

Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln¹⁴-ghrelin

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Abstract

The mechanisms underlying the cardiac activities of synthetic growth hormone secretagogues (GHS) are still unclear. The natural ligand of the GHS receptors, i.e. ghrelin, classically binds the GHS receptor and exerts endocrine actions in acylated forms only; its cardiovascular actions still need to be investigated further. In order to clarify these aspects, we studied the effects of either the synthetic peptidyl GHS hexarelin (1 μ M), or the natural ghrelin (50 nM) and the endogenous ghrelin derivatives des-Gln¹⁴-ghrelin (1–100 nM) and des-octanoyl ghrelin (50 nM), on the tension developed by guinea pig papillary muscle and on L-type Ca²⁺ current (I_{Ca}) of isolated ventricular cells. The binding of these molecules to ventricular cell membrane homogenates was also studied. We observed that all peptides reduced the tension developed at low frequencies (60–120 beats/min) in a dose-dependent manner. No alteration in cardiac contractility was induced by des-Gln¹⁴-ghrelin or des-octanoylated ghrelin when the endocardial endothelium had been removed or after cyclooxygenase blockade. Pretreatment with tyramine (2 μ M) had no effect on the inotropic response induced by des-Gln¹⁴-ghrelin. No significant effect on I_{Ca} of isolated ventricular cells was observed in the presence of des-Gln¹⁴-ghrelin (100 nM). The order of potency on the tension of papillary muscle was: des-octanoyl ghrelin>ghrelin=des-Gln¹⁴-ghrelin>hexarelin. This gradient of potency was consistent with the binding experiments performed on ventricular membranes where either acylated or unacylated ghrelin forms, and hexarelin, recognized a common high-affinity binding site. In conclusion, ghrelin, des-Gln¹⁴-ghrelin and des-octanoyl ghrelin, show similar negative inotropic effect on papillary muscle; as des-octanoyl ghrelin is peculiarly devoid of any GH-releasing activity, the cardiotropic action of these molecules is independent of GH release. The binding studies and the experiments performed both on the isolated cells and on papillary muscle after endothelium removal or cyclooxygenase blockade indicate that the cardiotropic action of natural and synthetic ghrelin analogues reflects the interaction with a novel GHS receptor (peculiarly common for ghrelin and des-octanoyl ghrelin), leading to release of cyclooxygenase metabolites from endothelial cells, as indicated by direct measurement of prostacyclin metabolite 6-keto-PGF_{1 α} .

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1. Introduction

Growth hormone (GH) and insulin-like growth factor-I (IGF-I) influence myocardial morphology and functions

acting on specific cardiac receptors (Colao et al., 2001). GH and IGF-1 excess or deficit are generally associated with deranged myocardial structure and performance (Broglia et al., 1999; Osterziel et al., 2000).

Cardiotropic activities are also exerted by other molecules known as GH secretagogues (GHS) that are distinct entities from GHRH, the endogenous hypophysiotropic neurohormone stimulating somatotroph secretion (Ghigo et al., 1997; Müller et al., 1999). GHS were discovered as a family of non-natural peptidyl and non-peptidyl molecules able to

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strongly stimulate GH secretion acting on a specific G-protein coupled receptor, namely GHS type 1a receptor, that is different from the GHRH-receptor (Smith et al., 1997; Muccioli et al., 1998, 2002). Theoretically, GHS would therefore influence cardiac structure and function via enhanced GH and IGF-I secretion. However, it has also been demonstrated that these molecules, at least the peptidyl ones, exert also direct and GH-independent cardiac actions. This is suggested by the observations that these effects occur also in hypopituitary patients (Muccioli et al., 2000) and that are not exerted by GHS analogues (i.e. EP-51389) with strong GH-releasing activity, but lacking cardiac binding sites (Locatelli et al., 1999; Müller et al., 2002). In particular, peptidyl GHS, such as hexarelin, improve the post-ischemic recovery of hearts of either aged or GH-deficient rats (Locatelli et al., 1999) and increase left ventricular ejection in patients with severe GH-deficiency (Bisi et al., 1999; Imazio et al., 2002). A stimulatory effect on cell proliferation (Pettersson et al., 2002) and an antiapoptotic action (Filigheddu et al., 2001) of GHS on the cardiomyocyte cell line H9c2 have also been shown. The natural ligand of the GHS type 1a receptor has more recently been discovered as a gastric-derived hormone named ghrelin (Kojima et al., 1999). It is an acyl-peptide consisting of 28 amino acids and esterified with octanoic acid on Ser³, though a form of 27 amino acids, des-Gln¹⁴-ghrelin (resulting from alternative splicing of the same gene) has also been isolated (Hosoda et al., 2000a). Both exist in either acylated and, more abundantly, non-acylated form (Hosoda et al., 2000b), but the latter have been shown inactive in term of endocrine activities (Kojima et al., 2001). They do not stimulate GH secretion in vivo (Torsello et al., 2002) because do not bind and activate the GHS type 1a receptor (Bednarek et al., 2001) neither displace radiolabelled ghrelin from its hypothalamo-pituitary binding sites (Muccioli et al., 2001; Torsello et al., 2002). The gastric peptide ghrelin is endowed with a strong stimulatory effect on GH release, gastric acid secretion and gut motility in rats and humans (Kojima et al., 2001). However, many recent studies have demonstrated that ghrelin is much more than a natural GH secretagogue and that its biological actions are not confined to the pituitary or to the gastro-intestinal tract. In fact, it has been reported that ghrelin increases food intake, adiposity and causes a positive energy balance, as well as modulates glucose metabolism and cell growth in non-tumoural and neoplastic tissues (Muccioli et al., 2002). In addition, as a result of studies conducted in vitro and in vivo, it has been already reported that ghrelin not only exerts cardiovascular effects in humans (Muccioli et al., 2000; Okumura et al., 2002; Nagaya and Kangawa, 2003), but it has also been demonstrated that ghrelin does not share all cardiac activities exerted by synthetic GHS (Torsello et al., 2003). Interestingly, besides GHS type 1a receptor mRNA expression (Nagaya and Kangawa, 2003) and specific binding sites labelled by [¹²⁵I]His⁹-ghrelin (Katugampola et al., 2001), animal and human myocardium possess also GHS receptors that are specific for peptidyl GHS and which do

not recognise ghrelin or the non-peptidyl GHS, MK-0677 (Bodart et al., 1999; Papotti et al., 2000). The recent finding that H9c2 cardiomyocytes do not express GHS type 1a receptor, but have high affinity binding sites, common for ghrelin and des-acyl ghrelin, involved in mediating their antiapoptotic activity, provides further support to the hypothesis that multiple ghrelin and GHS receptors exist in the cardiovascular system (Baldanzi et al., 2002). Each receptor may then contribute independently to the wide array of cardiovascular activities induced by synthetic GHS, ghrelin and endogenous ghrelin-derived molecules (Muccioli et al., 2000, 2002; Torsello et al., 2003).

