EXPERIMENTAL CELL RESEARCH 327 (2014) 264–275

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/yexcr

Research Article

The protective effect of hispidin against hydrogen peroxide-induced apoptosis in H9c2 cardiomyoblast cells through Akt/GSK-3β and ERK1/2 signaling pathway

Dae-Eun Kim[a,e], *Bokyung Kim*[c], *Hwa-Sup Shin*[d], *Ho Jeong Kwon*[a,b,f,*], *Eun-Seok Park*[e,**]

[a]*Department of Biomaterials Science and Engineering, Translational Research Center for Protein Function Control, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[b]*Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[c]*Department of Physiology, Institute of Functional Genomics, Konkuk University School of Medicine, Chungju, Chungbuk 380-701, Republic of Korea*
[d]*Department of Biomedical Chemistry, Konkuk University, Chungju, Chungbuk 380-701, Republic of Korea*
[e]*Department of Biomedical Laboratory Science, Kyungbok University, Sinbuk-myeon, Pochen, Gyeonggi 487-717, Republic of Korea*
[f]*Department of Internal Medicine, College of Medicine, Yonsei University, Seoul 120-752, Republic of Korea*

ARTICLE INFORMATION

Article Chronology:
Received 1 March 2014
Received in revised form
28 July 2014
Accepted 30 July 2014
Available online 14 August 2014

Keywords:
Hispidin
Oxidative stress
Myocardial apoptosis
PKC inhibitor

ABSTRACT

Hispidin, a phenolic compound from *Phellinus linteus* (a medicinal mushroom), has been shown to possess strong anti-oxidant, anti-cancer, anti-diabetic, and anti-dementia properties. However, the cardioprotective efficacy of hispidin has not yet been investigated. In the present study, we investigated the protective effect of hispidin against oxidative stress-induced apoptosis in H9c2 cardiomyoblast cells and neonatal rat ventricular myocytes. While the treatment of H9c2 cardiomyoblast cells with hydrogen peroxide caused a loss of cell viability and an increase in the number of apoptotic cells, hispidin significantly protected the cells against hydrogen peroxide-induced cell death without any cytotoxicity as determined by XTT assay, LDH release assay, Hoechst 33342 assay, and Western blotting of apoptosis proteins such as caspase-3, Bax, and Bcl-2. Our data also shows that hispidin significantly scavenged intracellular ROS, and markedly enhanced the expression of antioxidant enzymes such as heme oxygenase-1 and catalase, which was accompanied by the concomitant activation of Akt/GSK-3β and ERK1/2 phosphorylation in H9c2 cardiomyoblast cells. The effects of hispidin on Akt and ERK phosphorylation were abrogated by LY294002 (a PI3K/Akt inhibitor) and U0126 (an ERK1/2 inhibitor). The effect of hispidin on GSK-3b activities was also blocked by LY294002. Furthermore, inhibiting the

Abbreviations: CAT, catalase; DMEM, Dulbecco's modified Eagle's media; DMSO, dimethylsulfoxide; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; JNK, c-Jun N-terminal protein kinase; MAPKs, mitogen-activated proteins kinases; XTT, 2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase

*Corresponding author at: Chemical Genomics National Research Laboratory, Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea. Fax: +82 2 362 7265.
**Corresponding author. Fax: +82 31 570 9929.
E-mail addresses: kwonhj@yonsei.ac.kr (H.J. Kwon), eunspark@kbu.ac.kr (E.-S. Park).

http://dx.doi.org/10.1016/j.yexcr.2014.07.037

Akt/GSK-3β and ERK1/2 pathway by these inhibitors significantly reversed the hispidin-induced Bax and Bcl-2 expression, apoptosis induction, and ROS production. These findings indicate that hispidin protects against apoptosis in H9c2 cardiomyoblast cells exposed to hydrogen peroxide through reducing intracellular ROS production, regulating apoptosis-related proteins, and the activation of the Akt/GSK-3β and ERK1/2 signaling pathways.

Introduction

Cardiovascular diseases are the leading causes of death and disability worldwide [1]. One of the most important presentations of cardiovascular diseases is ischemia, which leads to tissue hypoxia, cellular apoptosis, and organ dysfunction in severe situations [2]. Cardiac myocyte apoptosis, which causes massive cell loss and eventually affects the long-term prognosis, is involved in the pathogenesis of various cardiovascular diseases such as myocardial infarction, ischemia/reperfusion (I/R) injury, and heart failure. It has always been a major focus of medical research [3,4].

Oxidative stress, which is caused by the accumulation of intracellular reactive oxygen species (ROS) or reactive nitrogen species (RNS), is one of the leading factors triggering cardiomyocyte apoptosis [5]. These species are generated constantly *in vivo*, and can cause oxidative damage to DNA, proteins, and lipids, resulting in cellular apoptotic death [6]. I/R is a major cause of oxidative stress in cardiomyocytes. This oxidative stress damages secondary cardiomyocytes during reperfusion therapy in acute myocardial infarction cases [7]. Hence, the modulation of intracellular ROS levels and regulation of apoptotic cascade are considered crucial therapeutic strategies for treating CVD.

Phellinus linteus is a species of fungus belonging to the *Hymenochaetaceae* family native mainly to tropical America, Africa, and East Asia (in particular China, Japan, and Korea) [8]. It has been reported that polyphenols, polysaccharides, and other components of *P. linteus* have antioxidant activities [9,10]. Furthermore, in an attempt to employ *P. linteus* in western therapies, many researchers have investigated the effects of the extracts from the fruit bodies or mycelia of *P. linteus* on *in vitro* and *in vivo* activities [8]. Among these extracts hispidin, a PKC inhibitor, has been shown to possess strong antioxidant, anticancer, and antidiabetic properties [11,12]. However, no study has examined the action of hispidin against oxidative stress or the detailed molecular mechanism underlying its cardioprotective efficacy.

Therefore, in this study, we investigated the protective effect of hispidin on H9c2 cardiomyoblasts against H_2O_2 and its effects on signaling pathways involved in cell death and survival. We found that hispidin protected against H_2O_2-induced H9c2 cardiomyoblast oxidative apoptotic injury mainly by activating the Akt/GSK-3β and ERK1/2 signaling pathways, which played an important role in mediating the anti-apoptotic effect of hispidin.

Materials and methods

Materials and reagents

Hispidin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.05% DMSO, followed by further dilution in DMEM without fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cell culture materials were purchased from Thermo Fisher Scientific. Hydrogen peroxide was purchased from Sigma-Aldrich. All antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). General laboratory reagents were purchased from Sigma-Aldrich.

Cell culture

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin under an atmosphere of 95% air and 5% CO_2 at 37 °C, and trypsinized every 3–5 days. Two days before experiments, the cells were seeded on glass coverslips or plastic wells at a quantity to achieve ~90% confluency. DMSO (0.05%) was present in the buffer to dissolve the hispidin, and had no effect on the H_2O_2-induced cytotoxicity at this concentration.

Isolation and culture of neonatal rat ventricular myocytes

Primary culture of cardiomyocytes was performed according to previous methods [13]. Cells were isolated from 1- to 2-day-old Sprague-Dawley rats by enzymatic digestion with 0.1% trypsin and 0.03% collagenase. After isolation, cells were plated onto laminin-coated 35-mm dishes at a density of 1×10^3 cells/mm^2 and cultured for 48 h in Dulbecco's modified Eagle's medium and Medium 199 (4:1) containing 10% fetal calf serum, 4 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 0.1 mM 5-bromo-2-deoxyuridine. 5-Bromo-2-deoxyuridine was used to inhibit fibroblast proliferation. The animal experiments were conducted according to protocols that follow the National Institutes of Health standards and the guidelines for the Care and Use of Experimental Animals.

