Fmr1 protects cardiomyocytes against lipopolysaccharide-induced myocardial injury

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Abstract. The present study explored the mechanisms by which fragile X mental retardation 1 (fmr1) overexpression inhibits lipopolysaccharide (LPS)-induced cardiomyocyte injury. Factors including oxidative stress reaction, mitochondrial membrane potential variation and cell apoptosis were evaluated. The viability of H9c2 cells was evaluated with a Cell Counting Kit-8 assay after cells were treated with LPS at different concentrations (0, 1, 3, 6 and 9 μg/ml) for various durations (4, 12 and 24 h). Flow cytometry was used to determine variations in reactive oxygen species (ROS), mitochondrial membrane potential and cell apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to detect the levels of apoptosis-associated factors, and western blot analysis was used to determine the phosphorylation levels of phosphoinositide-3 kinase (PI3K), Akt and forkhead box (Fox)O3a. The results indicated that LPS decreased the viability of H9c2 cells in a dose- and time-dependent manner. Overexpression of fmr1 inhibited the LPS-induced decrease in the mitochondrial membrane potential and the production of ROS as well as apoptosis in H9c2 cells. Fmr1 also inhibited LPS-induced reductions in antioxidant enzyme activities, including those of superoxide dismutase and reduced/oxidized glutathione ratio, and decreased LPS-associated increases in the lipid peroxidation product malondialdehyde. Apoptosis-associated factors were identified to be involved in the effects of Fmr1. Overexpression of Fmr1 attenuated LPS-associated increases in the apoptosis-activating factors B-cell lymphoma 2 (Bcl-2)-associated X protein and caspase-3 and decreases in apoptosis inhibitors, including Bcl-2 and X-linked inhibitor of apoptosis protein. Fmr1 overexpression also reduced LPS-induced increases in the phosphorylation levels of PI3K, Akt and FoxO3a. In conclusion, fmr1 overexpression alleviated oxidative stress and apoptosis in H9c2 cardiomyocytes injured by LPS via regulating oxidative stress and apoptosis-associated factors, as well as the PI3K/Akt pathway. This information may provide a novel and effective therapeutic strategy for heart diseases.

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Introduction

The mortality rate associated with cardiovascular disease (CVD) is increasing year by year. CVD is the most prevalent cause of mortality, according to World Health Organization's statistics. Myocardial dysfunction, the major cause of mortality in intensive care units (1), is a major clinical manifestation of CVD and is easily induced by endotoxin.

Lipopolysaccharides (LPS), also known as endotoxin, are a prevalent component of the cell walls of most Gram-negative bacteria (2). LPS is a pathogen that may induce endotoxemia, septic shock and multiple organ failure (3,4). The heart is the most common organ to be adversely affected by endotoxemia (5,6). The prevalence of myocardial dysfunction in endotoxemia patients is >60%. However, the detailed molecular mechanisms remain undefined and require further elucidation (7).

The fragile X mental retardation protein (FMRP) gene, which encodes the protein fragile X mental retardation 1 (fmr1), is a determinant of normal cognitive development and female reproductive function, and is highly prevalent in the brain (8). Mutations of fmr1 may lead to fragile X syndrome (9). Studies have demonstrated that fmr1 is essential for a normal heart rate in Drosophila (10) and cardiac function during development (11). The specific function of fmr1 in the cardiovascular system has remained largely elusive. It is therefore worthwhile to investigate whether and how fmr1 is able to inhibit LPS-induced cardiac injury.

The massive release of inflammatory cytokines, oxidative stress and mitochondrial dysfunction are the major characteristics of endotoxemia (12). LPS combines to serum endotoxin-binding protein to produce massive inflammatory cytokines, which induce oxidative stress and overproduction of reactive oxygen species (ROS), causing damage of the mitochondrial structure and cardiomyocyte dysfunction (13-15),

Oxidative stress and excessive ROS accumulation result in cytotoxicity and cell apoptosis. Previous studies have indicated that moderate oxidative stress is one of the major causes of cardiomyocyte apoptosis (16), which is an important mechanism of cardiomyocyte death (17). Therefore, inhibition of oxidative stress-induced cardiomyocyte apoptosis is an important strategy to prevent cardiomyocyte damage and protect subjects at risk from cardiac disease.

The phosphatidylinositide-3kinase (PI3K)/Akt pathway is involved in the regulation of myocardial contraction, revascularization, physiological or pathological myocardial hypertrophy and congestive heart failure, having critical roles in cell proliferation, differentiation and apoptosis (18). In the present study, it was speculated that the PI3K/Akt pathway also participates in the processes by which fmr1 inhibits LPS-induced cardiomyocyte injury.

In the present study, an *in vitro* model of LPS-induced cardiomyocyte damage was constructed using the H9c2 cell line, which was subjected to vector-mediated ectopic overexpression of fmr1, in order to study how fmr1 inhibits oxidative stress, myocardial injury and cell apoptosis, as well as the implication of the PI3K/Akt pathway. It is of significant value to unveil a novel potential biomarker of fmr1 in cardiovascular systems, which may provide a novel method for the diagnosis and prognosis of patients with CVD.

Materials and methods

Cell culture. H9c2 rat embryo cardiomyocytes purchased from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. Cells in the logarithmic growth phase were used in all experiments. The morphology of H9c2 cells were observed under an optical microscope at 48 h after inoculation.

Cell viability assay. H9c2 cells were divided into 5 groups: LPS groups treated with LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at different concentrations (1, 3, 6 and 9 μg/ml), and a control group without any treatment (n=5 per group). The cell viability was measured with a Cell Counting Kit-8 (CCK8; Beyotime Institute of Biotechnology, Haimen, China) after LPS treatment for the set durations (4, 12 and 24 h). In brief, cells were seeded in 96-well plates at an initial density of $5x10^3$ cells/well and incubated with different concentrations of LPS (1, 3, 6 and 9 μg/ml) for the indicated times. CCK-8 stain (20 μl) was then added to each well of the plate, followed by further incubation for 1 h. The optical density values at 450 nm (OD_{450}) were read with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Values were expressed as the percentage of viable cells as follows: Relative viability (%)=[OD_{450}(treated)-OD_{450} (blank)]/[OD_{450}(control)-OD_{450}(blank)] x100%.