In order to clarify the mechanisms underlying the cardiac activities of ghrelin and to verify whether its different molecular forms are cardioactive or not, we have studied the effects of ghrelin (50 nM), des-Gln¹⁴-ghrelin (1–100 nM) and des-octanoyl ghrelin (50 nM), as well as of the synthetic peptidyl GHS hexarelin (1 µM), on the tension developed by guinea pig papillary muscle, a model that shows a biphasic force–frequency relationship similar to that reported for human cardiac muscle (Brixius et al., 1999). The effects of ghrelin and its derivatives on L-type calcium current (I_{Ca}) of isolated ventricular cells and the binding of all these molecules to ventricular cell membrane homogenates have also been studied.

2. Materials and methods

2.1. Materials

Human ghrelin (Gly-Ser-Ser-(O *n*-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg-NH₂) and des-octanoyl ghrelin were purchased from Phoenix Pharmaceuticals (Belmont CA, USA), whereas des-Gln¹⁴-ghrelin was both from Tocris (Pallwin, MO, USA) and Phoenix Pharmaceuticals, Tyr⁴-ghrelin, Tyr⁴-des-octanoyl ghrelin, and hexarelin (His-D-2Me-Trp-Ala-Trp-D-Phe-Lys-NH₂) were purchased from Neosystem (Strasbourg, France). [¹²⁵I]Tyr⁴-ghrelin (1800–2100 Ci/mmol) and [¹²⁵I]Tyr⁴-des-octanoyl ghrelin (1700–2000 Ci/mmol) were iodinated using a lactoperoxidase method and purified by reverse-phase high-performance liquid chromatography, as previously described (Muccioli et al., 2001). The release of PGI₂ was measured by evaluating the concentration of 6-keto-PGF_{1α} in perfusate samples, with an enzymeimmunoassay kit (Amersham International, UK).

Solutions used in the experiments on papillary muscles or on isolated ventricular cells were freshly prepared as previously described (Bedendi et al., 2001).

2.2. Animals and tissue preparation

Animal care, sacrifice and experiments were conducted according to the European Community guidelines for care

and use of animals and the projects supervised by the local ethical committee. Young adult guinea pigs of either sex were anaesthetized with ether and killed by stunning and cervical dislocation.

Papillary muscle or isolated ventricular cells were used to study the effects of hexarelin and different ghrelin molecules on contractility or calcium current. Binding studies on ventricular tissue homogenates of male guinea pigs were also performed.

2.3. Isolated papillary muscle and contractility determination

As previously detailed (Bedendi et al., 2001), papillary muscles were driven at various frequencies (from 60 to 320 beats/min). Peak tension, the maximum rate of rise ($+dT/dt_{\max}$) and of fall ($-dT/dt_{\max}$) of developed tension were acquired, recorded and measured by a Power Mac computer, using the Labview Software (National Instruments, Texas, USA).

The effects of the following drugs were tested: hexarelin (1 μ M), ghrelin and des-octanoyl ghrelin (50 nM), des-Gln¹⁴-ghrelin (1–100 nM). Endocardial endothelium was removed as previously described (Bedendi et al., 2001). To study the role of cyclooxygenase metabolites and of the possibly released catecholamines by nerve endings, papillary muscles were pretreated with indomethacin (1 μ M) or tyramine (2 μ M) for 30 min, respectively.

2.4. I_{Ca} measurements in isolated ventricular cells

Ventricular myocytes were obtained from hearts of adult guinea pig by enzymatic dissociation using methods described previously (Bedendi et al., 2001). To measure I_{Ca} , the cell was depolarised every 5 s from -100 to -40 mV for 50 ms, to inactivate both fast Na current and T-type calcium current, and from there for 300 ms to $+10$ mV, that is the potential at which I_{Ca} is maximal. All control and analysis of the experiments were performed with the Axo-data and Axograph-4 programs (Axon Instruments, CA, USA) on PowerMac computers (Apple Computer, CA, USA). The experiments were performed at 35°C under thermostatic control.

2.5. Ghrelin binding assay

Binding of ghrelin and des-octanoyl ghrelin to tissue membranes was studied using as radioligands [¹²⁵I]Tyr⁴-ghrelin and [¹²⁵I]Tyr⁴-des-octanoyl ghrelin. Tyr⁴-ghrelin has been reported to have the same GH-releasing potency of native ghrelin in rats and to be a reliable probe for labelling ghrelin receptors in the hypothalamus and pituitary gland (Muccioli et al., 2001; Torsello et al., 2002). To perform binding studies, the atria were removed and ventricles were minced in small pieces and stored at -40°C . Afterwards, the membrane fractions ($30,000 \times g$ pellet) were prepared

from frozen pieces of ventricular myocardium using the method previously described for brain and pituitary gland (Muccioli et al., 2001). Membranes were resuspended in ice-cold buffer (50 mM Tris-HCl, 2.5 mM EGTA, 0.002% bacitracin, pH 7.4) and immediately used to determine protein content by the method of Lowry and for binding studies. For saturation binding studies, tissue membranes (corresponding to 100 μ g protein) were incubated in triplicate at 23°C for 2 h under constant shaking with increasing concentrations (0.035–3 nM) of [¹²⁵I]Tyr⁴-ghrelin or [¹²⁵I]Tyr⁴-des-octanoyl ghrelin in a final volume of 0.5 ml assay buffer (50 mM Tris-HCl, 2.5 mM EGTA, 0.002% bacitracin, 0.1% bovine serum albumin, pH 7.4). Parallel incubations, where 2.0 μ M unlabelled ghrelin or des-octanoyl ghrelin was also present, were used to determine nonspecific binding, which was subtracted from total binding to yield specific binding values. The binding reaction

