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Original Article

Tetramethylpyrazine confers cardioprotection against I/R injury by the Hsp70/NF-κB dependent pathway

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Abstract: Background and Objective: Tetramethylpyrazine (TMP), one of the active ingredients of the Chinese herb Lingusticum Wallichii Frantchat (Chuan Xiong), plays an important role in cardioprotection against I/R injury. However, the underling mechanisms remain unknown. This study investigated the effects of TMP on myocardial ischemia-reperfusion (I/R) injury in rats and explored its possible related mechanism. Methods: The mice were anesthetized with pentobarbital intraperitoneally and mice were subjected to myocardial ischemia (6 h) and reperfusion (24 h). Animals were randomly classified into 3 groups (n = 6 each). ① sham-operated mice ② ischemia (6 h) and reperfusion (24 h) ③ 10 mg/kg TMP iv into the right jugular vein at the time of reperfusion. A ischemia reperfusion model was constructed *in vitro* using H9c2 cells. The hearts were taken for histopathological examination and infarct size was detected with 2, 3, 5-triphenyltetrazolium chloride staining. Expression of Hsp70, NF-κB p65, PUMA, pro-caspase-3, cleaved caspase-3, IL-6, IL-1β, and TNF-α protein and mRNA were detected by Western blot and RT-PCR assay. Caspase-3 activity was detected along with IL-6, IL-1β, and TNF-α protein determined by ELISA. NF-κB activity was detected by EMSA. Cell apoptosis was detected using terminal deoxynucleotidyl nick-end labeling (TUNEL). Results: The mice displayed larger infarct sizes and enhanced cardiac myocyte apoptosis following I/R. The effects were associated with a marked activation of NF-κB and increased IL-6, IL-1β. and TNF-α protein as well as pro-apoptotic PUMA protein. TMP significantly reduced infarct size compared to the sham-treated group (14.6% ± 2.3% *vs.* 41.3% ± 7.4%, $P < 0.01$). These beneficial effects were associated with a marked increase of Hsp70 in the cardiac myocyte *in vivo* and *in vitro*, followed by inactivation of myocardial induced I/R NF-κB and PUMA as well as reduction of IL-6, IL-1β, and TNF-α level. TMP increased Hsp70 expression and reduced expression of NF-κB and PUMA genes. Conclusions: TMP protects myocardial I/R injury by inhibiting cell apoptosis and reducing inflammatory infiltration. The protective effects of TMP are associated with activation of Hsp70, resulting in the inactivation of NF-κB and PUMA, as well as reduction of inflammatory factors. Drug development strategies specifically targeting Hsp70 may be beneficial in treating ischemic heart disease.

Keywords: Ischemia-reperfusion injury, apoptosis, myocardium, tetramethylpyrazine, Hsp70, NF-κB, PUMA

Introduction

Current acute myocardial infarction (AMI) treatment focuses on reperfusion of the ischemic myocardium. This reperfusion, however, results in the induction of inflammation in the heart, which has been shown to inflict additional damage to the myocardium, hence termed ischemia/reperfusion (I/R)-injury [1-3]. Different forms of reperfusion therapy, including coronary angioplasty, coronary stenting, and coronary revascularization, as well as pharmacological adjuvants are widely used in clinical practice for the treatment of this disease [4]. However, I/R-induced injury due to restoration of the blood supply to ischemic areas occurs subsequent to reperfusion therapy, leading to acute tissue damage [5]. Present therapeutic strategies are not efficient at preventing I/R-induced injury. Therefore, development of novel strategies focused on prevention of I/R-induced injury is crucial for the treatment of ischemic heart disease.

Tetramethylpyrazine (TMP), first extracted in 1973, is a biologically active alkaloid isolated from the traditional herbal medicine *Ligusticum wallichii* (Chuanxiong). Previous studies reported that platelet aggregation, inflammatory response, microembolization, and cell

death contribute to myocardial ischemia-reperfusion injury [6]. A low dose of TMP (20 mg/10 mg·kg^{-1}·d^{-1}) can reduce myocardial pathology injury, increase the Ca^{2+}-ATPase activity of myocardial mitochondria, improve cardiac function, and antagonize calcium overload in rats [7]. Lv et al. [8] reported that TMP has anti-apoptotic and cardioprotective effects against myocardial ischemia/reperfusion injury, which is mediated by the PI3K/Akt pathway. In addition, TMP can promote phosphorylation of eNOS to increase NO production. Liu et al. [9] demonstrated that TMP can reduce the scope of myocardial infarction induced by long-term ischemia, while decreasing hemorheological indices of myocardial ischemia in rats and protecting acute ischemic myocardium and ischemia-reperfusion injured myocardium. Another study [10] showed that TMP reduces the size of infarcts resulting from ischemia/reperfusion injury *in vivo*, which might be associated with its antioxidant activity via the induction of heme oxygenase-1 (HO-1) and with its capacity for neutrophil inhibition. Despite the wide application of TMP, there is no consensus among scholars regarding the mechanisms underlying this compound.

Heat shock proteins (HSPs) are a group of phylogenetically conserved proteins found in all prokaryotic and eukaryotic cells and are categorized into five major families named on the basis of their approximate molecular weight [11]. The most studied and highly conserved HSPs is the HSP70 family, including both constitutively expressed and stress-inducible members. Inducible HSP70 accelerates cellular recovery by enhancing the ability of stressed cells to cope with increased concentrations of unfolded/denatured proteins upon many different types of stresses [12, 13], and they also contribute to the mechanisms of cell protection against a variety of human diseases and cytotoxic factors [14].

Pharmacological inhibitors of NF-κB activation provide protection against cardiac I/R injury, attenuate release of TNF-α, IL-6, and the p53-upregulated modulator of apoptosis (PUMA), inhibit inflammation and apoptosis, reduce infarct size, and improve functional recovery [15-19]. Thus inflammation and apoptosis mediated through NF-κB play a critical role in infarct development following I/R. Previous studies have reported that overexpression of Hsp70 could inhibit the translocation of NF-κB, attenuate the release of inflammatory factors, and reduce the apoptosis of myocardium [20]. Yao et al. observed that pretreatment with low-dose LPS resulted in significantly increased levels of Hsp70 in the myocardium, which could dramatically inhibit NF-κB translocation and reduce release of inflammatory cytokines in the following I/R injury [21], suggesting that increase of Hsp70 reduced myocardial I/R injury by inhibition of NF-κB and release of inflammatory factors and pro-apoptotic factors.

Wang et al. reported that TMP can protect the myocardium by activating SOD and GSH-Px and stimulating Hsp70 mRNA and the corresponding protein expression [22]. Chen and colleagues reported that TMP could suppress ischemia-induced ventricular arrhythmias and reduce infarct size resulting from ischemia-reperfusion injury *in vivo by* upregulation of Hsp70 [23]. Chen et al. also reported that TMP treatment protected against anoxia-reoxygenation injury in neonatal rat cardiomyocytes by increasing Hsp70 expression [24]. However, the effect and mechanisms of TMP on cardioprotection against I/R injury remain unknown. Therefore, in the present study we aimed to evaluate the cardioprotective effects of TMP during myocardium I/R injury and the mechanisms underlying such effects.

Materials and methods

Reagents

Tetramethylpyrazine (TMP) (purity > 99%) was obtained from Zelang Pharmaceutical Co. (Jiangsu, China). TMP was solubilized in dimethyl sulfoxide (DMSO) and stored at -80°C. It was made up fresh each time and diluted in PBS to the desired concentration.

Animals

C57BL/6J mice were purchased from Shanghai laboratory animal research center. Animals were housed under conventional conditions at the Animal Care Facility, the affiliated hospital of Qingdao University, and were cared for in accordance with the guidelines established by the China Council on Animal Care. All animal experiments in this study were performed with the approval of the Animal Experiment Committees of the Affiliated Hospital of Qingdao

University. All experiments were conducted in strict adherence to the relevant international guidelines. Every effort was made to minimize suffering of the animals.

Cell line and cell culture

The rat heart cell line H9C2 were purchased from ATCC (Shanghai, China), cultured and maintained in DMEM medium (Invitrogen, China) which was supplemented with 10% fetal bovine serum (Sigma, Oakville, ON, Canada) and 100 U penicillin and streptomycin.

Transient transfection of siRNA

H9c2 cells (1×10^5) were seeded on six-well plates and transfected at the time of 70-80% confluence with a Hsp70 siRNA or p65 siRNA or PUMA siRNA or non-binding control siRNA for 24 h using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions.

In vitro ischemia reperfusion model and TMP treatment

Hsp70 siRNA or p65 siRNA or PUMA siRNA or control siRNA transfected H9c2 cells were plated in a 6 well plate (80,000 cells/well) and cultured in DMEM medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin overnight. DMEM culture medium was replaced by deoxygenized PBS and then placed in an *in vivo* 2 hypoxia workstation with 0% O_2 at 10°C for 16 h. After 24 h of the hypoxia treatment, PBS was removed and new complete culture medium was added to the cells. Cells were moved to a normal culture environment with 5% CO_2 and 28% O_2 at 37°C for 24 h. The H9c2 cells above were treated with 100 μM TMP for 24 h.

Model of ischemia-reperfusion injury and delivery of TMP in vivo

Mice were subjected to myocardial ischemia-reperfusion as described previously [25]. Briefly, after anesthetization (10 mg/kg pentobarbital intraperitoneally) and intubation, the left anterior descending coronary artery was ligated for 60 min with a suture using a snare occluder and then loosened. At 24 h after reperfusion, the suture was retied and Evans blue was systemically injected into mice to determine the non-ischemic tissue. In some experiments, we administered 10 mg/kg TMP or vehicle (PBS) into the right jugular vein at the time of reperfusion.

Determination of myocardial infarct size

The heart was excised, cut, and incubated with 2,3,5-triphenyltetrazolium chloride to determine infarction. The ischemic area [i.e., area at risk (AAR)] and the infarct area (IA) were assessed by computerized planimetry using NIH ImageJ. Ratios of infarct area vs. risk area (IA/AAR) were calculated and expressed as a percentage.

Serum troponin T

The blood concentrations of troponin T were measured as an index of cardiac cellular damage using a quantitative rapid assay kit (Roche Diagnostics GmbH, Mannheim, Germany).

TUNEL assay in vivo

Hearts were harvested by fixing with 4% paraformaldehyde. Paraffin sections (5 μM thickness) were treated as instructed with an *in situ* cell death detection kit (Roche; 1684817) and counterstained with 4', 6-diamidino-2-phenylindole (DAPI). The entire population was visualized under a fluorescence microscope with the DAPI filter (330-380 nm) and the FITC filter (465-495 nm). Apoptotic cells with green fluorescence in the infarction border zone were counted.

Caspase-3 activity assay

The assay was performed as described using the extract of hearts and H9c2 cells, with the results expressed as absorbance at 405 nm per milligram protein. Each experiment was performed in triplicate and repeated at least twice.

Western blotting

Total protein or nuclear extracts were extracted by centrifuging the tubes at 4°C for 15 min at maximum speed to remove debris. A total of 20 mg of protein was loaded into a sodium dodecyl sulfate/polyacrylamide electrophoresis gel for separation, and the proteins were then transferred for 1 h to a nitrocellulose (PVDF) membrane. Hsp70, p65, PUMA, pro-caspase-3,

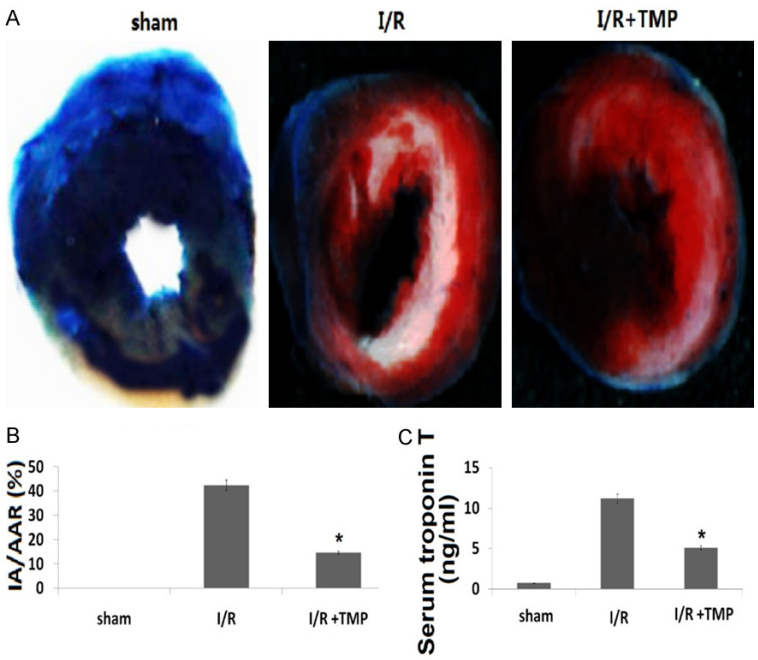

Figure 1. Myocardial infarct size in the different treatment groups. A: Representative images of Evans blue and triphenyltetrazolium chloride (TTC) staining in sham-operated mice, I/R, and TMP treatment groups. Blue-stained portion: non-ischemic, normal region; red-stained portion: ischemic/reperfused, but not infarcted region; unstained portion (white area): ischemic/reperfused, infarcted region. B: The infarct size of the left ventricle (LV) was expressed as a percentage of the area at risk (AAR) of each group. C: Serum cardiac troponin T (cTnT) was measured in different groups at 24 h after I/R. vs. I/R, $^{*}P < 0.05$.

cleaved caspase-3, TNF-α, IL-1β and IL-6 were detected with a primary antibody against Hsp70, p65, PUMA, cleaved caspase-3, TNF-α, IL-1β and IL-6. GAPDH served as a loading control. Protein bands were detected using an enhanced chemiluminescence detection system (Millipore, USA).

