

Original Article

Theaflavin decreases hypoxia/reoxygenation-induced inflammation and apoptosis by suppressing TLR4/NF- κ B signaling in neonatal rat cardiomyocytes

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Abstract: Objective: Theaflavin (TF1), a major component of black tea extract, possesses biological properties such as anti-inflammation and anti-apoptosis. The purpose of the present work was to verify whether TF1 plays a protective role in hypoxia/reoxygenation (H/R)-injured cardiomyocytes by inhibiting inflammation and apoptosis. Moreover, the potential molecular mechanisms are highlighted in association with the TLR4/NF- κ B pathway. Methods: Primary neonatal rat cardiomyocytes (NRCMs) were isolated and randomized into four groups: a control + vehicle group, a control + TF1 group, an H/R + vehicle group, and an H/R + TF1 group. The H/R injury model was constructed using the administration of 2 hours of hypoxia followed by 4 hours of reoxygenation. The cell vitality and myocardial enzymes were measured to evaluate the myocardial damage. A flow cytometric assay was used to assess the apoptotic response. The inflammatory mediators TNF α , IL-1 β , and IL-6 were measured to assess the inflammatory response. Western blotting and RT-PCR were used to evaluate the TLR4, NF- κ B, Bax, and caspase-3 protein and mRNA expressions. Results: The results showed that the H/R caused significant myocardial damage as shown by the increased release of LDH and CK, but it also caused a reduction in cell vitality. In addition, inflammation and apoptosis were dramatically promoted by H/R in the NRCMs, as indicated by the increased levels of TLR4, NF- κ B, Bax, caspase-3, TNF α , IL-1 β , and IL-6. However, the TF1 administration reversed the H/R-induced inflammation and apoptosis and reduced the TLR4 and NF- κ B levels. Conclusion: TF1 can reduce inflammation and apoptosis by inhibiting TLR4/NF- κ B signaling during H/R injury, and this reduction provides a therapeutic approach for treating myocardial ischemia/reperfusion injury (MIRI).

Keywords: Theaflavin1, hypoxia-reoxygenation, inflammation, apoptosis, TLR4/NF- κ B

Introduction

According to the World Health Organization, the number of acute myocardial infarction (AMI) cases is around 32.4 million per year worldwide. Timely revascularization is the most effective clinical intervention for AMI [1]. However, reperfusion therapy can cause myocardial ischemia/reperfusion (I/R) injury (MIRI) in turn, a therapy shown to be a complicated pathophysiological process that can cause additional myocardial damage [2]. In recent years, abundant evidence has shown that inflammation and apoptosis exert crucial roles in the pathogenesis of MIRI [3, 4]. Hence, it is necessary to explore and find effective approaches to reduce inflammation and apoptosis to attenuate MIRI.

Black tea, a popular tea, has various health benefits, including cancer prevention, anti-aging activity, refreshing effects, and assistance in lipid digestion [5, 6]. Theaflavins are natural polyphenols and are the main ingredient of black tea, including theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3-gallate (TF2B) and theaflavin-3,3-gallate (TF3) [7]. Some studies have shown that theaflavins can regulate the inflammatory response and apoptosis in various diseases, such as periodontitis [8], acute ischemic stroke [9], rheumatism [10], Parkinson's disease, [11] etc. Recently, many studies have demonstrated that theaflavins, especially TF1, is a potential protective agent for I/R injury [9, 12-15]. Luo *et al.* showed that TF1 has protective effects against liver I/R injury through its anti-

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inflammatory and anti-apoptotic mechanisms [14]. Li *et al.* indicated that TF1 can attenuate cerebral I/R injury by abolishing miRNA-128-3p-mediated Nrf2 inhibition and reducing oxidative stress [9]. In fact, Ma *et al.* also have indicated that TF1 can protect MIRI by opening the K⁺ ATP channels, particularly on the mitochondrial membrane, and inhibiting mPTP opening [15]. But the effect of TF1 on MIRI has not been studied sufficiently. Further study into the physiological function and underlying mechanism of TF1 in MIRI is required.

