Tropical Journal of Pharmaceutical Research August 2022; 21 (8): 1601-1606
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i8.4

Original Research Article

Tectorigenin ameliorates myocardial cell injury caused by hypoxia/reoxygenation by inhibiting autophagy via activation of PI3K/AKT/mTOR pathway

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Sent for review: 9 May 2022 *Revised accepted: 16 July 2022*

Abstract

Purpose: *To investigate the protective role of tectorigenin in myocardial ischaemia/reperfusion.*
Methods: *Myocardial cells (H9c2) were treated with different concentrations of tectorigenin and exposed to hypoxia/reoxygenation. Cell viability and apoptosis were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining, respectively. Oxidative stress and inflammation were evaluated using enzyme-linked immunosorbent assay (ELISA), while autophagy and the underlying mechanisms of action were evaluated by Western blot.*
Results: *Tectorigenin enhanced the proliferative activity of H9c2 under hypoxia/reoxygenation conditions, and significantly reduced the apoptotic activity ($p < 0.001$) through decrease in Bax and increase in Bcl-2. Tectorigenin also significantly up-regulated SOD (superoxide dismutase) and GSH (glutathione) levels ($p < 0.01$), and down-regulated MDA (malondialdehyde) and MPO (myeloperoxidase) in hypoxia/reoxygenation-induced H9c2. TNF-α (tumor necrosis factor-α), IL(interleukin)-1β, and IL-6 levels were also inhibited by tectorigenin by down-regulating p-p65. Hypoxia/reoxygenation-induced increase in p62 and decrease in Beclin-1 and LC3-II/LC3-I were reversed by tectorigenin. Protein expressions of p-mTOR, p-AKT, and p-PI3K in hypoxia/reoxygenation-induced H9c2 were elevated by tectorigenin.*
Conclusion: *Tectorigenin exerts anti-oxidant, anti-inflammatory, and anti-autophagic effects on hypoxia/reoxygenation-induced H9c2 through the activation of PI3K/AKT/mTOR pathway, thus suggesting that it is a potential agent for the management of myocardial ischaemia/reperfusion.*

Keywords: *Tectorigenin, Oxidative stress, Inflammation, Autophagy, Hypoxia/reoxygenation, Myocardial cells, PI3K/AKT/mTOR*

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INTRODUCTION

Myocardial infarction, which is caused by blood flow interruption and occlusion of coronary artery, is characterized by necrosis, hypoxia and ischemia of myocardial cells [1]. It is also a leading cause of morbidity and mortality in coronary heart diseases [2]. Reperfusion is an efficient therapy for the treatment of myocardial infarction, while ischemia/reperfusion injury

aggravates myocardial ischaemia and results in massive myocardial cell death [3]. Myocardial cell apoptosis, excessive accumulation of free radicals or reactive oxygen species, and myocardial inflammatory pathways are implicated in the pathogenesis of myocardial ischemia/reperfusion injury [3]. Therefore, anti-oxidant and anti-inflammatory strategies are considered to be effective therapies for the disease [4].

Ischemia/reperfusion injury induces inflammatory responses and excessive oxidative stress in myocardial cells, and leads to an accumulation of damaged proteins and organelles [5]. Autophagy, the physiological process that removes damaged cytoplasmic organelles and protein aggregates, is involved in the pathogenesis of myocardial ischemia/reperfusion injury [5]. The activation of autophagy exerts a detrimental effect on myocardial ischemia/reperfusion injury through the promotion of cell apoptosis [6]. Autophagy is also regarded as a potential target in the treatment of myocardial ischemia/reperfusion injury [5].

Tectorigenin is a bioactive flavonoid in the traditional Chinese herb, *Belamcanda chinensis*, and exhibits anti-inflammatory and anti-oxidant activities in a variety of diseases [7, 8]. For example, tectorigenin reduced oxidative stress in ultraviolet B-induced keratinocytes and suppressed apoptosis and collagen degradation to attenuate skin damage [9]. Tectorigenin also represses lipopolysaccharide-induced inflammation to ameliorate acute lung injury [7]. Moreover, tectorigenin mediates autophagy, thereby attenuating experimental fulminant hepatic failure [10].

