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Ghrelin inhibits apoptosis induced by high glucose and sodium palmitate in adult rat cardiomyocytes through the PI3K-Akt signaling pathway

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ABSTRACT

Objective: Ghrelin is a gastric acyl-peptide that has been identified as an endogenous ligand for the growth hormone secretagogue receptor. It has been reported to have cardioprotective activities independent of growth hormone release. We investigated the effect of ghrelin on apoptosis induced by high glucose and sodium palmitate and the mechanisms underlying the cardioprotective activities of ghrelin.
Research design and methods: Cardiomyocytes were isolated from hearts of adult rats and cultured in serum-free MEM. High glucose (30 mM) or sodium palmitate (0.5 mM) were used to induce apoptosis. Apoptosis was detected using an annexin V-FITC/PI binding assay and a caspase 3 activity assay. Reactive oxygen species were detected using a DCFH-DA fluorescent probe. Phospho-Akt, phospho-ERK, and NFκB levels were determined using ELISA. The transcription of genes was analyzed using real-time PCR.
Results: Ghrelin can inhibit apoptosis induced by oxidative stress in cardiomyocytes from adult rats through the activation of the PI3K-Akt signaling pathway. In addition, ghrelin does not decrease intracellular oxidative stress. Activation of the MEK-ERK1/2 signaling pathway has no influence on the inhibition of apoptosis. Finally, ghrelin activates NFκB and subsequently increases the transcription of survival genes such as Bcl-2, Bcl-xL, c-*iap*, and c-*fos*.
Conclusion: Our research provides evidence that ghrelin may act as a survival factor under oxidative stress in cardiomyocytes. This may provide a clue for therapy for myocardial disease in diabetes mellitus.

1. Introduction

Ghrelin is a 28-amino acid acyl-peptide that is esterified with octanoic acid on Ser 3 and is a ligand for the growth hormone secretagogue receptor (GHS-R) [1]. Ghrelin is widely expressed, but is mainly produced in the stomach of adults. Ghrelin acts centrally to stimulate growth hormone secretion and food intake, and acts peripherally to regulate energy homeostasis [2].

Recent evidence indicates that ghrelin also has a cardiovascular function. mRNAs coding GHS-R and ghrelin were detected in the heart and aorta [3]. An isotype-labeled ghrelin, [125I-His9] ghrelin, was shown to bind to heart and vascular tissues of normal humans and rats [4]. The intravenous injection of ghrelin into healthy volunteers decreased blood pressure without changing heart rate [5], and ghrelin increased the cardiac index and stroke volume. Ghrelin administered intravenously to patients with chronic heart failure increased the LV ejection fraction in association with an increase in the LV mass and a decrease in the LV end-systolic volume [6]. Furthermore, the subcutaneous administration of ghrelin increased the diastolic thickness of the non-infarcted posterior wall, inhibited LV enlargement, and increased the LV fractional shortening in chronic heart failure in rats [7]. Thus, ghrelin can improve LV dysfunction and attenuate the development of LV remodeling and cardiac cachexia.

Heart failure is a frequent cardiovascular complication of patients with diabetes. Diffuse coronary artery disease (CAD) can lead to nontransmural infarction with patchy necrosis and myocardial fibrosis, which leads to congestive heart failure [8,9]. However, CAD is not the only cause of congestive heart failure in diabetes: cardiomyopathy also plays an important role. In the 35- to 64-year-old Framingham cohort, diabetes increased the risk of congestive heart failure in men four-fold and in women eight-fold, even after adjustment for a history of CAD [10]. The more recent Washington DC Dilated Cardiomyopathy Study used case-controlled analyses to determine that there was an association between diabetes and idiopathic cardiomyopathy [11]. The loss of myocytes is a feature of cardiomyopathy that contributes to a progressive decline in left ventricular function and congestive heart failure [12,13]. During the last few years there has been increasing evidence from human and animal models suggesting that cardiomyocyte apoptosis could be a key modulator in heart failure. Failing hearts classified as NYHA class III–IV typically display apoptotic rates ranging anywhere between 0.12% and 0.70% in human biopsies [14–16]. However, one should take into account that the documented apoptotic rates were obtained at a single time point in the disease, and cellular apoptosis is a process that

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can take at most 24 h to complete. As heart failure is a condition that only manifests itself after many years, it is conceivable that the chronic loss of small numbers of cardiomyocytes on a daily basis can have dramatic consequences on myocardial function and integrity. Therefore, cardiomyocyte apoptosis may have a pivotal role in heart failure.

High glucose and free fat acids are thought to be important risk factors of non-ischemic heart failure in diabetes mellitus [17]. They induce cardiomyocyte apoptosis by oxidative stress, which is the main mechanism of non-ischemic heart failure [18]. Therefore, the inhibition of apoptosis caused by oxidative stress may be a therapeutic target for prevention of non-ischemic heart failure in diabetes mellitus. However, the role of ghrelin in cardiomyocyte apoptosis induced by oxidative stress has not yet been determined.

Based on the cardiac protection afforded by ghrelin, we hypothesized that ghrelin may protect cultured cardiomyocytes from apoptosis induced by high glucose or free fatty acids through a specific survival signaling pathway. Here, we demonstrate that ghrelin inhibits the apoptosis of cardiomyocytes through the activation of a survival signaling pathway, and not through a decrease in intracellular oxidative stress.

