Abstract. Ginkgolide B (GB) is a diterpene lactone found in the leaves of the traditional Chinese medicinal plant Ginkgo that has been shown to have various pharmacological effects. However, the anti-apoptotic properties of GB in cardiovascular disease remain poorly understood. The present study aimed to investigate the effect of GB on hydrogen peroxide-induced cell injury in cardiac H9c2 cells, and to further clarify its protective mechanism of action. An in vitro hydrogen peroxide-treated H9c2 cell model was used in order to mimic myocardial ischemia-reperfusion (I/R) injury. Cell viability was assessed by the Cell Counting Kit-8 assay. The induction of apoptosis was determined by flow cytometry and staining was performed using Hoechst 33342. In addition, the effect of GB on the expression levels of apoptosis-associated proteins was evaluated by western blot analysis. The present study demonstrated that GB protected against hydrogen peroxide-induced cytotoxicity and cell apoptosis in H9c2 cardiac cells. GB upregulated the expression level of the anti-apoptotic protein Bcl-2 and down-regulated the expression levels of the pro-apoptotic proteins cleaved caspase-3 and Bax in hydrogen peroxide-treated H9c2 cells. The molecular mechanism underlying the anti-apoptotic effects of GB was subsequently detected. GB pretreatment activated the PI3K/Akt/mTOR signaling pathway and caused an increase in the phosphorylation levels of Akt and mTOR in hydrogen peroxide-treated H9c2 cells. These results revealed that GB inhibited hydrogen peroxide-induced apoptosis in H9c2 cells via activation of the PI3K/Akt/mTOR signaling pathway.

Introduction
Acute myocardial infarction (AMI), resulting from sudden interruption of blood flow in the main coronary arteries remains a leading cause of morbidity and mortality worldwide (1). Currently, percutaneous coronary intervention (PCI) is the most effective minimally invasive treatment for AMI. PCI is performed in order to reopen the affected coronary artery and restore adequate blood flow to the ischemic myocardium. It aims to reduce the myocardial infarct size and to maintain heart function (2,3). However, following PCI treatment, ~4-7% of the patients experience recurrent chest pain, elevated troponin I/T levels and onset of hemodynamic disorders that are attributed to myocardial ischemia-reperfusion (I/R) injury (4). Cardiac I/R is characterized by restricted blood flow to the myocardium followed by reoxygenation associated with blood reperfusion when the coronary artery reopens (5). I/R injury is caused by mitochondrial dysfunction, calcium overload and excessive generation of mitochondrial reactive oxygen species (ROS), which eventually contribute to cardiomyocyte apoptosis and necrosis (6). Therefore, the prevention of myocardial I/R injury is essential for improving prognosis following PCI treatment.

Ginkgolide B (GB) is extracted from the leaves of an ancient Chinese medicinal plant Ginkgo. This compound is a diterpene lactone, which has been documented as a strong antagonist of platelet-activating factor receptor (7). Previous studies have shown that the anti-inflammatory, antioxidant and anti-apoptotic properties of GB have rendered it beneficial in ischemic and hemorrhagic stroke (8-11). Furthermore, it has been shown to improve cognitive function and the disease outcome of different types of cancer. Moreover, previous studies have demonstrated that GB plays a therapeutic role in cardiovascular diseases (12,13). For instance, GB can ameliorate oxidized low-density lipoprotein-induced endothelial cell dysfunction in human umbilical vein endothelial cells and inhibit inflammatory cascades in murine RAW264.7 macrophage-like cells (12). In addition, GB inhibits the production of ROS in doxorubicin-induced cardiotoxicity in H9c2 cells (13). However, the effect of GB on myocardial I/R injury remains unclear.

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PI3Ks are a family of lipid kinases involved in the regulation of cellular activation, inflammatory responses and apoptosis (14). PI3K activation induces the formation of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) (15). PIP3 provides an Akt docking site for Akt phosphorylation and activation (16). As the central mediator of the PI3K signaling pathway, Akt promotes cell survival, apoptosis and proliferation by inducing the downstream mTOR complex (17). The mTOR signaling pathway is also involved in regulating cell growth, cell survival and protein synthesis (18). Therefore, the regulation of the PI3K/Akt/mTOR signaling pathway could affect cell viability and the induction of apoptosis on different cell types.

Hydrogen peroxide (H$_2$O$_2$) is a key metabolite in oxidative stress that is widely used to simulate myocardial I/R injury (19). The H9c2 cell line, derived from a rat embryonic heart ventricle, is considered a close surrogate for cardiomyocytes and has been proven to be ideal for cardiomyocyte signal transduction studies (20). Therefore, in the present study, H$_2$O$_2$ treatment in H9c2 cardiac cells was used to simulate myocardial I/R. In addition, the present study investigated the cardioprotective effect of GB on H$_2$O$_2$-induced apoptosis in H9c2 cells and its interaction with the PI3K/Akt/mTOR signaling pathway.