Oxygen-glucose deprivation/reperfusion-induced apoptosis and inflammation in HT22 cells are suppressed by tectorigenin, and tectorigenin ameliorates cognitive impairments and chronic cerebral ischemia [11]. Therefore, tectorigenin might also exert a protective effect against myocardial ischemia/reperfusion injury. The effects of tectorigenin on oxidative stress, inflammation and autophagy in hypoxia/reoxygenation-induced H9c2 were investigated in this study.

EXPERIMENTAL

Cell culture and treatment

The H9c2 was cultured in Dulbecco's Modified Eagle's Medium containing 10 % fetal bovine serum (Gibco, Gaithersburg, MD, USA). To induce ischemia/reperfusion injury, H9c2 cells were cultured in serum-free medium under hypoxic condition (5 % CO_2 and 95 % N_2) for 3 h. Cells were then reoxygenated under condition (5 % CO_2 and 95 % O_2) for another 3 h. To investigate the cytotoxicity of tectorigenin, H9c2 was treated with 1, 10, 25, 50 or 100 μM tectorigenin (Feiyu Biotechnology Corporation, Jiangsu, China) for 3 h. The H9c2 cells were also treated with 10, 50 or 100 μM tectorigenin, and then subjected to hypoxia/reoxygenation condition to assess the effect of tectorigenin on ischemia/reperfusion injury.

Cell viability and apoptosis assays

The H9c2 ($1X10^6$) was seeded into 96-well culture plates for 24 h and then subjected to different treatments. Cells were incubated with 5 mg/mL MTT solution (20 μL; Sigma-Aldrich, St. Louis, MI; USA) for 4 h. The cells were then treated with DMSO, and absorbance at 490 nm was determined in a Microplate Autoreader (Thermo Fisher, Waltham, MA, USA). To determine cell apoptosis, H9c2 cells were fixed in 4 % paraformaldehyde and permeabilized with 0.3 % Triton-X 100, and then stained with TUNEL solution of One Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology, Beijing, China). The nucleus was stained with DAPI, and the cells were examined under a fluorescence microscope (Nikon, Tokyo, Japan).

ELISA and western blot

H9c2 cells were lysed in RIPA buffer (Beyotime), and the lysates were harvested post-centrifugation at 12000 g for 1 h. SOD, MDA, GSH and MPO levels were assessed by ELISA assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The culture medium of H9c2 was collected, and ELISA kits were used to determine the TNF-α, IL-1β, and IL-6 levels. For western blot, cell lysates were separated using SDS-PAGE, and then transferred onto nitrocellulose membranes. Membranes were blocked in 5 % dry milk at 37 ℃ for 1 h, and incubated with primary antibodies: anti-Bcl-2 and anti-Bax (1:2000), anti-INOS and anti-COX-2 (1:2500), anti-p-p65 and anti-p65 (1:3000), anti-LC3 and anti-p62 (1:3500), anti-Beclin1 and anti-β-actin (1:4000), anti-p-mTOR and anti-mTOR (1:4500), anti-p-AKT and anti-AKT (1:5000), anti-p-PI3K and anti-PI3K (1:5500). The membranes were then incubated with secondary antibodies (1:5000), and subjected to chemiluminescence reagent kit (Beyotime) to determine the intensity of each band using ImageJ software v.1.52a. All the proteins were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

The data were expressed as mean ± SEM and analyzed by Student's t-test or one-way analysis of variance (ANOVA) using SPSS 11.5 software (IBM, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Tectorigenin alleviated hypoxia/reoxygenation-induced cell apoptosis in H9c2 cells

To investigate the cytotoxicity of tectorigenin, H9c2 cells with the viability of H9c2 was not decreased after the incubation of tectorigenin (Figure 1 A). The H9c2 cells were pretreated with tectorigenin and then subjected to hypoxia/reoxygenation condition. Hypoxia/reoxygenation induced a decrease in the cell viability of H9c2 (Figure 1 B). However, tectorigenin enhanced cell viability in hypoxia/reoxygenation-treated H9c2 in a dose-dependent way (Figure 1 B). Moreover, tectorigenin reduced the protein expression of Bax and enhanced Bcl-2 in hypoxia/reoxygenation-treated H9c2 (Figure 1 C) in order to suppress cell apoptosis (Figure 1 D), suggesting an anti-apoptotic effect of tectorigenin on hypoxia/reoxygenation-treated H9c2 cells.

