

Article

Isoquercitrin Inhibits Hydrogen Peroxide-Induced Apoptosis of EA.hy926 Cells via the PI3K/Akt/GSK3 β Signaling Pathway

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Academic Editor: Maurizio Battino

Received: 23 January 2016; Accepted: 9 March 2016; Published: 16 March 2016

Abstract: Oxidative stress plays a critical role in endothelial injury and the pathogenesis of diverse cardiovascular diseases, including atherosclerosis. Isoquercitrin (quercetin-3-glucoside), a flavonoid distributed widely in plants, exhibits many biological activities, including anti-allergic, anti-viral, anti-inflammatory, and anti-oxidative effects. In the present study, the inhibitory effect of isoquercitrin on H₂O₂-induced apoptosis of EA.hy926 cells was evaluated. MTT assays showed that isoquercitrin significantly inhibited H₂O₂-induced loss of viability in EA.hy926 cells. Hoechst33342/PI and Annexin V-FITC/PI fluorescent double staining indicated that isoquercitrin inhibited H₂O₂-induced apoptosis of EA.hy926 cells. Western blotting demonstrated that isoquercitrin prevented H₂O₂-induced increases in cleaved caspase-9 and cleaved caspase-3 expression, while increasing expression of anti-apoptotic protein Mcl-1. Additionally, isoquercitrin significantly increased the expression of p-Akt and p-GSK3 β in a dose-dependent manner in EA.hy926 cells. LY294002, a PI3K/Akt inhibitor, inhibited isoquercitrin-induced GSK3 β phosphorylation and increase of Mcl-1 expression, which indicated that regulation of isoquercitrin on Mcl-1 expression was likely related to the modulation of Akt activation. These results demonstrated that the anti-apoptotic effect of isoquercitrin on H₂O₂-induced EA.hy926 cells was likely associated with the regulation of isoquercitrin on Akt/GSK3 β signaling pathway and that isoquercitrin could be used clinically to interfere with the progression of endothelial injury-associated cardiovascular disease.

Keywords: isoquercitrin; EA.hy926 cells; apoptosis; oxidative stress; PI3K/Akt/GSK3 β

1. Introduction

Cardiovascular disease is a primary cause of morbidity and mortality worldwide. It is estimated that the annual number of deaths due to cardiovascular disease will increase from 17 million in 2008 to 25 million in 2030 [1]. The endothelium is the inner layer of the vasculature, which represents the interface between blood and organ systems, secretes numerous vasoactive substances, and regulates blood flow [2,3]. A large body of research has shown that endothelial dysfunction is related to the pathogenesis of cardiovascular diseases such as atherosclerosis, peripheral artery disease, and glomerulosclerosis [4–6]. Reactive oxygen species (ROS) generated excessively in the endothelium, including H₂O₂, HO \cdot , and O₂⁻, are major mediators responsible for oxidative stress which results in endothelial dysfunction [7–9]. Therefore, to protect endothelial cells from ROS-induced apoptosis

has proven to be an effective intervention against the progress of some cardiovascular diseases such as atherosclerosis.

Isoquercitrin, also known as quercetin-3-glucoside, is a glucose-bound derivative of quercetin that is commonly found in medicinal herbs, fruits, vegetables, and plant-derived foods and beverages [10–14]. In recent studies, isoquercitrin has been reported to possess anti-Mayaro virus, anti-inflammatory, anti-oxidative, anti-allergic, anti-hypertensive, anti-hyperglycemic, and saluretic/diuretic effects [15–23]. Although the antioxidative and neuroprotective effects of isoquercitrin have been established, whether isoquercitrin could exert an anti-apoptotic effect on endothelial cells is not clear. Thus, the present study was designed to evaluate the effect of isoquercitrin on H₂O₂-induced EA.hy926 cell apoptosis.

2. Results

2.1. Isoquercitrin Inhibits H₂O₂-Induced Loss of Cell Viability in EA.hy926 Cells

The protective effect of isoquercitrin against H₂O₂-induced loss of viability in EA.hy926 cells was measured using the MTT assay. Cell viability was not significantly affected by exposure to 2.5–80 μmol/L isoquercitrin and decreased by 54.08% ± 7.02% after exposure to 200 μmol/L H₂O₂ for 4 h (Figure 1A,B). As illustrated in Figure 1C, the cells treated with 20 μmol/L isoquercitrin for 24 h before the addition of H₂O₂ significantly attenuated the viability loss induced by H₂O₂ ($p < 0.01$). These findings suggest that isoquercitrin inhibits H₂O₂-induced EA.hy926 cell death.

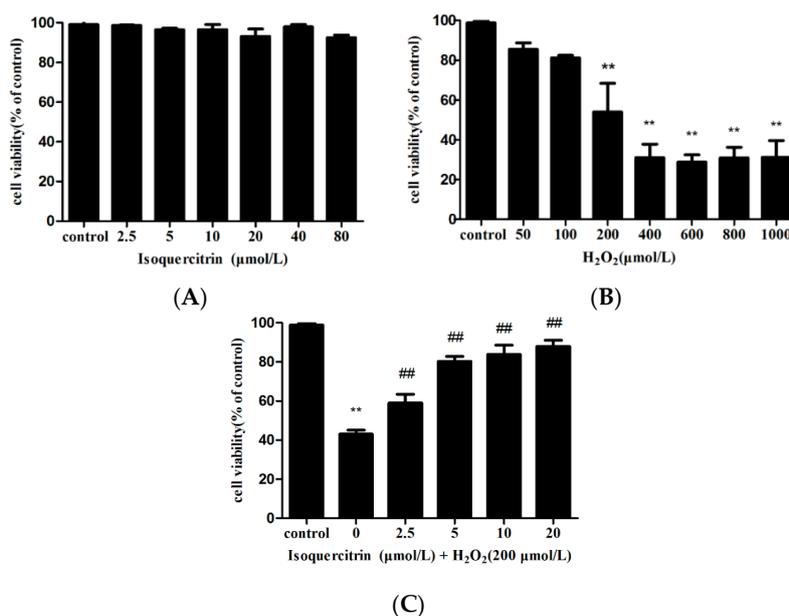


Figure 1. Protective activity of isoquercitrin measured by the MTT assay. (A) Cell viability of EA.hy926 cells treated with different concentrations of isoquercitrin; (B) Cell viability of EA.hy926 cells treated with different concentrations of H₂O₂; (C) Cell viability of EA.hy926 cells treated with isoquercitrin followed by H₂O₂ treatment. ** $p < 0.01$ vs. control; ## $p < 0.01$ vs. H₂O₂ group.

2.2. Isoquercitrin Inhibits H₂O₂-Induced Apoptosis of EA.hy926 Cells

Hoechst 33342 dye causes bright blue fluorescence in apoptotic cells because of the high permeability of cell membranes, while propidium iodide (PI) dye causes red fluorescence in the nuclei of dead cells. Hoechst 33342/PI double staining was performed to compare the apoptotic rates of EA.hy926 cells treated with H₂O₂ alone and those treated with H₂O₂ and isoquercitrin (Figure 2). The apoptotic rate of the control group was 7.16% ± 1.18%, whereas that of the H₂O₂-treated group was 47.09% ± 3.93% ($p < 0.01$ vs. the control group). However, the treatment of different concentrations of

isoquercitrin significantly attenuated H₂O₂-induced apoptosis from 47.09% ± 3.93% to 17.60% ± 1.15% in EA.hy926 cells. These results showed that isoquercitrin exhibited inhibitory effects on H₂O₂-induced EA.hy926 cell apoptosis.

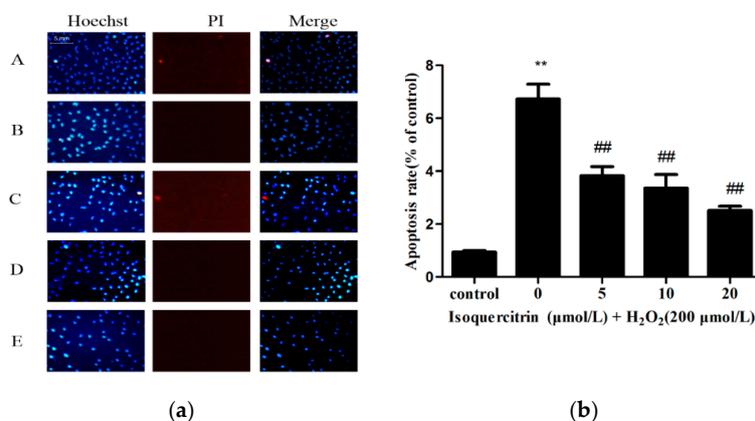


Figure 2. Hoechst 33342 and PI staining in EA.hy926 cells. (a) Representative fluorescence images obtained after Hoechst 33342/PI staining (A) Control group; (B) H₂O₂ treatment group; (C–E) 5, 10, and 20 μmol/L isoquercitrin, respectively, followed by the treatment of 200 μmol/L H₂O₂; (b) Percentages of apoptotic cells in total EA.hy926 cells. ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. H₂O₂ treatment group.

To further evaluate the inhibitory effect of isoquercitrin on H₂O₂-induced apoptosis in EA.hy926 cells, apoptotic rates were measured by Annexin V-FITC/PI double staining using flow cytometry. As shown in Figure 3, the percentage of apoptotic cells was 5.65% ± 0.35% in the control group, whereas that of the group treated with H₂O₂ alone was 47.75% ± 1.95% ($p < 0.01$, vs. the control group). However, the treatment with 5, 10 or 20 μmol/L isoquercitrin significantly decreased apoptotic rates to 30.60% ± 0.90%, 24.45% ± 0.95%, and 17.55% ± 0.85%, respectively ($p < 0.01$, vs. the H₂O₂-treated group), in EA.hy926 cells treated with H₂O₂. These results suggested that isoquercitrin exhibited inhibitory effects on H₂O₂-induced apoptosis in EA.hy926 cells.

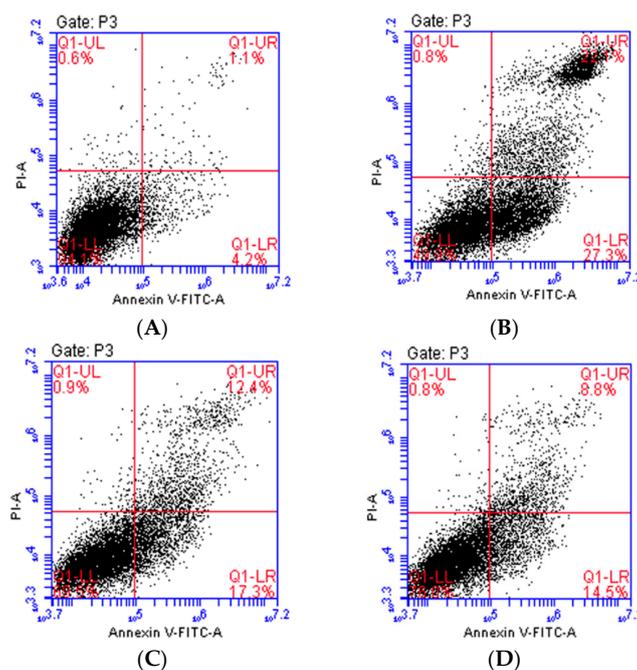


Figure 3. Cont.

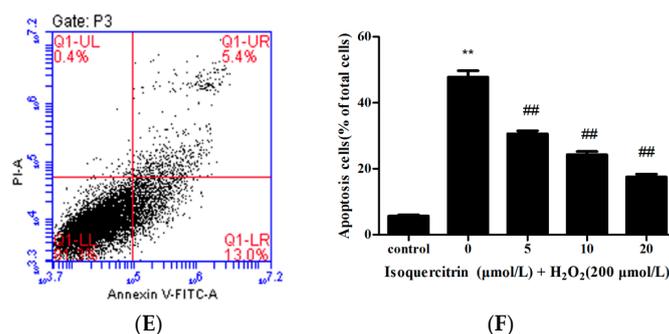


Figure 3. Effects of isoquercitrin on H₂O₂-induced apoptosis in EA.hy926 cells measured by flow cytometry. (A) Control group; (B) H₂O₂ treatment group; (C–E) 5, 10, and 20 μmol/L isoquercitrin, respectively, followed by the treatment of 200 μmol/L H₂O₂; (F) isoquercitrin decreased the percent of apoptotic cells induced by 200 μmol/L H₂O₂ in a dose-dependant manner. Data are presented as the mean ± SD ($n = 3$); ** $p < 0.01$ vs. the control; ## $p < 0.01$ vs. the H₂O₂-treated group.

2.3. Isoquercitrin Inhibits H₂O₂-Induced Decreases in Mitochondrial Membrane Potential in EA.hy926 Cells

Mitochondrial membrane potential was assessed with JC-1 dye, a cationic lipophilic dye widely utilized in apoptosis studies using flow cytometry. As shown in Figure 4A, the ratio of red to green fluorescence intensity was significantly decreased in the group treated with H₂O₂ alone in comparison with that of the control group ($p < 0.01$). However, the groups pretreated with 5, 10 or 20 μmol/L isoquercitrin showed a significantly increased ratio of red/green fluorescence intensity in comparison with that of the group treated with H₂O₂ alone ($p < 0.05$). These results suggested that isoquercitrin inhibited H₂O₂-induced early apoptosis in EA.hy926 cells.

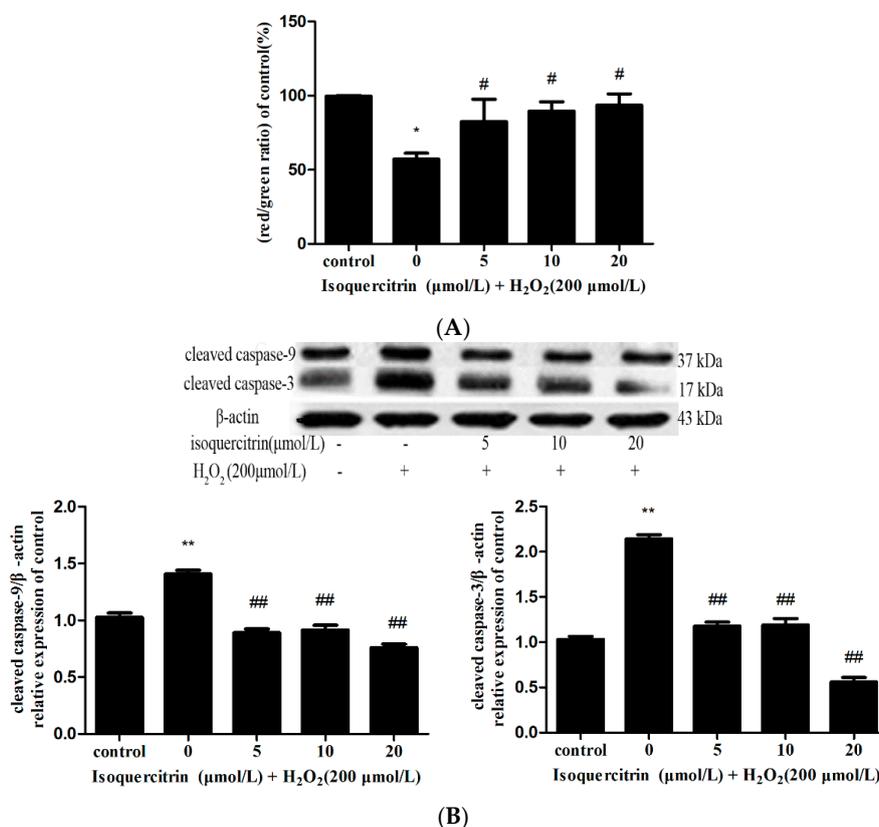


Figure 4. Cont.

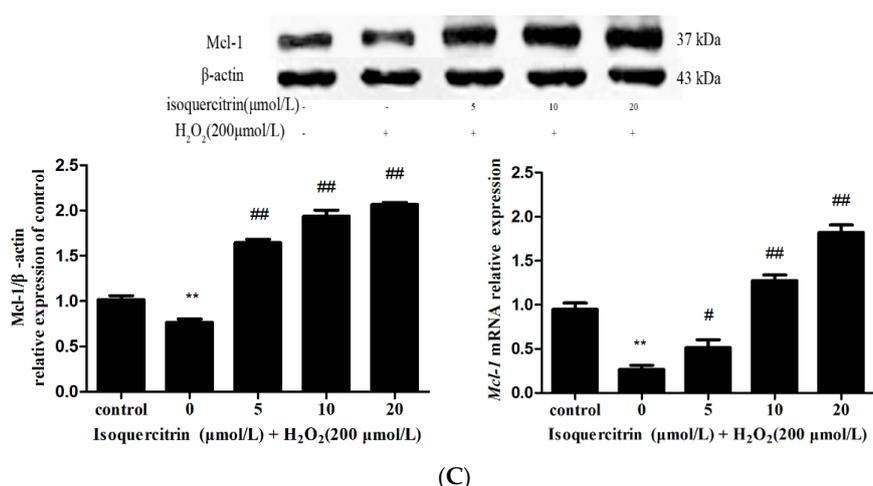


Figure 4. Effects of isoquercitrin on mitochondrial membrane potential and expression of cleaved caspase-3,-9 and Mcl-1 in H₂O₂-induced EA.hy926 cells. (A) Effects of isoquercitrin on mitochondrial membrane potential in H₂O₂-induced EA.hy926 cells; (B) Effects of isoquercitrin on the expression of cleaved caspase-3, cleaved caspase-9 in H₂O₂-induced EA.hy926 cells; (C) Effects of isoquercitrin on the expression of Mcl-1 in H₂O₂-induced EA.hy926 cells. * $p < 0.05$, ** $p < 0.01$ vs. the control; # $p < 0.05$, ## $p < 0.01$ vs. the H₂O₂-treated group.

2.4. Isoquercitrin Inhibited H₂O₂-Induced Increases in Cleaved Caspase-3 and Cleaved Caspase-9 Expression in EA.hy926 Cells

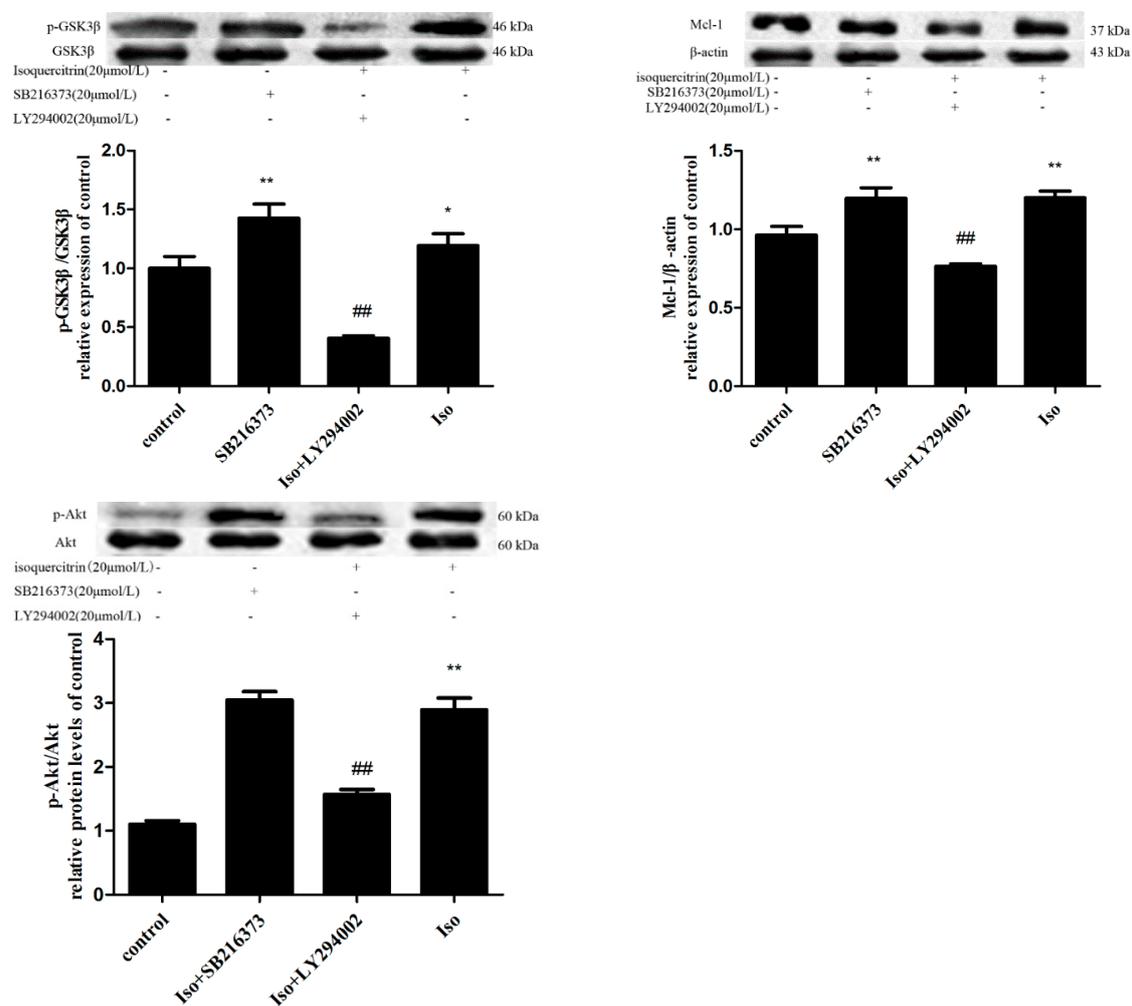
In response to mitochondrial membrane injury induced by apoptosis, cytochrome c is released from mitochondria to the cytosol and binds to Apaf-1, which result in Apaf-1/cytochrome c complex formation and subsequent activation of caspase-9. Activated caspase-9 then initiates processing of caspase-3 as well as caspase-7. Activated caspase-3 in turn activates caspases-2 and -6 and also appears to be capable of acting in a feedback loop on caspase-9 to ensure complete activation of the latter. Activated caspase-6 was found to be required for the activation of caspases-8 and -10 [24,25]. Therefore, caspase-9 is key apoptosis initiator and caspase-3 is significant apoptosis executioner and the cleaved form of caspase-9, -3 can serve as a biomarker for the existence of cell apoptosis. Then, we investigated the effect of isoquercitrin on caspase-9 and caspase-3 activity by detecting the expression of cleaved caspase-9, -3 in EA.hy926 cells using western blotting. As shown in Figure 4B, H₂O₂ treatment significantly increased cleavage of caspase-3 and caspase-9 in comparison with that of the control group ($p < 0.01$). In contrast, pretreatment with isoquercitrin significantly decreased the expression of cleaved caspase-3 and caspase-9 in a dose-dependent manner compared with that of cells treated with H₂O₂ only ($p < 0.01$ vs. the H₂O₂-treated group). These results showed that isoquercitrin inhibited H₂O₂-induced apoptosis in EA.hy926 cells by decreasing activation of caspase-3 and caspase-9.

2.5. Isoquercitrin Enhanced Mcl-1 Expression in H₂O₂-Induced EA.hy926 Cells

Mcl-1 appears to function at an apical step in many regulatory programs that control cell survival and death which can associate with Bcl-2 family pro-apoptosis (Bax or Bak) control cytochrome c release from mitochondria [26]. To evaluate the effect of isoquercitrin on Mcl-1 in EA.hy926 cells, we investigated the expression of Mcl-1 protein and mRNA. As shown in Figure 4C, the Mcl-1 protein and mRNA levels of the group treated with H₂O₂ were reduced in comparison with that of the control group ($p < 0.01$). However, H₂O₂-treated cells pretreated with isoquercitrin showed significantly increased Mcl-1 protein and mRNA expression levels in comparison with those of H₂O₂-treated group ($p < 0.01$). These results indicate that isoquercitrin regulated expression of Bcl-2 family anti-apoptotic protein Mcl-1.

2.6. Isoquercitrin Treatment Increased Mcl-1 Expression in a PI3K/Akt/GSK3 β -Dependent Manner

Mcl-1 has a short-life, and is quickly degraded in response to a variety of apoptosis-inducing signals. GSK3 β physically associated with and phosphorylated Mcl-1 and the phosphorylated Mcl-1 was then ubiquitinated and degraded by the E3 ligase β -TrCP [27]. Thus, we investigated whether GSK3 β inactivation was involved in the increase in Mcl-1 expression produced by isoquercitrin treatment in EA.hy926 cells. A cell-permeable inhibitor of GSK-3 β , SB216763 was used to determine if GSK3 β activation is required for the reduction in Mcl-1 levels. As shown in Figure 5A, pretreatment of EA.hy926 cells with SB216763 or isoquercitrin alone increased expression levels of Mcl-1 and p-GSK3 β in comparison with those of the control group ($p < 0.01$), while expression levels of Mcl-1 and p-GSK3 β in EA.hy926 cells treated with isoquercitrin alone were similar to those of cells pretreated with SB216763. These data suggest that the inhibitory effect of isoquercitrin on GSK3 β activation was similar to that of SB216763.



(A)

Figure 5. Cont.

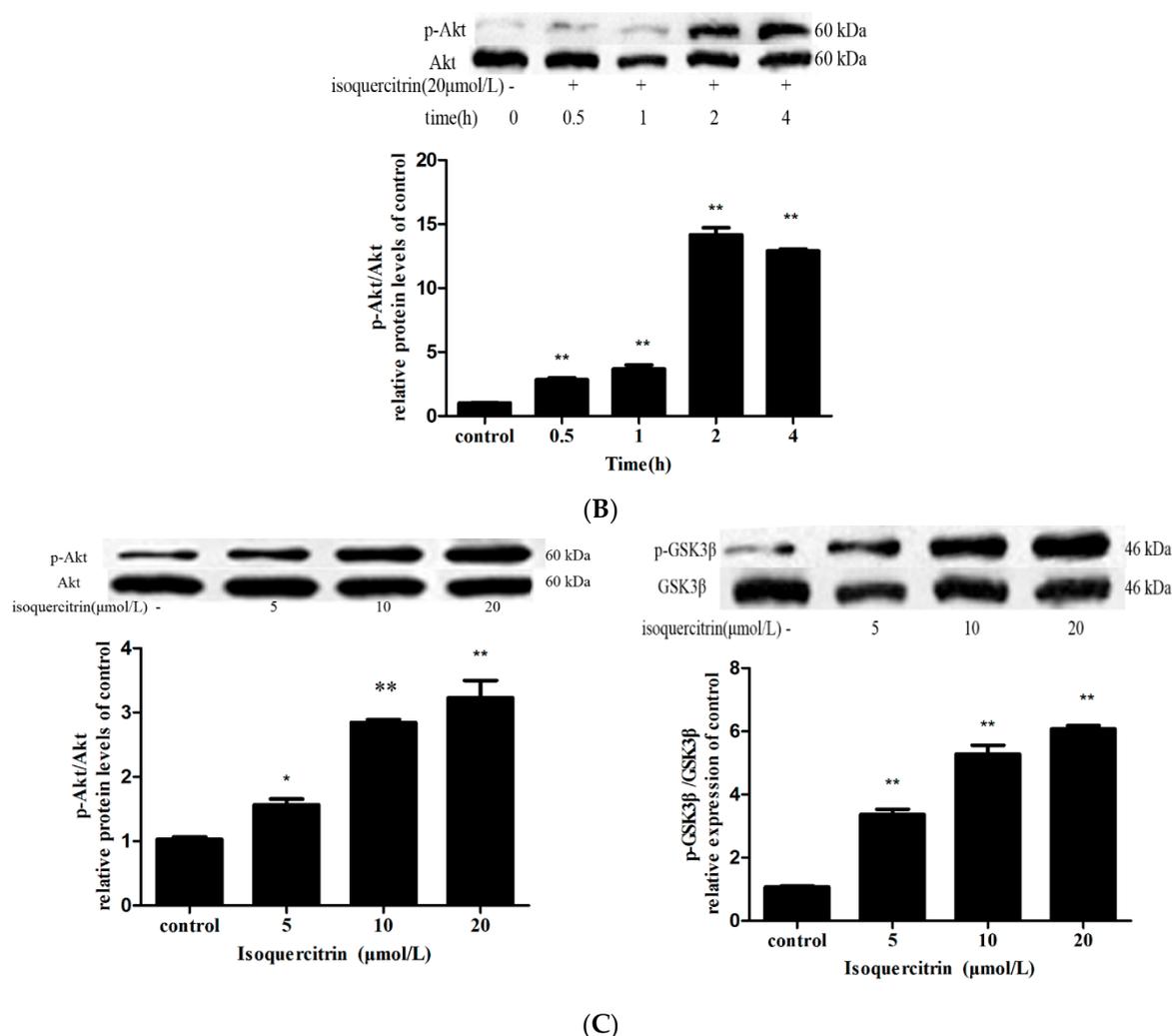


Figure 5. Effects of isoquercitrin on the expression of p-Akt, p-GSK3 β , and Mcl-1 in EA.hy926 cells. (A) Effects of LY294002 and SB216373 on the expression of p-GSK3 β , Mcl-1 and p-Akt in EA.hy926 cells. * $p < 0.05$, ** $p < 0.01$ vs. the control; ### $p < 0.01$ vs. isoquercitrin (20 $\mu\text{mol/L}$) group; (B) Effects of isoquercitrin (20 $\mu\text{mol/L}$) on the expression of p-Akt in EA.hy926 cells in different time, ** $p < 0.01$ vs. the control; (C) Effects of different concentrations of isoquercitrin on the expression of p-Akt, p-GSK3 β in EA.hy926 cells, ** $p < 0.01$ vs. the control.

As shown above, isoquercitrin increased the stability of Mcl-1 by inactivating GSK3 β . GSK3 β is a ubiquitously expressed protein serine/threonine kinase, which can be inactivated by phosphorylation at Ser⁹ through a PI3K/Akt-dependent mechanism. To explore if isoquercitrin phosphorylated GSK3 β through PI3K/Akt pathway. We examined phosphorylation of Akt and GSK3 β by western blot analysis. First, EA.hy926 cells were incubated with 20 $\mu\text{mol/L}$ isoquercitrin for 30 min, 1 h, 2 h and 4 h. As shown in Figure 5B, exposure of EA.hy926 cells to 20 $\mu\text{mol/L}$ for 2 h obviously induced phosphorylation of Akt ($p < 0.01$). Therefore, EA.hy926 cells were incubated with 5, 10 and 20 $\mu\text{mol/L}$ isoquercitrin for 2 h. As shown in Figure 5C, treatment of EA.hy926 cells with 5, 10, and 20 $\mu\text{mol/L}$ isoquercitrin for 2 h significantly increased levels of phosphorylated Akt and GSK3 β in a dose-dependent manner in comparison with those of the control group ($p < 0.01$). Additionally, cells pretreated with PI3K/Akt inhibitor LY294002 showed significantly reduced levels of GSK3 β phosphorylation and Mcl-1 expression in comparison with those of cells treated with isoquercitrin (Figure 5A, $p < 0.01$). Taken together, these results indicated that induction of isoquercitrin on Mcl-1 expression is likely associated with activation of the PI3K/Akt/GSK3 β signaling pathway.

3. Discussion

Reactive oxygen species (ROS) including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), and hydrogen peroxide (H_2O_2) are physiologically produced at a basal rate *in vivo* [28]. The cellular levels of ROS are controlled by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) and glutathione (GSH) [29]. However, different physiological or pathophysiological stimuli can generate abundant ROS which lead to oxidative stress characterized by an imbalance between the generation ROS and the capacity of the intrinsic antioxidant defense system. Oxidative stress can damage DNA, lipids, and proteins, and ultimately lead to tissue injury [30]. An increasing body of evidence suggests that oxidative stress induced by H_2O_2 is involved in the pathogenesis of cardiovascular diseases, such as cardiac ischemic-reperfusion injury and hypertrophy [31,32]. Therefore, inhibition of H_2O_2 -induced oxidative stress was regarded as an important strategy for cardiovascular disease prevention.

Flavonoids are widely distributed in plants. Quercetin, one of these flavonoids, is the most abundant and widely distributed in Nature. It has been reported that quercetin has antioxidant activity by scavenging radical and preventing GSH or SOD depletion [33]. Isoquercitrin, known as quercetin-3-glucoside, also stands out among the flavonols with anti-inflammatory and antioxidant effects [34,35]. In this study, we found that isoquercitrin could inhibit oxidative stress-induced endothelial cells apoptosis. Exposure of EA.hy926 cells to 200 $\mu\text{mol/L}$ H_2O_2 caused a decrease in cell viability and remarkably increased the apoptotic rate. However, isoquercitrin treatment attenuated the H_2O_2 -induced decrease in cell viability and increase in apoptotic rate in a dose-dependent manner, which suggested that isoquercitrin exhibited anti-apoptotic effects in H_2O_2 -induced EA.hy926 cells.

In addition, we explored the mechanisms underlying inhibition of H_2O_2 -induced apoptosis by isoquercitrin. It has been defined that there is two main routes of caspase-dependent cell apoptosis. One is the death receptor-dependent pathway (extrinsic pathway) and the other is the mitochondria-dependent pathway (intrinsic pathway). The death receptor-dependent pathway triggered mainly by Fas system, for example, Fas L is able to bind to its receptor which results in the recruitment and activation of procaspase-8/-10. Caspase-8, -10, in turn, promote activation of downstream executioner caspase-3 [36]. The intrinsic mitochondrial pathway of apoptosis can be initiated by a variety of stimuli (such as radiation and free radicals), which cause changes in mitochondrial membrane and result in pro-apoptosis protein cytochrome c releasing from mitochondrial matrix to cytosol [37,38]. The intrinsic pathway was controlled by the Bcl-2 protein family, which included anti-apoptotic proteins Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1, as well as proapoptotic proteins such as Bax, Bak, and Bok [39,40]. The hallmark of the intrinsic pathway was the release of cytochrome c into the cytosol and subsequent activation of procaspase-9 through the formation of the apoptosome, after which caspase-9 cleaved and activated effector caspase-3 [41,42]. In the present study, we found that H_2O_2 decreased mitochondrial membrane potential and increased expression of cleaved caspase-9 and caspase-3 in EA.hy926 cells. The effect of H_2O_2 was likely mediated by damage to the mitochondrial membrane, which led to release of cytochrome c and activation of procaspase-9. However, isoquercitrin pretreatment reversed this effect. Mcl-1 is a key member of the Bcl-2 family that blocks the progression of apoptosis by binding and sequestering pro-apoptotic proteins Bcl-2 Bak and Bax, which are capable of forming pores in the mitochondrial membrane, allowing release of cytochrome c into the cytoplasm [43,44]. We explored the mechanism by which isoquercitrin inhibited H_2O_2 -induced apoptosis via the intrinsic pathway by detecting expression of Bcl-2 family anti-apoptotic protein Mcl-1. In this study, FQ-PCR and western blotting showed that isoquercitrin significantly prevented H_2O_2 -induced decreases in Mcl-1 mRNA and protein expression. Therefore, we speculate that isoquercitrin regulate intrinsic pathway apoptosis by inducing over expression of anti-apoptotic protein Mcl-1.

Furthermore, we explored the manner in which isoquercitrin affected expression of Mcl-1. It has been reported that Mcl-1 protein levels seem to be dynamically regulated by growth factor signaling at the protein level. Mcl-1 was positively regulated by Akt and negatively regulated by

GSK3 β [45,46]. Phosphorylation of Mcl-1 (Ser159) by GSK3 β may regulate proteasome-dependent degradation [47]. GSK-3 β is a ubiquitously expressed serine/threonine protein kinase that is subject to multiple regulatory mechanisms [48,49]. The activity of GSK3 β can be reduced by phosphorylation at Ser9 in a PI3K/Akt dependent manner [50,51]. To determine whether GSK3 β inactivation was required for the increase in Mcl-1 expression produced by isoquercitrin treatment in EA.hy926 cells, the cells were exposed to a highly selective, cell-permeable inhibitor of GSK3 β , SB216763. Expression levels of p-GSK3 β and Mcl-1 were significantly increased in the SB216763-treated and isoquercitrin-treated groups in comparison with those of the control group, while there was no difference in the p-GSK3 β expression levels of the isoquercitrin-treated and SB216763-treated groups. Therefore, isoquercitrin increased Mcl-1 expression by inactivating GSK3 β . To further explore the manner in which isoquercitrin inactivated GSK3 β , we measured phosphorylation of Akt and GSK3 β . As shown in Figure 5C, isoquercitrin alone induced phosphorylation of Akt^{ser473} and GSK3 β ^{ser9} in a dose-dependent manner. We proposed that isoquercitrin induced inactivation of GSK3 β by activating the PI3K/Akt pathway. To examine this hypothesis, LY294002 was used to block activation of the PI3K pathway. LY294002 effectively abrogated the effects of isoquercitrin on Mcl-1 expression, which indicated that the PI3K/Akt signaling pathway was involved in regulation of GSK3 β phosphorylation and Mcl-1 expression in the present study.

4. Experimental Section

4.1. Materials

Isoquercitrin was purchased from the National Institutes for Food and Drug Control (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Antibodies against Mcl-1 and β -actin were obtained from Bioworld Technology (Nanjing, China). Antibodies against Akt, p-Akt, GSK3 β , p-GSK3 β , cleaved caspase-3, and cleaved caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Dimethyl sulfoxide (DMSO), H₂O₂, and 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The Annexin V-FITC apoptosis detection kit, Hoechst33342/PI apoptosis detection kit, and JC-1 detection kit were purchased from Nanjing Key Gen Biotech (Nanjing, China). All other chemicals used in the study were of analytical grade.

4.2. Cell Culture

The EA.hy926 cell line is an immortalized human umbilical vein endothelial cell (HUVEC) line derived from fusion of HUVECs and lung adenocarcinoma cells. EA.hy926 cells were obtained from the Shanghai Institute Cell Bank (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1% penicillin/streptomycin in a 5% CO₂ atmosphere at 37 °C, after which they were divided into four groups: a "control group", an "H₂O₂ group", an "isoquercitrin alone group", and an "isoquercitrin group". Isoquercitrin was dissolved in DMSO, which was present at a concentration less than 0.1% in all tested solutions. For all isoquercitrin group experiments, EA.hy926 cells were grown to 70%–80% confluence and treated with the designated concentrations of isoquercitrin for 24 h, after which they were exposed to 200 μ mol/L H₂O₂ for 4 h in fresh medium and harvested for further analysis.

