Quercetin inhibits TNF-α induced HUVECs apoptosis and inflammation via downregulating NF-kB and AP-1 signaling pathway in vitro

Tielong Chen, PhDa, b, Xudong Zhang, MDa,b, Guangli Zhu, MDa, Hongfei Liu, MDa, Jinru Chen, MDa, Yu Wang, MDa, Xiaolong He, MDa

Abstract

Background: Quercetin, a major flavonol, wildly exists in plantage, which has been reported to have an anti-apoptosis and anti-inflammation effects on vascular endothelial cells, but its underlying molecular mechanisms remain unclear.

Objective: The aim of this study was to investigate the mechanisms of how quercetin inhibits tumor necrosis factor alpha (TNF-α) induced human umbilical vein endothelial cells (HUVECs) apoptosis and inflammation.

Methods and Results: HUVECs were preconditioned with quercetin for 18 hours, and subsequently treated with TNF-α for 6 hours to induce apoptosis. The expression of intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, β-actin mRNA was then detected by RT-PCR. Flow cytometry was used to estimate the apoptosis rates, and the expression of activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) was measured by Western blot. TNF-α induced elevated apoptosis rates and upregulation of VCAM-1, ICAM-1, and E-selectin were meaningfully reduced in HUVECs by pretreatment with quercetin. In addition, quercetin also inhibited the activation of AP-1 and NF-κB.

Conclusion: Results indicate that quercetin could suppress TNF-α induced apoptosis and inflammation by blocking NF-κB and AP-1 signaling pathway in HUVECs, which might be one of the underlying mechanisms in treatment of coronary heart disease.

Abbreviations: AP-1 = activator protein 1, CHD = coronary heart disease, FasL = Fas ligand, HUVECs = human umbilical vein endothelial cells, ICAM-1 = intercellular cell adhesion molecule-1, NF-κB = nuclear factor kappa B, PVDF = polyvinylidene difluoride, TNF-α = tumor necrosis factor alpha, TRAIL = TNF-related apoptosis-inducing ligand, VCAM-1 = vascular cell adhesion molecule 1.

Keywords: AP-1, apoptosis, cell adhesion molecules, HUVECs, NF-κB, quercetin

1. Introduction

Coronary heart disease (CHD), lifetime of which at age 40 years is 1 in 3 for women and 1 in 2 for men in the United States, remains the major cause of death in developed countries. The underlying mechanism of CHD is initiated by inflammatory processes in the endothelium of the vessel wall.

Inflammation plays a key role in the development of atherosclerosis. Tumor necrosis factor-alpha (TNF-α) is one of cytokines involved in systemic inflammation, which could alter leukocyte adhesion molecule expression on cultured endothelial cells such as human umbilical vein endothelial cells (HUVECs). Cellular adhesion molecule responding to pathophysiological stimuli can influence the progress of atherosclerosis by mediating the interaction between blood cells (such as leukocytes) and endothelial cells. Inflammation could be promoted by TNF-α via upregulating expression of adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin.

In addition, TNF-α could activate NF-κB, which could mediate the transcription of a vast array of proteins involved in cell survival and inflammatory response, and anti-apoptotic factors. It has been proven that inhibiting the NF-κB and AP-1 pathway could exert anti-inflammatory effects.
abundant polyphenolic compounds is quercetin, which has antiatherosclerotic effects in human and animals,\textsuperscript{14,15} and has been shown to have the ability to fight against inflammation.\textsuperscript{16,17} 

Studies have shown that quercetin could attenuate cell adhesion molecules expression, such as ICAM-1, VCAM-1, and E-selectin.\textsuperscript{18-20} It has been proven that the mechanism of leukocyte adhesion in inflammatory lesion might be upregulating the expression of ICAM-1 and VCAM-1 via activation of NF-κB and AP-1.\textsuperscript{21,22} The protective mechanism against inflammation and atherosclerosis of quercetin might be the inhibitory effect on NF-κB activation and AP-1.\textsuperscript{23,24} We hypothesize that inhibiting NF-κB and AP-1 activation might be an anti-inflammatory way in the progress of CHD.

