Original Paper

Troloxerutin Protects Against Myocardial Ischemia/Reperfusion Injury Via PI3K/Akt Pathway in Rats

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Key Words

Myocardial ischemia/reperfusion injury • Troloxerutin • PI3K/Akt pathway

Abstract

Background/Aims: Troloxerutin, also known as vitamin P4, has been commonly used in the treatment of chronic venous insufficiency (CVI) disease. However, its effect on *in vivo* myocardial ischemia/reperfusion (I/R) injury, a model that closely mimics acute myocardial infarction in humans, is still unknown. **Methods:** The myocardial I/R injury rat model was created with troloxerutin preconditioning. Myocardial infarct size was evaluated by the Evans blue-TTC method. Hemodynamic parameters, including the heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximal rate of rise in blood pressure in the ventricular chamber (+dp/dt max), and maximal rate of decline in blood pressure in the ventricular chamber (-dp/dt max) were monitored. Serum TNF- α and IL-10 were determined by ELISA kit. Cell apoptosis was detected by MTT method. **Results:** Troloxerutin preconditioning significantly reduced myocardial infarct size, improved cardiac function, and decreased the levels of creatine kinase (CK), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in the I/R injury rat model. The serum and mRNA levels of TNF- α and IL-10 as well as some apoptosis markers (Bax, Caspase 3) also decreased. Moreover, troloxerutin pretreatment markedly increased the phosphorylation of Akt, and blocking PI3K activity by LY294002 abolished the protective effect of troloxerutin on I/R injury. **Conclusion:** Troloxerutin preconditioning protected against myocardial I/R injury via the PI3K/Akt pathway.

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Introduction

Ischemic heart disease secondary to acute myocardial infarction is a severe health problem in the world, which is the leading cause of death worldwide. Although timely reperfusion is necessary to limit the cell death resulting from cardiac ischemia, sudden

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restoration of blood flow to ischemic myocardium may exaggerate the myocardial injury. This phenomenon is known as myocardial ischemia/reperfusion (I/R) injury [1]. Some factors contribute to the pathological process of myocardial I/R injury, such as oxidative stress [2], autophagy [3], calcium overload [4], inflammation [5], and mitochondrial dysfunction [6].

The PI3K/Akt pathway plays a crucial role in cell growth and cell survival. The activated Akt pathway can regulate cell survival through phosphorylation of a variety of downstream targets, such as pro-apoptotic protein, transcription factors and endothelial nitric oxide synthase (eNOS)[7-9]. The activation of the PI3K/Akt pathway protects the brain from cerebral I/R injury by preventing neuronal apoptosis [10]. Activation of the PI3K/Akt pathway also protects against myocardial I/R injury by decreasing oxidative stress, repressing the inflammatory cascade, and inhibiting apoptosis *in vivo* and *in vitro* [11-14].

Troxerutin, also known as vitamin P4, is natural flavonoid rutin present in tea, coffee, cereal grains and a variety of fruits and vegetables. It has various biological functions, including improving capillary function, reducing capillary fragility and abnormal leakage, and anti-erythrocytic and anti-thrombotic effects [15]. Troxerutin has protective effects in several tissues, including kidney [16], liver [17] and brain [18]. In the context of myocardial I/R injury, troxerutin has a protective effect against reperfusion injury of diabetic myocardium by attenuating GSK-3 β activity and subsequently reducing apoptosis [19]. The PI3K/Akt pathway is important in mediating the effect of troxerutin [20]. However, there is no report on a troxerutin preconditioning effect on myocardial I/R injury in healthy bodies or the role of the PI3K/Akt pathway in troxerutin's effects *in vivo*.

In this study, we identified the protective properties of troxerutin in a myocardial I/R injury rat. Our findings provide *in vivo* and *in vitro* evidence that the cardioprotective effects of troxerutin are mediated through PI3K/Akt signaling pathways.

Materials and Methods

Animals

Male Wistar rats (40 rats, weighing 250-300 g) were supplied by the medical animal center of our institute. The animals were fed a standard rodent chow diet with free access to water and housed in a temperature-controlled (25 $\pm$ 3°C), pathogen-free room with a 12-hour light, 12-hour dark cycle. The procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 80-23, revised 1996) and approved by the local animal care committee.

Experimental group

After acclimation to their environment for 7 days, all rats were randomly allocated into four groups (n=10/group): 1) sham group; 2) I/R group; 3) I/R+Troxerutin group; 4) I/R+Troxerutin+LY294002 group. For those undergoing I/R surgery, the rats were administered with troxerutin (150 mg/kg) by oral gavage or LY294002 (an inhibitor of PI3K, 0.2 mg/kg) by subcutaneous injection (S.C.) for 4 weeks before IR operation. The sham group was given saline by oral gavage for 4 weeks before I/R operation.

