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Hexarelin Protects H9c2 Cardiomyocytes from Doxorubicin-Induced Cell Death

Nicoletta Filigheddu,[1] Alberto Fubini,[2] Gianluca Baldanzi,[1] Santina Cutrupi,[1] Corrado Ghè,[3] Filomena Catapano,[3] Fabio Broglio,[2] Amalia Bosia,[1] Mauro Papotti,[4] Giampiero Muccioli,[3] Ezio Ghigo,[2] Romano Deghenghi,[5] and Andrea Graziani[1]

[1]Department of Genetics, Biology and Biochemistry, [2]Department of Internal Medicine, Divisions of Cardiology and of Endocrinology, [3]Department of Pharmacology and Forensic Medicine, [4]Department of Biomedical Science and Human Oncology, University of Torino, Italy and [5]Europeptides, Argenteuil, France

Growth hormone secretagogues (GHSs) are synthetic peptidyl and nonpeptidyl molecules that possess strong growth hormone-releasing activity acting on specific pituitary and hypothalamic receptor subtypes. Differently from nonpeptidyl GHSs, peptidyl molecules such as hexarelin, a hexapeptide, possess specific high-affinity binding sites in animal and human heart and, after prolonged treatment, protect rats in vivo from ischemia-induced myocardial damage. To verify the hypothesis that peptidyl GHSs protect heart cells from cell death, we have investigated the cellular effects of hexarelin on H9c2 cardiomyocytes, a fetal cardiomyocyte-derived cell line, and on Hend, an endothelial cell line derived from transformed murine heart endothelium. We show that (i)H9c2 cardiomyocytes show specific binding for ^{125}I-Tyr-Ala-hexarelin, which is inhibited by peptidyl GHSs such as Tyr-Ala-hexarelin and hexarelin but not by the nonpeptidyl GHS MK-0677, (ii) hexarelin promotes survival of H9c2 cardiomyocytes induced to die by doxorubicin, and (iii) that hexarelin inhibits apoptosis, as measured by DNA fragmentation, induced in both H9c2 myocytes and endothelial cells. In conclusion, our findings show that peptidyl GHSs such as hexarelin act as survival factors for cardiomyocytes and endothelium-derived cells in culture. These findings suggest that the inhibitory activity of hexarelin on cardiomyocytes and endothelial cell death could explain, at least partially, its cardioprotective effect against ischemia recorded in rats in vivo.

Key Words: Hexarelin; growth hormone–releasing peptides; doxorubicin; cardiomyocytes; apoptosis.

Author to whom all correspopndence and reprint requests should be addressed: Prof. A. Graziani, Dept. of Medical Sciences, University A. Avogadro of Piemonte Orientale v. Solaroli 17, 28100 Novara, Italy. E-mail: graziani@med.unipmn.it

Introduction

The family of synthetic growth hormone secretagogues (GHSs) includes peptidyl molecules (growth hormone–releasing peptides [GHRPs] such as GHRP-6, GHRP-1, GHRP-2, hexarelin, Tyr-Ala-hexarelin and ipamorelin) and their nonpeptidyl analogs (benzolactam, spiroindoline) (*1*). GHSs possess strong growth hormone (GH)-releasing effects and other neuroendocrine activities: a stimulatory effect on prolactin and adrenocorticotorpic hormone secretion, influence on sleep and food intake, which are mediated by specific receptors mainly located at the pituitary and hypothalamic level but also in other areas of the central nervous system (CNS) *(2)*.

A specific GHS receptor (GHS-R), GHS-R, has recently been cloned *(3)*. GHS-R is a G-protein-coupled receptor with sequence homology to the neurotensin receptor. The existence of a specific GHS-R suggested that GHSs may act as synthetic agonists of an endogenous factor. Indeed, such an endogenous factor has been recently identified, ghrelin *(4)*. Ghrelin is an acylated peptide expressed in the stomach. Other receptors with partial homology with both GHS and neurotensin receptors have also been characterized—GPR 38, GPR 39, and FM-3—and are present also in peripheral tissues (reviewed in ref. *5*). The ligand for GPR38 has been identified as motilin, a gastrointestinal peptide, but the others still remain orphans.

