Jujuboside A Protects H9C2 Cells from Isoproterenol-Induced Injury via Activating PI3K/Akt/mTOR Signaling Pathway

Dandan Han,¹ Changrong Wan,¹ Fenghua Liu,² Xiaolong Xu,³ Linshu Jiang,² and Jianqin Xu¹

¹CAU-BUA TCVM Teaching and Researching Team, College of Veterinary Medicine, China Agricultural University (CAU), Beijing 100193, China
²Beijing Key Laboratory of Dairy Cow Nutrition, College of Animal Science and Technology, Beijing University of Agriculture (BUA), Beijing 102206, China
³Beijing Institute of Traditional Chinese Medicine, Beijing 100010, China

Correspondence should be addressed to Fenghua Liu; liufenghua1209@126.com, Linshu Jiang; jls@bac.edu.cn, and Jianqin Xu; xujianqincau@126.com

Received 25 February 2016; Accepted 26 April 2016

1. Introduction

Activation of β-adrenoceptors (β-AR) is a main reason for myocardial injury [1]. Isoproterenol (ISO), a synthetic catecholamine and the β-AR agonist used to treat bradycardia or heart block, could produce severe myocardial stress and infarct like necrosis with decreased myocardial compliance and inhibition of diastolic and systolic function when administered in supramaximal dose [2–4]. It has been reported that isoproterenol produced free radicals and stimulated lipid peroxidation, which is one of the causative factors for irreversible damage to the myocardial membrane [3, 5, 6]. It is also documented that the metabolic, morphological, and pathophysiological alterations are induced by ISO in experimental animals [7, 8]. Therefore, ISO-induced changes can be used as a model for studying the effect of protective agents on the processing of myocardial injury.

Semen ziziphi spinosae (SZS), the mature seed of Ziziphus jujuba Mill. var. spinosa (Bunge) Hu ex H. F. Chow, has been used as a sedative medicine in China, Japan, Korea, and other oriental countries for over one thousand years [9, 10]. Jujuboside A (JUA), the triterpene saponins isolated from SZS, has been proved to be a major active component of SZS [11, 12]. Previous studies showed that JUA can exert antioxidant, antianxiety, anti-inflammatory, and hypnotic-sedative activities and reduce the cell apoptosis [13–15]. However, there is no investigation focusing on the protective effect of JUA on ISO-induced myocardial injury.
In this study, ISO-induced H9C2 cell damage was used as an experimental model. We performed a preliminary assessment on cytotoxicity of JUA in cardiomyocytes and investigated the protective effect of JUA against ISO-induced injury of H9C2 cell.

2. Materials and Methods

2.1. Reagents and Antibodies. JUA (>98% high-performance liquid chromatography (HPLC) purity) was purchased from National Institutes for Food and Drug Control (Beijing, China). ISO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical (Sigma, USA). Dulbecco’s modified Eagle medium (DMEM) high glucose, fetal bovine serum (FBS), and antibiotic-antimycotic were purchased from Gibco (Grand Island, NY, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Antibodies for β-actin (#8457), P13K (#4292), p-P13K (#4228), Akt (#4685), mTOR (#2972), p-mTOR (#5536), and LC3 (#12741) were purchased from Cell Signaling Technology (Danvers, MA, USA). The goat anti-mouse antibody was purchased from Li-cdr Odyssye® (Lincoln, NE, USA).

2.2. Cell Culture and Treatment. H9C2 cells were purchased from the Cell Resource Center of Chinese Academy of Medical Sciences (Peking Union Medical College, China). Cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37°C in a humidified atmosphere of 5% CO2 and subcultured in 0.05% trypsin. The medium was replenished every 1-2 days. JUA and ISO were diluted in DMEM (1 mg/mL). All cells were washed with phosphate buffered saline (PBS) and serum-starved for 2 h before incubation with JUA or ISO. JUA was dissolved in dimethylsulfoxide (DMSO), and the final DMSO concentration was ≤0.05% (v/v).

