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Ghrelin protects H9c2 cells from hydrogen peroxide-induced apoptosis through NF-κB and mitochondria-mediated signaling

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ABSTRACT

Oxidative stress is a major mechanism underlying the pathogenesis of cardiovascular disease. Herein we investigate the protective effects of ghrelin in H_2O_2-induced apoptosis of H9c2 cells, as well as the possible molecular mechanisms involved. To study apoptosis, the cells were assessed by morphologic examination, MTS assay, Annexin V–propidium iodide dual staining and TUNEL analysis. Intracellular reactive oxygen species (ROS) production and mitochondrial membrane potential were also measured. To investigate the underlying molecular mechanisms, the expression of Bcl-2, Bax, active caspase-9 and NF-κB were assessed by Western blotting, and caspase-3 activity was determined by a colorimetric activity assay kit. After stimulation with H_2O_2 for 18 h, H9c2 cells viability decreased significantly; a large fraction of cells underwent apoptosis. We observed a dose-dependent rescue of H9c2 cells from H_2O_2-induced apoptosis in the presence of different ghrelin concentrations. Preincubation with ghrelin also restored the ROS and mitochondrial membrane potential levels that had been altered by H_2O_2 treatment. Moreover, ghrelin decreased H_2O_2-induced Bax production and caspase-9 activation, and increased Bcl-2 levels. NF-κB phosphorylation was also significantly inhibited by ghrelin in H_2O_2-treated cells. Caspase-3 activation was suppressed by ghrelin in H_2O_2-treated H9c2 cells in a dose-dependent manner. In summary, ghrelin protects H9c2 cells from oxidative stress-induced apoptosis through downregulation of Bax expression, caspase-9 activation and NF-κB phosphorylation, and upregulation of Bcl-2 expression. Caspase-3 activation was also reduced in a dose-dependent manner. These data suggest that ghrelin might protect against cardiovascular disease by protecting the mitochondria.

1. Introduction

Ghrelin, a novel 28 amino acid peptide, is a newly discovered hormone identified as an endogenous ligand for the growth hormone secretagogue receptor, which regulates energy balance and body weight homeostasis (Kojima et al., 1999; Tschop et al., 2000; Bowers, 2001; Inu, 2001; Nakazato et al., 2001; Van der Lely et al., 2004). Recently, growing evidence has indicated that the cardiovascular system is a target for ghrelin and that it is also synthesized and secreted by human cardiomyocytes in a paracrine/autocrine fashion (Iglesias et al., 2004). Ghrelin has various cardiovascular effects, including increased myocardial contractility (Bisi et al., 1999; Nagaya et al., 2001a), protection of the vascular endothelium (Rossi et al., 2007; Tesauro et al., 2005), and improved myocardial energy metabolism (Chang et al., 2003, 2004; Garcia and Korbonits, 2006; Xu et al., 2007). Some indirect evidence suggests that the cardioprotective activity of ghrelin is independent of growth hormone secretion (Locatelli et al., 1999; Tivesten et al., 2000). The effect of ghrelin on the cardiovascular system may be attributed to its various beneficial anti-inflammatory (Dixit et al., 2004; Li et al., 2004), antioxidant (Kawczynska-Drozdz et al., 2006) and anti-apoptotic effects in myocardial and endothelial cells (Baldanzi et al., 2002). However, few studies have identified the cellular and molecular cardioprotective mechanisms of ghrelin.

Considerable evidence has demonstrated that oxidative stress promotes apoptosis (Crow et al., 2004), and is implicated in the pathogenesis of various cardiovascular diseases including myocardial ischemia, arteriosclerosis, cardiomyopathy, transplant rejection, and heart failure (Gill et al., 2002). Cardiomyocyte apoptosis causes loss of contractile tissue, compensatory hypertrophy and reparative fibrosis, all of which contribute to the development of cardiovascular diseases (Takemura and Fujiwara, 2006). Therefore, modification of cardiomyocyte apoptosis is a major area of clinical interest, and it is important to identify the signaling pathways that mediate survival and/or apoptosis in cardiomyocytes. Many chemical and physiological inducers of oxidative stress cause apoptosis (O'Brien et al., 2000).

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The main oxygen species responsible for oxidative stress are hydrogen peroxide (H_2O_2), the free radical superoxide anion (O^{2-}) and the hydroxyl radical (OH^-). Doxorubicin, high glucose and sodium palmitate were used in previous studies as inducers in cardiomyocytes (Baldanzi et al., 2002; Liu et al., 2009; Xu et al., 2008).

In this study, H_2O_2 was used to induce apoptosis in H9c2 cells, as it is a well-established model to study oxidative stress-induced cardiomyocyte apoptosis. H9c2 cardiac myoblasts, a clonal cell line derived from the embryonic rat heart ventricle (Kimes and Brandt, 1976), were used as an experimental model, since they have been proven to be ideal for signal transduction studies (Su et al., 1999; Turner et al., 1998). Herein we examine whether ghrelin protects H9c2 cells from H_2O_2-induced cell death. The effects of ghrelin on Bcl-2, Bax , caspase-9 and NF-κB expression were detected and the caspase-3 activity was also determined.