Specific binding sites for peptidyl GHSs also have been shown in peripheral endocrine and nonendocrine human tissues including the cardiovascular system *(6,7)*. Interestingly, peptidyl GHSs such as hexarelin, but not the nonpeptidyl MK-0677, inhibit the binding of radiolabeled Tyr-Ala-hexarelin in the heart *(6,7)*, indicating that these cardiac receptors have pharmacologic and biochemical properties different from those of GHS-R in the pituitary and the hypothalamus. This evidence implies that the GHS-R family seems to include multiple receptors. Indeed, several independent observations reported in the past two years

suggest that peptidyl GHSs, namely hexarelin, exert cardiovascular activity. For example, prolonged pretreatment with hexarelin protects from myocardial damage induced by ischemia reperfusion in normal aged as well as in GH-deficient rats *(8)* and improves cardiac contractility in rats with myocardial infarction. In addition, in the isolated and perfused rat heart, acute administration of hexarelin induces prompt though transient coronary vasoconstriction *(6)*. Furthermore, the acute administration of hexarelin increases the left ventricular ejection fraction in normal humans and even in hypopituitary patients with severe GH deficiency *(9)*. Altogether these findings led to the hypothesis that peptidyl GHSs could have cardiovascular activities mediated by specific receptors independently of their GH-releasing activity.

The cellular mechanisms underlying the diverse cardiotropic actions of GHSs are still unknown, and, in order to identify such mechanisms, we have investigated the actions of hexarelin in cultures of cardiomyocyte-derived cells in vitro.

Cardiomyocyte death has been demonstrated to play a crucial role in ischemia-reperfusion myocardial damage as well as in the development of dilated cardiomyopathy *(10)*. Thus, we verified the hypothesis that hexarelin may exert its cardioprotective action by preventing cardiomyocyte cell death. To this goal we have investigated the ability of hexarelin in preventing cell death of H9c2 myocytes triggered by doxorubicin, an antitumoral drug, highly cytotoxic in the heart.

The data reported herein show that peptidyl GHSs such as hexarelin act as survival factors for cardiomyocyte-derived cells in culture. These findings suggest that the inhibitory activity of hexarelin on cardiomyocyte cell death could explain, at least in part, its cardioprotective effect against ischemia recorded in rats in vivo.

Results

Binding of ^{125}I-labeled Tyr-Ala-Hexarelin to Membranes from H9c2 Cells

To evaluate the suitability of H9c2 cells as a model to investigate the biologic action of hexarelin, we assessed the ability of Tyr-Ala-hexarelin, a hexarelin analog suitable for iodination, to recognize specific binding sites in these cells. Tyr-Ala-hexarelin has been reported to have the same GH-releasing potency of hexarelin in rats *(11)* and humans *(12)* and to be a reliable probe for labeling in vitro GHRP receptors in the brain *(13)*, pituitary gland *(11,13)* and heart *(6,7)*. Experiments using increasing concentrations of radioiodinated Tyr-Ala-hexarelin, ranging from 0.15×10^{-9} to 10×10^{-9} mol/L, revealed evidence of saturable specific binding in H9c2 cardiomyocytes (**Fig. 1A**). Scatchard analysis (**Fig. 1A**, top) indicated the presence of a single class of high-affinity sites with an apparent dissociation constant (K_d) of $1.5 \pm 0.4 \times 10^{-9}$ mol/L and a concentration (B_{max}) of 1035 ± 112 fmol/mg of protein. The specificity of ^{125}I-labeled Tyr-Ala-hexarelin binding to H9c2 membranes was established by determining the ability of different compounds to compete with the radioligand for the binding sites (**Fig. 1B**). All unlabeled GHRPs tested (Tyr-Ala-hexarelin, hexarelin, GHRP-2, and GHRP-6) completely displaced radiolabeled Tyr-Ala-hexarelin from binding sites. The IC_{50} values (mean ± SEM of three separate experiments) were $(1.2 \pm 0.6) \times 10^{-8}$ mol/L for Tyr-Ala-hexarelin, $(1.7 \pm 0.3) \times 10^{-8}$ mol/L for hexarelin, $(1.9 \pm 0.3) \times 10^{-8}$ mol/L for GHRP-2, and $(2.2 \pm 0.4) \times 10^{-7}$ mol/L for GHRP-6. By contrast, no competition was observed in the presence of nonpeptidyl GHSs such as MK-0677 or peptides structurally unrelated to GHRPs (GH-releasing hormone [GHRH], somatostatin, and tumor necrosis factor-α [TNF-α]). Thus, H9c2 cells constitute a suitable model to investigate the mechanisms of the cardiotropic action of a peptidyl GHS such as hexarelin.

