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Effect of ischemic postconditioning on cell apoptosis and expression of relevant genes in non-culprit coronary arteries

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ABSTRACT

This study was performed to determine the effect of ischemic postconditioning on cell apoptosis and angiotensin II receptor type 1 (AT1), connexin 43 (Cx43), and β -tubulin mRNA expression in non-culprit arteries. Non-culprit arterial tissues were isolated from a rabbit myocardial ischemia-reperfusion model and randomly divided into sham, ischemia-reperfusion, and ischemic postconditioning groups. Cell apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Expression of angiotensin II, AT1, Cx43, and β -tubulin mRNA was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). TUNEL analysis indicated significantly higher ratios of apoptotic cells in the ischemia-reperfusion group than in the sham group. However, significantly fewer apoptotic cells were observed in the ischemic postconditioning group than in the ischemia-reperfusion group. The qRT-PCR results indicated significantly higher expression of AT1, Cx43, and β -tubulin mRNA in the ischemia-reperfusion group than in the sham group. However, expression of AT1, Cx43, and β -tubulin was lower in the ischemic postconditioning group than in the ischemia-reperfusion group. The ratios of apoptotic cells and mRNA expression of AT1, Cx43, and β -tubulin in non-culprit arteries were increased after ischemia-reperfusion. Ischemic postconditioning may decrease these features and inhibit the progression of non-culprit arteries.

INTRODUCTION

The fundamental treatment strategy of ST-segment elevation myocardial infarction (STEMI) is the earliest possible restoration of myocardial perfusion.¹ Primary percutaneous coronary intervention (PPCI) is the most efficient treatment for STEMI. PPCI has good success rates in restoring blood flow and low rates of infarction or recurrent ischemia, which significantly improves patients' quality of life and prevents further myocardial necrosis.^{2,3} However, approximately 40%–65% of patients with STEMI present with three-vessel lesions. Additionally, recent clinical studies have shown that PPCI can lead to the progression of non-culprit lesions, which might be the

Significance of this study

What is already known about this subject?

- ▶ Primary percutaneous coronary intervention can lead to the progression of non-culprit lesions in patients with ST-segment elevation myocardial infarction, which might be the most significant factor influencing the prognosis after primary percutaneous coronary intervention.
- ▶ Non-culprit lesion progression can be affected by many factors, such as increased levels of catecholamines and activation of the angiotensin II/mitogen-activated protein kinase/connexin 43 (AgII/MAPK/Cx43) pathway.
- ▶ Ischemic postconditioning may inhibit the AgII/MAPK/Cx43 pathway and non-culprit lesion progression. However, the mechanism by which ischemic postconditioning affects the progression of non-culprit lesions has not been examined.

What are the new findings?

- ▶ The ratios of apoptotic cells and mRNA expression of AgII receptor type 1 (AT1), Cx43, and β -tubulin in non-culprit arteries were increased after ischemia-reperfusion.
- ▶ Ischemic postconditioning may decrease these features and inhibit the progression of non-culprit lesions.

How might these results change the focus of research or clinical practice?

- ▶ Apoptosis and mRNA expression of AT1, Cx43, and β -tubulin in non-culprit coronary arteries were increased after ischemia-reperfusion.
- ▶ Ischemic postconditioning may decrease apoptosis and the expression of these factors in non-culprit coronary arteries, thus inhibiting their pathological progression.
- ▶ In clinical practice, ischemic postconditioning may be used to inhibit non-culprit lesion progression.

most significant factor influencing the prognosis after PPCI.^{4,5} The molecular mechanisms of myocardial ischemia-reperfusion (IR) involve myocyte apoptosis, increased

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Figure 1 Experimental flowchart. IP group: ischemic postconditioning group; IR group: ischemia-reperfusion group.

catecholamine levels, and activation of the angiotensin II/mitogen-activated protein kinase/connexin 43 (AgII/MAPK/Cx43) pathway.⁶ The reason for the progression of non-culprit lesions in patients with STEMI after PPCI is not clear, but chronic inflammation and sustained stress may be involved in the progression of non-culprit lesions.⁴ Our recent experimental study showed that non-culprit lesion progression can be affected by many factors, such as increased levels of catecholamines and activation of the AgII/MAPK/Cx43 pathway.⁶ In addition, Sun *et al*⁷ found that ischemic postconditioning (IP) may inhibit the AgII/MAPK/Cx43 pathway. Our recent clinical study⁸ showed that IP may inhibit non-culprit lesion progression. However, the mechanism by which IP affects the progression of non-culprit lesions has not been examined. We herein report the effect of IP on cell apoptosis and the expression of angiotensin II receptor type 1 (AT1), Cx43, and β -tubulin mRNA in non-culprit coronary arteries of rabbits.