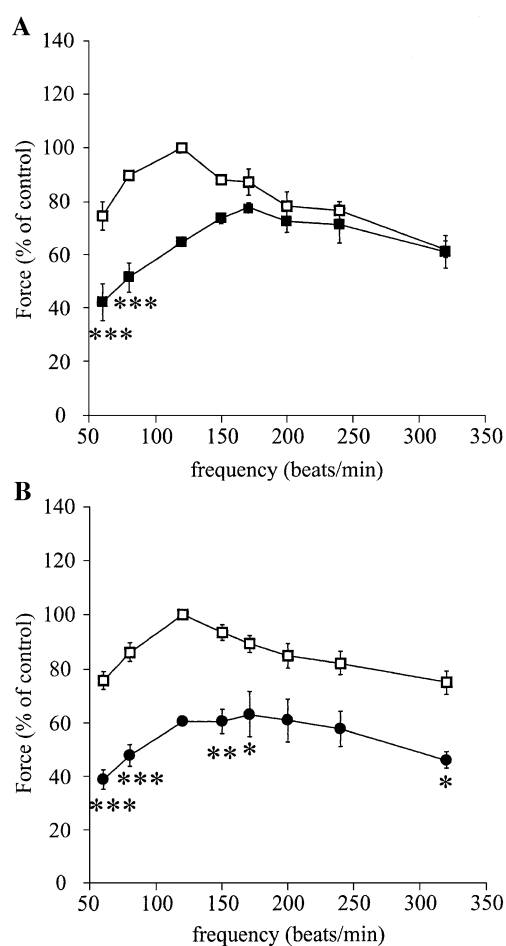


Fig. 1. Effects of the synthetic peptide hexarelin (1 μ M) (A) and of the endogenous peptide ghrelin (50 nM) (B) on isolated papillary muscle. The peak tension was recorded from papillary muscles stimulated at different beating frequencies in control conditions (□) and after treatment with hexarelin (■, $n=4$), or ghrelin (●, $n=4$). Data are expressed as the mean \pm S.E.M. % of the values measured at 120 beats/min before treatment. Statistical analysis was performed between data recorded at each frequency, before and after application of the drug (ANOVA followed by Student–Newman–Keuls test; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

was terminated following the procedure previously described (Muccioli et al., 1998, 2001) and the radioactivity remaining bound to the filters was measured by a Packard gamma counter A5003. Specific binding was expressed as fmol/mg protein. Saturation isotherms were transformed using the method of Scatchard and the maximal number of binding sites (B_{\max}) and the dissociation constant (K_d) were calculated with the GraphPAD Prism 3 program (GraphPAD Software, San Diego, CA, USA). To show binding site specificity, increasing concentrations ghrelin, des-Gln¹⁴ ghrelin, des-octanoyl ghrelin and hexarelin were tested in competition assays with [¹²⁵I]Tyr⁴-ghrelin or [¹²⁵I]Tyr⁴-des-octanoyl ghrelin and the IC₅₀ values for the various competitors were calculated by iterative nonlinear curve fitting program.

2.6. Statistical analysis

All results are expressed as mean values \pm S.E.M. Statistical analysis was carried out with one-way analysis of variance followed by Student's two-tailed *t*-test or Newman–Keuls multiple range test depending on the experiments. $P < 0.05$ was chosen as level of significance. The number of experiments is indicated by *n*.

3. Results

3.1. Contractile effect on isolated papillary muscle

In previous experiments, we observed that hexarelin modifies the force–frequency relationship in rat papillary muscle, reducing the tension developed at low frequencies, while at higher frequencies contractile force was unaltered (Bedendi et al., 2001). We observed that, similarly to the rat, also in the guinea pig papillary muscle hexarelin (1 μ M) reduces the tension developed at low frequencies (60–120 beats/min), while the tension at higher frequencies (150–320 beats/min) is unaffected (Fig. 1A; at 120 beats/

min, contractile force = $64.7 \pm 10.9\%$ of the control, $n = 4$). The negative inotropic effect induced at low beating frequencies was accompanied by a decrease of both $+dT/dt_{\max}$ (all $+dT/dt_{\max}$ values are summarized in Table 1) and $-dT/dt_{\max}$. Like hexarelin, the endogenous ligands for GHS receptors, ghrelin and des-Gln¹⁴-ghrelin, reduced peak tension (at 120 beats/min, 60.8 ± 7.6 and $75.4 \pm 9.1\%$ of the control; $n = 5$ and 6 , respectively; Figs. 1B and 2A), as well as $+dT/dt_{\max}$ and $-dT/dt_{\max}$ at low stimulation rates. Guinea pig papillary muscle was more sensitive to the action of both ghrelin and des-Gln¹⁴-ghrelin, in comparison with hexarelin. Indeed, the effects exerted by addition of 50 nM ghrelin or des-Gln¹⁴-ghrelin were comparable to those caused by a 20-fold higher concentration of hexarelin. Dose–response experiments, performed on papillary muscles stimulated at constant frequency (120 beats/min), showed that 50 nM des-Gln¹⁴-ghrelin was the half-maximal effective concentration, the tensions measured being: $101.6 \pm 1.3\%$, $91.9 \pm 3.7\%$, $85.7 \pm 5.2\%$ and $84.1 \pm 1.9\%$, at 0.5 ($n = 3$), 5 ($n = 4$), 50 ($n = 5$) and 100 nM ($n = 5$), respectively (all values expressed as a percentage of the control). Papillary muscles treated with tyramine, to exclude any interference by catecholamines released from nerve endings, showed a negative inotropic response to des-Gln¹⁴-ghrelin (50 nM), which was superimposable to that observed in untreated preparation (at 120 beats/min, $78.6 \pm 19.1\%$, $n = 3$, vs. $75.4 \pm 9.1\%$, $n = 6$).

In order to verify if also des-octanoyl ghrelin, the form of ghrelin inactive on GH release was able to modulate inotropism, we compared its effect to those exerted by ghrelin and des-Gln¹⁴-ghrelin. Surprisingly, we observed a significant response to this non-acylated peptide, which caused a negative inotropic effect extended also to higher frequencies (150–240 beats/min) (Fig. 2A; at 120 beats/min the tension was $51.0 \pm 11.8\%$ of the control, $n = 4$).

To study the role of endocardial endothelium and of prostacyclin in the inotropic effect induced by these peptides, we performed further experiments on guinea pig-

Table 1

Effect of synthetic hexarelin and endogenous peptides ghrelin, des-octanoyl ghrelin and des-Gln¹⁴-ghrelin on $+dT/dt_{\max}$, in papillary muscles untreated (control), or pretreated with Triton X-100 or indomethacin, at different beating frequencies (60–200 beats/min)

