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A novel peptide ghrelin inhibits neural remodeling after myocardial infarction in rats

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ABSTRACT

Ghrelin is a newly discovered peptide as an endogenous ligand for the growth hormone secretagogue receptor, and has been demonstrated to exert beneficial effect in the cardiovascular system. In the present study, we investigated whether ghrelin administration could inhibit cardiac neural remodeling and sympathetic hyperinnervation after myocardial infarction. Sprague–Dawley rats underwent coronary ligation to induce myocardial infarction and receiving ghrelin chronically (100 μg/kg s.c., twice daily) or saline control for 4 weeks after onset of ischemia. Four weeks after treatment, rats were sacrificed. We examined the expression of nerve growth factor and never markers as well as the mRNA expressions of inflammatory mediators in the infarcted border and non-infarcted left ventricular free wall. We also examined the NF-κBp65 protein and I-κBα protein levels by Western blot analysis. Compared to the control group, ghrelin administration significantly decreased the density of nerve fibers with positive immunostaining for GAP43 and TH, and decreased NGF mRNA and protein levels in the infarcted border and the non-infarcted area. Ghrelin also significantly suppressed interleukin-1β, tumor necrosis factor-α, and endothelin-l mRNA expression, and inhibited NF-κB activation. In conclusion, treatment with ghrelin inhibited neural remodeling and sympathetic hyperinnervation, the process that may be associated with the inhibition of proinflammatory response and NGF signaling.

1. Introduction

Ventricular tachycardia and fibrillation are major causes of morbidity and mortality in patients with coronary artery diseases and myocardial infarction. Previous studies have shown that heterogeneous cardiac nerve sprouting and sympathetic hyperinnervation contribute to ventricular arrhythmogenesis and sudden cardiac death in both patients and animal models of myocardial infarction (Cao et al., 2000a,b; Swissa et al., 2002; Zhou et al., 2001). Therefore, preventing the neural remodeling process will provide effective ways to treat and prevent ventricular arrhythmia and sudden cardiac death.

Ghrelin is a newly discovered peptide as an endogenous ligand for the growth hormone secretagogue receptor (GHSR), predominantly produced by the stomach (Kojima et al., 1999). Growth hormone (GH) and its mediator, insulin-like growth factor-1 (IGF-1), have been shown to decrease left ventricular wall stress and improve cardiac performance (Isgaard et al., 1999; Volterrani et al., 2000). Studies in animal models (Nagaya et al., 2001) and humans (Nagaya et al., 2004) with heart failure have shown that ghrelin significantly inhibits ventricular remodeling and attenuates cardiac cachexia, implying the cardiac effects of ghrelin may be mediated in part by GH. Apart from stimulating GH secretion, ghrelin may have additional therapeutic potential in cardiovascular system because it has GH-independent effects, such as vasodilatory actions (Shimizu et al., 2003) and anti-inflammatory effects (Dixit et al., 2004; Li et al., 2004). Recent research has demonstrated that treatment of ghrelin can prevent left ventricular remodeling and suppress cardiac sympathetic activity following myocardial infarction in rats (Soeki et al., 2008). However, the effect of ghrelin on sympathetic hyperinnervation following myocardial infarction remains unclear.

Nerve growth factor (NGF) is a neurotrophin that regulates the differentiation, survival, and synaptic activity of the peripheral sympathetic nervous system. The level of NGF synthesized in the target organ determines its innervation density (Korsching, 1993). Emerging evidences indicate that excessive local NGF production is responsible for initiating the nerve sprouting (Zhou et al., 2004b); while the inflammatory cells induced by myocardial infarction express large amount of NGF, which is temporo-spatially consistent with nerve sprouting and sympathetic hyperinnervation (Hasan et al., 2006). These findings indicate a correlation between the levels of NGF and the innervation density in the heart. It is well known that myocardial infarction is associated with an inflammatory reaction that is regulated by cytokines (such as IL-1β and TNF-α) (Guillen et al., 1995; Marx et al., 1997), the proinflammatory cytokines may activate the nuclear transcription factor NF-κB that mediates tissue inflammatory response and neurotrophin-regulated signaling pathways (Baeuerle and Henkel, 1994; Reichardt, 2006). In addition, NF-κB

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activity is also relevant with endothelin-1 expression, which induces NGF production specifically (Ieda et al., 2004).