2. Materials and methods

2.1. Materials

All cell culture medium components were purchased from Invitrogen Life Technologies unless otherwise noted. The H9c2 cell line was obtained from the Shanghai cell library of China (originally from ATCC, Manassas, VA, USA). H_2O_2 was purchased from Sigma and prepared in PBS at 500 μM immediately before use. Ghrelin peptide was purchased from Phoenix (Burlingame, CA, USA) and dissolved in Dulbecco's modified Eagle's medium (DMEM); it was subsequently diluted to 0.01, 0.1 and 1 μM. MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt] was purchased from Promega (San Luis Obispo, CA, USA). The Annexin V/FITC Kit was purchased from Bender MedSystems (Vienna, Austria). The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) In Situ Apoptosis Detection kit was purchased from Trevigen (Gaithersburg, MD, USA). 2′–7′dichlorodihydrofluorescin diacetate (DCFHDA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorescent dye were obtained from Molecular Probes (Eugene, OR, USA). Antibodies used for the Western blot analysis include rabbit anti-active caspase-9 polyclonal antibody (Chemicon, CA, USA) which recognizes only the cleaved large subunit (37 kDa). Rabbit anti-Bax, anti-Bcl-2, anti-NF-κB and anti-phospho-NF-κB polyclonal antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA), while actin and tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-3 activity was determined by the Caspase colorimetric activity assay kit (Chemicon).

2.2. Cell culture

H9c2 cells were cultured in DMEM supplemented with 10% calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, in a humidified 5% CO_2 atmosphere at 37 °C. The cells were passaged every 3 days. The cells were seeded at a density of 2×10^6 cells/dish in 100 mm dishes with 2% calf serum. When cells were nearly 80% confluent, the cells were pretreated with different ghrelin concentrations for 2 h prior to the addition of H_2O_2.

2.3. MTS assay

The H9c2 cells (5000 cells/well) were seeded in 96-well microtiter plates. After incubation with 0.01, 0.1, or 1 μM ghrelin for 2 h, the cells were treated with 500 μM H_2O_2 for another 18 h. Subsequently, 20 μl MTS solution was added to each well, and the plates were incubated for 3 h at 37 °C. The absorbance measured was measured at 470 nm and used to calculate the relative ratio of cell viability.

2.4. Flow cytometry analysis

Cell recovery was monitored by examining the levels of apoptosis at 18 h after the H_2O_2 treatment. Annexin V binding and propidium iodide staining were determined by flow cytometry. After incubation with 0.01, 0.1 or 1 μM ghrelin for 2 h, the cells were treated with 500 μM H_2O_2 for 18 h, washed with ice-cold PBS and double stained with propidium iodide and FITC-coupled annexin V for 20 min. Flow cytometry was performed with a 488 nm laser coupled to a FacsCalibur cell sorter (BD Biosciences, San Jose, CA, USA). Cells stained with both propidium iodide and annexin V were considered necrotic and cells stained only with annexin V were considered apoptotic.

2.5. TUNEL analysis

H9c2 cells, grown on coverslips (1×10^5 cells/well), were cultured under various experimental conditions and then washed with 1× PBS and fixed in Proteinase K Solution for 30 min at room temperature. The cells were permeabilized with 50 μl Cytonin for 30 min, then immersed in Quenching Solution for 5 min and then in 1× TdT Labeling Buffer for 5 min . Samples were covered with 50 μl of Labeling Reaction Mix and incubate at 37 °C for 1 h in a humidity chamber, and then immersed the samples in 1× TdT Stop Buffer for 5 min at room temperature to stop the labeling reaction. The samples were covered with 50 μl of Strep-HRP solution incubated for 10 min at room temperature, and then immersed in DAB solution for 5 min prior to washing in several changes of deionized water for 2 min each. Slides were then evaluated under a light microscope.

2.6. Intracellular reactive oxygen species (ROS) measurement

ROS production was measured using the cell permeant probe DCFDA. H9c2 cells cultured under various experimental conditions were loaded with DCFDA for 30 min at 37 °C in the dark. The samples were pelleted by centrifugation and resuspended. ROS production was determined by measuring the fluorescence resulting from the incorporation of the dye at the appropriate excitation and emission wavelengths with a spectrofluorimeter.

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

H9c2 cells cultured under various experimental conditions were incubated with 5 μM JC-1 dye for 30 min at 37 °C. After incubation, the cells were washed with PBS. The emission signals at 590 and 527 nm elicited by excitation at 485 nm were measured using a fluorimeter. The ratio of the signal at 590 nm to that at 527 nm was calculated.

2.8. Western blotting

Cytoplasmic extracts were obtained by lysing cells in lysis buffer [150 mM NaCl, 10 mM Tris–HCl (pH 7.9), 0.5% Triton X-100, 0.6% NP-40, and protease inhibitors (1 mg/ml leupeptin, 1 mg/ml pepstatin A, and 2 mg/ml aprotinin)]. The protein content was determined using the DC protein assay kit (Bio-Rad, Richmond, CA, USA). Protein samples (40 μg) were mixed with 2× SDS sample buffer and then separated on a 10% polyacrylamide gel and blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked for 1.5 h in Tris-buffered saline (TBS) with 5% non-fat dry milk, and incubated with primary antibodies (1:800 in TBS-Tween) overnight at 4 °C. Subsequently, the membranes were washed in TBST, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Pierce, 1:3000 in TBST) for 1 h at room temperature, washed in TBST five times and developed using the ECL chemiluminescence detection system (Amersham). Ponceau staining was performed in all experiments to control for loading. Protein bands were quantified by densitometric analysis. Results were