**Materials and methods**

**Cell culture.** H9c2 rat cardiomyocyte cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO$_2$. The culture medium was replaced every 2 days.

**Establishment of H9c2 cell oxidative stress model.** H9c2 cells (5x10$^3$ cells per well) were seeded in 96-well plates overnight and were pretreated with different concentrations of LY294002, GB and H$_2$O$_2$. To measure cell viability, 10 µl CCK-8 assay solution was added into each well, containing 90 µl medium, and the cells were then incubated in the dark at 37°C for additional 2 h. Absorbance was measured at 450 nm in a microplate reader (BioTek Instruments, Inc.).

**Hochevst 33342 staining.** Typical morphological features of apoptotic cells were observed by Hochevst 33342 staining (Nanjing KeyGen Biotech Co., Ltd.). Briefly, the cells were washed twice with PBS following H$_2$O$_2$ incubation with or without GB pretreatment and stained with Hochevst 33342 for 15 min in a 5% CO$_2$ incubator at 37°C in the dark. Finally, the cells were washed once with PBS and the images were captured by fluorescence microscopy (magnification, x200; Zeiss Axiovert A1). The apoptotic cells with nuclear chromatin condensation and fragmentation were stained bright blue. A total of three fluorescence images per well were captured randomly, and the percentage of apoptotic cells was calculated using the following equation: (The number of apoptotic cells/the total number of cells) x100.

**Annexin V-FITC/propidium iodide (PI) assay.** The induction of apoptosis was examined using the Annexin V-FITC/PI double staining assay (BD Biosciences; Becton-Dickinson and Company). Briefly, H9c2 cells were gently washed with ice-cold PBS, digested with 0.25% Trypsin without ethylenediaminetetraacetic acid (Gibco; Thermo Fisher Scientific, Inc.) and resuspended in complete culture medium. The cells were then centrifuged at 120 x g for 5 min at 4°C and the cell pellet was resuspended in ice-cold PBS and centrifuged at the same conditions. Finally, the cells were resuspended in 500 µl 1X binding buffer supplemented with 5 µl Annexin V-FITC and 5 µl PI. The cells were incubated at room temperature for 15 min in the dark and were analyzed by flow cytometry (BD FACSCalibur™; BD Biosciences; Becton-Dickinson and Company). Data was analyzed using FlowJo version 7.6.1 software (FlowJo LLC).

**Western blot analysis.** H9c2 cells were washed with ice-cold PBS, pelleted by cell scraper and lysed in 100 µl lysis buffer containing 1 µl phosphatase inhibitor, 0.1 µl protease inhibitor and 0.5 µl PMSF. The extracted proteins were quantified with the bicinchoninic acid Protein assay kit (Thermo Fisher Scientific, Inc.). A total of 20 µg protein extracts per lane were separated by 6-15% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes or nitrocellulose filter membrane. The membranes were blocked with 5% BSA (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 2 h and incubated overnight at 4°C with primary antibodies against rabbit anti-Bax (1:1,000; cat. no. 2772), rabbit anti-Bcl-2 (1:1,000; cat. no. 2870), rabbit anti-cleaved caspase-3 (1:1,000; cat. no. 9664), rabbit anti-caspase-3 (1:1,000; cat. no. 9662), rabbit anti-Akt (1:1,000; cat. no. 9272), rabbit anti-phosphorylated (p)-Akt (p-Akt; 1:1,000; cat. no. 4060), rabbit anti-mTOR (1:1,000; cat. no. 2983), rabbit anti-p-mTOR (p-mTOR; 1:1,000; cat. no. 2097), rabbit anti-p21 (p21; 1:1,000; cat. no. 6524).
Results

GB decreases H2O2-induced cytotoxicity in H9c2 cells. H2O2 reduced cell viability in a dose- and time-dependent manner (Fig. 1A). For instance, treatment of H9c2 cells with 600 µM H2O2 for 4 h reduced cell viability to 63.11% compared with that noted in untreated cells. The H9c2 cell state in this condition was not too poor to conduct subsequent experiments. Therefore, 600 µM H2O2 was selected as the optimal treatment concentration for subsequent experiments. The effect of GB alone on cell viability was also investigated. Cell viability was not significantly affected by GB treatment for 24 h even at concentrations up to 100 µM (Fig. 1B). Subsequently, the effects of H9c2 cell-GB pretreatment in the protection of the cells against H2O2-induced cytotoxicity were examined. GB pretreatment at 10 µM concentration for 20 h prior to co-incubation with 600 µM H2O2 for 4 h revealed the highest inhibitory effect on the induction of cell cytotoxicity caused by treatment of 600 µM H2O2 for 4 h (Fig. 1C). Therefore, 10 µM GB was used in subsequent experiments.