In the present study, we used a rat model of myocardial infarction to determine whether ghrelin inhibits sympathetic hyperinnervation after myocardial infarction and, if so, whether inhibition of inflammatory response and NGF signaling by ghrelin are involved in such a process.

2. Materials and methods

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the protocol was approved by the Institutional Animal Care Committee from Wuhan University, People's Republic of China.

2.1. Animas and experimental designs

Adult male Sprague–Dawley rats (200–250 g) were used in this study. Animals were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg) and underwent left coronary artery ligation through a left thoracotomy to induce myocardial infarction ($n=45$) by regional ischemia or sham coronary ligation ($n=12$). The operation was performed with electrocardiogram monitoring. Successful ligation of the left coronary artery was verified by the color change in the ischemic area and ECG lead Iand aVL S–T segment elevations after the occlusion. In sham-operated rats, a suture was tied loosely around the left coronary artery without ligating it.

Fifteen rats died within 24 h after coronary ligation. All rats surviving 24 h after operation were randomly assigned to three groups, receiving either ghrelin (100 μg/kg, s.c.) or saline (sham and control groups) twice daily for 4 weeks.

2.2. Tissue collected

At the end of the experimental period, the heart was removed from the chest under pentobarbital anesthesia (100 mg/kg, i.p.), and left ventricular myocardium was cut in half through the center of the infarct along the baso-apical axis; one half was immediately stored in −80 °C for further processing, and the other half for immunohistochemistry. The infarcted region was visually identified by a mottled and pale appearance. The myocardium extending 0.5–1.0 mm from the infarct scar was considered to represent the infarcted border myocardium. To avoid contamination of the non-infarcted LVFW with infarcted border, a myocardial area extending 1–2 mm from the border zone area was not included.

2.3. Real-time PCR

Total RNA was prepared from the infarcted border and non-infarcted left ventricular free wall (LVFW) with Trizol reagent (Invitrogen, USA), and reversely transcribed to cDNA using TaqMan Reverse Transcription Reagents (Biosystems). The expression levels of candidate genes were measured by real-time quantitative RT-PCR using a SYBR Green PCR Master mix (Biosystems). For the PCR amplification, 10 pmol primers were used in a total volume of 20 μl. The PCR parameters were as follows: 1 cycle at 94 °C for 10 min followed by 50 cycles of 94 °C for 10 s, 55–60 °C for 15 s, and 72 °C for 5 s. For each sample, both glyseraldehyde-3-phosphate dehydrogenase (GAPDH used as endogenous control) and target gene were amplified in duplicate in separate tubes. A single dissociation peak was detected in each reaction by the dissociation curve. The expected size amplicons were confirmed by gel electrophoreses. The mRNA levels of each gene were calculated using the $2^{-\ CT}$ method (Livak and Schmittgen, 2001). The specific primer sequence and amplicon size of the selected genes were listed in Table 1.

2.4. Western blot analysis

Nuclear and cytoplasmic extracts were prepared for Western blot analysis of NF-κBp65 (nuclear), I-kBα (cytoplasmic) and NGF (cytoplasmic) expression, using Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, USA). Equal amounts (40 μg) of denatured proteins were fractionated on 4–15% Bis–Tris gel and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in TBST (containing 0.05% Tween20), and incubated overnight at 4 °C with the primary antibody (NF-κB p65, 1:500, Santa Cruz Biotechnology; NGF, 1:1000, Abcam; I-κBα, 1:1000, Beyotime Inc, China). Then the blots were washed and incubated with horseradish peroxidase-conjugated second antibody (goat anti-rabbit IgG, 1: 2000, Beyotime Inc, China) for 1 h under room temperature. Immunoreactivity was enhanced by chemiluminescence kit (Beyotime, Inc, China) and exposed to film. The density of bands on Western blots was quantified by using a Bio-Rad image system (Hercules, CA).