2.3. Cell Viability Assay. Cell viability was determined by MTT reduction assay. In brief, H9C2 cells were preincubated with DMEM containing 10% FBS overnight in 96-well plates at a density of 5 x 10^4 cells per well. After cells were grown to 90% confluence, all cells were washed twice with PBS and serum-starved for 2 h. According to the experimental design, the culture medium was replaced (with or without ISO or the other compounds) and then cultured for 6 h. The mediums then were removed, and solution containing 10% MTT and DMEM was added to each well. The cells were incubated at 37°C for 4 hours, the supernatants were removed, and the formazan crystals were dissolved in 150 μL DMSO (Sigma, USA). Absorbance was recorded at a wavelength of 490 nm and reference wavelength of 630 nm using a microplate reader (BIO-RAD, Foster, California, USA). The cell viability is expressed as the ratio of the absorbance in control cultures.

2.4. Treatment and Morphology Observation. Briefly, cells were treated with JUA or ISO according to the experimental design. Following each specific treatment, cell morphology was observed using a phase-contrast inverted biological microscope (IX71/IX2, Olympus, Japan), scanning electron microscopy (SEM; S-3400N, Hitachi, Tokyo, Japan), and transmission electron microscopy (TEM; J2300, JEOL, Tokyo, Japan). For ultrastructure studies, cardiomyocytes were harvested and fixed with 3.0% glutaraldehyde and 1.5% paraformaldehyde, washed three times in PBS, and postfixed in cold 1% osmium tetroxide. After dehydration with graded series of alcohol, all samples were freeze-dried, coated in a sputter-coater with a layer of gold embedded in epoxy resin (EPON812, Emicron, Shanghai, China), and examined using a Hitachi S-3400N scanning electron microscope operated at 15 kV. Other ultrathin sections were stained with saturated uranyl acetate in 50% ethanol and lead citrate, and the ultrastructure of the H9C2 cardiomyocytes was examined by TEM.

2.5. Western Blot Analysis. H9C2 cells (1 x 10^6), cultured in tissue culture flasks for 24 h, were pretreated with JUA (5, 10, or 20 μM) 3 h prior to treatment with ISO (100 μM) for 6 h in a 37°C, 5% CO2 incubator. Cells were then harvested on ice, washed twice using ice-cold PBS, and suspended in 500 μL lysis buffer supplemented with protease inhibitor. Cells were extracted using a total protein extraction kit (Biochain, Hayward, CA, USA) and quantified using a BCA protein assay kit (Pierce, Rockford, USA) according to the manufacturer’s instructions. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Pierce, Rockford, USA). Membranes were blocked using SuperBlock T20 (TBS) blocking buffer (#37536, Pierce) for 2.5 h at room temperature and incubated overnight at 4°C with specific primary antibodies. The secondary antibody was at a dilution of 1:15000, and the proteins were incubated with the secondary antibody for 1 h. Then, the antigen was visualized and analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Blots were normalized by use of β-actin to correct for differences in loading of the proteins because it did not change significantly in the proteome profiles. Quantification of digitized images of western blot bands from three biological replicates was performed using Image J (National Institutes of Health, NY, USA).

2.6. Statistical Analysis. Unless otherwise indicated, all data were obtained from at least 3 independent experiments performed in triplicate. Data was expressed as mean and standard error of the mean and assessed by the one-way analysis of variance (ANOVA) followed by Duncan’s test for multiple comparisons and post hoc tests using SPSS 19.0 (SPSS, Inc., and IBM Company, Chicago, IL, USA). P value of 0.05 or 0.01 was considered statistically significant.

3. Results

3.1. Effect of JUA on Cell Viability In Vitro. In order to examine the cytotoxic effect of various concentrations (5, 10, 20, 50, and 100 μM) of JUA on H9C2 cells, cell viability was determined using the MTT assay. Results showed that JUA, in concentrations from 0 to 100 μM, had no cytotoxic effect on H9C2 cells (Figure 1).
3.2. JUA Improves Cell Viability after ISO Exposure. To determine the effect of H9C2 cell injury induced by ISO, cell viability was tested by MTT assay after cells were treated with various concentrations of ISO for 3, 6, 12, and 18 h. As shown in Figure 2(a), cell viability was not changed after being treated with 10, 20, 50, and 100 μM of ISO for 3 h. After being stimulated with 200 μM of ISO for 3 h, cell vitality was significantly inhibited ($P < 0.05$). An obvious decrease ($P < 0.01$) in cell viability occurred after treatment with 100 and 200 μM of ISO for 6, 12, and 18 h, and the effect of cell viability inhibition induced by ISO was more serious. According to the results, concentration of 100 μM of ISO for 6 h was chosen to be the model condition of H9C2 damage for further research. To assess the protective effect of JUA on ISO-induced cytotoxicity, the H9C2 cells were treated with JUA (5, 10, and 20 μM) for 3 h, and, then, ISO (0 or 100 μM) was added and incubated for 6 h. Subsequently, cell viability was also detected by MTT assay. The results (Figure 2(b)) demonstrated that JUA significantly enhanced the survival rates of the cells after exposure to ISO, and with increased amounts of JUA, the cell survival rates showed an increasing trend.