The purpose of the present investigation was to study the association between quercetin and adhesion molecules expression. HUVECs were treated with TNF-α to mimic the inflammatory situation. Flow cytometry assay was used to measure apoptosis rates of HUVECs presence or absence of quercetin. PT-PCR and Western blot were used to detect the expression of ICAM-1, VCAM-1, and E-selectin in HUVECs presence or absence of quercetin. We also detected the expression of NF-κB and AP-1 by Annexin-V staining.

2. Materials and methods

2.1. Materials

HUVECs were obtained from Chinese Academy of Sciences (Shanghai, China). Quercetin was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); our previous study, which was not published, found that quercetin could reverse upregulation of ICAM-1, VCAM-1 and E-selectin expression induced by TNF-α, and the effects were not in a dose-dependent manner, the optimal concentration of quercetin was 30 μg/mL. In the present study, quercetin was diluted at the concentration of 30 μg/mL. Antibodies of hCAM-1, hVCAM-1, E-selectin, NF-κB, and AP-1 were obtained from R&D systems (Minneapolis, Minnesota, USA). The antibody of β-actin was provided by Santa Cruz Biotechnology (Dallas, Texas, USA). This study was not conducted directly on humans, so ethical approval and informed consent statement were not necessary.

2.2. Primers

Primers were designed to assess gene expression of adhesion molecules by RT-PCR analysis. The primer sequences for VCAM-1 were 5'-AGT GGT GGC TTC TCT AAT GG-3' (upstream) and 5'-CTG TGT CTC TGG TCT CCG CT-3' (downstream), yielding a 700-bp fragment. Primer sequences for ICAM were 5'-TAT GGC AAC GAC TCC TGC T-3' (upstream) and 5'-CAT TCA GGC TCA CCT TGG-3' (downstream), yielding a 220-bp fragment. Primer sequences for E-selectin were 5’-CAA GAA GAT GCC TTC CCT CTT-3’ (upstream) and 5’-CAT TCA GAC CAC GAG CT-3’ (downstream), yielding a 325-bp fragment.

2.3. HUVECs culture

The HUVECs were supplemented with antibiotics in a humidiﬁed 37°C incubator and 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium (DMEM; Gibco (California, USA)) with an atmosphere of 5% CO2 and 95% air. The HUVECs were seeded in 24- or 96-well plates in DMEM, and subsequently randomly divided into 3 groups: control group: HUVECs were cultured with DMEM; TNF-α group: HUVECs were cultured with TNF-α for 6 hours at the dose of 200 ng/mL; quercetin group: HUVECs were cultured with quercetin for 18 hours at the dose of 30 μg/mL and then cocultured with TNF-α for 6 hours at the dose of 200 ng/mL. Before treatments, cells were washed with PBS to remove TNF-α or quercetin.

2.4. Annexin V assay

We decanted supernatant from a ﬂask of the control group, washed ﬂask twice in warm PBS to remove serum, then detached cells in trypsin without EDTA, washed the ﬂask twice in PBS. Five hundred microliter Annexin VB Blinding Buffer was added to suspend the cells, and the cells were subsequently stained with FITC and PI. We analyzed with ﬂow cytometry to estimate the apoptosis rates. The same progress was done in the TNF-α group and the quercetin group.

2.5. RNA extractions and RT-PCR analysis

HUVECs were cultured with TNF-α presence or absence of quercetin. Total RNA from HUVECs was then extracted and analyzed by RT-PCR using a kit. Twenty microliter reaction mixture contained 4-μL dNTP Mix, 2-μL Primer Mix, 4-μL 5×RT buffer, 2-μL DTT, 1-μL HiFi-MMLV, and Rnase-Free Water. RT was performed at 42°C for 50 minutes and then inactivated at 70°C for 15 minutes. PCR reaction was performed in 50-μL containing 2×Taq MasterMix, 10-μM of Forward Primer, 10-μM of Reverse Primer, Template DNA, and Rnase-Free Water. Basic PCR consists of 3 steps: thermal denaturation of the target DNA, primer annealing of synthetic oligonucleotide primers, and extension of the annealed primers by a DNA polymerase. RT-PCR products were then resolved in 1.7% agarose gels and stained with ethidium bromide. The expression of VCAM-1, ICAM-1, E-selectin, β-actin mRNA was then detected with the electrophoretic analysis and the semi-quantitative analysis.

