Original Research Article

Quercetin attenuates lipopolysaccharide-induced myocardial cell apoptosis via modulation of cAMP-Epac pathway

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Abstract

Purpose: To investigate the effects and mechanism of action of quercetin (QUE) on sepsis-induced apoptosis of myocardial cells in vitro.
Methods: Lipopolysaccharide (LPS) was used to induce apoptosis H9c2 myocardial cells. Apoptosis of H9c2 cells was determined by propidium iodide staining. Knock down of Epac1 was achieved using small interfering RNA (SiEpac1). The levels of associated proteins (Epac1 and Rap1) were evaluated by western blotting.
Results: Lipopolysaccharide promoted apoptosis of H9c2 cells and inhibited the activity of cAMP-Epac pathway ($p < 0.001$ vs. control). Quercetin inhibited caspase 3 activity and apoptosis ($p < 0.05$ vs. LPS) induced by LPS via activation of cAMP-Epac1 signaling pathway. Moreover, Epac1 knockdown decreased the anti-apoptosis effect of Que, which indicates that Que attenuated apoptosis partly via cAMP-Epac pathway.
Conclusion: Que attenuated LPS-induced apoptosis in myocardial cells via activation of cAMP-Epac1 pathway. Therefore, quercetin treatment may serve as a promising strategy in the treatment of sepsis-induced myocardial injury.

Keywords: Quercetin, cAMP-Epac pathway, Apoptosis, Sepsis, Myocardial injury

INTRODUCTION

Sepsis, a leading cause of death worldwide, is a systemic dysregulated host response caused by infection and may lead to multiple organ dysfunction syndrome (MODS) [1]. Organ dysfunction is considered an important characteristic of severe sepsis, and recent research has shown the importance of using myocardial depression and cardiac dysfunction as criterion for diagnosis of severe sepsis [2]. About 50% of patients with sepsis have myocardial injury [3]. Myocardial cell apoptosis, which is associated with sepsis-induced myocardial injury, is believed to be an important mechanism of myocardial dysfunction [4].

Quercetin (Que, 3,5,7,3′,4′-pentahydroxyflavone), a potent plant-derived flavonoid, exerts various physiological effects including anti-oxidation,

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anti-inflammation, and anti-proliferation [5,6]. Studies have demonstrated the protective effects of Que on multiple organs in inflammatory disorders, such as myocardial diseases[7]. Moreover, intake of Que has been correlated with reduced incidence of cardiovascular diseases [8].

Myocardial cell death is one of the main causes of heart failure induced by sepsis. Previous research demonstrated that Que pretreatment protect myocardial cells against ischemia-reperfusion injury via anti-oxidation, anti-inflammation, and anti-apoptotic mechanisms *in vivo* through the PI3K/Akt pathway[9].Although the protective effects of Que in myocardial cells were previously reported, the mechanism by which Que protects cells from sepsis-induced myocardial cell apoptosis remains poorly understood. This study investigated the effects of Que on lipopolysaccharide (LPS)-induced apoptosis of myocardial cells, and the associated mechanism.

EXPERIMENTAL

Chemicals

Primary antibodies against caspase 3 (no. #9662), Epac1 (no. #4155), Rap1 (no. #2326), and tubulin (no. #2128) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Lipopolysaccharide (no. L2880) was purchased from Sigma-Aldrich (St Louis, MO, USA). Quercetin (no.100081, purity exceed 98 %) was purchased from National Institutes for Food and Drug Control (Beijing, China).

Cell culture

The H9c2 cells were purchased from the ATCC (Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 1 mM HEPES buffer, according to manufacturer's instructions. The cells were incubated in FBS-free basal medium overnight before treatment.

Apoptosis assay

Apoptosis of H9c2 cells was analyzed by propidium iodide (PI) staining. Cells were treated with LPS plus Que or 8-pCPT-2′-O-Me-cAMP (8-pCPT, Epac1 agonist) for 24 h. After another 24 h, the cells were collected and fixed in cold ethanol (70%), stained with PI for 30 min in the dark, and PI-positive cells (apoptotic cells) were identified using a fluorescence microscope (excitation at 535 nm, and emission at 615 nm).

