Role of neuronal nitric oxide synthase in cardiac ischemia preconditioning in mice

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Abstract: objective: Several studies have demonstrated the role of endothelial and inducible nitric oxide synthase in cardiac ischemia preconditioning (IPC). However, the role of neuronal nitric oxide synthase (nNOS) in IPC is still controversial; the present study was designed to explore the possible involvement of nNOS in cardiac IPC. Methods: nNOS-/- knockout (KO) and wild type C57 (WT) mice were subjected to 45 minutes of ischemia by left descending branch of coronary artery ligation followed 3 hours reperfusion. IPC was induced by 3 cycles of 5 minutes ischemia and reperfusion before 30 minutes ischemia. After 3 hours reperfusion, which plasma was collected for creatine kinase (CK) and lactate dehydrogenase (LDH) measurements, terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) staining and and measurements of activities of caspase-3, -8, -9, phospho-p38, -ERK, -JNK mitogen-activated protein kinase (MAPK). Results: IR induced cardiac tissue apoptosis by increases of TUNEL staining and activities of caspase-3, -8, and -9, accompanied with increase of CK and LDH concentration and phosphorylation of p38, ERK and JNK MAPK in both mouse strains. IPC protected cardiac tissue from apoptosis by reducing TUNEL staining and activities of caspase-3, -8, and -9, with activation of p38 MAPK. However, reduction of TUNEL staining and activities of caspase-3, -8, and -9 was disappeared in KO mice accompanied with no activation of p38 MAPK. Conclusions: The data obtained suggest that nNOS mediates IPC-induced protection, maybe involved p38 MAPK activation.
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1. Introduction

Ischemic preconditioning (IPC) is the phenomenon whereby a mild ischemic stress enhances the tolerance of the heart to a subsequent ischemic stress (Ferdinandy et al., 2007), reducing infarct size, attenuating the incidence and severity of reperfusion-induced arrhythmias and preventing endothelial cell dysfunction. Several mechanisms are involved in IPC including activation of G-coupled membrane receptors (Gross and Gross 2006) and generation of ROS (Saini et al., 2004).

Nitric oxide synthase (NOS) plays an important role in the control of coronary tone and regulation of myocardial contractility, as well as exerting effects on platelet aggregation, neutrophil activation, and free radical production through the generation of nitric oxide (NO) (Snyder et al., 1992). consistent literatures showed NO mediated the IPC with different isoforms of NOS (iNOS and eNOS) in rapid and delayed stages (Cohen et al., 2006; Liu et al., 2008). However, the potential involvement of the neuronal NOS (nNOS) in cardiac IPC has so far not been fully investigated, although this enzyme has emerged as a key regulator of critical cardiac functions such as heart rate, calcium cycling, sodium transport, and energy metabolism (Kanai et al., 2001).

Stress-activated protein kinases (SAPKs) including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38 MAPK play a crucial role in IPC (Sato et al., 2000). Among them p38MAPK has been extensively studied as an effector (Maulik et al., 1998). its activation appears to be an obligatory step for IPC. Increasing studies have demonstrated that NO is involved in the activation of SAPKs, which mediated cardiac IPC protection (Hausenloy et al., 2006); however, the possible involvement of nNOS in activation of SAPKs during IPC is remained unclear.

The availability of genetically engineered mice in which the nNOS gene is selectively disrupted offers an opportunity to more conclusively establish whether nNOS is involved in cardiac IPC protection. In the present study, we used this model to elucidate the role of nNOS in cardiac IPC in vivo, furthermore, to investigate the possible involvement of nNOS in activation of SAPKs during cardiac IPC.

2. Material and Methods

Animal preparation for IPC

Ten to twelve weeks-old male nNOS KO mice (Jackson Laboratories, Bar Harbor, ME, USA) and its littermate C57BL/6 mice (Animal Center of China Medical University, ShenYang, China) were used in this study. Mouse cardiac IR and IPC models are followed as previously described with modification (Lim et al., 2007). In brief, animals were anesthetized by pentobarbital sodium (50 mg/kg

m, i.p.) and were ventilated using a rodent Minivent (type 845, Harvard Apparatus, Kent, UK). A left anterior thoracotomy and a chest retractor were used to expose the heart. Ligation of the left anterior descending (LAD) coronary artery was performed using a 7/0 nylon suture. Successful LAD coronary artery occlusion is confirmed under the microscope. IPC group is obtained by 3cycles of 5 minutes ischemia and 5 minutes reperfusion before 45 minutes ischemia. After 45 min of ischemia, the slipknot was released, and the myocardium was reperfused for 3 h. All surgical and experimental procedures were performed according to the guidelines for the care and use of animals established by China Medical University.

