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Effect of Diazoxide Preconditioning on Cultured Rat Myocardium Microvascular Endothelial Cells against Apoptosis and Relation of PI3K/Akt Pathway

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Background: Anti-apoptotic mechanism for cell protection on reperfusion may provide a new method to reduce reperfusion injury.
Aims: The aim of the present study is to explore the effect of mitochondrial ATP sensitive potassium channel (Mito-KATP) opener diazoxide (DZ) preconditioning on hypoxia/ reoxygen (H/R) injury of rat myocardium microvascular endothelial cells (MMECs) against apoptosis and relation of PI3K/Akt pathway.
Study Design: Animal experimentation.
Methods: The rat MMECs were cultivated, and H/R model was made to imitate ischemia-reperfusion injury. The cells were seeds in 96-wellplates (100μL/hole) or in 6cm diameter dishes (2 mL/dish) with the density of 1×106/mL and randomly divided into 4 groups (n=6 each): control group (Group N), hypoxia-regoxygen group (Group H/R), Diazoxide preconditioning+H/R group (Group DZ) and Diazoxide preconditioning +mitochondrial KATP blocker 5-hydroxydecanoate (5-HD) + H/R group (Group DZ+5-HD). The cells were exposed to 2h hypoxia followed by 2h reoxygenation. Diazoxide 100μmol/L and diazoxide 100μmol/L+ 5-HD100μmol/L were added to the culture medium 2h before hypoxia in DZ and DZ+5-HD groups respectively. Each group was observed the proliferation in MTT, apoptotic rate in Annexin V-FITC/PI double standard, cell structure of Hoechst staining, and the levels of PI3K, Akt and p53 mRNA by RT-qPCR.
Results: Compared with Group N, apoptotic rate of Group H/R increased ($p<0.01$) and the vitality decreased significantly ($p<0.05$), and the expression of PI3K, Akt and p53 mRNA elevated in Group H/R ($p<0.05$). Compared with Group H/R, apoptotic rate and p53 mRNA level of Group DZ depressed significantly ($p<0.01$, $p<0.05$), while the vitality, PI3K and Akt mRNA levels increased ($p<0.05$). Compared with Group DZ, apoptotic rate and p53 mRNA level of Group DZ+5-HD increased significantly ($p<0.01$, $p<0.05$), but the vitality, PI3K and Akt mRNA levels decreased ($p<0.05$).
Conclusion: Under the condition of H/R, mito-KATP opened by DZ may depend on PI3K/Akt pathway to regulate expression level of the downstream p53 mRNA to inhibit apoptosis and improve viability of MMECs at the same time. (*Balkan Med J* 2014;31:83-87).
Key Words: Apoptosis, diazoxide, Mito KATP, MMECs, p53, PI3K/Akt

The cell viability decreased when reperfusion occurred, and it is an important factor that leading to cardiac ischemia-reperfusion injury. In the past, most attention was focused on cardiac cells. While in recent years, myocardiac microvascular endothelial cells (MMECs) attracted scholar's attention. The one hand, endothelial cells participate in many important body balance regulation, and can synthesize and secrete a variety of biologically active substances, the other hand they regulate blood coagulation, leukocyte activity and the platelet aggregation and may be the first spreading site of reperfusion injury (1, 2). Anti-apoptotic mechanism for cell protection on reperfusion may provide a new method to reduce reperfusion injury. Papers have reported that PI3K/Akt signaling and mitochondrial ATP sensitive potassium channel (Mito-KATP) have protective effect on the heart, brain, blood vessels, and they may play important roles in endothelial cells, such as angiogenesis, proliferation, microvascular permeability, etc (3-6). In this study, we chose MMECs as the research object, and made hypoxia/ reoxygen (H/R) model to imitate ischemia-reperfusion injury, pretreated with diazoxide (DZ) to open mito-KATP, observed the effect of PI3K/Akt signaling pathway on the aspect of inhibiting MMECs apoptosis to explore mechanisms of reduced myocardial ischemia-reperfusion injury due to opening of mito-KATP.