| (Beats/min) | 60 | 80 | 120 | 150 | 171 | 200 |
|---|------------------------|-------------------------|------------------|------------------|------------------|------------------|
| Control | 69.8 ± 3.9 | 78.0 ± 1.8 | 100.0 | 102.5 ± 1.5 | 111.1 ± 6.6 | 105.4 ± 11.2 |
| Hexarelin | $40.4 \pm 5.9^*$ | $48.1 \pm 2.9^*$ | 67.6 ± 11.9 | 84.0 ± 3.5 | 89.9 ± 2.3 | 90.8 ± 6.2 |
| Ghrelin | $44.7 \pm 3.1^*$ | 56.4 ± 2.8 | 76.85 ± 11.9 | $79.7 \pm 7.3^*$ | $90.6 \pm 7.2^*$ | $91.5 \pm 8.6^*$ |
| Des-octanoyl ghrelin | $33.5 \pm 4.1^\dagger$ | $48.0 \pm 5.3^\diamond$ | 64.8 ± 17.7 | 73.9 ± 7.4 | 80.9 ± 12.5 | 70.7 ± 3.5 |
| Des-Gln ¹⁴ -ghrelin | 53.8 ± 3.9 | 66.1 ± 5.6 | 84.5 ± 8.7 | 86.6 ± 2.3 | 94.8 ± 3.6 | 99.5 ± 7.4 |
| Triton | 66.4 ± 4.9 | 73.0 ± 5.2 | 100.0 | 118.6 ± 4.3 | 132.2 ± 5.9 | 140.4 ± 10.6 |
| Triton + des-octanoyl ghrelin | 64.8 ± 6.2 | 70.8 ± 6.1 | 98.9 ± 12.3 | 111.3 ± 8.0 | 128.5 ± 12.8 | 141.6 ± 15.5 |
| Triton + des-Gln ¹⁴ -ghrelin | 55.2 ± 3.1 | 64.1 ± 2.7 | 85.2 ± 17.7 | 100.7 ± 6.2 | 112.4 ± 8.0 | 127.6 ± 11.4 |
| Indomethacin | 60.9 ± 2.5 | 80.0 ± 7.6 | 100.0 | 98.4 ± 5.9 | 98.8 ± 4.3 | 95.1 ± 4.4 |
| Indomethacin + des-octanoyl ghrelin | 54.7 ± 6.2 | 74.7 ± 5.5 | 104.4 ± 14.3 | 99.9 ± 7.6 | 116.2 ± 5.0 | 103.1 ± 7.1 |

Values are expressed as the mean \pm S.E.M. % of the value at 120 beats/min before treatment. Concentration of the drugs and number of experiments are the same as indicated in Figs. 1 and 2. Statistical analysis was performed between data recorded at each frequency, before and after application of the drug (ANOVA followed by Student–Newman–Keuls test; $^*P < 0.05$; $^\diamond P < 0.01$; $^\dagger P < 0.001$).

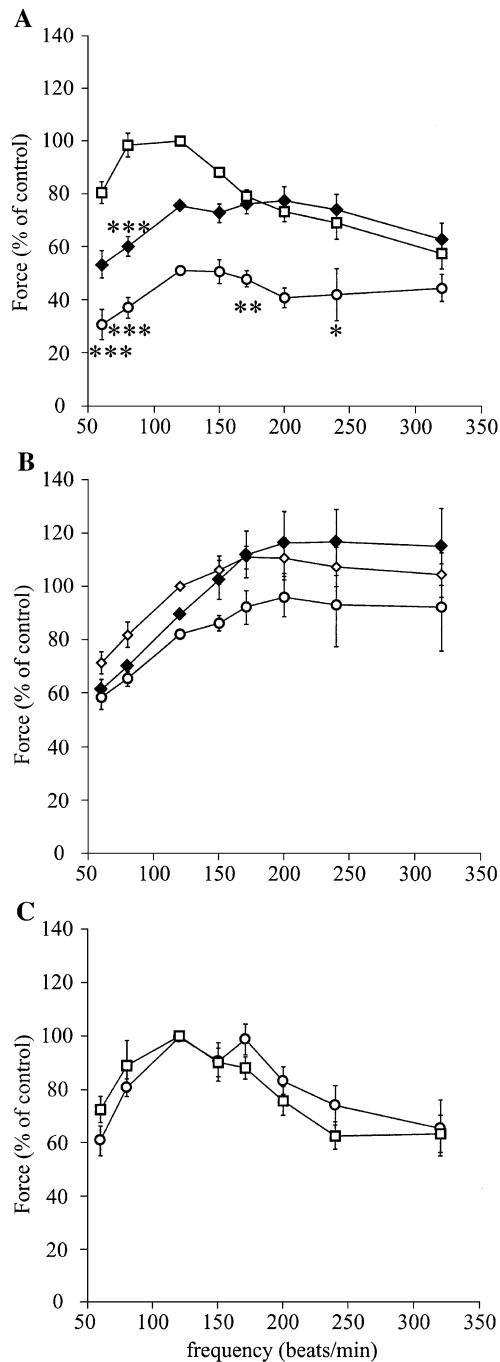


Fig. 2. Relationship between beating frequency and peak tension developed by papillary muscles: (A) perfused with Tyrode solution alone (\square , $n=12$), containing 50 nM des-octanoyl ghrelin (\circ , $n=6$) or 50 nM des-Gln¹⁴-ghrelin (\blacklozenge , $n=6$); (B) deprived of endocardial endothelium perfused with Tyrode solution alone (\diamond , $n=12$), containing 50 des-octanoyl ghrelin (\circ , $n=6$) or 50 nM des-Gln¹⁴-ghrelin (\blacklozenge , $n=6$); (C) pretreated with indomethacin (1 μ M), before (\square) and after treatment with 50 nM des-octanoyl ghrelin (\circ , $n=4$). Values are expressed as mean \pm S.E.M. Statistical significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

papillary muscles deprived of endocardial endothelium or pretreated with indomethacin (1 μ M). Both treatments did not significantly alter the shape of the force–frequency relationship. As shown in Fig. 2B, removal of endocardial

endothelium markedly reduced the inotropic response of guinea pig papillary muscle to both des-Gln¹⁴-ghrelin and des-octanoyl ghrelin ($89.4 \pm 12.8\%$ and $82.0 \pm 6.7\%$; $n=6$ and 5, respectively). A similar result was obtained in papillary muscles in which des-octanoyl ghrelin was applied after cyclooxygenase blockade (Fig. 2C; $99.6 \pm 13.7\%$, $n=4$).

The hypothesis that the inotropic effect of ghrelin is mainly due to PGI₂ released from endothelial cells, was further supported by direct measurement of the prostacyclin metabolite, 6-keto-PGF_{1 α} . In our preparations, baseline production of 6-keto-PGF_{1 α} was 52.5 ± 3.5 pg/ml; des-octanoyl ghrelin (50 nM) induced a significant enhancement of 6-keto-PGF_{1 α} release ($130.7 \pm 4.9\%$), which was completely blocked by 1 μ M indomethacin ($86.0 \pm 2.3\%$).