Determination of plasma creatine kinase and lactate dehydrogenase levels

Blood samples (1 ml) were drawn at the end of 3 h of reperfusion. Plasma CK and LDH levels were measured spectrophotometrically (DU 640; Beckman Coulter, Brea, CA) in a blinded manner. All measurements were assayed in duplicates.

Determination of myocardial apoptosis

Detection of apoptotic cells was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) assay kit. At the end of reperfusion, Tissues from the area at risk were fixed in optimal tissue compound, 4-μm thick slices were stained using the in situ cell death detection kit (Promega Technology, Madison, WI, USA) according to the manufacturer's instructions. In each field, nuclei were counted and the percentage of TUNEL-positive nuclei was calculated. Cardiac tissue apoptosis was also confirmed by caspase-3, -8 and -9 activity assays (Chemicon International Inc., Temecula, CA, USA) according to the manufacturer's instructions.

Determination of MAPK activation by western blotting

At the end of reperfusion, Ischemic myocardium tissue samples were lysed and protein concentrations were determined. Equal amount of protein from tissue homogenates was separated on SDS-PAGE gels and transferred to nitrocellulose membranes. membrane was incubated with primary antibodies against phospho-p38, phospho-ERK, phospho-JNK MAPK (Cell Signaling Technology, Beverly, MA， Inc. USA) overnight. And then incubated with HRP-conjugated secondary antibody (Cell Signaling Technology), for 1 h and visualized with enhanced chemiluminescence system (ECL kit, Amersham Pharmacia, GE Healthcare, Little Chalfont, Buckinghamshire, UK). Membranes then were re-probed with an antibody against p38, ERK, JNK (Cell Signaling Technology.) as an indicator for equal loading of samples. Western blotting data and density of blots were quantified by densitometric analysis using NIH image software. Data are expressed as the relative differences after normalization to p38, ERK and JNK expression.

Statistical analysis

All values are presented as means ± SE. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test where appropriate. Probabilities of <0.05 were considered to be statistically significant. All statistical tests were performed with the SPSS software 13.0.

3. Results

Plasma Creatine Kinase and Lactate Dehydrogenase levels

IPC induced decreases in CK and LDH levels in WT mice compared with IR group($P < 0.05$). However,these decreases were disappear in KO mice (Table 1).

Table 1. Plasma creatine kinase and lactate dehydrogenase levels in each group.

GROUP	CK（U/L）	LDH（U/L）
WT IR	5480±810$^{\#}$	3970±480$^{\#}$
WT IPC	3630±380	2020±250
KO IR	3890±590$^{*\triangle}$	2780±310$^{*\triangle}$
KO IPC	5210±480	4050±390

$^{*}P < 0.05$ (KO IR vs. WT IR). $^{\#}P < 0.05$ (WT IPC vs. WT IR). $^{\triangle}P < 0.05$ (KO IPC vs. KO IR).

IR and IPC-induced apoptosis

IPC induced decrease of TUNEL staining and activities of caspase-3, -8 and -9 in WT mice compared with IR group ($P < 0.05$). However, there was no reduction of TUNEL staining and caspase activities after IPC in KO mice compared with KO IR group ($P < 0.05$). (Figs.1 and 2).

MAPK activation

As shown in Figure 3, IR increased phospho-p38, -ERK and JNK MAPK as compared with the sham group in WT mice ($P < 0.05$), although IR also induced increase of phospho-p38 compared with WT sham mice in KO mice, but it was significantly lower compared with the WT IR group ($P < 0.05$). Phospho-ERK and JNK also increased in the KO mice after IR, but there is no reduction of phospho-ERK and JNK compared with the WT IR group. Further more, IPC also increased phospho-p38, -ERK and JNK MAPK as compared with sham group in WT mice. However, in KO group, increase of phospho-p38 was significantly lower compared with IR ($P < 0.05$), without any reduction of phospho-ERK and -JNK compared with IR.