Cell transfection. Cell transfection experiments were performed after the construction of an fmr1 overexpression plasmid using the pGEM-T/pFLAG Vector (Promega Corp., Madison, WI, USA) and transfection reagent Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The empty vector was transfected into another batch of cells in parallel. In brief, cells were inoculated in DMEM without antibiotics. When the cell density reached 90-95%, the plasmid expressing fmr1 and Lipofectamine® 2000 were added into DMEM without FBS, and mixed gently at the ratio of DNA toLipofectamine® 2000 of 1:2. The culture media was changed to DMEM with FBS after incubation for 6 h at 37˚C with 5% CO_2. The cell transfection rates were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis after further culture for 48 h.

RT-qPCR. The mRNA expression levels were determined by RT-qPCR. Total RNA was extracted from cells with an RNeasy kit (Qiagen, Valencia, CA, USA), and complementary DNA was reversely transcribed with 1 μg RNA using the Quantiscript Reverse Transcriptase kit (Qiagen) according to the manufacturer's protocol. PCR amplification was performed in an ABI 7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) using Fast SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following reaction conditions: 15 sec at 95˚C, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 15 sec. The oligonucleotide primer sequences are displayed in Table I.

Western blot analysis. Cells were lysed using lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02% NaN_2, 100 μg/ml phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100), and the lysate was centrifuged at a speed of 12,000 x g for 30 min at 4˚C, and the supernate containing proteins was collected. The protein concentrations were determined with the bicinchoninic acid assay (Beyotime Institute of Biotechnology). Subsequently, the total protein (20 μg) was subjected to each lane of15% SDS-PAGE gel electrophoresis (SDS-PAGE) and then electroblotted onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Little Chalfont, UK). Following blocking with 5% non-fat dry milk in PBS for 1 h at room temperature, the blotting membranes were probed overnight at 4˚C with the following primary antibodies obtained from Abcam (Cambridge, UK): Rabbit anti-Fmr1 (cat. no. ab17722; 1:1,000), anti-Bax (cat. no. ab53154; 1:1,000), anti-Bcl-2 (cat. no. ab196495; 1:1,000), anti-cleaved caspase-3 (cat. no. ab2305; 1:200), anti-XIAP (cat. no. ab2541; 1:200), anti-p-PI3K (cat. no. ab182651; 1:1,000), anti-PI3K (cat. no. ab227204; 1:2,000), anti-p-AKT (cat. no. ab38449; 1:1,000), anti-AKT (cat. no. ab64148; 1:200), anti-p-FoxO3a (cat. no. ab47285; 1:1,000), anti-FoxO3a (cat. no. ab23683; 1:1,000) and anti-GAPDH (cat. no. ab9485; 1:2,500). Samples were then probed with horseradish peroxidase conjugated Goat anti-rabbit immunoglobulin G secondary antibodies (Abcam; cat. no. ab6721, 1:5,000) for 2 h at room temperature. GAPDH was used as loading control. The PVDF membrane was exposed to X-ray film (Kodak, Rochester, NY, USA) and immunoreactive bands were detected using enhanced chemiluminescence detection system of GE ECL Start (GE Healthcare, USA). Western blot bands were quantified using the Bio-Rad ChemiDoc MP system with Image Lab™ Software version 4.1 (each, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. Primers used for polymerase chain reaction.

Name	Type	Sequence (5'-3')
Fmr1	Forward	GAGGGTGAGGATCGAAGCTG
	Reverse	GTACCATCCCCCTCTGGACT
Bcl-2	Forward	CCCCTGGCATCTTCTCCTTCC
	Reverse	GGGTGACATCTCCCTGTGACG
Bax	Forward	GGATGCGTCCACCAAGAA
	Reverse	ACGGAGGAAGTCCAGTGT
Caspase3	Forward	GCCTCTGCCCGGTTAAGAAA
	Reverse	CATCTGTACCAGACCGAGCG
XIAP1	Forward	TGGATCTGAATGCCCGATCT
	Reverse	TCCAACCAGTGTGGAACAGT
XIAP2	Forward	TGTTGTACCTGCAGACACCA
	Reverse	AGCTGAGTCTCCATACTGCC
GAPDH	Forward	GGTCATGAGTCCTTCCACGATA
	Reverse	ATGCTGGCGCTGAGTACGTC

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; XIAP, X-linked inhibitor of apoptosis protein; Fmr1, fragile X mental retardation 1.

Treatment groups. In the present study, $1x10^5$ cells were inoculated onto each well of a 6-well plate and divided into 5 experimental groups as follows: H9c2 cells treated with 9 μg/ml LPS for 24 h after being transfected with fmr1 overexpression plasmid for 12 h (Fmr1+LPS group), H9c2 cells treated with 9 μg/ml LPS for 24 h after being transfected with empty plasmid vector for 12 h (Vect+LPS group), H9c2 cells only transfected with empty plasmid vector for 12 h (Vect group), H9c2 cells only treated with 9 μg/ml LPS for 24 h (LPS group) and H9c2 cells without any treatment (Control group).

ROS detection. ROS were detected with the oxygen-sensitive fluorescence probe dichloro-dihydro-fluorescein diacetate (DCTH-DA) assay. Following culture and pre-treatment as aforementioned, 10 μmol/lDCFH-DA was added to the wells of a 6-well plate and incubated at 37˚C for 20 min. Then, the cells were washed with PBS3 times. Analysis was immediately performed with a flow cytometer (BD Biosciences, San Diego, CA, USA). BD CellQuest™ Pro software version 5.1 (BD Biosciences) was used to analyze ROS levels.

Mitochondrial membrane potential detection. The mitochondrial membrane potential was determined with the JC-1 probe assay. In brief, the floating and trypsinized adherent cells ($5x10^5$) of these 5 cell groups (Fmr1+LPS, Vect+LPS, Vect, LPS and Control group) were collected and resuspended in 500 μl PBSJC-1 dye (10 μmol/l) was added, followed by incubation for another 20 min at 37˚C. Cells were resuspended in phosphate buffer and the fluorescence was detected with a flow cytometer (BD Biosciences). BD CellQuest™ Pro software version 5.1 (BD Biosciences) was used to analyse the mitochondrial membrane potential.

Apoptosis detection. The apoptotic rate was determined with the Annexin-V/propidium iodied (PI) double-staining assay (Biovision, Mountain View, CA, USA) according to the manufacturer's protocol. In brief, floating and trypsinized adherent cells ($5x10^5$) of the 5 cell groups (Fmr1+LPS, Vect+LPS, Vect, LPS and Control group) were collected and resuspended in 500 μl phosphate buffer containing 5 μl Annexin-V fluorescein isothiocyanate and 5 μl PI, followed by incubation for 5 min in the dark at the room temperature. Analysis was immediately performed with a flow cytometer (BD Biosciences). BDCellQuest™ Pro software version 5.1 (BD Biosciences) was used to analyze the apoptotic rate.