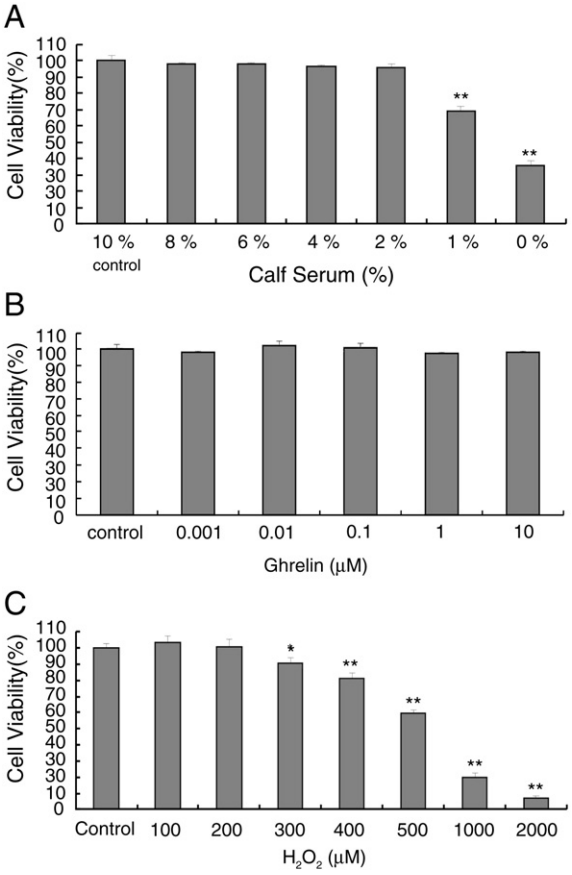

Fig. 1. Determination of working conditions. H9c2 cells were cultured with different concentrations of calf serum (A), ghrelin (B) or H_2O_2 (C) for 18 h and viability assessed by the MTS assay. *P<0.05 compared with control group, **P<0.01 compared with control group.

calculated as the relative ratio between the band of interest and the β-actin or tubulin band.

2.9. Caspase-3 activity assay

Treated H9c2 cells were resuspended in 500 μl of cell lysis buffer, and then incubated on ice for 10 min. After centrifugation at 10,000×*g* for 5 min, the supernatant was transferred to a fresh tube. Caspase-3 activity was determined by a caspase colorimetric activity assay kit, which is based on spectophotometric detection of p-nitroaniline (pNA) after cleavage from the labeled substrate LEHD-pNA. We quantified free pNA using a microtiter plate reader at 405 nm.

2.10. Statistical analysis

All the experiments were performed at least three times. The data are expressed as the mean ± S.D. Statistical significance was analyzed by one-way analysis of variance. Values with $P<0.05$ were considered statistically significant.

3. Results

3.1. Working concentration of calf serum, ghrelin and H_2O_2

H9c2 cells were cultured in DMEM supplemented with different concentrations of calf serum for 18 h, which revealed that cell viability significantly decreased with less than 2% calf serum (Fig. 1A). Therefore, we used 2% calf serum in subsequent experiments. To determine the working concentration of ghrelin, H9c2 cells were treated with 0.001, 0.01, 0.1, 1 or 10 μM ghrelin, none of which had any damaging effects on cells (Fig. 1B). We chose three concentrations of ghrelin (0.01, 0.1 and 1 μM) for further experiments. To determine the working concentration of H_2O_2, we performed a series of dose–response assays using the MTS test. Treatment with increasing concentrations of H_2O_2 for 18 h caused a dose-dependent loss of cell viability (Fig. 1C). Five hundred micromolar H_2O_2 reduced cell viability (survival rate was 59.8 ± 2.0%); we used this concentration in subsequent experiments.

3.2. Morphologic changes of H9c2 cells

The morphology of H9c2 cells treated with H_2O_2 (500 μM) in the absence or presence of ghrelin (0.01, 0.1 or 1 μM) was assessed by light microscopy (Fig. 2). In the control group, almost no abnormal cells were observed. After H_2O_2 treatment, a high number of cells displayed typical apoptosis-like morphological changes including detachment, irregular shape, nuclear fragmentation or apoptotic body formation (blebbing). However, in the three ghrelin-pretreated groups (0.01, 0.1 or 1 μM), the proportion of apoptotic cells decreased