Determination of caspase-3 activity

The activity of caspase-3 was measured using a EnzChek Caspase-3 Assay Kit (Thermo Fisher, Scotts Valley, CA, USA). About 1×10^6 cells for each group were collected and lysed with lysis buffer. After centrifugation, the reaction buffer and substrate solution were added to each sample sequentially. The activity of caspase-3 was determined using a fluorescence microplate reader (excitation/emission 342/441 nm).

Epac1 knockdown by siRNA transfection

Small interfering RNA against Epac1 (siEpac1) was synthesized by Invitrogen (Carlsbad, CA, USA). H9c2 cells were transfected with siEpac1 or negative control using Lipofectamine 2000 (Invitrogen).

Western blotting

The cells of different groups were collected and lysed in RIPA buffer with protease and phosphatase inhibitor cocktails. Protein concentrations were measured by Bradford method (Thermo Scientific, Rockford, IL, USA). Proteins were separated and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with the respective primary antibodies (at a dilution of 1:1000), and subsequently with secondary antibodies (1:10,000). The proteins were detected using an enhanced chemiluminescence system.

Statistical analysis

Data were analyzed by one-way ANOVA using Prism 6 (GraphPad Software CA, USA), and are presented as mean ±standard deviation (SD).

RESULTS

LPS promoted apoptosis in H9c2 cells and inhibited activation of cAMP-Epac

As shown in Figure 1 A, the number of apoptotic cells (PI$^+$) was increased in the LPS group (about seven-fold more than control; $p < 0.001$), and the 8-pCPT Epac1 agonist inhibited LPS-induced apoptosis. The activity of caspase-3 (Figure 1 B) was increased about 12-fold when compared with control ($p < 0.001$) but was inhibited by 8-pCPT ($p < 0.01$ vs. LPS group). The expressions of cAMP-Epac signaling proteins (Epac1 and Rap1) were decreased in the LPS group, and 8-pCPT reversed the effects of LPS.
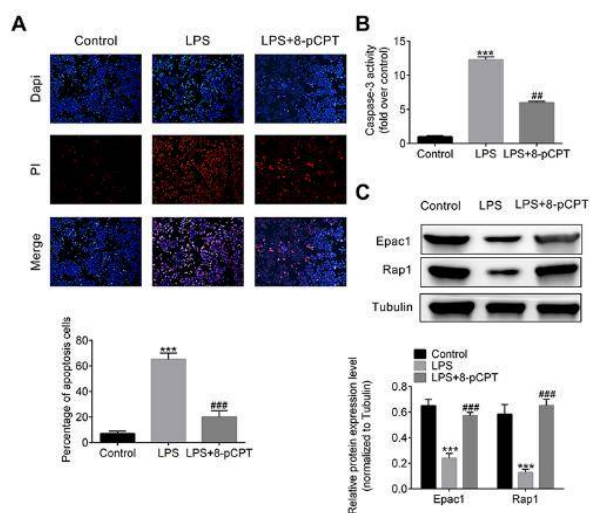
Figure 1: Apoptosis and associated protein expressions of LPS-treated H9c2 cells. (A) Apoptosis analysis by PI staining of H9c2 cells. (B) Activity of caspase-3 in the LPS-treated H9c2 group with or without 8-pCPT treatment. (C) expressions of Epac1 and Rap1 in theH9c2 group measured by western blotting; $^{***}p < 0.001$ vs. control, $^{##}p < 0.01$, $^{###}p < 0.001$ vs.control group

Que inhibited LPS-triggered H9c2 cell apoptosis

To investigate the effects of Que on H9c2 cell apoptosis, the cells were treated with 10, 30, or 60μM Que after LPS treatment. The results are shown in Figure 2. Propidium Iodide staining indicated that Que inhibited LPS-triggered H9c2 cell apoptosis, and the number of apoptotic cells in the 30 μM Que group decreased the most ($p < 0.01$). The activity of caspase-3 decreased by about 50 % in the Que group (30 μM) when compared with the LPS group ($p < 0.01$), which was in agreement with the apoptosis results.

