Chronic administration of hexarelin attenuates cardiac fibrosis in the spontaneously hypertensive rat

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1Department of Physiology and Pathophysiology, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College; Beijing, China; and 2School of Biomedical Sciences, University of Queensland, Brisbane, Australia.

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Xu X, Ding F, Pang J, Gao X, Xu R, Hao W, Cao J, Chen C. Chronic administration of hexarelin attenuates cardiac fibrosis in the spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 303: H703–H711, 2012. —Cardiac fibrosis is a hallmark of heart disease and plays a vital role in cardiac remodeling during heart diseases, including hypertensive heart disease. Hexarelin is one of a series of synthetic growth hormone secretagogues (GHSs) possessing a variety of cardiovascular effects via action on GHS receptors (GHS-Rs). However, the role of hexarelin in cardiac fibrosis in vivo has not yet been investigated. In the present study, spontaneously hypertensive rats (SHRs) were treated with hexarelin alone or in combination with a GHS-R antagonist for 5 wk from an age of 16 wk. Hexarelin treatment significantly reduced cardiac fibrosis in SHRs by decreasing interstitial and perivascular myocardial collagen deposition and myocardial hydroxyproline content and reducing mRNA and protein expression of collagen I and III in SHR hearts. Hexarelin treatment also increased matrix metalloproteinase (MMP)-2 and MMP-9 activities and decreased myocardial collagen deposition and myocardial hydroxyproline content and reducing mRNA and protein expression of collagen I and III in SHR hearts. Hexarelin treatment also increased matrix metalloproteinase (MMP)-2 and MMP-9 activities and decreased myocardial matrix metalloproteinase (TIMP)-1 in SHR hearts. In addition, hexarelin treatment significantly attenuated left ventricular (LV) hypertrophy, LV diastolic dysfunction, and high blood pressure in SHRs. The effect of hexarelin on cardiac fibrosis, blood pressure, and cardiac function was mediated by its receptor, GHS-R, since a selective GHS-R antagonist abolished these effects and expression of GHS-Rs was upregulated by hexarelin treatment. In summary, our data demonstrate that hexarelin reduces cardiac fibrosis in SHRs, perhaps by decreasing collagen synthesis and accelerating collagen degradation via regulation of matrix metalloproteinase (TIMP) and blood pressure. These findings provide novel insights into the cardiac effect of hexarelin, which explores the potential of hexarelin and other GHSs to cardiac fibrosis in spontaneously hypertensive rats (SHRs) using multiple approaches at different levels. Here, we demonstrate GHS-R-dependent attenuation of cardiac fibrosis by long-term in vivo treatment with hexarelin via regulation of matrix metalloproteinase (MMPs)/tissue inhibitor of metalloproteinase (TIMP) and blood pressure. These findings provide novel insights into the cardiac effect of hexarelin, which explores the potential of hexarelin and other GHSs as therapeutic agents for the treatment of cardiac fibrosis.

MATERIALS AND METHODS

Experimental design. Sixteen-week-old male SHRs (n = 39) and age-matched Wistar rats (n = 22) were randomly assigned to treatment with either hexarelin (100 μg·kg body wt−1·day−1, subcutaneous injection, twice a day) or saline for 5 wk. Animals were divided into five groups: saline-treated Wistar rats, hexarelin-treated Wistar rats, saline-treated SHRs, hexarelin-treated SHRs, and hexarelin + (D-Lys3)-GHRP-6-treated SHRs (n = 13 rats/group). Systolic blood pressure was measured before and each week after the administration of hexarelin in all rats by a standard tail-cuff method. Five weeks after the first injection of hexarelin, cardiac diastolic function was measured, and arterial blood samples were extracted under anesthesia with pentobarbital sodium (40 mg/kg ip). Animals were then killed by decapitation, and cardiac tissues were harvested for further experiments. All animals received regular access to rat chow and humane care in compliance with the standards set by the Ethics Committee of Peking Union Medical College, by which the protocols in this study were approved.

Measurements of cardiac diastolic function. Anesthetized rats were subjected to LV cannulation via the left femoral artery with a silicone catheter. The catheter was connected to a pressure transducer, and the transducer was connected to the BL420S data-acquisition system available; thus, there is a urgent need for the development of antifibrotic agents.