3.3. JUA Alleviates ISO-Induced Cell Damage in H9C2 Cells. To evaluate the degree of characteristic morphological changes of H9C2 cells, we monitored cytomorphology using phase-contrast inverted biological microscope, scanning electron microscope, and transmission electron microscope. As shown in Figure 3, treatment with ISO (100 μM) for 6 h induced obvious morphological changes in H9C2 cells. ISO induced pronounced cell damage as displayed by cell shrinkage and gradual detachment from culture dishes (Figure 3(a)). SEM showed normal elongated spindle cells and may help in electrophysiological properties [16], characterized with complete grain, dense, and delicate surface. ISO induced abnormal cellular microstructures characterized with irregular-arranged cell shape; cell surface protein became sparser and increased nuclear gap (Figure 3(b)). Electron microscopy analysis of H9C2 cells revealed obvious alterations in the structure of cardiomyocytes and the cellular architecture. Ultrastructural images of H9C2 cells using TEM showed obvious nuclear chromatin...
3.4. Influence of JUA on ISO-Induced PI3K/Akt Signaling Pathway. To further expound the mechanism of protective effect of JUA on ISO-induced myocardial injury, we then investigated the activation of PI3K/Akt/mTOR signaling pathway after intervention of JUA on ISO-induced H9C2 cells. Cell proteins were extracted for western blotting analysis. We assessed the effect of JUA on ISO-induced phosphorylation of Akt, PI3K, and mTOR using three different phosphospecific antibodies. Results indicated that the expression of phosphorylation of Akt and mTOR was significantly strengthened by ISO \((P < 0.05)\), and the phosphorylation of PI3K, Akt, and mTOR levels was all enhanced to some degree in JUA-pretreated cells compared with ISO-treated cells \((P < 0.01, \text{ Figure 4})\).

3.5. Influence of JUA on ISO-Induced LC3 Conversion. LC3 is essential for final autophagosome formation and thus serves as an autophagosome marker. The ratio of LC3-II/I most likely indicated the levels of autophagy. Therefore, western blot analysis was performed to detect LC3-II/I level. Correspondingly, the ratio of LC3-II/I in JUA-pretreated cells decreased remarkably \((P < 0.01)\) compared to control group and ISO-stimulated group (Figure 5).

4. Discussion
ISO, a \(\beta\)-adrenergic agonist and synthetic catecholamine, can be employed at submaximal dose as a noninvasive method to induce myocardial lesions in rodents [17, 18]. Recent studies have been conducted to evaluate myocyte damage following exposure to this drug in rat and monkey species [4]. In this study, H9C2 cells were exposed to ISO to perform a myocardial toxicity model triggered by persistent excitation of \(\beta_1\) receptor; we found a significant inhibition of cell viability of H9C2 cells induced by ISO in...
a time- and dose-dependent manner (Figure 2(b)). In addition, we found that ISO-induced morphology of H9C2 was changed and cell microstructure was destroyed. In model group, morphological changes including fuzzy cell boundary, cellular atrophy, disorderly or missing microvilli, and different degrees of cell collapse were observed by SEM. Subcellular structure changes including nuclear cavitation, swelling or rupture of microvilli, hazy mitochondrial structures, and loose cytoplasmic matrix structure were studied by means of TEM (Figure 3). Therefore, an excessive stress response stimulated by ISO has been confirmed in cardiomyocytes, which will cause damage to cells and reduce cell viability.