Hexarelin Prevents Apoptotic Cell Death of H9c2 Cardiomyocytes Induced by Doxorubicin

To investigate in vitro the cardioprotective activity of GHSs, we induced cell death of H9c2 by treatment with doxorubicin in the presence or absence of hexarelin. Treatment with 1 μ*M* doxorubicin for 18 h induced cell death of H9c2 cardiomyocytes (40% survival), as measured by the MTT cell viability assay (**Fig. 2**). Hexarelin alone did not affect H9c2 cell survival. On the other hand, 6 h of pretreatment with 1μ*M* hexarelin prevented doxorubicin-induced cell death (**Fig. 2**). Furthermore, we investigated whether hexarelin could inhibit doxorubicin-induced nucleosomal DNA fragmentation, a hallmark of cell death by apoptosis. Doxorubicin treatment in 5% Fetal calf serum (FCS) induced DNA fragmentation in H9c2 cardiomyocytes, as measured by an enzyme-linked immunosorbent assay (ELISA) (**Fig. 3A**). Hexarelin alone did not stimulate DNA fragmentation. However, pretreatment of H9c2 cells with hexarelin reduced doxorubicin-induced DNA fragmentation. In another experiment, H9c2 cells were treated with doxorubicin in the absence of serum (**Fig. 3B,C**). Under these experimental conditions, DNA fragmentation was detected by DNA staining in agarose gel. Serum starvation potentiated the doxorubicin-induced DNA fragmentation, which, however, was not prevented by hexarelin treatment.

Hexarelin Inhibits Doxorubicin-Induced Apoptosis in Endothelial Cells

Endothelial cells lining along the coronary vessel may play a major role in regulating heart function and homeostasis. Previous data suggested that part of hexarelin binding in the heart was localized on the endothelium *(7)*. We then investigated whether pretreatment with hexarelin affected endothelial cell survival. The experiment was carried out on Hend cells, a cell line derived from heart endothelium of middle T transgenic mice. Treatment with