Cell viability and morphological changes

Cell viability was determined by XTT (2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Biological Industries Co. Beit Haemek, Israel) and lactate dehydrogenase (LDH) assays (Roche Diagnostics, Ltd., Lewes, East Sussex, UK). Briefly, H9c2 cells were seeded at 1×10^4 cells/well in 96-well plates. After 1 h of treatment with different hispidin concentrations followed by 6 h of incubation with 600 μM H_2O_2, 50 μl of XTT solution was added to each well. After an additional 2 h, the cell viability was determined by measuring the absorbance at 460 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For the LDH assay, after H9c2 cells were seeded into 96-well plates and subjected to the aforementioned treatment with hispidin and H_2O_2, the reaction buffer was added to each well, and the whole mixture was incubated for 30 min at room temperature. The reaction was then stopped by adding 2 mol/L HCl, and the absorbance was

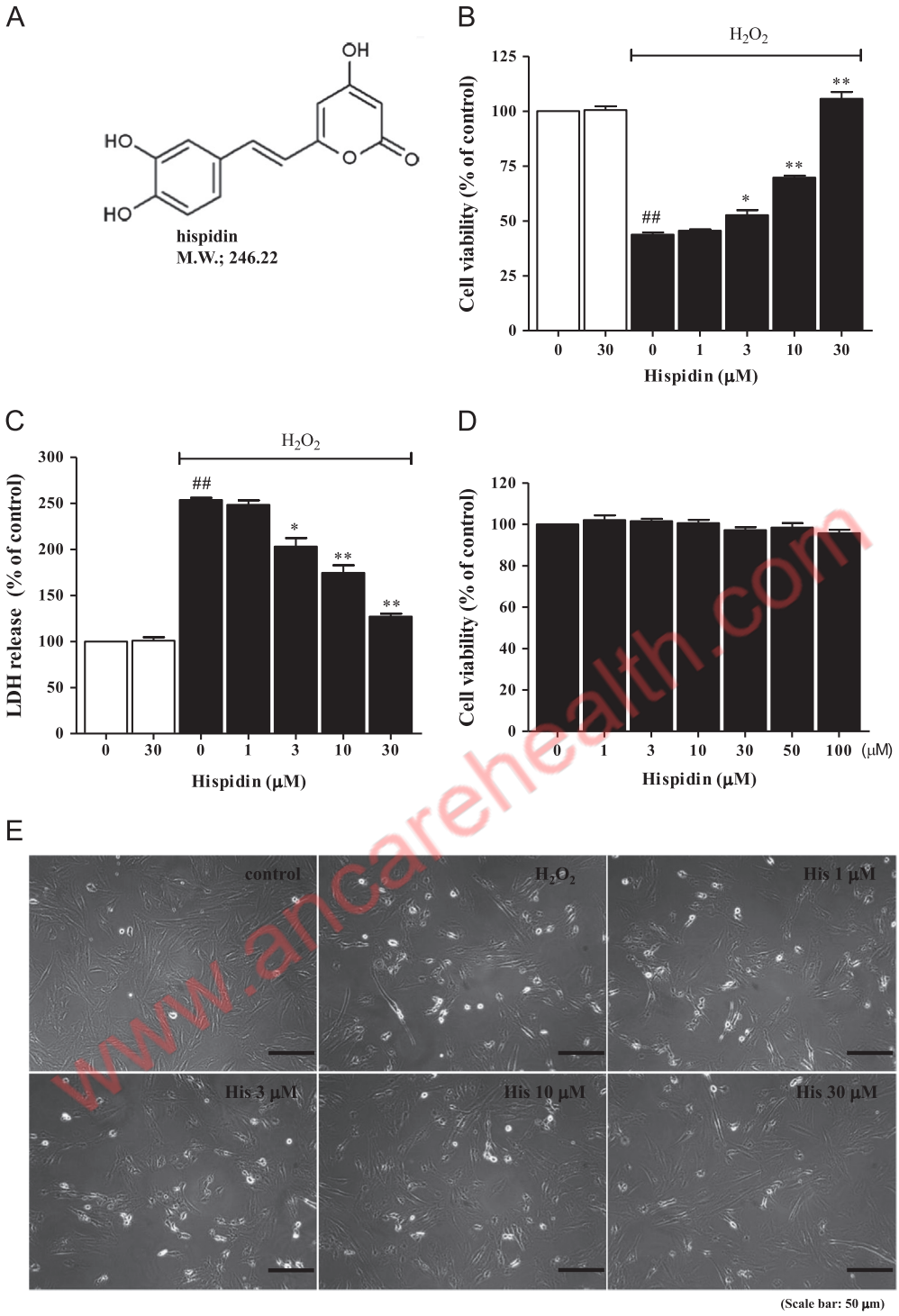


Fig. 1 – *The protective effect of hispidin on H_2O_2-treated H9c2 cells.* (A) Chemical structure of hispidin. (B) Effect of hispidin on H_2O_2-induced cell death. H9c2 cells were pre-cultured in serum-free medium in the presence or absence of hispidin (1, 3, 10 and 30 μM) for 1 h and then stimulated further with 600 μM H_2O_2 for an additional 6 h, during which the XTT reagent was added at the end of hour 4, and the entire culture mixture was further incubated for 2 h. The absorbance was determined with an enzyme-linked immunosorbent assay reader at a wavelength of 460 nm. (C) Effect of hispidin on lactate dehydrogenase (LDH) release in H_2O_2-treated H9c2 cells. H9c2 cells were pretreated with different concentrations of hispidin for 1 h and further incubated with 600 μM H_2O_2 for 6 h, during which LDH reagent was added at the end of hour 4, and the entire culture mixture was further incubated for the remaining 2 h. Absorbance was determined with a spectrophotometer at a wavelength of 420 nm. (D) The cytotoxic effect of hispidin (1–100 μM) on H9c2 cells was measured by the XTT assay following a 24 h incubation. (D) Cell morphology was observed after 6 h of H_2O_2 exposure. H_2O_2 resulted in abnormal cell morphology, whereas hispidin pretreatment resulted in dose-dependent protection from the H_2O_2-induced morphological changes. Representative images were taken from three independent experiments (magnification × 40). Data are mean ± standard error ($n=3$). $^{\#\#}P<0.01$ vs. untreated cells; $^{*}P<0.05$ and $^{**}P<0.01$ vs. H_2O_2 alone.

measured with a spectrophotometer at a wavelength of 420 nm. Morphological changes in the cells were observed, and images were captured under an inverted microscope connected to a digital camera (Canon Inc., Tokyo, Japan).

Assessment of apoptotic cell death

Hoechst 33342 staining (BD Biosciences, Franklin Lakes, NJ, USA), which distinguishes apoptotic cells from normal cells based on nuclear chromatin condensation and fragmentation, was used for the qualitative and quantitative analyses of the apoptotic cardiomyoblast. H9c2 cells were cultured at 1×10^4 cells/well on 8-well chamber slides for 24 h. After the aforementioned treatment with hispidin and H_2O_2, the cells were incubated with 2 μg/mL Hoechst 33342 for 15 min, washed twice with phosphate-buffered saline, and visualized by fluorescence microscopy (FV-1000 spectral, Olympus, Tokyo, Japan).

Measurement of intracellular ROS

The generation of intracellular ROS in H9c2 cells was measured using the green fluorescence probe 6-carboxy-2′,7′-dichlorofluoroscein diacetate (DCF-DA; Invitrogen, Rockville, MD, USA). Cells were seeded at 1×10^4 cells/well in 8-well cell culture slides (SPL, Seoul, Korea) and subjected to the aforementioned hispidin and H_2O_2 treatment. Then, the cells were stained with 10 μM DCF-DA at 37 °C for 30 min and observed with a confocal microscope system (FV-1000 spectral, Olympus, Tokyo, Japan). The cells were plated onto clear-top, black-welled 96-well plates (SPL) at 1×10^4 cells/well to measure ROS by fluorometric analysis. After the cells were treated with hispidin and H_2O_2 they were loaded with 10 μM DCF-DA at 37 °C for 30 min, and fluorescence was measured with a fluorometer (Victor 3, Perkin-Elmer, Waltham, MA, USA; excitation = 485 nm, emission = 535 nm).

Western blotting

Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis containing 12% acrylamide gels according to a method described previously [14]. The supernatant protein concentrations were determined using Bio-Rad DC Protein Assay Reagents (Hercules, CA, USA). The proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), which were blocked overnight at 4 °C in Tris-buffered saline containing 0.1% Tween 20 and 5% BSA. The membranes were incubated overnight at 4 °C with specific antibodies diluted to 1:1000. Immune complexes were incubated for 1 h with a peroxidase-conjugated secondary antibody diluted to 1:5000. After applying the secondary antibody, the blots were incubated in an Enhanced Chemiluminescence Western Blotting Detection System (Thermo Fisher) and exposed to photographic film. The band intensity was measured using quantification software (Bio-Rad). The band intensities were quantified using Scion-Image software for Windows.

Statistical analysis

The experimental results are expressed as mean ± standard error. A one-way analysis of variance was used for multiple comparisons (GraphPad Prism version 4.00 for Windows, San Diego, CA, USA). Dunnett's test was applied if there was a difference among the treated groups. $P < 0.05$ was considered significant.