JUA, a classic natural product extracted from SZS, has been efficiently used for insomnia relief and considered to be the major effective pharmacological active constituent [13, 19]. In our study, MTT assay showed that JUA did
It participated in the regulation of cellular signaling networks linked to the survival, growth, proliferation, metabolism, and differences in cell functions and controlled major cell functions through a number of downstream effectors, including p70S6K/p85S6K, 4E-BP1, NF-κB, and mammalian target of rapamycin (mTOR) [27–30]. mTOR is a highly conserved serine-threonine protein kinase that belongs to the PI3K family and plays an important role in signal transduction pathways that control cell proliferation, survival, angiogenesis, and protein translation [31–34]. Therefore, we focus on the role and regulatory mechanisms of PI3K/Akt/mTOR signaling pathway in the process of JUA exhibited proliferation on H9C2 cell. We found that JUA promotes H9C2 cardiomyocytes cell proliferation through strengthening the activation of PI3K/Akt/mTOR signaling pathway.

mTOR played a vital role in regulating cell proliferation, growth, differentiation, and survival, controlling a cell that undergoes programmed cell death type I (apoptosis) or type II (autophagy) [35]. Activated Akt is the downstream effector of PI3K, which can stimulate mTOR to negatively regulate autophagy [36]. Activation of the PI3K-Akt-mTOR and Akt-tuberous sclerosis complex- (TSC-) mTOR pathways inhibits autophagy, whereas the loss of signaling through this cascade removes the negative repression of mTOR. Therefore, there is a direct link between autophagy and the mTOR signaling pathway [37]. Microtubule-associated protein light chain 3 (LC3), a mammalian homologue of yeast Apg8p, was used as a marker of autophagy induction, because cytosolic LC3-I is processed to its lipidated LC3-II form upon autophagy induction [38]. LC3 is initially synthesized in an unprocessed form, pro-LC3, which is converted into the proteolytically processed form LC3-I, lacking amino acids from the C-terminus, and is finally modified into a phosphatidylethanolamine-conjugated form LC3-II [39]. LC3-II is the protein marker that is reliably associated with phagosomes, sealed autophagosomes, and mature autophagosomes/lysosomes [40]. Once the autophagosome and lysosome integrated, LC3-II in autophagosome degraded by hydrolase in lysosome. The expression of LC3-II is proportional to the number of autophagic vacuoles, and LC3-II is regarded as a marker of autophagosome formation [41]. That is to say, we can judge the activity of autophagy by detecting the expression of LC3-II or the ratio of LC3-II/LC3-I [42]. Western blot analysis revealed significantly increased levels of p-Akt and p-mTOR and decreased LC3 conversion in H9C2 cells, indicating that the PI3K/Akt/mTOR pathway may be involved in the regulation of autophagy by JUA.

5. Conclusions

In conclusion, our investigation indicated that JUA has potential protective effect on ISO-induced damage in H9C2 cells by accelerating the activation of PI3K/Akt/mTOR signaling pathway and decreasing LC3 conversion. These observations therefore suggest that JUA, a saponin from semen ziziphi spinosae, possesses potential anti-injury activity and beneficial characteristics for cardiovascular diseases. However, other possible pathways and targets related to the anti-injury effect of JUA need to be researched in the future.

not exhibit cytotoxic effect in concentrations from 0 to 100 µM when treating H9C2 cells (Figure 1). Moreover, JUA effectively reversed the inhibition of cell viability caused by ISO (Figure 2(a)). Recent researches also found that JUA has certain effect on anti-injury effect and neuroprotective beneficial characteristics for cardiovascular diseases. However, other possible pathways and targets related to the anti-injury effect of JUA need to be researched in the future.

FIGURE 5: Inhibition of ISO-induced LC3 conversion by JUA. Effect of JUA in different concentrations on ISO-induced LC3-II/I protein level in H9C2 cells. Cells were pretreated with various concentrations (5, 10, and 20 µM) of JUA and exposed to 100 µM of ISO for 6 h; proteins samples were prepared at the indicated time points and analyzed by western blotting. LC3 was analyzed using specific anti-LC3 antibody. Ratio of LC3-II/I protein levels was calculated and relative protein quantification in treated versus control extracts was calculated by densitometric analysis. Beta-actin was probed as a loading control. Data represent the mean ± SEM of three independent experiments, and differences between mean values were assessed by one-way ANOVA. *P < 0.01 versus group treated with ISO only.