Hexarelin is one of a series of synthetic growth hormone (GH) secretagogues (GHSs) possessing a variety of cardiovascular effects via action on GHS receptors (GHS-Rs). Recent evidence has indicated that GHSs improve LV dysfunction, diminish LV pathological remodeling (10, 21, 39), and alleviate infarction-enhanced and workload-induced heart failure (15, 26). Our previous study (38) has shown that hexarelin significantly suppressed ANG II- and transforming growth factor (TGF)-β-induced cardiac fibroblast proliferation and collagen synthesis in cultured rat cardiac fibroblasts, despite that fact that ANG II and TGF-β are two potent and key profibrotic factors. It has also been reported that ghrelin, an endogenous analog of hexarelin, exerts antifibrotic effects in the liver (20). However, the role of hexarelin in cardiac fibrosis has not yet been reported. The present study was therefore designed to examine the effects of hexarelin on cardiac fibrosis in spontaneously hypertensive rats (SHRs) using multiple approaches at different levels. Here, we demonstrate GHS-R-dependent attenuation of cardiac fibrosis by long-term in vivo treatment with hexarelin via regulation of matrix metalloproteinase (MMPs)/tissue inhibitor of metalloproteinase (TIMP) and blood pressure. These findings provide novel insights into the cardiac effect of hexarelin, which explores the potential of hexarelin and other GHSs as therapeutic agents for the treatment of cardiac fibrosis.

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**Table 1. Primer sequences**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Collagen I</td>
<td>Sense 5'-TCT TGG TCG TTG TCC GCA GGG TAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-TTC TGG TAG CAG GCT TCT TCC-3'</td>
</tr>
<tr>
<td>Collagen III</td>
<td>Sense 5'-TGC CCA CAG CTT TCT ACA CCT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CAC CCA TTC CTC CCA CAG-3'</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase-1</td>
<td>Sense 5'-GGA GAG CCT CTG TCG AT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GT TTT CAG GTC GGT TTT GC-3'</td>
</tr>
<tr>
<td>CD36</td>
<td>Sense 5'-GGA TAA CAT AAG CAA GGT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CAT AAA AGC AAC AAA CAT-3'</td>
</tr>
<tr>
<td>GHS-R</td>
<td>Sense 5'-GAG ATC GCT CAT ATC AGC CAG TAC-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GGA TAC CCT GCA GAG CCT GGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5'-TCA AAG TCG TGG TCA ACG GAT TTG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-AGC AGC TAC TCA GCA GCA CCA TCA C-3'</td>
</tr>
</tbody>
</table>

GHS-R, growth hormone (GH) secretagogue receptor.

Serum nitric oxide (NO) concentration was measured with a NO assay kit (Nanjing Jiancheng Biotechnique Institute, Nanjing, China) (23). This assay involves the conversion of nitrate to nitrite by the enzyme nitrate reductase. The concentration of NO is indirectly measured by determining both nitrate and nitrite levels in the sample, as detailed in the manufacturer’s instruction manual.

**Measurement of myocardial hydroxyproline content.** Myocardial hydroxyproline content was measured with a hydroxyproline detection kit (Nanjing Jiancheng Biotechnique Institute) and normalized to total protein. Because hydroxyproline is incorporated only into collagen, and it is assumed that collagen contains 14% of total hydroxyproline, myocardial hydroxyproline content can be considered as a proportional estimation of myocardial collagen content.

**RNA isolation and quantitative real-time PCR.** Total RNA was isolated from the respective LV tissues of each group by TRIzol reagent (Invitrogen). The concentration of total RNA was determined by UV spectrophotometry at 260/280 nm. Total RNA (2 μg) from each sample was reverse transcribed into cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). Relative quantitative real-time PCR was performed on a sequence detection system (Prism 7000 with 29 SYBR Green PCR Master Mix, Applied Biosystems) according to the manufacturer’s protocol. PCR was performed using primers specific for rat target genes (Table 1), with GAPDH as an inner control. Briefly, in each assay both GAPDH and a target gene from the same samples were amplified in duplicate in separate tubes. mRNA levels of each gene were calculated using the relative standard curve method, normalized against the corresponding GAPDH mRNA level, and then expressed as the relative change over control. A single dissociation peak was detected in each reaction by the dissociation curve. The results are the average of three independent experiments at each time point.