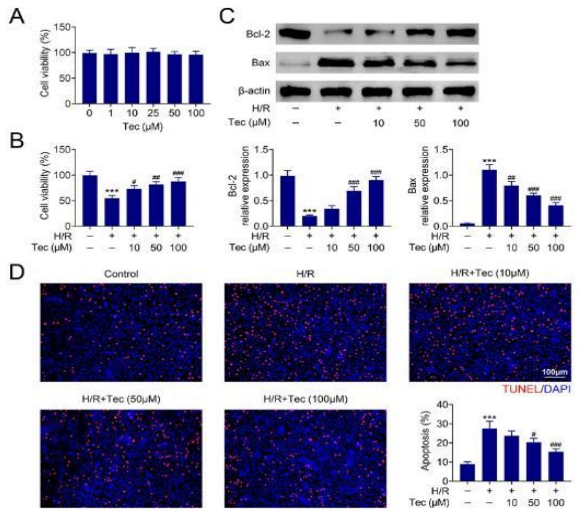

Figure 1: Tectorigenin relieved hypoxia/reoxygenation-induced cell apoptosis in H9c2. (A): Incubation with tectorigenin did not affect the cell viability of H9c2. (B): Tectorigenin enhanced the cell viability of hypoxia/reoxygenation-treated H9c2 in a dose dependent way. (C): Tectorigenin reduced the protein expression of Bax and enhanced Bcl-2 protein in hypoxia/reoxygenation-treated H9c2. (D): Tectorigenin suppressed cell apoptosis of hypoxia/reoxygenation-induced H9c2. # vs. H/R$P < 0.05$, ##$p < 0.01$, ###, ***$p < 0.001$

Tectorigenin lowered hypoxia/reoxygenation-induced oxidative stress in H9c2 cells

Hypoxia/reoxygenation condition induced increase in MDA and MPO, and down-regulated SOD and GSH in H9c2 (Figure 2 A). However, tectorigenin reduced MDA and MPO levels, and increased SOD and GSH in hypoxia/reoxygenation-treated H9c2 (Figure 2 A). Moreover, tectorigenin attenuated hypoxia/reoxygenation-induced increases in INOS and COX-2 in H9c2 (Figure 2 B), indicating that tectorigenin exerted anti-oxidant effect on hypoxia/reoxygenation-treated H9c2 cells.

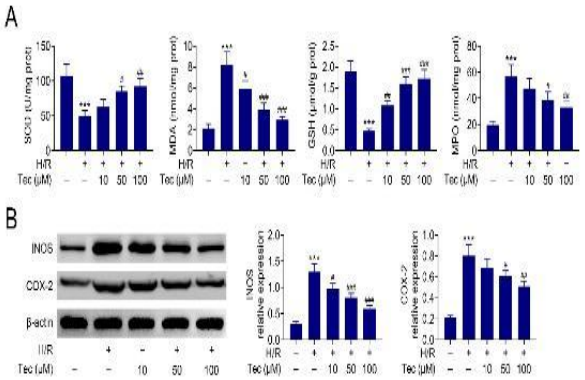

Figure 2: Tectorigenin relieved hypoxia/reoxygenation-induced oxidative stress in H9c2. (A): Tectorigenin reduced MDA and MPO, but increased SOD and GSH in hypoxia/reoxygenation-induced H9c2. (B): Tectorigenin attenuated hypoxia/reoxygenation-induced increases in INOS and COX-2 in H9c2. # vs. H/R$P < 0.05$, ##$p < 0.01$, ###, ***$p < 0.001$

Tectorigenin ameliorated hypoxia/reoxygenation-induced inflammation in H9c2 cells

Tectorigenin reduced the TNF-α, IL-1β, and IL-6 levels in hypoxia/reoxygenation-treated H9c2 (Figure 3 A). the protein expression of p-p65 in hypoxia/reoxygenation-treated H9c2 was also decreased by tectorigenin (Figure 3 B) in a dose dependent way, revealing an anti-inflammatory effect of tectorigenin on hypoxia/reoxygenation-induced H9c2.