Oxidative stress factor detection. The levels of antioxidant enzymes, including superoxide dismutase (SOD) and reduced glutathione/oxidized glutathione (GSH/GSSG), and the lipid peroxidation product malondialdehyde (MDA) were determined with specific kits. Activities of SOD were determined using the Total Superoxide Dismutase Assay kit with WST-8 (Beyotime Institute of Biotechnology). The GSH/GSSG ratio was measured with a GSH and GSSG Assay kit (Beyotime Institute of Biotechnology) with 2,2-dithio-bis-nitrobenzoic acid. The levels of MDA were detected with a Lipid Peroxidation MDA Assay kit (Beyotime Institute of Biotechnology); in this assay, MDA reacts with thiobarbituric acid. All measurements were performed according to the manufacturers' protocols.

Statistical analysis. All results were expressed as mean ± standard deviations of five independent experiments. Statistical analysis was performed using the SPSS 18.0 statistical package (SPSS, Inc., Chicago, IL, USA) and data were subjected to one-way analysis of variance, followed by Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effect of LPS on H9c2 cell viability. The viability of H9c2 cells treated with LPS at different concentrations (0, 1, 3, 6 and 9 μg/ml) for determined durations (4, 12 and 24 h) was evaluated with a CCK8 assay in order to indicate the damage effect of LPS on H9c2 cells. The results indicated that the viability of H9c2 cells was decreased by LPS in a dose- and time-dependent manner. Compared with the control, treatment with 6 or 9 μg/ml LPS for 24 h significantly decreased the cell viability by 35 and 50%, respectively ($P<0.05$; Fig. 1A). Treatment with 9 μg/ml LPS for 24 h was selected as the conditions for the subsequent experiments (LPS group) due to the severe damage effect.

Transfection rates of overexpression of fmr1. The ectopic overexpression rates of fmr1 were determined by RT-qPCR and western blot analysis. The mRNA and protein levels of fmr1 were significantly increased by ~3-fold in the fmr1 overexpression group compared with those in the control group ($P<0.01$). As expected, mRNA and protein levels in the Vect group transfected with empty vector were similar to those in the control group (Fig. 1B and D).

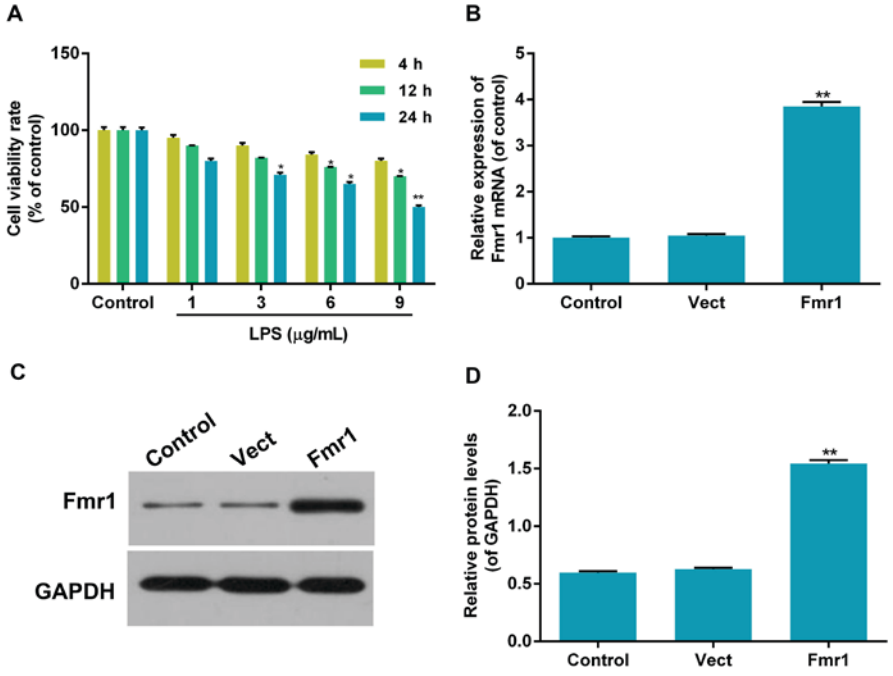

Figure 1. Effect of LPS on cell viability of H9c2 cells and the efficacy of vector-mediated overexpression of fmr1. (A) The inhibitory effect of LPS (0, 1, 3, 6 or 9 μg/ml for 4, 12 and 24 h) on H9c2 cell viability was detected with a Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation (n=5 per group). *P<0.05, **P<0.01 vs. control. (B) The mRNA expression levels of fmr1 were detected by reverse transcription-quantitative polymerase chain reaction analysis. (C and D) Fmr1 protein levels were determined by western blot analysis. Values are expressed as the mean ± standard deviation (n=5 per group). **P<0.01 vs. control. Vect, empty vector-transfected group; LPS, lipopolysaccharide; fmr1, fragile X mental retardation 1.

Fmr1 overexpression inhibits oxidative stress induced by LPS in H9c2 cells. To determine the effect of fmr1 overexpression on ROS levels when H9c2 cells were injured by LPS, an assay utilizing the oxygen-sensitive fluorescence probe DCTH-DA was performed to detect ROS levels in the following 5 cell groups: Fmr1+LPS, Vect+LPS, Vect, LPS and Control group. The results indicated that ROS levels in the LPS group increased significantly compared with those in the control group (P<0.01), and ROS levels in the Fmr1+LPS group decreased significantly in the Vect+LPS group (P<0.01), in which the levels were similar to those in the LPS group. It was demonstrated that fmr1 overexpression inhibited the promotion effect of LPS on ROS levels in H9c2 cells (Fig. 2A).