MATERIAL AND METHODS

Culture and identification of MMECs

SD rats that born 8 to 10 days of clean grade were provided by the Experimental Animal Center of Nantong University. The rat hearts

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were resected in ice-cold PBS, then their left and right atriums and right ventricles were removed, and the left ventricular anterior walls were remained. After PBS flushing to remove blood stains, the endocardium and epicardium were stripped under a dissecting microscope. The remaining myocardial tissues were cut into the size of 1mm×1mm×1mm, and inoculated them into the petri dishes that prewetting with complete medium. Cultured them at 37°C, in 5% CO2 incubator for 4 hours, then added 2.0 mL DMEM (Carlsbad, California, USA) high glucose complete medium that containing 20% FBS (Sijiqing , Hangzhou , China) to continue to foster. After 60 hours, removed tissues and changed culture medium. Then change the medium every 3 days until the cells reached 80% confluence. The cells were arranged like the cobblestone mosaic under the inverted phase contrast microscope and identified by using immunocytochemistry SABC method to check VIII factor related antigen, the cytoplasm of MMECs would appear brown coloration.

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee in Nantong Medical College, Jiangsu, China.

Experimental groups

The MMECs were seeded in 96-well plates (100 μL/hole) or in 6cm diameter dishes (2 mL/dish) with density of 1×106/mL, and divided into four groups randomly (n=6 each): control group (Group N), hypoxia-regoxygen group (Group H/R), Diazoxide preconditioning+H/R group (Group DZ) and Diazoxide preconditioning + mitochondrial KATP 5-hydroxydecanoate (5-HD)+H/R group (Group DZ+5-HD).

Preparation of hypoxia-reoxygenation model and administration

In Group N, replaced for a sugar-based medium when hypoxia and for complete medium when reoxygenation. In H/R, DZ and DZ+5-HD groups, replaced for a sugar-based medium when hypoxia, continued passed into the 94% N2-5% CO2-1% O2 gas mixture in the three gas incubator for two hours, and replaced for complete medium when reoxygenation, then incubated in 5% CO2 incubator for two hours. Diazoxide (St.Louis.MO, USA) was added into Group DZ with density of 100 μmol/L, Group DZ+5-HD was added into 100 μmol/L 5-HD (St.Louis.MO, USA) two hours before diazoxide pretreatment and incubated for two hours, then hypoxia-reoxygenation, respectively. Then indicators were measured two hours after reoxygenation.

Hoechst Staining

Sterile coverslips were placed in 24-well plates, coated with polylysine, then inoculated with the cells and cultured until cells covered an area of about 50% to 80% of the coverslip. Then after hypoxia-reoxygenation, replaced cultured medium with 0.5 mL fixing agent for ten minutes or more, got rid of the fixative and washed two times with PBS. Then 0.5 mL of Hoechest 33258 staining liquid was added in for five minutes. Mounted a drop of anti-fluorescence quenching liquid on glass slide, and covered with cover slip that affixed with the cells. Then nucleus of normal and apoptotic cells were observed under the fluorescence microscope.

Determination of cell activityl

20 μL MTT solution (5 mg/mL) was added into each well, incubated for four hours at 37°C, then aspirate supernatant carefully, added 150 μL DMSO (St.Louis.MO, USA), and oscillated for ten minutes to fully dissolved the formazan. Determined absorbance values at wavelength of 490 nm by ELX8W Enzyme-linked immunosorbent assay.

Detection of apoptosis

The Annexin V-FITC apoptosis kit was from Boyou Biotechnology Co., Ltd., China. The cells were digested with trypsin free of EDTA (Carlsbad, California, USA), centrifugated, washed them with pre-cold PBS, then added 500 μL Annexin V to bind suspension cells in buffer, added 5 μL Annexin V-FITC and blended, then added 5 μL propidium iodide, mixed, protected from light, reacted for ten minutes at room temperature, and then detected apoptotic rate with flow cytometry.