Tectorigenin decreased hypoxia/reoxygenation-induced autophagy in H9c2 cells

Hypoxia/reoxygenation repressed the protein expression of p62, but increased LC3-II/LC3-I and Beclin1 in H9c2 (Figure 4). However, tectorigenin increased p62, decreased LC3-II/LC3-I ratio and Beclin1 level in hypoxia/reoxygenation-induced H9c2 (Figure 4).

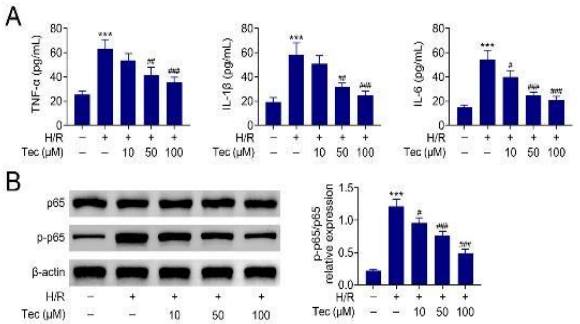

Figure 3: Tectorigenin relieved hypoxia/ reoxygenation-induced inflammation in H9c2. (A): Tectorigenin reduced the TNF-α, IL-1β, and IL-6 levels in hypoxia/reoxygenation-induced H9c2. (B): Tectorigenin reduced the protein expression of p-p65 in hypoxia/reoxygenation-treated H9c2. # vs. H/RP < 0.05, ##p < 0.01, ###, ***p < 0.001

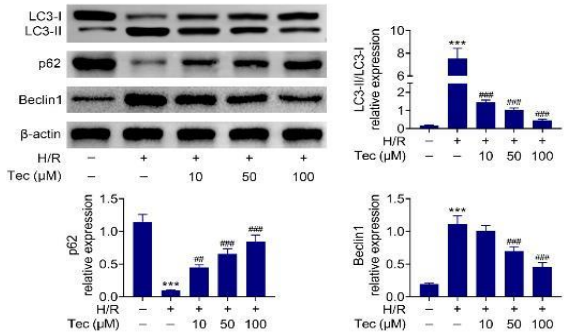

Figure 4: Tectorigenin relieved hypoxia/reoxygenation-induced autophagy in H9c2. Tectorigenin increased p62, decreased the LC3-II/LC3-I ratio and Beclin1 in hypoxia/reoxygenation-treated H9c2. ##p < 0.01, ###, ***p < 0.001, vs. H/R

Tectorigenin enhanced the activation of PI3K/AKT/mTOR in hypoxia/reoxygenation-induced H9c2

Hypoxia/reoxygenation did not affect the protein expressions of PI3K, AKT and mTOR in H9c2 (Figure 5), but decreased p-mTOR, p-AKT and p-PI3K (Figure 5). Moreover, tectorigenin enhanced p-mTOR, p-AKT and p-PI3K expressions in hypoxia/reoxygenation-induced H9c2 cells (Figure 5), leading to the activation of the PI3K/AKT/mTOR signaling.

DISCUSSION

Traditional Chinese medicine has been widely used in the treatment of myocardial ischaemia/reperfusion injury with safety and efficacy [12]. The bioactive flavonoids of Chinese medicine also exerted a cardioprotective effect against myocardial ischaemia/reperfusion injury [13].

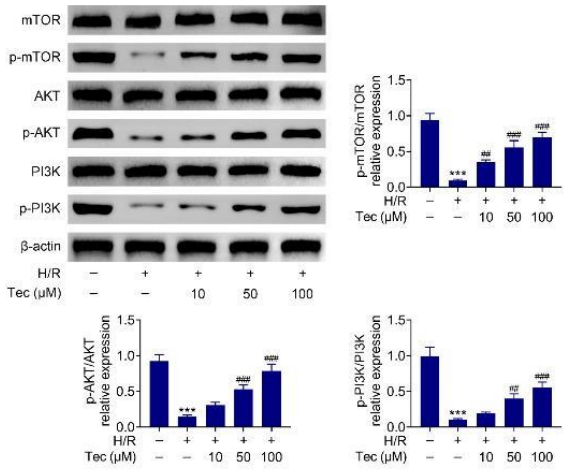

Figure 5: Tectorigenin enhanced the activation of PI3K/AKT/mTOR in hypoxia/reoxygenation-treated H9c2. Tectorigenin enhanced the p-mTOR, p-AKT and p-PI3K expressions in hypoxia/reoxygenation-induced H9c2. ##P < 0.01, ###, *** p < 0.001 vs. H/R

This study found that tectorigenin, a flavonoid from *Belamcanda chinensis*, protected against myocardial ischaemia/reperfusion injury through the inhibition of oxidative stress, inflammation and autophagy.