Que promotes activation of cAMP-Epac1 signaling pathway

To investigate the mechanism of Que on H9c2 cell apoptosis induced by LPS, the concentration of cAMP, and expressions of Epac1 and Rap1 were evaluated and the results are shown in Figure 3. The levels of cAMP, Epac1 and Rap1 were decreased by over 50 % ($p < 0.001$ for cAMP, and $p < 0.01$ for Epac1 and Rap1) when compared with the control group. Quercetin treatment increased the levels of cAMP, Epac1and Rap1. Furthermore, 8-CPT markedly increased the expression of Epac1, and Rap1 in LPS-stimulated H9c2 cells, which was similar to the effects of Que treatment. Together, these results demonstrated that Que promoted the activation of cAMP-Epac1 signaling.
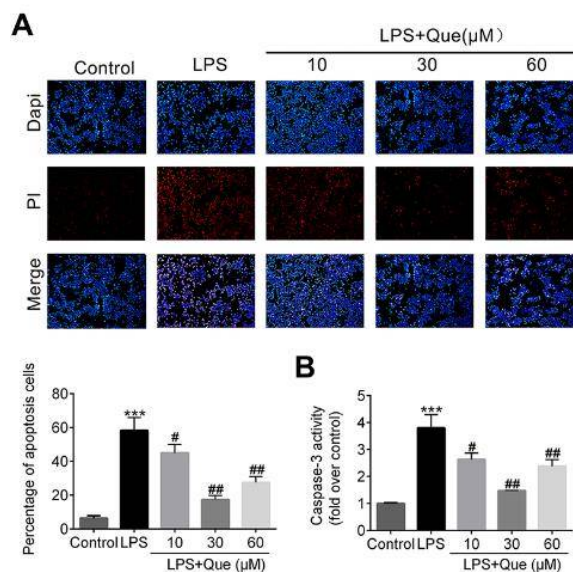

Figure 2: Effect of Que on LPS-induced H9c2 cell apoptosis. (A) Apoptosis of H9c2 cells was evaluated by Propidium Iodide staining. (B) Effect of QUE on caspase-3; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with controls
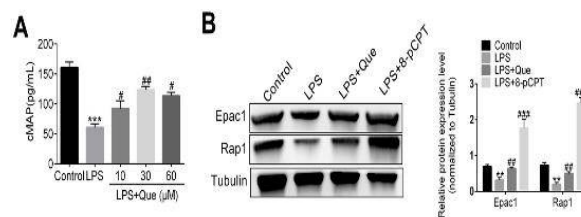

Figure 3: cAMP level (A) and expression levels of Epac1 and Rap1 (B). Relative protein expressions are shown in the right panel; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control, $^{##}p < 0.01$ and $^{###}p < 0.001$ vs. control group

QUE inhibited LPS-induced apoptosis of H9c2 cells via Epac1 pathway

To further investigate the role of Epac1 in the inhibitory effects of Que on apoptosis, siRNA was used to knockout the Epac1 proteins, and the cells were then treated with LPS with or without the addition of Que. As shown in Figure 4 A, the number of apoptotic cells was increased in the LPS group and the effects were inhibited by Que. However, the inhibitory effects of Que on apoptosis were reversed by Epac1 knockout. The activity of caspase-3 is shown in Figure 4 B. Quercetin treatment decreased the LPS-induced activity of caspase-3, and Epac1 knockout partly inhibited the effects of Que. The levels of Epac1 and Rap 1 are shown in Figure 4 C. Lipopolysaccharide-induced increased expressions of Epac1 and Rap 1, and Que treatment enhanced the effects of LPS. However, the effect of Que was inhibited in Epac1 knockout cells. Therefore, Que inhibited the LPS-