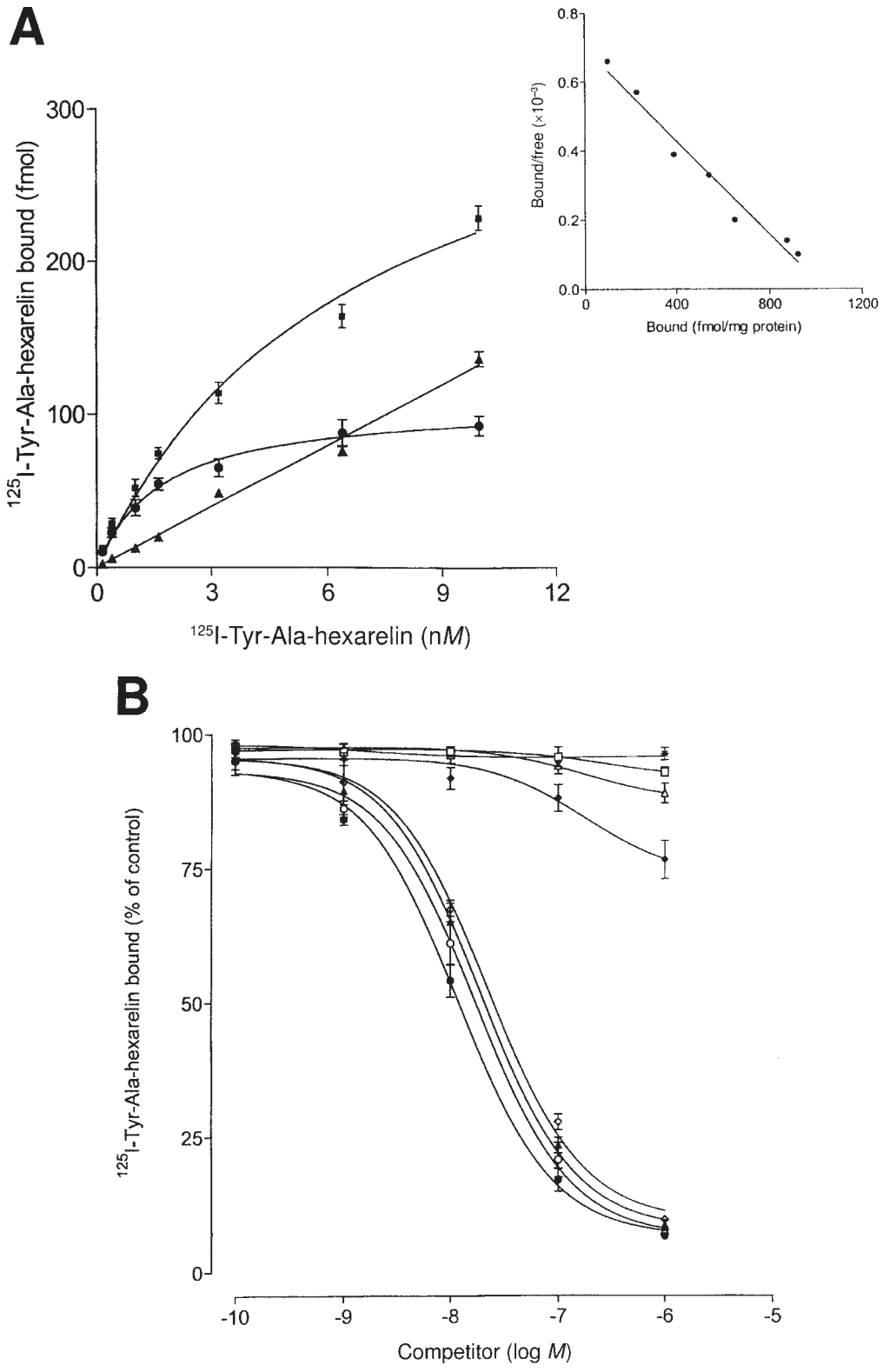

Fig. 1. ***Tyr-Ala-hexarelin binds in a specific and saturable manner to H9c2 cells.*** **(A). (Bottom)**. Saturation curve of ^{125}I-Tyr-Ala-hexarelin binding to membranes of H9c2 cardiomyocytes: ■ total binding; ▲, nonspecific binding; ●, specific binding. **(Top)** Scatchard plot (B_{max} :1035±112 fmol/mg protein; K_d: 1.5± 0.4 nmol/l). **(B)** Displacement curves of ^{125}I-Tyr-Ala-Hexarelin by Tyr-Ala-hexarelin (●), hexarelin, (○), GHRP-2 (▲), GHRP-6 (◊), MK 0677 (◆), GHRH (□), somatostatin (△) and TNF-α (*). Values are the mean ± SEM of three separate experiments.

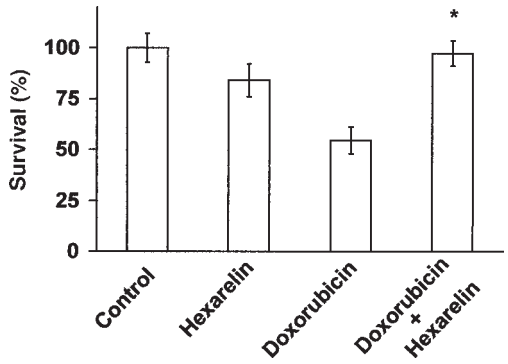

Fig. 2. ***Hexarelin protects H9c2 cardiomyocytes from doxorubicin-induced cell death.*** Cell survival, measured by MTT assay, of H9c2 cells following 18 h treatment of 1μM Doxorubicin in presence or absence of 1 μM Hexarelin (6 h pretreatment). Values are the mean ± SEM of three separate experiments. (* *t*-test, $p < 0.02$.)