2.5. Immunohistochemical studies

Samples taken from the infarcted border and non-infarcted LVFW were routinely processed. Three sections of each heart were used for immunohistochemical study. The primary antibodies used for immunohistochemical staining were anti-growth associated protein 43 (GAP43, 1:100, Abcam) and anti-tyrosine hydroxylase (TH, 1:100, Abcam). Nerve densities were determined by a computer assisted image analysis system (Image-Pro Plus3.0, Media Cybernetics, USA). The computer automatically calculated the number and area occupied by the nerves in the field. The density of stained nerve was expressed as the nerve area divided by the total area examined ($\mu m^2/mm^2$). The nerve density of each slide was determined by the average of 3 fields with the highest nerve density.

2.6. Statistical analysis

All data were expressed as the Mean ± S.D. ANOVA with Newman–Keuls test was used for multiple comparisons. $P<0.05$ was considered statistically significant.

Table 1
Primer sequence and amplicon size of genes.

Genes name	Accession no	Primer sequence	Amplicon size, bp
IL-1β	NM_031512	F:5′-GGGATGATGACGACCTGC-3′ R:5′-CCACTTGTTGGCTTATGTT-3′	150
TNF-α	NM_012675	F:5′-GCCACCACGCTCTTCTGTC-3′ R:5′-GCTACGGGCTTGTCACTCG-3′	149
Endothelin-1	NM_012548	F:5′-CACCTGGACATCATCTGG-3′ R:5′-GTCTGTGGTCTTTGTGGG-3′	114
NGF	XM_227525	F:5′-AAGGCTTTGCCAAGGACG-3′ R:5′-TCTGGGACATTGCTATCTG-3′	139
GAPDH	NM_017008	F:5′-GCAAGTTCAACGGCACAG-3′ R:5′-CATTTGATGTTAGCGGGAT-3′	100

3. Result

3.1. Effect of ghrelin on mRNA expression of IL-1β, TNF-α and endothelin-1

To investigate whether ghrelin inhibited proinflammatory response and endothelin-1 activity, IL-1β, TNF-α and endothelin-1 mRNA levels were measured at the infarcted border and non-infarcted LVFW. Real-time RT-PCR revealed that ghrelin significantly decreased these factors compared with the control group (Fig. 1).

3.2. Effect of ghrelin on NF-κB translocation

To study whether ghrelin has any direct effect on NF-κB translocation, the Western blot analysis was performed. These studies showed that myocardial infarction induced nuclear translocation of p65 protein in the infarcted border, which was markedly attenuated by ghrelin ($P<0.01$). Cytoplasmic I-κBα protein levels were significantly higher ($P<0.01$) in myocardial infarcted rats treated with ghrelin than in those given saline. As demonstrated in Fig. 2, myocardial infarction induced a clear NF-κB nuclear translocation in an IκBα-dependent manner. Ghrelin administration inhibited the degradation of IκBα, and prevented the translocation of NF-κB into the nucleus.

3.3. Effect of ghrelin on NGF expression

The relative NGF mRNA levels in the infarcted heart were shown in Fig. 3A. Compared with the control, ghrelin-treated rats had a significant decrease ($P<0.01$) in NGF mRNA levels at the infarcted border and non-infarcted LVFW. Similar to qRT-PCR results, Western blot analysis showed that NGF protein levels were significantly lower at different locations of heart in ghrelin-treated rats compared with the control (Fig. 3B and C).