METHODS

Animal model of hyperlipidemia

The requirement for informed consent in this study was exempted by the board. As shown in [figure 1](#), 40 healthy male rabbits were randomly divided into a hyperlipidemia group (n=30) and control group (n=10). The rabbits in the hyperlipidemia group were fed a high-fat diet for 80 days, while the rabbits in the control group were fed a normal diet for 80 days. Auricular vein blood samples were taken on the 81st day after a 12 hours fast. The serum was separated after centrifugation to measure the serum level of total cholesterol. Rabbits with a serum total cholesterol level of three times higher than the control group (1–2 mmol/L) were selected from the hyperlipidemia group and used for myocardial IR modeling.

Animal model of myocardial IR

An animal model of acute myocardial ischemia was prepared according to the following operation. Because of the long and steady maintenance of anesthesia, all rabbits were administered intraperitoneal anesthesia using urethane sodium at a dosage of 1 g/kg. In addition, all rabbits were administered 1 mg/kg of lidocaine to prevent

ventricular fibrillation. In the control group, thoracotomy was performed on 10 hyperlipidemic rabbits without coronary artery ligation. Another 10 hyperlipidemic rabbits underwent thoracotomy alone. After exposing the pericardium and heart, the left anterior descending coronary artery (LAD) was clamped with 5/0 string and maintained for 30 min. An electrocardiographic monitor was connected subcutaneously (25 mm/s, 10 mm/mV). The model was defined as successfully prepared and thereafter maintained for 30 min under the following conditions: (1) ST elevation or necrotic Q wave observed in corresponding lead on the ECG and (2) dark purple myocardium in the corresponding region. After reperfusion was performed by releasing the clamp, the abdominal incisions were closed. All rabbits were fed a normal diet 1 week after the operation.

Experimental animal groups were as follows: sham group (n=10, thoracotomy without coronary artery ligation), IR group (n=10, thoracotomy, LAD ligation for 30 min, and reperfusion for 1 week), and IP group (n=10, thoracotomy, LAD ligation for 30 min, six cycles of 10 s reperfusion and 10 s ischemia, and reperfusion for 1 week).

H&E staining and observation

Coronary arterial tissues were harvested for H&E staining and observation. The tissues were embedded in paraffin, discontinuously and serially sectioned, stained with H&E, and observed under a light microscope. The thickness of each section was 4 μ m. All sections were analyzed with a medical imaging analysis system to measure the thickness of the plaques.

Ratios of apoptotic cells in non-culprit coronary arterial tissues

The right coronary arterial tissues were subsequently embedded in paraffin. The coronary arterial smooth muscle tissues were cut into 10- μ m-thick slices with a freezing microtome and immediately fixed to a glass slide. The slices were stored at room temperature for 5 min, fixed in 4% paraformaldehyde, sealed with methanol solution containing 3% hydrogen peroxide, and immersed into 0.1% Triton X-100 and 0.1% sodium

nitrate. The slices were then washed with 1×phosphate-buffered saline (137mmol/L NaCl, 2.7mmol/L KCl, 4.3mmol/L Na₂HPO₄, and 1.4mmol/L KH₂PO₄; pH 7.4). Next, the slides were drained and blocked with goat serum for 30 min and then washed with 1× phosphate-buffered saline for 30 min. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) reaction solution was prepared by mixing an end-labeling enzyme and labeling the liquid at a ratio of 1:9. The slices were covered with the TUNEL reaction solution and incubated for 60 min in a moist chamber at 37°C. Negative controls were incubated with labeling liquid without an end-labeling enzyme instead of the TUNEL reaction solution. Positive controls were incubated with DNaseI at a concentration of 5.1 U/mL for 10 min at room temperature before adding the TUNEL reaction solution. The samples were blocked with glycerol and observed using a fluorescence microscope (Olympus IX71; Olympus, Tokyo, Japan). Normal nuclei were labeled with blue fluorescence by 4',6-diamidino-2-phenylindole (DAPI) dye, whereas apoptotic nuclei were labeled with red fluorescence by the TUNEL reagent. Each sample was counted in at least three fields (200× magnification). Apoptosis was determined by the number of positive nuclei per muscle cell. The ratio of apoptotic cells was obtained as the ratio of TUNEL-positive to DAPI-positive cells.