Results

Effects of hispidin on cell viability, LDH release, and morphological changes

To evaluate whether hispidin protects H9c2 cardiomyoblast cells from oxidative stress, we examined the direct cytotoxic effect of 600 μM H_2O_2 on H9c2 cells in the present and absence of hispidin for 6 h. The data shown in Fig. 1B indicates that hispidin protects against 600 μM H_2O_2-induced cytotoxicity in a concentration-dependent manner. The cell viability was significantly decreased after 600 μM H_2O_2-treatment (43.7 ± 3.1%, $P < 0.01$) compared to the non-treated group. As the concentration of hispidin was increased to 3, 10, and 30 μM, the cell viability was significantly increased to 52.7 ± 7.2% ($P < 0.01$), 69.7 ± 3.0% ($P < 0.01$), and 105.7 ± 7.6% ($P < 0.01$), respectively (Fig. 1B). As shown in Fig. 1C and E, pretreatment with 3, 10, and 30 μM hispidin reduced H_2O_2-induced LDH release while recovering the H_2O_2-induced abnormal morphological changes in a concentration-dependent manner. However, hispidin did not influence cell viability in cultured H9c2 cells at all concentrations up to 100 μM (Fig. 1D).

Inhibitory effect of hispidin on intracellular ROS generation by H_2O_2

Hispidin was found to exhibit quenching effects against free radicals [11,12]. Therefore, we determined whether hispidin shows a potent ROS-scavenging effect. Using confocal imaging (Fig. 2A and B, 6.5-fold vs. the normal control group, $P < 0.01$) and a fluorescence assay (Fig. 2C, 155 ± 2.8%, vs. the normal control group, $P < 0.01$) we observed an increased level of intracellular ROS production in H9c2 cells exposed to H_2O_2, which was reduced 6.1-fold ($P < 0.05$), 3.6-fold (vs. H_2O_2-treated group, $P < 0.01$), and 1.8-fold ($P < 0.01$) and decreased to 149.5 ± 4.2% ($P > 0.05$), 131 ± 3.1% (vs. H_2O_2-treated group, $P < 0.01$), and 105.1 ± 4.2.% ($P < 0.01$), respectively, following hispidin pretreatment at concentrations of 3, 10, and 30 μM.

Next, the expression levels of antioxidant proteins such as heme oxygenase (HO-1), catalase (CAT) and superoxide dismutase (SOD, Cu/ZnSOD and MnSOD) were examined in the H9c2 cells under H_2O_2 exposure by Western blotting analysis to determine whether the regulation of antioxidant enzymes is involved in the scavenging properties of hispidin against H_2O_2-induced oxidative stress. As shown in Fig. 2D, hispidin doses of 3, 10, and 30 μM significantly increased the expression of HO-1 and CAT compared to those in cells treated with H_2O_2 alone. However, Cu/ZnSOD and MnSOD protein expression did not increase with hispidin treatment.

Inhibitory effects of hispidin on H_2O_2-induced apoptosis in H9c2 cells

Hoechst 33342 staining was performed to improve the XTT and LDH results and characterize the type of cell death involved in the experiments. We evaluated the effect of hispidin regarding DNA fragmentation, which is a hallmark of apoptosis [15]. As shown in Fig. 3A and B, Hoechst 33342 assay revealed that H_2O_2 increased DNA fragmentation which is in accordance with a previous study that showed that ROS induces apoptotic damage such as DNA fragmentation [14,16]. H_2O_2 caused an increase in Hoechst-positive cells (bright blue, 74.7 ± 8.5% vs. the normal control group, $P < 0.01$), whereas the

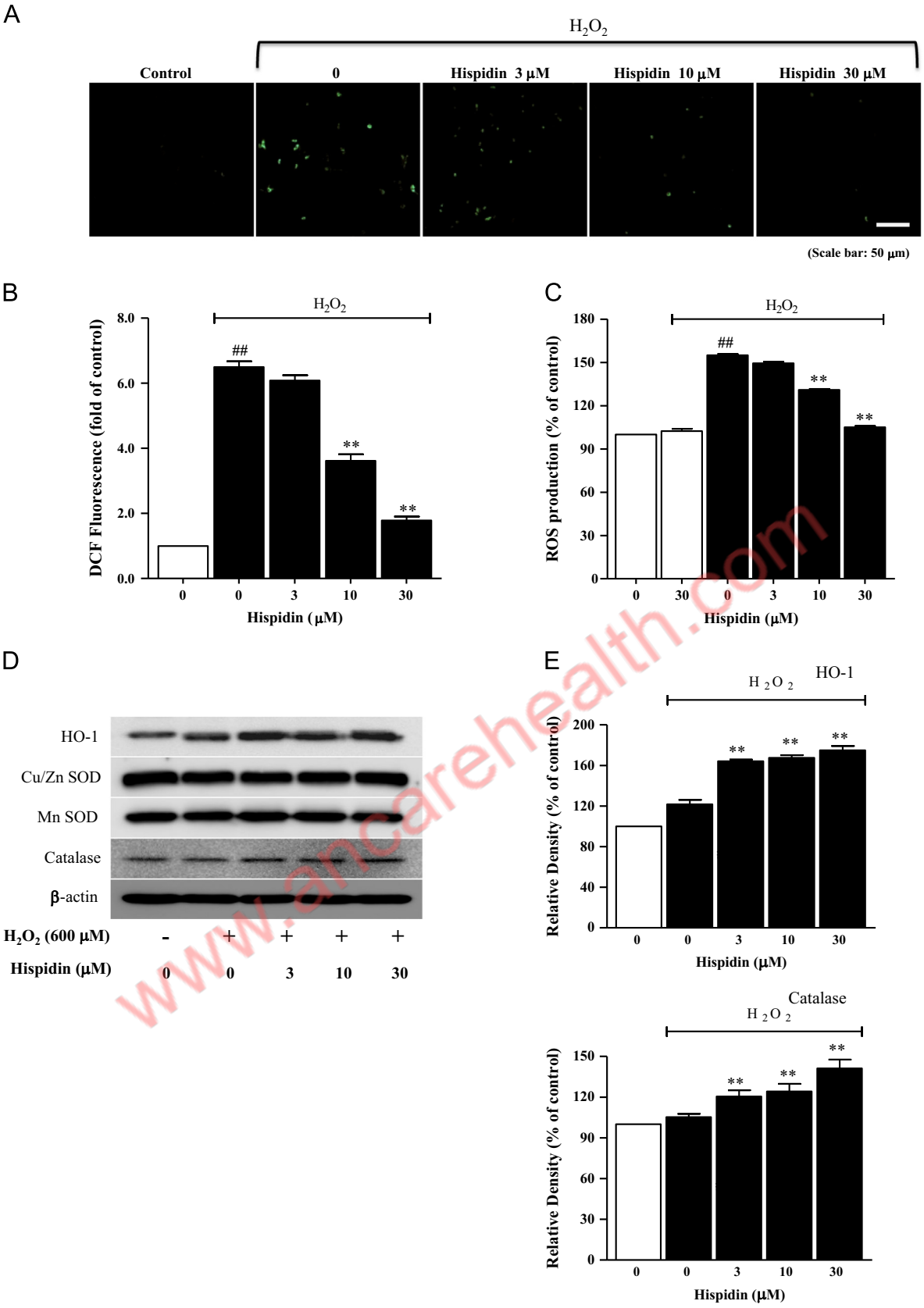


Fig. 2 – *Effect of hispidin on intracellular reactive oxygen species (ROS) generation.* After 1 h of pretreatment with or without hispidin (3, 10, and 30 μM), the cells were exposed to H_2O_2 for 6 h and assayed for ROS generation using DCF-DA fluorescence. (A) Confocal microscopy images of cells fluorescently stained with DCF-DA (magnification ×40, scale bar=50 μm). (B) Bar graphs depict the percentage of fluorescent-positive cells detected by confocal images upon staining with fluorescent dye. (C) Fluorescence was measured with a fluorometer (excitation=485 nm, emission=535 nm). (D) Effects of hispidin on expression of antioxidant proteins in H_2O_2-treated H9c2 cells. The cells were pre-incubated with various concentrations of hispidin for 1 h and stimulated with 600 μM H_2O_2 for 6 h. The cells were then lysed, and the proteins were detected by immunoblotting. (E) Densitometry scanning analysis of HO-1 and catalase. β-actin was used for normalization. Microscopic images and Western blot images are representative of three independent experiments. Data are mean±standard error ($n=3$). $^{\#\#}P<0.01$ vs. untreated cells; $^{**}P<0.01$ vs. H_2O_2 alone.