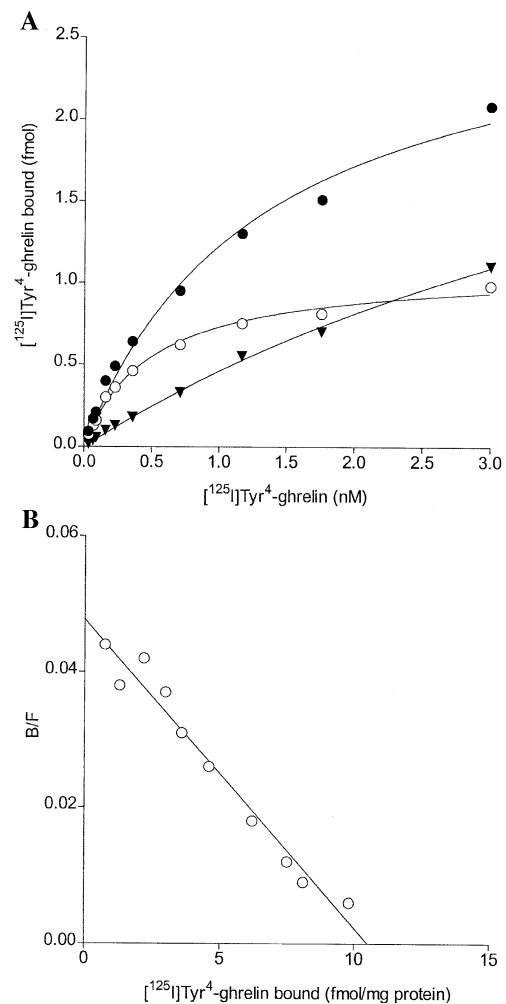


Fig. 3. Representative saturation isotherms (A) and Scatchard plot (B) of [¹²⁵I]Tyr⁴-ghrelin binding to membranes from ventricular myocardium of guinea pig. Experiments were performed by incubating a fixed amount of membrane protein (100 μ g/tube) with increasing concentrations of radio-labelled ghrelin (total binding, \bullet) or plus 2.0 μ M unlabelled ghrelin to define nonspecific binding (\blacktriangledown). Specific binding values (\circ) were obtained by subtracting nonspecific binding from total binding. The saturation curve of specific binding was analyzed by Scatchard analysis in order to calculate the maximal binding capacity (B_{\max}) and the dissociation constant (K_d).

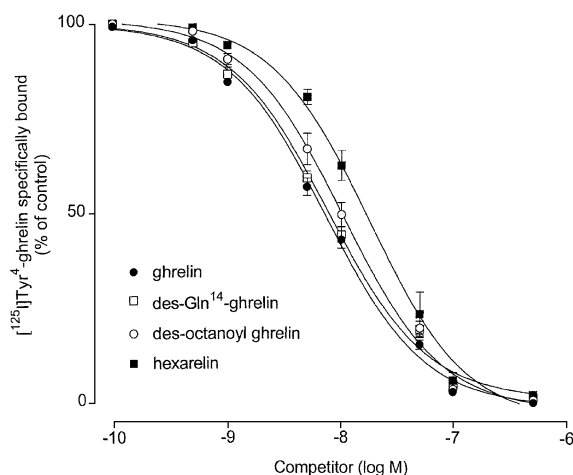


Fig. 4. Competition for [125 I]Tyr⁴-ghrelin binding to membranes of ventricular myocardium by ghrelin, des-Gln¹⁴-ghrelin, des-octanoyl ghrelin and hexarelin. Binding assays were conducted as described in Materials and methods. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor). Each point represents the mean \pm S.E.M. of three separate experiments.

3.2. Lack of effect on I_{Ca} in isolated ventricular cells

After the patch membrane was broken to achieve the whole-cell condition, the isolated cells were allowed to equilibrate for at least 5 min. The application of des-Gln¹⁴-ghrelin at a concentration (100 nM) able to reduce significantly the contractile force developed by papillary muscle, did not modify the L-type calcium current on isolated ventricular cells. In eight cells, I_{Ca} was -1.40 ± 0.16 nA in control conditions, and -1.36 ± 0.15 nA in the presence of des-Gln¹⁴-ghrelin ($p = \text{n.s.}$). However, the fact that in the same cells I_{Ca} promptly increased after application of isoproterenol (1 μM), suggests that they were still responsive to stimulation of membrane receptors.

3.3. Binding of [125 I]Tyr⁴-ghrelin and [125 I]Tyr⁴-des-octanoyl ghrelin to membranes from ventricular myocardium

In order to investigate the identity of the receptor mediating the effects of ghrelin and des-octanoyl ghrelin on guinea pig heart, we have performed binding studies of [125 I]Tyr⁴-ghrelin and [125 I]Tyr⁴-des-octanoyl ghrelin to membranes of guinea pig ventricular myocardium. Experiments using increasing concentrations of [125 I]Tyr⁴-ghrelin revealed the presence of a saturable specific binding (associated with a low nonspecific binding) that increased linearly with the radioligand concentrations (Fig. 3A). Scatchard analysis of the specific binding data (Fig. 3B) demonstrated the existence of a single class of binding sites with a dissociation constant (K_d) of 0.51 ± 0.06 nM and a maximal binding capacity (B_{max}) of 10.9 ± 1.8 fmol/mg of protein ($n = 4$). Unlabelled ghrelin, des-Gln¹⁴ ghrelin, hexarelin) and, surprisingly, des-octanoyl ghrelin, an endogenous ghrelin precursor which is

unable to bind the hypothalamo-pituitary receptor for GH secretagogues (Muccioli et al., 2001; Torsello et al., 2002) competed with [125 I]Tyr⁴-ghrelin for cardiac binding sites (Fig. 4). The IC_{50} values, all expressed as nM concentrations, were 8.1 ± 0.9 for ghrelin, 7.4 ± 0.4 for des-Gln¹⁴ ghrelin, 12.5 ± 1.7 for des-octanoyl ghrelin and 20.8 ± 2.3 for hexarelin (mean \pm S.E.M. of three separate experiments). Experiments using increasing concentrations of [125 I]Tyr⁴-des-octanoyl ghrelin also provided consistent evidence of a saturable specific binding in ventricular myocardium of guinea pig (Fig. 5A). Scatchard analysis demonstrated the