Quantitative real-time polymerase chain reaction for expression of AT1, Cx43, and β-tubulin mRNA in non-culprit coronary arterial tissues

TRIzol and chloroform reagents were used to extract total RNA from vascular smooth muscle cells according to the manufacturer's instructions. Briefly, after the addition of 2 mL of TRIzol reagent to lyse vascular smooth muscle cells for 20 min, the sample was transferred to a 2 mL Eppendorf tube. Next, 400 μL of chloroform was added. The tube was shaken vigorously for 30 s and allowed to stand for 15 min. Next, the sample was centrifuged at 13,000 ×g for 15 min at 4°C. The supernatant from the final extraction step was transferred to a clean 2 mL Eppendorf tube, in which RNA was precipitated with 500 μL of isopropanol at −20°C for 2 h. Precipitated RNA was collected by centrifugation at 13,000 ×g for 15 min at 4°C, and the pellet was washed with 1 mL of 75% ice-cold ethanol. The RNA pellet was resuspended in 20 μL of nuclease-free water, and the two duplicate tubes were combined. The RNA concentration was measured using a spectrophotometer (Biolab ND-1000; Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm. RNA purity was assessed by determining the A260/A280 ratio. Purified RNA with an A260/A280 ratio of 1.7–2.0 was used in this study. Complimentary DNA was synthesized from RNA using reverse transcriptase and a PrimeScript

reverse transcriptase kit (Cat. No. AB-1455/A, Fermentas; Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a 20 μL reaction volume containing 10 μL of SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), 0.4 μL of ROX Reference Dye II, 0.8 μL each of forward and reverse primer (table 1), 2 μL of complementary DNA, and 6 μL of nuclease-free water. Reactions were run on a 7500 RT-PCR system (Thermo Fisher Scientific) for 45 cycles at 95°C for 15 s, followed by 60°C for 1 min. Specific PCR products were confirmed by melting-curve analysis with glyceraldehyde 3-phosphate dehydrogenase as an internal control. Gene expression levels were standardized against glyceraldehyde 3-phosphate dehydrogenase.

Cx3 expression in smooth muscle cells in non-culprit arteries

Smooth muscle cells from the four groups were separated, and total proteins were isolated from the smooth muscle cells. Equal amounts (20 μg) of protein samples were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% milk in Tris-buffered saline and 0.1% Tween 20 (TBS-T) for 1 hour and then incubated with primary antibody to Cx43 (1:200; USCN Life Science, Wuhan, China) or β-actin (1:200; USCN Life Science) as an internal control overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:10,000; USCN Life Science) for 1 hour at 25°C. After washing three times with TBS, the membrane was developed using an enhanced chemiluminescent detection system (Amersham Biosciences, Piscataway, New Jersey, USA). Signal intensities were quantitated using Quantity One software (Bio-Rad, Hercules, California, USA). Cx43 expression was quantitated by the Cx43/β-actin optical absorption ratio.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 statistical software (IBM, Armonk, New York, USA). Descriptive statistical data are expressed as mean±SD. Statistical significance was determined using one-way analysis of variance and the least significant difference test. P<0.05 was considered statistically significant.

RESULTS

H&E staining of non-culprit arterial tissues

H&E staining indicated that the structures of non-culprit artery tissues were clear and that the alignment of vascular smooth muscle fibers was regular in each group. However,

Table 1 Primer sequences for RT-PCR			
Gene name	GenBank accession no.	Forward primer 5'–3'	Reverse primer 5'–3'
AT1	NM_030985.4	TCTGACATCGTGGACACTGC	CGTAGACAGGCTTGAGTGGG
Cx43	NM_012567.2	GACTGCTTCTCTCACGTCC	CGCGATCCTTAACGCCCTTG
β-tubulin	NM_139254.2	CTGCTCATCAGCAAAGTGCG	TGCGGAAGCAGATGTCGTAG

Figure 2 H&E staining of non-culprit arterial tissues (400×). (A) Sham group. (B) Ischemia-reperfusion group. (C) Ischemic postconditioning group.

significant atherosclerotic plaques were present in the IR and IP groups compared with the sham group (figure 2).

Ratios of apoptotic cells in non-culprit coronary arterial tissues

As shown in figure 3, a higher ratio of apoptotic cells was observed in non-culprit coronary arterial tissues of the IR group than sham group (46.11 ± 5.92 vs 3.77 ± 6.31 , $p < 0.001$). However, a lower ratio of apoptotic cells was observed in non-culprit coronary arterial tissues in the IP group than IR group (28.36 ± 9.41 vs 46.11 ± 5.92 , $p < 0.001$).