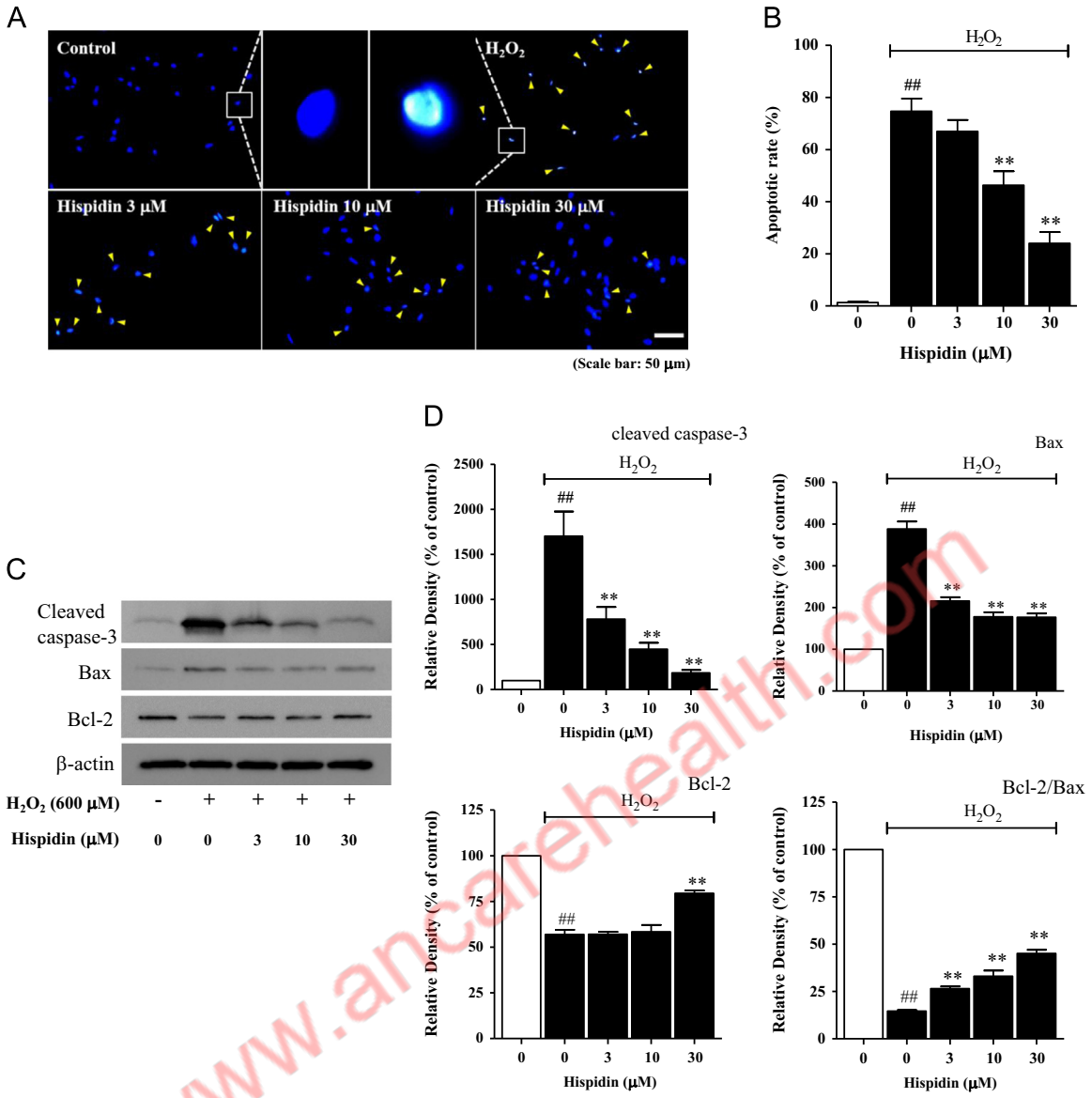


Fig. 3 – *Effect of hispidin on H_2O_2-induced apoptosis in H9c2 cells.* Apoptotic cells were examined under a fluorescence microscope at 200× magnification after Hoechst 33342 staining (scale bar=50 μm). (A) After 1 h of pretreatment with or without different concentrations of hispidin the cells were exposed to H_2O_2 and stained using Hoechst 33342, and then the cells were visualized under a fluorescence microscope. Apoptotic cells were identified as those with nuclei exhibiting brightly stained condensed chromatin (Hoechst-positive cells). (B) The apoptotic rate was determined by calculating the percentage of Hoechst-positive cells over total cells. Arrows indicate apoptotic cell nuclei (Hoechst-positive cells). (C) Effects of hispidin on the expression of apoptosis-related proteins in H_2O_2-treated H9c2 cells. The cells were pre-incubated with various concentrations of hispidin for 1 h and stimulated with 600 μM H_2O_2 for 6 h. The cells were then lysed, and the proteins were detected by immunoblotting. (D) Densitometry scanning analysis of cleaved caspase-3, Bax, and Bcl-2 expression, and Bcl-2/Bax ratio. β-actin was used for normalization. Microscopic images and Western images are representative of three independent experiments. Data are mean±standard error ($n=3$). $^{\#\#}P<0.01$ vs. untreated cells; $^{**}P<0.01$ vs. H_2O_2 alone.

number of Hoechst-positive cells was reduced to 67.0±7.5% ($P>0.05$), 46.3±9.3% ($P<0.01$), and 24.0±7.5% ($P<0.01$) when cells were pretreated with 3, 10, and 30 μM hispidin, respectively.

The regulatory effects of hispidin on the expression of apoptotic regulatory proteins such as caspase-3, Bax, and Bcl-2 were examined to further confirm the anti-apoptotic effect of hispidin on H_2O_2-mediated apoptosis. As shown in Fig. 3C and D, H_2O_2 treatment is associated with a significant increase in cleaved caspase-3 and Bax and decreased Bcl-2 compared to those in the control. Hispidin significantly decreased cleaved caspase-3 and Bax expression in H_2O_2-treated H9c2 cells, while increasing Bcl-2 expression. In addition H_2O_2 treatment resulted in a decreased Bcl-2/Bax ratio compared to the normal control group, while hispidin recovered the ratio of Bcl-2/Bax proteins in a dose-dependent manner (Fig. 3D).

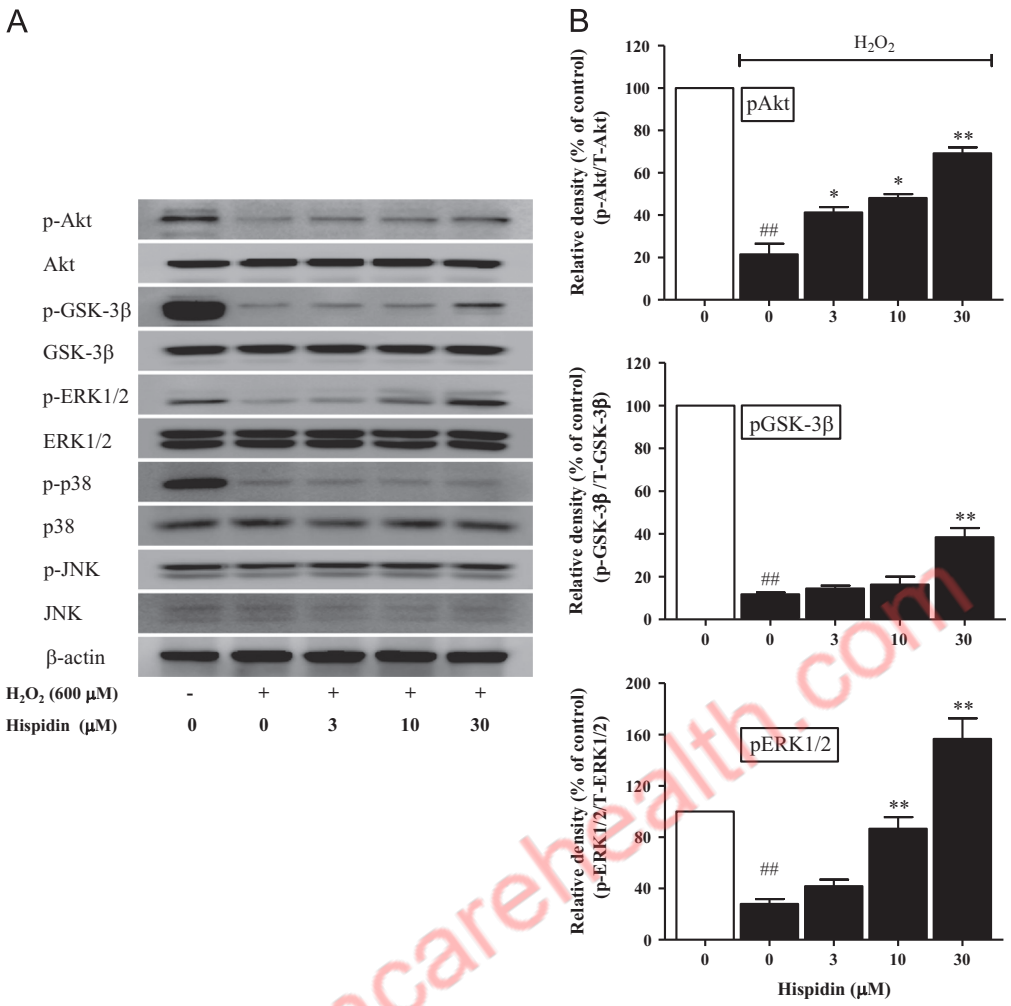


Fig. 4 – Effects of hispidin on phosphorylation of Akt, glycogen synthase kinase-3β (GSK-3β), extracellular regulated kinase (ERK)1/2, p38 and JNK in H_2O_2-treated H9c2 cells. (A) Cells were cultured in 6-well plates until confluence, and the medium was replaced with serum-free medium in the presence or absence of hispidin (3, 10 and 30 μM) for 1 h. The cells were then stimulated with 600 μM H_2O_2 for 30 min. The cells were then lysed, and the proteins were detected by immunoblotting. (B) Densitometry scanning analysis of ratio of p-Akt/T-Akt, p-GSK-3β/T-GSK-3β, p-ERK1/2/T-ERK1/2, p-p38/T-p38, and p-JNK/T-JNK. Total proteins were used for normalization. Western images are representative of three independent experiments. Data are mean ± standard error ($n=3$). $^{\#\#}P<0.01$ vs. untreated cells; $^{*}P<0.05$ and $^{**}P<0.01$ vs. H_2O_2 alone.