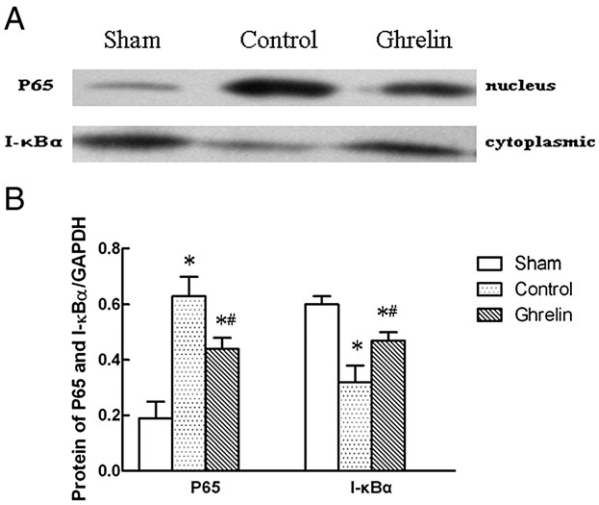

Fig. 2. Effect of ghrelin on NF-κB nuclear translocation at the infarcted border. (A) Representative Western blots of NF-κBp65 and I-κBα. (B) The quantitative comparison between the groups. Data are expressed as mean ± S.D. ($n=12$ to 14 for each group). *$P<0.01$ vs. sham group; #$P<0.01$ vs. control group.

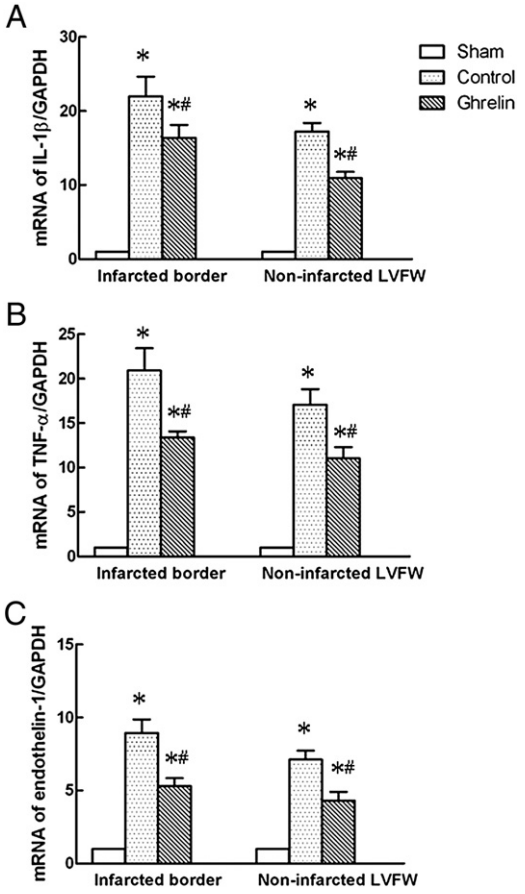

Fig. 1. Expression of mRNA for IL-1β (A), TNF-α (B) and endothelin-1 (C) by real-time PCR at the infarcted border and non-infarcted LVFW of Sham rats and infarcted rats treated with saline or ghrelin. Data are corrected by GAPDH and expressed as mean ± S.D. ($n=12$ to 14 for each group). *$P<0.01$ vs. sham group; #$P<0.01$ vs. control group.