**Western blot analysis.** Total protein extracts were prepared by homogenizing myocardial biopsies in lysis buffer. Protein (40 μg/sample) electrophoresis was performed using a SDS-polyacrylamide gel with transfer onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk, incubated in a 1:1,000 dilution of primary antibody overnight, and incubated with a 1:2,000 dilution of the corresponding secondary antibody for 1 h, dehydrated, and then embedded in paraffin for histological experiments.

**Histological experiments.** For histomorphological analysis, coronal sections (5 μm thick) of the LV were taken from the paraformaldehyde-fixed paraffin embedded specimens. The equator was selected as being representative of the whole ventricle. Each section was stained with Sirius red F3BA (0.5% in saturated aqueous picric acid, 30–60 min) or Van Gieson’s solution (15 min or longer). The collagen volume fraction was determined as a percentage by quantitative morphometry with an automated image-analysis system (Visilog 4.1.5, Noesis) as previously described (33). For the qualitative assessment of fibrosis, Sirius red F3BA-stained sections were viewed through a polarized-light microscope.

**Serum GH and nitric oxide assays.** Serum GH levels were measured with a human GH radioimmunoassay kit (Jiuding Medical and Bioengineering, Tianjin, China) as previously described by us (39). Our previous study (39) showed that the primary antibody (rabbit anti-human GH serum) in the kit interacted with rat GH. The secondary antibody was donkey anti-rabbit IgG. The radiation intensity of the antibody complex was measured with a human GH radioimmunoassay kit (Jiuding Medical and Bioengineering, Tianjin, China). Intra-assay and interassay variability were determined as 10.8% and 12.6%, respectively.

**Measurement of end-diastolic pressure (LVEDP) and LV weight/tibial length.** End-diastolic pressure (LVEDP) was measured in conscious rats with a ramped balloon catheter (Radionics, Burlington, MA) introduced into the left ventricle through the carotid artery, as previously described (5). LV weight/tibial length was calculated by dividing the LV weight by the length of the tibial bone.

**MMP activity assay by gelatin zymography.** Zymography was performed to determine the activity of MMPs in the rat heart as previously described (24). Briefly, heart tissues were homogenized in 400 μl lysis buffer [50 mM Tris-HCl (pH 7.4) with protease inhibitors] on ice. Proteins (100 μg) were then mixed with an equal volume sample buffer [80 mM Tris-HCl (pH 6.8) 4% SDS, 10% glycerol, and 0.01% bromophenol blue] and then loaded onto a 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin, which was overlaid with 5% stacking gel. After electrophoresis, gels were rinsed in distilled water and washed three times for 15 min each in 150 ml of 2.5% Triton X-100 solution. Gels were then incubated in 100–150 ml of 50 mM Tris-HCl (pH 7.5), 10 mM CaCl2, 1 μM ZnCl2, 1% Triton X-100, and 0.02% NaN3. After incubation, gels were stained with 100 ml Coomasie blue R-250 for 3 h and then destained 1 h with destaining solution (50% methanol and 10% acetic acid). Gels were washed in distilled water for 20 min and then scanned.

**Table 2. Characterization of animal and serum GH levels**

<table>
<thead>
<tr>
<th></th>
<th>Saline-Treated Wistar Rats</th>
<th>Hexarelin-Treated Wistar Rats</th>
<th>Saline-Treated SHRs</th>
<th>Hexarelin-Treated SHRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent increase in body weight</td>
<td>19.2 ± 3.0</td>
<td>25.4 ± 3.4*</td>
<td>9.3 ± 2.9*</td>
<td>14.8 ± 6.2†</td>
</tr>
<tr>
<td>LV weight/body weight, g/kg</td>
<td>2.06 ± 0.11</td>
<td>1.99 ± 0.08</td>
<td>3.66 ± 0.56*</td>
<td>3.19 ± 0.22†</td>
</tr>
<tr>
<td>LV weight/tibial length, mg/mm</td>
<td>13.1 ± 1.1</td>
<td>13.5 ± 0.2</td>
<td>18.7 ± 2.0*</td>
<td>17.0 ± 2.0†</td>
</tr>
<tr>
<td>Serum GH level</td>
<td>2.40 ± 0.51</td>
<td>2.87 ± 0.39*</td>
<td>2.41 ± 0.41*</td>
<td>2.87 ± 0.17†</td>
</tr>
</tbody>
</table>

