentrations needed to stimulate a dose-dependent increase in coronary perfusion pressure when hexarelin is administered to rat hearts in a Langendorff perfusion system (Bodart *et al.* 1999). The plasma concentration of ghrelin in both the human (Ariyasu *et al.* 2001) and rat (Hosoda *et al.* 2000b) is around 0.2 nM and a single injection of 10  $\mu$ g/kg to healthy males resulted in a marked decrease in systemic vascular resistance (Nagaya *et al.* 2001a). The plasma concentration was 45 nM after 1 min and decreased to 4.5 nM after 100 min, with a remaining effect on

vascular resistance. The ghrelin concentrations for these effects are lower than the concentrations that were needed to obtain thymidine incorporation effects *in vitro*. However, *in vitro* studies are difficult to compare with *in vivo* experiments since culturing conditions may influence the concentration needed for an effect. Also, since GHS-R expression has been shown in the whole heart and in the aorta (Nagaya *et al.* 2001a) these hemodynamic effects may very well be mediated through the cloned GHS-R and our effects through another different GHS subtype receptor.

In order to test the specificity of the proliferative effect on H9c2 cells, several GHRPs and MK-0677, a non-peptidyl GHS, were tested in two different concentrations: 10 and 100  $\mu$ M. Interestingly, no stimulatory effect of MK-0677 was observed, suggesting that the effect on thymidine incorporation may be specific for peptidyl GHS. Moreover, a truncated GHRP, EP51389, was also ineffective in binding to and stimulating H9c2 thymidine incorporation. These findings are in agreement with Bodart *et al.* (1999), who also reported a lack of effect on coronary perfusion pressure by MK-0677 and EP51389. In our study, both MK-0677 and EP51389 had a negative effect on thymidine incorporation, resulting in decreased values compared with controls. MK-0677 was able to completely blunt the effect of hexarelin when the cells were co-incubated with both agents. In contrast, EP51389 was only able to partially inhibit the stimulatory effect of hexarelin on thymidine incorporation. The reason for this inhibitory effect and possible differences in the mechanism

of action of these compounds are unclear and need to be further studied.

The modified hexarelin, Tyr-Ala-hexarelin, that was used for  $^{125}\text{I}$  iodination was found to be effective in stimulating thymidine incorporation. Another peptide, EP80317, which has a structure similar to hexarelin and binds to the GHS-R (Muccioli *et al.* 2001) but is characterized by a D-Lys in the third position, which previously has been suggested to have a negative impact on GH release from the pituitary (Momany *et al.* 1981), was also found to have a dose-dependent stimulatory effect on thymidine incorporation.

Overall, the results indicate specificity of the stimulatory effect on thymidine incorporation by peptidyl GHS and ghrelin and are in agreement with the hypothesis that a different subtype of the GHS-R found in the pituitary is present in cardiomyocytes. This proposed novel cardiac receptor seems to bind full-length GHRP-6 derivatives such as hexarelin, Tyr-Ala-hexarelin, EP80317 and also ghrelin, but not the non-peptidyl GHS MK-0677 or the truncated GHRP, EP51389. The GHS-R was cloned from its ability to bind MK-0677 (Howard *et al.* 1996) and has been found to be expressed in whole heart tissue (Nagaya *et al.* 2001a). However, whether it is expressed in cardiomyocytes or in other cell types in the heart remains to be investigated. We were not able to show binding of MK-0677 or expression of the GHS-R in H9c2 cardiac cells. The positive effect we found on thymidine incorporation seems to be separate from the GH-releasing effect of GHS and suggests a subtype receptor that is expressed in cardiomyocytes.

