

Post-conditioning anti-PUMA treatment protects mice against mice heart I/R injury

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Abstract. – OBJECTIVE: PUMA is a pro-apoptotic gene, which has been found to be critical to the pathogenesis during heart ischemia-reperfusion injury (IRI). We investigate whether anti-PUMA protect mice from acute heart failure.

MATERIALS AND METHODS: Mice were subjected to 30 min ischemia and 24 hrs reperfusion in the presence or absence of anti-PUMA. Treated mice were evaluated for heart PUMA protein and mRNA expression, and apoptosis by terminal deoxy nucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining.

RESULTS: In mice, anti-PUMA post conditioning markedly reduced PUMA mRNA and protein expression in the heart 4-fold. Hearts from mice that received anti-PUMA had substantially fewer heart muscles apoptosis by TUNEL staining. Anti-PUMA post-conditioning greatly reduced infarct size to 14.4±3.7%, from 38.2±3.9% in the untreated I/R group. Furthermore, survival experiments revealed that more than 90% of control mice died from lethal I/R, whereas 20% of the anti-PUMA post-treated mice survived until the end of the 10-day observation period.

CONCLUSIONS: This study confirms the importance of PUMA-mediated apoptosis in heart ischemia-reperfusion injury. Silencing PUMA by recombinant PUMA has therapeutic promise to limit ischemia-reperfusion injury.

Key Words:

Heart, Ischemia-reperfusion injury, PUMA, Apoptosis.

el therapeutics^{3,4}. However, none of these methods has yet been transferred to clinical practice or tested for their potential untoward complications.

PUMA (p53 upregulated modulator of apoptosis) is a pro-apoptotic member of the BH3-only subgroup of the Bcl-2 family and a critical mediator of p53-dependent and -independent apoptosis induced by a wide variety of stimuli⁵⁻⁶. It has reported that PUMA was the critical for cardiomyocyte death upon ischemia-reperfusion (I/R) of the heart⁷. Nickson et al⁸ has found that PUMA is a critical component of ER stress-induced apoptosis in cardiac myocytes, and inhibition of PUMA activity by adenoviral delivery of shRNA or eliminated by PUMA deletion in knockout mice may be used to treat cardiac infarcts or prevent heart failure by blocking ER stress-induced apoptosis. Recently, it has found⁹ that ischemia and reperfusion (I/R) can induce PUMA regulation, followed by the apoptosis of astrocytes. However, targeted down-regulation of PUMA by siRNA transfection significantly decreased the I/R-induced apoptosis of primary cerebral astrocytes.

In the present study, we aimed to examine whether PUMA silencing by anti-PUMA as a pharmacological post conditioning agent at the onset of reperfusion to provoke cardio protection.

Materials and Methods

Ethics Statement

All animal protocol was performed according to the guide for the Care and Use of Laboratory Animals in the Affiliated Hospital of Qingdao University. We strictly conform to the guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH, Bethesda, MD, USA).

Materials

Anti-PUMA antibody was purchased from R&D Systems (Minneapolis, MN, USA). The an-

Introduction

Acute myocardial infarction (AMI) is one of the important causes of mortality and morbidity in the world¹. Myocardial ischemia/reperfusion (I/R) injury has been the important clinical problem with significant morbidity and mortality². Current reperfusion therapies included thrombolysis, coronary angioplasty, and coronary bypass surgery. Novel pharmacological post-conditioning or molecular interventions have generated considerable interest for the development of nov-

ti-PUMA was purchased from Sigma Company (St Louis, MO, USA) and anti- β -actin was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA).

Animals

Male Wistar rats were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were housed in cages with free access to water and food. 40 male mice weighing 220-230 g were used.

Ischemia/Reperfusion

The mice were anesthetized using intraperitoneal pentobarbitone (50 mg/kg), then subjected to I/R or sham operation as described before¹⁰. Briefly, mice were anesthetized intraperitoneally injected with 100 mg/kg ketamin, and each mouse was subjected to left thoracotomy. An 8-0 suture was looped under the left descending coronary artery for the induction of a coronary artery occlusion for 30 min followed by 24 hrs of reperfusion. Anti-PUMA agents (100 ng/mL) were started at the time of reperfusion. The anti-PUMA used in this study was chosen according to our published observations.