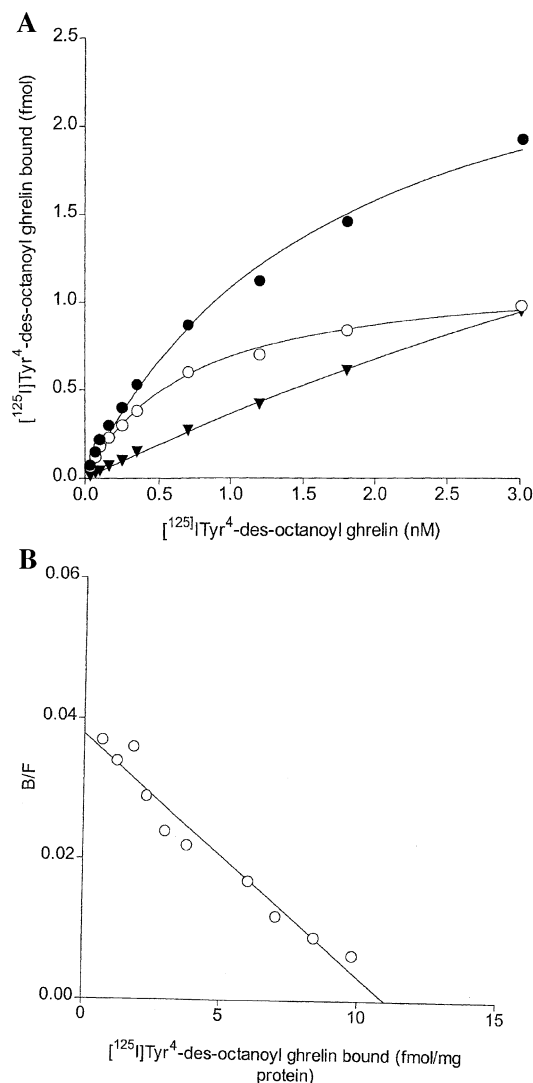


Fig. 5. Representative saturation isotherms (A) and Scatchard plot (B) of [125 I]Tyr⁴-des-octanoyl ghrelin binding to membranes from ventricular myocardium of guinea pig. Experiments were performed by incubating a fixed amount of membrane protein (100 $\mu\text{g}/\text{tube}$) with increasing concentrations of radiolabelled des-octanoyl ghrelin (total binding, \bullet) or plus 2.0 μM unlabelled des-octanoyl ghrelin to define nonspecific binding (\blacktriangledown). Specific binding values (\circ) were obtained by subtracting nonspecific binding from total binding. The saturation curve of specific binding was analyzed by Scatchard analysis in order to calculate the maximal binding capacity (B_{max}) and the dissociation constant (K_d).

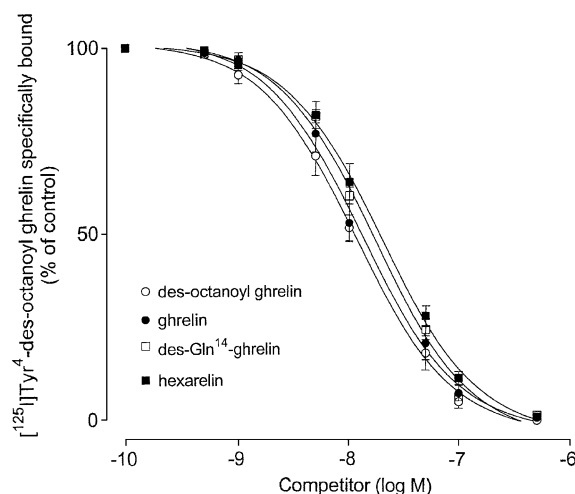


Fig. 6. Competition for [125 I]Tyr 4 -des-octanoyl ghrelin binding to membranes of ventricular myocardium by des-octanoyl ghrelin, ghrelin, des-Gln 14 -ghrelin and hexarelin. Binding assays were conducted as described in Materials and methods. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor). Each point represents the mean \pm S.E.M. of three separate experiments.

existence of a single class of binding sites that showed values of B_{\max} (12.2 ± 1.4 fmol/mg protein, $n=4$) and K_d (0.74 ± 0.12 nM, $n=4$), very close to those detected by radio-labelled ghrelin (Fig. 5B). Both unlabelled ghrelin and des-octanoyl ghrelin, as well as des-Gln 14 -ghrelin and hexarelin competed with [125 I]Tyr 4 -des-octanoyl ghrelin for binding sites (Fig. 6). IC $_{50}$ values, all expressed as nM concentrations (mean \pm S.E.M. of three separate experiments) of 10.4 ± 0.8 for des-octanoyl ghrelin, 12.2 ± 1.8 for ghrelin, 16.5 ± 0.8 for des-Gln 14 -ghrelin and 22.0 ± 2.1 for hexarelin.

4. Discussion

The results of present study demonstrate that ghrelin, des-Gln 14 -ghrelin or des-octanoyl ghrelin, as well as hexarelin, a synthetic peptidyl growth hormone secretagogue (GHS), all show similar negative inotropic effect on the guinea pig papillary muscle. Interestingly, the most significant influence on contractility of the papillary muscle was exerted by des-octanoyl ghrelin, an unacylated form devoid of any endocrine action (Kojima et al., 2001; Torsello et al., 2002) because this form, unlike octanoylated ghrelin and des-Gln 14 -ghrelin (Hosoda et al., 2000a), is unable to bind the classical GHS type 1a receptor (Bednarek et al., 2001). The binding studies performed here with either acylated and unacylated ghrelin demonstrated that there is a specific receptor recognized by all ghrelin forms and by hexarelin as well; these findings therefore indicate the existence of a cardiac non-GHS type 1a receptor. No change in cardiac contractility was induced by either acylated or unacylated ghrelin when the endocardial endothelium had been re-

moved or after pretreatment with indomethacin, suggesting that these effects are mediated by cyclooxygenase metabolites produced by endothelial cells. In contrast, ghrelin did not modify L-type calcium current of isolated ventricular cells and this finding further supports the hypothesis that the effects of ghrelin are mainly due to endothelium-released prostaglandins.

The existence of GH-independent cardiotropic activities of GHS had also been demonstrated by previous works (Locatelli et al., 1999; Muccioli et al., 2000) showing that these effects also occur in hypopituitary patients (Bisi et al., 1999) and that they are not produced by GHS analogues (EP-51389) with strong GH-releasing activity, but lacking cardiac binding sites (Locatelli et al., 1999; Müller et al., 2002). In particular, peptidyl GHS, such as hexarelin, improve the post-ischemic recovery of hearts from either aged or GH-deficient rats (Locatelli et al., 1999; Müller et al., 2002) and increase left ventricular ejection in patients with severe GH deficiency (Bisi et al., 1999; Imazio et al., 2002). An antiapoptotic action of synthetic GHS has also been shown on H9c2 cardiomyocytes (Filigheddu et al., 2001). Moreover, we have previously reported that the peptidyl GHS hexarelin has negative inotropic effect on rat papillary muscle (Bedendi et al., 2001). We have now shown that the same action of hexarelin is exerted by an endogenous ligand of GHS type 1a receptor, such as ghrelin, a 28 amino acid gastric peptide with an octanoyl modification at Ser 3 essential for the hormone's binding and bioactivity (Kojima et al., 2001). The same action was found to be exerted by des-Gln 14 -ghrelin, another natural acylated form of ghrelin derived from an alternative splicing of the ghrelin gene also capable of binding the GHS type 1a receptor (Hosoda et al., 2000a). However, we have now found unexpectedly that non-acylated ghrelin was able to exert the same action at even greater extent than the acylated forms of ghrelin, even though unacylated ghrelin is generally assumed to be devoid of biological activity (Kojima et al., 2001), as it cannot bind and activate the GHS type 1a receptor (Bednarek et al., 2001). Although GHS type 1a receptor expression at the myocardial level has been demonstrated (Nagaya and Kangawa, 2003), the existence of GHS receptor subtypes in the heart has been already shown (Muccioli et al., 2000, 2002). At the myocardial level and within the vasculature, a receptor that binds synthetic peptidyl GHS only, but not ghrelin or non-peptidyl GHS, has been demonstrated (Bodart et al., 1999; Papotti et al., 2000). In this present work, we have demonstrated the existence of a cardiac receptor that binds a peptidyl GHS, namely hexarelin, as well as ghrelin either in acylated or unacylated form. This is therefore another GHS receptor subtype that could well mediate the inotropic action of all these molecules on the papillary muscle of both the guinea pig and the rat. It is important to note that the existence of a GHS receptor subtype able to bind either acylated or unacylated ghrelin and hexarelin had been already demonstrated in cultured cardiomyocytes and endothelial cells