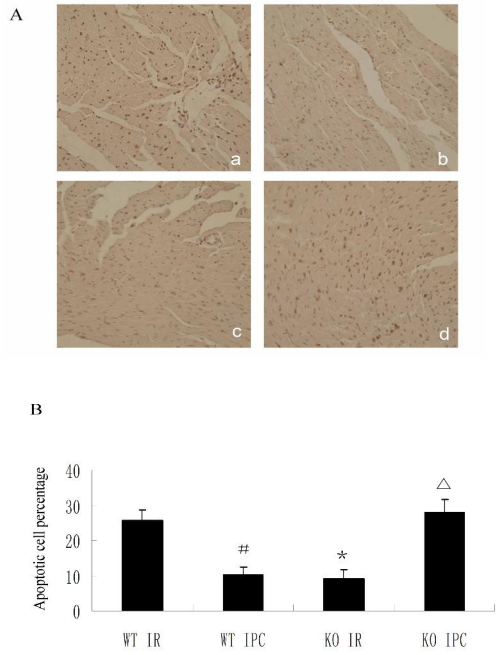

Figure 1: TUNEL staining to show the tissue apoptosis. Staining was done at 3 h of reperfusion after 45 min of ischemia. Percentage of positive apoptosis nuclei and total nuclei were expressed as mean ± SE (n = 6 for each group). WT and KO indicate wild-type and KO mice, respectively; IR and IPC represent ischemia reperfusion group and ischemia preconditioning respectively. (A) TUNEL staining. a, WT IR group; b, WT IPC group; c, means KO IR group and d, KO IPC group. (B) Percentage of apoptotic cells. *P < 0.05 (KO IR vs. WT IR). # P < 0.05 (WT IPC vs. WT IR). △P < 0.05 (KO IPC vs. KO IR)

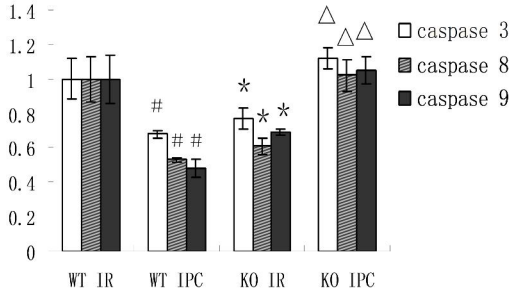

Figure 2: Caspases-3, -8 and -9 activities measured by spectrophotometry. Results are expressed as fold increase. Data were shown as mean ± SE (n = 6 for each group). WT and KO indicate wild-type and KO mice, respectively; IR and IPC represent ischemia reperfusion group and ischemia preconditioning respectively. *P < 0.05 (KO IR vs. WT IR). # P < 0.05 (WT IPC vs. WT IR). △P < 0.05 (KO IPC vs. KO IR)

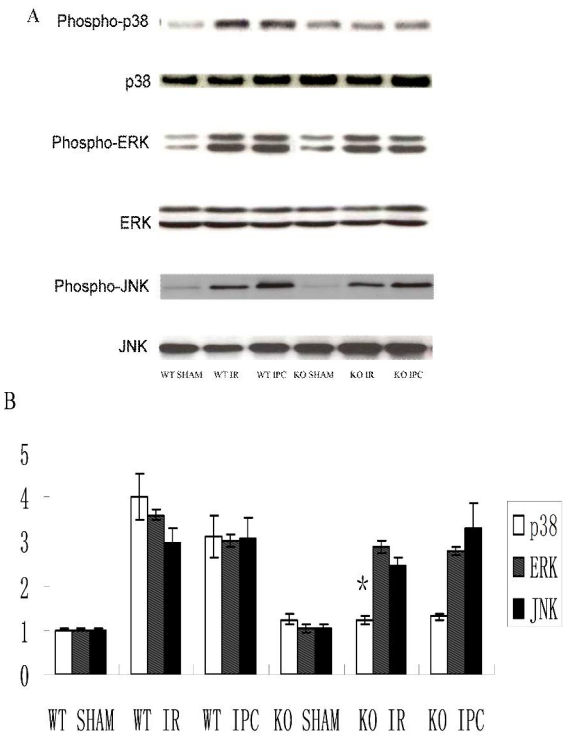

Figure 3: Analysis of MAPK activation. The phosphorylated-p38, -ERK and JNK in the ischemic area of the heart after 45 min ischemia followed by 3 h of reperfusion were analyzed. (A) Western blot bands of phosphorylated-p38, -ERK, JNK and their total protein at each group. (B) Densitometric values normalized by their total protein, respectively. Results are expressed as fold increase compared with the wild-type sham group. Data are shown as mean ± SE (n = 6 for each group). WT and KO indicate wild-type and KO mice, respectively; IR and IPC represent ischemia reperfusion group and ischemia preconditioning respectively. *P < 0.05 (KO IR vs. WT IR).