Studies have indicated that I/R can induce pro-inflammatory cytokines and apoptosis-related proteins in cardiomyocytes by triggering TLR4 and its downstream NF- κ B signaling [4, 16-18]. The administration of a TLR4 or NF- κ B inhibitor has been shown to decrease I/R-induced pro-inflammatory cytokines and apoptosis, further ameliorating cardiac dysfunction [4]. Interestingly, NF- κ B-related signaling transduction can also be suppressed by theaflavins [10, 13, 19]. However, whether TF1 can decrease inflammation and apoptosis by suppressing TLR4/NF- κ B activation during MIRI is largely unknown. The present study showed that TF1 administration represses myocardial inflammation and apoptosis during 2 hours of hypoxia followed by 4 hours of reoxygenation (H/R) injury, which may be related to the inhibition of the TLR4/NF- κ B pathway. Thus, this study provides novel insights into the benefits and potential mechanisms of TF1 against MIRI and provides a prospective therapeutic option for MIRI.

Materials and methods

Chemicals and reagents

The theaflavin (TF1) used in the experiment was purchased from Chromadex, Inc. Some experimental reagents, such as DMEM, PBS, trypsin, and FBS were purchased from Gibco. The CCK-8 kits were purchased from Dojindo (Japan). LDH and CK were obtained from Xitang Company (Shanghai, China). The primary antibodies were from Abcam (Cambridge, UK): Bax (1:500 dilution), caspase-3 (1:500 dilution), TLR4 (1:1000 dilution), NF- κ B (1:500 dilution), and GAPDH (1:1000 dilution). Secondary antibodies, the horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG, were obtained from BIOSS (Beijing, China).

Neonatal rat cardiomyocyte (NRCM) culture

The NRCMs were isolated from SD rats (about 2-days old). All the rats were sacrificed by decapitation and were immersed in 75% alcohol. Afterward, their hearts were quickly removed, and their large blood vessels were excised carefully. The obtained heart tissues were rinsed in ice-cold PBS to remove any residual blood. Then, we used 0.08% collagenase type II and 0.125% trypsin to digest the tissues. Finally, the NRCMs were centrifuged and re-suspended in DMEM containing 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂ and 95% O₂.

The establishment of the H/R injury model

The H/R injury model was established as previously described. Basically, the cultured NRCMs were preserved in serum-free DMEM medium. Next, the cells were incubated in an anaerobic chamber for 2 hours at 37°C. Then, the cells were moved into a normal incubator for an additional 4 hours to constitute the reoxygenation. To determine the possible function of TF1 in MIRI, different dosage gradients of TF1 (0.1, 1, 10, 20 μ M) were first carried out to determine the most effective dosage against MIRI. Next, another experiment was designed to explore the exacted roles and molecular mechanisms of TF1 in response to H/R. The primary cardiomyocytes were randomly divided into four groups: the control + vehicle group, the cultured NRCMs in normoxic conditions without TF1 treatment group, the control + TF1 group, the NRCMs in normoxic conditions with TF1 treatment group, the H/R + vehicle group, the NRCMs in H/R condition without TF1 treatment group, the H/R + TF1 group, and the NRCMs in H/R condition with TF1 treatment group. Each experiment was repeated at least five times.

Cell viability assay

We used CCK-8 assays to measure the cell viability in each group as described previously. The optical density values were measured using a microplate reader at the 450 nm wavelength. The cell viability was recorded as a percentage relative to the control group.