Data are means ± SD. SHRs, spontaneously hypertensive rats; LV, left ventricular. *P < 0.05 vs. saline-treated Wistar rats; †P < 0.05 vs. saline-treated SHRs.
The heart weight-to-body weight ratio is a useful index for the evaluation of ventricular hypertrophy (18), with a higher ratio representing greater cardiac hypertrophy (2). In the present study, both LV weight-to-body weight and LV weight-to-tibial length ratios in SHRs were significantly higher than those in Wistar rats, suggesting an increase of LV mass and/or a decrease of body growth. Chronic administration of hexarelin significantly decreased the LV weight-to-body weight ratio and LV weight-to-tibial length ratio in SHRs (Table 2). In addition, hexarelin increased serum GH levels in both SHRs and Wistar rats (Table 2).

LV diastolic function (LV \( \frac{dP}{dt} \max \)) was impaired and LVEDP was increased in SHRs compared with Wistar rats (Table 3). However, LV diastolic dysfunction was significantly improved and LVEDP was markedly reduced in SHRs after treatment with hexarelin for 5 wk, and pretreatment with the GHS-R antagonist abolished the effect of hexarelin on improving cardiac diastolic dysfunction (Table 3).

Systolic blood pressure in SHRs was significantly higher than that in Wistar rats (Fig. 1). After the administration of hexarelin, blood pressure decreased in both Wistar rats and SHRs (Fig. 1B). Daily injection of (D-Lys3)-GHRP-6, an antagonist of growth hormon secretogogue receptors (GHS-R), with injection of hexarelin partially abolished the antihypertensive effect of hexarelin in SHRs (Fig. 1B). Serum NO levels were lower in SHRs than in Wistar rats and increased after hexarelin treatment in SHRs but not in Wistar rats (Fig. 1C). Data represent means ± SD. *\( P < 0.05 \) vs. saline-treated Wistar rats; †\( P < 0.05 \) vs. saline-treated SHR group; ‡\( P < 0.05 \) vs. the hexarelin-treated SHR group.

RESULTS

**Hexarelin attenuated LV hypertrophy and systolic blood pressure and improved LV diastolic dysfunction in SHRs.** Natural body growth (as reflected by body weight gain and tibial length) was significantly affected in SHRs compared with Wistar rats (Table 2), whereas chronic administration of hexarelin improved the body weight gain in both SHRs and Wistar rats (Table 2). The heart weight-to-body weight ratio is a useful index for the evaluation of ventricular hypertrophy (18), with a higher ratio representing greater cardiac hypertrophy (2). In the present study, both LV weight-to-body weight and LV weight-to-tibial length ratios in SHRs were significantly higher than those in Wistar rats, suggesting an increase of LV mass and/or a decrease of body growth. Chronic administration of hexarelin significantly decreased the LV weight-to-body weight ratio and LV weight-to-tibial length ratio in SHRs (Table 2). In addition, hexarelin increased serum GH levels in both SHRs and Wistar rats (Table 2).

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**Table 3. Effects of hexarelin and GHS-R antagonist on cardiac diastolic function in rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>LV End-Diastolic Pressure, mmHg</th>
<th>LV ( \frac{dP}{dt} \max ), mmHg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-treated Wistar rats</td>
<td>4 ± 2</td>
<td>5,462 ± 154</td>
</tr>
<tr>
<td>Saline-treated SHRs</td>
<td>19 ± 4*</td>
<td>2,835 ± 136*</td>
</tr>
<tr>
<td>Hexarelin-treated SHRs</td>
<td>9 ± 4†</td>
<td>4,248 ± 162†</td>
</tr>
<tr>
<td>Hexarelin + (D-Lys3)-GHRP-6-treated SHRs</td>
<td>14 ± 5‡</td>
<td>3,026 ± 164‡</td>
</tr>
</tbody>
</table>

Data are means ± SD; \( n = 4 \) rats/group. Data were obtained after 5 wk of drug administration. *\( P < 0.01 \) vs. the saline-treated Wistar rat group; †\( P < 0.05 \) vs. the saline-treated SHR group; ‡\( P < 0.05 \) vs. the hexarelin-treated SHR group.