2. Materials and methods

2.1. Animal, chemicals, peptides, and reagents

Adult male Sprague–Dawley rats aged eight to twelve weeks were provided by the Animal Center of Fudan University. Mouse ghrelin, sodium palmitate (spa), glucose, 2,3-butandione monoxime, and ITS (5 mg/ml insulin, 5 mg/ml transferrin, 5 mg/ml selenium) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Joklik minimal essential medium, bovine serum albumin, collagenase I, and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). The Annexin-v-FITC/PI Apoptosis Detection Kit was purchased from BD Biosciences, and wortmannin and PD98059 were provided by the Beyotime Institute of Biotechnology. The Akt (Ser473) Dual Detect CELISA Assay Kit, the ERK 1/2 (Thr202/Tyr204)/(Thr185/Tyr187) Dual Detect CELISA Assay Kit, and the CleavLite Caspase-3 Activity Assay Kit were purchased from Millipore Corporation (Billerica, MA, USA). The BCA Protein Assay Kit was purchased from Pierce (Appleton, WI, USA). The SuperSignal West Femto maximum sensitivity substrate was purchased from Thermo Fisher Scientific (Rockford, IL, USA). The mouse anti-rat GAPDH and anti-human Bcl-2 monoclonal antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). The goat anti-human IAP-1 monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The real-time PCR Master Mix Kit and the ReverTra Ace Kit were purchased from TOYOBO (Osaka, Japan).

2.2. Isolation and culture of adult rat ventricular myocytes

Adult rat ventricular myocytes were isolated as described previously, with little modification [19,20]. Male Sprague–Dawley rats were anesthetized with 10% chloral hydrate (0.4 ml/100 g; ip). The heart was quickly removed from the chest and retrogradely aortic-perfused with perfusion buffer (137.0 mM NaCl, 5.0 mM KCl, 1.2 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 5.6 mM glucose, 10 mM HEPES, 10 mM 2,3-butanedione monoxime, pH 7.4) at 10 ml/min for 5 min. It was then switched to a digestion buffer (perfusion buffer with 1 mg/ml collagenase type I) and digested for 8–10 min, when the heart became swollen and turned slightly pale.

The ventricles were placed in a 60 mm chamber containing 20 ml of stopping buffer (70 mM KOH, 40 mM KCl, 20 mM KH_2PO_4, 50 mM glutamine, 3 mM $MgCl_2$, 20 mM taurine, 0.5 mM EGTA, 10 mM HEPES, and 5.6 mM glucose), and the tissue was cut into chunks. The supernatant containing the dispersed myocyte suspension was filtered into a 60 mm dish. After a gradient calcium reintroduction to 1.5 mM, the supernatant was transferred to a sterile tube and the myocytes were allowed to sediment by gravity for 8–10 min. The pelleted cardiomyocytes were suspended in plating medium (Joklik minimal essential medium, 100 U/l streptomycin, 100 U/l penicillin, 1.5 mM $CaCl_2$, 10 μl/ml ITS, 10 mM/l 2,3-butandione monoxime, and 0.1 mg/ml BSA). The cardiomyocytes were plated at $0.5–1 \times 10^4$ cells/cm^2 in 10 μg/ml laminin-coated dishes or plates. The dishes or plates were immediately placed in a 5% CO_2 incubator at 37 °C in order to allow for myocyte attachment. After 1 h, the medium was changed to MC culture medium (Joklik minimal essential medium, 100 U/l streptomycin, 100 U/l penicillin, 1.5 mM $CaCl_2$, and 0.1 mg/ml BSA). The unattached cardiomyocytes were removed with the plating medium, whereas the attached cardiomyocytes were cultured in MC culture medium. All solutions were sterilized and kept at 37 °C during the experiment. The criteria for viable rat cardiomyocytes were: (a) rod shape, (b) clearly defined sarcomeric striations, (c) quiescent state (i.e., no spontaneous contractile waves) for at least 5 min during observation, and (d) rejection of trypan blue. Before use, cardiomyocytes were cultured in MC culture medium lacking BDM and ITS for 24 h in order to eliminate the effects of BDM and ITS on ghrelin signal transduction.

2.3. Measurement of intracellular reactive oxygen species formation

The cardiomyocytes were pretreated with 1 μM ghrelin for 24 h and then treated with high glucose (30 mM) or sodium palmitate (0.5 mM) for 1 h. The level of intracellular reactive oxygen species (ROS) was monitored using the peroxide-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) and a fluorescence plate reader. DCFH-DA is converted by intracellular esterases to DCFH, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant. Samples were loaded with 1 mM DCFH-DA in phosphate buffered saline (PBS) in the dark for 10 min at 37 °C. After incubation, the cells were washed with PBS three times and analyzed immediately on the fluorescence plate reader (Model 450, BioRad, Hercules, USA). The DCF fluorescence intensity of cells in 96-well plates was quantified at an excitation of 485 nm and an emission of 538 nm. Fluorescence photomicrographs of cardiomyocytes on glass-bottom dishes were obtained with an Olympus inverted fluorescence microscope.

2.4. Assessment of apoptosis by annexin V-FITC staining

Apoptotic cells were detected by annexin V-FITC/PI staining. In apoptotic cells, the membrane phospholipid phosphatidylserine is exposed to the extracellular environment. Annexin V binds to cells with exposed phosphatidylserine. After treatment with high glucose (30 mM) or sodium palmitate (0.5 mM) for 1 h, cardiomyocytes plated on glass-bottom dishes were incubated with 5 μl annexin V in 500 μl MC culture medium at room temperature (27 °C) in the dark for 10 min and were then washed three times with PBS. The total number of cardiomyocytes and annexin V-bound cardiomyocytes was counted using an inverted fluorescence microscope. At least three randomly selected fields were counted and the average number of cardiomyocytes in the three fields for each dish was counted. The total number of cardiomyocytes or annexin V-bound cardiomyocytes in each dish equaled the product of the mean number of cardiomyocytes or annexin V-bound cardiomyocytes and the area factor. The "area factor" was empirically determined for each microscope objective/eyepiece that was used. In order to calculate the area factor, we used a stage micrometer to measure the diameter of the microscopic field for a 20× objective and eyepiece. We calculated the area of the circular microscopic field and then divided the area of the dish (supplied by the manufacturer and measured directly) by the area of the field in order to derive the area factor.