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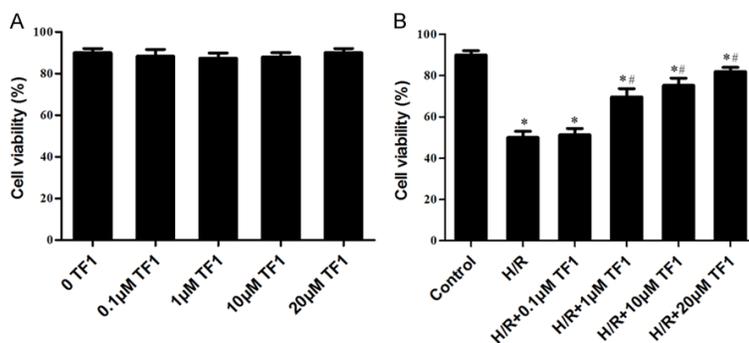


Figure 1. The effect of TF1 on cell viability. A. The cytotoxicity of TF1 (0.1, 1, 10, 20 μ M) on NRCMs assessed by CCK-8. B. The influence of TF1 on cell viability after H/R insult. The data are shown as the means \pm SD ($n=5$). * $P<0.05$, compared with the control; # $P<0.05$, compared with the H/R group.

Measurement of the myocardial injury markers

Myocardial enzymes are released when cardiomyocytes are seriously damaged, and their levels represent the severity of the myocardial injury under MIRI. LDH and CK are commonly used as molecular markers for myocardial injuries. After the indicated procedures, the supernatant of the cultured NRCMs were collected, and commercially available biochemical kits were used in line with the kits' instructions to determine the LDH and CK.

Apoptosis quantification

The apoptosis of the NRCMs was measured using a flow cytometric assay as previously described. Following the standard protocols, the cells were obtained through corresponding treatments, then, these cells were maintained in 100 μ l of binding buffer, which includes 5 μ l of annexin V-APC and 5 μ l of PI. Afterward, the stained cells were observed using a flow cytometer (Beckman, Boulevard Brea, CA, USA). The NRCMs, which were tagged positively for PI or annexin V-APC, were recognized as apoptotic cells.

Measuring the TNF- α , IL-1 β , and IL-6 levels

The ELISA method was used to determine the TNF α , IL-1 β , and IL-6 levels. The detailed process was performed according to the standard protocol. TNF α , IL-1 β , and IL-6 kits (Xitang Company Shanghai, China) were used.

Western blotting (WB)

WB was used to determine the protein expressions as described earlier. The myocardial sam-

ples were homogenized and lysed in RIPA lysis buffers. The protein was then extracted, and the protein concentration was determined using a bicinchoninic acid protein assay (BCA, Beyotime, China). We used 10% SDS-PAGE to separate the proteins, and the extracted proteins were electrophoretically transferred onto PVDF membranes. Thereafter, the membranes were incubated with antibodies against Bax, caspase-3, TLR4, and NF- κ B overnight. The protein bands were visualized

using an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc.).

Quantitative real-time PCR (RT-PCR)

RT-PCR was used to determine the mRNA levels as previously described. Basically, the total RNA was extracted from the NRCMs using Trizol Reagent (Invitrogen, Carlsbad, CA) after the indicated treatments. qRT-PCR was manipulated using a SYBR green Master Mix kit (Thermo Fisher, USA) on the 7500 ABI Prism system. The PCR condition was set as follows: 2 min at 50 $^{\circ}$ C, 95 $^{\circ}$ C for 10 min and immediately following 40 cycles of 95 $^{\circ}$ C for 30 seconds and 60 $^{\circ}$ C for 30 seconds. The mRNA expressions of Bax, caspase-3, TLR4, and NF- κ B were normalized to that of GAPDH.

Statistical analysis

SPSS 17.0 (IBM Corp.) was used for statistical analysis. The data were expressed as the means \pm SD. Student's t-tests were used for the between-group comparisons. One-way ANOVA was used for the comparisons among groups and Tukey's post hoc test was used for multiple comparisons. A P value <0.05 was considered statistically significant.

Results

The effect of TF1 on cell viability

CCK-8 was measured in each group to assess the cell viability. The four different concentrations of TF1 (0.1, 1, 10, 20 μ M) had no influences on cell viability and exhibited a very low cytotoxicity on the control NRCMs (**Figure 1A**).

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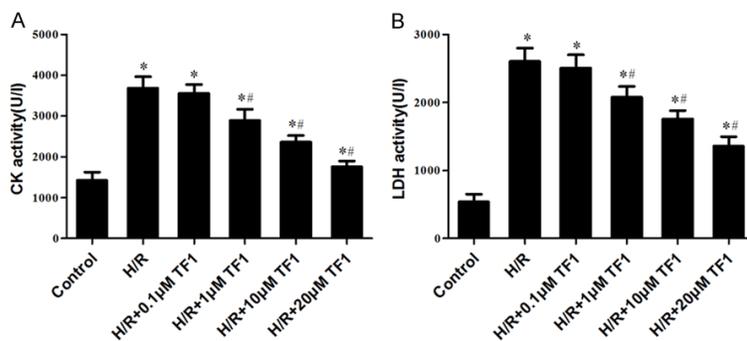


Figure 2. TF1 attenuated NRCMs injury induced by H/R. CK (A) and LDH (B) were reduced apparently after the TF1 pretreatment after the H/R. The data are exhibited as the means \pm SD ($n=5$). * $P<0.05$, compared with the control; # $P<0.05$, compared with the H/R group.

In order to exploit the possible contributions of TF1 on H/R injury, the NRCMs underwent 2 hours of hypoxia and 4 hours of reoxygenation. As displayed in **Figure 1B**, the cell viability was repressed after the H/R injury. In addition, the gradient doses of TF1 (0.1, 1, 10, 20 μ M) increased the cell viability significantly. Basically, the cell viability was strengthened in the 1 μ M and 10 μ M groups, and the optimal data were obtained at 20 μ M compared with the H/R group.

TF1 attenuated the NRCMs injury induced by H/R

The LDH and CK releases, as the common markers for cell damage, were determined to assess the NRCMs deaths. As shown in **Figure 2**, the LDH and CK releases caused by H/R were limited starting at 1 μ M with the minimum level at 20 μ M. Moreover, there was no statistical difference between the LDH/CK releases and the cell viability between the 0.1 μ M group and the H/R group. Consequently, 20 μ M of TF1 was used for the following experiments. The above-mentioned results suggest that TF1 can decrease damage in NRCMs with H/R injury.

TF1 treatment alleviated inflammation during H/R injury

MIRI is tightly related to the excessive inflammatory response. In addition, TF1 has been shown to be involved in the progression of inflammation. The ELISA method was used to determine the TNF α , IL-1 β , and IL-6 expressions. We observed that the levels of TNF α ,

IL-1 β , and IL-6 were up-regulated after H/R ($P<0.05$, **Figure 3**). However, TF1 pretreatment significantly decreased the expressions of the above inflammatory factors under H/R conditions, when compared with the vehicle-treated cells after H/R ($P<0.05$, **Figure 3**).

TF1 treatment alleviated apoptosis during H/R injury

To estimate whether TF1 affected H/R induced apoptosis, the apoptosis of NRCMs was measured using flow cytometric assays and the protein expressions of Bax and caspase-3 were determined using WB. As shown in **Figure 4A**, compared with the control group, the minimal apoptotic rates were observed in the control-vehicle and control-TF1 groups, but H/R accelerated the apoptosis of the cardiomyocytes. Of note, compared with H/R + vehicle group, the TF1 pretreatment significantly decreased the apoptotic cells under H/R conditions ($P<0.05$). Consistent with the above results, the TF1 administration significantly decreased Bax and caspase-3 during the H/R process ($P<0.05$, **Figure 4B, 4C**).

TF1 pretreatment inhibits TLR4/NF- κ B signaling

To further investigate the underlying mechanism of TF1 in alleviating H/R injury, we determined the molecular expressions involved in TLR4/NF- κ B signaling. As shown in the **Figure 5A and 5B**, compared with the control group, the H/R significantly up-regulated the TLR4 and NF- κ B levels ($P<0.05$). However, compared to the H/R-vehicle group, the pretreatment with TF1 at the onset of H/R decreased the expressions of TLR4 and NF- κ B. Therefore, it can be speculated that the H/R injury was ameliorated by TF1 possibly by inhibiting the TLR4/NF- κ B signaling.

Discussion

Previous studies have found that TF1 can protect neurons from cerebral I/R injury significantly [9, 12, 13]. However, the pharmacological role of TF1 in MIRI and the possible

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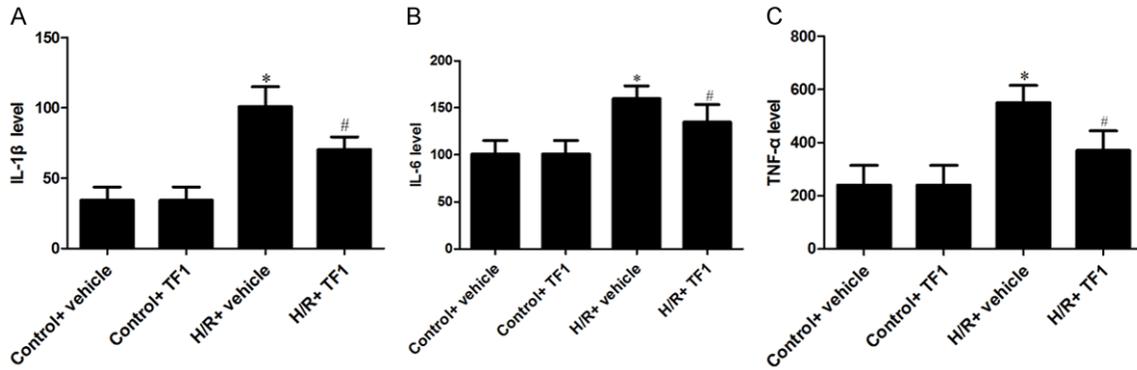


Figure 3. The TF1 treatment alleviated inflammation during H/R injury. The ELISA method was employed to measure the IL-1 β , IL-6, and TNF- α levels. The inflammatory factors increased sharply but TF1 pretreatment can reduce (A) IL-1 β , (B) IL-6, and (C) TNF- α . The data are shown as the means \pm SD ($n=5$). * $P<0.05$, compared with the control; # $P<0.05$, compared with the vehicle-H/R.

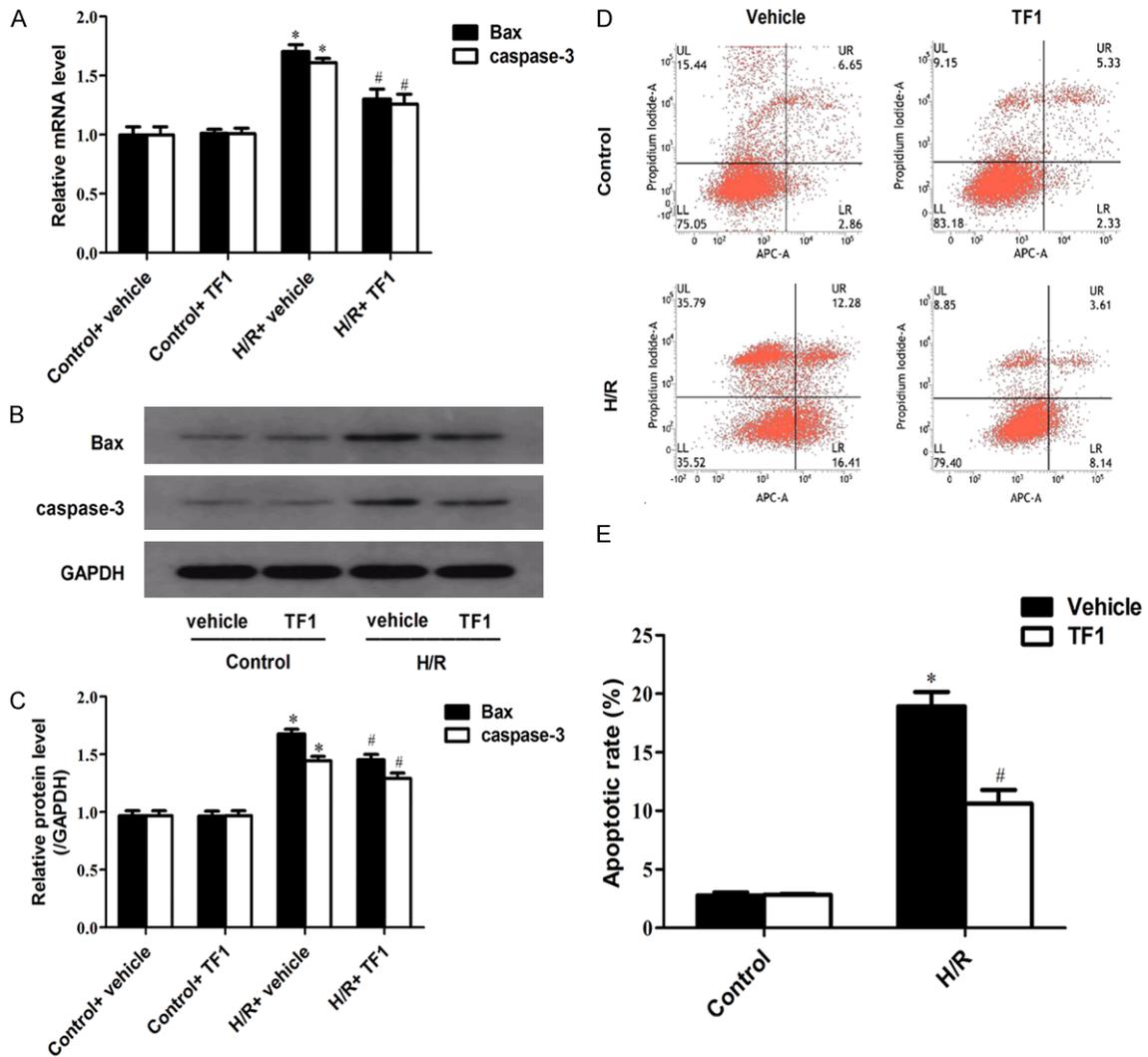


Figure 4. TF1 treatment alleviates apoptosis during H/R injury. A. The mRNA levels of Bax and caspase-3 measured using qRT-PCR. B. The protein levels of Bax and caspase-3 were determined using WB. C. Result of the quantitative protein level. D. The apoptotic cells were monitored using flow cytometry. E. Result of the quantitative apoptotic rate. The data are exhibited as the means \pm SD ($n=5$). * $P<0.05$, compared with the control; # $P<0.05$, compared with the vehicle-H/R.

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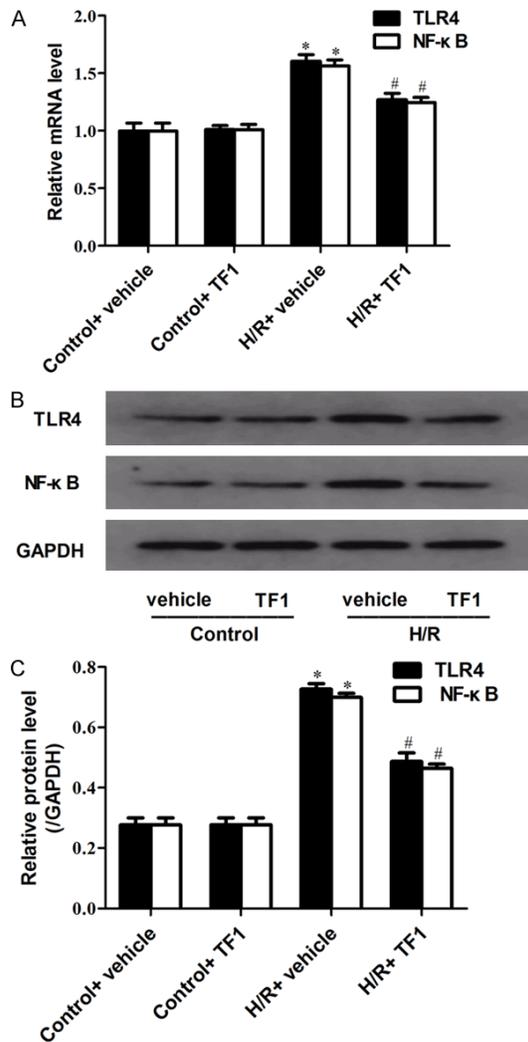


