Ghrelin prevents doxorubicin-induced cardiotoxicity through TNF-alpha/NF-κB pathways and mitochondrial protective mechanisms

Zhiwei Xu a, Shiqing Lin b, Weikang Wu a,∗, Hongmei Tan a, Zhi Wang c, Chao Cheng d, Lihe Lu a, Xuanhong Zhang a

a Department of Pathophysiology, Zhongshan Medical College, Sun Yat-sen University, Guangzhou 510080, China
b Department of Anesthesiology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China
c Department of Anesthesiology, The Second Affiliated Hospital, Sun Yat-sen University, Guangzhou 510120, China
d Department of Thoraces, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China

A B S T R A C T

We had reported that increased levels of endogenous ghrelin during the progression of doxorubicin-induced cardiomyopathy and heart failure might provide a compensatory self-protective effect. We investigated which pathway(s) produced these protective effects in vitro. Primary cultured cardiomyocytes were induced with doxorubicin in the presence or absence of ghrelin or a tumor necrosis factor-alpha (TNF-alpha) antagonist (etanercept). Ghrelin up-regulated TNF-alpha in a time- and dose-dependent manner. It significantly reduced cell apoptosis and markers of oxidative stress, such as malondialdehyde (MDA) content and lactate dehydrogenase (LDH) activity; it also increased anti-oxidative enzyme activity such as superoxide dismutase (MnSOD) and catalase (CAT), retained mitochondrial membrane potential and energy metabolism compared with doxorubicin alone. Moreover, ghrelin increased mitochondrial anti-apoptosis related gene expression such as bcl-2 and MnSOD, reduced cytochrome c (Cyt C) release and strengthened the activation of NF-κB. All these effects were abrogated by etanercept. This suggests ghrelin affects the TNF-alpha/NF-κB activation pathways, up-regulating TNF-alpha, to produce anti-oxidative and anti-apoptotic effects that protected cardiomyocytes from doxorubicin-induced cytotoxicity.

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1. Materials and methods

1.1. Materials

Sera and media (GIBCO, U.S.A.); gluthamine (Pheonix Co., U.S.A.); doxorubicin (Wanle Pharma Co., Shenzhen, China); MTT assay kit (Sigma, U.S.A.); nucleotides standards for HPLC (Amresco, U.S.A.); Mitochondria/Cytosol Fractionation Kit (BioVison Co., U.S.A.); other chemicals (from commercial suppliers unless otherwise stated).

1.2. Primary cardiomyocytes culture

One-to-three-day old Wistar rats were sterilized and sacrificed in accordance with Chinese Council on Animal Care guidelines. The beating heart was immediately surgically removed, kept in cold Hanks buffer, washed, and minced with fine scissors into 1-3 mm³ pieces. The minced tissue was transferred to a 40 ml flask containing trypsin (0.08%, 0.5 ml per rat). The flask was sealed on ice for 20 min for preconditioning, as previously described (Harary and Farley, 1960; Fu et al., 2006), and the cardiomyocytes were isolated by serial enzymatic digestion and centrifugation. Finally, the harvested cells were incubated in flasks at 37°C in a humidified atmosphere (5% CO₂, 95% air) to allow the attachment of non-myocardocytes.

The major type of myocardocytes remained in culture medium, which were then collected and plated at a density of 5 x 10⁵ cells into a new culture flask or plate. 5-Bromo-2′-deoxyuridine (BrdU, 0.1 mmol/l) was added to the culture medium for the first 48 h to prevent proliferation of non-myocardocytes. At the 6th days cells were exposed in serum-free conditions with or without ghrelin and with or without exogenous TNF-α for indicated experimental time.

1.3. Detection of TNF-α level in medium by ELISA

The cardiomyocytes (5 x 10⁵ cells/well) were treated with 0.1–1 µM doxorubicin in the presence or absence of 10⁻⁹ to 10⁻¹⁰ M ghrelin for 1, 4, 8, or 16 h. The culture media were collected and centrifuged (1000 rpm) at 4°C for 5 min, and the supernatant was collected. Quantitative levels of TNF-α in the culture media were obtained according to the manufacturer’s instructions (ELISA Kit, Jingmei Biotech, China). As previously described (El Eter et al., 2007), the second antibodies were conjugated to streptavidin peroxidase. The optical density was measured using an automatic plate reader (Spectra Image; Tecan) at 450 nm, and values obtained according to the manufacturer’s instruction using a computer.