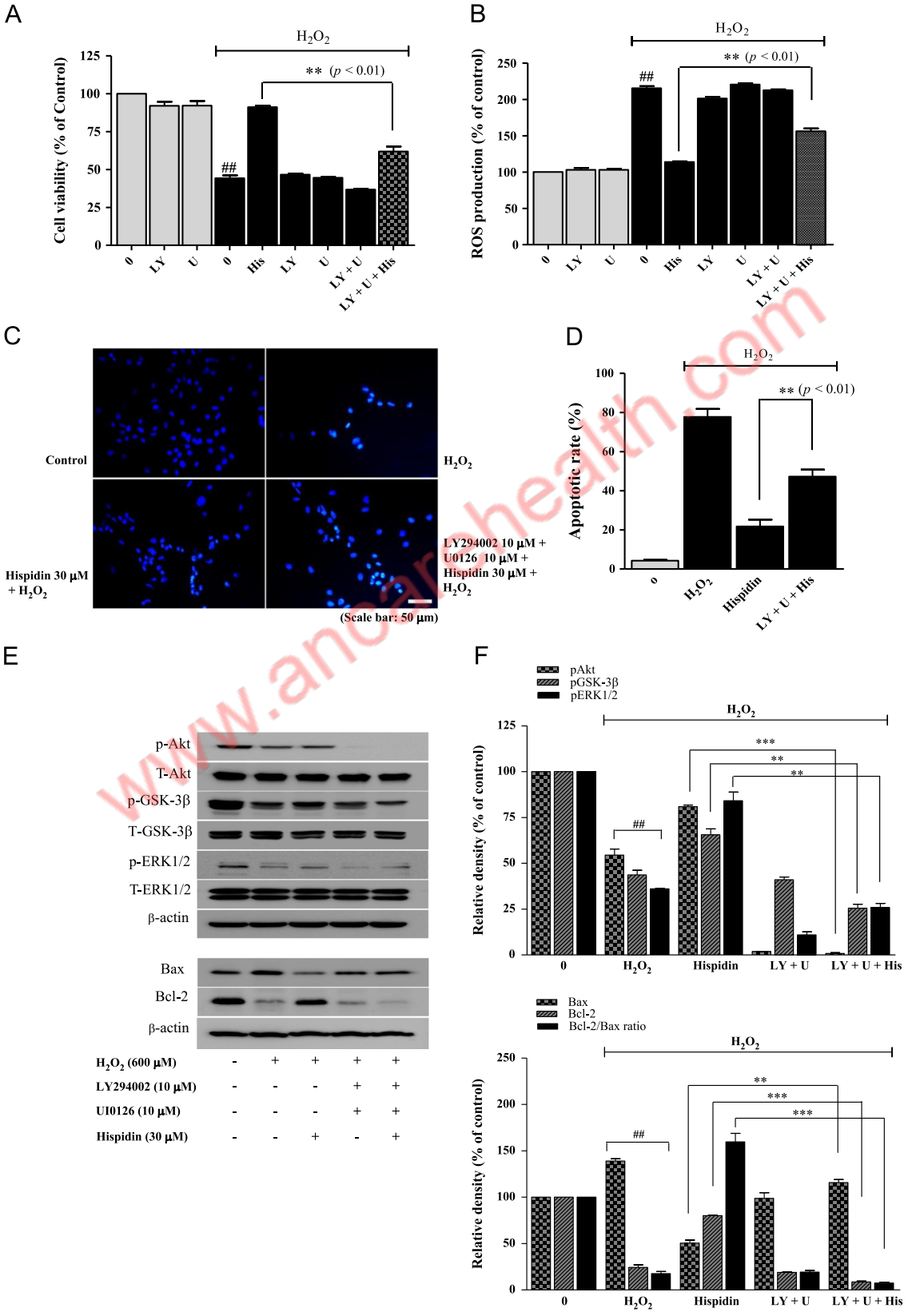
Effects of hispidin on H_2O_2-induced dephosphorylation of Akt, extracellular regulated kinase (ERK)1/2, glycogen synthase kinase-3β (GSK-3β), p38 mitogen activated protein kinase (MAPK) and JNK

The activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway in H9c2 cells suppresses apoptosis and promotes cell survival, and MAPKs are involved in the regulation of apoptosis and anti-apoptosis [17,18]. Thus, to explore the potential signaling pathways contributing to the anti-apoptotic effect of hispidin, we examined Akt/GSK-3β and MAPK activation. As shown in Fig. 4A H_2O_2 treatment decreased Akt, GSK-3β and, ERK1/2 phosphorylation compared to the control, but the decreased phosphorylation of these kinases was recovered by hispidin in a concentration-dependent manner. However, no significant alterations in p38 and JNK phosphorylation were observed following hispidin pretreatment of H_2O_2-exposed H9c2 cells. These results indicate that the Akt/GSK-3β and EKR1/2 signaling pathways may participate in the anti-apoptotic function of hispidin on H_2O_2-exposed H9c2 cells.

Fig. 5 – *Effects of PI3K/Akt and ERK1/2 inhibitors on hispidin-induced apoptosis and ROS generation in H9c2 cells*. H9c2 cells were pre-cultured in serum-free medium in the presence or absence of PI3K/Akt inhibitor (LY294002, 10 μM) and ERK1/2 inhibitor (U0126, 10 μM) with or without 30 μM hispidin for 1 h, and then stimulated further with 600 μM H_2O_2 for an additional 6 h. (A) Cell death and ROS generation were assessed by XTT assay and DCF-DA assay, as described in Figs. 1 and 2, respectively. (C) Apoptotic cells were examined under a fluorescence microscope at 200× magnification after Hoechst 33342 staining (scale bar = 50 μm). (D) Apoptotic index was determined by calculating the percentage of Hoechst-positive cells over total cells. (E) The cells were then lysed, and the proteins were detected by immunoblotting. (F) Data are mean ± S.E.M. for at least three independent experiments after densitometric quantification. $^{\#\#}P<0.01$ vs. untreated cells; $^{**}P<0.01$ and $^{***}P<0.001$ vs. hispidin-treated cells.

Effect of pharmacological inhibition of Akt/GSK-3β and ERK1/2 signaling pathway on protection of hispidin against H_2O_2-induced apoptosis in H9c2 cells

To determine whether the increased phosphorylation of Akt/GSK-3β and ERK1/2 pathways contributes to the cardioprotective effect of hispidin we pretreated H9c2 cells with LY294002 (PI3K inhibitor, 10 μM) and U0126 (MEK inhibitor, 10 μM), and applied H_2O_2 for 6 h in the absence or presence of hispidin (30 μM) treatment. As shown in Fig. 5 A and B, both LY294002 and U0126 alone did not alter cell viability or ROS production. However, pretreatment with both LY294002 and U0126 antagonized the



