Hexarelin protects rat cardiomyocytes from angiotensin II-induced apoptosis in vitro

Jin-Jiang Pang, Rong-Kun Xu, Xiang-Bin Xu, Ji-Min Cao, Chao Ni, Wen-Ling Zhu, Kamlesh Asotra, Meng-Chin Chen, and Chen Chen

1Department of Physiology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing 100005; 2Division of Cardiology, Department of Medicine, Peking Union Hospital, Beijing 100005, China; 3Division of Cardiology, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, California 90048-1865; 4Endocrine Cell Biology, Prince Henry’s Institute of Medical Research, Clayton, Victoria 3168, Australia

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Pang, Jin-Jiang, Rong-Kun Xu, Xiang-Bin Xu, Ji-Min Cao, Chao Ni, Wen-Ling Zhu, Kamlesh Asotra, Meng-Chin Chen, and Chen Chen. Hexarelin protects rat cardiomyocytes from angiotensin II-induced apoptosis in vitro. Am J Physiol Heart Circ Physiol 286: H1063–H1069, 2004. First published December 11, 2003; 10.1152/ajpheart.00648.2003.—Loss of cardiomyocytes by apoptosis is proposed to cause heart failure. Angiotensin II (ANG II), an important neurohormonal factor during heart failure, can induce cardiomyocyte apoptosis. Inasmuch as hexarelin has been reported to have protective effects in this process, we examined whether hexarelin can prevent cardiomyocytes from ANG II-induced cell death. Cultured cardiomyocytes from neonatal rats were stimulated with ANG II. Apoptosis was evaluated using fluorescence microscopy, TdT-mediated dUTP nick-end labeling (TUNEL) method, flow cytometry, DNA laddering, and analysis of cell viability by (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). It was found that incubation with 0.1 μmol/l ANG II for 48 h increased cardiomyocyte apoptosis. Administration of 0.1 μmol/l hexarelin significantly decreased this ANG II-induced apoptosis and DNA fragmentation and increased myocyte viability. To further investigate the underlying mechanisms, caspase-3 activity assay and mRNA expression of Bax, Bcl-2, and growth hormone secretagogue receptor (GHS-R; the supposed hexarelin binding site) were examined. GHS-R mRNA was abundantly expressed in cardiomyocytes and was upregulated after administration of hexarelin. These results suggest that hexarelin abates cardiomyocytes from ANG II-induced apoptosis possibly via inhibiting the increased caspase-3 activity and Bax expression induced by ANG II and by increasing the expression of Bcl-2, which is depressed by ANG II. Whether the upregulated expression of GHS-R induced by hexarelin is associated with this antiapoptotic effect deserves further investigation.

Address for reprint requests and other correspondence: C. Chen, Prince Henry’s Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia (E-mail: chen.chen@phimr.monash.edu.au).

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ever, H9c2 cell line is not a “real” cardiomyocyte. Although H9c2 cells conserve biological features of myocytes, they are not terminally differentiated cardiomyocytes and they do not possess organized sarcomeres (10, 35, 36). H9c2 cells have both cardiomyocyte and skeletal muscle properties and seem to become more dedifferentiated over cell passaging. With exponential division of H9c2 cells, myoblast fusion could not be found, thus indicating a differentiation toward a more skeletal muscle cell-like phenotype (28). It is necessary, therefore, to determine whether hexarelin exerts an antipototic action on cardiomyocytes. In the present study, we observed the effects of hexarelin on apoptosis induced by ANG II in cultured neonatal cardiomyocytes and further investigated the underlying signaling.

**MATERIALS AND METHODS**

**Cell culture and treatment.** Primary cultures of neonatal rat cardiomyocytes were prepared by the method originally described by Iwaki et al. (15) with minor modifications. Briefly, hearts were isolated from 1- to 3-day-old Sprague-Dawley rats. The myocardial cells were dispersed by digestion with collagenase (type II, 0.4 mg/ml, GIBCO-BRL) and pancreatin (0.6 mg/ml, Sigma) with agitation for 10 min at 37°C. This digestion step was repeated six times, and cells were collected by centrifugation. Isolated cells were replated into 150-mm petri dishes for 2 h in DMEM/F-12 (GIBCO-BRL) with 10% FCS (GIBCO-BRL) in a 37°C incubator. Cardiomyocytes were purified with differential attachment technique and further identified by sarcomeric α-actin immunostaining. Briefly, myocytes that did not attach to the dish during this time were collected and replated (~25,000–30,000 cells/cm²) in 96-well plates for (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazonium bromide (MTT) assay, 24-well plates for TdT-mediated dUTP nick-end labeling (TUNEL) assay, and 100-cm² dishes for examination using alternate culture medium (21) (DMEM/F-12 with 10% FCS and 0.1 mmol/l bromodeoxyuridine). Three days after cells were seeded, the culture medium was changed to serum-free DMEM/F-12 (DMEM/F-12 supplemented with 1% BSA, 5 µg/ml human transferrin, and antibiotics), and cells were cultured for 12 h before stimulation. Hexarelin (hexarelin group), ANG II (0.1 µmol/l, Sigma; ANG II group), both agents (hexarelin + ANG II group), or culture medium (control group) were added, respectively, to culture dishes for 48 h. The purity of cardiomyocytes, assessed by immunocytochemical analysis with an antisarcomeric α-actin antibody, was 94.5 ± 0.15% (n = 3).

**Morphological assessment of myocyte apoptosis.** After stimulation with hexarelin, ANG II, or vehicle, cultured cells on slides were washed three times with PBS and stained with acridine orange (AO; 200 µg/ml) for 3 min. Morphological features of apoptotic death (cell shrinkage, chromatin condensation, and fragmentation) were monitored by fluorescent microscopy. The percentage of myocytes with apoptotic death was measured by counting these cells at ×20 power in 10 randomly chosen fields (1 mm²) in each slide. Three slides were checked in each group.

**TUNEL assay.** The TUNEL assay was performed on cells plated on glass coverslips with an in situ cell death detection kit (Roche; Philadelphia, PA) according to the manufacturer’s instructions. Briefly, the cells were washed with PBS and fixed in 4% paraformaldehyde for 1 h. After being washed with PBS, the cells were incubated with blocking solution (3% H₂O₂ in methanol) for 10 min at 15–25°C and then permeable solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. The cells were then rinsed with PBS and incubated with the TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. As a positive control, fixed and permeated cells were treated with DNase I (1 mg/ml, Sigma) for 10 min to introduce nicks in the genomic DNA. After converter-peroxidase (POD) was added, the slides were incubated for 30 min at 37°C in a humidified chamber. 3′,3′-Diaminobenzidine (DAB) substrate was added for analysis by light microscopy. The percentage of myocytes with DNA nick end-labeling was measured by counting the cells exhibiting brown nuclei at ×20 power in 10 randomly chosen fields (1 mm²) in triplicate plates.

**Agarose gel electrophoresis for DNA fragmentation.** Cells (5 × 10⁶) were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 s with 50 µl lysis buffer (1% Nonidet P-40 in 20 mmol/l EDTA and 50 mmol/l Tris-HCl; pH 7.5). After 5 min, the centrifugation at 1,600 g was carried out and the supernatants were collected and extracted with 50 µl lysis buffer. SDS (1%) was added to the mixture, and the supernatant was treated for 2 h with 5 µg/ml RNase A (at 37°C), followed by digestion with 2.5 µg/ml protease K for 2 h at 37°C (14). After the addition of 1/2 vol of 10 mol ammonium acetate, DNA was precipitated with 2.5 vol ethanol and dissolved in gel loading buffer. The DNA fragments were then separated by electrophoresis with a 1.5% agarose gel.

**Flow cytometric study.** Flow cytometric studies were performed on a FACScan with Lysis II software (Becton-Dickinson). The cells were washed with d-Hanks, trypsinized, washed with PBS, and fixed in 70% ethanol for 30 min at 4°C. The cells were again rinsed with PBS and resuspended in 1 ml PBS containing 50 µg/ml RNase A and 50 µg/ml propidium iodide (Sigma). The samples were kept in the dark at 4°C for 30 min and then analyzed by flow cytometry with excitation at 488 nm and emission measured at 560–640 nm (FL2 mode). Cells undergoing apoptosis stain with propidium iodide and exhibit a reduced DNA content with a peak in the hypodiploid region (8, 12). The percentage of cells with apoptosis was taken as the fraction of cells with hypodiploid DNA content.

**MTT assay for cell viability.** The MTT assay is based on the transformation of the tetrazolium salt MTT by active mitochondria to an insoluble formazan salt. Cardiomyocytes were treated in 96-well plates, MTT was added to each well under sterile conditions (with a final concentration of 0.5 mg/ml), and the plates were incubated for 4 h at 37°C. Untransformed MTT was removed by aspiration, and formazan crystals were dissolved in dimethyl sulfoxide (150 µl/well). Formazan was quantified spectroscopically at 540 nm using a Bio-Rad automated ELA Analyzer.

**RT-PCR analysis.** RT-PCR was performed using Bcl-2 and Bax mRNA assays. Several members of the Bcl-2 family such as Bcl-2, Bcl-xL, Mcl-1, A1, and Bag-1 promote cell survival, whereas other members such as Bax, Bcl-xS, and Bak induce cell death (23, 25). The Bcl-2 family proteins form homodimers and/or heterodimers, and, depending on the balance between homodimers and heterodimers, cells can undergo apoptosis (9, 35). Unphosphorylated Bad is thought to induce cell death by forming heterodimers with Bcl-2 and by concomitantly generating Bax homodimers (9).

Total cellular mRNA was obtained using TRizol reagent (Invitrogen; Carlsbad, CA) according to the manufacturer’s protocol. One microgram of total RNA was reverse transcribed using random primers and Superscript II RT. The single-strand cDNA was amplified by PCR with Taq DNA polymerase. The sequences of sense and antisense primers, corresponding PCR conditions, and cycle counts were as follows: GHS receptor (GHS-R): 5’-GAGATGCAGTCAGT-CAGCCAGTAC-3’ and 5’-TAAATCCAAAACGTAGGTCTCGC-3’; 5 s at 98°C, 10 s at 65°C, and 1 min at 72°C, 35 cycles (23); Bax: 5’-GCAAGGAGATTGCTAGTG-3’ and 5’-CTCAGGCCATCCTTCC-3’; 5 s at 98°C, 10 s at 65°C, and 1 min at 72°C, 35 cycles (23); and Bcl-2: 5’-TCCATTAAAGTCGTACAG-3’ and 5’-GAGAGTTCCTCCACCAC-3’. 5 s at 94°C, 1 min at 55°C, and 1 min at 72°C, 35 cycles (27). GAPDH cDNA was amplified as a control.

**Caspase-3 (ICE-like protease) activity assay.** Recent work has supported a central role for caspase family members, especially caspase-3, as effectors of apoptosis (17). Two characteristic features of the caspase family have been described. Briefly, caspases cleave their target proteins after specific aspartic acid and caspase are
themselves activated by cleavage after specific aspartic acids, resulting in two subunits, which in vitro have been shown to combine together as an activated form (17). Some caspases activate other family members, suggesting that they act in a cascade, which may account for many biochemical and morphological changes occurring during apoptosis. Among the caspase family, caspase-3 (CPP32) has been considered as a central component of the proteolytic cascade during apoptosis and has thus been shown to play a key role in this family (17). In the present caspase-3 activity assay, the caspase-3 substrate rhodamine-110 (Z-DEVD-R110) was used as a fluorescent substrate. Activity of the ICE-like protease caspase-3 was determined using a commercial kit (Promega; Madison, WI) according to the manufacturer’s instructions. Briefly, after 48-h treatments with ANG II, hexarelin, ANG II + hexarelin, or vehicle, caspase-3 reagent was added and incubated for 10 h. Levels of release of rhodamine-110 were measured with a luminescence spectrometer LS55 (PerkinElmer) at an excitation wavelength of 499 nm and an emission wavelength of 521 nm (34).

Statistics. Data are reported as means ± SD. Comparisons were made between different treatment groups using ANOVA, followed by the Dunnett post hoc test for differences. Data for percent changes were analyzed using the Kruskal-Wallis H-test. A value of $P < 0.05$ was considered statistically significant. All experiments conformed to the Chinese Academy of Medical Sciences ethics code of practice.

RESULTS

Dose-response and time-response relationships of ANG II and hexarelin. In our laboratory, using MTT assay, TUNEL, flow cytometry; and DNA laddering, we observed the effects of ANG II and hexarelin at a series of concentrations ($10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol/l). Neither ANG II nor hexarelin at $10^{-10}$–$10^{-6}$ mol/l showed significant dose-response patterns in inducing apoptotic cell death (ANG II) or in depressing ANG II-induced apoptotic cell death (hexarelin). ANG II or hexarelin at $10^{-7}$ mol/l appeared to have the strongest action. Therefore, we present the data obtained at $10^{-7}$ mol/l for both ANG II and hexarelin in this study. The absence of dose-response patterns for ANG II in the present study is consistent with the results reported by Kajstura et al. (16). We also observed the effects of ANG II and hexarelin at

![Fluorescence photomicrographs of cultured neonatal rat cardiomyocytes treated with vehicle (A), ANG II (0.1 μmol/l; B), hexarelin (Hex; 0.1 μmol/l; C), and Hex + ANG II (D). Cells were stained with acridine orange (AO). E: percentages of apoptotic cardiomyocytes in the different groups. Original magnification: ×40. *P < 0.05 vs. control; **P < 0.05 vs. ANG II.](image1)

![Hex protected cardiomyocytes from ANG II-induced apoptosis. TdT-mediated dUTP nick-end labeling (TUNEL) analysis was performed as described in MATERIALS AND METHODS. A: vehicle-treated cardiomyocytes. B: cardiomyocytes incubated with 0.1 μmol/l ANG II for 48 h. C: cardiomyocytes incubated with 0.1 μmol/l Hex for 48 h. D: cardiomyocytes incubated with 0.1 μmol/l Hex and 0.1 μmol/l ANG II for 48 h. Magnification: ×40; bar = 20 μm. E: bar graph showing the percentage of cardiomyocytes undergoing apoptosis in the different groups. One hundred cells were counted. Number of TUNEL-positive cells was presented as a percentage from 3 independent experiments (means ± SD). *P < 0.05 vs. control; **P < 0.05 vs. ANG II.](image2)
different incubation times and found that ANG II and hexarelin exerted the most significant actions in inducing apoptotic cell death (ANG II) or in depressing ANG II-induced apoptotic cell death (hexarelin) at an incubation time of 48 h.

Morphological characterization of cardiomyocyte apoptosis. As shown in Fig. 1, visual inspection of ANG II-treated myocytes by fluorescence microscopy demonstrated that myocytes displayed morphological features characteristic of cell apoptosis. Compared with vehicle-treated cells, myocytes in the presence of ANG II became shrunken and retracted and had condensed cytoplasm. When the myocytes were stained with AO and assessed by fluorescence microscopy, cells with highly condensed chromatin or fragmented nuclei were more clearly visualized in ANG II-treated myocytes (15.22 ± 1.89%) compared with vehicle-treated cells (4.1 ± 1.04%), hexarelin-treated cells (3.42 ± 0.91%), and ANG II + hexarelin-treated cells (10.34 ± 1.82%) (Fig. 1E).

In situ TUNEL staining of apoptotic cardiomyocytes and flow cytometry study. Cardiomyocytes cultured in serum-free media for 48 h and stained by the TUNEL method showed that ~4% of cardiomyocytes stained positive for apoptosis. When cardiomyocytes were incubated with 0.1 μmol/l ANG II for 48 h, some nuclei of these TUNEL-positive cells appeared condensed and contracted, suggesting that ANG II induced apoptosis in cardiomyocytes. When cardiomyocytes were treated with both hexarelin (0.1 μmol/l) and ANG II (0.1 μmol/l), the number of TUNEL-positive cardiomyocytes was significantly decreased, by ~8% (Fig. 2).

Agarose gel electrophoresis for DNA fragmentation. To confirm that ANG II induces apoptosis in cardiomyocytes, we examined DNA ladder formation by agarose gel electrophoresis. Cardiomyocytes cultured in serum-free medium for 48 h showed faint DNA laddering. When cardiomyocytes were exposed to 1 or 0.1 μmol/l ANG II for 48 h, extracted genomic DNA showed a prominent DNA ladder characteristic of apoptosis. DNA ladder formation was significantly decreased after cardiomyocytes were incubated with 0.1 μmol/l hexarelin for 48 h. These results suggest that hexarelin prevents cardiomyocytes from ANG II-induced apoptosis (Fig. 3).

Flow cytometry. Flow cytometric analysis revealed 3.17 ± 0.32% of control cells had hypodiploid DNA content typical of apoptosis. Treatment with ANG II (0.1 μmol/l) for 48 h increased the percentage of apoptotic cells to 24.97 ± 4.64% (P < 0.05 vs. the control group, n = 6). A decrease in the percentage of cell apoptosis was observed in cells treated with both ANG II and hexarelin (14.25 ± 2.15%) and in cells treated with hexarelin alone (3.97 ± 0.21%) (Fig. 4).

MTT assay. Treatment with ANG II (0.1 μmol/l) for 48 h induced cell death in cardiomyocytes, as measured by the MTT cell viability assay. Hexarelin treatment alone did not affect normal cell survival (94.34 ± 8.87%). In contrast, administration of hexarelin to ANG II-treated cells (98.7 ± 5.86%, P < 0.05 vs. the ANG II group) was shown to prevent

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**Fig. 3.** ANG II-induced DNA fragmentation in cardiomyocytes with or without Hex. Cardiomyocyte lysate was first incubated with RNase and then with proteinase K. By this method, only fragmented DNA was extracted. DNA was separated by electrophoresis in 1.5% agarose gels and stained by ethidium bromide. Lane A, vehicle-treated cardiomyocytes; lane B, cardiomyocytes incubated with 1 μmol/l ANG II; lane C, cardiomyocytes incubated with 0.1 μmol/l ANG II for 48 h; lane D, cardiomyocytes incubated with 0.1 μmol/l Hex for 48 h without ANG II; lane E, cardiomyocytes incubated with 0.1 μmol/l Hex for 48 h with 0.1 μmol/l ANG II.

**Fig. 4.** Flow cytometric analysis of DNA contents in different groups. Neonatal cardiomyocytes were exposed to 0.1 μmol/l ANG II for 48 h, stained with propidium iodide (50 mg/ml), and sorted for DNA content with fluorescence-activated flow cytometry. A–D: DNA profiles of control cells (A), ANG II-treated cells (B), cells treated with 0.1 μmol/l Hex (C), and cells treated with 0.1 μmol/l Hex and ANG II (D), respectively. Note that an increased quantity of hypodiploid DNA in ANG II-treated cells was observed. PMT4 indicates the fluorescence intensity detected by the photomultiplier. E: summary data. Data depicted are mean ± SD of 3–6 experiments, each performed in duplicate. *P < 0.05 vs. control; #P < 0.05 vs ANG II.
ANG II-induced cell death (84.13 ± 6.26%, P < 0.05 vs. the control group) (Fig. 5).

RT-PCR assay. Expression of Bax and Bcl-2 mRNA were compared between vehicle- and drug-treated cells. The mRNA of GSH-R was abundantly expressed in neonatal cardiomyocytes of rats. Administration of hexarelin significantly increased the expression of GHS-R. The levels of mRNA encoding for GAPDH were not found to be significantly different between each group (n = 3 per group), whereas the expression of Bax was significantly upregulated and that of Bcl-2 was downregulated in ANG II-treated cardiomyocytes compared with other groups (Fig. 6).

Caspase-3 activity assay. The amount of fluorescent product generated is proportional to the amount of caspase-3 cleavage activity present in the sample. As shown in Fig. 7, caspase-3 activity in ANG II-treated cells (221.77 ± 14.78, P < 0.05 vs. the control group) was significantly increased compared with vehicle-treated cells (160.33 ± 25.83), hexarelin-treated cells (166.69 ± 23.02), and ANG II + hexarelin-treated cells (191.32 ± 16.66, P < 0.05 vs. the ANG II group).

DISCUSSION

The results of this study indicate for the first time that apoptosis in cardiomyocytes induced by ANG II can be considerably reduced (but not totally prevented) by hexarelin. This mechanism involves inhibiting apoptosis-related activation of caspase-3, inhibiting Bax mRNA expression, and increasing the expression of Bcl-2. We also found that hexarelin upregulated mRNA expression of GHS-R, an interesting self-gain signaling that may possibly magnify the effect of hexarelin. The causal relationship between upregulated GHS-R expression and hexarelin action, however, needs further investigations.

The development of heart failure is characterized by progressive worsening of LV function. The concept of apoptosis of cardiomyocytes is currently discussed as a mechanism that reduces the contractile cell mass of the heart and thereby leads to impaired LV performance. It can thus be argued that the inhibition of apoptosis in cardiomyocytes might conserve the number of contractile cardiomyocytes and delay or even prevent LV dysfunction and the development of heart failure. On the basis of the cardioprotective action of hexarelin, we investigated the hypothesis that hexarelin may prevent death of cardiomyocytes. The results of this study suggest that hexarelin may offer a practicable approach to the reduction of apoptosis of cardiomyocytes and may merit further investigation. Initially, the results of our study demonstrated that ANG II caused cardiomyocyte apoptosis. This finding is consistent with those of previous studies showing that ANG II acts as an efficient inducer of apoptosis in adult and neonatal cardiomy-
ocytes (6, 16). Because a single assay for apoptosis is difficult to interpret, care was taken in the present study to assess cardiomyocyte death via four independent assays. At first, morphological assessment of cardiomyocytes showed that shrinkage, particular to apoptosis, was encountered less frequently in cells treated with ANG II + hexarelin than in cells treated with ANG II alone. Second, a statistically significant reduction of TUNEL-positive cardiomyocytes was observed when hexarelin was added to ANG-II treated cells. In addition, using flow cytometry analysis, it was found that cells with hypodiploid DNA content were markedly increased in ANG II-treated cells but decreased in the cell groups treated with hexarelin. Furthermore, the MTT assay demonstrated that cell viabilities in ANG II + hexarelin-, hexarelin-, and vehicle-treated groups were much higher than that in cells treated with ANG II alone. The data obtained through these four methods indicate that neonatal myocyte apoptosis can be induced by ANG II and that hexarelin may abate cardiac cell apoptotic death.

Bax and Bcl-2 play important roles in apoptotic cell death, whereas caspase-3 is a key downstream effector of apoptosis (17, 23, 25). To investigate the underlying mechanism(s) of the antiapoptotic effect of hexarelin, we examined mRNA expression of Bax, Bcl-2, and caspase-3 activity. The results showed that hexarelin inhibited the expression of Bax and increased the expression of Bcl-2 in cardiomyocytes. These observations suggest that hexarelin may modify the imbalance of Bax and Bcl-2 in apoptotic cardiac cells. We also found that caspase-3 activity of myocardial cells is significantly increased when cells were treated with ANG II and that hexarelin (0.1 μmol/l) greatly reduced this activation. The mRNA expression of Bax, Bcl-2, and caspase-3 activity is consistent with the results obtained by morphological analysis, flow cytometry, and DNA fragmentation.

GHS-R, the only receptor for GHS identified so far, is expressed exclusively in the pituitary and hypothalamus. However, specific high-affinity binding sites for both peptidyl and nonpeptidyl GHS in tissues other than the hypothalamus and pituitary have been demonstrated, namely, in other areas of the CNS and in peripheral endocrine and nonendocrine tissues (3, 22). The highest specific binding for peptidyl GHS was detected in cardiac cell membrane from both rat and human tissues. In the present study, mRNA of GHS-R in neonatal cardiomyocytes was abundantly expressed. Furthermore, administration of hexarelin increased the expression of GHS-R mRNA. Thus we postulate that the antiapoptotic effect of hexarelin might be associated with GHS-R, as the increased expression of GHS-R mRNA may increase GHS-R protein expression and therefore intensify the effect of hexarelin. However, this mechanism, particularly the causal relationship between the increased expression of GHS-R and the antiapoptotic effect of hexarelin, warrants further investigation.

Clinical implications. The present study demonstrates strongly that hexarelin protects neonatal cardiomyocytes from ANG II-induced apoptosis. In another study, we have also observed that chronic treatment with hexarelin protected cardiomyocytes from apoptotic death, improved cardiac dysfunction, LV structural remodeling, and the development of cardiac cachexia in a rat model of pressure overload-induced heart failure (unpublished data). Taken together, these studies suggest that the antiapoptotic effect of hexarelin may be an important underlying mechanism accounting for its cardioprotectant action. Hexarelin might potentially be developed to treat heart failure or other apoptosis-related heart diseases if further studies were performed to define and clarify rationale for its clinical use.

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