The levels of oxidative stress-associated factors, including lipid peroxidation product MDA, the antioxidant enzyme SOD and the GSH/GSSG ratio were determined by specific kits. The content of MDA increased by >2-fold in the LPS group compared with that in the control group (P<0.01), and decreased by 42% in the Fmr1+LPS group compared with that in the Vect+LPS group (P<0.01; Fig. 2B). The activity of SOD decreased by 41% in the LPS group compared with that in the control group (P<0.01), and increased again in the Fmr1+LPS group compared with that in theVect+LPS group (P<0.01; Fig. 2C). Under normal conditions, mitochondrial thiols, most notably GSH, prevent mitochondrial DNA from oxidation, but the administration of LPS decreases mitochondrial GSH. The ratio of GSH/GSSG decreased significantly in the LPS group compared with that in the control group, and increased again in the Fmr1+LPS group compared with that in the Vect+LPS group (P<0.01; Fig. 2D).

Fmr1 overexpression inhibits cell apoptosis induced by LPS in H9c2 cells. To assess the function of fmr1 overexpression on the mitochondrial membrane potential in H9c2 cells after injury by LPS, the JC-1 probe assay was performed to detect mitochondrial membrane potential levels in the 5 experimental groups. The results indicated that the disrupted mitochondrial membrane potential in the LPS group increased significantly compared with that in the control group (P<0.01), and decreased significantly in the Fmr1+LPS group compared with that in the Vect+LPS group (P<0.01). The values in the Vect+LPS and LPS group were similar. Overall, the results indicated that fmr1 overexpression reduced the disruption of the mitochondrial membrane potential following LPS-induced injury in H9c2 cells (Fig. 3A).

To identify whether LPS induces apoptosis in H9c2 cells and whether fmr1 overexpression inhibits this, an Annexin-V/PI double-staining assay was performed to detect the apoptotic rates in the 5 experimental groups. The results indicated that the apoptotic rate increased significantly in the LPS group compared with that in the control group (P<0.01), and decreased to 46% in the Fmr1+LPS group compared with that in the Vect+LPS group (P<0.01), while the values in the Vect+LPS and LPS group were similar. It was demonstrated that fmr1 overexpression inhibited the apoptotic effect of LPS on H9c2 cells (Fig. 3B). These results indicated an inhibitory effect of fmr1 overexpression on LPS-induced apoptosis in H9c2 cells.

To detect the mechanism by which fmr1 overexpression protects H9c2 cells from the LPS-induced reduction of cell viability and promotion of apoptosis, RT-qPCR and western blot analysis were performed to detect the mRNA and protein levels of apoptosis-associated factors, including B-cell

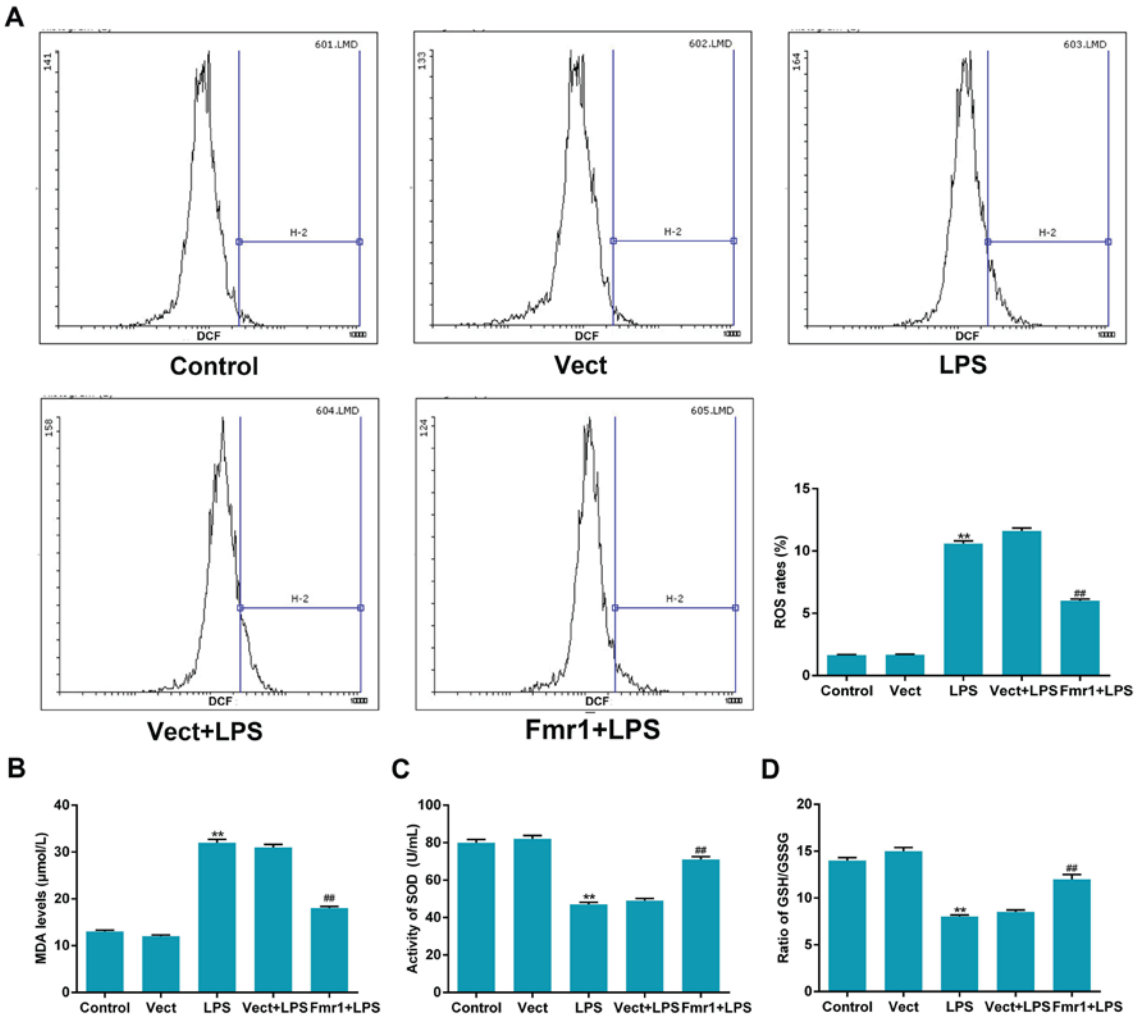

Figure 2. Fmr1 overexpression alleviates oxidative stress induced by LPS in H9c2 cells. (A) A dichloro-dihydro-fluorescein diacetate assay and flow cytometry were applied to detect ROS levels in the Fmr1+LPS group, Vect+LPS group, Vect group, LPS group and Control group. (B) The content of the lipid peroxidation product MDA, (C) the activity of SOD and (D) the ratio of GSH/GSSG were also detected in these 5 cell groups. Values are expressed as the mean ± standard deviation (n=5 per group). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. Vect+LPS control. LPS, lipopolysaccharide; Vect, empty vector; fmr1, fragile X mental retardation 1; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; GSH/GSSG, reduced vs. oxidized glutathione.

lymphoma 2 (Bcl-2)-associated X protein (Bax), Bcl-2, cleaved caspase-3 and X-linked inhibitor of apoptosis protein (XIAP) in the 5 experimental groups (Fig. 4). The results indicated that, at the mRNA and protein level, the apoptosis-activating factors Bax and cleaved caspase-3 increased significantly in the LPS group compared with those in the control group (P<0.01), and decreased significantly in the Fmr1+LPS group compared with those in the Vect+LPS group (P<0.05), while levels in the Vect+LPS and LPS groups were similar. By contrast, the mRNA and protein levels of the apoptosis inhibitors Bcl-2 and XIAP decreased significantly in the LPS group compared with those in the control group (P<0.01), and increased significantly in the Fmr1+LPS group compared with those in the Vect+LPS group (P<0.05), while levels in the Vect+LPS and LPS groups were similar (Fig. 4).

Effect of fmr1 overexpression on the PI3K/Akt/forkhead box (Fox)O3a pathway in H9c2 cells injured by LPS. To detect whether the protective function of fmr1 overexpression on LPS-associated inhibition of H9c2 cell viability and induction of apoptosis were associated with the PI3K/Akt/FoxO3a pathway, western blot analysis was performed to detect the phosphorylation levels of PI3K, Akt and FoxO3a in H9c2 cells treated with LPS (LPS group), which had been optionally transfected with Fmr1 overexpression vector (Fmr1+LPS group). The phosphorylation levels were compared with those in the control and Vect+LPS groups. As displayed in Fig. 5, the phosphorylation levels of PI3K, Akt and FoxO3a in the LPS group were all significantly increased compared with those in the control group (P<0.01). In addition, the phosphorylation levels of PI3K, Akt and FoxO3a in the Fmr1+LPS group were all significantly decreased by 9, 22 and 37%, respectively, compared with those in the Vect+LPS group (P<0.05). However, the total protein levels of PI3K, Akt and FoxO3a were not significantly affected (P>0.05).

Discussion

LPS, also called endotoxin, induces endotoxemia and myocardial dysfunction, which is a major clinical manifestation of CVD (11). Recently, fmr1 was identified to be associated with a normal heart rate and cardiac function (11,19). However, the

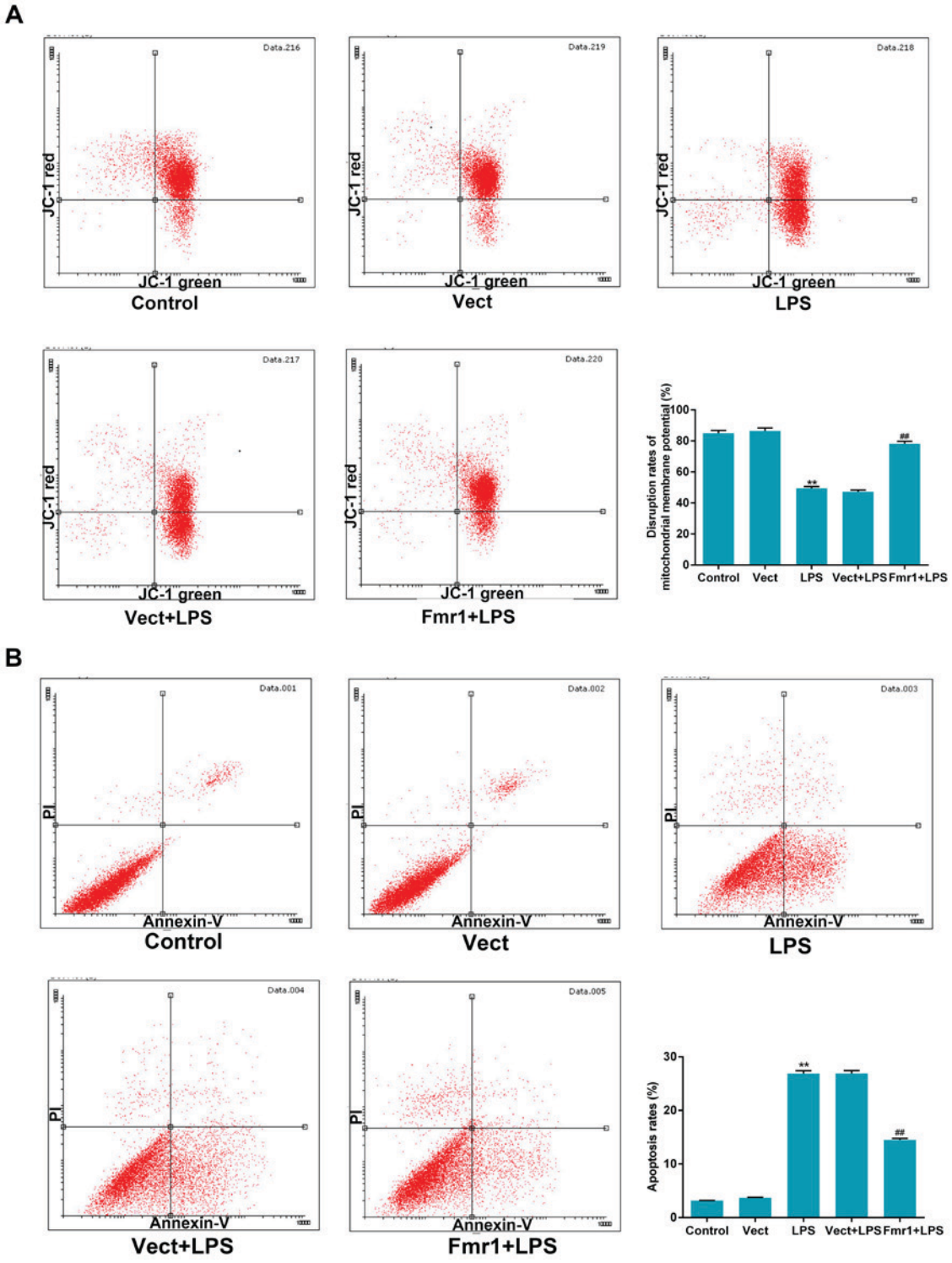

Figure 3. Fmr1 overexpression inhibits the induction of cell apoptosis by LPS in H9c2 cells. (A) The JC-1 probe assay and flow cytometric analysis were applied to detect mitochondrial membrane potential in the different experimental groups. (B) The Annexin-V/propidium iodide double-staining assay and flow cytometric analysis were applied to detect apoptotic rates in these 5 cell groups. Values are expressed as the mean ± standard deviation (n=5 per group). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. Vect+LPS control. LPS, lipopolysaccharide; Vect, empty vector; fmr1, fragile X mental retardation 1.

specific mechanism has remained to be fully elucidated and requires further study.

The present study explored the effect of fmr1 overexpression on LPS-induced cardiomyocyte injury, including oxidative stress reaction, mitochondrial membrane potential variation and cell apoptosis. H9c2 cardiomyocytes were used in the present study. The viability of LPS-injured H9c2 cardiomyocytes was evaluated with a CCK8 assay and was observed to be decreased by LPS in a dose- and time-dependent manner. Treatment of H9c2 cells with 9 μg/ml LPS for 24 h was employed in the subsequent experiments due to the severe damage associated with it.

Mitochondrial dysfunction and oxidative stress are the major characteristics of endotoxemia (20). Injury of

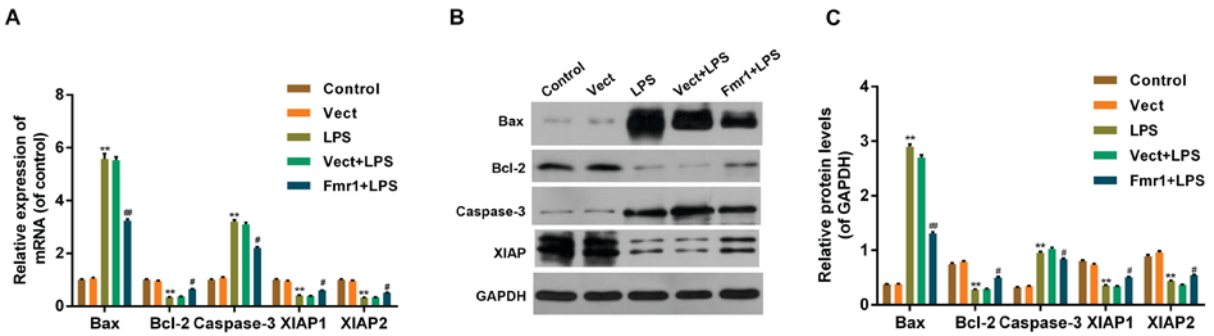

Figure 4. Effect of fmr1 on apoptosis-associated factors in H9c2 cells damaged by LPS. (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA levels of apoptosis-associated factors, including Bax, Bcl-2, Caspase-3 and XIAP in the following experimental cell groups: Fmr1+LPS, Vect+LPS, Vect, LPS and Control group. (B and C) Western blot analysis was performed to detect protein levels of the abovementioned apoptosis-associated factors in the 5 cell groups. Values are expressed as the mean ± standard deviation (n=5 per group). **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Vect+LPS control. LPS, lipopolysaccharide; Vect, empty vector; fmr1, fragile X mental retardation 1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; XIAP, X-linked inhibitor of apoptosis protein.

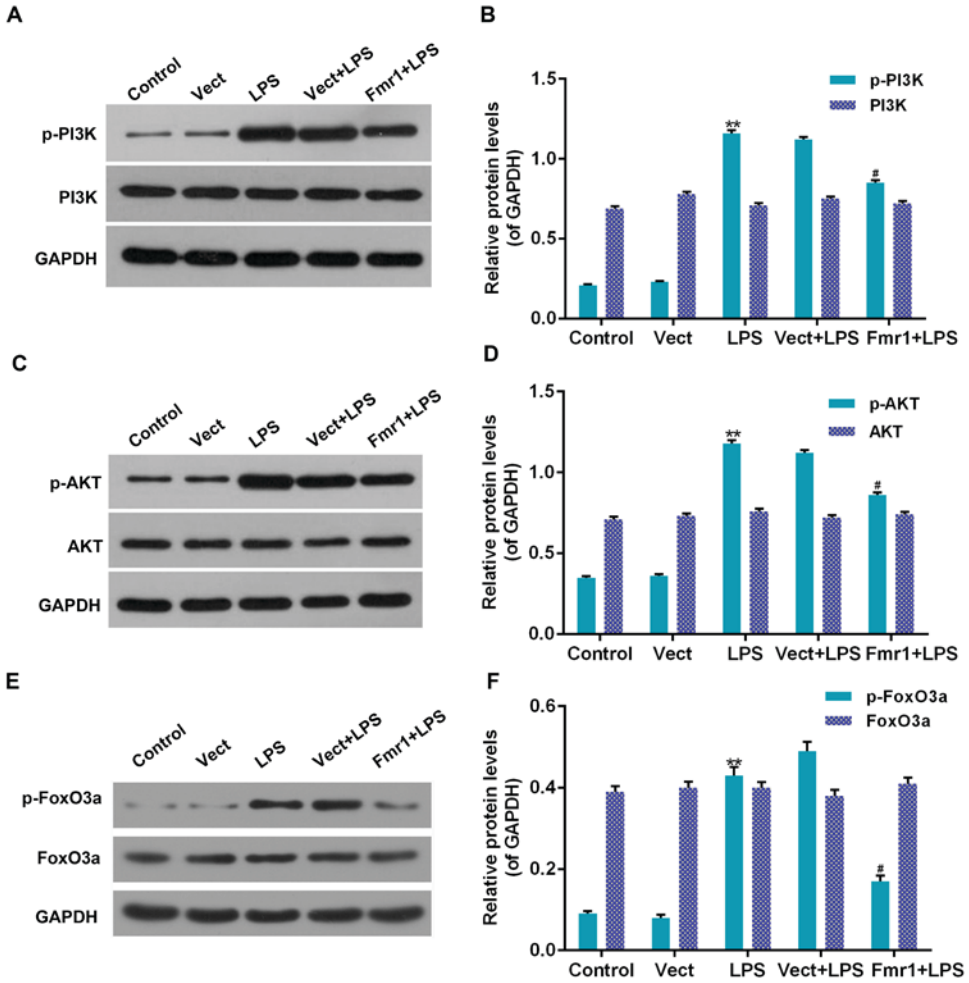

Figure 5. Effect of fmr1 on the PI3K/Akt/FoxO3a pathway in H9C2 cells injured by LPS. Levels of total and phosphorylated (A and B) of PI3K, (C and D) Akt and (E and F) FoxO3a were detected by western blot in these following cell groups: Fmr1+LPS, Vect+LPS, Vect, LPS and Control group. Values are expressed as the mean ± standard deviation (n=5 per group). **P<0.01 vs. control; $^{\#}$P<0.05 vs. Vect+LPS control. LPS, lipopolysaccharide; Vect, empty vector; fmr1, fragile X mental retardation 1; p-PI3K, phosphorylated phosphoinositide-3 kinase; Fox, forkhead box.

the structural integrity of mitochondria induces oxidative stress and abundant ROS release, resulting in mitochondrial dysfunction, cardiomyocyte injury and cardiac structure damage. The present results indicated that LPS increases the disruption rate of the mitochondrial membrane potential, and fmr1 overexpression reduced the increased rates of mitochondrial membrane potential collapse to repair mitochondrial dysfunction, which may influence the release of cytochrome *c* and mitochondrial ATP production in H9c2 cells treated with LPS.

ROS are highly reactive oxygenated chemical substances. Under normal conditions, the antioxidant enzymes, including SOD and GSH-peroxidase (GSH-Px), eliminate ROS during cell metabolism, maintaining a balance of ROS generation and elimination (21). However, excessive amounts of ROS are generated to resist bacterial infection-mediated cellular damage. When ROS accumulates ceaselessly and the endogenic anti-oxygen defence system cannot eliminate it in time, it induces oxidative stress, cellular disorders including upregulated lipid peroxidation, and also cell apoptosis (22). Therefore, LPS induces cardiomyocyte injury mostly through excessive ROS generation (23). The present study proved that the levels of ROS and MDA, the representative product of the lipid peroxidation process, increased, while activities of antioxidant enzymes, including SOD and GSH-Px (GSH/GSSG ratio) declined inH9c2 cells treated with LPS, which was attenuated by fmr1overexpression. Hence, fmr1 overexpression alleviated oxidative stress induced by LPS, with decreased peroxide levels and improved antioxidant enzyme activities.

Partial oxidative stress is one of the major reasons of cardiomyocyte apoptosis, and apoptosis is an important mechanism of cardiomyocyte death (24-26). The abnormal expression of bcl-2 family proteins serve important roles in cell apoptosis. Bax facilitates apoptosis by forming a heterodimer with Bcl-2 and inhibiting the activities of Bcl-2. Caspase-3 is the downstream activator of apoptosis, actively causes the dismemberment of the cell. On the contrary, XIAP has critical roles in inhibiting apoptosis. The present study provided evidence that the mechanism underlying the effects of LPS and the inhibitory role of fmr1is associated with apoptosis and apoptosis-associated factors. The results indicated that LPS increased the mRNA and protein levels of apoptosis-activating factors, including Bax and caspase-3, while overexpression of fmr1 significantly decreased them. Furthermore, LPS inhibited the mRNA and protein expression of the apoptosis inhibitors Bcl-2 and XIAP, while overexpression of fmr1 caused a significant upregulation of these factors. It was indicated that fmr1 inhibited apoptosis and associated factors to alleviate LPS-induced oxidative stress in H9c2 cells.

In addition to apoptosis-associated signaling, the PI3K/Akt/FoxO3a pathway was also indicated to have a role in the effect of fmr1 to alleviate LPS-induced injury of H9c2 myocardial cells. The activation of the PI3K/Akt pathway has critical roles in cell proliferation and differentiation, and alleviates oxidative stress-induced apoptosis (18). FoxO3a is a critical transcriptional factor downstream of the PI3K/Akt pathway, and also has important roles in regulating oxidative stress-induced apoptosis (27). Consistent with the activating effect of LPS on Akt by stimulating its phophorylation in macrophages demonstrated in RAW 264.7 macrophages by Nishina *et al* (28), in HT29 cells by Shi *et al* (29) and in chondrogenic cells by Wu *et al* (30), the present results indicated that the phosphorylation levels of PI3K, Akt and FoxO3a increased in the LPS group, which was significantly reduced when fmr1 was overexpressed. It was confirmed that the alleviating function of fmr1 on LPS-induced oxidative stress in H9c2 cells was mediated via the PI3K/Akt/FoxO3a pathway.

In conclusion, in the present study, a model of LPS-induced H9c2 cardiomyocyte injury was constructed to determine how the overexpression of fmr1 attenuated LPS-induced oxidative stress and apoptosis, during which peroxide levels, antioxidant enzyme activities and levels of apoptosis-associated factors were regulated via reducing the phosphorylation levels of PI3K, Akt and FoxO3a. Fmr1may be of significant value as a novel potential biomarker and an important endogenous protection factor in the cardiovascular system. The present results provide novel insight into the cardioprotective effects of fmr1, which may also have potential use as a diagnostic and prognostic biomarker for CVD.

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Availability of data and materials

The analyzed data sets generated during the study are included in this published article.

Authors' contributions

JB and ZZho conceived the study; JB performed the oxidative stress reaction, and assessed mitochondrial membrane potential variation and cell apoptosis; CY performed RT-qPCR and western blotting; and ZZhe treated cells.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

References

1. Fattahi F and Ward PA: Complement and sepsis-induced heart dysfunction. Mol Immunol 84: 57-64, 2017.
2. Murphy E and Steenbergen C: What makes the mitochondria a killer? Can we condition them to be less destructive? Biochim Biophys Acta 1813: 1302-1308, 2011.
3. Chen JQ, Cammarata PR, Baines CP and Yager JD: Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. Biochim Biophys Acta 1793: 1540-1570, 2009.
4. Lin WJ and Yeh WC: Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. Shock 24: 206-209, 2005.
5. Vieillard-Baron A, Caille V, Charron C, Belliard G, Page B and Jardin F: Actual incidence of global left ventricular hypokinesia in adult septic shock. Crit Care Med 36: 1701-1706, 2008.