2.6. Western blot analysis

Proteins from HUVECs were separated according to the molecular weight of the proteins using 10% SDS–PAGE gel. The separated gel was transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 5% non-fat milk powder (blocking agent) in tris buffered saline (TBS). The membrane was then washed three times with TBS containing 0.1% Tween 20 and incubated with a protein-specific antibody at room temperature followed by a secondary antibody. Antibodies to hVCAM-1, hICAM-1, E-selectin, nuclear factor kappa-B (NF-κB), AP-1, β-actin were used as follows. The antibodies of β-actin were used as follows. The membranes were rinsed after the secondary antibody incubation, and the bound antibodies were then detected using enhanced chemiluminescence (ECL; Santa Cruz Biotechnology followed by autoradiography. We used the Image pro plus 5 software to semi-quantify protein in every lane.

2.7. Flow cytometry

The flow cytometry was used to measure the expression of AP-1 and NF-κB in the 3 groups. We collected cells and suspend them
with the PBS and centrifuge for 5 minutes at the speed of 1500 rpm after added with fixation/permeabilization solutions for 20 minutes, and then decanted the supernatant and added 2 mL Perm/Wash Butter to wash the cells. The NF-κB-PE or AP-1-FITC antibodies were then incubated with the cells for 30 minutes. We finally washed cells with 2 mL Perm/Wash Butter and collected the cells by centrifuge (2000 rpm, 5 minutes), decanted the supernatant, and added 500 µL PBS for concussion, and analyzed on FACS can flow cytometer using BD CellQuest Pro software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA).

2.8. Statistical analysis
Data are presented as mean±standard deviation. Multiple comparisons between the groups were performed by 1-way analysis of variance (ANOVA). P values of .05 or less were considered to indicate statistical significance for all subgroup and interaction analyses.

3. Results
3.1. Quercetin decreases apoptosis rates of endothelial cells
To determine how quercetin reverses TNF-α induced down-regulation of apoptosis rates, we used flow cytometry assay to measure apoptosis rates of HUVECs. Apoptosis rates of HUVECs were increased in TNF-α group compared with control group (10.005±0.903 vs 3.602±0.833%, P<.01), and were decreased with quercetin pretreatment compared with TNF-α group (7.154±0.862 vs 10.005±0.903, P<.01) in Figure 1D. In

![Image](image-url)

Figure 1. Quercetin decreased TNF-α induced upregulation of apoptosis rates. (A) Apoptosis rates in the control group were measured by flow cytometry assay; (B) Apoptosis rates in the TNF-α group were measured by flow cytometry assay; (C) Apoptosis rates in the quercetin group were measured by flow cytometry assay. (D) Quantitation of apoptosis rates in HUVECs. Results were expressed as mean±standard deviation. *P<.01 vs TNF-α group.
this study, we could see that quercetin had the protective effects on apoptosis of HUVECs induced by TNF-α.

3.2. Effects of quercetin on VCAM-1, ICAM-1, E-selectin mRNA expression

Cell adhesion molecules such as VCAM-1, ICAM-1, and E-selectin could mediate the process of leukocytes adhering to endothelial cells, which might be downregulated by quercetin. Figure 2A showed the expression of adhesion molecules detected by RT-PCR. As shown in Figure 2B, the expressions of VCAM-1/β-actin, ICAM-1/β-actin, and E-selectin/β-actin in TNF-α group compared with control group were 0.982 ± 0.047 vs 0.422 ± 0.31 (P < .01), 1.038 ± 0.024 vs 0.314 ± 0.378 (P < .01), and 1.09 ± 0.051 vs 0.75 ± 0.068 (P < .01), respectively. Compared with the TNF-α group, the expressions of VCAM-1/β-actin, ICAM-1/β-actin, and E-selectin/β-actin in the quercetin group were 0.338 ± 0.0562 vs 0.982 ± 0.047 (P < .01), 0.412 ± 0.061 vs 1.038 ± 0.024 (P < .01), and 0.812 ± 0.060 vs 1.09 ± 0.051 (P < .01), respectively. It could be speculated that pretreatment with quercetin could downregulate the upregulation of these adhesion molecules induced by TNF-α.