2.5. Assessment of apoptosis by caspase-3 activity

The catalytic activity of caspase-3 was determined using a CleavLite Caspase-3 Activity Assay Kit, according to the manufacturer's instructions. Cardiomyocytes that were cultured in 6-well plates were scraped off in PBS after treatment and collected by centrifugation at 1000 ×*g* for 10 min at 4 °C. The cells were resuspended to 1×10^5/ml with ice-cold cell lysis buffer, incubated for 5 min on ice bath, and centrifuged at 10,000 ×*g* for 10 min at 4 °C. The protein concentration in the supernatant was determined using the BCA Protein Assay Kit. The cell lysate (50 µl), 2× reaction buffer (50 µl), and caspase-3 fluorogenic substrate (5 µl; DEVD-AFC) were loaded a well of a 96-well plate and the plate was incubated at 37 °C in the dark for 1 h. The plates were read on a fluorescence plate reader set at an emission of 505 nm (to detect the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (AFC) that represented caspase-3 activity).

2.6. ELISA for ERK1/2 and Akt

Phospho-ERK1/2 (Thr202/Tyr204)/(Thr185/Tyr187) and phosphor-Akt (Ser473) were detected using the Fluorogenic Detection CELISA Assay Kits, according to the manufacturer's instructions. Briefly, cardiomyocytes were plated in 96-well plates. Following stimulation with 1 µM ghrelin, the cardiomyocytes were immediately fixed with 4% formaldehyde for 20 min at room temperature. After washing in PBS, cells were permeabilized with Quenching Buffer (1% H_2O_2 in TBST) for 20 min at room temperature, blocked in Blocking Buffer (10% BSA in TBS), and then incubated with a mouse anti-Akt (Total) antibody, a rabbit anti-phospho Akt (Ser473) antibody, a mouse anti-ERK (Total) antibody, or a rabbit anti-phospho-ERK1/2 (Thr202/Tyr204,Thr185/Tyr187) antibody in TBST overnight at 4 °C with gentle agitation. The plates were subsequently incubated with a secondary HRP-conjugated goat anti-mouse antibody and an AP-conjugated goat anti-rabbit antibody for 1 h at room temperature. The substrate mixture (100 µl) was added and the plate was incubated for 20–60 min at room temperature, in the dark. The plate was read using fluorescence plate reader to detect the HRP signal (590 nm) and the AP signal (460 nm). The readings at 590 nm represented the amount of total Akt or ERK1/2 in the cells, whereas the readings at 460 nm represented the amount of phosphorylated Akt or ERK1/2 in the cells.

2.7. ELISA NFκB

The nuclear translocation of NFκB was detected by using the NFκB p50/p65 EZ-TFA Transcription Factor Assay kit, according to the manufacturer's instructions. A total of 0.5×10^5 cardiomyocytes were plated in dishes for each sample. Following stimulation with 1 µM ghrelin, the cells were collected in a microcentrifuge tube. The cells were pelleted for 10 s and resuspended in cold Buffer A (10 mM HEPES–KOH pH 7.9 at 4 °C, 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM dithiothreitol, 0.2 mM PMSF). The cells were incubated on ice for 10 min and then vortexed for 10 s. Samples were centrifuged for 10 s. The pellet was resuspended in cold Buffer B (20 mM HEPES–KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min. The samples were then centrifuged at 4 °C for 10 s. The supernatant fraction (the nuclear protein extract) was stored at −80 °C or used immediately. The capture probe, a double-stranded biotinylated oligonucleotide containing the DNA-binding consensus sequence for NFκB (5′-GGGACTTTCC-3′), was incubated with the nuclear protein extract solution for 30 min at room temperature. The extract/probe/buffer mixture was then transferred to a streptavidin-coated plate and incubated for 1 h. The active NFκB protein was then immobilized to the biotinylated double-stranded oligonucleotide capture probe, which was bound to the streptavidin plate well, and any inactive, unbound material was washed away. The bound NFκB transcription factor subunits were detected using specific rabbit anti-NFκB p50 and p65 antibodies and, subsequently, a secondary HRP-conjugated antibody. The plate was read using a fluorescence plate reader to detect the HRP signal at 590 nm, which represented the amount of NFκB in the nucleus.

2.8. Western blot analysis

Cardiomyocytes were stimulated with 1 µM ghrelin for 24 h and then lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1% NP-40, 10 mM NaF, 100 µM leupeptin, 2 µg/ml aprotinin, and 1 mM PMSF) by pipetting repeatedly. The samples were then centrifuged at 20,000 ×*g* for 30 min at 4 °C and the supernatant was collected. The cell lysates (10 µg of total protein) were electrophoresed in 12% SDS-PAGE gels and the proteins were transferred to a nitrocellulose filter membrane. The membranes were blocked with 5% nonfat dry milk in TBST at room