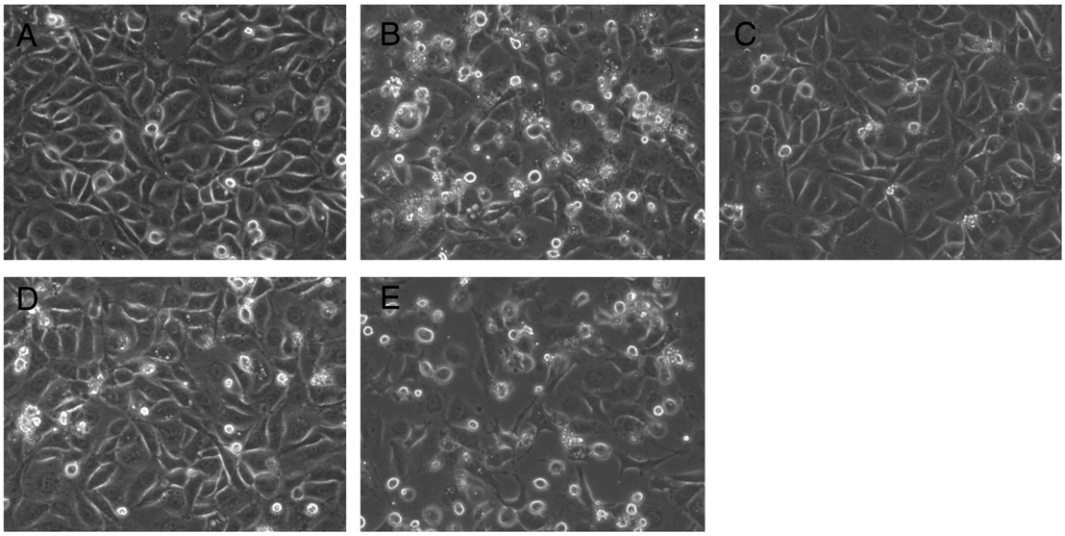

Fig. 2. Ghrelin decreased the proportion of apoptosis-like morphology induced by H_2O_2 in H9c2 cells. (A) H9c2 cells without ghrelin or H_2O_2 (Control); (B) H9c2 cells exposed to 500 μM H_2O_2 alone; (C–E) H9c2 cells pretreated with 1, 0.1 or 0.01 μM ghrelin followed by treatment of 500 μM H_2O_2. Magnification: 200×.

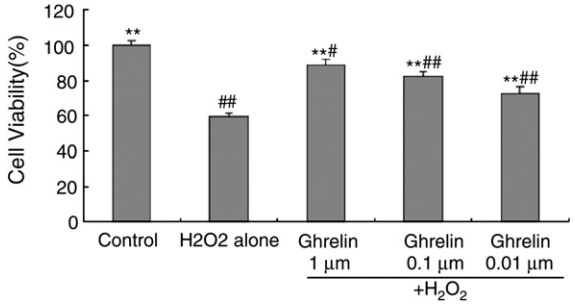

Fig. 3. Protection of ghrelin on H9c2 cells from H_2O_2-induced oxidative stress in a dose-dependent manner. Cell viability was measured by MTS assay. **P<0.01 compared with H_2O_2 only treatment group, #P<0.05 compared with control, ##P<0.01 compared with control.

with increasing ghrelin concentration, indicating a concentration-dependent protective effect.

3.3. Ghrelin protects H9c2 cells from H_2O_2-induced cell death

H9c2 cells were treated with H_2O_2 (500 μM) in the presence or absence of ghrelin (0.01, 0.1 or 1 μM), and cell viability measured by the MTS assay (Fig. 3). Viability of H_2O_2-treated H9c2 cells significantly decreased compared to control (survival rate was 59.8 ± 2.0%). When the cells were pretreated with 0.01, 0.1, or 1 μM ghrelin for 2 h, the cell viability increased significantly to 72.5 ± 4.1%, 82.4 ± 2.5% and 88.6 ± 3.5%, respectively. To quantify the effects of different concentrations of ghrelin on H_2O_2-induced H9c2 cells apoptosis, the percentage of apoptotic cells was detected by Annexin V-FITC and PI double staining (Fig. 4). The number of apoptotic cells was increased significantly when cells were treated with 500 μM H_2O_2 compared with the controls (40.74 ± 2.75% vs 3.33 ± 0.79%). When H9c2 cells were cultured with 500 μM H_2O_2 and ghrelin (0.01, 0.1 and 1 μM respectively) for 18 h, the percentages of apoptotic cells were 29.93 ± 3.06%, 16.53 ± 3.37% and 9.06 ± 2.07%, respectively.

Apoptotic cells were also analyzed by TUNEL staining, an immunostaining method widely used for detecting DNA fragmentation *in situ*. The effect of ghrelin on H_2O_2-induced apoptosis was examined by the TUNEL assay to determine the extent of DNA fragmentation. Treatment with 500 μM H_2O_2 significantly increased the proportion of TUNEL-positive cells (Fig. 5). Following preincubation with ghrelin (0.01, 0.1 or 1 μM), the number of TUNEL-positive cells was markedly reduced; this protective effect was dose-dependent. These results demonstrate that ghrelin significantly reduces apoptosis cells induced by H_2O_2 in a dose-dependent manner.

3.4. Ghrelin attenuated the generation of intracellular ROS

One potential mediator of H_2O_2-induced cell damage is the secondary generation of ROS. To determine whether ghrelin attenuates cell death by blocking ROS generation, the intracellular ROS concentration was measured using the DCFDA assay. Treatment of H9c2 cells with H_2O_2 significantly increased intracellular ROS generation, but this was significantly reduced by ghrelin pretreatment in a concentration-dependent manner (Fig. 6).

3.5. Ghrelin rescued the loss of ΔΨm

The loss of mitochondrial ΔΨm is an important event associated with apoptosis. To determine whether mitochondria participated in H_2O_2-induced apoptosis in H9c2 cells, the ΔΨm was assessed using the JC-1 assay. H9c2 cells were cultured with 500 μM H_2O_2 and ghrelin (0.01, 0.1 and 1 μM respectively) for 18 h. The ΔΨm markedly decreased in H9c2 cells treated with 500 μM H_2O_2 for 18 h, indicating that H_2O_2 treatment induced mitochondrial dysfunction (Fig. 7). Ghrelin pretreatment significantly improved the H_2O_2-induced impairment of the ΔΨm in a dose-dependent manner.