1.4. Cell viability and apoptosis assay

Cell viability was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] with a method described previously (Baldanzi et al., 2002). MTT is absorbed into cells and transformed into formazan by mitochondrial succinate dehydrogenase. Formazan accumulation directly reflects mitochondrial activity, which is an indirect measure of cell viability. Cells (5 x 10⁵ cells/well) were seeded on 96-well plates. After treatment with doxorubicin in the presence or absence of ghrelin or exogenous TNF-α for 16 h, the cells were incubated with 0.5 mg/ml MTT for 30 min at 37°C. The medium was aspirated, and the formazan product solubilized with 100 µl DMSO. Viable cells were detected using an ELISA plate reader, and values were normalized to OD values for an identical condition with no cells. Results were expressed as percentages of control group.

For cells (5 x 10⁵ cells) seeded on 6-well plates were harvested after treatment. Apoptotic cells can be recognized and distinguished from necrotic cells using flow cytometric analysis of the cellular DNA (Nicolleti et al., 1991). The percentage of apoptotic cells was determined from a DNA histogram as the ratio of the hypo-diploid cell population (the sub-G1 peak) to the total number of cells.

1.5. Determination of oxidative stress and cell damage state

After 4h treatment, cytotoxicity induced by doxorubicin was reflected by spectrophotometric measurement of MDA content and LDH activity in the medium. Briefly, the content of MDA, served as an indicator of lipid peroxidation (LPO), was determined using the thiobarbituric acid (TBA) method as described previously (Xu et al., 2007). LDH activity in the supernatant was neutralized with 0.5 M potassium hydroxide. HPLC (Agilent chemstation for LC, Germany) separation was carried out using a BDS C18 (2) 5 μm column (300 mm × 4.6 mm); Hypersil, U.S.A.) with a stainless-steel frit filter (2 μm). Detect signals (absorbance at 254 nm for ATP, ADP, and AMP) were calculated with a computer.

1.6. RT-PCR for TNF-α, MsdSOD, and bcl-2 mRNA

As previously described (Wu et al., 2002), total RNA was isolated using TRIzol reagent (Sigma) and 2 µg total RNA was back-transcribed into cDNA using a RETRO-script Kit (Promega, U.S.A.). The primers of bcl-xl/xs, bcl-2, MsdSOD, and beta-actin were designed as follows: bcl-xl/xs sense: AACAGTTCTCAAGAAGCCGG and anti-sense: TAATCCGAGCTGAGGGTA (705 bp); TNF-alpha sense: TACTAAGCTTGGGTGATGTC and anti-sense: CGCGC-CTTGGCTCTTGAAGCAAC (295 bp); MsdSOD sense: GCGCTCCAAATGTTGGTCGTG and anti-sense: ATATCCGTCGACAATCTCTCC (372 bp), bcl-2 sense: GTATGATAAC- CGGAGATGAC and anti-sense: TAATCCGCGACATTGAAGGG (554 bp), β-actin sense: CTGCGACCTCAGCTCAGCACA and anti-sense: TAGACCGACCAACCCACAGAAC (444 bp). The PCR amplification protocol accompanying the RT-PCR Kit (Promega) was followed. Next, 6 µl of the final reaction product was separated by electrophoresis in a 1.5% agarose gel. The bands were visualized and quantified (Bio-Rad), using β-actin as control.

1.7. Western blots

Cells were collected and homogenized according to the manufacturer’s instructions using the Mitochondria/Cytosol Protein Extraction Kit (BioVison Co.). Briefly, cell lysates were centrifuged at 10,000 × g for 30 min, and then the supernatants (as cytoplasmic protein) and the pellet (as mitochondrial protein) were stored at -80°C until use. The isolated protein amount of the samples was measured with a Bio-Rad protein assay using bovine serum albumin as a standard, and lysates (30 µg per lane) were analyzed by SDS-12% polyacrylamide gel and transferred to nitrocellulose paper (pore size: 0.2 µm, AMC Co., Ltd) The membranes were immunoblotted with anti-rat MsdSOD antibody and anti-Cyt C antibody (Chemicon, U.S.A.), -rat COX-IV antibody (Cellsigna, U.S.A.), and anti-rat actin antibody (Beyotime Biotech), followed by the addition of horseradish peroxidase-conjugated secondary antibody. After the final wash, the membranes were probed using ECL (Beijing Biotech) and autoradiographed by the method of Zhou (Zhou et al., 2007). The intensity of the bands was determined using densitometric analysis.