Reperfusion therapy induced a massive apoptosis of myocardial cells by stimulating ischemia/reperfusion injury [3]. Inhibition of myocardial cell apoptosis appears to be the mechanism operating in anti-myocardial ischemia/reperfusion injury [14]. Tectorigenin has been shown to repress oxygen-glucose deprivation/reperfusion-induced apoptosis in HT22 cells, thereby ameliorating chronic cerebral ischemia [11]. The results of this study also showed that tectorigenin enhanced cell viability and reduced the apoptosis of hypoxia/reoxygenation-induced H9c2 cells, leading to improvement in myocardial cell apoptosis.

Reperfusion therapy with blood flow restoration to an ischemic heart leads to the accumulation of reactive oxygen species and INOS expression, thus promoting oxidative and nitrosative stress, and generating ischaemia/reperfusion injury [15]. Excessive oxidative stress up-regulated COX-2, thus stimulating ischemia/reperfusion injury [16]. Antioxidants exhibited a cardioprotective effect through the restoration of antioxidant defense system [15]. Tectorigenin reduced the expressions of INOS and COX-2, thus suppressing lipopolysaccharide-induced inflammatory responses [17]. The results of this study demonstrated that tectorigenin attenuated hypoxia/reoxygenation-induced decrease in SOD and GSH levels and increase in MDA and MPO

levels in H9c2 cells, but relieved myocardial cell oxidative stress via the down-regulation of INOS and COX-2.

Excessive accumulation of reactive oxygen species induced the release of pro-inflammatory factors, and contributed to the inflammatory injury in myocardial ischaemia/reperfusion [18]. The NF-κB signaling, which is essential for the secretion of pro-inflammatory factors, was activated, and promoted oxidative stress-induced necrosis in myocardial ischaemia/reperfusion injury [19]. Tectorigenin blocked the activation of NF-κB signaling in lipopolysaccharide-stimulated macrophages [17]. This study indicates that tectorigenin reduced TNF-α, IL-1β, and IL-6 in hypoxia/reoxygenation-stimulated H9c2 through the down-regulation of p65 which ameliorated myocardial inflammation.

Ischaemia/reperfusion induced excessive autophagy and promoted autophagic cardiomyocyte death, and the suppression of autophagy with the knockdown of Beclin1 reduced myocardial cell death in ischaemia/reperfusion injury [20]. The activation of mTOR was associated with autophagy in myocardial ischaemia/reperfusion injury [6], and the activation of PI3K/AKT/mTOR inhibited autophagy and reduced hypoxia/reoxygenation injury [21].

Tectorigenin has been reported to promote the activation of PI3K/AKT signaling and ameliorate oxygen-glucose deprivation/reperfusion-induced oxidative stress and inflammation in HT22 cells [22]. Moreover, tectorigenin enhanced p62 expression, thereby regulating autophagy and protecting against experimental fulminant hepatic failure [10]. Furthermore, tectorigenin up-regulated the expression of p62, down-regulated LC3-II/LC3-I and Beclin1 in hypoxia/reoxygenation-induced H9c2. p-mTOR, p-AKT, and p-PI3K in hypoxia/reoxygenation-induced H9c2 cells were up-regulated by tectorigenin, thus contributing to the amelioration of ischaemia/reperfusion injury.

CONCLUSION

Tectorigenin exerts anti-oxidant, anti-inflammatory and anti-autophagic effects on hypoxia/reoxygenation-stimulated H9c2 cells. Therefore, tectorigenin might be a potential agent for the treatment of ischaemia/reperfusion injury. However, the effect of tectorigenin in ischaemia/reperfusion injury should be also be investigated *in vivo* to ascertain its potential for application in clinical practice.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest 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. All authors contributed to the study conception and design. Material preparation and the experiments were performed by Jijun Wu and Yingli Zhou; Data collection and analysis were performed by Ming Guo and Jie Yang. The first draft of the manuscript was written by Feifei Wu and Jialin Wang, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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