To date two different suggested endogenous ligands for the GHS-R have been isolated and characterized from rat stomach, the 28 amino acid peptide ghrelin (Kojima *et al.* 1999) and des-Gln<sup>14</sup>-ghrelin whose sequence is identical to ghrelin except for one glutamine in position 14 (Hosoda *et al.* 2000a). Both peptides have GH-releasing properties *in vivo* (Hosoda *et al.* 2000a). As well as stomach, it was recently reported that ghrelin is expressed in human and rat placenta (Gualillo *et al.* 2001). So far the physiological role of ghrelin is poorly understood but a role in the regulation of metabolic balance has been suggested (Tschöp *et al.* 2000). However, it was recently reported that a single injection of ghrelin to healthy volunteers had rapid cardiovascular effects including decreased blood pressure, increased stroke volume and increased cardiac output (Nagaya *et al.* 2001a). Moreover, administration of ghrelin to rats with experimental myocardial infarction improved systolic function (Nagaya *et al.* 2001b). Since ghrelin administration in this last study also elicited a potent increase in circulating GH and IGF-I levels, it is not clear whether the effects of ghrelin were direct on cardiovascular tissue or mediated by GH. The finding in the present study that ghrelin stimulates thymidine incorporation in H9c2 cells suggests potential effects on the cardiovascular system which merit further investigation.

Although our findings demonstrate direct effects of peptide GHS on thymidine incorporation, it should be pointed out that it is not possible to directly compare these effects with previously reported cardioprotective effects *in vivo* and further studies directed at intracellular signaling and anti-apoptotic effects are needed to clarify this issue.

Several studies have addressed the binding of GHS to specific receptors in the cardiovascular system. Using  $^{125}\text{I}$ -labeled Tyr-Bpa-Ala-hexarelin, a photoactivatable derivative of hexarelin, Ong and collaborators were able to demonstrate in competitive binding studies that hexarelin, MK-0677 (Ong *et al.* 1998b, Bodart *et al.* 1999) and EP51389 (Bodart *et al.* 1999) had very different binding patterns. Hexarelin could completely displace the radioligand in heart membranes, although it showed a lower affinity than in the pituitary. EP51389 and MK-0677 showed no displacement at the concentrations used. The results in the present study confirm specific binding of iodinated Tyr-Ala-hexarelin with complete displacement with hexarelin, Tyr-Ala-hexarelin and to a lesser extent ghrelin. The neuropeptide SRIF-14, which has a known negative effect on GH release, did not show any displacing effect. MK-0677 and EP51389 were not as potent in displacing Tyr-Ala-hexarelin at comparable concentrations. Together with the finding that H9c2 cells do not express the GHS-R1a receptor demonstrated with RT-PCR, this would add further support for an alternative subtype binding site in the heart compared with the pituitary.

There are several advantages in using the H9c2 cell line and it has been shown that H9c2 cells express specific cardiac markers. Hescheler *et al.* (1991) characterized the H9c2 cells as immature embryonic cardiomyocytes with electrical and hormonal signal pathways preserved as in adult cardiac cells. They also reported that 95% of H9c2 cells are mononucleated and that they express L-type calcium channels which are typical for cardiomyocytes. The L-type calcium channels were also found by Sipido & Marban (1991), who also pointed out that H9c2 cells had been neglected as potential surrogates for primary cardiac cells. H9c2 cells also show expression of N-cadherin, a specific component of intercalated discs in cardiac muscle tissue (Wu *et al.* 1996). However, it should also be pointed out that the H9c2 cell line has both cardiomyocyte and skeletal muscle properties and seems to become more dedifferentiated over passages. Under the culturing conditions used in the present paper, with exponentially dividing cells, we could not detect any myoblast fusion, which would indicate differentiation towards a more skeletal muscle cell-like phenotype. Another rationale for using H9c2 cells is that they express specific surface receptors for IGF-I, which has been shown to stimulate H9c2 cell proliferation (Chen *et al.* 1995, Bahr *et al.* 1997) and inhibit apoptosis (Wang *et al.* 1998). This would be an advantage when choosing H9c2 as a cardiomyocyte cell line to investigate stimulatory effects of endocrine factors.

Nevertheless, even if H9c2 cells have many cardiomyocyte-like properties, one should bear in mind that the GHRP binding properties of this cell type may to some extent differ from adult rat cardiac tissue.

In conclusion, accumulating data suggest a number of GH-independent effects of synthetic GHRPs and ghrelin on the cardiovascular system, also at the cellular level on cardiomyocytes and vascular smooth muscle cells. However, very little is known about mechanisms of action of these potential cardiovascular therapeutic agents and further studies are clearly warranted to clarify this.

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