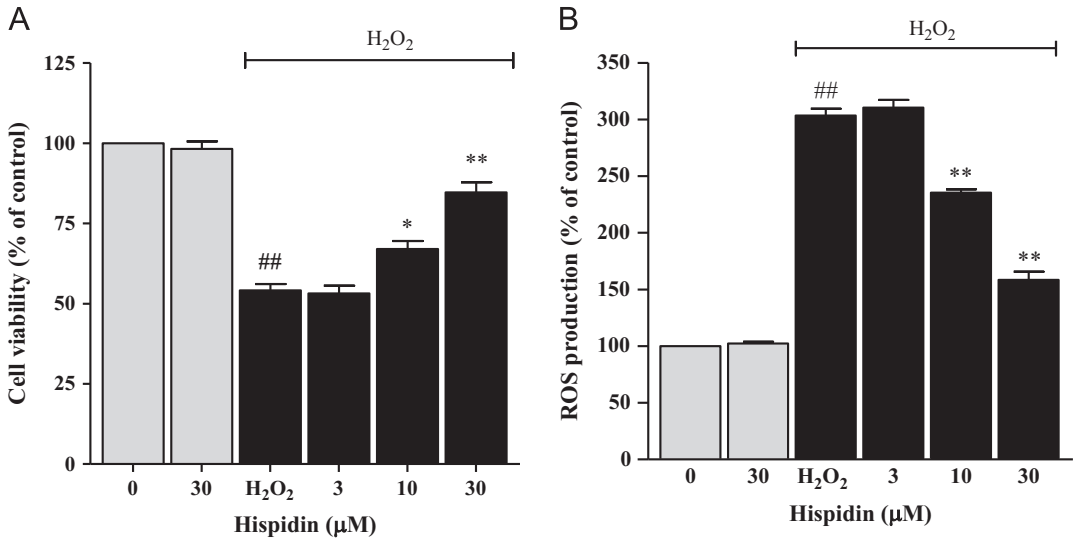


Fig. 6 – *Effects of hispidin in neonatal rat ventricular myocytes.* (A) Neonatal rat ventricular myocytes were pre-cultured in the presence or absence of hispidin (3, 10, and 30 μM) for 1 h and then stimulated further with 600 μM H_2O_2 for an additional 6 h, during which the XTT reagent was added at the end of hour 4, and the entire culture mixture was further incubated for 2 h. The absorbance was determined with an enzyme-linked immunosorbent assay reader at a wavelength of 460 nm. (B) After 1 h of pretreatment with or without hispidin (3, 10, and 30 μM), neonatal rat ventricular myocytes were exposed to H_2O_2 for 6 h and assayed for ROS generation using DCF-DA fluorescence. Fluorescence was measured with a fluorometer (excitation=485 nm, emission=535 nm). Representative images were taken from three independent experiments. Data are mean±standard error ($n=3$). $^{\#\#}P<0.01$ vs. untreated cells; $^{*}P<0.05$ and $^{**}P<0.01$ vs. H_2O_2 alone.

protective effect of hispidin against H_2O_2-induced cell viability and ROS production. When cells were pretreated with LY294002, U0126, and hispidin, the cell viability of cells exposed to H_2O_2 decreased by 32.1±8.9% ($P<0.01$, $n=3$), ROS production increased by 37.1±5.7% ($P<0.01$, $n=3$), and apoptosis increased by approximately 2.2-fold ($P<0.01$, $n=3$) compared to the levels of cells treated with hispidin alone. This was further examined by Western blot analysis, in which the effect of each inhibitor was confirmed by reduced Akt/GSK-3β and ERK1/2 phosphorylation. As shown in Fig. 5E, pretreatment with LY294002 and U0126 prevented the increased phosphorylation of Akt (99.0±0.9% reduction, $P<0.01$), GSK3-β (61.1±5.7% reduction, $P<0.01$) and ERK1/2 (69.3±4.7% reduction, $P<0.01$) by hispidin in H_2O_2-treated H9c2 cells compared to cells treated with hispidin alone. In addition, LY294002 and U0126 reversed the protective effects of hispidin on H_2O_2-inudced apoptosis as determined by apoptosis-related proteins such as Bax and Bcl-2 expression. These results suggest that the cytoprotective effect of hispidin on the H_2O_2-induced apoptosis of H9c2 cells is mediated at least in part through the Akt/GSK-3β and ERK1/2 signaling pathways.

Effect of hispidin on cell viability, intracellular ROS generation of cultured neonatal rat ventricular myocytes

The protective properties of hispidin in H9c2 cells may also be different from those functionally expressed in cardiac myocytes. Therefore, the effect of hispidin in neonatal rat ventricular myocytes was further evaluated. As shown in Fig. 6, hispidin protected against H_2O_2-induced cytotoxicity of neonatal rat ventricular myocytes in a concentration-dependent manner. The cell viability was significantly decreased after 600 μM H_2O_2-treatment (54.1±6.9%, $P<0.01$) compared to the non-treated group. As the concentration of hispidin was increased to 3, 10, and 30 μM, the cell viability was increased to 53.2±8.4% ($P>0.05$, NS), 67.0±8.4% ($P<0.05$), and 84.6±5.8% ($P<0.01$), respectively (Fig. 6A). Similarly, the results of intracellular ROS production showed the same protective pattern in neonatal rat ventricular myocytes (Fig. 6B).

Discussion

Growing evidence indicates that reactive oxygen species (ROS) play a critical role in many disorders of the cardiovascular system, such as ischemia–reperfusion (I/R) injury, myocardial stunning, and atherosclerosis [19]. Furthermore the accumulation of ROS in mitochondria can lead to apoptotic cell death and ROS may also have direct effects on cellular structure and function, including myocardial remodeling and failure [20]. Accordingly, inhibiting ROS production or the enhancement of ROS scavenging could be used for treating these cardiovascular disorders. Antioxidant compounds may be useful for ROS-related diseases, which has resulted in increasing demand for alternative and safe antioxidants isolated from natural sources [21]. Hispidin has already been proven to exert potent free-radical scavenging effects via its antioxidant properties, which might be used to eliminate the key mediators for the oxidative stress [22,23]. We examined the effect of hispidin against the cytotoxicity activity of H_2O_2 in H9c2 cardiomyoblast cells and neonatal rat ventricular myocytes (Figs. 1 and 6). Our study demonstrates a beneficial effect of hispidin on the myocardial response to oxidative stress. *In vitro* studies suggest a potential mechanism of this helpful effect in which hispidin attenuates H_2O_2-induced apoptosis in H9c2 cardiomyoblast cells by upregulating

specific survival proteins such as Akt/GSK-3β and ERK1/2, and scavenging ROS generation via the activation of heme oxygenase-1 (HO-1) and catalase (CAT).

The chemical structure of hispidin, 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone, is very similar to the structure of strong antioxidants such as resveratrol derivatives found in a number of plant species [24]. The strong radical scavenging activities of resveratrol-like stilbene, which is a flavonoid found in grapes, are associated with the dihydroxyl group in the A ring [25]. It has been reported that the catechol moiety of hispidin may contribute to its antioxidative activity [12]. In the present study, hispidin pretreatment reduced ROS production and increased HO-1 and CAT expression in H_2O_2-treated H9c2 cells using H_2O_2 to generate intracellular ROS (Fig. 2). These results suggest that the protective effects of hispidin are due to its role as an ROS scavenger by increasing the amounts of antioxidant enzymes such as HO-1 and CAT. Several enzymes convert ROS into compounds that are either harmless or rapidly metabolized. These enzymes prominently include SODs that convert superoxide into oxygen and hydrogen peroxide such as CAT, which converts H_2O_2 into O_2 and water, thereby aiding in the maintenance of cell membrane integrity and preventing the activation of cellular signaling processes that may lead to diseases such as oxidative cardiovascular injury [26–28]. Our findings indicate that hispidin exerts its protective effect in part by enhancing antioxidant enzyme activity, thereby attenuating the oxidative damage.

In the present study, cells exposed to H_2O_2 exhibited distinct morphological features of apoptosis such as an increase in Hoechst 33342 staining (Fig. 3). However cells that were pretreated with hispidin had a significantly reduced proportion of apoptotic cells, as shown by morphological changes (Fig. 1E) and Hoechst 33342 staining (Fig. 3A). The Bcl-2 proteins are the major regulators of mitochondria permeabilization, which includes pro-apoptotic (e.g., Bax and Bak) and anti-apoptotic (e.g., Bcl-2 and Bcl-xL) members. Bax oligomerization at the outer mitochondrial membrane causes transmembrane pore formation, leading to the release of pro-apoptotic molecules into the cytoplasm, whereas Bcl-2 forms heterodimers with a variety of pro-apoptotic proteins, thereby preventing Bax oligomerization [29]. Evidence in the present study indicated that hispidin pretreatment increased the expression of Bcl-2, as it decreased the expression of pro-apoptotic Bax. Therefore hispidin might exert protective effects by maintaining mitochondrial function and modulating the balance of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, thus inhibiting the release of pro-apoptotic molecules from the mitochondrial intermembrane space into the cytoplasm [30].