In vivo myocardial I/R model

The rats were anesthetized with isoflurane inhalation. A left thoracotomy was performed to expose the beating heart. The I/R animal model was established by ligation of the left anterior descending (LAD) coronary artery for 30 min followed by reperfusion for 45 min (by reopening of LAD) [21]. The chest was closed in layers, and the animals were allowed to recover. The successful performance of coronary occlusion and reperfusion was verified by an immediate fall in coronary flow at the onset of index ischemia and its recovery upon reperfusion.

Determination of myocardial infarct size

Myocardial infarct size was evaluated by the Evans blue-TTC method as previously described [22]. Briefly, the ligature around the left anterior descending coronary artery was reoccluded after reperfusion. Four milliliters of 1% Evans blue dye was injected into the vena cava to track the perfused region and the area at risk (AAR). The myocardial AAR area was identified as the region lacking blue staining. The ventricles of the hearts were sliced transversely into 2-mm-thick slices and incubated in 1% tetrazolium chloride solution (TTC) for 20 min to identify the infarct area. The weights of the infarct area (white), AAR (red), and nonischemic zones of the LV (blue) were measured. Infarct size is reported as a percentage of the AAR mass.

Hemodynamic examination

Hemodynamic examination was performed as previously described [22]. The right carotid artery was exposed via a neck dissection. Hemodynamic parameters, including the heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximal rate of rise in blood pressure in the ventricular chamber (+dp/dt max), and maximal rate of decline in blood pressure in the ventricular chamber (-dp/dt max), were measured and recorded.

Serum parameters measurement

Serum CK and LDH were measured in the clinical laboratory of our institution according to the manufacturer's protocols. The results are reported as U/L. Serum TNF- α and IL-10 were determined by an ELISA kit following the manufacturer's instructions.

Cell culture

The H9c2 cells, a rat cardiomyoblast line, were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines had been passaged for less than 6 months when used for experiments. H9c2 cells were maintained in DMEM containing 4.5 g/L glucose with Glutamax from Invitrogen (Basel, Switzerland) and supplemented with 10% FBS, 5 mmol/L HEPES, 1 mmol/L sodium pyruvate and 500 μ g/ml penicillin-streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO₂ in air.

Other standard methods

RNA isolation, qPCR, MTT Assay and Western blot (WB) were performed as described previously [23-26]. For WB, equal amounts of proteins from five mouse livers in each group (n=5/group) were pooled, and single or duplicate loading was used.

Statistical Analysis

All data are presented as the mean $\pm$ SEM. Statistical analysis was carried out using Student's t test between two groups and one-way ANOVA between multiple groups. In all cases, values of P < 0.05 were considered statistically significant.

Results

Troxerutin preconditioning decreases the infarct size induced by I/R operation

As shown in Fig. 1A, compared with the sham group, AAR area was increased dramatically by I/R operation, while there was no significant difference between the three groups with I/R operation. The infarct size induced by I/R operation was decreased in the I/R+troxerutin group (P < 0.05), while the effect of troxerutin on infarct size was blocked by LY294002 (Fig. 1B).

Further, we detected the serum levels of several myocardial infarction markers. As shown in Table 1, similar to the results of infarct size, I/R significantly increased cTnI, CK and LDH. Pre-treatment with troxerutin (I/R+troxerutin) significantly reduced these markers compared with the I/R group (P<0.05), which effect was also blocked by LY294002.

Fig. 1. Troxerutin preconditioning decreases the infarct size induced by I/R operation. (A) AAR area and (B) infarct size were evaluated in all the groups. Group 1, sham group; group 2, I/R group; Group 3, I/R+troxerutin group; Group 4, I/R+troxerutin+LY294002 group. Data are shown as Mean $\pm$ SEM. * $P < 0.05$ vs I/R group.

Fig. 2. Effect of troxerutin on hemodynamics of I/R injury rat. The hemodynamic parameters LVSP, LVEDP, +dp/dt max and -dp/dt max were measured. Group 1, sham group; group 2, I/R group; Group 3, I/R+troxerutin group; Group 4, I/R+troxerutin+LY294002 group. Data are shown as Mean $\pm$ SEM. * $P < 0.05$ vs sham group; # $P < 0.05$ vs I/R group. & $P < 0.05$ vs I/R+troxerutin group.