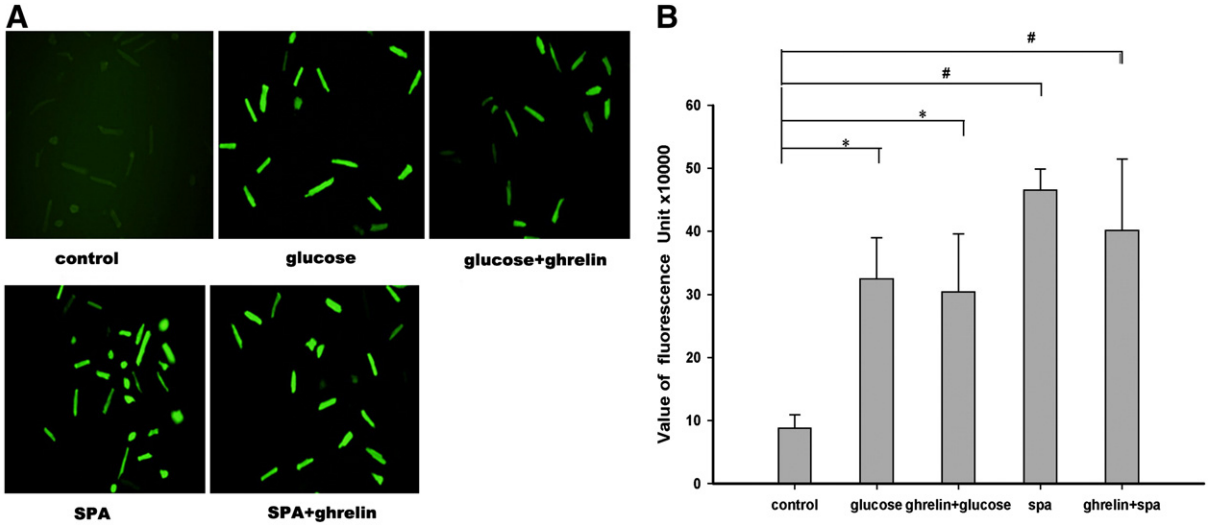

Fig. 1. High glucose and sodium palmitate (spa) induce oxidative stress in cardiomyocytes. Cardiomyocytes were treated with high glucose (30 mM) or spa (0.5 mM) for 1 h. The glucose + ghrelin and spa + ghrelin groups were pretreated with 1 µM ghrelin for 24 h. (A) Intracellular oxidative stress was detected using DCFH-DA following treatment with high glucose or spa for 1 h. (B) Fluorescence intensity measured by fluorescence plate reader. The values are presented as the mean ± SE of eight samples ($^{\#}t$-test, $P<0.01$; $^{*}t$-test, $P<0.05$).

temperature for 1 h and then incubated with a 1:500 dilution of mouse anti-human Bcl-2 or rabbit anti-rat IAP-1 for 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG were used as the secondary antibodies at a 1:2000 dilution. Bands were visualized using the ECL chemiluminescence detection method with the SuperSignal West Femto maximum sensitivity substrate and analyzed using a Fujifilm LAS-3000 Imaging System (FUJIFILM Corporation, Tokyo, Japan).

2.9. Relative real-time qPCR

Total mRNA was extracted by Trizol and reverse transcribed into cDNA at 42 °C for 1.5 h and at 72 °C for 15 min using the ReverTra Ace Kit. Real-time qPCR was performed with a 5 min incubation at 95 °C and 40 amplification cycles (15 s at 94 °C, 15 s at 63 °C, and 45 s at 72 °C). The primer sequences were: c-*iap* Sense primer 5′-TTCGGAAGAATAGAATGG-3′, c-*iap* Anti-sense primer 5′-TGCTTGTAAGGGTATCTG-3′; c-*fos* Sense primer 5′-GCCTTTCCTACTACCATTCC-3′, c-*fos* Anti-sense primer 5′-ATCT-TATTCCTTTCCCTTCG-3′; Bcl-2 Sense primer 5′-CCCCAGA-AGAAACTGAACC-3′, Bcl-2 Anti-sense primer 5′-GCATCTCCTTGTCTACGC-3′; Bcl-xL Sense primer 5′-CGGGAGAACAGGGTATGA-3′, Bcl-xL Anti-sense primer 5′-CAGGCTGGAAGGAGAAGAT-3′; GAPDH Sense primer) 5′-GCCT-TCCGTGTTCCTACC-3′, GAPDH Anti-sense primer) 5-GCCCCTCCTGTTGT-TATG-3′. The amplification and data acquisition were run on a real-time PCR system (ABI Prism 7700, Applied Biosystems, Foster City, CA, USA).

2.10. Statistical analysis

The results are presented as the mean ± SE. The data was analyzed using a one-way ANOVA with Bonferroni post hoc tests for multiple comparisons. Student's *t*-test was used for two unpaired groups. Any *P*-value less than 0.05 was considered statistically significant.