Determination of PI3K, Akt and P53 mRNA by RT-qPCR

Following the manufacturer's instructions, total RNA from myocardium microvascular endothelial cells (MMECs) was extracted with Trizol (Carlsbad, California, USA), the concentrations were about 100-120ug/mL and ODs were quantified by UV spectrophotometry (1.8-2.0). cDNA was synthesized by PrimeScriptTM RT kit. RT system included 1 μL total RNA, 2 μL 5×RT buffer, 0.5 μL oligo dT, 0.5 μL PrimeScriptTM RT Enzyme Mix I, then added DEPC water to a total volume of 10 μL. Reaction conditions were as follows: 37°C, 15 min, then 85°C, 5s. The cDNA was stored at -. Primers were designed by ourselves, and synthesized by Sangon Bioscience and Technology Co., Ltd., Shanghai, China. The sequences of primers and probes were as follows: (1) PI3K, 5'-GCCCAGGCTTACTACAGAC-3' (sense), 5'-AAGTAGGGAGGCATCTCG -3' (antisense), and the amplified product was 236 bp. (2) Akt, 5'-TGCATTGCCGAGTCCAGAA -3' (sense), 5'-GCATCCGAGAAACAAAACATCA -3' (antisense), and the amplified product was 262 bp. (3) P53 (Exon 5-6), 5'-CTGGCCTCATCTTGGGCCTG-3' (sense), 5'-CTCGCTTAGTGCTCCCTGGG-3', and the amplified product was 467 bp. (4) β-actin, 5'-CACGAAACTACCTTCAACTCC -3' (sense), 5'-CATACTCCTGCTTGCTGATC -3' (antisense), and the amplified product was 265 bp.

Extraction of 2 μL cDNA as template to RT-qPCR, adding SYBR green 1 and PCR premix (TaKaRa) 12.5 μL and primers 0.5 μL, respectively, then adding sterile distilled water to the reaction volume of 25 μL. Two-step PCR reaction were used, and the conditions were as follows: pre-denaturation 95°C, 30s, 95°C 5s, 60°C, 30s, for 40 cycles, then into the melting curve determination procedure, calculated ΔCt and RQ values at last.

Statistical analysis

Data were expressed as mean±SD. Statistical analyses were done using the SPSS 15.0 software package. T-test and one-way analysis of variance (ANOVA) were performed for comparing with the differences between H/R, DZ, DZ+5-HD and N groups. $p<0.05$ was regarded statistically significant.

RESULTS

Impact of Diazoxide on MMECs morphology

Cells of group N were polygonal with abundant cytoplasm, round or oval nucleus, and were tightly adherent (Figure 1a).