In addition, a group of survival protein kinases which include Akt and ERK1/2 that confer powerful cytoprotection provides an amenable pharmacological target for cardioprotection [31]. Therefore we speculate that the anti-apoptotic mechanism of hispidin may be responsible for the activation of Akt and ERK1/2.Akt, a serine/threonine kinase, is activated subsequently to the production of PIP3 by phosphatidylinositol-3-kinase (PI3K), and mediates several functions through the phosphorylation and inactivation of the pro-apoptotic kinase, glycogen synthase kinase-3 (GSK-3α/β) [32]. Akt directly phosphorylates GSK-3β at Ser9, which negatively regulates its kinase activity. Thereafter, phosphorylated GSK-3β inhibits the opening of mitochondrial permeability transition pores [33]. A number of studies have shown that GSK-3β is the phosphorylation target of PI3K/Akt [34,35]. Persistent inhibition of GSK-3β induces compensatory hypertrophy, inhibits apoptosis and fibrosis, and increases cardiac contractility [36]. Ischemic preconditioning, an endogenously triggered cardioprotective stimulus, has also been shown to enhance the phosphorylation of Akt, which further leads to phosphorylation and the subsequent inactivation of GSK-3β [37]. So far, three major MAPK signaling pathways – ERK1/2, p38 MAPK, and JNK – have been identified. They are activated in response to myocardial I/R, and the activation of ERK1/2 (beneficial effect) and p38 MAPK–JNK (a deleterious effect) exerts opposite effects on myocardial apoptosis and cardiac function recovery [38]. ERK is known to be a signal of the cardiac reperfusion injury salvage kinase (RISK) pathway [31]. When activated in the setting of cardiac I/R, the ERK1/2 cascade plays an important role in preventing cellular apoptosis [39]. The activation of either the PI3K/Akt or the ERK1/2 pathway inhibits the conformational change in Bax required for its translocation to the mitochondria, therefore preventing apoptosis [40,41]. Furthermore the up-regulation of ERK1/2 and Akt inactivates the caspase cascade, a proposition which is supported by inhibiting caspase-3 and caspase-9 activation [42,43]. In the present study hispidin increased the phosphorylation of Akt/GSK-3β and ERK1/2 but not p38 MAPK and JNK, which are reduced by H_2O_2 (Fig. 4). We clearly demonstrated that LY294002, a specific inhibitor of PI3K, can inhibit the functions of downstream target kinases of PI3K, and U0126, a specific inhibitor of ERK1/2, significantly reversed the anti-apoptotic effect of hispidin (Figs. 4 and 5). These findings suggest an essential role of the Akt/GSK-3β and ERK1/2 pathways in hispidin anti-apoptotic activity.

One could argue that some proportion of the ability of hispidin to reduce cardiomyocyte damage caused by oxidative stress could be due to PKC inhibition. The role of PKC in cardiac I/R is well documented [32,44,45]. The selective activation of PKCδ causes increased damage from ischemic insults, both in neonatal cardiac myocytes and in adult isolated rat cardiac myocytes, whereas its inhibition results in protection [46]. Furthermore, the disruption of the PKCε gene abolishes the infarct size reduction that follows ischemic preconditioning [44]. Drugs that target PKC include Gö6983, a broad spectrum PKC inhibitor, which restores cardiac function and attenuates the deleterious effects in isolated perfused rat heart after PMN-induced I/R injury [47]. PKC inhibition using Chelerythrine not only inhibited the pro-apoptotic pathways such as bax and bcl-2 but also led to an activation of anti-apoptotic signals, including Akt- and Bad-phosphorylation [48]. It is plausible that hispidin may inhibit PKC activity by binding to its ATP binding sites, and hence attenuate cardiac contractile dysfunction, a process similar to the mechanism of action of other PKC inhibitors [49,50]. However, the cardiopharmacological effects of hispidin remain to be elucidated.

In summary, this study has demonstrated that hispidin protects cardiomyoblast cells against oxidative stress by regulating apoptosis-related proteins such as caspase-3, Bax, and Bcl-2, as well as by the activation of Akt/GSK-3β and ERK1/2 signaling pathways, and by enhancing anti-oxidant enzyme systems. Although an animal study is needed hispidin is a promising agent for the treatment of oxidative-induced heart injury such as myocardial I/R injury and may have implications for other diseases associated with ROS, such as neurodegenerative disorders [4].

Conflicts of interest

The authors have no conflicts of interest to report.

Acknowledgments

This work was supported by Kyungbok University, National Research Foundation of Korea 322 (NRF) grant funded by the Korea government (MSIP; 2009-0092964, 2009-0083522, 2010-323 0017984, and 2012M3A9D1054520), Center for Food and Drug Materials of Agriculture Science and Technology Development 324 (PJ0079772012), Rural Development Administration 325, the National R&D Program, Ministry of Health and Welfare (0620360-1), and the Brain Korea 21 Plus Project, Republic of Korea.

REFERENCES

[1] S. Mendis, L.H. Lindholm, S.G. Anderson, A. Alwan, R. Koju, B.J. Onwubere, A.M. Kayani, N. Abeysinghe, A. Duneas, S. Tabagari, W. Fan, N. Sarraf-Zadegan, P. Nordet, J. Whitworth, A. Heagerty, Total cardiovascular risk approach to improve efficiency of cardiovascular prevention in resource constrain settings, J. Clin. Epidemiol. 64 (2011) 1451–1462.

[2] E. Murphy, C. Steenbergen, Mechanisms underlying acute protection from cardiac ischemia–reperfusion injury, Physiol. Rev. 88 (2008) 581–609.

[3] R.A. Gottlieb, K.O. Burleson, R.A. Kloner, B.M. Babior, R.L. Engler, Reperfusion injury induces apoptosis in rabbit cardiomyocytes, J. Clin. Invest. 94 (1994) 1621–1628.

[4] B. Sun, G.B. Sun, J. Xiao, R.C. Chen, X. Wang, Y. Wu, L. Cao, Z.H. Yang, X.B. Sun, Isorhamnetin inhibits H(2)O(2)-induced activation of the intrinsicapoptotic pathway in H9c2 cardiomyocytes through scavenging reactive oxygen species and ERK inactivation, J. Cell. Biochem. 113 (2012) 473–485.

[5] H. Takano, Y. Zou, H. Hasegawa, H. Akazawa, T. Nagai, I. Komuro, Oxidative stress-induced signal transduction pathways in cardiac myocytes: involvement of ROS in heart diseases, Antioxid. Redox Signal. 5 (2003) 789–794.

[6] N. Tuteja, P. Ahmad, B.B. Panda, R. Tuteja, Genotoxic stress in plants: shedding light on DNA damage, repair and DNA repair helicases, Mutat. Res. 681 (2009) 134–149.

[7] A. Rodriguez-Sinovas, Y. Abdallah, H.M. Piper, D. Garcia-Dorado, Reperfusion injury as a therapeutic challenge in patients with acute myocardial infarction, Heart Fail. Rev. 12 (2007) 207–216.

[8] T. Zhu, S.H. Kim, C.Y. Chen, A medicinal mushroom: *Phellinus linteus*, Curr. Med. Chem. 15 (2008) 1330–1335.

[9] H.G. Kim, D.H. Yoon, W.H. Lee, S.K. Han, B. Shrestha, C.H. Kim, M.H. Lim, W. Chang, S. Lim, S. Choi, W.O. Song, J.M. Sung, K.C. Hwang, T.W. Kim, *Phellinus linteus* inhibits inflammatory mediators by suppressing redox-based NF-kappaB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage, J. Ethnopharmacol. 114 (2007) 307–315.

[10] S.F. Ye, Z.Q. Hou, Q.Q. Zhang, Protective effects of *Phellinus linteus* extract against iron overload-mediated oxidative stress in cultured rat hepatocytes, Phytother. Res. 21 (2007) 948–953.

[11] C. Gonindard, C. Bergonzi, C. Denier, C. Sergheraert, A. Klaebe, L. Chavant, E. Hollande, Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells in vitro, Cell Biol. Toxicol. 13 (1997) 141–153.

[12] I.H. Park, S.K. Chung, K.B. Lee, Y.C. Yoo, S.K. Kim, G.S. Kim, K.S. Song, , An antioxidant hispidin from the mycelial cultures of *Phellinus linteus* 27 (2004) 615–618Arch. Pharm. Res. 27 (2004) 615–618.

[13] K.J. Won, H.Y. Lin, S. Jung, S.M. Cho, H.C. Shin, Y.M. Bae, S.H. Lee, H.J. Kim, B.H. Jeon, B. Kim, Antifungal miconazole induces cardiotoxicity via inhibition of APE/Ref-1-related pathway in rat neonatal cardiomyocytes, Toxicol. Sci. 126 (2012) 298–305.

[14] E.S. Park, J.C. Kang, D.H. Kang, Y.C. Jang, K.Y. Yi, H.J. Chung, J.S. Park, B. Kim, Z.P. Feng, H.S. Shin, 5-AIQ inhibits H_2O_2-induced apoptosis through reactive oxygen species scavenging and Akt/GSK-3beta signaling pathway in H9c2 cardiomyocytes, Toxicol. Appl. Pharmacol. 268 (2013) 90–98.