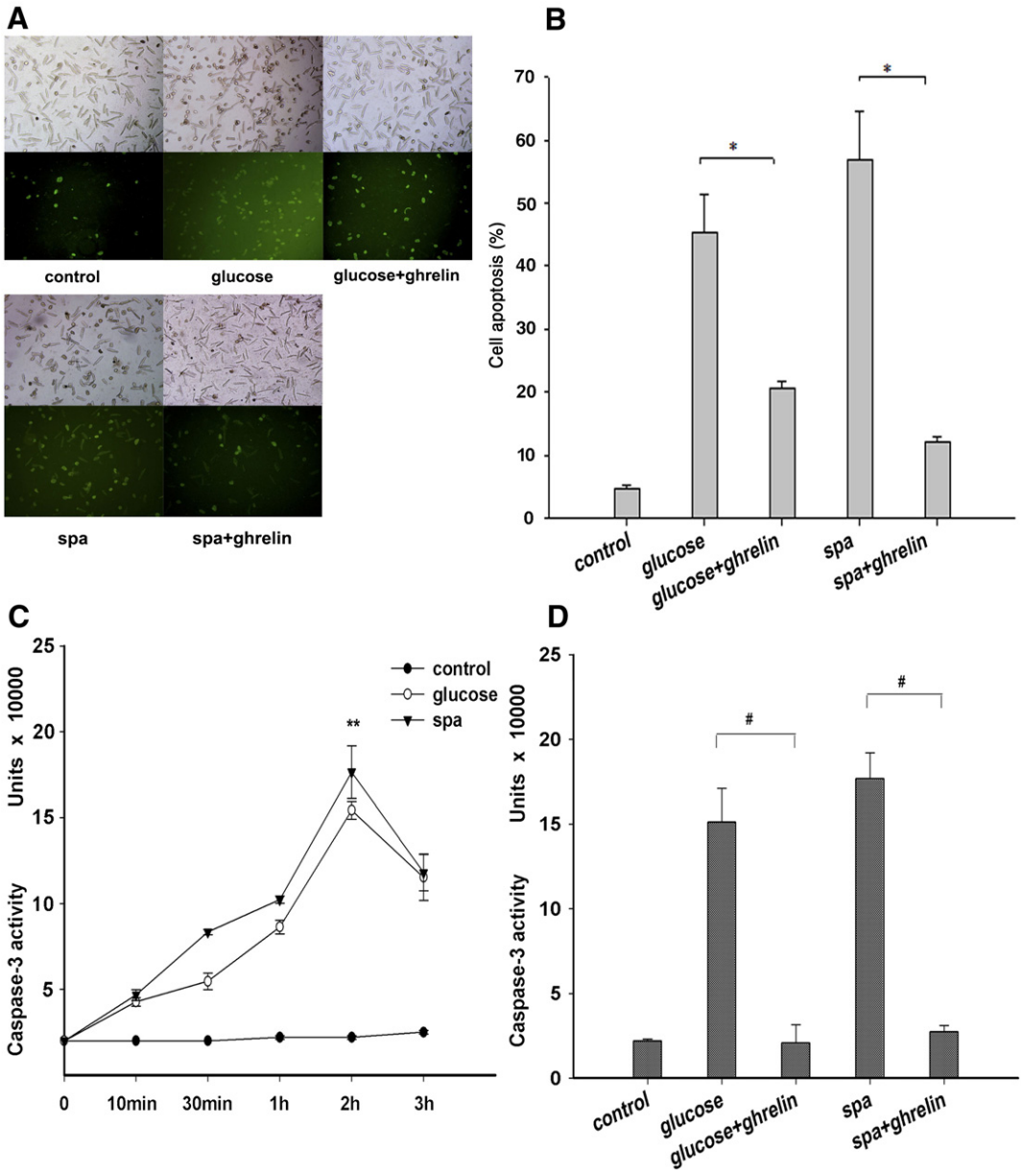

Fig. 2. Ghrelin inhibits apoptosis induced by high glucose and spa. Cardiomyocytes were treated with high glucose (30 mM) or spa (0.5 mM) for 2 h. The glucose + ghrelin and spa + ghrelin groups were pretreated with 1 μM ghrelin for 24 h. (A) Apoptosis was detected using FITC-conjugated annexin V. (B) The rate of apoptosis was detected using annexin V. Data were calculated as a percentage of apoptotic cells. The values are presented as the mean ± SE of three samples (*t-test, $P<0.05$). (C) Time course of caspase-3 activity in apoptotic cardiomyocytes. Values are presented as the mean ± SE of three samples (**one-way ANOVA, $P<0.05$). (D) Apoptosis was measured using a caspase-3 assay and is expressed as the activity of caspase-3. The values are presented as the mean ± SE of three samples ($^{\#}t$-test, $P<0.05$).

3. Results

3.1. High glucose and sodium palmitate induce oxidative stress in cardiomyocytes

Reactive oxygen species induced by high glucose and sodium palmitate (spa) in diabetes is one of the important causes of cardiac complications. In order to investigate the effect of high glucose and spa on oxidative stress in cardiomyocytes, we treated cardiomyocytes that had been preincubated with ghrelin or media alone for 24 h with high glucose (30 mM) or spa (0.5 mM). After a 1 h treatment with high glucose and spa, intracellular oxidative stress was measured using a DCFH-DA fluorescent probe. Compared to the control group, the fluorescence intensity of the high glucose and spa groups was significantly higher ($P<0.01$; Fig. 1B). However, there was no difference when compared to ghrelin-pretreated groups (glucose + ghrelin and spa + ghrelin). These data demonstrate that high glucose and spa can induce oxidative stress in primary cardiomyocytes from adult rats and that ghrelin does not affect the generation of oxidative stress.

3.1.1. Ghrelin inhibits apoptosis induced by high glucose and spa

Ghrelin has been reported to inhibit apoptosis in H9c2 cardiomyocytes and endothelial cells [21]. Therefore, we examined the effect of ghrelin on oxidative stress-induced apoptosis in primary cardiomyocytes. Cardiomyocytes that were pretreated with ghrelin or media alone for 24 h were incubated with high glucose (30 mM) or spa (0.5 mM). Following a 2 h incubation, high glucose- and spa-induced apoptosis was observed using phase contrast microscopy (Fig. 2A). Viable cells were rod-shaped with sarcomeric striations, whereas apoptotic cells became round and detached from the plate. When observed using a fluorescence microscopy, apoptotic cells stained with FITC-conjugated annexin V and emitted green light, while living cells were not stained (Fig. 2A). In the ghrelin-pretreated groups, the number of apoptotic cells detected by FITC-conjugated annexin V was significantly lower than in the groups not pretreated with ghrelin (Fig. 2B). In order to confirm that ghrelin could inhibit apoptosis induced by high glucose and spa, we measured caspase-3 activity, which is higher in apoptotic cells. When the cells were stimulated with high glucose and spa, caspase-3 activation occurred 10 min after stimulation, peaked at 2 h, and lasted for 3 h (Fig. 2C). In the ghrelin-pretreated groups, the activation of caspase-3 was significantly lower than that in the groups not pretreated with ghrelin (Fig. 2D). Although ghrelin did not affect cell survival in the absence of high glucose or spa, 25–44% of the cardiomyocyte apoptosis was inhibited in the presence of 1 μM ghrelin. Thus, these data indicate that ghrelin inhibits experimentally induced cardiomyocyte apoptosis.

3.1.2. Ghrelin activates a cell survival signaling pathway

In order to explore the possible mechanism responsible for the anti-apoptotic effect of ghrelin, we investigated the ability of ghrelin to stimulate intracellular anti-apoptotic signaling pathways. Survival factors, such as insulin and insulin growth factor-1, inhibit apoptosis by stimulating several intracellular signaling pathways, including tyrosine phosphorylation and the activation of extracellular signal-regulated kinase (ERK)-1/2 and Akt. Ghrelin may function in a similar manner. Wortmannin and PD98059 are highly specific inhibitors of PI3-kinase and ERK1/2-kinase, respectively. The apoptosis rate of the cells treated with wortmannin was much higher than that of the untreated cells, whereas in cells treated with PD98059, the apoptosis rate was the same as in untreated cells (Fig. 3A). This demonstrates that wortmannin abolished the anti-apoptotic activity of ghrelin after 10 min of treatment. In order to provide more evidence, we investigated the effects of ghrelin on the phosphorylation of signaling molecules. Ghrelin activated both Akt and ERK in primary cardiomyocytes from adult rats. The phosphorylation of Akt at Ser473 occurred 5 min after stimulation with 1 μM ghrelin, peaked at 20 min, and lasted for at least 30 min thereafter (Fig. 3B). The phosphorylation of ERK1/2 at (Thr202, Tyr204)/(Thr185, Tyr187) reached a small peak after 10 min of stimulation with 1 μM ghrelin and quickly returned to the baseline level. The peak phosphorylation of ERK1/2 was very low when compared to Akt. However, when compared to phosphorylation at 0 min, 5 min, 20 min, and 30 min, this peak was statistically significant. Therefore, these data suggest that ghrelin can activate the

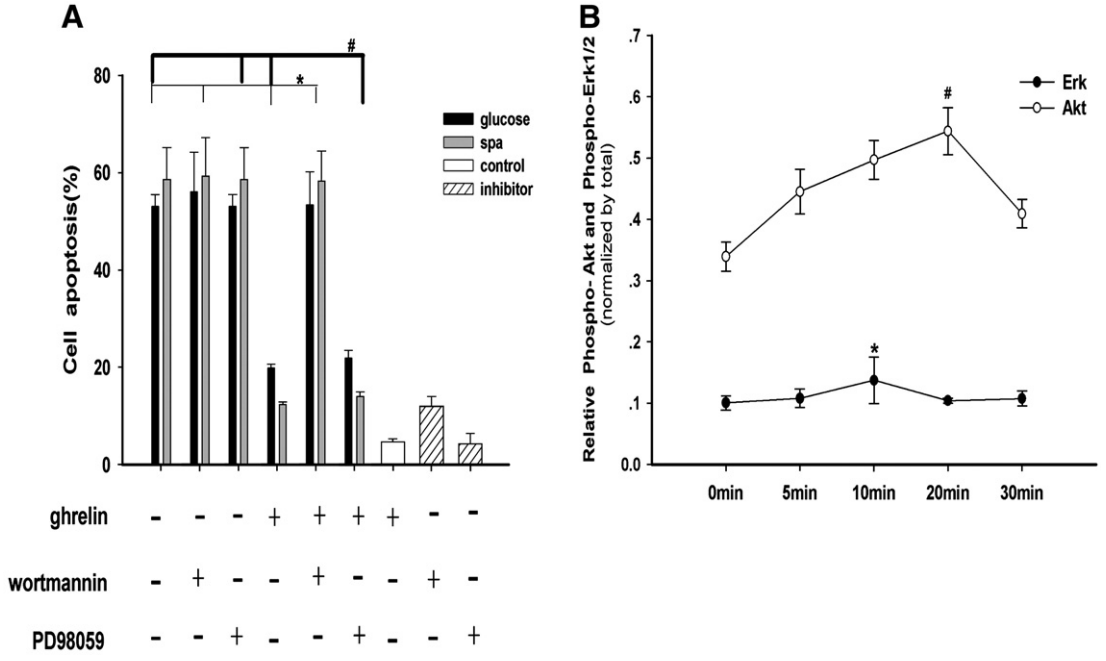

Fig. 3. The effect of pharmacological inhibition on cardiomyocyte apoptosis and the activation of Akt and ERK1/2 by ghrelin. (A) Primary cardiomyocytes were pretreated with 100 nM wortmannin and 100 nm PD950898 for 10 min before adding 1 μM ghrelin to the medium. Twenty-four hours later, the cardiomyocytes were treated with 30 mM glucose and 0.5 mM spa. Apoptosis was detected using FITC-conjugated annexin V and fluorescence microscopy. The values are presented as the mean ± SE of three samples (* # one-way ANOVA, $P<0.05$). (B) The phosphorylation of ERK1/2 and Akt following stimulation with 1 μM ghrelin. Values are presented as the mean ± SE of four samples (* # one-way ANOVA $P<0.05$).

PI3K-Akt and the MEK-ERK1/2 signaling pathways, that blocking the PI3K pathway abolishes the anti-apoptotic activity of ghrelin, and that blocking the MEK-ERK1/2 has no influence on oxidative stress-induced apoptosis in primary cardiomyocytes from adult rats.

3.1.3. Ghrelin promotes NFκB nuclear translocation and increases the expression of survival genes

NFκB is a critical transcription factor in the suppression of apoptosis through its transactivation of anti-apoptotic genes, which are regulated by cellular anti-apoptotic signaling pathways. In order to determine if ghrelin activates NFκB, we analyzed the level of NFκB in the nucleus following ghrelin stimulation. In cardiomyocytes the nuclear translocation of NFκB occurred 20 min after ghrelin stimulation, peaked at 30 min, and lasted for 2 h (Fig. 4A). In order to provide further evidence on relationship between NFκB and the anti-apoptotic signaling pathway, we used the pharmacological inhibitors wortmannin (PI3K inhibitor) and PD98059 (MEK inhibitor) to investigate the specific signaling pathway through which NFκB was activated. In cardiomyocytes, wortmannin inhibited the activation of NFκB by 79.5%. When used in combination with PD98059, wortmannin almost totally blocked the activation of NFκB. Independently, PD98059 demonstrated no effect on the nuclear translocation of NFκB. These data suggested that ghrelin induced the nuclear translocation of NFκB mainly through the PI3K-Akt pathway. The nuclear translocation of NFκB may induce the expression of survival genes. In order to confirm this hypothesis, we analyzed the expression of survival genes in ghrelin-treated cardiomyocytes by real-time qPCR and western blot. The expression of survival genes increased after stimulation with 1 μM ghrelin (Fig. 4B). The expression of bcl-2 and bcl-xL started to increase after 8 h of stimulation and at 20 h of stimulation increased 11.4- and 3.4-fold, respectively, when compared with the control group. The expression of c-*iap* was 17.9-fold that of the control group at 8 h and it began to decrease following 20 h of stimulation. The expression of c-*fos* was unchanged during the first 8 h but increased rapidly after 20 h of ghrelin stimulation, when it was 4.3-fold greater than the control group. In order to have protective activity, the mRNA of survival genes must be translated into proteins. Therefore, we used western blots to detect the expression of IAP-1 and Bcl-2 protein following ghrelin stimulation. After stimulation for 24 h, the protein levels of IAP-1 and Bcl-2 in cardiomyocytes were 3.67- and 2.59-fold higher than that of control group, respectively. These data suggest that activated Akt promotes the nuclear translocation of NFκB and subsequently increases the expression of survival genes.