4. Discussions

In the present study, we found that nNOS mediated the protective effect in IPC, and the effects of nNOS in IPC maybe involved in p38 MAPK activation. Observations related the role of NOS in cardiac IP is consistent. IP involves exposing the heart to brief periods of ischemic insult that, in turn, generates a cardioprotective effect against later, more prolonged ischemic episode. This phenomenon exhibits two distinct phases: an early phase, that is protective for 2–3 hours after the initial insult; and a late phase, a second window of protection, which is effective 1–3 days following the initial ischemic insult (Vinten-Johansen et al., 2007). Thus, ischemic preconditioning serves to protect against myocardial dysfunction normally associated with IR events. It is

generally accepted that the second window of protection is triggered by eNOS and activated by iNOS (Liu et al., 2007). But the precise mechanism by which NOS provides cardioprotection in early phase has not been unequivocally established, studies have shown that early ischemic preconditioning was able to preserve eNOS protein expression and function in the ischemic/reperfused myocardium, which suggest that it may be useful to accelerate the complete recovery of endothelial function by preserving the level of cardiac eNOS and stimulating the basal production of NO (Muscari et al., 2004), and in a cellular model of early preconditioning in cultured neonatal rat ventricular myocytes, IPC was blocked by N(G)-monomethyl-L-arginine monoacetate and pretreatment with the NO donor S-nitroso-N-acetyl-L,L-penicillamine (SNAP) resulting in significant protection. iNOS protein expression was not detected, which suggested that early IPC was produced via activation of constitutive NOS isoforms (Rakhit et al., 2000). Our results showed that the protective effects induced by IPC in WT mice was disappeared in nNOS KO mice, which indicates that in early stage, IPC-induced protection was, at least in part, mediated by nNOS.

Numerous studies supported the involvement of various MAPK cascades in the protective mechanisms of IPC (Strohm et al., 2000). Positive role of ERK cascade in the mechanisms of IPC-mediated cardioprotection has been demonstrated in pig myocardium, when inhibition of ERK pathway during the IPC protocol inhibited both, the IPC-induced limitation of infarct size and the stimulation of ERK activities during IPC (Strohm et al., 2000). Other studies also confirmed the positive role of ERK in regulation of both the 'classical' early (Fryer et al., 2001) and late (Ping et al., 1999) phase of IPC-mediated cardioprotection. On the contrary, the role of p38 MAPK cascade in IPC remains still unclear. In some studies, a down-regulation of the p38 MAPK during repeated periods of short ischemia and reperfusion was observed with a subsequent further decrease in p38 MAPK activities during sustained ischemia (Gysembergh et al., 2001). However, in some studies it was demonstrated that IPC mediated a subsequent increase in p38 MAPK activities (Nakano et al., 2006). These discrepancies suggest that, apart from species differences, specific p38 MAPK isoforms might play a role. Recent studies revealed a different role for p38α and β isoforms in apoptotic responses and cell survival (Martin et al., 2001): a negative role of p38α and positive of p38β (mediating hypertrophic response). It may be explained that ischemia and IPC differentially activate p38 MAPK isoforms and that this may account for the controversies obtained in various observations. Our results showing that the activation of p38 was decreased in KO mice after IR and IPC, which is accompanied with the obviously different manifestation, revealing that p38 MAPK is potentially involved in nNOS mediated cardiac IR injury and IPC protection in mice.

In conclusion, our present data demonstrated that IPC-induced cardioprotective effects were not reserved in nNOS KO mice compared with WT mice. Our results suggest that nNOS mediates IPC-induced protection, maybe mediated by p38 MAPK.