(Baldanzi et al., 2002), as well as in neoplastic breast carcinoma cell lines (Cassoni et al., 2001). On the cardiomyocytes, this receptor is probably involved in mediating the cytoprotective effect exerted by ghrelin (in acylated or unacylated form) and by hexarelin (Baldanzi et al., 2002).

In vivo studies have demonstrated that ghrelin exerts cardiac actions also in humans (Okumura et al., 2002; Nagaya and Kangawa, 2003). Ghrelin administration induces hemodynamic effects in man either in normal subjects or in patients with dilated cardiomyopathy, reducing cardiac afterload and increasing cardiac output, without an increase in heart rate (Nagaya and Kangawa, 2003). In addition, ghrelin is an effective endothelium-independent vasodilator of the long-lasting constrictor endothelin-1 in human arteries (Wiley and Davenport, 2002) and acts, at least in part, on the central nervous system decreasing arterial pressure and renal sympathetic activity in conscious rabbits (Matsumura et al., 2002). However, it is not mandatory that ghrelin and synthetic GHS share all the cardiotropic actions, as indicated by recent data showing that, differently from hexarelin, ghrelin exert minimal protective action against cardiac ischemia in hypophysectomized rats (Torsello et al., 2003). Some different cardiotropic actions exerted by natural and synthetic GHS could be well explained by the existence of various GHS receptor subtypes (see above).

Regarding the negative inotropic effect demonstrated in the present and a previous study (Bedendi et al., 2001), it is important to note that all ghrelin forms and the peptidyl GHS hexarelin loose their activity when tested on papillary muscles deprived of endocardial cells or in isolated ventricular cells. These findings strongly indicate a major role of endothelial cells in mediating GHS inotropic action. This assumption is strengthened by a previous observation obtained studying the effect of hexarelin on the rat papillary muscle (Bedendi et al., 2001). Endothelial cells synthesize and release many potential mediators, among these prostacyclins (Mebazaa et al., 1993). In agreement with the hypothesis that prostacyclin mediates the inotropic effect of GHS, in our study, a pretreatment of papillary muscle with indomethacin completely blocked the action of des-octanoyl ghrelin. The involvement of prostacyclin was also confirmed by direct detection of increased amounts of 6-keto-PGF_{1α} released after des-octanoyl ghrelin application. On the other hand, ghrelin effects are neither accompanied by alterations of plasma cyclic guanosine 3,5-monophosphate level nor inhibited by pretreatment with nitric oxide synthase inhibitors, suggesting NO-independent mechanism of action (Okumura et al., 2002). It has been reported that ghrelin may modulate sympathetic nerve activity (Matsumura et al., 2002). However, we observed that the negative inotropic effect of des-octanoyl ghrelin was unaltered after pretreatment with tyramine. This finding excludes a relevant role of endogenous catecholamines released by nerve endings within cardiac tissue.

In conclusion, ghrelin either in acylated or unacylated form, as well as peptidyl GHS show similar inotropic effect

on guinea pig papillary muscle. The cardiotropic action of natural and synthetic ghrelin analogues probably reflects the interaction with a novel GHS receptor (peculiarly common for ghrelin and des-octanoyl ghrelin) and is mediated by cyclooxygenase metabolites produced by endothelial cells.

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References

- Baldanzi, G., Filigheddu, N., Cutrupi, S., Catapano, F., Bonisconi, S., Fubini, A., Malan, D., Baj, G., Granata, R., Broglio, F., Papotti, M., Surico, N., Bussolino, F., Isgaard, J., Deghenghi, R., Sinigaglia, F., Prat, M., Muccioli, G., Ghigo, E., Graziani, A., 2002. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J. Cell Biol.* 159, 1029–1037.
- Bedendi, I., Gallo, M.P., Malan, D., Levi, R.C., Alloati, G., 2001. Role of endothelial cells in modulation of contractility induced by hexarelin in rat ventricle. *Life Sci.* 69, 2189–2201.
- Bednarek, M.A., Feighner, S.D., Pong, S.S., McKee, K.K., Hreniuk, D.L., Silva, M.V., Warren, V.A., Howard, A.D., Van Der Ploeg, L.H., Heck, J.V., 2001. Structure–function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *J. Med. Chem.* 43, 4370–4376.
- Bisi, G., Podio, V., Valetto, M.R., Broglio, F., Bertuccio, G., Amaretti, G., Pelosi, E., Del Rio, G., Muccioli, G., Ong, H., Boghen, M.F., Deghenghi, R., Ghigo, E., 1999. Cardiac effects of hexarelin in hypopituitary adults. *Eur. J. Pharmacol.* 381, 31–38.
- Bodart, V., Bouchard, J.F., McNicoll, N., Escher, E., Carrière, P., Ghigo, E., Sejlitz, T., Sirois, G., Lamontagne, D., Ong, H., 1999. Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. *Circ. Res.* 85, 796–808.
- Brixius, K., Pietsch, M., Schwinger, R.H., 1999. The intracellular Ca(2+)-homeostasis influences the frequency-dependent force-generation in man. *Basic Res. Cardiol.* 94, 152–158.
- Broglio, F., Fubini, A., Morello, M., Arvat, E., Amaretti, G., Gianotti, L., Boghen, M.F., Deghenghi, R., Mangiardi, L., Ghigo, E., 1999. Activity of GH/IGF-I axis in patients with dilated cardiomyopathy. *Clin. Endocrinol.* 50, 417–430.
- Cassoni, P., Papotti, M., Ghè, C., Catapano, F., Sapino, A., Graziani, A., Deghenghi, R., Reissmann, T., Ghigo, E., Muccioli, G., 2001. Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. *J. Clin. Endocrinol. Metab.* 86, 1738–1745.
- Colao, A.M., Marzullo, P., Di Somma, C., Lombardi, G., 2001. Growth hormone and the heart. *Clin. Endocrinol.* 54, 137–154.
- Filigheddu, N., Fubini, A., Baldanzi, G., Cutrupi, S., Ghè, C., Catapano, F., Broglio, F., Bosia, A., Papotti, M., Muccioli, G., Ghigo, E., Deghenghi,