4. Discussion

During mitochondrial respiration, molecular oxygen is essential for the production of ATP. Under physiological conditions, 0.4–4% of the consumed oxygen is converted to superoxide free radicals [22]. These superoxide free radicals are derivatized to ROS and reactive nitrogen species, which lead to intracellular oxidative stress [22]. Under normal conditions, ROS are cleared by the oxidant defense system. In the

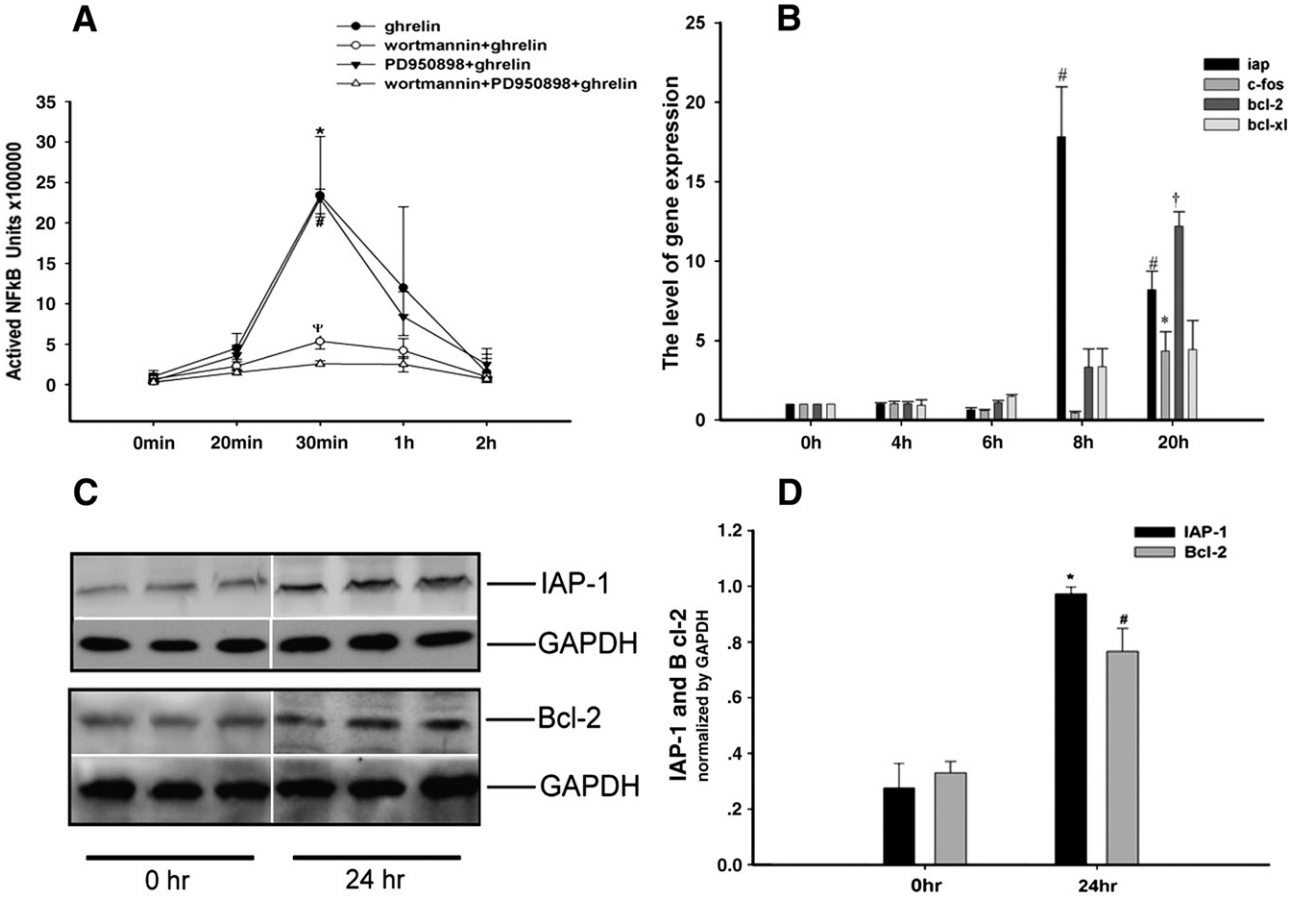

Fig. 4. Ghrelin promotes the nuclear translocation of NFκB and increases the expression of survival genes. (A) Primary cardiomyocytes were pretreated with 100 nM wortmannin and 100 nM PD950898 for 10 min before adding 1 μM ghrelin to the medium. Nuclear extracts were isolated from cardiomyocytes stimulated with 1 μM ghrelin for different lengths of time. NFκB was detected in the nuclear extracts with an ELISA kit. The values are presented as the mean ± SE of three samples (*one-way ANOVA $P<0.05$, one-way ANOVA $P<0.05$, #one-way ANOVA Bonferroni post hoc tests $P>0.05$) (B) The expression of survival genes following stimulation with 1 μM ghrelin for different lengths of time. The values are presented as the mean ± SE of three samples (#one-way ANOVA $P<0.01$, one-way ANOVA *$P<0.05$). (C) Total protein was isolated from cardiomyocytes following a 24 h stimulation with 1 μM ghrelin. Bcl-2 and IAP-1 protein levels were analyzed by western blot using specific antibodies. (D) The western blot intensities were quantified by densitometric analysis of the relative protein levels. The values are presented as the mean ± SE of three samples(* # *t*-test $P<0.05$).