6. Niederbichler AD, Westfall MV, Su GL, Donnerberg J, Usman A, Vogt PM, Ipaktchi KR, Arbabi S, Wang SC and Hemmila MR: Cardiomyocyte function after burn injury and lipopolysaccharide exposure: Single-cell contraction analysis and cytokine secretion profile. Shock 25: 176-183, 2006.
7. Akira S, Takeda K and Kaisho T: Toll-like receptors: Critical proteins linking innate and acquired immunity. Nat Immunol 2: 675-680, 2001.
8. Van't Padje S, Chaudhry B, Severijnen LA, van der Linde HC, Mientjes EJ, Oostra BA and Willemsen R: Reduction in fragile X related 1 protein causes cardiomyopathy and muscular dystrophy in zebrafish. J Exp Biol 212: 2564-2570, 2009.
9. Jiraanont P, Hagerman RJ, Neri G, Zollino M, Murdolo M and Tassone F: Germinal mosaicism for a deletion of the FMR1 gene leading to fragile X syndrome. Eur J Med Genet 59: 459-462, 2016.
10. Novak SM, Joardar A, Gregorio CC and Zarnescu DC: Regulation of heart rate in drosophila via fragile X mental retardation protein. PLoS One 10: e0142836, 2015.
11. Klusek J, Roberts JE and Losh M: Cardiac autonomic regulation in autism and Fragile X syndrome: A review. Psychol Bull 141: 141-175, 2015.
12. Schechter MA, Southerland KW, Feger BJ, Linder D Jr, Ali AA, Njoroge L, Milano CA and Bowles DE: An isolated working heart system for large animal models. J Vis Exp, 2014.
13. Chen L, Li W, Qi D and Wang D: Lycium barbarum polysaccharide protects against LPS-induced ARDS by inhibiting apoptosis, oxidative stress, and inflammation in pulmonary endothelial cells. Free Radic Res 23: 1-11, 2018.
14. Dong Z and Yuan Y: Accelerated inflammation and oxidative stress induced by LPS in acute lung injury: Inhibition by ST1926. Int J Mol Med 41: 3405-3421, 2018.
15. Zhang H, Zhang W, Jiao F, Li X, Zhang H, Wang L and Gong Z: The nephroprotective effect of MS-275 on lipopolysaccharide (LPS)-induced acute kidney injury by inhibiting reactive oxygen species (ROS)-oxidative stress and endoplasmic reticulum stress. Med Sci Monit 24: 2620-2630, 2018.
16. Tavares AM, da Rosa Araujo AS, Llesuy S, Khaper N, Rohde LE, Clausell N and Belló-Klein A: Early loss of cardiac function in acute myocardial infarction is associated with redox imbalance. Exp Clin Cardiol 17: 263-267, 2012.
17. Selvaraju V, Joshi M, Suresh S, Sanchez JA, Maulik N and Maulik G: Diabetes, oxidative stress, molecular mechanism, and cardiovascular disease-an overview. Toxicol Mech Methods 22: 330-335, 2012.
18. Deng C, Sun Z, Tong G, Yi W, Ma L, Zhao B, Cheng L, Zhang J, Cao F and Yi D: α-Lipoic acid reduces infarct size and preserves cardiac function in rat myocardial ischemia/reperfusion injury through activation of PI3K/Akt/Nrf2 pathway. PLoS One 8: e58371, 2013.
19. Ma Y, Tian S, Wang Z, Wang C, Chen X, Li W, Yang Y and He S: CMP-N-acetylneuraminic acid synthetase interacts with fragile X related protein 1. Mol Med Rep 14: 1501-1508, 2016.
20. Maximilian Buja L: Mitochondria in ischemic heart disease. Adv Exp Med Biol 982: 127-140, 2017.
21. Wang X, Lai R, Su X, Chen G and Liang Z: Edaravone attenuates lipopolysaccharide-induced acute respiratory distress syndrome associated early pulmonary fibrosis via inhibition of oxidative stress and transforming growth factor-β1/Smad3 signaling. Biochem Biophys Res Commun 495: 706-712, 2018.
22. Ding ZM, Jiao XF, Wu D, Zhang JY, Chen F, Wang YS, Huang CJ, Zhang SX, Li X and Huo LJ: Bisphenol AF negatively affects oocyte maturation of mouse in vitro through increasing oxidative stress and DNA damage. Chem Biol Interact 278: 222-229, 2017.
23. Gölz L, Memmert S, Rath-Deschner B, Jäger A, Appel T, Baumgarten G, Gotz W and Frede S: LPS from P. gingivalis and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis. Mediators Inflamm 2014: 986264, 2014.
24. Liu XR, Li T, Cao L, Yu YY, Chen LL, Fan XH, Yang BB and Tan XQ: Dexmedetomidine attenuates H2O2-induced neonatal rat cardiomyocytes apoptosis through mitochondria- and ER-medicated oxidative stress pathways. Mol Med Rep 17: 7258-7264, 2018.
25. Wang Y, Lei T, Yuan J, Wu Y, Shen X, Gao J, Feng W and Lu Z: GCN2 deficiency ameliorates doxorubicin-induced cardiotoxicity by decreasing cardiomyocyte apoptosis and myocardial oxidative stress. Redox Biol 17: 25-34, 2018.
26. Wang Z, Wang M, Liu J, Ye J, Jiang H, Xu Y, Ye D and Wan J: Inhibition of TRPA1 attenuates doxorubicin-induced acute cardiotoxicity by suppressing oxidative stress, the inflammatory response, and endoplasmic reticulum stress. Oxid Med Cell Longev 2018: 5179468, 2018.
27. Li D, Qu Y, Mao M, Zhang X, Li J, Ferriero D and Mu D: Involvement of the PTEN-AKT-FOXO3a pathway in neuronal apoptosis in developing rat brain after hypoxia-ischemia. J Cereb Blood Flow Metab 29: 1903-1913, 2009.
28. Nishina A, Shimizu K, Koketsu M, Ninomiya M, Sato D, Suzuki T, Hayakawa S and Kimura H: 5,7-Dihydroxyflavone analogues may regulate lipopolysaccharide-induced inflammatory responses by suppressing iκbα-linked AKT and ERK5 phosphorylation in raw 264.7 macrophages. Evid Based Compl Altern Med 2017: 7898973, 2017.
29. Shi J, Shan S, Li H, Song G and Li Z: Anti-inflammatory effects of millet bran derived-bound polyphenols in LPS-induced HT-29 cell via ROS/miR-149/Akt/NF-κB signaling pathway. Oncotarget 8: 74582-74594, 2017.
30. Wu DP, Zhang JL, Wang JY, Cui MX, Jia JL, Liu XH and Liang QD: MiR-1246 promotes LPS-induced inflammatory injury in chondrogenic cells ATDC5 by targeting HNF4γ. Cell Physiol Biochem 43: 2010-2021, 2017.