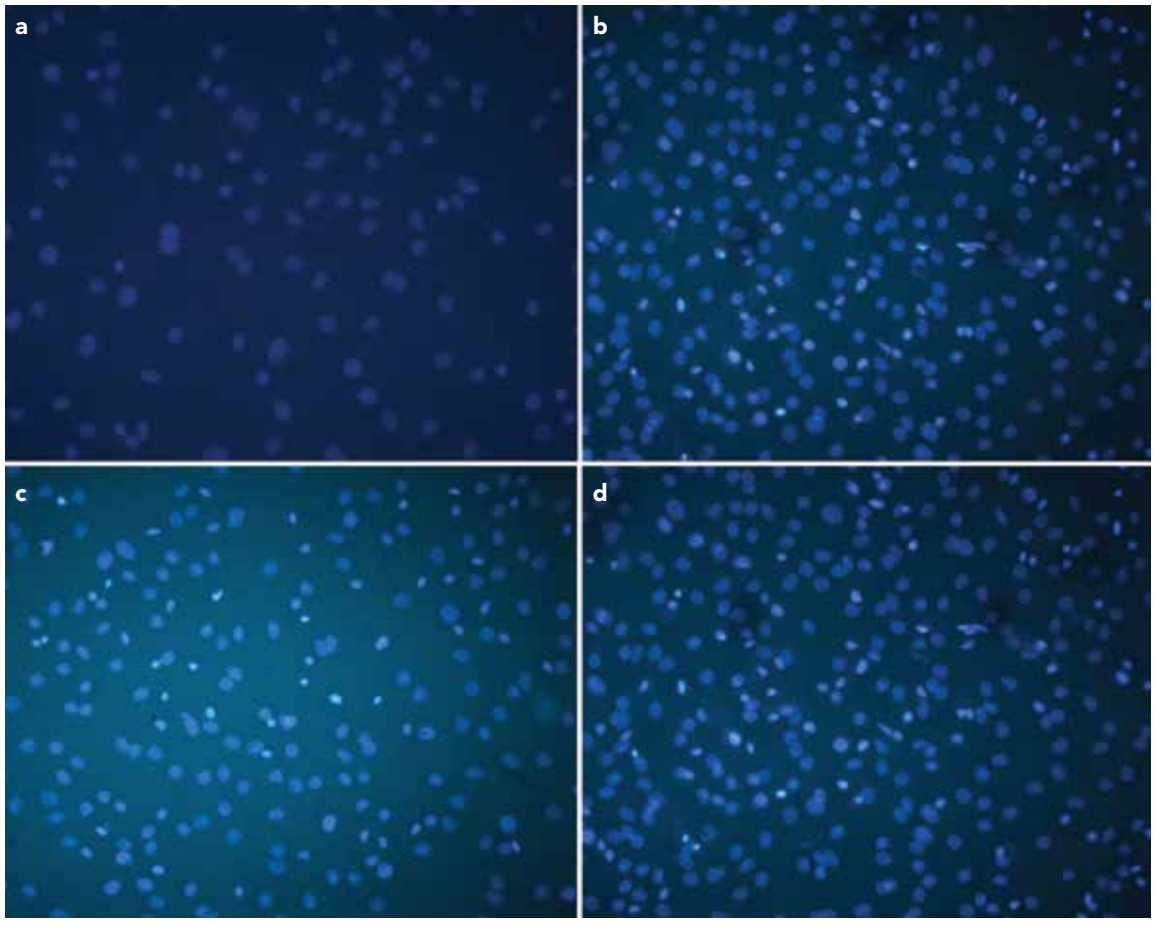

FIG. 1. a-d. Impact of Diazoxide on MMECs morphology (Hoechest staining, 200×). control group (Group N) (a), hypoxia-regoxygen group (Group H/R) (b), Diazoxide preconditioning+H/R group (Group DZ) (c), Diazoxide preconditioning + mitochondrial KATP 5-hydroxydecanoate (5-HD)+H/R group (Group DZ+5-HD) (d). Red arrows indicate the normal MMECs and green arrows apoptotic MMECs

Group H/R occurred a large number of cells necrosis and the cells retracted to round lost their normal shape, the gap turned larger, refractive index enhanced (Figure 1b). Group DZ appeared part of the cells necrosis, but shape the remaining cells were normal (Figure 1c). Group DZ+5-HD showed a large number of floating necrotic cells, and the residual cells retracted to round, lost their normal shape, the gap turned larger, refractive index enhanced (Figure 1d).

Apoptotic and proliferation rate of MMECs in each group

Figures 2 and 3 showed apoptotic and proliferation rate of MMECs in each group. Compared with Group N, apoptotic rate of Group H/R increased significantly ($p<0.01$), and proliferation rate decreased significantly ($p<0.05$). Compared with Group DZ, apoptotic rate of Group DZ+5-HD increased significantly ($p<0.01$), and proliferation rate decreased significantly ($p<0.05$).

Expression of PI3K, Akt and p53 mRNA in each group

Compared with Group N, levels of Akt and p53 mRNA in Group H/R increased significantly ($p<0.05$). Compared with Group H/R, PI3K and Akt mRNA levels of Group DZ increased significantly ($p<0.05$), while p53 level decreased significantly ($p<0.05$). Compared with Group DZ, PI3K and Akt mRNA levels of Group DZ+5-HD decreased significantly ($p<0.05$), while p53 level increased significantly ($p<0.05$) (Figure 4).