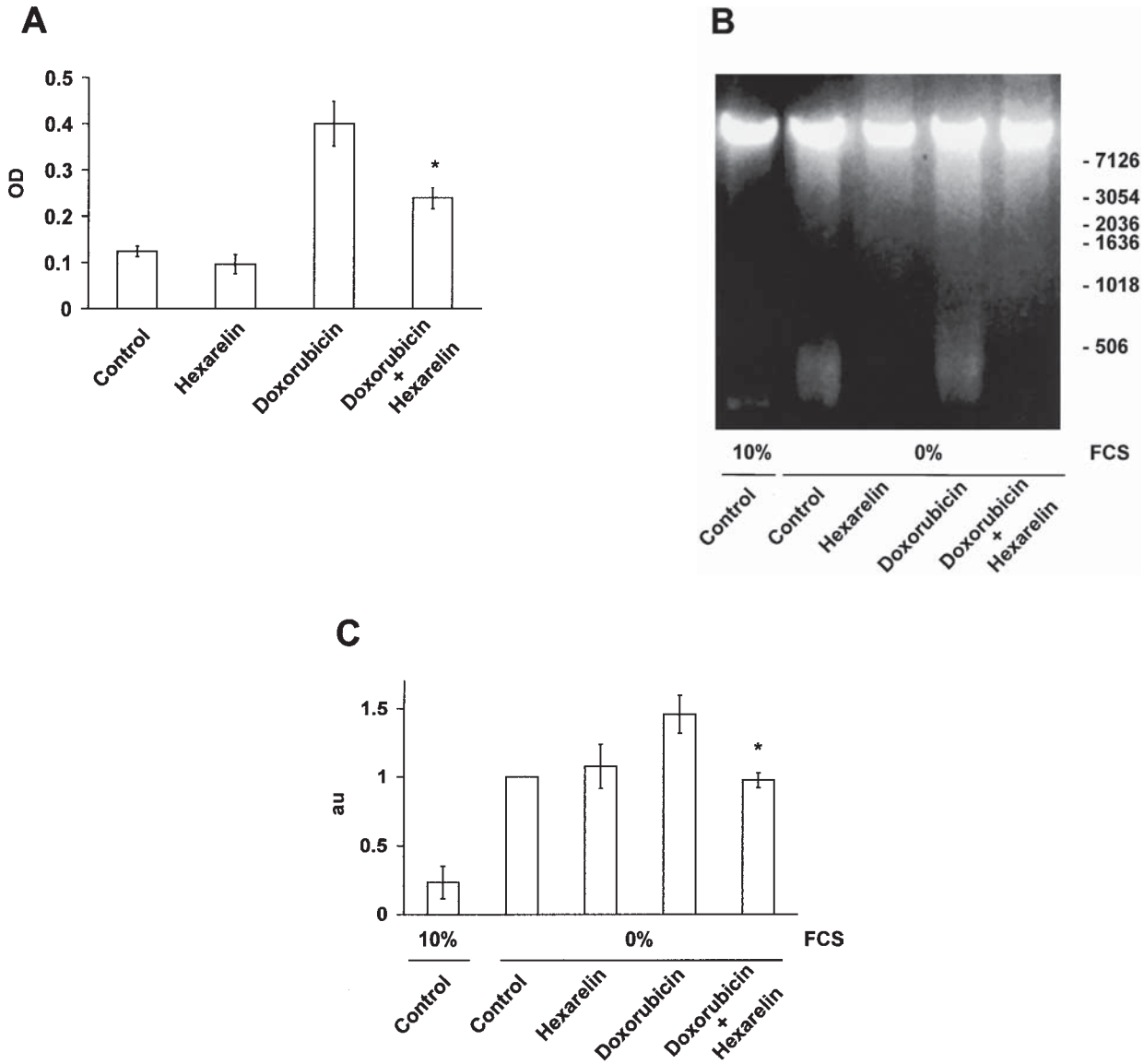

Fig. 3. ***Hexarelin inhibits doxorubicin induced DNA fragmentation in H9c2 cardiomyocytes.*** DNA fragmentation of H9c2 cardiomyocytes following 18 h treatment with 1mM doxorubicin, in either 5% FCS (**A**) or 0.1% FCS (**B**), in presence or absence of 1 mM hexarelin (6 h. pretreatment). DNA fragmentation was measured by eitherELISA assay (**A**) or DNA staining in agarose gel (**B,C**). (**A**) Mean value of a representative experiment performed in quadruplicate; **(B,C)** fragmented DNA from four separate agarose gels were quantified as described and expressed as the mean ± SEM (*t-test, $p < 0.02$).

doxorubicin induces cell death and apoptosis, which was detected as DNA nucleosomal fragmentation by an ELISA assay (**Fig. 4**). Overnight pretreatment with hexarelin significantly reduced the extent of DNA fragmentation elicited by doxorubicin in these cells. Thus, these data suggest that the endothelium also may be a cellular target, along the cardiomyocytes, for the cardioprotective activity reported in vivo.

Discussion

In this study we report that hexarelin, a peptidyl GHS, binds to specific high-affinity receptors on H9c2 cardiomyocytes, protects H9c2 cells from cell death triggered by doxorubicin and inhibits doxorubicin-induced apoptosis in heart endothelium-derived cells. To the best of our knowledge, this is the first report showing a specific cellular function for a GHS outside the endocrine system.

GHS-R, the only receptor for GHSs identified so far, is expressed exclusively in the pituitary and the hypothalamus. However, specific high-affinity binding sites for both peptidyl and nonpeptidyl GHSs in tissues other than the hypothalamus and the pituitary have