Western Blot

Proteins were extracted from the heart ventricles of wild-type and ApoE2/2 mice in modified RIPA buffer [Tris-HCl (pH8.0) 20 mmol/L, NaCl 1 mmol/L, NP-40 1%, NaF 50 mmol/L, Na-ortho-vanadate 10 mmol/L, phenylmethylsulfonyl fluoride 1 mmol/L, ethylenediaminetetraacetic acid 5 mmol/L, sodium dodecyl sulfate 0.25%, Na-deoxycholate 1%, and cocktail of protease inhibitors), as previously described¹¹. Western blotting was performed using antibodies against Cx43 (1/1000, BD Transduction Laboratories, Franklin Lakes, NJ, USA), Cx40 (1/500, Alpha Diagnostic International, San Antonio, TX, USA), or Cx37 (1/1000, Alpha Diagnostic International).

Quantitative RT-PCR

Total RNA was extracted using a Trizol reagent (Qiagen, Valencia, CA, USA) from the heart tissue and quantified using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA)¹². DNA (cDNA) was synthesized through random hexamer primed reactions using the TaqMan Universal Master Mix according to the manufacturer's protocol. Quantitative real-time polymerase chain (qRT-PCR) reactions were carried out in a 7300

Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The following primers and TaqMan probes (Applied Biosystems) were used: PUMA: 5'-AGTTGAGGGGACTTTCCAG-GC-3' 3'-TCAACTCCCCTGAAAGGGTCCG-5'; β -actin: 5-CAA CTG GGA CGA CAT GGA GA-3 and 5-CAG GCA GCT CGT AGC TCT TC-3. qRT-PCR (20 μ l reaction volume) was performed as follows: 95°C for 30 sec followed by 36 cycles at 95°C for 5 sec, 56°C for 32 sec, and 72°C for 42 sec, and then melt curve analysis at 95°C for 15 sec then 60°C for 1 min. Each PCR reaction was conducted in triplicate, and controls without template were included. All measurements were performed in duplicate. In addition, melting curve analysis was performed in each assay in order to detect non-specific amplifications.

Terminal Deoxynucleotidyl Transferase dUTP nick-end Labeling (TUNEL) Assay

Cell apoptosis was detected by TUNEL staining as Ootsuji et al's method¹³. Briefly, specimens were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C, permeabilized with 0.1% Triton X-100, then incubated with anti-sarcomeric actinin antibody for 60 minutes followed by incubation with Alexa Fluor 594-conjugated anti-mouse IgG antibody (Life Technologies). Specimens were then incubated with TUNEL staining solution for 1 hour according to the manufacture's protocol. DAPI was used for nuclear staining. TUNEL-positive cardiomyocytes were counted in randomly selected three fields of the slide.

Infarct Size

Infarct size was determined by triphenyl tetrazolium chloride (TTC) technique¹⁴. In brief, at the end of the experimental protocol, slices were exposure to 1% TTC in sodium phosphate buffer (pH 7.4) at 37°C for 10 min to stain the survival cells in the zone at risk in red, and the slices were post-fixed in 10% formaldehyde solution for another 24 h. The heart slices were then digitally photographed for planimetry using NIH Image 1.62. The area at risk was expressed as a percentage of whole ventricular volume in this global ischemic mode, and the infarcted area was expressed as a percentage of the area at risk.

Statistical Analysis

SPSS 11.0 (SPSS, Inc., Chicago, IL, USA) statistical software was used in the statistical analysis. Results are expressed as mean \pm SD. Fish-