DISCUSSION

Coronary recanalization is the effective measure to treat coronary heart disease (CHD). However, reperfusion is main reason that leading to myocardial injury after coronary recanalization. Postoperative apoptosis is an important factor that impact on recovery of cardiac function. Most attention was

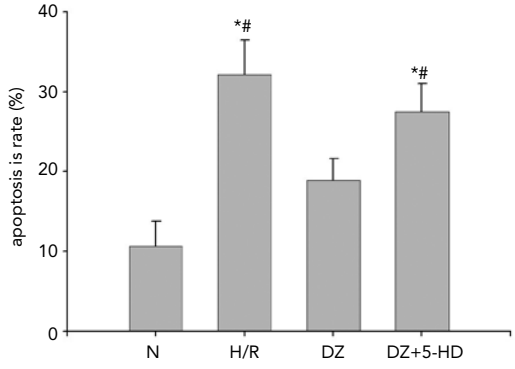

FIG. 2. Apoptosis of MMECs in each group ($\bar{x}$±s). *Compared with group N, p<0.01; # Compared with Group DZ, p<0.01. N: control group, H/R: hypoxia-regoxygen group, DZ: Diazoxide preconditioning+H/R group, DZ+5-HD: Diazoxide preconditioning + mitochondrial KATP 5-hydroxydecanoate(5-HD)+H/R group

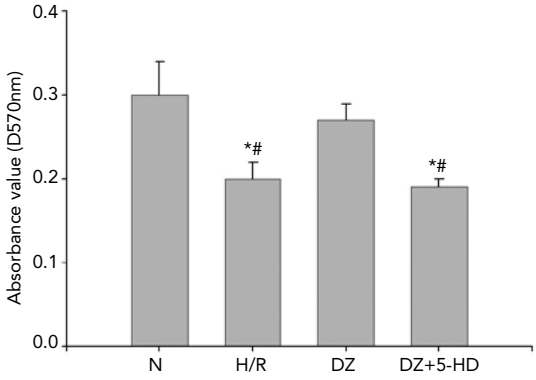

FIG. 3. Vitality of MMECs in each group ($\bar{x}$ ±s). *Compared with group N, p<0.05; # Compared with group DZ, p<0.05. N: control group, H/R: hypoxia-regoxygen group, DZ: Diazoxide preconditioning+H/R group, DZ+5-HD: Diazoxide preconditioning + mitochondrial KATP 5-hydroxydecanoate(5-HD)+H/R group

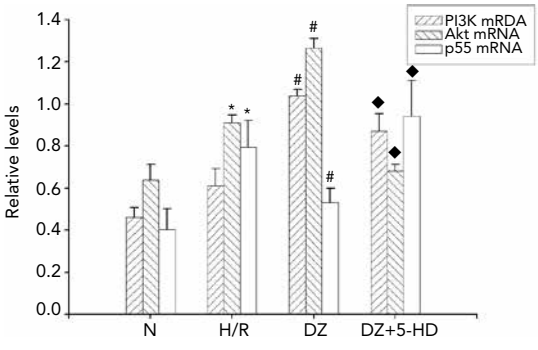

FIG. 4. Expression of PI3K, Akt, P53 mRNA in each group ($\bar{x}$ ±s). The image is from four independent experiments.*Compared with Group N, p<0.05; # Compared with Group H/R, p<0.05; ◆Compared with Group DZ, p<0.05. N: control group, H/R: hypoxia-regoxygen group, DZ: Diazoxide preconditioning+H/R group, DZ+5-HD: Diazoxide preconditioning + mitochondrial KATP 5-hydroxydecanoate(5-HD)+H/R group

focused on cardiac cells in the past. While in recent years, myocardiac microvascular endothelial cells (MMECs) also attracted scholar's attention.

PI3K and Akt are important intracellular signal transduction molecules. PI3K catalyze inositol ring of phosphoinositide 3-hydroxy phosphorylation to generate a variety of 3-phosphate inositol phospholipids, and participate in angiogenesis (7). Akt is a major downstream target of PI3K. Studies have confirmed that transient ischemia and hypoxia can activate Akt phosphorylation (8, 9), and activated Akt can activate or inhibit its downstream target proteins by phosphorylation, then regulate cell proliferation, differentiation and glucose metabolism, etc. (10, 11). In our results, H/R induced PI3K, significantly increased the expression of Akt mRNA, which showed that PI3K/Akt signaling pathway was activated by some of the physical and chemical factors that H/R produced, and regulated the cell's basic functions, thus could play an important role in MMECs involved in the regulation of H/R injury.