1.8. Statistical analysis

Results are shown as means ± S.D. for at least three independent experiments. Data were analyzed using SPSS version 11.0 (SPSS Inc., Chicago, IL). Statistical significance was determined using one-way ANOVA followed by the Student-Newman-Keuls test for comparison of two groups. A P value <0.05 was considered statistically significant.

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2. Results and discussion

2.1. Ghrelin prevents the decrease of TNF-alpha in cardiomyocytes induced by doxorubicin

The pathophysiology of doxorubicin-induced cardiotoxicity is multifactorial and complex, but the main mechanisms are increased damage from oxidative stress and cardiomyocyte loss from apoptosis (Li et al., 2000). We found a time- and dose-dependent decrease in TNF-alpha secretion following exposure to doxorubicin (Fig. 1), which is consistent with a previous report that TNF-alpha levels dropped in the late stage of doxorubicin-induced heart failure (Lou et al., 2004). TNF-alpha is known to exert anti-apoptotic effects through the activation of NF-kB signaling (McGowan et al., 2003), which suggests that, at least to a certain extent, TNF-alpha may play a protective role in AIC and heart failure.

TNF-alpha is generally considered to be harmful, but there are studies reporting that TNF-alpha administration can improve left ventricle systolic performance (Murray and Freeman, 1996), be involved in cardiac adaptation to stress by stimulating the production of heat shock proteins (Haudek et al., 2001), and provoke a hypertrophic growth response (Dibbs et al., 2003). TNF-alpha may, therefore, have different effects depending on its mode of secretion, the species and type of cell, and the nature of the stimulus. Our findings are the first evidence that ghrelin can reverse the decrease in TNF-alpha secretion that follows myocyte exposure to doxorubicin and, in vitro; it does this in a time- and dose-dependent manner (Fig. 1). The highest TNF-alpha levels occurred after 4 h of treatment with ghrelin, after which there was a plateau (Fig. 1). Further investigation is needed to explore the regulatory mechanisms of TNF-alpha secretion in cardiomyocytes damaged by doxorubicin.

2.2. Ghrelin prevents the cell apoptosis and oxidative stress damage induced by doxorubicin

In this doxorubicin-induced cardiotoxicity study, cell apoptosis and oxidative stress damage – reflected as depressed cell viability (Fig. 2A), increased cell apoptosis (Fig. 2B and C), decreased MnSOD and CAT activity (Table 1), and increased MDA content and LDH activity (Table 1) – were all prevented by ghrelin. Ischemia and reperfusion increases production of myocardial MDA and LDH, but this was attenuated by ghrelin in one study (Chang et al., 2004). Another study showed that peripherally administered ghrelin was protective during gastric ischemia and reperfusion injury and that this protection was provided by an antioxidant pathway: ghrelin inhibited reactive oxygen species (ROS) generation by human polymorphonuclear cells in a dose-dependent manner (El Eter et al., 2007). Ghrelin can also protect endothelial cells from high glucose by inhibiting ROS generation (Zhao et al., 2007).

Correlation analysis of a previous in vitro study performed by our group found that...
myocardial apoptosis was significantly related to oxidative stress in doxorubicin-induced heart failure (Xu et al., 2007). That study suggested that ghrelin’s anti-apoptotic effect was partly attributable to its anti-oxidative effect. An increase in the intrinsic anti-oxidative activity of MnSOD and CAT (Table 1) may have been the mechanism by which ghrelin prevented doxorubicin-induced oxidative stress in cardiomyocytes in the present study.