RT-PCR

Total RNA was extracted by TriPure Reagent (Aidlab Biotechnologies Co., Ltd., Beijing, China) following the manufacturer's instructions and quantified with a Ultramicro ultraviolet visible light meter (Gene Company, Ltd., Hong Kong, China). cDNA was synthesized from total RNA with an EasyScript First-Strand cDNA synthesis supermix (TransGen Biotech Co., Ltd.) following the manufacturer's instructions. Semi-quantitative PCR was performed using Taq PCR star mix (Beijing GenStar Biosolutions Co., Ltd.) with the following reaction conditions: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. A 10 μl sample of the PCR product was analyzed by electrophoresis, and the gene expression levels were calculated using GAPDH as the internal standard. The primers were designed using Primer 5.0 software and are shown as below: Hsp70: Forward 5'-CCGCCTACTTCAACGACTC-3', Reverse 5'-TCTTGAACTCCTCCACGAAG-3; PUMA: Forward 5'-ATG GCG GAC GAC CTC AAC-3', Reverse 5'-AGT CCC ATG AAG AGA TTG TAC ATG AC-3'; IL-6: forward: 5'-CAAAGCCAGAGTCCTTCAGAG-3', reverse: 5'-GTCCTTAGCCACTCCTTCTG-3'; IL-1β: forward: 5'-ACGGACCCCAAAAGATGAAG-3', reverse: 5'-TTCTCCACAGCCACAATGAG-3'; TNF-α: forward: 5'-CTTCTGTCTACTGAACTTCGGG-3', reverse: 5'-CAGGCTTGTCACTCGAATTTTG-3'; GAPDH: Forward 5'-GCCACATCGCTCAGACAC-3', Reverse 5'-CATCACGCCACAGTTTCC-3'.

ELISA assay

Blood was collected before and after ischemia/reperfusion injury, and serum was prepared. The concentration of TNF-α, IL-1β and IL-6 in serum was determined by ELISA kits (R&D Systems) according to the instructions of the manufacturer. Experiments were repeated three times for verification of results.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from hearts were processed to obtain nuclear extracts. Binding reactions containing 5 μg nuclear extracts, 10 mmol/L Tris-HCl (pH 7.6), 5% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT, 50 mmol/L NaCl, and 3 mg poly(dI-dC) were incubated for 30 min with 5,000 cpm of α-^{32}P-end-labeled double-stranded oligonucleotide in a total volume of 20 μL. The probe was 5'-AGTTGAGGGGACTTTCCAGGC3'. Labeling of the probe was obtained by incubating 5 pmol of oligonucleotide with 10 pmol [α-^{32}P] ATP and 3 UT4 polynucleotide kinase for 30 min at 37°C. The probe was then purified with MicroBIO-Spin P-30 columns.

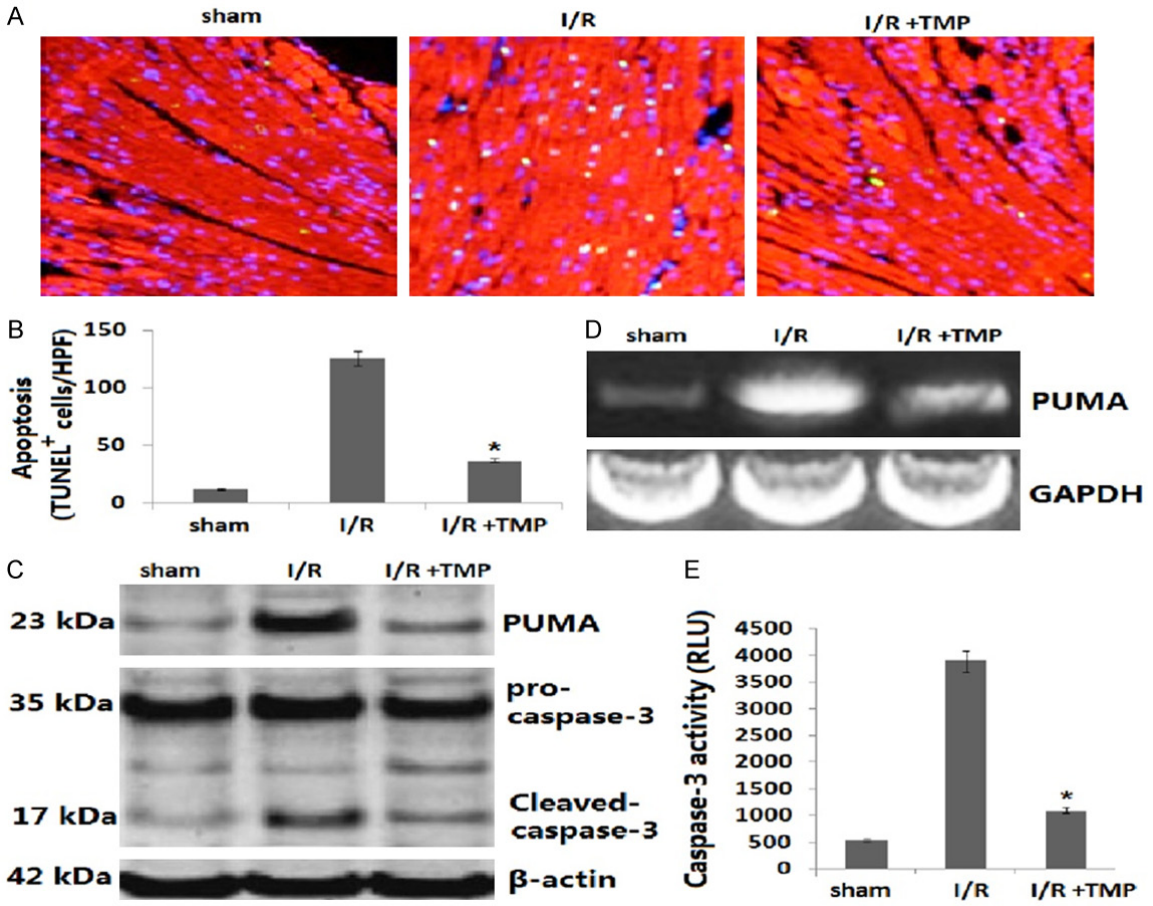

Figure 2. TMP treatment attenuates myocardial apoptosis following I/R injury. A: Representative photomicrographs of TUNEL-positive cells in ischemic cardiac muscle cells (× 200). B: Cardiac myocyte apoptosis was increased after ischemic/reperfusion injury. TMP treatment significantly decreased the number of TUNEL-positive cells in the heart following I/R injury; C: Western blot assay for PUMA and caspase-3. Corresponding β-actin blots are shown as a control for sample loading; D: RT-PCR assay for PUMA, Corresponding GAPDH blots are shown as a control for sample loading; E: Caspase-3 activity assay. Data are mean ± SEM. **P* < 0.01 compared with I/R groups.

Complexes were separated on 60 g/L polyacrylamide gels with 45 mmol/L Tris-borate, 1 mmol/L EDTA, pH 8 buffer. After fixation and drying, gels were exposed on phosphor screens which were then analyzed by phosphor/fluorescence imager STORM 840 (Molecular and Dynamics, Sunnyvale, CA, USA). The intensity of the revealed bands was directly quantified by Image QuaNT software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analyses

All experimental data were replicated a minimum of three times and are expressed as mean ± SEM. Statistical analysis was performed by one way ANOVA using SPSS version 11.0 software, and multiple comparisons were done by Student *t* test. Differences were considered significant at $P \leq 0.05$.

Results

TMP reduces myocardial infarct size following myocardial I/R injury

At the end of a 24-hour reperfusion, myocardial infarct size was assessed using the Evans blue/TTC staining method. As illustrated in **Figure 1A**, **1B**, ischemia followed by reperfusion resulted in development of substantial myocardial infarcts, which were significantly attenuated by TMP treatment (14.6% ± 2.3% *vs.* 41.3% ± 7.4%, $P < 0.05$). The cardiac troponin T (cTnT) level in the serum, a direct index of myocyte damage, was also signifi-

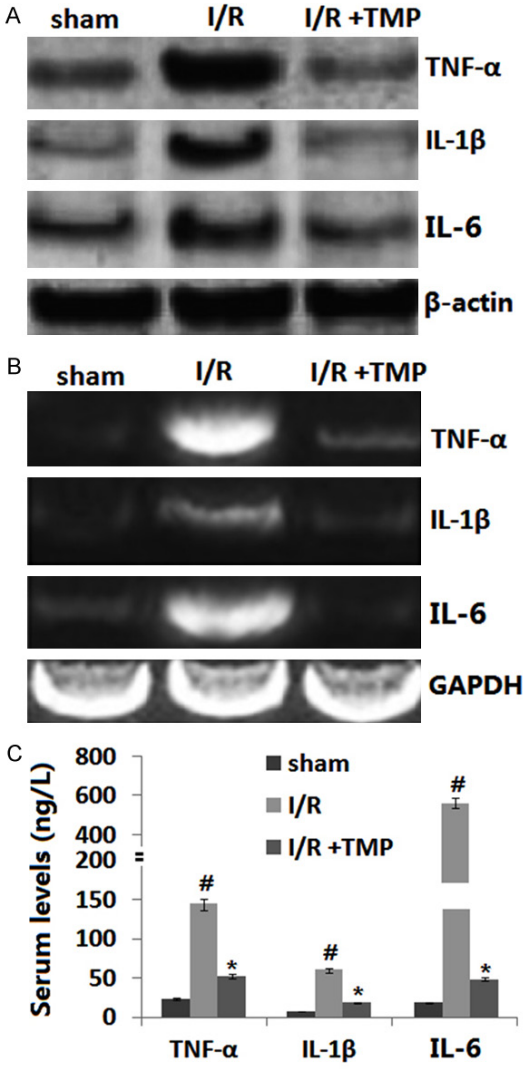

Figure 3. TMP reduces inflammatory cytokine expression. A: Protein expression of TNF-α, IL-6 and IL-1β was detected by Western blot; B: mRNA expression of TNF-α, IL-6, and IL-1β was detected by Western blot; C: Protein level of TNF-α, IL-6 and IL-1β was detected by ELISA assay *vs.* I/R, $^{*}P < 0.01$; *vs.* sham, $^{\#}P < 0.01$.

cantly lower in the TMP treatment group (**Figure 1C**).

TMP attenuates myocardial apoptosis following I/R injury

In order to study myocardial apoptosis following I/R injury and anti-apoptotic effect of TMP treatment, TUNEL assay was performed. As shown in **Figure 2A**, **2B**, cardiac myocyte apoptosis was greatly increased in the I/R group compared with the sham group (26.5 ± 2.8% vs. 1.6 ± 0.3%, $P < 0.01$). TMP treatment significantly decreased the number of TUNEL-positive cells in the I/R group compared with that in I/R group (8.36 ± 1.5% vs. 27.3 ± 3.4%, $P < 0.01$).

TMP blocks pro-apoptotic PUMA signaling

It has been shown that PUMA plays an essential role in apoptosis induced by a variety of stimuli in different tissues through a mitochondrial pathway. In the present study, the expression of PUMA was measured to identify pro-apoptotic cell signaling. PUMA expression was significantly increased at 24 hours following *in vivo* I/R ($n = 6$, $P < 0.01$) but was reduced to TMP treatment at reperfusion ($n = 6$, $P < 0.01$) (**Figure 2C**, **2D**). As an executioner caspase, caspase-3 is activated in apoptotic cells both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. The caspase-3 zymogen exhibits virtually no activity until it is cleaved by an initiator caspase following apoptotic signaling events. As shown in **Figure 2E**, the caspase-3 activity was significantly reduced in the TMP group compared with the I/R group. In addition, cleaved caspase-3 expression was significantly reduced in the TMP group compared to the I/R group (**Figure 2C**). These results demonstrated that administration of TMP significantly alleviates the I/R-induced myocardial cell apoptotic injury in the rats via the activation of PUMA signaling pathway.

TMP treatment reduces IL-1β, IL-6, and TNF-α levels following I/R injury in vivo

Inflammatory cytokines TNF-α, IL-6 and IL-1β were detected by Western blot (**Figure 3A**) and RT-PCR (**Figure 3B**). All the inflammatory cytokine expression levels above were decreased in TMP treated group compared with the I/R group. Serum levels of inflammatory cytokines following I/R were analyzed by ELISA. **Figure 3C** shows that I/R injury increased the levels of serum TNF-α, IL-1β and IL-6, and TMP treatment significantly reduced the TNF-α (144.6 ± 17.4 *vs.* 52.6 ± 10.3 pg/mL), IL-1β (60.4 ± 9.8 *vs.* 18.7 ± 3.6 ng/L) and IL-6 levels (564.4 ± 72.5 *vs.* 198.3 ± 40.6 ng/L) ($P < 0.01$, respectively).

TMP treatment increases Hsp70 expression and reduces NF-κB activity in vivo

To elucidate the mechanisms for *TMP* to regulate the transcriptional gene PUMA and inflam-

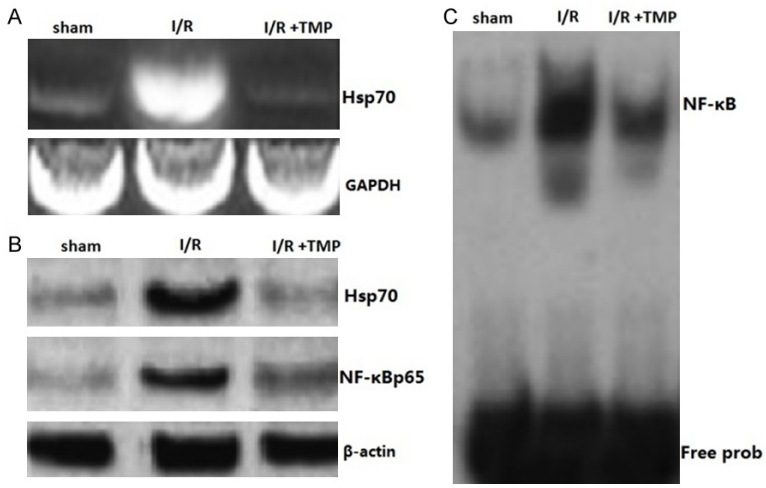

Figure 4. TMP treatment increases Hsp70 expression and reduces NF-κB activity. A: RT-PCR assay; B: Western blot assay; C: ESMA assay.

matory cytokine expression, we examined activation of Hsp70 and NF-κB, which play critical roles in controlling the expression of PUMA and inflammation-related genes. Our results showed that under TMP treatment, Hsp70 was increased by RT-PCR (**Figure 4A**) and Western blot (**Figure 4B**) assay. However, p-65 phosphorylation protein (**Figure 4B**) and NF-κB activity (**Figure 4C**) were reduced. Together, these results suggest that TMP treatment induces Hsp70 expression, resulting in NF-κB inactivity and downregulation of PUMA.

H9c2 cell apoptosis induced by I/R in vitro by NF-κB/PUMA signal

To further verify the protective effect of TMP to reduce apoptosis during I/R, we cultured rat H9c2 cells, the most commonly used heart cell line for *in vitro* studies of I/R. We confirmed that PUMA and NF-κB p65 expression was up-regulated both at the mRNA and protein levels in H9c2 cells during I/R *in vitro* (**Figure 5A**, **5B**). Cleaved caspase-3 expression was also significantly increased in the H9c2 cells during I/R *in vitro* (**Figure 5B**).