In human trials, about 22% of endothelial cells can cause significant reduction of microvessel density (12). In our study, characteristic changes of apoptosis occurred after H/R, and under optical microscopy, it showed a large number of cells apoptosis, chromatin condensation, nuclear dense stain, activity decreased. The results showed that cardiac ischemia and reperfusion lead to increased apoptosis, and inhibition of increased apoptosis and decreased proliferation caused by ischemia-reperfusion could be an important measure to prevent and treat the injury.

Oxidative stress inhibits KATP (13, 14). In this study, the MMECs were pretreated with diazoxide to open mito-KATP channel, and were carried on H/R, we found that the apoptosis of Group DZ was significantly reduced compared with Group H/R, while compared with Group N, the apoptosis increased. This effect excluded from the role of H/R, and indicated that opened mito-KATP channel could only partly inhibited apoptosis caused by H/R. After usage of the mito-KATP channel blocker 5-HD, the apoptosis rate increased significantly as well as proliferation increased. It showed the above changes were related to the activation of mito-KATP channel, and opening of mito-KATP channel depended on PI3K/Akt signaling pathway and participated in protection of H/R injury in rats.

As we all know, p53 is an important transcription factor, which participate in the mitochondrial membrane damage under the action of Bcl-2 (15). It is recognized as one of the pro-apoptotic genes, and p53 transcriptional activity increased when ischemia and hypoxia (16), mediating apoptosis of p53-dependent myocardial cells (17). In this study, we found that expression level of p53 mRNA enhanced significantly after H/R, which showed p53 have participated in the apoptosis of MMECs caused by H/R. We suppose p53 may be the downstream molecule of the PI3K/Akt signaling pathway, and PI3K/Akt signaling pathway may induce apoptosis by p53 that promotes apoptosis via the regulation of H/R. It showed

that mito-KATP openers could activate the PI3K/Akt signaling pathway, while inhibited p53 gene expression, inhibited apoptosis and enhanced cell viability. It suggested indirectly that the PI3K/Akt signaling pathway could inhibited apoptosis by inhibiting the pro-apoptotic gene p53, and increased microvessel density to improve microcirculation.

Therefore, we hypothesized that under H/R environment, mito-KATP opening depended on PI3K/Akt signaling pathway to reduce reactive oxygen species generation, calcium overload, and destruction of mitochondrial potential and energy metabolism, etc. (18-20). The change of these factors could regulate transcription and expression of the downstream p53 mRNA, and regulate cell growth and apoptosis, which provided a new experimental evidence for clinical ischemia and reperfusion injury therapeutic targets, and has important significance for exploring treatments of MMECs apoptosis and finding effective molecular intervention target to suppress cardiac ischemia-reperfusion injury. We believed that external factors that could activate the PI3K/Akt signaling pathway, lowered expression of p53, could possibly become one of the measures to prevent ischemia and reperfusion injury.

In conclusion, under the condition of H/R, mito-KATP channel opened by DZ may depend on PI3K/Akt pathway to regulate expression level of the downstream p53 mRNA to inhibit apoptosis and improve viability of MMECs at the same time, which will have important implications to explore treatments for MMECs apoptosis, and find effective molecular intervention target from inhibition of cardiac H/R injury.

Ethics Committee Approval: Ethics committee approval was received for this study from the local ethics committee of Nantong University.

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept – X.Z., S.C., X.T.; Design – X.Z., X.T., S.C.; Supervision – X.Z.; Resource – S.R.S.; Materials – Q.S., J.J.D.; Data Collection&/or Processing – X.T., X.L.H.; Analysis&/or Interpretation – S.C., Y.B.Q.; Literature Search – Y.Z.; Writing – S.C., X.T.; Critical Reviews – X.Z.

Cao Su and Tao Xia contributed equally to this study.

Conflict of Interest: No conflict of interest was declared by the authors.

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