AT1, Cx43, and β -tubulin mRNA expression in non-culprit coronary arterial tissues

Expression of AT1 (30.576 ± 1.760 vs 1.002 ± 0.068 , $p < 0.01$), Cx43 (15.171 ± 1.736 vs 1.009 ± 0.133 , $p < 0.01$), and β -tubulin (1.361 ± 0.042 vs 1.003 ± 0.083 , $p < 0.01$) mRNA in the IR group was significantly higher than that in the sham group. However, expression of AT1 (4.697 ± 0.227 vs 30.576 ± 1.760 , $p < 0.01$), Cx43 (2.267 ± 0.312 vs 15.171 ± 1.736 , $p < 0.01$), and β -tubulin (1.083 ± 0.098 vs 1.361 ± 0.042 , $p < 0.01$) mRNA was significantly lower in the IP than IR group (figure 4).

Expression of Cx43 protein was significantly higher in the IR than sham group (1.69 ± 0.21 vs 1.05 ± 0.11 , $p < 0.0001$). However, expression of Cx43 protein was significantly lower in the IP than IR group (0.81 ± 0.15 vs 1.69 ± 0.21 , $p < 0.0001$) (table 2, figure 5).

Figure 3 Effect of IP on the apoptotic cells in non-culprit coronary arterial tissues. (A) TUNEL and DAPI staining of non-culprit arterial tissues. (B) Percentage of apoptotic cells in each group. $n=10/\text{group}$. * $P < 0.05$ and ** $p < 0.01$. DAPI, 4',6-diamidino-2-phenylindole; IP, ischemic postconditioning; IR, ischemia-reperfusion; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Figure 4 Effect of IP on the mRNA expression of AT1, Cx43, and β -tubulin in non-culprit coronary arterial tissues. $n=10/\text{group}$. * $P < 0.05$ and ** $p < 0.01$. AT1, angiotensin II receptor type 1; Cx43, connexin 43; IP, ischemic postconditioning; IR, ischemia-reperfusion.

DISCUSSION

PPCI in a culprit artery is the preferred strategy for the treatment of patients with acute STEMI. However, approximately 40%–65% of patients with STEMI present with three-vessel lesions. A clinical follow-up study of such patients after successful PPCI suggested that non-culprit lesions may progress, which may be the most important factor influencing the prognosis of patients with acute myocardial infarction after successful PPCI.⁴

Few studies have examined the progression of non-culprit lesions. Hanratty *et al*⁹ demonstrated exaggeration of non-culprit lesions during acute myocardial infarction and indicated that inflammatory and spastic mechanisms may be involved in non-culprit lesion progression. On follow-up angiography of 117 patients with acute coronary syndrome, Tsiamis *et al*¹⁰ found that non-culprit lesions may progress and that acute myocardial infarction may be an independent predictive factor for this progression. Our previous study¹¹ suggested that non-culprit lesion progression may be the most important prognostic factor in patients with STEMI after successful PPCI. This indicates that inflammation and stress might contribute to the progression of non-culprit lesions. In addition, the AgII-MAPK-Cx43 pathway might be involved in the progression of non-culprit lesions according to the findings in a rabbit model of IR.⁶ Sun *et al*⁷ demonstrated that IP can inhibit the MAPK-Cx43 pathway, indicating that IP may inhibit non-culprit lesion progression.

In the present study, we investigated the effect of IP on cell apoptosis and mRNA expression of AT1, Cx43, β -tubulin, and Cx43 protein in non-culprit coronary arteries of a rabbit IR model. H&E staining of non-culprit arterial tissues showed that the structures of the non-culprit arterial tissues were clear and that the alignment of vascular smooth muscle fibers was regular in each group. There were no significant pathological changes in the IR or IP group compared with the sham group (figure 1).

Quantitative analysis of apoptosis in non-culprit coronary arterial tissues indicated significantly higher ratios of apoptotic cells in the IR than sham group (46.11 ± 5.92 vs 3.77 ± 6.31 , $p < 0.001$). Moreover, the ratios of apoptotic cells were significantly lower in the IP than IR group (28.36 ± 9.41 vs 46.11 ± 5.92 , $p < 0.001$). These results suggest that IR may increase cell apoptosis in non-culprit coronary arterial tissues and that IP may prevent non-culprit lesion progression by inhibiting this apoptosis.

Table 2 Effect of IP on the expression of Cx43 in non-culprit coronary arterial tissues (Cx43/ β -actin optical absorption ratio)

n=10	The control group	The sham group	IR group	IP group
Cx43/ β -actin optical absorption ratio	0.51 $\pm$ 0.13	1.05 $\pm$ 0.11*	1.69 $\pm$ 0.21†	0.81 $\pm$ 0.15‡

*Compared with normal control group, $p<0.0001$.