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References

1. Ferdinandy P, Schulz R, Baxter GF. Interaction of Cardiovascular Risk Factors with Myocardial Ischemia/Reperfusion Injury, Preconditioning, and Postconditioning. Pharmacol Rev 2007; 59: 418-458.
2. Gross ER, Gross GJ. Ligand triggers of classical preconditioning and postconditioning. Cardiovasc Res 2006; 70: 212-221.
3. Saini HK, Machackova J, Dhalla NS. Role of reactive oxygen species in ischemic preconditioning of subcellular organelles in the hearts. Antioxid Redox Signal 2004; 6: 393-404.
4. Snyder SH, Bredt DS. Biological roles of nitric oxide. Sci Am. 1992; 266: 68-71, 4-7
5. Cohen MV, Yang XM, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. Cardiovasc Res 2006; 70: 231-239.
6. Liu VW, Huang PL. Cardiovascular roles of nitric oxide: A review of insights from nitric oxide synthase gene disrupted mice. Cardiovasc Res 2008; 77:19-29.
7. Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, et al. Identification of a neuronal nitric oxidesynthase in isolated cardiac mitochondria using electrochemical detection. Proc Natl Acad Sci USA 2001; 98:14126–14131.
8. Sato M, Cordis GA, Maulik N, Das DK. SAPKs regulation of ischemic preconditioning. Am J

Physiol Heart Circ Physiol 2000; 279:H901-H907.
9. Maulik N, Sato M, Price BD, Das DK. An essential role of NFkappaB in tyrosine kinase signaling of p38 MAP kinase regulation of myocardial adaptation to ischemia. FEBS Lett 1998; 429:365-369.
10. Hausenloy DJ, Yellon DM. Survival kinases in ischemic preconditioning and postconditioning. Cardiovasc Res 2006; 70:240-253.
11. Lim SY, Davidson SM, Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. Cardiovasc Res 2007; 75:530-535
12. Vinten-Johansen J, Zhao ZQ, Jiang R, Zatta AJ, Dobson GP. Preconditioning and postconditioning: innate cardioprotection from ischemia-reperfusion injury. J Appl Physiol 2007; 103:1441-1448.
13. Liu X, Chen H, Zhan B, Xing B, Zhou J, Zhu H, et al. Attenuation of reperfusion injury by renal ischemic postconditioning: the role of NO. Biochem Biophys Res Commun 2007; 359:628-634.
14. Muscari C, Bonafe' F, Gamberini C, Giordano E, Tantini B, Fattori M, et al. Early preconditioning prevents the loss of endothelial nitric oxide synthase and enhances its activity in the ischemic/reperfused rat heart. Life Sci 2004; 74:1127-1137.
15. Rakhit RD, Edwards RJ, Mockridge JW, Baydoun AR, Wyatt AW, Mann GE, et al. Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes. Am J Physiol Heart Circ Physiol 2000; 278:H1211-H1217.
16. Cohen MV, Baines ChP, Downey JM: Ischemic preconditioning: From adenosine receptor to KATP Channel. Annu Rev Physiol 2000; 62:79–109.
17. Strohm C, Barancik M, v. Bruehl ML, Kilian SAR, Schaper W. Inhibition of the ER-Kinase by PD98059 and UO126 counteracts ischemic preconditioning in pig myocardium. J Cardiovasc Pharmacol 2000; 36:218–229.
18. Fryer RM, Pratt PF, Hsu AK, Gross GJ. Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. J Pharmacol Exp Ther 2001; 296:642–649.
19. Ping P, Zhang J, Cao X, Kong D, Tang XL, Qiu Y, et al. PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. Am J Physiol Heart Circ Physiol 1999; 276:H1468–H1481.
20. Gysembergh A, Simkhovich BZ, Kloner RA, Przyklenk K. p38 MAPK activity is not increased early during sustained coronary artery occlusion in preconditioned versus rabbit heart. J Mol Cell Cardiol 2001; 33:681-699.
21. Nakano A, Baines CP, Kim SO, Pelech, Downey JM, Cohen MV, et al. Ischemic precomditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. Circ Res 2000; 86:144-151.
22. Martin JL, Avkiran M, Quinlan RA, Cohen P, Marber MS: Antiischemic effects of SB203580 are mediated through the inhibition of p38alpha mitogen-activated protein kinase: Evidence from ectopic expression of an inhibition-resistant kinase. Circ Res 2001; 89:750–752.

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