To detect cell apoptosis and death, we double stained cells with Annexin-V-FITC and PI, and then conducted flow cytometry. As shown in **Figure 5C**, cell apoptosis in the 24 h I/R group was increased compared to the control group. The data suggests that I/R induced H9c2 cells apoptosis during I/R *in vitro*. A recent study reported that I/R induced apoptosis by activation of PUMA expression. We transfected H9C2 cells with PUMA siRNA for 24 h and then exposed these transfected cells to the I/R environment. PUMA siRNA significantly down-regulated PUMA expression (**Figure 5A**, **5B**). PUMA siRNA also decreased I/R induced cell apoptosis/death (**Figure 5C**). It has been reported that activation NF-κB leads to cell apoptosis by up-regulation of PUMA. Therefore, we transfected H9C2 cells with p65 siRNA for 24 h and then exposed these transfected cells to the I/R environment. P65 siRNA significantly down-regulated the expression of p65 and PUMA (**Figure 5A**, **5B**). p65 siRNA also decreased I/R induced cell apoptosis/death (**Figure 5C**). The data further demonstrate the effect of I/R induced cell apoptosis by activation of NF-κB/PUMA signal.

TMP protects H9c2 cell from I/R induced apoptosis in vitro by activation of Hsp70 and inhibits the NF-κB/PUMA signal

We found that Hsp70 was increased in H9c2 cells during I/R injury when treated with 100 uM TMP (**Figure 5D**, **5E**). We transfected H9C2 cells with Hsp70 siRNA for 24 h and then exposed these transfected cells to I/R environment and treated with TMP. Hsp70 siRNA significantly down-regulated expression of Hsp70 in the TMP treated H9c2 cells (**Figure 5D**, **5E**); Furthermore, p65 and PUMA were also decreased followed by Hsp70 inhibition (**Figure 5D**, **5E**).

We also found that TMP decreased I/R induced cell apoptosis/death in H9C2 cells (**Figure 5C**). However, Hsp70 siRNA restored I/R induced cell apoptosis/death in H9C2 cells treated with TMP (**Figure 5C**). The data further demonstrate that TMP prevents cell apoptosis against I/R is associated with activation of Hsp70 and inhibits NF-κB/PUMA signaling.

Discussion

The present study reports novel finding related to the signaling pathways by which TMP exerts its cardioprotective activity in experimental