been demonstrated *(6,7)*, namely, in other areas of the CNS and in peripheral endocrine and nonendocrine tissues *(7)*. The highest specific binding for peptidyl GHSs was detected in cardiac membranes from either rat or human tissues *(6,7)*. Interestingly, such binding was not displaced by MK-0677, a paradigmatic nonpeptidyl GHS, which binds GHS-R at high affinity *(3)*. Furthermore, Bodart et al. *(6)* have recently reported that Tyr-Ala-hexarelin, a peptidyl GHS, in the heart binds at high affinity to an 85-kDa glycosylated myocardial protein *(6)*; interestingly, such binding is not inhibited by MK-0677. Taken together these data led to the hypothesis that a second GHS-R, featuring a selective specificity for peptidyl GHSs and not for nonpeptidyl GHSs, may be expressed in the heart. However, neither the cellular distribution of GHS binding in the heart nor its biologic function were defined.

We report that hexarelin, a peptidyl GHS, binds to H9c2 myocytes and that such binding is not inhibited by MK-0677, a nonpeptidyl GHS. This is the first report demonstrating that cardiomyocytes, within the heart, have the ability to bind GHSs. Furthermore, these data suggest that the GHS binding sites detected on H9c2 membranes feature the same characteristic as the GHS binding sites detected in the whole myocardium, leading to the hypothesis that the cardiomyocytes may be the target of GHS myocardial activities. Such a finding implies also that H9c2 cells may be employed as an experimental system to investigate the biologic function of GHSs on the heart. Although H9c2 cells are not terminally differentiated cardiomyocytes and they do not possess organized sarcomeres, they conserve biologic features of myocytes *(14-16)*.