[15] N.N. Danial, S.J. Korsmeyer, Cell death: critical control points, Cell 116 (2004) 205–219.

[16] R. von Harsdorf, P.F. Li, R. Dietz, Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis, Circulation 99 (1999) 2934–2941.

[17] R. Amaravadi, C.B. Thompson, The survival kinases Akt and Pim as potential pharmacological targets, J. Clin. Invest. 115 (2005) 2618–2624.

[18] N.A. Turner, F. Xia, G. Azhar, X. Zhang, L. Liu, J.Y. Wei, Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH2-terminal kinase pathway in H9c2 cardiac muscle cells, J. Mol. Cell. Cardiol. 30 (1998) 1789–1801.

[19] C. Penna, D. Mancardi, F. Tullio, P. Pagliaro, Postconditioning and intermittent bradykinin induced cardioprotection require cyclooxygenase activation and prostacyclin release during reperfusion, Basic Res. Cardiol. 103 (2008) 368–377.

[20] D.B. Zorov, C.R. Filburn, L.O. Klotz, J.L. Zweier, S.J. Sollott, Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes, J. Exp. Med. 192 (2000) 1001–1014.

[21] A.T. Diplock, J.L. Charleux, G. Crozier-Willi, F.J. Kok, C. Rice-Evans, M. Roberfroid, W. Stahl, J. Vina-Ribes, Functional food science and defence against reactive oxidative species, Br. J. Nutr. 80 (Suppl. 1) (1998) S77–112.

[22] I.K. Lee, S.J. Seok, W.K. Kim, B.S. Yun, Hispidin derivatives from the mushroom *Inonotus xeranticus* and their antioxidant activity, J. Nat. Prod. 69 (2006) 299–301.

[23] J.S. Jang, J.S. Lee, J.H. Lee, D.S. Kwon, K.E. Lee, S.Y. Lee, E.K. Hong, Hispidin produced from *Phellinus linteus* protects pancreatic beta-cells from damage by hydrogen peroxide, Arch. Pharm. Res. 33 (2010) 853–861.

[24] A.R. Martin, I. Villegas, C. La Casa, C.A. de la Lastra, Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats, Biochem. Pharmacol. 67 (2004) 1399–1410.

[25] D.B. McPhail, R.C. Hartley, P.T. Gardner, G.G. Duthie, Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy, J. Agric. Food Chem. 51 (2003) 1684–1690.

[26] P. Chelikani, I. Fita, P.C. Loewen, Diversity of structures and properties among catalases, Cell. Mol. Life Sci. 61 (2004) 192–208.

[27] U. Forstermann, Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies, Nat. Clin. Pract. Cardiovasc. Med. 5 (2008) 338–349.

[28] T.B. Kryston, A.B. Georgiev, P. Pissis, A.G. Georgakilas, Role of oxidative stress and DNA damage in human carcinogenesis, Mutat. Res. 711 (2011) 193–201.

[29] T. Kuwana, D.D. Newmeyer, Bcl-2-family proteins and the role of mitochondria in apoptosis, Curr. Opin. Cell Biol. 15 (2003) 691–699.

[30] X. Sun, G.B. Sun, M. Wang, J. Xiao, X.B. Sun, Protective effects of cynaroside against H(2)O(2)-induced apoptosis in H9c2 cardiomyoblasts, J. Cell. Biochem. 112 (2011) 2019–2029.

[31] D.J. Hausenloy, D.M. Yellon, New directions for protecting the heart against ischaemia–reperfusion injury: targeting the

Reperfusion Injury Salvage Kinase (RISK)-pathway, Cardiovasc. Res. 61 (2004) 448–460.
[32] S.C. Armstrong, Protein kinase activation and myocardial ischemia/reperfusion injury, Cardiovasc. Res. 61 (2004) 427–436.
[33] M. Juhaszova, D.B. Zorov, Y. Yaniv, H.B. Nuss, S. Wang, S.J. Sollott, Role of glycogen synthase kinase-3beta in cardioprotection, Circ. Res. 104 (2009) 1240–1252.
[34] D.A. Cross, D.R. Alessi, P. Cohen, M. Andjelkovich, B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, Nature 378 (1995) 785–789.
[35] C. Badorff, H. Ruetten, S. Mueller, M. Stahmer, D. Gehring, F. Jung, C. Ihling, A.M. Zeiher, S. Dimmeler, Fas receptor signaling inhibits glycogen synthase kinase 3 beta and induces cardiac hypertrophy following pressure overload, J. Clin. Invest. 109 (2002) 373–381.
[36] S. Hirotani, P. Zhai, H. Tomita, J. Galeotti, J.P. Marquez, S. Gao, C. Hong, A. Yatani, J. Avila, J. Sadoshima, Inhibition of glycogen synthase kinase 3beta during heart failure is protective, Circ. Res. 101 (2007) 1164–1174.
[37] S. Kaga, L. Zhan, E. Altaf, N. Maulik, Glycogen synthase kinase-3beta/beta-catenin promotes angiogenic and anti-apoptotic signaling through the induction of VEGF, Bcl-2 and survivin expression in rat ischemic preconditioned myocardium, J. Mol. Cell. Cardiol. 40 (2006) 138–147.
[38] J.J. Jeong, Y.M. Ha, Y.C. Jin, E.J. Lee, J.S. Kim, H.J. Kim, H.G. Seo, J.H Lee, S.S. Kang, Y.S. Kim, K.C. Chang, Rutin from *Lonicera japonica* inhibits myocardial ischemia/reperfusion-induced apoptosis in vivo and protects H9c2 cells against hydrogen peroxide-mediated injury via ERK1/2 and PI3K/Akt signals in vitro, Food Chem. Toxicol. 47 (2009) 1569–1576.
[39] L.D. Mayo, D.B. Donner, A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus, Proc. Natl. Acad. Sci. USA 98 (2001) 11598–11603.
[40] H. Yamaguchi, H.G. Wang, The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change, Oncogene 20 (2001) 7779–7786.
[41] C.R. Weston, K. Balmanno, C. Chalmers, K. Hadfield, S.A. Molton, R. Ley, E.F. Wagner, S.J. Cook, Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways, Oncogene 22 (2003) 1281–1293.
[42] K. Terada, Y. Kaziro, T. Satoh, Analysis of Ras-dependent signals that prevent caspase-3 activation and apoptosis induced by cytokine deprivation inhematopoietic cells, Biochem. Biophys. Res. Commun. 267 (2000) 449–455.
[43] M.H. Cardone, N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, J.C. Reed, Regulation of cell death protease caspase-9 by phosphorylation, Science 282 (1998) 1318–1321.
[44] A.T. Saurin, D.J. Pennington, N.J. Raat, D.S. Latchman, M.J. Owen, M.S. Marber, Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts, Cardiovasc. Res. 55 (2002) 672–680.
[45] H.S. Hahn, M.G. Yussman, T. Toyokawa, Y. Marreez, T.J. Barrett, K.C. Hilty, H. Osinska, J. Robbins, G.W. Dorn 2nd, Ischemic protection and myofibrillar cardiomyopathy: dose-dependent effects of in vivo deltaPKC inhibition, Circ. Res. 91 (2002) 741–748.
[46] L. Chen, H. Hahn, G. Wu, C.H. Chen, T. Liron, D. Schechtman, G. Cavallaro, L. Banci, Y. Guo, R. Bolli, G.W. Dorn 2nd, D. Mochly-Rosen, Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC, Proc. Natl. Acad. Sci. USA 98 (2001) 11114–11119.
[47] E.E. Peterman, P. Taormina 2nd, M. Harvey, L.H. Young, Go 6983 exerts cardioprotective effects in myocardial ischemia/reperfusion, J. Cardiovasc. Pharmacol. 43 (2004) 645–656.
[48] G. Simonis, S. Wiedemann, K. Schwarz, T. Christ, D.G. Sedding, X. Yu, R. Marquetant, R.C. Braun-Dullaeus, U. Ravens, R.H. Strasser, Chelerythrine treatment influences the balance of pro- and anti-apoptotic signaling pathways in the remote myocardium after infarction, Mol. Cell. Biochem. 310 (2008) 119–128.
[49] H. Hug, T.F. Sarre, Protein kinase C isoenzymes: divergence in signal transduction?, Biochem. J. 291 (1993) 329–343.
[50] K. Numaguchi, H. Shimokawa, R. Nakaike, K. Egashira, A. Takeshita, PKC inhibitors prevent endothelial dysfunction after myocardial ischemia–reperfusion in rats, Am. J. Physiol. 270 (1996) H1634–H1639.