Statistical analysis. Data are shown as means ± SD. Whether parameters differed across groups was determined using one-way ANOVA followed by post hoc Newman-Keuls tests. To determine if there were any changes within groups during treatment, two-way ANOVA for repeated measures was performed followed by a post hoc Newman-Keuls test. \( P \) values of <0.05 were accepted as significant.
SHRs (Fig. 1A), but inhibition of GHS-R by its antagonist abolished this hexarelin-reduced blood pressure (Fig. 1B).

NO plays an important role in controlling blood pressure by regulating vasodilation, and it is also important in maintaining endothelial homeostasis. We found that serum NO levels in SHRs were lower than those in Wistar rats before hexarelin treatment. After 5-wk administration of hexarelin, serum NO levels increased significantly in SHRs but not in Wistar rats (Fig. 1C).

**Hexarelin attenuated ventricular fibrosis in SHRs.** To test the effect of hexarelin on ventricular fibrosis in SHRs, Wistar rats and SHRs were treated with hexarelin or saline for 5 wk,
and picrosirius red and Van Gieson staining was performed on ventricular sections of the heart. As shown in Fig. 2, there was only slight staining of interstitial (Fig. 2A) and perivascular (Fig. 2B) collagen by Sirius red F3BA in Wistar rats. In contrast, large areas of interstitial and perivascular staining were observed in cardiac sections from SHRs. After 5 wk of treatment, hexarelin significantly reduced interstitial and perivascular fibrosis in SHRs. In addition, administration of hexarelin together with (D-Lys3)-GHRP-6, a specific GHS-R antagonist, showed no inhibitory effect of hexarelin on myocardial fibrosis in SHRs, indicating that hexarelin acted on its specific receptor to inhibit cardiac collagen deposition in SHRs.

Van Gieson staining (Fig. 3), a widely used method to evaluate total collagen deposition, supported the results of Sirius red F3BA staining (Fig. 2). Hexarelin had no effect on collagen deposition in Wistar rats, as evidenced by all stainings.

We next investigated the effect of hexarelin on myocardial hydroxyproline content as a quantitative measure of collagen deposition. We found that myocardial hydroxyproline content in SHRs was significantly higher than that in Wistar rats, and it remarkably decreased after 5 wk of hexarelin treatment compared with the saline-treated SHR control group. No significant difference of myocardial hydroxyproline content was found between hexarelin-treated and saline-treated Wistar rat groups (Fig. 4).

We then studied the effect of hexarelin on the expression of collagen I and III at both mRNA and protein levels. Quantitative real-time PCR results revealed that mRNA expression levels of collagen I and III in SHRs were significantly higher than those in Wistar rats, and they significantly decreased after hexarelin treatment in SHR hearts (Fig. 5A). Consistent with the mRNA data, protein levels of collagen I and III were increased in SHRs and reduced by hexarelin treatment (Fig. 5B). As expected, administration of (D-Lys3)-GHRP-6, a selective GHS-R blocker, abolished the inhibitory effects of hexarelin on the expression of collagen I and III at both mRNA and protein levels, indicating a GHS-R-dependent effect of hexarelin. No significant differences in both mRNA and protein levels of collagen I and III were found between hexarelin-treated and untreated Wistar rats (Fig. 4).

Fig. 4. Effect of hexarelin on myocardial hydroxyproline content. Data represent means ± SD; n = 6. *P < 0.05 vs. saline-treated Wistar rats; #P < 0.05 vs. saline-treated SHRs.

Fig. 5. Effect of hexarelin on cardiac mRNA and protein expression of collagen I and III. A: relative mRNA expression of collagen I and III by quantitative PCR. B: protein expression of collagen I and III by Western blot analysis. Data represent means ± SD; n = 3. *P < 0.05; **P < 0.01.
Collectively, these data indicate that hexarelin attenuates cardiac fibrosis by inhibiting collagen deposition via the activation of GHS-Rs.