On the basis of the cardioprotective action of hexarelin *(8)*, we have investigated the hypothesis that hexarelin may

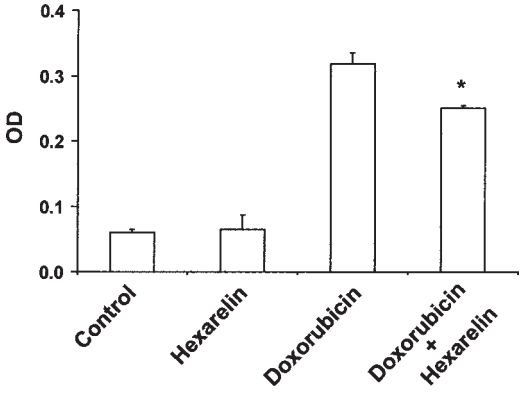

Fig. 4. ***Hexarelin protects Hend endothelial cells from doxorubicin-induced DNA fragmentation.*** DNA fragmentation of Hend heart-derived endothelial cells following 18 h of treatment with 1μ*M* doxorubicin in 5% FCS in the presence or absence of 1 μ*M* Hexarelin (6-h pretreatment). Values are the mean ± SEM of quadruplicates from a representative experiment of three (* *t*-test, $p < 0.02$).

prevent cell death of cardiomyocytes. Doxorubicin, an anthracyclin, is a well-known antitumoral molecule that induces apoptosis in cardiomyocytes in vitro *(17)* and possess marked cardiotoxic effects leading to dilated cardiomyopathy *(18,19)*. Indeed, doxorubicin induces cell death of H9c2 cells in a similar manner as it does with primary cultures of cardiomyocytes *(15,16)*. Hexarelin *per se* was not toxic to H9c2 myocytes, but it prevented doxorubicin-induced cell death and DNA fragmentation. Thus, these results suggest that peptidyl GHSs, such as hexarelin, by preventing cell death of cardiomyocytes, may be able to counteract the cardiotoxic effect of doxorubicin also in vivo. We can speculate that this finding may have potential therapeutic implications, taking into account that doxorubicin is widely used in clinical oncology but often elicits severe cardiovascular side effects by mechanisms still unclear *(18,19)*. Furthermore, we also report that hexarelin is able to reduce cell death of endothelial cells induced by doxorubicin, lending support to the hypothesis that hexarelin may elicit cardioprotection through multiple cellular mechanisms involving both the cardiomyocytes and the vasculature.

The mechanism by which hexarelin prevents doxorubicin-induced cell death still remains to be elucidated. The finding that hexarelin requires several hours to prevent cell death suggests that the cardioprotective action of hexarelin may require gene transcription and new protein synthesis. Doxorubicin has been shown to induce cell death of cardiomyocytes through a mechanism involving iron-mediated generation of reactive oxygen species *(20,21)*. We can hypothesize that hexarelin may activate a pathway stimulating antioxidant proteins, such as superoxide dismutase and cyclooxygenase-2, which have been shown to prevent free radical-mediated cell death in cardiomyocytes *(21,22)*. Alternatively, hexarelin may act as a survival factor, by either activation of its own antiapoptotic pathway or by an

autocrine loop through secretion of a second survival factor, e.g., insulin-like growth Factor-1 (IGF-1). Given the activity in the pituitary, we may speculate that GHSs in the periphery may stimulate directly IGF-1 secretion, the major mediator of GH function in the periphery. Indeed, IGF-1 has been reported to inhibit cell death induced by doxorubicin in both H9c2 myocytes and primary cultures of neonatal cardiomyocytes *(15,16)*.

Materials and Methods

Cell cultures

H9c2 cells were obtained from American Tissue Culture Collection and cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% FCS (Gibco). Cells were plated (~25,000–30,000 cells/cm^2) in 96-well slides for MTT and ELISA assays and in 10-cm dishes for other studies. Apoptosis was induced in 5% FCS cultured cardiomyocytes by incubation with 1 μ*M* doxorubicin (Pharmacia & Upjohn) for 18 h.

Chemicals, Peptides, and Reagents

GHRP-2, GHRP-6, hexarelin, Tyr-Ala-hexarelin, and MK-0677 were provided by Europeptides. Complete sequences or structures of all the GHS molecules used are reported in ref. *1*. Human GHRH (GHRH 1-44) and somatostatin (somatostatin 1-14) were purchased from Bachem. ^{125}I-Labeled Tyr-Ala-hexarelin (specific activity of 2000 Ci/mmol) was iodinated using a lactoperoxidase method and purified by reverse-phase high-performance liquid chromatography as previously described *(13)*.

