Soy isoflavones protect against H_2O_2-induced injury in human umbilical vein endothelial cells

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Abstract. The aim of this study was to investigate the effects of soy isoflavones on the injury of human umbilical vein endothelial cells induced by H_2O_2. EVC-304 cells were preincubated with soy isoflavones for 12 h, and then exposed to 100 μM H_2O_2 for 1 h. Cell viability was evaluated by a 3-(4,5-di-methylthiazol-2-yl) 2,5-diphenyltetrazolium bromide assay. The apoptosis of EVC-304 cells was detected by Hoechst 33258 and Annexin-V/propidium iodide staining. The oxidative stress-related biochemical parameters were detected and the expression of apoptosis-related proteins was examined by western blot analysis. The results showed that incubation with soy isoflavones caused a significant increase in the viability of EVC-304 cells and a decrease in cell apoptosis induced by H_2O_2. Soy isoflavones also markedly enhanced the activities of superoxide dismutase and glutathione peroxidase, and reduced the level of malondialdehyde. Western blot analysis results show that soy isoflavones can modulate the activation of nuclear factor-κB and the mitochondria-mediated apoptosis signaling pathway. The results of this study indicated the potential biological relevance of soy isoflavones in the therapy of cardiovascular diseases.

Introduction

Vascular endothelial cells (ECs) form the boundaries between circulating blood and vascular walls; they are necessary to maintain vascular homeostasis (1). As such, any injury of ECs induced by reactive oxygen species (ROS) is crucial in the development of the early stages of vascular diseases, such as high blood pressure, high cholesterol, diabetes and cancer. ROS can cause oxidative damage to lipids, proteins and enzymes in ECs; as a result, cellular function is impaired. This condition causes the apoptosis of severely damaged ECs (2-5). As one of the most important ROS, H_2O_2 can easily cross the plasma membrane and damage neighboring cells, including H_2O_2-producing cells. Thus, H_2O_2 has been extensively used to induce oxidative stress in *in vitro* models (6-8). Therefore, the inhibition of H_2O_2-induced damage in ECs has been considered as a potential therapeutic strategy of various cardiovascular diseases, such as atherosclerosis (9,10).

Since ancient times, natural plant extracts have been used to develop novel therapeutic agents. Among these agents, the phytochemicals, flavonoids, isoflavonoids and related compounds are the most useful as they are present in edible plants and exhibit broad pharmacological activities (11). For centuries, soybeans and their products have been a part of the staple diet of Asians (12). Isoflavones are major soybean flavonoids extensively investigated due to their beneficial properties in preventing coronary heart disease, cancer and osteoporosis (13). The antioxidant activity of soy isoflavones is essential for its cardiovascular protective effect.

In this study, H_2O_2 was utilized to mimic the effect of oxidative stress in human umbilical vein endothelial cells (HUVECs) and to investigate the pharmaceutical functions of soy isoflavones in oxidative stress-induced vascular endothelial cell damage.

Materials and methods

Reagents. Soy isoflavones (purity, >98%) were provided by Hubei Yuanceng Pharmaceutical Co., Ltd. (Wuhan, China). H_2O_2 was purchased from the Tianjin Guangfu Chemical Research Institute (Tianjin, China). Dimethylsulfoxide (DMSO), 3-(4,5-di-methylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), and streptomycin were obtained from Amresco (Solon, OH, USA). Penicillin, gelatin, glutamine, paraformaldehyde and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Roche Diagnostics (Mannheim, Germany). The rabbit anti-human polyclonal Bcl-2 (1:1,000; sc-492), rabbit

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anti-human polyclonal Bax (1:1,000; sc-6236), goat anti-human polyclonal Caspase-3 (1:1,000; sc-1226), goat anti-human Caspase-9 (1:1,000; sc-22182), rabbit anti-human polyclonal Cytochrome *c* (1:1,000; sc-7159) and rabbit anti-human polyclonal IκB (1:1,000; sc-371) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-human monoclonal nuclear factor (NF)-κB (1:1,000; SAB4502609) and rabbit anti-human monoclonal β-actin (1:1,000; SAB5500001) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical chemical grade.

Cell culture. The EVC-304 HUVECs were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin (Life Technologies, Carlsbad, CA USA) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Cells from the stock flask were suspended in phosphate-buffered saline, treated with trypsin (Sigma-Aldrich) and counted using a hemocytometer (Ningbo Biocotek Scientific Instrument Co., Ltd., Ningbo, China). After ~3 days from seeding, active growth of cells began and the experiment was started only after this period.

Cell viability assay. The MTT cytotoxicity assay was used to measure cell viability as described (14). EVC-304 cells were grown to ~80% confluence, maintained with fresh medium described above, and subcultured every 2-3 days. The cells were treated with soy isoflavones (25, 50, 100 and 200 μM) for 12 h prior to testing for the presence of 100 μM H_2O_2 for another hour. Each treatment condition was tested in 5 replicate wells. At the end of the treatment, cells were incubated with 100 μl of 0.5 mg/ml MTT at 37˚C for 4 h. Then, 100 μl DMSO was added to each well. Absorbance of each well was detected at 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative cell viability was expressed as the ratio of the microwave-treated cells to that of control cells. All experiments were performed in triplicate.

Morphological examination for Hoechst 33258 staining. EVC-304 cells were collected, washed with PBS and fixed with 2% paraformaldehyde at room temperature for 15 min. Cells were then were washed with PBS and stained with Hoechst 33258 staining solution (25 μg/ml; Sigma-Aldrich) for 30 min at room temperature. The stained nuclei were observed using a fluorescence photomicroscope (IX71; Olympus, Tokyo, Japan).

Flow cytometric evaluation of apoptosis. EVC-304 cells were double stained by an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Samples stained with Annexin V and propidium iodide (PI) were quantitatively analyzed at an emission wavelength of 488 nm and an excitation wavelength of 570 nm using the BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurements of intracellular superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) contents. The activities of SOD, GSH-Px and MDA were all determined using the SOD, GSH-Px and MDA Assay kits, and all the procedures complied with the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China). The activity of the enzymes was expressed as U/mg protein. The SOD activity assay was based on its ability to inhibit the oxidation of hydroxylamine by the O^{2-} produced from the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the quantity that reduced the absorbance at 550 nm by 50%. GSH-Px activity was measured using the enzyme-catalyzed reaction product (reduced glutathione) and the absorbance was recorded at 412 nm. The activities of GSH-Px were expressed as U/mg protein. The MDA content was measured at a wavelength of 532 nm by reaction with thiobarbituric acid (TBA) to form a stable chromophore. The values of the MDA level were expressed as nmol/mg protein.

Western blot analysis. For immunoblot analyses, 40 μg protein lysates per sample were denatured in 4X SDS-PAGE sample buffer (Tris-HCl 260 mM, pH 8.0; 40% (v/v) glycerol, 9.2% (w/v) SDS, 0.04% bromophenol blue and 2-mercaptoethanol as a reducing agent) and subjected to SDS-PAGE on 12% acrilamide/bisacrilamide gels (DingGuo ChangSheng Biotechnology Co., Ltd., Beijing, China). Separated proteins were transferred to nitrocellulose membranes (Hybond-P PVDF; Bio-Rad Laboratories, Inc.). Residual binding sites on the membrane were blocked by incubation in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% (w/v) non-fat milk powder overnight at 4˚C. Membranes were then probed with with anti-Bcl-2, anti-Bax, anti-Caspase-3, anti-Caspase-9, anti-Cytochrome *c*, anti-NF-κB, anti-IκB and anti-β-actin antibodies at a dilution of 1:1,000 for 16 h at 4˚C, after which they were incubated with the appropriate peroxidase-linked secondary antibody with a dilution of 1:5,000 for 1 h at room temperature. Chemiluminescence signals were visualized with an enhanced chemiluminescence ultra-sensitive light-emitting liquid (Beyotime Institute of Biotechnology, Shanghai, China) and quantified by Quantity One software, version 4.62 (Bio-Rad Laboratories, Inc.).

Statistical analysis . Results are expressed as the mean ± standard error of the mean. All data were analyzed using SPSS version 13 software (SPSS Inc., Chicago, IL, USA). For comparisons between groups of more than two unpaired values, one-way analysis of variance (ANOVA) was used. If an ANOVA F-value was significant, *post hoc* comparisons were performed between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Soy isoflavones inhibit H_2O_2-induced cytotoxicity in EVC-304 cells. Initially, the cytotoxicity of soy isoflavones on EVC-304 cells was examined using the MTT assay. Soy isoflavones did not show obvious cytotoxic effects up to 200 μM (Fig. 1A). Then it was further evaluated whether soy isoflavones had a protective effect. EVC-304 cells were pretreated with soy isoflavones for 12 h, then incubated with H_2O_2 for 1 h and

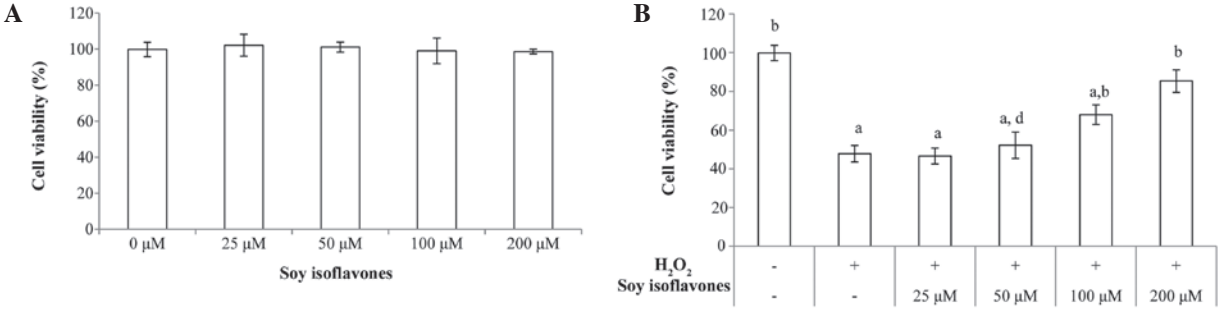

Figure 1. Soy isoflavones attenuated H_2O_2-induced cytotoxicity in EVC-304 cells. (A) The cytotoxicity of soy isoflavones (25, 50, 100 and 200 μM) on EVC-304 cells. (B) The EVC-304 cells pretreated with different concentration of soy isoflavones (25, 50, 100 and 200 μM) for 12 h were exposed to 100 μM H_2O_2 for 1 h. The viability was measured by 3-(4,5-di-methylthiazol-2-yl) 2,5-diphenyltetrazolium bromide assay. Values are represented as the mean ± standard error of the mean (n=5). [a]P<0.01 vs. the untreated group (control), [b]P<0.01 vs. the H_2O_2 alone group.

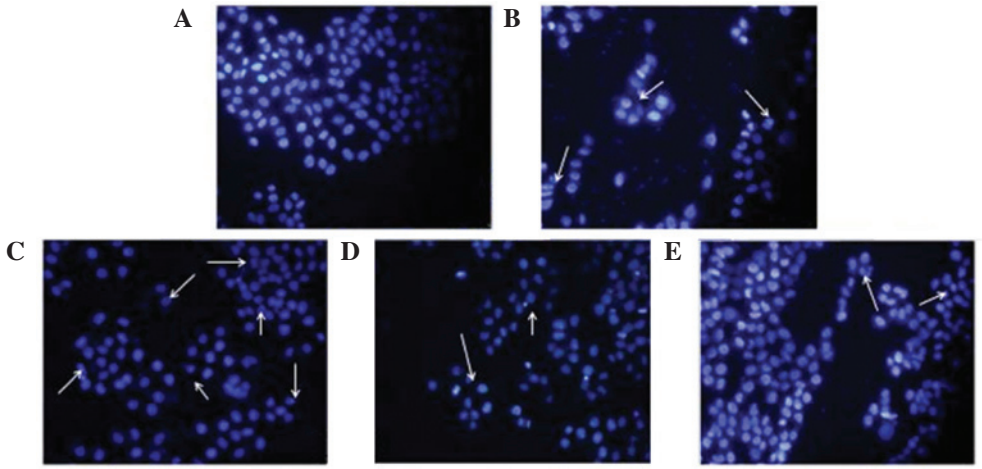

Figure 2. Morphological analysis of EVC-304 cells. The EVC-304 cells pretreated with different concentration of soy isoflavones (50, 100 and 200 μM) for 12 h were exposed to 100 μM H_2O_2 for 1 h, then cellular nuclei were fluorescence stained by Hoechst 33258 (magnification, x200). Arrows indicate apoptotic nuclei. (A) Non-treated cells. (B) Treatment with 100 μM H_2O_2 alone. Cells pretreated with (C) 50 μM, (D) 100 μM and (E) 200 μM soy isoflavones.

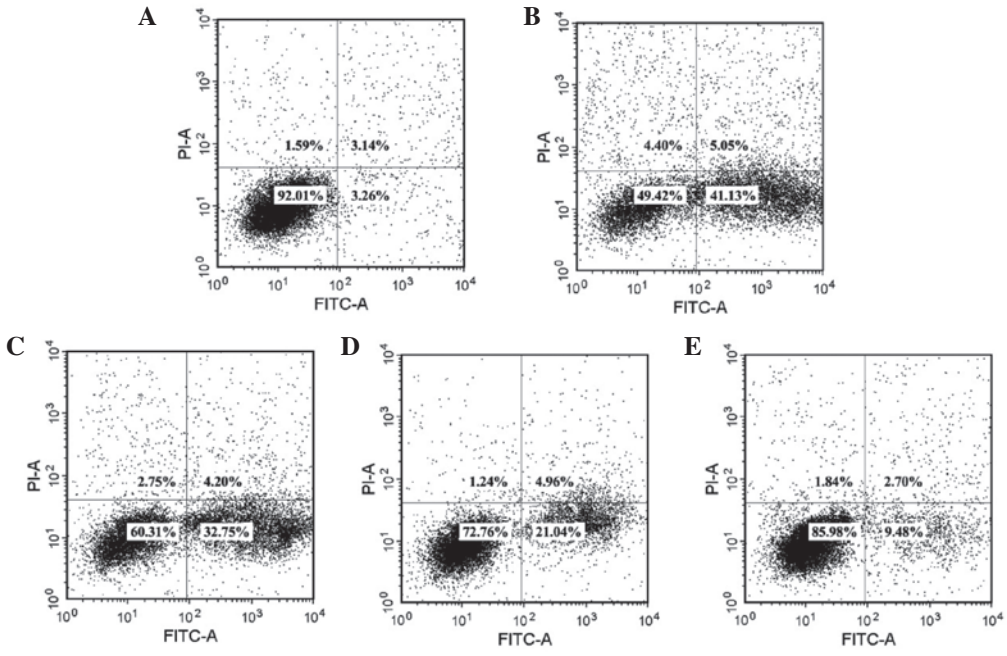

Figure 3. Soy isoflavones suppress the apoptosis of EVC-304 cells induced by H_2O_2 was observed by flow cytometric analysis. The EVC-304 cells pre-treated with soy isoflavones were exposed to 100 μM H_2O_2 for 1 h. Cells were then harvested and subjected to quantitative analysis of cell apoptosis by Annexin-V and PI double-stained flow cytometry. (A) Representative flow cytometry results of untreated group. (B) Representative of the H_2O_2 alone group. Representative of the cells pretreated with (C) 50 μM, (D) 100 μM and (E) 200 μM soy isoflavones. PI, propidium iodide; FITC, fluorescein isothiocyanate.

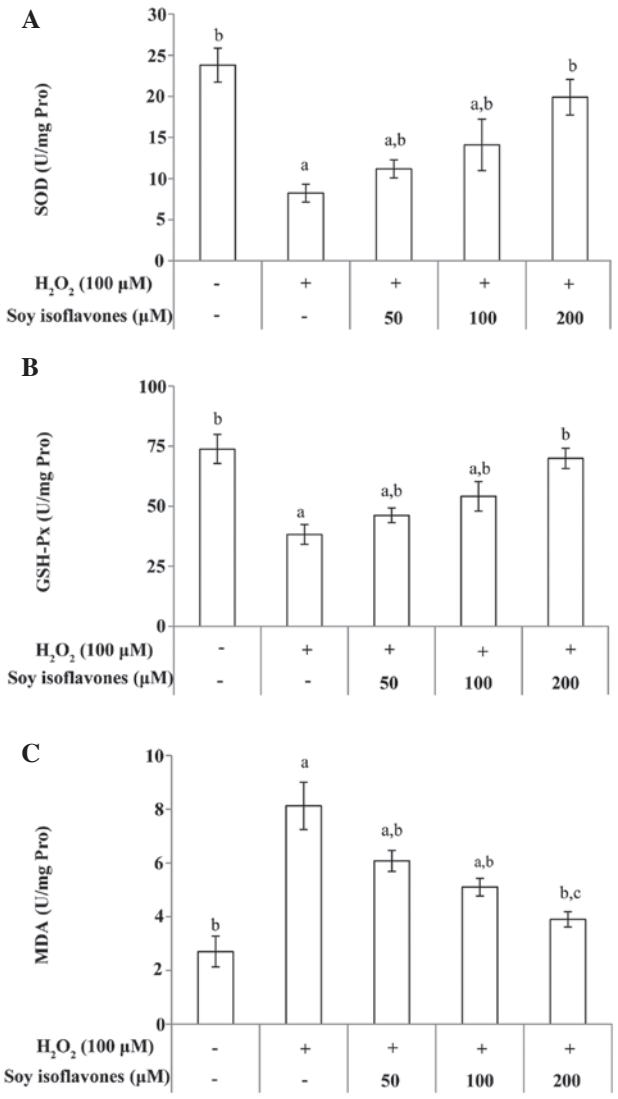

Figure 4. Effects of soy isoflavones on the intracellular SOD, GSH-Px, and MDA enzyme activities. The EVC-304 cells pre-treated with soy isoflavones were exposed to 100 μM H_2O_2 for 1 h. Cells were then harvested and subjected to analysis the effect on (A) SOD, (B) GSH-Px, and (C) MDA enzyme activities. The EVC-304 cells pretreated with soy isoflavones for 12 h were exposed to 100 μM H_2O_2 for 1 h. Values are represented as the mean ± standard error of the mean (n=5). [a]P<0.01 vs. the untreated group (control), [b]P<0.01 vs. the H_2O_2 alone group, [c]P<0.05 vs. the untreated group. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

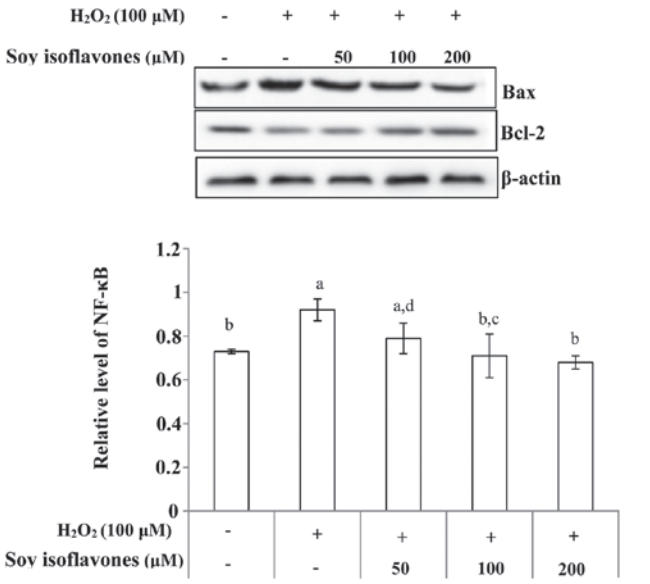

Figure 5. Western blot analysis of Bax and Bcl-2 in response to soy isoflavones treatment in EVC-304 cells. Cells were pretreated with soy isoflavones 50, 100 and 200 μM, followed by exposure to 100 μM H_2O_2 for 1 h. Protein expression was then analyzed by western blot analysis using anti-bcl-2 and anti-bax antibodies. The results are expressed as the mean ± standard error of the mean, n=5, [a]P<0.01 vs. the untreated group (control), [b]P<0.01 vs. the H_2O_2 alone group, [c]P<0.05 vs. the untreated group.

cell viability was measured by the MTT assay. The viability of cells was decreased significantly following treatment with 100 μM H_2O_2 for 1 h (P<0.001 vs. untreated group), while soy isoflavones protected cells from H_2O_2-induced cytotoxicity in a concentration-dependent manner (25 μM, 46.6±4.1%; 50 μM, 52.2±6.8%; 100 μM, 60±5.1%; and 200 μM, 68.3±5.8%), as shown in Fig. 1B.

Soy isoflavones improved morphological changes of EVC-304 cells. The uniform shape of EVC-304 nuclei and well-distributed deep blue fluorescence were revealed by Hoechst 33258 staining. Hoechst 33258 was used as an apoptosis marker, which detected apoptotic nuclei with condensed and/or fragmented DNA. The majority of nuclei in the control groups had uniform blue chromatin with an organized structure (Fig. 2A). Following treatment with 100 μM H_2O_2 for 1 h, EVC-304 cells exhibited typical features of apoptosis, as shown in Fig. 2B. Soy isoflavone pretreatment reduced H_2O_2-induced apoptosis, demonstrated by few apoptotic nuclei (Fig. 2C-E), similar to that of the control conditions. The data showed that the apoptotic index increased markedly in cells stimulated with H_2O_2; however soy isoflavone pretreatment reduced the level of EVC-304 cells induced by H_2O_2.

Soy isoflavones reduce EVC-304 cell apoptosis induced by H_2O_2. In order to quantitatively gain insight into the anti-apoptotic effects of soy isoflavones in H_2O_2-induced EVC-304 cells, the apoptosis rate of EVC304 cells after treatment with 100 μM H_2O_2 for 1 h, was measured by Annexin-V/PI staining. As shown in Fig. 3A and B, the apoptosis rate increased from 3.26±0.4 to 41.13±2.2%. By contrast, increased doses of soy isoflavones could evidently attenuate the apoptosis of EVC-304 cells to 32.75±2.4, 21.04±2.5 and 9.48±2.8%, respectively (Fig. 3C-E).

Soy isoflavones reduce oxidative stress in EVC-304 cells. Major antioxidant defenses include antioxidant scavengers, such as SOD and GSH-Px. An increase in MDA, which is a lipid peroxidation end-product, indicates reduced antioxidant capacity. Total SOD, GSH-Px and MDA activity in EVC-304 cells was measured. After treating the cells with H_2O_2 for 1 h, the SOD and GSH-Px levels decreased respectively, (Fig. 4A; P<0.001 vs. untreated group). However, incubation with soy isoflavones significantly attenuated the changes in the content of SOD and GSH-Px (Fig. 4A and B; P<0.01 vs. the H_2O_2 group). In addition, cells treated with H_2O_2 for 1 h showed increasing intracellular MDA release, (P<0.001 vs. untreated group); however, incubation with soy isoflavones produced a

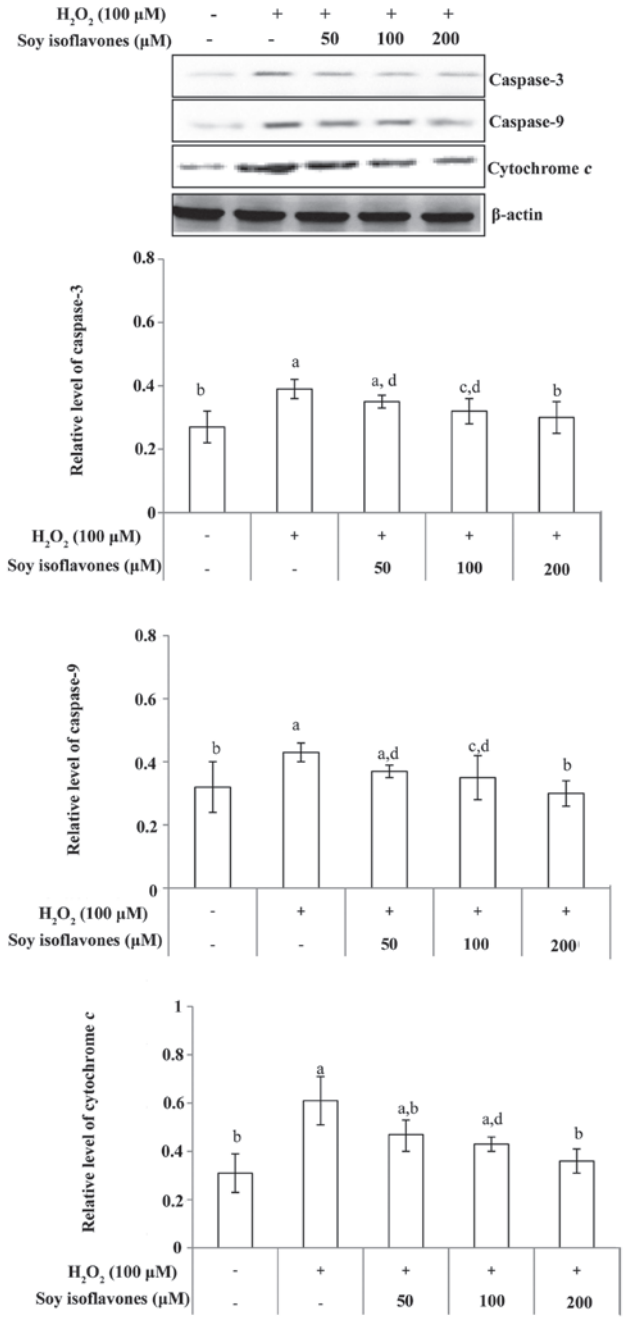

Figure 6. Effect of Soy isoflavones on H_2O_2 induced mitochondrial membrane potential in EVC-304 cells. Cells were pretreated with Soy isoflavones 50,100 and 200 μM, followed by exposure to 100 μM H_2O_2 for 1 h. Protein expression was then analyzed by western blot using anti-Caspase-3, anti-Caspase-9 and anti-Cyto C antibodies. The results are expressed as means ± SEM, n=5, [a]P<0.01 vs. untreated group (control), [b]P<0.01 vs. H_2O_2 alone group, [c]P<0.05 vs. untreated group, [d]P<0.05 vs. H_2O_2 alone group.

marked decrease in the intracellular level of MDA (Fig. 4C; P<0.01 vs. the H_2O_2 group).

Effect of soy isoflavones on the expression of H_2O_2-induced apoptosis-related proteins. To investigate whether soy isoflavones exhibit an effect on the apoptosis-related protein expression in EVC-304 cells, the expression of apoptosis-related proteins were analyzed by western blot analysis. The ratio of Bax/Bcl-2 was analyzed, which is crucial for the activation of the cell apoptosis. The level of Bax increased while the

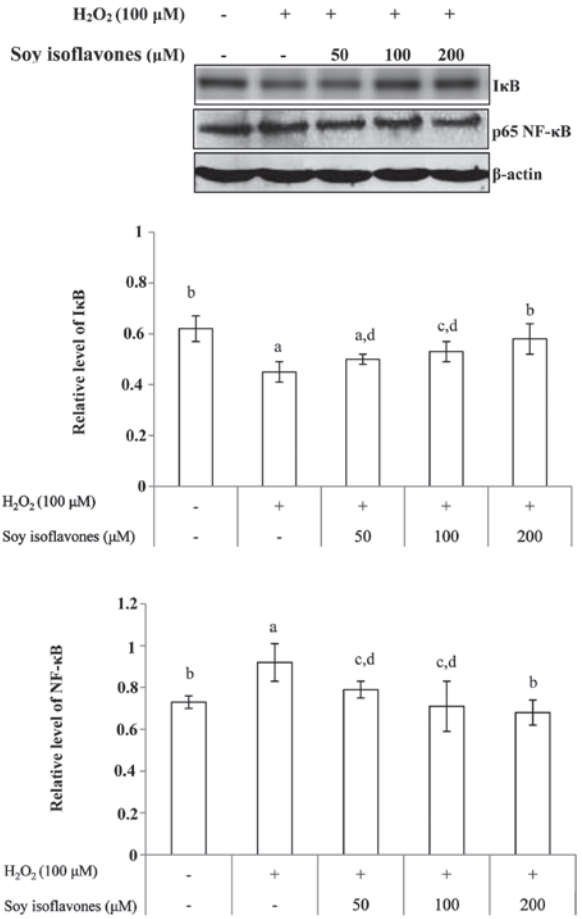

Figure 7. Effects of soy isoflavones on the protein expression of NF-κB and IκB. Cells were pretreated with soy isoflavones 50, 100 and 200 μM, followed by exposure to 100 μM H_2O_2 for 1 h. Western blot analysis was performed. The results are expressed as the mean ± standard error of the mean, n=5. [a]P<0.01 vs. the untreated group (control), [b]P<0.01 vs. the H_2O_2 alone group, [c]P<0.05 vs. the untreated group, [d]P<0.05 vs. the H_2O_2 alone group. NF-κB, nuclear factor-κB.

Bcl-2 level showed marginal decrease when the EVC-304 cells were treated with H_2O_2, but pretreatment with soy isoflavones decreased the ratio (Fig. 5). The protein expression of cytochrome *c* release, and caspase-3 and caspase-9 protein levels were also investigated. As expected, western blot analysis showed the protein levels were significantly higher in the H_2O_2 group than that in the control group; however, this effect was inhibited by soy isoflavones (Fig. 6). Thus, treatment with soy isoflavones decreased the protein expression induced by H_2O_2, and it was indicated that this occurred via inhibition of the mitochondria-mediated apoptosis signaling pathway.

Effects of soy isoflavones on H_2O_2-induced NF-κB expression. As noted, degradation of IκB allows the nuclear localization of NF-κB and subsequent transcriptional activation of target genes. Western blot analysis indicated that treatment with H_2O_2 resulted in a degradation of the IκB protein compared with that in control cells. The H_2O_2-induced degradation of IκB was inhibited following pretreatment with soy isoflavones. Western blot analysis also indicated that treatment with soy isoflavones prior to H_2O_2 treatment markedly abrogated H_2O_2-induced activation of NF-κB (Fig. 7).

Discussion

In the present study, the effects of soy isoflavones on H_2O_2-induced damage in EVC-304 cells *in vitro* were examined. Possible mechanisms underlying these effects were also investigated. Data indicated that soy isoflavones could protect EVC-304 cells from H_2O_2-induced damage. The underlying mechanisms may involve soy isoflavones functioning as a potent inhibitor of oxidative stress. Soy isoflavones could also modulate the activation of NF-κB and the mitochondria-mediated apoptosis signaling pathway in HUVECs.

Under physiological conditions, ROS are generated at low levels and involved in signaling and metabolic pathways. However, an increase in oxidative stress induced by different vascular risk factors is a key mechanism of EC injury (15). In the present study, the viability of EVC-304 cells was significantly decreased and cell apoptosis was induced when the cells were exposed to H_2O_2. However, cell viability and apoptosis were notably improved when soy isoflavones were added to the culture medium 12 h prior to H_2O_2 administration. To verify whether or not EC injury is correlated with oxidative stress, the activity of antioxidant enzymes was determined. The results showed that pre-treatment with soy isoflavones could reduce MDA content and enhance SOD and GSH-PX activity. Therefore, soy isoflavones could protect EVC-304 cells from cellular injury induced by oxidative stress.

Oxidative stress can directly induce EC apoptosis, which accelerates EC injury. EC apoptosis corresponds to an important process in the pathogenesis of vascular diseases (16). Pre-treatment with soy isoflavones could effectively alleviate H_2O_2-induced EC apoptosis. If the exact mechanism of the anti-apoptotic effect of soy isoflavone in oxidative stress-induced ECs can be identified, vascular diseases can be prevented or alleviated. A previous study suggested that changes in mitochondrial membrane potential can mediate committed cells to undergo apoptosis with oxidative stress (17). Apoptosis can be initiated via two pathways; the extrinsic and intrinsic pathways. The intrinsic pathway is mitochondrial dependent and involves caspases and the Bcl-2 protein family (18). Among various caspases, caspase-9 and -3 are important in cell apoptosis. These proteins are also the upstream regulators of mitochondrial membrane potential, inducing the release of cytochrome *c* into the cytosol. The translocation of cytochrome *c* from the mitochondria to the cytosol is required for activation of the apoptotic machinery in various cell death models (19). The Bax/Bcl-2 ratio determines whether or not a cell survives when it is exposed to an apoptotic stimulus (20,21). The pro-apoptotic protein Bax can promote the release of cytochrome *c*, where as the pro-survival protein Bcl-2 elicits anti-apoptotic effects. The mitochondria are also important in apoptosis or the programmed cell death pathway. The results indicated that exposure to H_2O_2 could upregulate the ratio of Bax/Bcl-2, enhance the release of cytochrome *c*, and activate the cleavage of caspase-3 and -9. However, pre-treatment with soy isoflavones could decrease the Bax/Bcl-2 ratio and prevent the translocation of cytochrome *c*, thereby inhibiting the cleavage of caspase-3 and -9.

The NF-κB family of transcription factors regulates multiple biological functions. For example, NF-κB is vital in inflammatory and innate immune responses. It is commonly activated by various agents, such as ROS (22). Conversely, agents that scavenge ROS can inhibit the activation of NF-κB. In this study, *in vitro* soy isoflavone treatment prevented the H_2O_2-induced generation of ROS in EVC-304 cells. To verify whether or not this protective effect is correlated with the inhibitory activation of NF-κB, NF-κB and IκB protein expression levels were detected by western blot analysis. The results showed that NF-κB was activated by H_2O_2-induced HUVEC injury. However, this activation was effectively inhibited by pre-treatment with soy isoflavones. H_2O_2-induced HUVEC injury also resulted in IκB protein degradation; however, the pre-treatment of soy isoflavones prevented this degradation.

Therefore, soy isoflavones elicited protective effects against H_2O_2-induced cytotoxicity and apoptosis of EVC-304 cells. Soy isoflavones could elicit anti-apoptotic effects by inhibiting ROS generation and modulating NF-κB and the mitochondria-mediated apoptosis signaling pathway activation. The current study suggests that soy isoflavone is a potential candidate for therapeutic application against cardiovascular diseases, however the precise mechanism of its action still requires further investigation.

Acknowledgements

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