Table 1. Effect of troxerutin preconditioning on serum cTnI, CK and LDH in myocardial I/R injury rat model. a, $P < 0.01$ vs sham group; b, $P < 0.01$ vs I/R group; c, $P < 0.01$ vs I/R+troxerutin group

Group	cTnI (ng/ml)	CK (U/L)	LDH (U/L)
Sham	0.24 $\pm$ 0.03	1293.51 $\pm$ 124.75	1075.28 $\pm$ 122.35
I/R	20.81 $\pm$ 2.43a	5236.24 $\pm$ 157.63a	3284.02 $\pm$ 326.93a
I/R+troxerutin	10.74 $\pm$ 1.79ab	2702.46 $\pm$ 195.27ab	1268.51 $\pm$ 147.28ab
I/R+troxerutin+LY294002	18.75 $\pm$ 2.23ac	4638.92 $\pm$ 268.35ac	3027.86 $\pm$ 203.17ac

Table 2. Effect of troxerutin preconditioning on myocardial apoptosis rate in myocardial I/R injury rat model. a, $P < 0.01$ vs sham group; b, $P < 0.01$ vs I/R group; c, $P < 0.01$ vs I/R+troxerutin group

Group	Myocardial apoptosis rate (%)
Sham	2.87 $\pm$ 0.32
I/R	31.25 $\pm$ 2.76 ^a
I/R+troxerutin	18.43 $\pm$ 2.53 ^{ab}
I/R+troxerutin+LY294002	25.32 $\pm$ 3.56 ^{ac}

Fig. 3. Effect of troxerutin on I/R induced myocardial apoptosis. (A) the mRNA and (B) protein levels of apoptosis markers (BAX, caspase 3 and BCL-2). (C) Caspase 3 activity. Group 1, sham group; group 2, I/R group; Group 3, I/R +troxerutin group; Group 4, I/R+troxerutin+LY294002 group. Data are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs sham group; # $P < 0.05$ ## $P < 0.01$ vs I/R group. & $P < 0.05$ && $P < 0.01$ vs I/R+troxerutin group.

Effect of troxerutin on hemodynamics of I/R injury rat

As shown in Fig. 2, the levels of LVSP, +dp/dtmax and -dp/dtmax were significantly decreased, whereas LVEDP was significantly increased, in the I/R group compared with the sham group. Troxerutin preconditioning significantly increased LVSP, +dp/dtmax and -dp/dtmax, whereas troxerutin preconditioning reduced LVEDP in the I/R+troxerutin group compared to the I/R group. However, the effects of troxerutin on hemodynamics were significantly reversed in the I/R+troxerutin+LY294002 group compared to the IR+troxerutin group.

Effect of troxerutin on I/R-induced myocardial apoptosis

We also detected the myocardial apoptosis in this study. As shown in Table 2, the myocardium apoptosis rate in the I/R group was significantly increased compared with the sham group. Troxerutin preconditioning significantly decreased the myocardium apoptosis rate in the I/R+troxerutin group compared to the I/R group. However, the decreased myocardium apoptosis rate caused by troxerutin was significantly reversed in the I/R+troxerutin+LY294002 group.

We also measured several apoptosis markers in myocardial tissues. As shown in Fig. 3A & B, compared with the sham group, the mRNA and protein levels of two apoptosis markers (Bax and Caspase 3) were increased and the anti-apoptosis marker (Bcl-2) was reduced in the I/R group. Troxerutin preconditioning significantly attenuated the increases in Bax and Caspase 3 and the decrease in Bcl-2 induced by I/R, which was reversed in the I/R+troxerutin+LY294002 group. We also detected the Caspase 3 activity in these groups, which showed a similar pattern with its mRNA expression (Fig. 3C).

Effect of troxerutin preconditioning on serum cytokine levels and heart ICAM-1 expression

As shown in Fig. 4, serum levels of CRP, TNF- α , IL-1 β and IL-10 as well as the mRNA level of ICAM-1, the indicator of neutrophil–endothelial cell interactions, were significantly increased in the I/R group over the sham group. These cytokines were significantly decreased in troxerutin-preconditioned rats compared with I/R rats. However, their levels were significantly increased in rats treated with troxerutin and LY29400 compared to troxerutin alone.

Fig. 4. Effect of troxerutin preconditioning on serum cytokine levels and heart ICAM-1 expression. Serum CRP, TNF- α , IL-10 and IL-1 β as well as heart ICAM-1 mRNA expression were measured. Group 1, sham group; group 2, I/R group; Group 3, I/R+troxerutin group; Group 4, I/R+troxerutin +LY294002 group. Data are shown as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs sham group; # $P < 0.05$ ## $P < 0.01$ vs I/R group. & $P < 0.05$ && $P < 0.01$ vs I/R+troxerutin group.

Fig. 5. Protective effect of troxerutin against I/R Injury is mediated via PI3K/Akt pathway. (A) The p-Akt and total Akt protein levels in I/R rat model with troxerutin and LY294002 treatment. The p-Akt and total Akt protein levels in H9c2 cells treated with troxerutin for different (B) doses and (C) times. (D) The cell viability of H9c2 cells detected by MTT assay with H_2O_2 and troxerutin treatment. Group 1, sham group; group 2, I/R group; Group 3, I/R+troxerutin group; Group 4, I/R+troxerutin+LY294002 group. Data are shown as Mean $\pm$ SEM. ** $P < 0.01$ vs con; ## $P < 0.01$ vs H_2O_2 treatment.

Protective effect of troxerutin against I/R Injury is mediated via PI3K/Akt pathway

The activation of the PI3K/Akt pathway has a protective effect against I/R injury, and troxerutin can activate the PI3K/Akt pathway. As shown in Fig. 5A, the t-Akt level had no

significant change between these groups, while p-Akt increased moderately in the I/R groups and increased more in the I/R+troxerutin group. The I/R+troxerutin + LY29400 group showed significantly lower p-Akt than the I/R+troxerutin group.

We also studied the effect of troxerutin *in vitro*. As shown in Fig. 5B, troxerutin significantly increased p-Akt in H9c2 cells in a dose- and time-dependent manner. ROS release is thought to be important in the pathogenesis of myocardial I/R injury [27]. Our study found that the treatment of H9c2 cells with troxerutin markedly reduced H₂O₂-induced cell death compared to H₂O₂ treatment alone. In addition, inhibition of the Akt pathway by LY294002 completely blocked the protective ability of troxerutin in H₂O₂-treated H9c2 cells (Fig. 5C). These results strongly suggest that Akt pathway activation is necessary for a protective effect of troxerutin in myocardial I/R injury.

Discussion

In the present study, preconditioning with troxerutin significantly ameliorated the myocardial I/R injury, including reduction of myocardial infarct size, decreased inflammatory cytokines, and inhibited cardiomyocyte apoptosis. Mechanistically, our results show that the protective effect of troxerutin on myocardial I/R injury is mediated by the PI3K/Akt pathway.

Flavonoids are a subgroup of the more extended family of polyphenols. More than 5000 flavonoids have been identified, each with a basic structure containing two benzene rings with a pyrane ring in the middle [28]. A number of flavonoid compounds have cardioprotective effects against I/R injury, such as tilianin [29], vitexin [30], icariin [22] and quercetin [11]. Here, we reported the protective effect of troxerutin on myocardial I/R injury, which is a naturally occurring flavonoid.

The inflammatory response is stimulated by I/R injury, leading to the release of inflammatory cytokines, which are critical mediators of infarct healing and cardiac remodeling [31]. In addition, inhibiting inflammation can decrease myocardial I/R injury and preserve cardiac function [32, 33]. In the present study, we found that preconditioning of troxerutin reduced the production of inflammatory cytokines induced by I/R injury.

Cardiomyocyte apoptosis is dominant in the pathogenesis of myocardial I/R injury [34] and is responsible for a significant amount of cardiomyocyte death in I/R injury [35, 36]. Suppressing cell apoptosis attenuates cardiomyocyte loss and prevents I/R injury [37-40]. In the present study, the cardiomyocyte apoptotic rate decreased, and myocardial infarction size was reduced in the I/R+troxerutin group compared with the I/R group. We also found that troxerutin treatment reduced H₂O₂-induced cell apoptosis *in vitro*. These results suggest that the protective effect of troxerutin might be related to a reduction of cellular apoptosis.

Cardiomyocyte necrosis is another type of cell death in myocardial I/R injury [34]. LDH is a stable cytosolic enzyme present in mammalian cells that is released when cell necrosis occurs [41]. In our study, we found that I/R induced serum LDH dramatically, while troxerutin preconditioning reduced LDH, which suggested that troxerutin might prevent myocardial I/R injury by also reducing cell necrosis.

Troxerutin also has been reported to block the endoplasmic reticulum stress and oxidative stress pathway in brain and liver of mice [18, 42, 43], and troxerutin could increase the activity of anti-oxidant enzymes and limiting the lipid peroxidation [44]. We need to do further study to explore if these pathways also participate the protective effect of troxerutin in myocardial I/R injury.

Conclusion

In summary, our study revealed the protective effect of troxerutin preconditioning on myocardial I/R injury, which was mediated by the PI3K/Akt pathway. Our results provide new evidence for the clinical use of troxerutin in ischemic heart diseases.

Disclosure Statement

The authors declare that there is no financial conflict of interest.

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