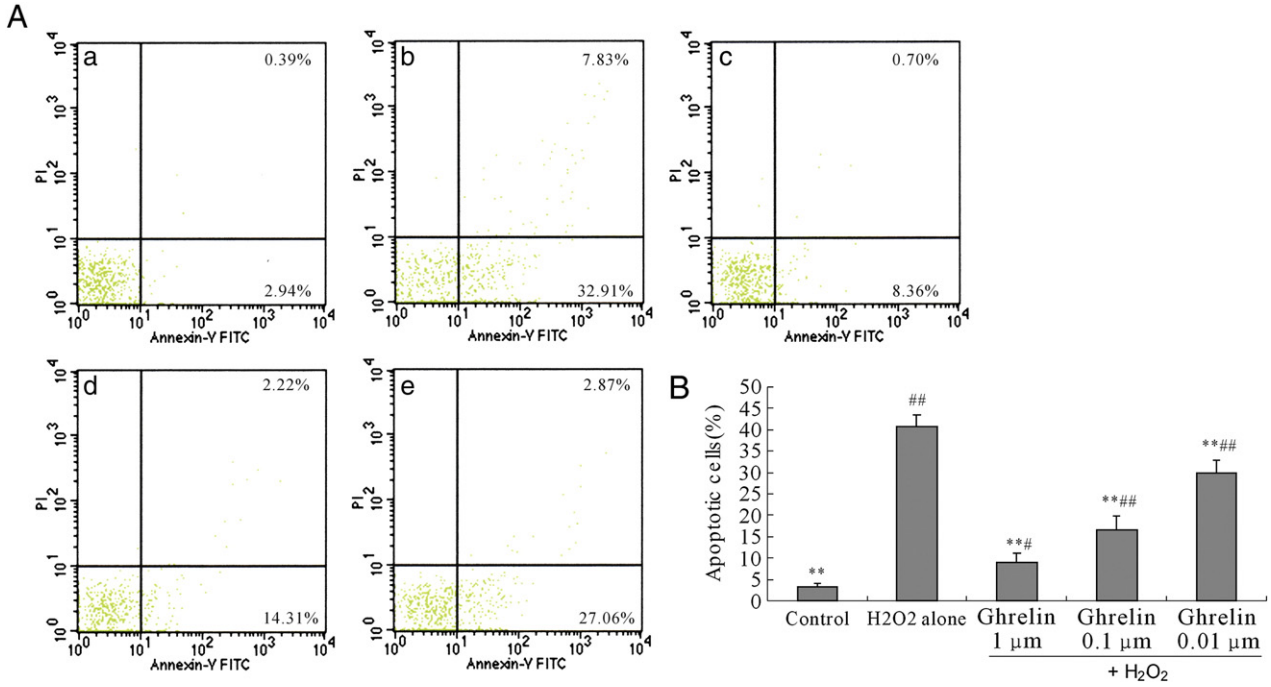

Fig. 4. Ghrelin blocked apoptosis of H9c2 cells induced by H_2O_2. Apoptotic cells were detected by Annexin V and propidium iodide double staining. The graph in (A) shows a representative experiment. In (B), data were calculated as a percentage of apoptotic cells. The data are presented as the mean ± S.D. **P<0.01 compared with H_2O_2 treatment alone, #P<0.05 compared with control, ##P<0.01 compared with control.