2.3. Ghrelin prevents the impaired mitochondrial bioenergetics induced by doxorubicin

Mitochondria, as the primary site of cellular energy generation and oxygen consumption, represent a likely pathway for doxorubicin-induced apoptosis and oxidative stress. In a previous in vivo study, we reported characteristic mitochondrial ultrastructural damage and energy metabolism dysfunction as early events in AIC and showed that endogenous ghrelin levels had a significantly positive correlation with energy reserves, such as myocardial ATP, in doxorubicin-induced heart failure. ATP/ADP and energy charge (EC) are crucial parameters reflecting the energy level and energetic situation of the cell. EC was calculated according to the following formula: EC = ([ATP + 0.5 ADP]*AMP + ADP + ATP]. All data were presented as mean ± S.D.

Table 1
Ghrelin prevents oxidative stress and impaired energy metabolism state in adriamycin-induced cardiotoxicity

<table>
<thead>
<tr>
<th>Group (n = 9)</th>
<th>Control</th>
<th>Adr</th>
<th>Adr + Ghr</th>
<th>Adr + Ghr + Anti-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative stress</td>
<td>MnSOD (U/ml)</td>
<td>1.884 ± 0.205</td>
<td>1.593 ± 0.254&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>1.889 ± 0.165&lt;sup&gt;□&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (U/ml)</td>
<td>6.373 ± 0.322</td>
<td>5.492 ± 0.203&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>6.081 ± 0.216&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>6.037 ± 0.356&lt;sup&gt;△△&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>0.312 ± 0.015</td>
<td>0.469 ± 0.040&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>0.320 ± 0.024&lt;sup&gt;□&lt;/sup&gt;</td>
<td>0.380 ± 0.032&lt;sup&gt;△△&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA (nmol/l)</td>
<td>0.555 ± 0.043</td>
<td>2.331 ± 0.182&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>0.829 ± 0.082&lt;sup&gt;□&lt;/sup&gt;</td>
<td>1.592 ± 0.134&lt;sup&gt;△△&lt;/sup&gt;</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>ATP/ADP</td>
<td>10.16 ± 0.455</td>
<td>4.809 ± 0.182&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>6.119 ± 0.252&lt;sup&gt;□&lt;/sup&gt;</td>
</tr>
<tr>
<td>EC</td>
<td>0.936 ± 0.003</td>
<td>0.802 ± 0.003&lt;sup&gt;△△&lt;/sup&gt;</td>
<td>0.900 ± 0.005&lt;sup&gt;□&lt;/sup&gt;</td>
<td>0.830 ± 0.001&lt;sup&gt;△△&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Adr, adriamycin; Ghr, ghrelin; Anti-T, TNF-alpha antagonist (etanercept); MnSOD, manganese superoxide dismutase; CAT, catalase; LDH, lactate dehydrogenase; MDA, malondialdehyde; EC = (ATP + 0.5 ADP)/([AMP + ADP + ATP]. All data were presented as mean ± S.D.

<sup>△</sup> P < 0.05 compared with control.
<sup>△△</sup> P < 0.01 compared with Adr group.
<sup>□</sup> P < 0.05 compared with Adr group.
<sup>△</sup> P < 0.05 compared with Adr + Ghr group.
<sup>△△</sup> P < 0.01 compared with Adr + Ghr group.

Fig. 3. Ghrelin inhibited doxorubicin-induced decreased mitochondrial membrane potential (∆Ψ<sub>m</sub>), decreased anti-oxidative protein manganese superoxide dismutase (MnSOD), and increased pro-apoptosis protein cytochrome C (Cyt C) release. A and B, Cultured cardiomyocytes were treated with 1 μM doxorubicin (Adr) in the presence or absence of 10⁻⁶ M ghrelin (Ghr) and in the presence or absence of 10 μg/ml anti-TNF-alpha antagonist, etanercept (Anti-T). After 4h of treatment, the cells were co-incubated with the fluorescence probe JC-1 for 30 min at 37 °C, images (600×) were scanned by confocal laser microscopy (A), and red/green fluorescence intensity value was calculated (B), (C and D) After 4-h treatment as indicated, cellular cytoplasmic (Cyt) and mitochondrial (Mito) proteins were extracted, respectively, from the cell lysate and analyzed by Western blot with specific anti-Cyt C antibodies, specific anti-MnSOD antibodies and anti-COX-IV antibodies (C), with densitometric analysis of the relative protein levels (D). Data are presented as means ± S.D. **P < 0.01 compared with control; *P < 0.05, **P < 0.01 compared with Adr group; △ P < 0.05, △△ P < 0.01 compared with Adr + Ghr group.