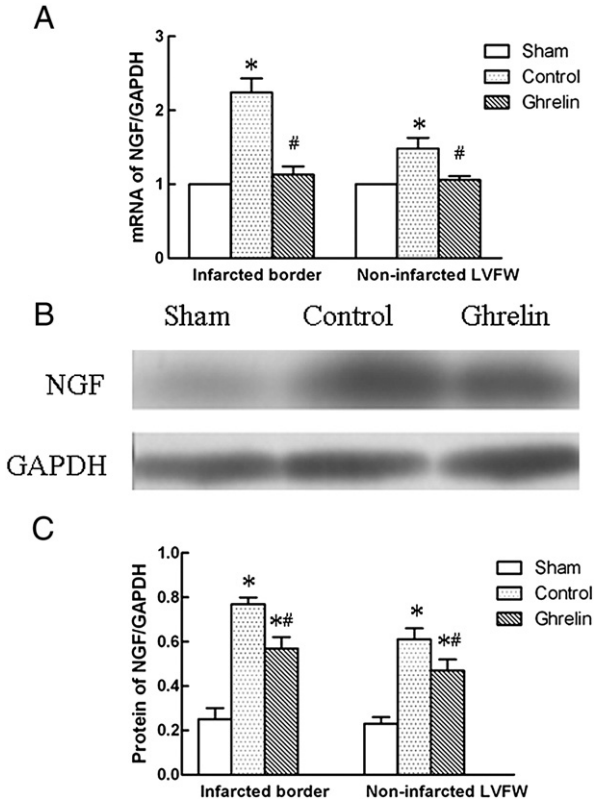

Fig. 3. Effect of ghrelin on NGF levels at different regions of heart after myocardial infarction. (A) Relative NGF mRNA levels. (B and C) Representative Western blots are aligned with the matching grouped data. Data are expressed as mean ± S.D. ($n=12$ to 14 for each group).*$P<0.01$ vs. sham group; #$P<0.01$ vs. control group.

3.4. Effect of ghrelin on cardiac neural remodeling

GAP43, a protein expressed in the growth cones of sprouting axons, is a marker for nerve sprouting. TH is a marker of sympathetic nerves. To investigate whether the decrease in infarcted myocardium NGF levels caused by ghrelin treatment contributed to the corresponding down-regulation of cardiac nerve density, immunohistochemical study was performed on samples taken from the infarcted border. Significant sympathetic nerve hyperinnervation and heterogeneous nerve sprouting were observed in the control group (Fig. 4B and E). Table 2 shows the results of nerve density measurements. There were more GAP43-positive and TH-positive nerves in the control group than in the sham group ($P<0.01$), and the sprouting nerves appeared to be mainly distributed at the periphery of infarcted zone. The distribution of nerves within the same fields also showed significant inhomogeneity (as showed in Fig. 4). However, in the sham rats, immunohistochemical staining showed few GAP43-positive nerves (Fig. 4A and D). The density of nerve fibers with positive immunostaining for GAP43 and TH was significantly lower ($P<0.01$) in myocardial infarcted rats treated with ghrelin than in those given saline.

Table 2
Result of immunohistochemical studies in different groups.

Group	GAP43 ($\mu m^2/mm^2$)		TH ($\mu m^2/mm^2$)	
	Infracted border	Non-infarcted LVFW	Infracted border	Non-infarcted LVFW
Sham	863 ± 242	783 ± 204	738 ± 157	721 ± 151
Control	3019 ± 497^{a}	2505 ± 559^{a}	2693 ± 464^{a}	2330 ± 458^{a}
Ghrelin	$1957 \pm 449^{a,b}$	$1752 \pm 313^{a,b}$	$1772 \pm 368^{a,b}$	$1524 \pm 287^{a,b}$

[a] $P<0.01$ vs. sham group.
[b] $P<0.01$ vs. control group.

4. Discussion

Ghrelin is a novel GH-releasing peptide, it is predominantly expressed in the gastric mucosa, but widespread expression is found through the body (Garcia and Korbonits, 2006). Some of the biological effects of ghrelin are mediated through receptor GHSR (Kojima et al., 1999). Recently, GHSR has been detected in cardiovascular tissues (Gnanapavan et al., 2002), suggesting that the peptide may play a direct role in cardiovascular regulation through GH-independent mechanisms. In the present study, we found that chronic ghrelin treatment of myocardical infarcted rats inhibited neural remodeling, attenuated inflammatory response and decreased NGF levels. Furthermore, we also demonstrated that ghrelin inhibited NF-κB activation and decreased endothelin-1 expression in infarcted heart. These findings support the potential beneficial effect of ghrelin in cardiovascular system.

Recent studies have indicated that NF-κB plays important roles in cardiac hypertrophy and remodeling (Grabellus et al., 2002), as well as proinflammatory cytokine, such as TNF-α (Murray and Freeman, 2003). Blockade of NF-κB improves cardiac function and survival after myocardial infarction (Kawano et al., 2006), while TNF-α overexpression in the myocardium contributes to adverse cardiac remodeling (Sakai et al., 1996; Stewart et al., 1992), and cardiac overexpression of endothelin-1 results in deterioration in cardiac function (Yang et al., 2004). In this study, we demonstrated that ghrelin inhibited TNF-α, endothelin-1 expression, as well as NF-κB activity. These effects may contribute to improvement of cardiac function. Therefore, it is possible that the beneficial effect of ghrelin in myocardial infarction is at least in part mediated by the inhibition of these factors. Ghrelin can also activate AMPK (Steinberg et al., 2009), and the activation of AMPK regulates the activity and trafficking of cardioprotective KAPT channels (Sukhodub et al., 2007) that are crucial for protecting the heart against myocardial infarction(Budas et al., 2004).