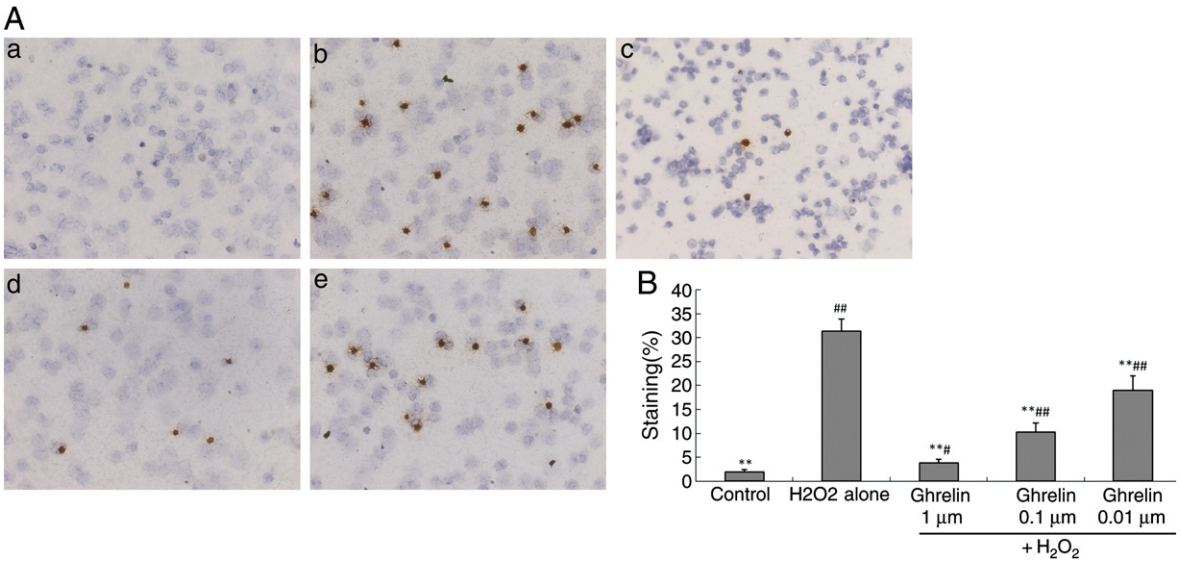

Fig. 5. Ghrelin blocked apoptosis of H9c2 cells induced by H_2O_2. TUNEL staining performed after H_2O_2 treatment, developed with stable DAB. Brown coloration indicates apoptotic cells. (A) H9c2 cells without H_2O_2 or ghrelin treatment (Control); (B) H9c2 cells with only 500 μM H_2O_2 treatment; (C–E) H9c2 cells with pretreated with 1, 0.1 and 0.01 μM ghrelin followed by the treatment of 500 μM H_2O_2. Images are representative of three separate experiments. In the graph, data were calculated as a percentage of brown coloration cells and are presented as the mean ± S.D. $^{**}P<0.01$ compared with H_2O_2 treatment alone, $^{\#}P<0.05$ compared with control, $^{\#\#}P<0.01$ compared with control. Magnification: 200×.

3.6. Ghrelin altered Bcl-2 and Bax expression, as well as caspase activation induced by H_2O_2

To investigate the molecular mechanisms by which ghrelin protects H9c2 cells from H_2O_2-induced oxidative stress injury, we examined Bcl-2, Bax, active caspase-9 and phosphor-NF-κB protein levels to determine whether the regulation of these cell death-associated proteins is responsible for the protective effect of ghrelin (Fig. 8). As expected, we found that ghrelin (0.01, 0.1 or 1 μM) prevented H_2O_2-induced downregulation of Bcl-2 and upregulation of Bax, caspase-9 activation and NF-κB phosphorylation, thus decreasing the Bax/Bcl-2 expression ratio. These changes in Bax, Bcl-2, active caspase-9 and phospho-NF-κB were dose-dependent.

The activation of caspase-3 is an important biomarker of the apoptosis process. A significant increase in caspase-3 activity was detected in H9c2 cells treated with 500 μM H_2O_2 compared with the control (0.84 ± 0.07 vs 0.11 ± 0.02; Fig. 9). When H9c2 cells were cultured with 500 μM H_2O_2 and ghrelin (0.01, 0.1 or 1 μM) for 18 h, caspase-3 activity decreased to 0.74 ± 0.08, 0.46 ± 0.06 and 0.20 ± 0.02, respectively. Ghrelin significantly suppressed caspase-3 activation in the H_2O_2-treated H9c2 cells in a dose-dependent manner.

4. Discussion

In addition to stimulating growth hormone secretion and regulating appetite and metabolism, many studies have showed that ghrelin has a protective action in many cardiac and vascular diseases (Matsumura et al., 2002; Shimizu et al., 2003; Wiley and Davenport, 2002; Enomoto et al., 2003; Nagaya et al., 2001b). However, the cellular and molecular mechanisms underlying ghrelin cardioprotective activity are not well known. Previous studies showed that ghrelin could decrease oxidative injury in the stomach (Eter et al., 2007), brain (Obay et al., 2008), blood vessels (Kawczynska-Drozdz et al., 2006) and liver (Iseri et al., 2008; Obay et al., 2008). In our study, cell morphology and the MTS assay revealed that the proportion of apoptotic cells induced by H_2O_2 decreased with increasing ghrelin concentration. Similarly, flow cytometry and TUNEL analysis illustrated that ghrelin blocks H9c2 apoptosis induced by H_2O_2. We also found that ghrelin pretreatment can reduce intracellular ROS generation and improved the H_2O_2-induced impairment of the ΔΨm. These protective effects may contribute to the antioxidant activity of this peptide.

Oxidative stress in cardiomyocytes has an important role in the pathogenesis of both heart failure and ischemic–reperfusion injury.

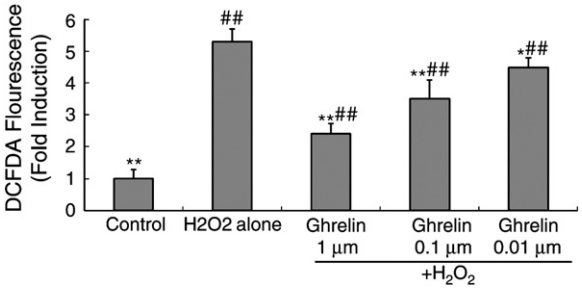

Fig. 6. Ghrelin attenuated the generation of intracellular ROS induced by H_2O_2. The intracellular ROS concentration was measured using the DCFDA assay. $^{*}P<0.05$ compared with H_2O_2 treatment alone, $^{**}P<0.01$ compared with H_2O_2 treatment alone, $^{\#\#}P<0.01$ compared with control.