**Hexarelin increased myocardial MMP activity and decreased the expression of TIMP-1 in SHRs.** MMPs, particularly MMP-9 and MMP-2, are involved in cardiac fibrosis. Activities of MMP-2 and MMP-9 in the heart did not show significant differences between SHRs and Wistar rats (Fig. 6). However, hexarelin treatment remarkably increased MMP-2 and MMP-9 activities in SHRs, especially MMP-9 activity, in a GHS-R-dependent manner, since blockade of GHS-R abolished the hexarelin-induced increase in MMP-2 and MMP-9 activities (Fig. 6A). MMP-2 and MMP-9 activities were also slightly increased in hexarelin-treated Wistar rats compared with untreated Wistar rats (Fig. 6A). mRNA expression of TIMP-1, the inhibitor of MMPs, was dramatically increased in SHRs compared with Wistar rats and was reduced by hexarelin in SHRs in a GHS-R-dependent manner (Fig. 6B). These data suggest that MMPs and TIMP-1 are involved in hexarelin-reduced cardiac fibrosis in SHRs.

**Hexarelin increased myocardial mRNA expression of GHS-R and CD36.** No significant differences in GHS-R expression levels in the heart were found between SHRs and Wistar rats. Hexarelin treatment, however, significantly increased GHS-R expression in myocardial tissues from both SHRs and Wistar rats (Fig. 7, A and B). CD36 expression levels were significantly lower in SHRs compared with Wistar rats, and hexarelin treatment increased CD36 mRNA expression in both SHRs and Wistar rats (Fig. 7, C and D).

**DISCUSSION**

In the present study, we identified, for the first time, hexarelin as a potential antifibrotic agent in the treatment of cardiac fibrosis in vivo. We showed that long-term chronic treatment with hexarelin significantly reduced cardiac fibrosis in SHRs and that the antifibrotic effect of hexarelin was mediated by its receptor, GHS-R, since a selective GHS-R antagonist abolished the antifibrotic effect of hexarelin in SHRs and the expression of GHS-Rs was upregulated by hexarelin. We also found that hexarelin increased MMP-2 and MMP-9 activities and decreased the myocardial mRNA expression of TIMP-1 in SHRs. In addition, long-term chronic treatment with hexarelin significantly attenuated LV hypertrophy, LV diastolic dysfunction, and high blood pressure in SHRs. These results indicate that hexarelin may act on GHS-Rs to regulate MMPs/TIMP and blood pressure and then to reduce cardiac fibrosis and improve cardiac dysfunction, which may lead to new therapeutic reagents for the treatment of cardiac fibrosis.

Of particular interest in this study is the identification of the novel action of hexarelin in reducing cardiac fibrosis in SHRs in vivo. Collagen, a fibrous protein constituting approximately one-third of the total protein in the body, is synthesized by fibroblasts and is a major component of the extracellular matrix (ECM) in multiple tissues, including those in the heart, and collagen deposition in the heart is significantly higher in SHRs than in normal control rats (19, 43). Collagen I and III represent 80% and ~10% of collagen protein in the heart, respectively, and high levels of collagen I and III expression are found in SHRs (41, 43). Hydroxyproline is incorporated only into collagen, and collagen contains 14% of total hydroxyproline, so myocardial hydroxyproline content has been widely used as a quantitative estimation of myocardial collagen content (3, 8, 9, 43). The myocardial hydroxyproline concentration is also higher in SHRs than that in control rats (8, 43). Similarly, we found that collagen deposition, collagen I and III expression, and myocardial hydroxyproline were all significantly increased in the SHR group compared with the control group, which were reversed by treatment with hexarelin for 5 wk, suggesting that hexarelin could exert an antifibrotic effect in the hypertensive heart.