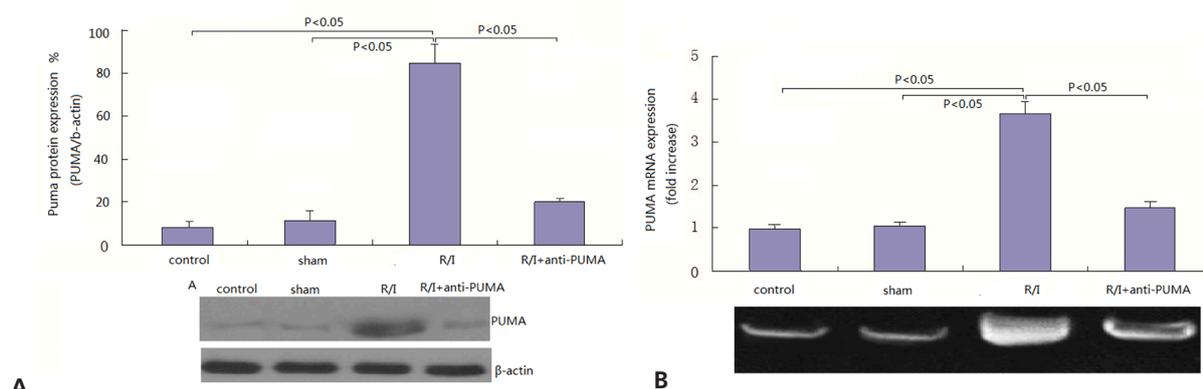


Figure 1. PUMA is activated in myocardial cells after 30-min ischemia and 24-hrs reperfusion (I/R). **A**, The PUMA expression in myocardial cells 24 hrs after I/R or anti-PUMA treatment was determined by Western blot assay. **B**, The PUMA mRNA expression in myocardial cells 24 hrs after I/R or anti-PUMA treatment was determined by quantitative RT-PCR assay. Data represent means from at least 5 mice each. * $p < 0.05$.

er exact test and Student's *t*-test were used for between-group comparisons. A *p*-value < 0.05 indicated that the difference was statistically significant.

Results

PUMA is Activated in Myocardial Cells After Ischemia-Reperfusion

We examined the impact of I/R injury on the PUMA expression in myocardial cells. Mice were subjected to 30 min of ischemia and a subsequent 24 hrs of reperfusion. The expression levels of PUMA protein and PUMA mRNA were upregulated significantly (Figure 1A-B). However, the expression levels of PUMA protein and PUMA mRNA were significantly less in the I/R with PUMA treatment groups (Figure 1A-B). These results suggest that PUMA is activated in myocardial cells following I/R, and treatment with recombinant PUMA could reduce the PUMA levels after I/R.

Post-Conditioning with Anti-PUMA Induces Cardioprotection

As shown in Figure 2A, anti-PUMA post-conditioning significantly reduced infarct size to $14.4 \pm 3.7\%$, from $38.2 \pm 3.9\%$ in the untreated control I/R group ($p < 0.01$, $n = 10$).

Post-Conditioning with anti-PUMA Inhibits Apoptosis in Myocardial Cells

As shown in Figure 2B, anti-PUMA post-conditioning significantly decreased apoptosis to $2.14 \pm 0.4\%$ from $7.28 \pm 1.3\%$ in the untreated control I/R group ($p < 0.01$, $n = 10$).

Post-Conditioning with Anti-PUMA Increased the Survival

After 10 days observation, survival experiments revealed that 90% of control mice died from lethal I/R, whereas 10% of the anti-PUMA post-treated mice died ($p < 0.01$).

Table I. Post-conditioning with anti-PUMA increased the survival.

	Survival mice (n)										
	0 d	1d	2d	3d	4d	5d	6d	7d	8d	9d	10d
I/R (n=10)	10	10	9	7	7	6	5	3	2	1	1
Anti-PUMA (n=10)	10	10	10	10	9	9	9	8	8	8	8*