mitochondria, superoxide free radicals are converted to H_2O_2 by SOD2 [23] and H_2O_2 is then decomposed into H_2O and O_2 by glutathione peroxidase [23]. In patients with diabetes mellitus, however, the balance between the oxidant system and the oxidant defense system is disturbed. High glucose and free fat acid lead to mitochondrial dysfunction, which increases the production of superoxide free radicals by inhibiting electron transfer and decreases the clearance super oxide radicals [24]. Additionally, oxidative stress can lead to a significant loss of the mitochondrial membrane potential [25], which can in turn trigger the opening of the mitochondrial permeability transition pore (MPTP) [26,27]. The leakage of cytochrome *C* through MPTP into the cytoplasm initiates the caspase cascade and induces apoptosis [28]. In patients with diabetes, oxidative stress is thought to be an important cause of non-ischemic heart failure [29,30]. Therefore, we cultured adult rat cardiomyocytes in hyperglycemic and free fatty acid conditions in order to mimic the state of patients with diabetes. We found that both high glucose and sodium palmitate could induce intracellular oxidative stress and apoptosis in cardiomyocytes from adult rats and that the oxidative stress and apoptosis induced by sodium palmitate are stronger than those induced by high glucose. In addition, the rate of apoptosis in cardiomyocytes pretreated with ghrelin was lower than those in cells not treated with ghrelin but the intensity of intracellular oxidative stress was the same in the presence or absence of ghrelin. This suggests that ghrelin has no anti-oxidative effect and that the inhibition of apoptosis by ghrelin is mediated through another signaling pathway. Gianluca et al. also found that ghrelin could inhibit Fas- and doxorubicin-induced apoptosis in H9c2 and adult pig cardiomyocytes [21]. Fas and doxorubicin induce apoptosis through an extrinsic pathway and the p53 signaling pathway, respectively [31,32]. Therefore, ghrelin may be a survival factor for cardiomyocytes and it may inhibit apoptosis through a specific anti-apoptotic signaling pathway.

The PI3K-Akt signaling pathway is considered to be one of the key pro-survival pathways within the cell [33]. It is activated by many types of cellular stimulation and regulates fundamental cellular functions such as growth, proliferation, and the cell cycle [34,35]. Notably, it has been shown to play a major role in the prevention of apoptosis [36–38] and we observed similar results in our experiments. Cardiomyocytes from adult rats that were pretreated with ghrelin could survive under high glucose conditions or treatment with sodium palmitate. However, this ability was abolished by wortmannin, a highly specific inhibitor of PI3K. This suggests that the activation of PI3K is necessary for the ghrelin-mediated inhibition of cardiomyocyte apoptosis induced by high glucose or sodium palmitate. Activated PI3K cannot inhibit apoptosis directly. It needs to phosphorylate Akt through activation of 3-phosphoinositide-dependent kinase. Akt regulates cell survival by phosphorylating different substrates that directly or indirectly regulate apoptosis. Akt phosphorylates BAD on Ser136 to promote cell survival by inhibiting its interaction with anti-apoptotic Bcl-2 family members like Bcl-xL, thereby further preventing cytochrome *C* release[39]. In cardiomyocytes, IGF-I and insulin inhibit apoptosis by activating the PI3K-Akt pathway to phosphorylate BAD [40,41]. Akt also phosphorylates caspase-9 on Ser196, which inhibits its proteolytic activity via a conformation change [42]. Our data suggest that ghrelin can activate the PI3K-Akt pathway in a manner similar to IGF-I and insulin and we have reason to believe that the anti-apoptotic effect of ghrelin may come from the phosphorylation of BAD. Akt also inhibits apoptosis via the activation of NFκB [43,44]. Active NFκB enters the nucleus and induces the transcription of NFκB-dependent anti-apoptotic genes [45]. We found that ghrelin not only stimulated the nuclear translocation of NFκB, but also increased the transcription of the anti-apoptotic genes Bcl-2, Bcl-xL, c-*iap*, and c-*fos*. The mitochondria are known to be a significant source of superoxide radicals and other ROS associated with oxidative stress. Once ROS are generated, they increase the mitochondrial permeability, which results in the release of cytochrome *C* and apoptosis inducing factor from the mitochondria to the cytosol with the assistance of Bax translocation [46,47]. The mitochondrial membrane permeability and the release of pro-apoptotic proteins are both regulated by Bcl-2 family proteins. Bcl-2 and Bcl-xL maintain the mitochondrial membrane potential and prevent the release of cytochrome *C* from the mitochondria by binding to the pro-apoptotic proteins Bad, Bax, and Bak [48,49]. So Bcl-2 and Bcl-xL may block apoptosis by preventing the release of mitochondrial cytochrome *C* during oxidative stress in cardiomyocytes. In addition, the anti-apoptotic protein IAP-1 can specifically inhibit the post-mitochondrial caspases 3, 7 and 9 [50]. Therefore, increasing the expression of anti-apoptotic genes is another way to inhibit apoptosis.

We also found that ghrelin can activate the MAPK signaling pathway but blocking MEK activation does not seem to have a significant effect on the anti-apoptotic effect of ghrelin. In the glomerulosa, 3T3-L1, and hepatoma cell lines, ghrelin promotes proliferation and growth via the activation of the MAPK signaling pathway [51–53]. Insulin, EGF, and PDGF also promote proliferation by activating the MAPK pathway, which in turn increases the transcription of c-*fos* [54–56]. Therefore, ghrelin may promote the proliferation of cardiomyocytes, while at the same time increasing the transcription of c-*fos*. The results of the ghrelin stimulation in our assays provide evidence of this effect of ghrelin, however, we did not observe cardiomyocyte growth in the presence of ghrelin. This can be explained by the fact that cardiomyocytes from adults have undergone a terminal differentiation and have lost the ability to proliferate.

In summary, the data presented here suggest that ghrelin can inhibit apoptosis induced by high glucose and sodium palmitate. In addition, ghrelin is not an anti-oxidant and it suppresses apoptosis by activating the PI3K-Akt signaling pathway. This leads to the phosphorylation of BAD, the activation of NFκB, and the increased expression of anti-apoptotic genes.

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