GB enhances the phosphorylation levels of Akt and mTOR. The expression levels of p-Akt and p-mTOR were downregulated in the H2O2-treated group, whereas GB pretreatment was able to reverse the effects of H2O2 (Fig. 3A and B).

Effect of the PI3K inhibitor LY294002 on the H2O2-induced cytotoxicity in H9c2 cells. To determine whether the activation of Akt and mTOR was associated with the protective effects of GB, the LY294002 inhibitor was used to investigate the expression levels of p-Akt and to assess cell viability. Pretreatment of the cells with LY294002 and GB downregulated the expression levels of p-Akt in a dose-dependent manner, compared with those noted in the single GB-pretreatment group. In addition, LY294002 and GB pretreatment reduced cell viability compared with that of the H2O2-treated group. Both the Bax/Bcl-2 and the cleaved caspase-3/caspase-3 ratios were significantly decreased in the GB-pretreatment group compared with those of the H2O2-treated group.

Discussion

The present study provides evidence regarding the protective effects of GB on H2O2-induced cytotoxicity in H9c2 cells.
These effects were mediated by the inhibition of cell apoptosis. Furthermore, it was shown that the GB-induced protective effect was mediated via activation of the PI3K/Akt/mTOR signaling pathway.
The process of apoptosis that was initially described by Kerr et al (21) is a form of programmed cell death with certain morphological features, such as narrowed cell volume, chromatin condensation, nuclear fragmentation and apoptotic body formation (22). In the present study, GB inhibited the induction of cell apoptosis by H₂O₂. Two major pathways of apoptosis, namely the death receptor-mediated and the mitochondrial-mediated apoptotic pathways have been identified. Both pathways result in caspase-dependent cell death (23). The members of the Bcl-2 family of proteins, which is composed of anti- and pro-apoptotic factors, are involved in the mitochondrial-mediated apoptotic pathway (24). Bax is a pro-apoptotic protein of the Bcl-2 family that is negatively regulated by Bcl-2 (anti-apoptotic protein). Consequently, the Bax/Bcl-2 ratio can act as an indicator that determines the cell susceptibility to apoptosis and the balance between anti- and pro-apoptotic factors (25). Caspase-3 is one of the most important members of the caspase family and is considered the central effector of apoptosis activated by upstream initiator caspases. Caspase-3 is cleaved to produce the final cleaved caspase-3 protein form (26). In the present study, GB pretreatment significantly decreased cleaved caspase-3 and
Bax expression levels, whereas it upregulated Bcl-2 expression levels in H2O2-treated H9c2 cells, resulting in a declined Bax/Bcl-2 ratio and increased cell viability. These results indicated that GB exerted protective effects against the H2O2-induced cytotoxicity in H9c2 cells partly through its anti-apoptotic properties.

Previous studies have shown that the activation of the PI3K/Akt/mTOR signaling pathway promotes cell proliferation and inhibits cell apoptosis (27,28). In the current study, GB pretreatment inhibited cell apoptosis by inducing Akt and mTOR phosphorylation. To further confirm this observation, H9c2 cells were treated with the PI3K inhibitor LY294002 and it was shown that the GB-induced Akt phosphorylation was partially blocked by the LY294002 inhibitor. In addition, LY294002 treatment partially reversed the protective effect of GB in maintaining cell viability. The aforementioned results suggested that GB exerted protective effects against cell apoptosis via the activation of the PI3K/Akt/mTOR signaling pathway.

All in vitro experiments were repeated three times in this study, this is a limitation of the study so in future experiments there should be a higher number of repeats. Additional in vitro and clinical studies are also required to support the in vitro results reported in the current study. Previous studies demonstrated that PCI treatment followed by remote ischemic preconditioning (RIPC) exhibited protective effects on myocardial I/R injury (29) and contrast-induced nephropathy (CIN) (30,31). Furthermore, the activation of Akt may mediate the target organ protection by RIPC (32). The present study suggested that GB pretreatment could trigger the activation of Akt during oxidative stress. In conclusion, GB pretreatment could be used to alleviate myocardial I/R injury and CIN following PCI treatment. However, additional clinical trials need to be conducted in the future in order to confirm this hypothesis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

JL and PW performed the majority of the experiments and drafted the manuscript; ZX, JZ and JL performed some of the experiments and collected the data; and JL and ZY designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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