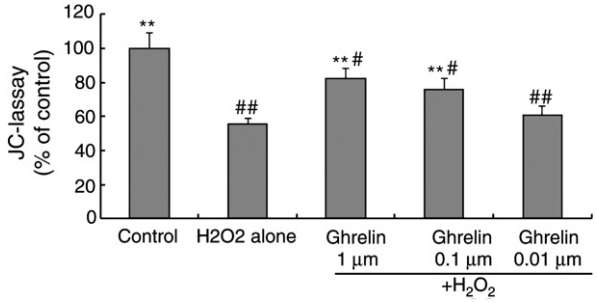

Fig. 7. Ghrelin restored the loss of ΔΨm induced by H_2O_2. H9c2 cells were cultured with 500 μM H_2O_2 and ghrelin (0.01, 0.1 or 1 μM) for 18 h. The ΔΨm was assessed using the JC-1 assay. $^{**}P<0.01$ compared with H_2O_2 treatment alone, $^{\#}P<0.05$ compared with control, $^{\#\#}P<0.01$ compared with control.

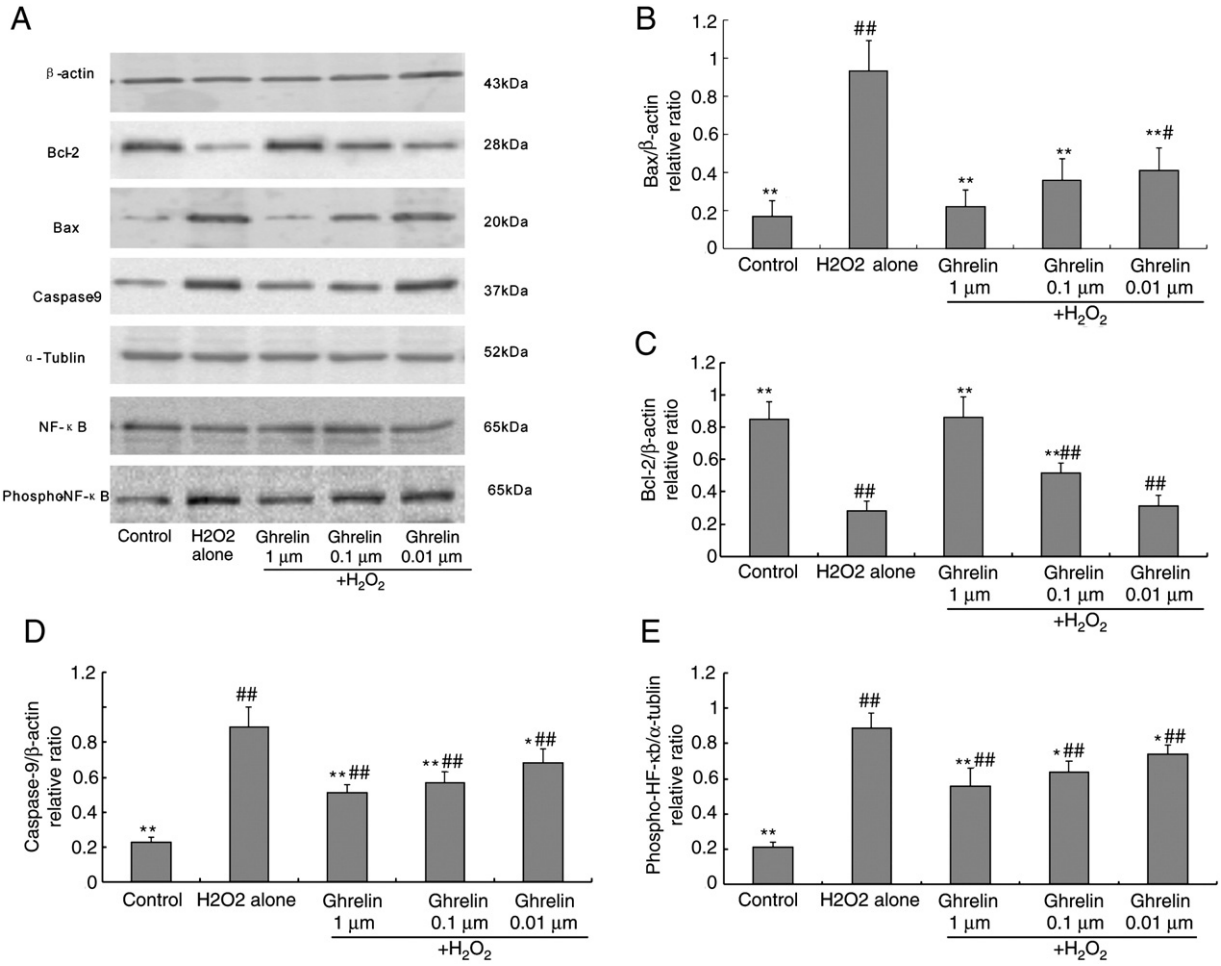

Fig. 8. Effect of ghrelin on the expression of proteins in H_2O_2-treated H9c2 cells. The expression of Bcl-2, Bax, active caspase-9, NF-κB and phospho-NF-κB in treated H9c2 cells was detected by Western blot analysis (A). (B–E) The ratio between the band of interest and the β-actin or α-tubulin band. *P<0.05 compared with H_2O_2 treatment alone, **P<0.01 compared with H_2O_2 treatment alone, #P<0.05 compared with control, ##P<0.01 compared with control.

Recent investigations demonstrated that ghrelin has antioxidant effects that prevented doxorubicin or high glucose and sodium palmitate-induced cardiotoxicity; these processes involved activation of extracellular signal-regulated kinase-1/2, Akt serine kinases and NF-κB, as well as up-regulation of TNF-α (Baldanzi et al., 2002; Xu et al., 2008; Liu et al., 2009). Most studies favor free radical-induced oxidative stress as pivotal in doxorubicin-induced cardiotoxicity, as doxorubicin generates ROS during its metabolism (Iarussi et al., 2001; Neilan et al., 2007; Wallace, 2003). But other contributors to doxorubicin-induced cardiotoxicity include dysregulation of calcium handling, adrenergic dysfunction, and selective inhibition of cardiomyocyte-specific genes expression (Iarussi et al., 2001; Takemura and Fujiwara, 2007). H_2O_2, superoxide and hydroxyl radicals, as the major ROS, have been used frequently in models of oxidative stress in H9c2 cardiomyocytes (Yasuoka et al., 2004; Murata et al., 2003; Han et al., 2004; Eguchi et al., 2008). Some studies implied that the induction of an apoptotic process during sustained H_2O_2 stimulation, which is not evident during transient stress. When cells were treated with increasing concentrations of H_2O_2 for 18 h, a dose-dependent reduction was detected in cell viability using the MTS assay. Five hundred micromolar of H_2O_2 was used in subsequent experiments. This concentration may be higher than previous studies, because the cells were cultured without calf serum in previous studies. We found that cell viability decreased significantly with less than 2% calf serum, so we used 2% calf serum in our experiments, which may influence the lethal concentration of H_2O_2.

Apoptosis is executed by caspases activated by signal transduction pathways. The investigations found that mitochondria play a prominent role in transduction and amplification of the apoptotic response (Suleiman et al., 2001). Once ROS are generated, they increase mitochondrial permeability, which results in cytochrome c release. Bcl-2 family member proteins may regulate the release of mitochondrial cytochrome c during oxidative stress in cardiomyocytes. Cell survival is enhanced when Bcl-2 expression is relatively high, but Bax expression is low. Caspases are downstream of Bcl-2 family in the apoptotic cascade. Pro-caspase-3 is cleaved by active caspase-9 to active caspase-3, which along with caspases-6 and -7 are executioner caspases, They activate a DNase which is responsible for the fragmentation of oligonucleosomal DNA. Our study found that ghrelin downregulated H_2O_2-induced

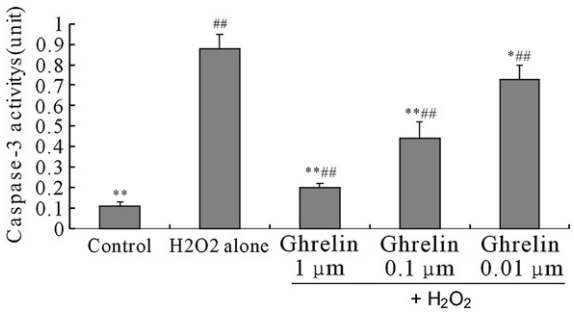

Fig. 9. Ghrelin suppressed the activation of caspase-3 in H_2O_2 treated H9c2 cells. Caspase-3 activity was measured with a caspase colorimetric activity assay kit. *P<0.05 compared with H_2O_2 treatment alone; **P<0.01 compared with H_2O_2 treatment alone. ##P<0.01 compared with control.

expression of the proapoptotic protein Bax and upregulated Bcl-2 expression, which was accompanied by downregulated active caspase-9 expression and delayed reduced caspase-3 activation. These results demonstrated that the antiapoptotic effect of ghrelin was probably due to mitigated H_2O_2 stress-induced mitochondrial dysfunction.

We also found that H_2O_2-induced NF-κB protein phosphorylation was attenuated by ghrelin in a concentration-dependent manner. The transcription factor NF-κB is a critical signaling molecule in oxidant stress responses. NF-κB is sequestered in the cytoplasm, bound by members of the I-κB family of inhibitor proteins. Phosphorylation of I-κB by an I-κB kinase complex exposes nuclear localization signals on the NF-κB subunits, such that it translocates to the nucleus. In the nucleus, NF-κB binds with consensus sequences of various genes, activating their transcription. Therefore, a potential mechanism whereby ghrelin could modulate antioxidant responses to H_2O_2 is by blocking activation of NF-κB. Our result is consistent with data demonstrating that ghrelin inhibits NF-κB activation in HUVECs and lung epithelial cell line A549 (Wei et al., 2004; Hou et al., 2009). The molecular mechanisms leading to inhibition of NF-κB activation by ghrelin and the relationship to the mitochondrial pathway remain to be determined.

In conclusion, our study demonstrates that ghrelin rescues H9c2 cells from H_2O_2-induced cell death, and that its protective effect may be related to its antioxidant effects. Notably, the mechanism whereby ghrelin prevents apoptosis appears to involve downregulated H_2O_2-induced Bax expression and caspase-9 activation, but upregulated Bcl-2 expression accompanied by reduced caspase-3 activation. We also found that ghrelin inhibited H_2O_2-induced NF-κB activation. Our current *in vitro* study suggests that ghrelin exerts a protective effect against H_2O_2-induced apoptosis in H9c2 rat cardiomyocytes by preventing activation of the mitochondrial pathway and the transcription factor NF-κB. Future investigations will be necessary to determine the upstream molecular mechanisms as well as the *in vivo* relevance of our findings. Our data suggest that ghrelin may have an important role in preventing oxidant-induced heart disease.

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