Vs control I/R, * $p < 0.01$

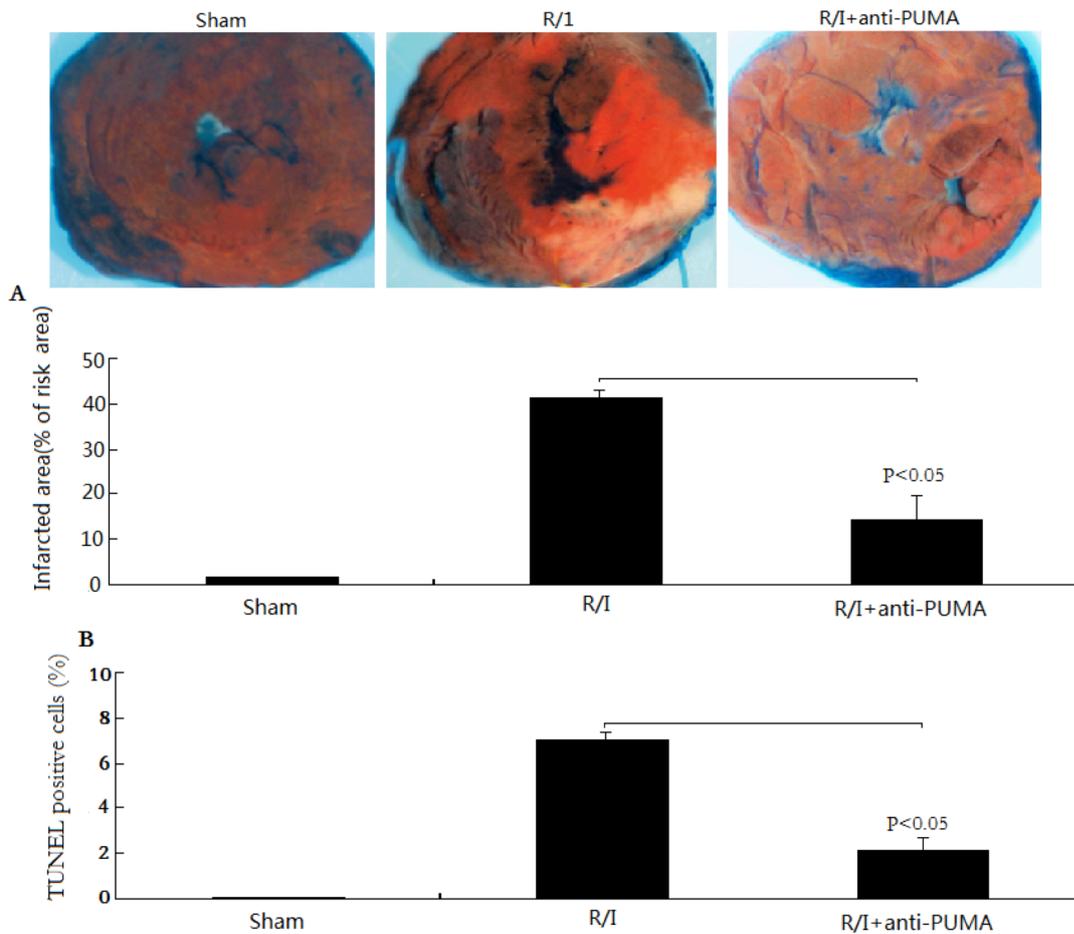


Figure 2. Anti-PUMA post-conditioning induced cardioprotection. A, Infarct size is expressed as a percentage of area at risk, which is the whole transverse section of the whole ventricle. Representative transversely sectioned heart slices are displayed on the top panel. Infarcted areas are seen after TTC staining. B, TUNEL was used to detect apoptosis in heart slices. Data are presented as mean \pm standard deviation (SD); $n=10$ per group.

Discussion

Perfusion is the main treatment method for ischemic myocardial disease, however, ischemia-perfusion (I/R induced injury has become a complicated obstacle for effective heart therapy⁴. Therefore, the mechanism underlying I/R injury has become a crucial focus of cardiovascular research.

NF- κ B is a well-known transcription factor that is intimately involved with inflammation and immunity¹⁵. It is also involved in the process of inflammation and apoptosis induced by I/R injury, by which the NF- κ B subunit p65 is activated¹⁶. Accordingly, the suppression of NF- κ B by its inhibitor BAY 11-7802 or the inhibition of I κ B phosphorylation has been validated to reduce the

inflammation and apoptosis induced by myocardial or cerebral I/R injury^{17,18}.

Wang et al¹⁹ has found that PUMA is a direct target of NF- κ B and mediates TNF- α -induced apoptosis in vitro and in vivo. Li et al²⁰ has found that H₂O₂ treatment led to increases in NF- κ B activity, PUMA protein levels in cardiac H9c2 cells, and the induction of PUMA was mediated by the p65 component of NF- κ B. We, therefore, suggested that knockdown of PUMA would inhibit NF- κ B activity-induced apoptosis.

In the present study, we have performed 30 mini ischemia followed by 24 h reperfusion in vivo. The results showed that the expression levels of PUMA protein and PUMA mRNA were significantly upregulated followed by increased apoptosis and myocardial infarct. However, an-

ti-PUMA post-conditioning markedly reduced PUMA mRNA and protein expression. Hearts from mice that received anti-PUMA had substantially fewer heart muscles apoptosis and reduced infarct size. Furthermore, anti-PUMA post-conditioning greatly increased the survival.

Conclusions

Anti-PUMA induces cardioprotection when infused into the heart during reperfusion, anti-PUMA treatment proves to be extremely and consistently beneficial whenever it is delivered to the heart, establishing its substantial promises for being developed into a robust therapeutic strategy for acute myocardial infarction.

Acknowledgement

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Conflicts of interest

The authors declare no conflicts of interest.

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