The PI3K/AKT/mTOR pathway is one of major signal transduction pathways responsible for regulating cell growth, proliferation, survival, apoptosis, and malignant transformation and is frequently hyperactivated in most cancers [22–24]. The lipid products of phosphoinositide 3-kinase (PI3K) provide localized membrane anchors for the assembly of various signaling proteins, which have domains that bind to D3 phosphorylated phosphoinositides [25, 26]. The well-established downstream target of PI3K is the serine-threonine kinase Akt (also known as protein kinase B, PKB), which is considered to be a key mediator of the PI3K signaling pathway and transmits survival signals from growth factors.

It participated in the regulation of cellular signaling networks linked to the survival, growth, proliferation, metabolism, and differences in cell functions and controlled major cell functions through a number of downstream effectors, including p70S6K/p85S6K, 4E-BP1, NF-κB, and mammalian target of rapamycin (mTOR) [27–30]. mTOR is a highly conserved serine-threonine protein kinase that belongs to the PI3K family and plays an important role in signal transduction pathways that control cell proliferation, survival, angiogenesis, and protein translation [31–34]. Therefore, we focus on the role and regulatory mechanisms of PI3K/Akt/mTOR signaling pathway in the process of JUA exhibited proliferation on H9C2 cell. We found that JUA promotes H9C2 cardiomyocytes cell proliferation through strengthening the activation of PI3K/Akt/mTOR signaling pathway.

mTOR played a vital role in regulating cell proliferation, growth, differentiation, and survival, controlling a cell that undergoes programmed cell death type I (apoptosis) or type II (autophagy) [35]. Activated Akt is the downstream effector of PI3K, which can stimulate mTOR to negatively regulate autophagy [36]. Activation of the PI3K-Akt-mTOR and Akt-tuberous sclerosis complex- (TSC-) mTOR pathways inhibits autophagy, whereas the loss of signaling through this cascade removes the negative repression of mTOR. Therefore, there is a direct link between autophagy and the mTOR signaling pathway [37]. Microtubule-associated protein light chain 3 (LC3), a mammalian homologue of yeast Apg8p, was used as a marker of autophagy induction, because cytosolic LC3-I is processed to its lipidated LC3-II form upon autophagy induction [38]. LC3 is initially synthesized in an unprocessed form, pro-LC3, which is converted into the proteolytically processed form LC3-I, lacking amino acids from the C-terminus, and is finally modified into a phosphatidylethanolamine-conjugated form LC3-II [39]. LC3-II is the protein marker that is reliably associated with phagosomes, sealed autophagosomes, and mature autophagosomes/lysosomes [40]. Once the autophagosome and lysosome integrated, LC3-II in autophagosome degraded by hydrolase in lysosome. The expression of LC3-II is proportional to the number of autophagic vacuoles, and LC3-II is regarded as a marker of autophagosome formation [41]. That is to say, we can judge the activity of autophagy by detecting the expression of LC3-II or the ratio of LC3-II/LC3-I [42]. Western blot analysis revealed significantly increased levels of p-Akt and p-mTOR and decreased LC3 conversion in H9C2 cells, indicating that the PI3K/Akt/mTOR pathway may be involved in the regulation of autophagy by JUA.
Competing Interests
The authors declare that there are no competing interests regarding the publication of this paper.

Authors’ Contributions
Jianqin Xu, Linshu Jiang, and Fenghua Liu designed the experiments. Dandan Han conducted the study and wrote the paper. Changrong Wan and Xiaolong Xu contributed to reagents, materials, and analysis tools. All authors read and approved the final paper.

Acknowledgments
This work was supported by grants from the Ministry of Agriculture, Public Service Sectors Agriculture Research Projects (no. 201403051-07), National Twelve-Five Technological Supported Plan of China (no. 2011BAD34B01), National Natural Science Foundation of China (no. 31272478), and Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&TD20130324). The authors are thankful for the help from the members of CAU-BUA TCVM Teaching & Research Team.

References
Evidence-Based Complementary and Alternative Medicine