3.3. Effects of quercetin on hVCAM-1, hICAM-1, E-selectin expression

Quercetin could reverse the upregulation of VCAM-1, ICAM-1, and E-selectin induced by TNF-α. Figure 3 showed the expression of hVCAM-1, hICAM-1, and E-selectin detected by Western blot. As shown in Figure 3, the expression of hVCAM-1/β-actin, hICAM-1/β-actin, and E-Selectin/β-actin in the TNF-α group compared with the control group were 1.122 ± 0.048 vs 0.093 ± 0.027 (P < .01), 0.958 ± 0.031 vs 0.059 ± 0.019 (P < .01), and 1.120 ± 0.060 vs 0.106 ± 0.016 (P < .01), respectively. Compared with the TNF-α group, the expression of hVCAM-1/β-actin, hICAM-1/β-actin, and E-selectin/β-actin in the quercetin group were 0.702 ± 0.038 vs 1.122 ± 0.048 (P < .01), 0.396 ± 0.032 vs 0.958 ± 0.031 (P < .01), and 0.743 ± 0.036 vs 1.120 ± 0.060 (P < .01), respectively. It could be speculated that pretreatment with quercetin could reverse the upregulation of these adhesion molecules induced by TNF-α.

3.4. Quercetin inhibited NF-κB and AP-1 pathway activation

NF-κB and AP-1 play important roles in the progress of inflammation. To evaluate the effects, we detected the expression of NF-κB and AP-1 by Annexin-V staining. As shown in Figure 4, NF-κB/β-actin expression was increased in the TNF-α group compared with the control group (45.41 ± 7.037 vs 25.03 ± 3.472, P < .01), whereas pretreatment with quercetin decreased the expression of NF-κB/β-actin compared with TNF-α group (30.21 ± 6.30 vs 45.41 ± 7.037, P < .01). Figure 5 showed that AP-1/β-actin expression was increased in the TNF-α group compared with the control group (49.19 ± 7.51 vs 28.75 ± 3.68, P < .01), and the expression was decreased in the quercetin group compared with the TNF-α group (30.16 ± 4.02 vs 49.19 ± 7.51, P < .01). In summary, quercetin might have the ability of reversing upregulation of NF-κB and AP-1 induced by TNF-α, which might be one of the mechanisms of anti-inflammation.

4. Discussion

In this study, TNF-α was used to induce cell apoptosis and upregulate expression of adhesion molecules, which contributed to the inflammation of HUVECs. We investigated the underlying mechanism of quercetin in the development of inflammation and the progress of cell apoptosis. Caspases are responsible for cells into apoptotic bodies. Apoptosis inducers [Fas ligand (FasL), TNF-α] activate the initiator caspases such as caspase-8 and sequentially initiate the apoptotic cascade. There are other apoptosis-related
proteins such as an apoptosis-inducing ligand as a TNF-related apoptosis-inducing ligand (TRAIL), and FasL and their receptors. TNF-α has an extra effect on Fas activation, which activates Fas signaling pathway while excited. The results revealed that apoptosis rate was increased by TNF-α, and was reduced by pretreatment with quercetin. With our present study, quercetin was found to be capable of protecting HUVECs against apoptosis.

Figure 3. Effects of quercetin on the expression of hVCAM-1, hICAM-1, E-selectin. 25 μg HUVECs lysates in control, TNF-α, and quercetin groups were loaded onto an SDS-polyacrylamide gel, and hVCAM-1 (A), hICAM-1 (B), and E-selectin (C) were detected by Western blotting. Equal loading of protein was loaded with antibodies against β-actin onto the three groups. Expressions of hVCAM-1, hICAM-1, and E-selectin were detected by Western blotting. Quantitation of hVCAM-1, hICAM-1, and E-selectin expression of HUVECs were detected by Annexin-V. Results were expressed as mean ± standard deviation. *P < .01 vs TNF-α group.