Myocardial infarction is associated with elevated NGF levels, and NGF protein and mRNA are localized primarily within macrophages and myofibroblasts (Hasan et al., 2006). Thus, it is possible that inflammatory response after myocardial infarction contributes to elevated NGF synthesis. TNF-α released following myocardial ischemia, is responsible for initiating the inflammatory cascade (Gordon and Galli, 1990); NF-κB is activated by a vast number of agents (such as IL-1β and TNF-α), and regulates the expression of multiple inflammatory and immune response genes (Brown and Jones, 2004). Lu (Lu et al., 2004) previously reported that NF-κB and proinflammatory mediators are activated in infarcted heart, our study is consistent with this observation. Given the biological effects of IL-1β, TNF-α, NF-κB, the anti-inflammatory effect of ghrelin shown in this study could partly help to explain the consequence of decreased NGF. Moreover, endothelin-1 can be stimulated by IL-1 and TNF-α, overexpression of NF-κBp65 significantly increases endothelin-1 production in cultured endothelia cells (Quehenberger et al., 2000), endothelin-1 also modulates leukocyte adhesion to endothelial cells and enhances production of cytokines in monocytes/macrophages (Helset et al., 1994; McMillen et al., 1995), it could directly induce NGF production in cardiomyocytes but not in cardiac fibroblasts (Ieda et al., 2004), which indicates that endothelin-1 regulation of NGF synthesis is

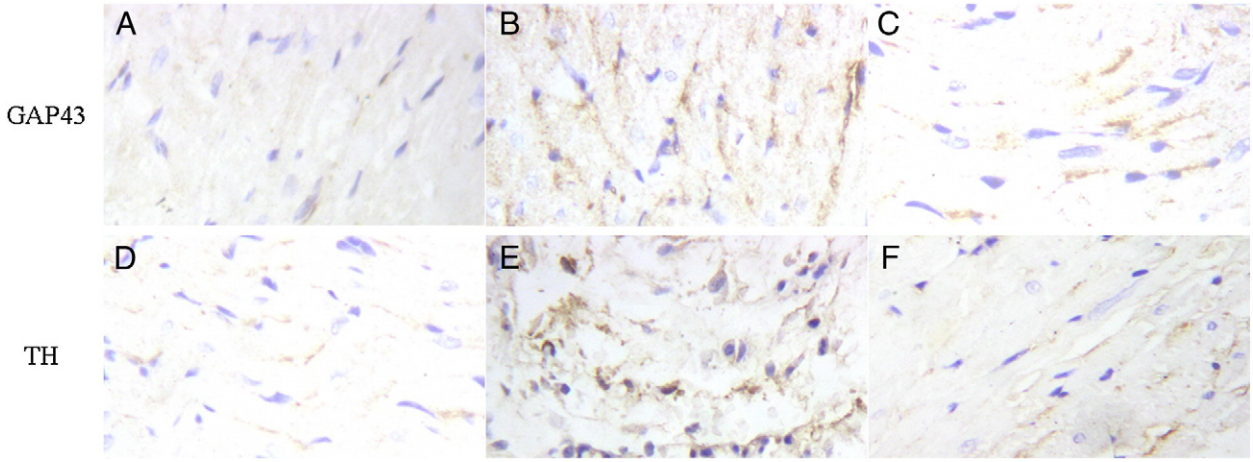

Fig. 4. Examples of immunohistochemical staining of cardiac nerves with anti-GAP43 and anti-TH at infarcted border. There were more GAP43-positive and TH-positive in the control group (B and E) than in the sham group (A and D). Ghrelin significantly decreased GAP43 and TH nerve density (C and F). Positive stain is marked by red color. Magnification of objective lens: ×40. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mediated in a cell type-specific manner. In the present study, we found that the increased NGF protein levels were associated with enhanced endothelin-1 expression in the infarcted heart. The spatial relationship between endothelin-1 and NGF supports stimulating role of endothelin-1 on NGF production. Zhou et al. (2004b) previously developed the nerve sprouting hypothesis, which assumes that myocardial infarction results in early local NGF release in the infarcted site, and the NGF proteins are then retrogradely transported to the left stellate ganglion where it stimulates the nerve sprouting in non-infarcted regions. Base on our results, we proposed that cardiac nerve sprouting in the non-infarcted region might be mediated in part by endothelin-1/NGF dependent pathway.

We showed that the dramatic increase in the markers of sympathetic nerve growth were 3 times higher after myocardial infarction while still twice as high in the presence of the given dose of ghrelin. One possible explanation is that the chronic presence of low dose subcutaneous ghrelin during the development of myocardial infarction in response to 4 weeks of experimental ischemic attenuated but did not abolish the marked expression of inflammatory markers (IL-1, TNF-α and endothelin-1) as well as NGF protein, although the rise in mRNA for NGF was completely reversed by ghrelin. A second explanation is that the length of the ischemic period, the cardiac dysfunction and other contributing factors influence sympathetic innervation.

In this study, we demonstrated that ghrelin significantly inhibited neural remodeling in the form of sympathetic nerve sprouting and hyperinnervation. The mechanism of ghrelin on neural remodeling is unclear. Neutrophic effects of NGF are mediated by binding to specific cell surface receptors, which include the high-affinity receptor tyrosine kinase A (TrkA) and the low-affinity receptor (P75NTR) (Reichardt, 2006). Immunostaining showed no TrkA within the myocardium in canine cardiac tissue or human hearts, indicating that p75NTR was the main cardiac NGF receptor (Zhou et al., 2004a). In the absence of TrkA, NGF selectively binds to P75NTR and activates NF-κB in rat Schwan cells (Carter et al., 1996), thereby promoting NF-κB dependent neuronal survival and development. It is possible that ghrelin inhibited the release of NGF in the infarcted myocardium, and then inhibited cardiac nerve sprouting. A second possible mechanism is that ghrelin directly blocked the neuronal survival signaling pathway through inhibition of NF-κB activation. Unfortunately, we only examined the effect of ghrelin on the translocation of the nuclear transcription factor in the infarcted border, further testing needed in the non-infarcted zone.

As reported previously, ghrelin suppresses cardiac sympathetic activity (Soeki et al., 2008), and significantly decreases plasma norepinephrine levels (Nagaya et al., 2004), implying that beneficial effects of ghrelin on cardiac performance may be due to inhibitory effects of ghrelin on sympathetic nerve activity. Our results showed that ghrelin inhibited sympathetic hyperinnervation, which is consistent with these studies. Since sympathetic activation is likely to play important roles in the risk of life-threatening arrhythmia after myocardial infarction (Vaseghi and Shivkumar, 2008), ghrelin may have a potential anti-arrhythmia effect.

In summary, we demonstrated that ghrelin inhibited neural remodeling in rats with myocardial infarction, likely mediated through NGF suppression. The expression of NGF and NF-κB mediated inflammatory mediator, as well as their relationship to cardiac nerve sprouting in the experimental infarcted rats, was addressed. The study suggested that ghrelin could serve as an effective strategy to inhibit neural remodeling after myocardial infarction. In addition, ghrelin has been shown to improve cardiac function (Enomoto et al., 2003), which is considered as an independent predictor of sudden cardiac death. Thus, our results suggested that ghrelin might be used as a new potential way to treat and prevent sudden cardiac death after myocardial infarction. Further studies are needed to elucidate the mechanisms underlying sympathetic nerve sprouting in the myocardium and explore the relationship between inhibition of cardiac nerve remodeling by ghrelin treatment and incidence of ventricular arrhythmia after myocardial infarction.

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