Of another interest in this study is the finding that the hexarelin-reduced cardiac fibrosis in SHRs may be via the regulation of MMPs/TIMP. In response to hormonal activation or hemodynamic overload, the ECM undergoes structural remodeling involving the production of new collagens, alterations in collagen structure, and matrix degradation, leading to progressive ventricular dilation and eventual congestive heart failure (11, 30). ECM remodeling is regulated by MMPs, which promote ECM degradation, such as collagen and elastin degradations (22). Approximately 20 different MMPs have been identified, and the predominant forms of MMPs involved in ECM alterations are MMP-9 and MMP-2, which degrade collagens. Reduced MMP-2 activity contributes to cardiac fibrosis in experimental diabetic cardiomyopathy (31). A study by Li et al. (12) showed that MMP-2 and MMP-9 activities by zymography in the heart of SHRs slightly increased at the age of 9 mo and dramatically increased at the age of 13 mo. In our study, we used SHRs at the age of 5 mo, and we did not find any significant differences.
in MMP-2 and MMP-9 activities in the heart between SHRs and Wister rats. Treatment with hexarelin significantly increased MMP-2 and MMP-9 activities. The activity of MMPs is tightly regulated by TIMPs (27). Four different types of TIMPs have been identified: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (6). MMP-9 activity has been shown to be preferentially inhibited by TIMP-1. mRNA expression of TIMP-1 is increased in the heart of SHRs at the ages of 9 and 13 mo (12). We also showed that TIMP-1 expression was higher in SHRs at the age of 5 mo; interestingly, hexarelin dramatically downregulated TIMP-1 expression, suggesting that hexarelin interrupted the inhibitory effect of TIMP-1 on MMP activity. This effect may lead to an increase in MMP activity and subsequently an increase in the degradation of collagen. Therefore, the accumulation of excess collagen in SHRs is reduced by hexarelin. The results of the present experiments demonstrate that hexarelin may increase the degradation of collagen by increasing MMP-2 and MMP-9 activities and abating the inhibitory effect of TIMP-1 on MMP activity in SHRs, which subsequently reduces cardiac fibrosis.

Of additional interest in this study is the possible involvement of the hemodynamic effect of hexarelin in regulating cardiac fibrosis in SHRs. Chronic arterial hypertension plays a vital role in the development of cardiac fibrosis and contributes to abnormalities in cardiac function (25). Chronic pressure overload stimulates both procollagen gene expression and collagen protein synthesis, leading to excessive collagen deposition and fibrosis (4). Therefore, the GHS-R-dependent reduction of systolic blood pressure by hexarelin in SHR might also contribute to hexarelin-reduced cardiac fibrosis in SHRs. The effect of hexarelin on blood pressure is supported by other studies (16, 28, 39). Our previous study (39) showed that treatment with hexarelin for 3 wk significantly reduced mean arterial pressure in a rat heart failure model induced by pressure overload for 3 mo (179 vs. 166 mmHg). Moreover, ghrelin administration decreases blood pressure in several animal models (28), and ghrelin plasma levels are negatively correlated with blood pressure in humans (16). The hexarelin-increased NO levels in SHR in this experiment might play a role in decreasing blood pressure in SHRs. It has also been reported that NO synthesis is impaired in hypertension (7) and in SHRs (17), and hexarelin increases serum NO levels in atherosclerotic rats (23). Ghrelin also increases endothelial NO synthase activation and NO production in endothelial cells (37).

CD36 is a fatty acid transporter and plays a key role in supplying the heart with its major energy substrate. CD36 has been reported as a receptor of GHS in mediating the cardiovascular action of GH-releasing peptides in the heart (5). It has been reported that CD36 is a novel and potential antifibrogenic target in albumin-induced renal proximal tubule fibrosis (42), and a CD36 synthetic peptide inhibits silica-induced lung fibrosis in mice (34). Our study showed that mRNA expression of CD36 is very low in SHRs and increased to the same level of wild-type rats after hexarelin treatment, indicating the involvement of CD36 in the antifibrotic effect of hexarelin.

In summary, hexarelin reduces cardiac fibrosis in SHRs, perhaps by decreasing collagen synthesis (38) and accelerating collagen degradation via regulation of MMPs/TIMP. In addition, hexarelin reduced systolic blood pressure through GHS-Rs, offering another mechanism for reducing cardiac fibrosis in SHRs. The present study not only provides novel insights into the mechanisms underlying the regulation of cardiac fibrosis but also suggests a new target to develop novel therapeutic strategies for the treatment of cardiac fibrosis.
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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES