Quercetin inhibits angiotensin II induced apoptosis via mitochondrial pathway in human umbilical vein endothelial cells

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Abstract. – OBJECTIVE: Quercetin exhibits various biological functions including anti-oxidation, anti-inflammatory, antiviral, immunity and anticancer, etc. It can also lower blood pressure and improve blood capillary elasticity. Angiotensin II (Ang II) has been showed to induce apoptosis of human umbilical vein endothelial cells. In this study, we attempted to clarify the effect of quercetin on Ang II induced apoptosis.

MATERIALS AND METHODS: Human umbilical vein endothelial cells (HUVECs) were cultured. The cytotoxicity was determined by MTT assay. HUVECs apoptosis was determined by DAPI staining and flow cytometry. The mitochondrial transmembrane potential was observed by JC-1 staining. The expression of cytochrome c, Bcl-2, Bax, activated-caspase-3 and activated-caspase-9 was measured by Western blot.

RESULTS: MTT assays showed Ang II decreased cell viability in a concentration-dependent manner in HUVECs. Notably, quercetin presented very little effect on HUVECs. Quercetin inhibited the effect of Ang II on HUVECs in a concentration- and time-dependent manner. Furthermore, the loss of mitochondrial membrane potential, upregulation of cytochrome c and Bax, downregulation of Bcl-2, and activation of caspase-9 and caspase-3 caused by angiotensin II were also recovered after treated with quercetin.

CONCLUSIONS: Quercetin could inhibit Ang II induced apoptosis of human umbilical vein endothelial cells via the mitochondrial pathway.

Key Words: Quercetin, Angiotensin II, Apoptosis, Mitochondrial pathway.

Introduction

Endothelial cells (ECs), which are involved in many vascular processes, including vasoconstriction, angiogenesis and vasodilation, play an essential role in maintaining endothelial homeostasis. Previous studies showed that endothelial dysfunction underlies the pathogenesis of various cardiovascular diseases. EC injury, which is accompanied by an increasing rate of apoptosis, has been regarded as a common feature of endothelial dysfunction and involved in the pathogenesis of cardiovascular disease such as hypertension. Angiotensin II (Ang II), which is involved in the regulation of blood pressure, cardiovascular physiology and electrolytes homeostasis, is a member of the renin-angiotensin system (RAS). Ang II activates several signaling pathways into the vasculature, which can lead to vascular inflammation, contraction, endothelial dysfunction, growth and migration of vascular smooth muscle cells, all these factors promote the development and progression of vascular damage. Ang II triggers excessive expression of reactive oxygen species (ROS), DNA damage, mitochondrial dysfunction and inflammation, as well as eventually inducing endothelial apoptosis. Although Ang II has been long recognized as the mediation of endothelial cell apoptosis, little is known about its underlying mechanism. It is necessary to look for effective agents to antagonism the effect on endothelial cell.

A lot of researches indicated many foods, which are rich in plant nutrients, could reduce the incidence of non-infectious chronic disease, including inflammation, cardiovascular disease, cancer, aging, etc. Many natural phenolic compounds have the biological activity: anti-aging, anti-inflammatory and involving in clear of drugs or carcinogens. Flavonoid compounds are the most common class of the natural phenolic compounds, and considered to be safe and good for your health in food additives. Quercetin is an effective ingredient of flavonoids to treat coronary heart disease, and a previous study showed that quercetin has the biological activity of expansion of crown and protection of ischemic brain injury. Quercetin, alias Quercetin, is a kind of natural flavonoid compounds, which widely exists in the main...
from cherry, tea, raspberry, red onions and hawthorn so on. Quercetin can lower blood pressure and improve blood capillary elasticity. In myocardial cells, quercetin can ameliorate the damage of adriamycin and promote survival by regulating mitochondrial apoptosis-related proteins\textsuperscript{11-14}. Given the effect of quercetin, we try to explore whether quercetin inhibits Ang II induced apoptosis in endothelial cells.

In this study, we explore the effect of quercetin on Ang II induced apoptosis. Results indicated quercetin could inhibit Ang II induced apoptosis of cell apoptosis via mitochondrial-dependent signaling pathway in HUVECs. Therefore, quercetin has great potential as an agent against cardiovascular disease.

**Materials and Methods**

**Reagents and Chemicals**

RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NJ, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl) 2-, 5-diphenyl tetrazolium bromide (MTT), BCA protein kit, JC-1 and Western lysis buffer were from Beyotime Institute of Biotechnology (Nantong, China). Enhanced chemiluminescence kit was purchased from Peirce (Tattenhall, UK). Annexin V-FITC/PI apoptosis detection kit was from Pharmingen-Becton Dickinson (San Diego, CA, USA). Bax and Bcl-2 antibodies were obtained from BBI (UK). Antibodies against activated-caspase-3, activated-caspase-9, Cytochrome-c and α-tubulin were purchased from Beyotime Institute of Biotechnology (Nantong, China).

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated from a fresh umbilical cord which was obtained from a healthy neonate under aseptic conditions in the Department of Gynecology and Obstetrics at the Shanxi Medical University. HUVECs were cultured in RPMI-1640 medium. All media were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated in a humidified atmosphere with 5% CO\textsubscript{2} at 37°C.

**MTT Assay**

The cytotoxicity was determined by MTT assay. HUVECs were seeded in 96-well plates at a density of 8000 cells/well. After 24h, cells were treated with Ang II (40, 60, 80, 100 and 120 μm) or quercetin (5, 10, 15 and 20 μm) for 24h. Based on previous dose response studies, effective Ang II (100 μm for 24h) was chosen as optimum, which was followed, for further studies. Quercetin (5, 10 and 15 μm) was added to the wells to determine alone and pre-treatment efficacy. The control group was added phosphate-buffered saline (PBS) in medium. MTT (20 μl 0.5 mg/μl in PBS) was added to each well and the plates were incubated at 37°C for 4 h. Then the medium was removed and DMSO (150 μl) was added to each well for 10 min to solute the purple formazan crystals. Absorbance at 570 μm was determined using a microplate reader. The percentage of cell viability was calculated based on the following formula: A_{570_{Experiment}}/A_{570_{Control}} × 100%. All assays were executed in three independent experiments.

**DAPI Staining of Apoptotic Cells**

HUVECs apoptosis were determined by DAPI staining. Briefly, HUVECs (2×10\textsuperscript{5} cells/well) were seeded in 12-well plates with glass slides. After 24 h, HUVECs were treated with Ang II (100 μm), quercetin (5, 10 and 15 μm) alone or composite processing. The control group was exposed in PBS in medium. HUVECs were washed twice with PBS and stained with DAPI for 20 min in the dark at room temperature. Then, HUVECs were washed with PBS and cell nuclei were observed under laser confocal scanning microscope (LCSM).

**Flow Cytometric Analysis**

HUVECs apoptosis were measured by Flow cytometry using the Annexin V-FITC and propidium iodide (PI) staining method. Briefly, HUVECs were seeded into 60 mm dishes (1×10\textsuperscript{6} cells/dish) and incubated overnight. After 24 h, HUVECs were treated with Ang II (100 μm), quercetin (5, 10 and 15 μm) alone or composite processing. Control group was with PBS in medium. Then cells were trypsinized, washed with cold PBS and re-suspended with binding buffer containing Annexin V-FITC (5 μl) and of PI (2.5 μl). Then cells were incubated in the dark for 30 min. Finally, cells were analyzed using a FAC Sort flow cytometer.

**Mitochondrial Transmembrane Potential Assay**

The mitochondrial transmembrane potential was observed by JC-1 staining. Briefly, HUVECs were plated in 12-well plates at a density of 2×10\textsuperscript{5}
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cells/well. After treated with Ang II, quercetin alone or composite processing, cells were washed with PBS and incubated with JC-1 (1 μg/mL) at 37°C in the dark for 20 min. The Δψm were analyzed for intracellular fluorescence by laser confocal scanning microscope (LCSM).

Western Blotting
Treated cells were lysed with western lysis buffer containing 1% PMSF for 15 min and centrifuged at 13000 rpm. Protein concentration was detected using a BCA protein kit. An equal amount of protein (80 μg) was loaded on 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked in TBS (Tris-buffered saline containing 5% milk) for 1 h. Then, the membranes were incubated with anti-Bcl-2 (1:500), anti-Bax (1:500), anti-Cytochrome-c (1:500), anti-Activated-caspase-3 (1:1000), anti-Activated-caspase-9 (1:1000) and anti-α-tubulin (1:500) overnight at 4°C. Then, the membranes were washed with TBST (Tris-buffered saline containing Tween-20) and incubated with the appropriate HRP-conjugated secondary antibody (1:1000). Finally, the amount of protein was detected with ECL western blotting detection system.

Statistical Analysis
Data were analyzed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance of differences of treatments was analyzed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison tests. A value of less than 0.05 (p < 0.05) and 0.01 (p < 0.01) were considered significant and highly significant, respectively.

Results
Quercetin Inhibits Ang II-induced Decline of Cell Viability in HUVECs
To detect whether quercetin could inhibit Ang II induced decline of cell viability, we firstly used MTT assay to assess the effects of Ang II, quercetin alone on HUVECs. HUVECs were treated with Ang II (40, 60, 80, 100 and 120 μm) or quercetin (5, 10, 15 and 20 μm) alone for 24h. The results showed Ang II inhibited the growth of HUVECs in dose-dependent (Figure 1A). Quercetin had no obvious effect on HUVECs during the range of 5 to 15 μm but inhibited growth significantly in 20 μm (Figure 1B). Based on the above dose response studies, effective Ang II (100 μm for 24 h) was chosen as optimum which was followed for further studies. Quercetin (5, 10 and 15 μm) was added to the wells to determine alone and pre-treatment efficacy. The results showed the viability of HUVECs which were pre-treated with quercetin significantly elevated and the effective time of quercetin is 3h (Figure 1C and D). All above results indicated quercetin could inhibit Ang II induced decline of viability of HUVECs.

Quercetin Inhibits Ang II Induced Cell Apoptosis in HUVECs
To detected whether quercetin inhibits Ang II induced decline of cell viability in HUVECs is related to cell apoptosis, HUVECs were treated with Ang II (100 μm), quercetin (5, 10 and 15 μm) alone or composite processing and then apoptosis was detected by DAPI staining and flow cytometric analysis. As shown in Figure 2A, Ang II treated HUVECs exhibited the apoptotic characteristics of chromatin condensation with typical apoptotic bodies. The apoptosis rate of HUVECs was significantly decreased after pre-treated with quercetin in dose-dependent compared with Ang II alone (Figure 2B). The degree of apoptosis was further measured by flow cytometry using Annexin V/PI double-staining. As indicated in Figure 3C and D, the HUVECs apoptosis rate which were treated with Ang II alone was 35.4% but decreased to 12.6% after composite processing. Taken together, quercetin could inhibit Ang II induced cell apoptosis in HUVECs.

Quercetin inhibited Ang II induced decrease of mitochondrial membrane potential (Δψm)
The mitochondria, which is often related to the loss of mitochondrial membrane potential (Δψm), plays an important role in the induction and control of apoptosis. To verify weather quercetin inhibited Ang II induced apoptosis via mitochondria pathway, HUVECs were treated with Ang II (100 μm), quercetin (5, 10 and 15 μm) alone or composite processing and then using JC-1 staining detected the change of Δψm. As showed in Figure 3A and B, the Δψm of HUVECs was decreased after with Ang II alone but elevated after pre-treated with quercetin in dose-dependent. These results indicated that quercetin inhibited Ang II induced decrease of mitochondrial membrane potential (Δψm) in HUVECs.
Quercetin Blocks the Ang II Induced Activation of the Mitochondrial Signaling Pathway

In order to further verified quercetin blocks the Ang II induced activation of the mitochondrial signaling pathway, key factors of mitochondria pathway including cytochrome c, Bcl-2, Bax, Activated-caspase-3 and Activated-caspase-9 were detected. HUVECs were treated with Ang II (100 μM), quercetin (15 μM) alone or composite processing, quercetin decreased the up-regulation of cytochrome c, Bax, Activated-caspase-3 and Activated-caspase-9 protein levels and increased the down-regulation of Bcl-2 expression (Figure 4 A, B and C). These data suggested that quercetin blocks the Ang II induced activation of the mitochondrial-dependent signaling pathway.

Discussion

Endothelial cells play an important role in many vascular processes and in maintaining endothelial homeostasis. EC injury has become a common feature of endothelial dysfunction because of many reasons such as apoptosis, inflammation. Ang II has founded to cause apoptosis in endothelial cells5. Furthermore, quercetin can reduce
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**Figure 2.** Protective effect of quercetin on Ang II induced apoptosis in HUVECs. **(A)** HUVECs were pre-treated with various concentrations of quercetin (5, 10 and 15 μM) for 3h and incubated with Ang II (100 nM) for 24h. Then cell nuclei were stained with DAPI and images were captured under a fluorescence microscope; **(B)** The percentage of apoptotic cells was measured; **(C)** HUVECs were pre-treated with various concentrations of quercetin (5, 10 and 15 μM) for 3h and incubated with Ang II (100 nM) for 24h and then cell apoptosis were determined with flow cytometry analysis using Annexin V/Propidium Iodide double-staining; **(D)** Apoptosis ratio from flow cytometry were shown in the bar graph. The data in (B) and (D) were expressed as mean ± SD and acquired from three biologically independent experiments. **##** *p < 0.01 versus control; **p < 0.01 versus Ang II alone.

**Figure 3.** Effects of quercetin on Ang II induced mitochondrial membrane potential. **(A)** HUVECs were pre-treated with various concentrations of quercetin (5, 10 and 15 μM) for 3h and incubated with Ang II (100 nM) for 24h. Then cells were stained with JC-1 and images were captured under a fluorescence microscope; **(B)** The Δψm were analyzed for intracellular fluorescence. The data in (B) was expressed as mean ± SD and acquired from three biologically independent experiments. **##** *p < 0.01 versus control; **p < 0.01 versus Ang II alone.
damage and promote survival by regulating mitochondrial dependent apoptosis.

Apoptosis plays an important role in drugs-induced cell death. Therefore, we explore whether quercetin inhibited Ang II induced decline of HUVECs viability by apoptosis. Results of DAPI staining and flow cytometry revealed apoptotic bodies and apoptosis ratio were significantly decreased after pre-treatment with quercetin compared with Ang II alone. These results showed quercetin decreased Ang II induced HUVECs apoptosis. Many studies showed mitochondrial damage plays an essential role in apoptosis process\textsuperscript{16-18}. The Δψm (the loss of mitochondrial membrane potential) is an important indicator in mitochondrial damage\textsuperscript{19,20}. Bcl-2 protein family, which is the central regulator of cell apoptosis, has the function of mitochondrial membrane potential function\textsuperscript{21,22}. Anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are two crucial members of Bcl-2 family\textsuperscript{23}. The loss of mitochondrial membrane potential could cause the release of cytochrome c alone with the activation of caspases which is involved in mitochondria-de-
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Conclusions

Quercetin inhibits Ang II induced apoptosis in HUVECs by modulating Bel-2 and Bax and inhibiting mitochondrial-dependent apoptosis pathway which includes the increasing of mitochondrial membrane potential, accumulation of cytochrome c and inhibition of caspase-3 and caspase-9. This study revealed quercetin could antagonism Ang II alone and pre-treated with quercetin. All these results revealed quercetin decreased elevated cytochrome c levels and inhibit Ang II induced mitochondrial-dependent pathway. Caspases which response to pro-apoptosis signals are a family of cysteine proteases. Caspase-3 and caspase-9 were both activated in mitochondrial-dependent apoptosis cells. In order to further detected quercetin inhibited Ang II induced apoptosis by mitochondrial-dependent pathway in HUVECs, the expression of Bcl-2, Bax, cytochrome c, caspase-3 and caspase-9 were detected after treated with Ang II alone and pre-treated with quercetin. Results found quercetin decreased the up-regulation of cytochrome c, Bax, Activated-caspase-3 and Activated-caspase-9 protein levels and increased the down-regulation of Bel-2 expression after pre-treatment with quercetin. All these results revealed quercetin decreased elevated cytochrome c levels and inhibit Ang II induced mitochondrial-dependent apoptosis in HUVECs.

Conflicts of interest

The authors declare no conflicts of interest.

References


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