4.3. Cell Viability Analysis

MTT assays were used in the first step of determining appropriate concentrations of isoquercitrin and H₂O₂. EA.hy926 cells were seeded in 96-well plates (1 \times 10⁴ cells/well) and cultured for 24 h. The culture medium was removed, after which the cells were pretreated with various concentrations of isoquercitrin for 24 h before exposure to H₂O₂ for 4 h. After the treatment, 10 μ L of 5 g/L MTT solution was added to each well, after which the cells were incubated for 4 h. Next, the culture medium was

replaced with 100 μ L of DMSO, after which formazan crystals were dissolved by shaking the plate at room temperature. The absorbance of the solution in each well was measured at 570 nm.

4.4. Hoechst33342/PI Fluorescent Staining

Apoptosis was assessed using Hoechst33342/PI double fluorescent staining detection kit. EA.hy926 cells were cultured in 6-well plates at a density of 5×10^5 cells per well, after which they were exposed various concentrations of isoquercitrin for 24 h, followed by exposure to 200 μ mol/L H_2O_2 for 4 h. Next, the cells were stained with 10 μ L of Hoechst 33342 solution at 37 °C for 10 min in the dark, followed by staining with 5 μ L of PI at 25 °C for 15 min in the dark. The stained cells were observed under a fluorescence microscope. Cells were counted using the Image pro-plus (version 6.0.0., Media Cybernetics, Rockville, MD, USA).

4.5. Flow Cytometric Analysis of Apoptotic Cells

To evaluate the percentage of apoptotic cells, a double-staining assay was performed using an Annexin V-FITC/propidiumiodide(PI) kit. EA.hy926 cells were cultured in 6-well plates at a density of 5×10^5 cells per well and treated with various concentrations of isoquercitrin for 24 h prior to exposure to 200 μ mol/L H_2O_2 for 4 h. Next, the treated cells were collected, washed twice with ice-cold PBS, and resuspended in binding buffer at a concentration of 1×10^6 cells/mL. Next, 5 μ L of Annexin V-FITC and 5 μ L of PI were added. The cells were cultured for 15 min in the dark, after which apoptotic cells were quantified using flow cytometry.

4.6. Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential was estimated using a mitochondrial membrane potential assay kit with JC-1, a lipophilic, cationic dye that changes color as the mitochondrial membrane potential increases. EA.hy926 cells (1×10^4 cells/well) were cultured in 6-well plates and treated with various concentrations of isoquercitrin for 24 h prior to exposure to 200 μ mol/L H_2O_2 for 4 h, after which the treated cells were washed and incubated with 500 μ L of JC-1 at 37 °C for 20 min in the dark. Next, the stained cells were collected, washed with JC-1 staining buffer, and resuspended in staining buffer. The fluorescence intensity of each sample was detected using flow cytometry.

4.7. Western Blotting

Total protein was extracted from the cells and prepared with RIPA buffer. Protein concentrations were estimated using a bicinchoninic acid protein assay kit. For western blot analysis, equal amounts of protein (40 μ g) from each sample were separated and electro transferred onto NC membranes (Invitrogen, CA, USA), which were blocked in 5% nonfat milk and incubated overnight at 4 °C with primary antibodies. After three washes with Tris-buffered saline containing 0.05% Tween-20 (TBST), each membrane was incubated for 1.5 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies at room temperature. The bands were visualized using an ECL detection kit. Quantification of the bands was performed by densitometric analysis using Image J software (Version 1.44p, National Institutes of Health, Bethesda, MD, USA).

4.8. Fluorescence Quantitative PCR

Fluorescence quantitative polymerase chain reaction (FQ-PCR) was applied to determine Mcl-1 transcript levels in EA.hy926 cells. Total RNA was extracted using TRIzol reagent, after which cDNA was synthesized according to the instructions included with the PrimeScript[®] RT reagent kit with gDNA Eraser. The reaction system was prepared according to the instructions included with the SYBR[®] Premix Ex Taq[™] kit. Following initial denaturation at 95 °C for 15 min, the amplification conditions were as follows: 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 20 s. All PCR data were checked for homogeneity by dissociation curve analysis.

4.9. Statistical Analysis

Each experiment was performed at least three times. The values were expressed as the mean \pm standard deviation (SD). Differences among experimental groups were evaluated by one way ANOVA. Values of $p < 0.05$ were considered statistically significant.

5. Conclusions

The present study provided evidences that isoquercitrin mediated antiapoptotic effects in H₂O₂-treated EA.hy926 cells were likely associated with inhibiting activation of caspase-3 and caspase-9, inducing Mcl-1 expression, and activating GSK3 β and PI3K/Akt. These data suggested that isoquercitrin could be potential for the treatment of oxidative stress-associated cardiovascular disease.

Acknowledgments: This work was financially supported by the National Natural Science Foundation of China (grant No. 81274132). We are grateful to Editage for providing editorial assistance.

Author Contributions: M.X.Z., X.L.H. and Q.S.L. designed the research; M.X.Z., J.K.L., K.W., and R.G. performed the experimental work; M.X.Z. wrote the manuscript. All authors discussed, edited and approved the final version.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

| | |
|--------|--|
| ROS | Reactive oxygen species |
| DMEM | Dulbecco's modified Eagle's medium |
| FBS | Fetal bovine serum |
| DMSO | Dimethyl sulfoxide |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide |
| HUVEC | Human umbilical vein endothelial cell |
| FBS | Fetal bovine serum |
| PI | Propidium iodide |
| TBST | Tris-buffered saline containing 0.05% Tween-20 |
| HRP | Horseshoe peroxidase |
| FQ-PCR | Fluorescence quantitative polymerase chain reaction |
| Iso | Isoquercitrin |

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Sample Availability: Samples of the compound isoquercitrin are available from the authors.



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