GHRP Receptor Assay

GHRP binding sites were assayed on cell membranes (30,000*g* pellet) isolated from H9c2 cells as previously described (*13*) using ^{125}I-labeled Tyr-Ala-hexarelin as ligand. For each point binding assay, cell membranes (100 μg of protein) were incubated in triplicate at 0ºC for 60 min with approx 4×10^{-9} mol/L of ^{125}I-labeled Tyr-Ala-hexarelin in a final volume of 0.5 mL assay buffer (50 mmol/L Tris, 2 mmol/L EGTA, 0.1% bovine serum albumin, 0.03% bacitracin, titrated with HCl to pH 7.3). The binding reaction was terminated by adding ice-cold assay buffer followed by filtration over Whatman GF/B filters. Filters were rinsed three times with assay buffer and radioactivity bound to membranes was measured using a Packard auto–gamma counter. Specific binding was calculated as the difference between total binding and nonspecific binding measured in the presence of excess unlabeled Tyr-Ala-hexarelin (2.5×10^{-6} mol/L) and was expressed as a percentage of the total radioactivity added. To establish binding site specificity, increasing concentrations of various competitors were tested in displacement assays with ^{125}I-labeled Tyr-Ala-hexarelin. Receptor binding saturation studies were conducted by incubating tissue membranes with increasing concentrations (0.15 to 10×10^{-9} mol/L) of radioligand in the absence and presence of a fixed amount (2.5×10^{-6} mol/L) of unlabeled Tyr-Ala-hexarelin. Saturation isotherms were transformed using the method of Scatchard, and the dissociation constant (K_d) and number of binding sites (B_{max}) were calculated with the GraphPAD Prism 3 program (GraphPAD Software).

MTT Assay for Cell Viability

The assay is based on the reduction of the tetrazolium salt MTT by active mitochondria to produce insoluble formazan salt. Cells were treated in 96-well plates, MTT (Sigma) was added to each well under sterile conditions (final concentration of 0.5 mg/mL), and the plates were incubated for 4 h at 37ºC. Untransformed MTT was removed by aspiration, and formazan crystals were dissolved in dimethyl sulfoxide (100 μL/well). Formazan was quantified spectroscopically at 540 nm using a microplate reader (Bio-Tek).

ELISA System for DNA Fragmentation

DNA fragmentation was determined by an ELISA assay (Roche Molecular) performed according to the manufacturer's instructions. The test is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones present in the cytoplasmatic fraction of cell lysates. In brief, cytoplasmatic fraction of cell lysates are placed into a streptavidin-coated plate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. After removal of the unbound antibodies and nucleosomes, the amount of peroxidase retained is determinated photometrically (405 nm) with ABTS as substrate.

DNA Fragmentation

Cells were washed in phosphate-buffered saline and lysed in 2 mL of lysis buffer (10 m*M* Tris-HCl, 100 m*M* NaCl, 25 m*M* EDTA, 0.5% sodium dodecyl sulfate, 10 mg/mL of proteinase K, and 12.5 μg/mL of ribonuclease A). Cells were scraped, mixed gently, and incubated at 50°C for 1 h. DNA was purified using 1 mL of phenol:chloroform: isoamyl alcohol (25:24:1), washed with 1 mL of chloroform, and precipitated with 4.4 mL of ethanol and 0.2 mL of sodium acetate (3 *M*), and the pellet was washed in 70% ethanol. The desiccated pellet was solubilized in 200 μL of TE (10 m*M* Tris, pH 7.6; 1 m*M* EDTA). DNA was quantified and assessed for purity by absorbance measurement at 260 and 280 nm. Equal amounts of DNA were loaded in each lane, separated by 1.5% agarose gel electrophoresis, stained by ethidium bromide, and photographed. DNA fragmentation was quantified by measuring the intensity of the staining on gel photography between 100 and 7000 bp (Scion Image software), and values were normalized for control 0% FSC equal to 1.

Statistical Analyses

The data are presented as the mean ± SEM. The statistical significance was tested using Student's *t*-test.

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