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have become so permeable that they are unable to maintain a sufficient $\Delta W_m$ to retain cytochrome C (Cyt C). Cyt C, released from the mitochondria into the cytoplas, kills cells in two ways: (i) by activating caspases via Apaf-1 and (ii) by interrupting the electron transport chain, thus preventing oxidative phosphorylation, promoting free-radical production, and eventually depriving the cell of ATP (Reed, 1997). In this study, we observed that ghrelin significantly prevented impaired mitochondrial bioenergetics by retaining $\Delta W_m$ (Fig. 3A and B), increasing anti-oxidative enzyme MnSOD protein expression (Fig. 3C and D), and reducing pro-apoptotic protein Cyt C release induced by doxorubicin (Fig. 3C and D). This prevention of impaired mitochondrial bioenergetics by ghrelin was TNF-alpha dependent.

2.4. Ghrelin reduces mitochondrial-related apoptosis, involves TNF-alpha/NF-κB activation

A key finding was that ghrelin could strengthen doxorubicin-induced NF-κB nuclear translocation. Doxorubicin and TNF-alpha both proved to be potent activators of NF-κB (Fig. 4A and B). It is well known that over-expression of TNF-alpha activates both anti-apoptotic and pro-apoptotic pathways in the myocardium (Kubota et al., 2001). By activating NF-κB, TNF-alpha induces a number of anti-apoptotic genes (including c-FLIP, cIAP1, cIAP2, A1, A20, TRAF1 and TRAF2, and MnSOD) that strongly oppose its pro-apoptotic potential (Pinkus et al., 1996; Bouwmeester et al., 2004). Induction of MnSOD by TNF-alpha is a possible protective mechanism (Wong and Goeddel, 1998). Over-expression of MnSOD has been shown to protect against AIC in animals, via increased anti-oxidative activity (Yen et al., 1996). It is likely in our study that ghrelin-induced up-regulation of mRNA and protein expression of MnSOD (Fig. 4C and D, and Fig. 3C and D) corresponded with up-regulated TNF-alpha/NF-κB activation (Fig. 1, and Fig. 4A and B). Up-regulation of intrinsic anti-oxidants such as MnSOD may explain why the protective effects of ghrelin were TNF-alpha dependent (Figs. 2 and 3).

NF-κB activation is critical for mitochondrial MnSOD and for the mitochondrial Bcl-2 family, whose members are key regulators of apoptosis and which includes anti-apoptotic proteins (such as bcl-2 and bcl-xl) and pro-apoptotic proteins (such as bcl-xx) (Barkett and Gilmore, 1999). In our study, mRNA expression of bcl-2 and bcl-xl was reduced by doxorubicin (Fig. 4C and D), consistent with other in vitro experiments (Wu et al., 2002). Bcl-2 and Bcl-xl can control apoptosis by blocking pro-apoptotic Cyt C release from mitochondria (Skulachev, 1998). We found that ghrelin up-regulated the mRNA expression of protective proteins from the Bcl-2 family and that this process was TNF-alpha dependent (Fig. 4C and D).

Increased TNF-alpha gene expression (Fig. 4C and D) by ghrelin may be attributable to NF-κB activation (Fig. 4A and B), something which needs further research. Doxorubicin could activate NF-κB nuclear translocation, which is known to be pro-apoptotic (Wang et al., 2002). However, ghrelin could enhance TNF-alpha signaling and so strengthen NF-κB translocation, which appeared to be anti-apoptotic in our in vitro study. Another study reported that ghrelin up-regulated the expression of Bcl-2/Bax, which then had a neuro-protective effect (Miao et al., 2007). Whether ghrelin up-regulation of protective members of the Bcl-2 family is NF-κB dependent is unknown.

In conclusion, this study showed that ghrelin had anti-oxidative and anti-apoptotic effects that prevented doxorubicin-induced cardiotoxicity and that these processes involved up-regulation of TNF-alpha. The mechanisms by which ghrelin prevented impairment of mitochondrial bioenergetics and reduced mitochondrial pathways of apoptosis involved TNF-alpha/NF-κB activation.
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