†Compared with the sham group, $p<0.0001$.

‡Compared with IR group, $p<0.0001$.

Cx43, connexin 43; IP, ischemic postconditioning; IR, ischemia-reperfusion.

Quantitative analysis of AT1, Cx43, and β -tubulin mRNA expression in non-culprit coronary arterial tissues indicated higher expression of AT1 and β -tubulin in the IR than sham group. In addition, lower expression of AT1 and β -tubulin mRNA was observed in the IP than IR group. These results indicate that AT1, Cx43, and β -tubulin may participate in the progression of non-culprit lesions and that IP may prevent this progression by inhibiting mRNA expression of AT1, Cx43, and β -tubulin and activation of the AgII-MAPK-Cx43- β -tubulin pathway in non-culprit coronary arterial tissues.^{11–14}

We observed lower mRNA expression of AT1, Cx43, β -tubulin, and Cx43 protein in non-culprit coronary arteries in the IP than IR group. However, He *et al*¹⁵ found that IP increased Cx43 expression in the left ventricular myocardial cellular membrane and attenuated reperfusion injury in a rat model of acute myocardial infarction. Although this result was not in conflict with our findings, He *et al*¹⁵ investigated the role of IP on Cx43 expression in the left ventricular myocardial cellular membrane during an early stage (1 and 3 hour after reperfusion), whereas we investigated the role of IP on expression of non-culprit coronary arteries at a later stage (1 week after reperfusion).

Schulz *et al*¹⁶ reported that in contrast to its importance for ischemic or pharmacological preconditioning, Cx43 does not impact the cardioprotection achieved by IP. The results of their study also implicated a role for IP on Cx43 expression in the left ventricular myocardial cellular membrane during the early stage, which is not in conflict with our findings.

Cx43 is present in the cell membrane and in mitochondria. Indeed, Cx43 in the inner mitochondrial membrane may participate in functional coordination between subsarcolemmal and interfibrillar mitochondria. Cx43 in the subsarcolemmal mitochondria is involved in regulation of reactive oxygen species production through modulation of potassium permeability and oxidative phosphorylation. Notably, subsarcolemmal mitochondria appear to play a prominent role in superoxide production. In addition, Cx43

in subsarcolemmal mitochondria is involved in calcium uptake from the sarcoplasmic reticulum and antioxidant regeneration. In contrast, interfibrillar mitochondria are important players in energy demand—supply matching, cytosolic calcium buffering, and antioxidant regeneration as a result of their intimate communication with the sarcoplasmic reticulum. In the present study, we aimed to determine the effect of IP on cell apoptosis and mRNA expression of AT1, Cx43, β -tubulin, and Cx43 protein in non-culprit coronary arteries of rabbits. As such, differences in the distribution and function of Cx43 in various cellular locations, as well as the role of individual mitochondrial subpopulations, should be investigated in future studies.

In our recent study, we investigated the expression of β -tubulin in non-culprit arteries and the effect of ramipril on lesion progression. Our results indicated that a sympathetic nervous system catecholamine/AgII/Cx43/ β -tubulin pathway may participate in the progression of non-culprit lesions¹⁵. In this study, we also found increased expression of AT1, Cx43, and β -tubulin mRNA in non-culprit coronary arteries after IR. IP may decrease the ratio of apoptotic cells and expression of AT1, Cx43, and β -tubulin mRNA in non-culprit coronary arteries, thus inhibiting non-culprit lesion progression. Moreover, our results indicated that IP inhibited the sympathetic catecholamine/AgII/Cx43/ β -tubulin pathway.

CONCLUSION

Apoptosis and mRNA expression of AT1, Cx43, and β -tubulin in non-culprit coronary arteries were increased after IR. However, IP may decrease apoptosis and expression of these factors in non-culprit coronary arteries, thus inhibiting their pathological progression. In clinical practice, IP may be used to inhibit non-culprit lesion progression.

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Contributors JW designed and coordinated the study, JW wrote the main manuscript text, S-YH collected samples. All authors reviewed the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by a priori approval by the Ethics Committee of Peking University (Beijing, China), and the study protocol was approved by the Institutional Ethics Committee of Peking University.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. All data were available in database of Aerospace Center Hospital, Peking University Aerospace School of Clinical Medicine.

Figure 5 Effect of ischemic postconditioning (IP) on the expression of Cx43 in non-culprit coronary arterial tissues. n=10/group. From left to right: normal control group, sham group, IR group, IP group. Cx43, connexin 43; IP, ischemic postconditioning; IR, ischemia-reperfusion.

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