Figure 5. The TF1 pretreatment inhibited the TLR4/NF-κB signaling. A. The mRNA levels of TLR4 and NF-κB determined using qRT-PCR. B. The protein levels of TLR4 and NF-κB determined by WB. C. The result of the quantitative protein level. The data are exhibited as the means \pm SD ($n=5$). * $P<0.05$, compared with the control; # $P<0.05$, compared with the vehicle-H/R.

mechanisms have not been elucidated. In the present work, we observed that TF1 treatment can reduce myocardial H/R injury, which is characterized by the up-regulation of cell activity and the decreased releases of LDH/CK. In addition, TF1 administration significantly alleviates myocardial inflammation and apoptosis, and inhibits the protein expressions of Bax, caspase-3, and the inflammatory factors. At the same time, it is observed that TF1 markedly inhibits the TLR4/NF-κB signaling pathway. The above results therefore indicate

that TF1 possesses a strong ability to ameliorate H/R-caused inflammation and apoptosis mainly through the TLR4/NF-κB-dependent pathway.

MIRI is an important cause of myocardial damage and is an unsolved problem in clinical practice [20]. Inflammation and apoptosis to MIRI exert crucial roles, so a persistent pro-inflammatory and apoptotic reaction would cause a series of complications following reperfusion therapy [21]. Thus, reducing inflammation and apoptosis effectively has been an important therapeutic goal to improve outcomes for MIRI [22]. Previous studies have found that TF1 can regulate the inflammatory response and apoptosis in various diseases [14, 23, 24]. In recent years, the multiple bioactivities of TF1 have become a research hotspot and has received much attention. Consistent with the above, our experiments here indicate that TF1 can ameliorate H/R-caused inflammation and apoptosis.

H/R can directly decrease cardiomyocyte contractility and induce inflammatory and apoptotic related protein expressions in cardiomyocytes by activating the TLR4/NF-κB pathway [18]. As a transcription factor, NF-κB has been confirmed to be closely related to inflammation and apoptosis [25]. Ample evidence has suggested that theaflavins have pleiotropic impacts on a variety of pathological conditions by inhibiting NF-κB activation. Wu *et al.* demonstrated that theaflavin-3,3-gallate can inhibit the LPS-induced inflammation by blocking NF-κB [8]. Ukil *et al.* demonstrated that theaflavin-3,3-gallate exerts protective effects in experimental colitis and inhibits inflammation by suppressing NF-κB activation [26]. Liu *et al.* put up evidence showing that theaflavin-3,3-gallate exerts protection on rheumatism by inhibiting the activation of NF-κB [10]. Lin *et al.* demonstrated nitric oxide synthase by down-regulating the activation of NF-κB in macrophages [19]. Cai *et al.* showed that theaflavin-3,3-gallate exerts protective effects against cerebral I/R injury by reducing inflammation by modulating NF-κB activation [12, 13]. In terms of theaflavin's possible involvement in the activation of NF-κB, we thus hypothesize that TF1 can also exert a protective role on cardiomyocytes and has an anti-inflammatory and anti-apoptotic effect in

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response to H/R insult by inhibiting the downstream signaling transduction pathway of NF- κ B. Consequently, our research provides evidence that TF1 has an advantageous role on H/R-injured NRCMs by suppressing TLR4-mediated NF- κ B activation.

In summary, this work indicated that pretreatment with TF1 alleviates MIRI by attenuating inflammation and apoptosis in a TLR4/NF- κ B dependent manner. Moreover, many other signaling pathways are involved in the pathophysiological process of MIRI. Further studies are still needed to explore whether there are any other signaling pathways involved in the protective effect of TF1 in MIRI. Taken together, our results here indicate that TF1 may be a prospective therapeutic option for MIRI.

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Disclosure of conflict of interest

None.

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