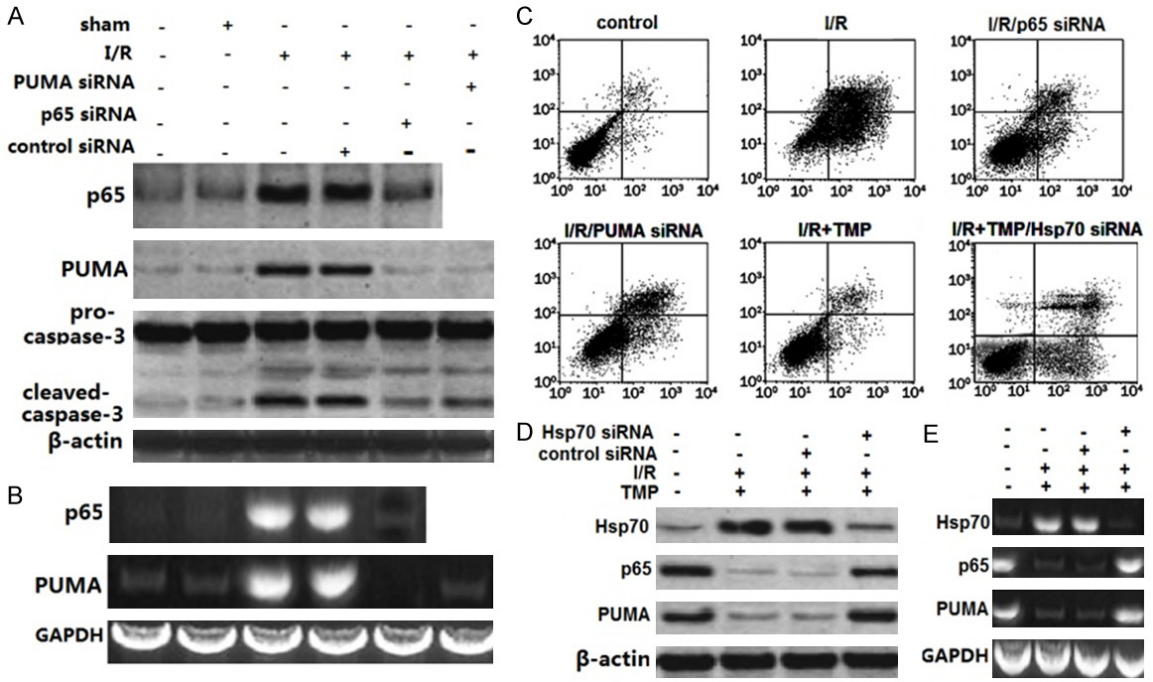

Figure 5. TMP prevents H9c2 cell apoptosis induced by I/R *in vitro* by Hsp70/NF-κB/PUMA signal. H9C2 cells (80,000 cells/well) were plated in six well plates and allowed to culture at 37°C 5% CO_2 overnight. Cells were infected with Hsp70 siRNA or p65 siRNA or PUMA siRNA for 24 h. Cells were then subjected to a hypoxia chamber with 0% O_2 and 15% CO_2 at 10°C for 16 h, followed by 24 h reperfusion at 5% CO_2, 28% O_2 at 37°C. Then the cells were treated with 100 Um TMP for 24 h. A, D: Protein expression by Western blot assay; B, E: Gene expression was detected by RT-PCR; C: Cell apoptosis was detected by double staining with FITC labeled Annexin-V and PI and flow cytometry. $^{*}P < 0.01$.

model of IR *in vivo*. We have shown that TMP prevents the development and progression of IR injury primarily through the activation of Hsp70 pathway. Moreover, our study provides substantial evidence that TMP exerts potent anti-inflammation response and antiapoptotic effects as inferred by reduced of NF-κB p65 dependent TNF-α, IL-1β and IL-6 levels and downregulation of NF-κB p65 dependent pro-apoptotic PUMA expression.

Recently, the relationship between the inflammatory reaction and myocardial R/I has become well-established. The influence of TMP on decreasing myocardial I/R-induced effects on activation of production of inflammatory cells and pro-inflammatory mediators has been confirmed [26]. In myocardial I/R, high levels of free radicals activates p38 mitogen activated protein kinase (p38MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-B), both of which induce the generation of TNF-α and IL-6 [27]. Shang et al. reported that TMP could decrease p38MAPK activity, inhibit the expression of TNF-α and IL-6, and thereby protect the myocardium [28].

Our study showed that pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) were impeded by the TMT treatment in an I/R model. Furthermore, we observed that TMP increased Hsp70 expression and inhibited phosphorylation of RelA (p65), a member of the NF-κB family. A previously study has reported that TMP treatment protected against anoxia-reoxygenation injury in neonatal rat cardiomyocytes by increasing the Hsp70 expression [24]. Yao et al. have reported that LPS-induced cardioprotection is mediated partly through inhibition of NF-κB via increased HSP70, and LPS pretreatment could provide a means of reducing myocardial I/R injury [21]. Our data suggest that the attenuation of inflammation by TMP is mediated by the increase of Hsp70 expression and the inhibition of the NF-κB signaling pathway. Overall, our study shows a new circumstance in which TMP protects against inflammation, and further supports TMP as a cardio protective agent against inflammation under ischemic conditions.

It is thought that apoptosis has a key role in mediating cell death after I/R [29, 30]. For quantitative analyses of myocardial apoptosis,

TUNEL staining is used widely to detect DNA damage. Caspases are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis. Cells possess multiple caspases, of which caspase-3 activity is required at the step at which a protease cascade pathway converges [31]. Hence, caspase-3 is another widely used biochemical marker for detecting apoptosis. In our study, we found that the number of apoptotic cells was decreased in TMP groups as detected by TUNEL assays. In addition, caspase-3 was activated by TMP treatment. The expression of apoptotic genes PUMA were also reduced in hearts with TMP treatment, compared to controls. Additionally, our *in vitro* result with H9C2 cells showed that, TMP treatment reduced H9C2 cell apoptosis under hypoxia/reperfusion stress.

In addition, *in vitro I/R in* H9C2 cells activated NF-κB and increased PUMA expression and cleaved caspase-3 expression. Targeting NF-κB p65 or PUMA inhibited I/R-induced apoptosis in H9C2 cells. In addition, targeting NF-κB p65 reduced PUMA expression in H9C2 cells, suggesting that I/R induced apoptosis in H9C2 cells by activation of NF-κB/PUMA signal. Our study also showed that TMP treatment increased the Hsp70 expression in protein and mRNA levels as compared to the controls. Cell apoptosis can be reversed by targeting Hsp70 expression in TMP treated H9C2 cells. Additionally, our *in vitro* result with H9C2 cells showed that targeting Hsp70 restored I/R-induced NF-κB activation and PUMA upregulation. According to our *in vivo* and *in vitro* studies, we found that TMP could reduce myocardial injury induced by I/R by inhibiting apoptosis and pro-inflammatory mediators, which was associated with activation of Hsp70 and inhibited NF-κB and NF-κB/PUMA signaling.

In conclusion, TMP protects heart cells against apoptosis induced by the myocardial I/R model. The ability of TMP to prevent cell apoptosis suggests that TMP has potential therapeutic value in preventing myocardial I/R.

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Disclosure of conflict of interest

None.

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