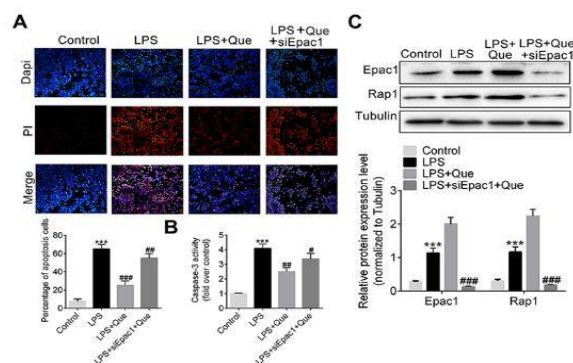induced apoptosis of H9c2 cells partly through the Epac1 pathway.

Figure 4: Apoptosis and associated protein expressions. (A) Apoptosis analysis by PI staining of H9c2 cells. (B) Caspase-3 activity in H9c2 cells (transfected with siEpac1) treated with LPS with or without Que. (C). Expression of Epac1 and Rap1 in H9c2 cells, as measured by western blotting. Quantitative scans are shown in the bottom right panel; [***]$p < 0.001$ vs. control, [##]$p < 0.01$ and [###]$p < 0.001$ vs. control group

DISCUSSION

Myocardial injury is a common complication of sepsis and septic shock, with an incidence rate of up to 40 %. Myocardial injury is also an important cause of poor prognosis of sepsis [4]. Many factors lead to myocardial injury in sepsis, including cytokines, oxidative stress, calcium overload, and excessive activation of the renin angiotensin system (RAS) [10]. Recent studies indicated that apoptotic pathways are activated by LPS, which plays a vital role in the myocardial injury caused by sepsis [11]. In the present study, LPS was used to induce apoptosis of H9C2 cells to model sepsis-induced myocardial injury. The number of apoptotic cells and the level of pro-apoptotic protein caspase-3wereincreased after LPS treatment.

As a common second messenger, cAMP, which regulates many cellular processes, is a key signaling molecule in the cardiovascular system and the myocardial injury response pathway [12].Cyclic adenosine monophosphate is involved in a variety of cellular processes mostly through activation of protein kinase A (Epac) [13]. Epac isoforms, Epac1 and Epac2, are considered to be the important downstream components of the cAMP pathway due to their ability in activating small GTPase Rap [14]. Elevated intracellular cAMP has been shown to produce protective effects, both in clinical observations and animal experiments, and in various pathological situations such as during ischemia/reperfusion injury [15]. Xin Wang *et al*

demonstrated that the Epac1-Rap1 pathway is involved in the pathogenesis of myocardial ischemia/reperfusion injury *in vitro* and *in vivo* [16].The present study is in agreement with previous reports that showed that the levels of cAMP, Epac1, and Rap1 were all decreased after LPS treatment. The cAMP analogs, including 8-pCPT, are selective activators of Epac [13,17]. In this study, the Que-triggered effects of apoptosis and cAMP-Epac1 signaling were similar to 8-pCPT treatment.

Moreover, the effects of Que on sepsis *in vivo* have been reported. Que ameliorates systemic inflammation due to LPS-induced sepsis of mice via promoting the expression of IL-10 (an anti-inflammatory cytokine) [18]. Quercetin alleviated sepsis-induced cardiac insufficiency in mice and improved survival rates via regulating nitrous oxide production and the apoptosis pathway [19].Quercetin also exerts protective effects on other organ dysfunctions induced by sepsis. Chang *et al* found that Que protect mice from LPS-induced sepsis via the NF-κB signaling pathway, and reduced the inflammatory response of tissues [20]. *In vivo* experiments are needed to further confirm the effects of Que on sepsis-induced myocardial injury.

CONCLUSION

Quercetin attenuates LPS-induced apoptosis of myocardial cells via activation of cAMP-Epac1 pathway, which highlights the protective effects of Que against myocardial injury induced by sepsis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. YuHui Wang designed all the experiments and revised the paper. Qian Fu performed the experiments. While Baning Ye and Yanpei Liu wrote the manuscript.

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