Figure 4. Quercetin inhibited NF-κB pathway activation. (A–C) Expressions of NF-κB were detected by Annexin-V staining in control group, TNF-α group, and quercetin group, respectively. (D) NF-κB from HUVECs in control, TNF-α, and quercetin groups were detected by Western blot. Equal loading of proteins was confirmed by reprobing blots with antibodies against β-actin. (E) Quantitation of the expression of NF-κB was measured by Annexin-V. *P < .01 vs TNF-α group. Results were expressed as mean ± standard deviation.
In addition, as a cell signaling protein involved in systemic inflammation, TNF-α could promote expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) in HUVECs. Inflammation plays a critical role in the progress of atherosclerosis. The molecules associated with cell adhesion molecules are proteins located on the cell surface helps cells stick to each other and to their surroundings. When stimulated by TNF-α, leukocytes adhere to endothelium via ICAM-1, an endothelial-and leukocyte-associated transmembrane protein, and then transmigrate to the inside tissue.\(^{[27]}\) E-selectin and VCAM-1 could also be found increased via induction by TNF-α, and helped leukocyte roll on microvascular endothelium.\(^{[28]}\) Studies showed a different train of thoughts in adhesion mechanisms of atherosclerosis, such as activation of NF-κB and IFN regulatory factor-1, and the downregulation of CCL8 and CXCL10 chemokines.\(^{[29–31]}\) Expression of ICAM-1, VCAM-1, and E-selectin could be upregulated via modulated by AP-1 and NF-κB.\(^{[21,32]}\)

NF-κB, a protein complex that controls transcription of DNA, could combine with the target site of κB and promote transcription. When impelled by oxygen stress, NF-κB regulates the expression of proinflammatory cytokines, transcription factors, and cell adhesion molecules, which play a key role in the development of microcirculatory disturbance.\(^{[33]}\) Activation of NF-κB induced by TNF-α could upregulate the expression of ICAM-1, VCAM-1, and E-selectin, which could integrate with leukocytes, helping leukocytes stick to the vascular wall.

Special attention has been paid to the study on AP-1, another transcription factor, as a key factor in the development of cardiovascular disease. It is a heterodimeric protein composed of proteins belonging to the ATF and JDP, c-Jun, c-Fos families, which could modulate the expression of gene in response to stimuli, such as TNF-α. One of the mechanisms is the MAPK signaling pathway, after being phosphorylated, the c-Fos and c-jun proteins will be activated, following the activation of AP-1.

Quercetin, a major bioflavonoid widely present in fruits and vegetables, has been found to fight against inflammation via suppressing I-κB-phosphorylation, NF-κB translocation, AP-1, and reporter gene transcription.\(^{[24,34]}\) Studies has shown that quercetin could exert its biological action in other fields via modulating JNK, AP-1, NF-κB signaling pathway.\(^{[35–37]}\) TNF-α is a key proinflammatory cytokine that acts to provoke inflammation, which induced by TNF-α was reduced with treatment of quercetin.\(^{[38,39]}\)

Our results showed that the expression of NF-κB and AP-1 were significantly increased by the treatment of TNF-α in the HUVECs compared with the control group. Simultaneously, ICAM-1, VCAM-1, and E-selectin expression were also increased, and both the translation factors and cell adhesion molecules were downregulated after the pretreatment with TNF-α.
quercetin. Hence, the results of our research indicate that quercetin might reverse the upregulation of ICAM-1, VCAM-1, and E-selectin via inhibiting the activation of NF-kB and AP-1. These mechanisms could all relieve the progress of atherosclerosis. The deeper mechanisms of quercetin on atherosclerosis should be further studied.

Author contributions

Designed the study: Tielong Chen. Performed the study: Xudong Zhang, Guangli Zhu, Hongfei Liu, and Jinru Chen. Analyzed the data: Yu Wang and Xiaolong He. Wrote the paper: Xudong Zhang. Revised the article: Tielong Chen.

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