- R., Graziani, A., 2001. Hexarelin protects H9c2 cardiomyocytes from doxorubicin-induced cell death. *Endocrine* 14, 113–119.
- Ghigo, E., Arvat, E., Muccioli, G., Camanni, F., 1997. Growth hormone-releasing peptides. *Eur. J. Endocrinol.* 136, 445–460.
- Hosoda, H., Kojima, M., Matsuo, H., Kangawa, K., 2000a. Purification and characterization of rat des-Gln¹⁴-ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *J. Biol. Chem.* 275, 21995–22000.
- Hosoda, H., Kojima, M., Matsuo, H., Kangawa, K., 2000b. Ghrelin and des-octanoyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem. Biophys. Res. Commun.* 279, 909–913.
- Imazio, M., Bobbio, M., Broglio, F., Benso, A., Podio, V., Valetto, M.R., Bisi, G., Ghigo, E., Trevi, G.P., 2002. GH-independent cardiotropic activities of hexarelin in patients with severe left ventricular dysfunction due to dilated and ischemic cardiomyopathy. *Eur. J. Heart Fail.* 4, 185–191.
- Katugampola, S.D., Pallikaros, Z., Davenport, A.P., 2001. [¹²⁵I-His⁹]-ghrelin, a novel radioligand for localizing GHS orphan receptors in human and rat tissue: up-regulation of receptors with atherosclerosis. *Br. J. Pharmacol.* 134, 143–149.
- Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402, 656–660.
- Kojima, M., Hosoda, H., Matsuo, H., Kangawa, K., 2001. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. *Trends Endocrinol. Metab.* 12, 118–122.
- Locatelli, V., Rossoni, G., Schweiger, F., Torsello, A., De Gennaro Colonna, V., Bernareggi, M., Deghenghi, R., Müller, E.E., Berti, F., 1999. Growth hormone-independent cardioprotective effects of hexarelin in the rat. *Endocrinology* 140, 4024–4031.
- Matsumura, K., Tsuchihashi, T., Fujii, K., Abe, I., Iida, M., 2002. Central ghrelin modulates sympathetic activity in conscious rabbits. *Hypertension* 40, 694–699.
- Mebazaa, A., Martin, L.D., Robotham, J.L., Maeda, K., Gabrielson, E.W., Wetzel, R.C., 1993. Right and left ventricular cultured endocardial endothelium produces prostacyclin and PGE₂. *J. Mol. Cell. Cardiol.* 25, 245–248.
- Muccioli, G., Ghé, C., Ghigo, M.C., Papotti, M., Arvat, E., Boghen, M.F., Nilsson, M.H., Deghenghi, R., Ong, H., Ghigo, E., 1998. Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland. *J. Endocrinol.* 157, 99–106.
- Muccioli, G., Broglio, F., Valetto, M.R., Ghé, C., Catapano, F., Graziani, A., Papotti, M., Bisi, G., Deghenghi, R., Ghigo, E., 2000. Growth hormone-releasing peptides and the cardiovascular system. *Ann. Endocrinol. (Paris)* 61, 27–31.
- Muccioli, G., Papotti, M., Locatelli, V., Ghigo, E., Deghenghi, R., 2001. Binding of ¹²⁵I-labelled ghrelin to membranes from human hypothalamus and pituitary gland. *J. Endocrinol. Invest.* 24, RC7–RC9.
- Muccioli, G., Tschöp, M., Papotti, M., Deghenghi, R., Heiman, M., Ghigo, E., 2002. Neuroendocrine and peripheral activities of ghrelin: implication in metabolism and obesity. *Eur. J. Pharmacol.* 440, 235–254.
- Müller, E.E., Locatelli, V., Cocchi, D., 1999. Neuroendocrine control of growth hormone secretion. *Physiol. Rev.* 79, 511–607.
- Müller, E.E., Rigamonti, A.E., De Gennaro Colonna, V., Locatelli, V., Berti, F., Cella, S.G., 2002. GH-related and extra-endocrine actions of GH secretagogues in aging. *Neurobiol. Aging* 23, 907–919.
- Nagaya, N., Kangawa, K., 2003. Ghrelin improves left ventricular dysfunction and cardiac cachexia in heart failure. *Curr. Opin. Pharmacol.* 3, 146–151.
- Okumura, H., Nagaya, N., Enomoto, M., Nakagawa, E., Oya, H., Kangawa, K., 2002. Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *J. Cardiovasc. Pharmacol.* 39, 779–783.
- Osterziel, K.J., Ranke, M.B., Strohm, O., Dietz, R., 2000. The somatotrophic system in patients with dilated cardiomyopathy: relation of insulin-like growth factor-1 and its alterations during growth hormone therapy to cardiac function. *Clin. Endocrinol.* 53, 61–68.
- Papotti, M., Ghé, C., Cassoni, P., Catapano, F., Deghenghi, R., Ghigo, E., Muccioli, G., 2000. Growth hormone secretagogue binding sites in peripheral human tissues. *J. Clin. Endocrinol. Metab.* 85, 3803–3807.
- Petersson, I., Muccioli, G., Granata, R., Deghenghi, R., Ghigo, E., Ohlsson, C., Isgaard, J., 2002. Natural (ghrelin) and synthetic (hexarelin) GH secretagogues stimulate H9c2 cardiomyocyte cell proliferation. *J. Endocrinol.* 175, 201–209.
- Smith, R.G., Van der Ploeg, L.H.T., Howard, A.D., Feighner, S.D., Cheng, K., Hickey, G.J., Wyvrat Jr., M.J., Fisher, M.H., Nargund, R.P., Patchett, A.A., 1997. Peptidomimetic regulation of growth hormone secretion. *Endocr. Rev.* 18, 621–645.
- Torsello, A., Ghé, C., Bresciani, E., Catapano, F., Ghigo, E., Deghenghi, R., Locatelli, V., Muccioli, G., 2002. Short ghrelin peptides neither displace ghrelin binding in vitro nor stimulate GH release in vivo. *Endocrinology* 143, 1968–1971.
- Torsello, A., Bresciani, E., Rossoni, G., Avallone, R., Tulipano, G., Cocchi, D., Bulgarelli, I., Deghenghi, R., Berti, F., Locatelli, V., 2003. Ghrelin plays a minor role in the physiological control of cardiac function in the rat. *Endocrinology* 144, 1787–1792.
- Wiley, K.E., Davenport, A.P., 2002. Comparison of vasodilators in human internal mammary artery: ghrelin is a potent physiological antagonist of endothelin-1. *Br. J. Pharmacol.* 136, 1146–1152.