

Role of Neuronal Nitric Oxide Synthase in the Cardiac Ischemia Reperfusion in Mice

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Abstract

Several studies have demonstrated the role of endothelial and inducible nitric oxide synthase (iNOS) in cardiac ischemia reperfusion (IR). However, the role of neuronal nitric oxide synthase (nNOS) in IR is still controversial. The present study was designed to explore the possible involvement of nNOS in cardiac IR. nNOS^{-/-} knockout (KO) and wild type C57 (WT) mice were subjected to 45 min of ischemia by left descending branch of coronary artery ligation followed by 3 h reperfusion, which plasma was collected for creatine kinase (CK) and lactate dehydrogenase (LDH) measurements, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and measurements of activities of caspase-3, -8, -9, phospho-p38, -ERK, -JNK mitogen-activated protein kinase (MAPK) and phospho-nNOS, phospho-eNOS and iNOS. 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 and phospho-nNOS, phospho-eNOS and iNOS in both mouse strains. However, IR induced increases of TUNEL staining and activities of caspase-3, -8 and -9, and CK and LDH concentrations and activation of p38 MAPK were markedly lower in KO mice compared with WT mice. But the phosphorylation of eNOS was significantly higher compared with WT IR group ($P < 0.05$). The data obtained suggest that nNOS exacerbates IR-induced injury maybe involving p38 MAPK activation.

Key Words: ischemia reperfusion, mitogen-activated protein kinase, neuronal nitric oxide synthase

Introduction

Myocardial infarction is one of the most frequent cardiovascular events worldwide. Restoring blood flow to the heart to regain its function may experience additional damage due to the reperfusion process (17). 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) (7). Dysregulation of NO production has been implicated in the pathogenesis of hypertension, atherosclerosis, allograft rejection, myocardial infarction, cardiomyopathy and septic shock. However, the nature of NOS involvement in ischemia

reperfusion injury is still controversial. Most data support the concept that NOS is protective during cardiac IR (10). Approaches to remove NO by pharmacological inhibiting NOS and transgenic inducible NOS (iNOS) or endothelial (eNOS) knockout (KO) mice have been shown to exacerbate IR injuries in the heart (11, 31, 34). In contrast to these findings, other studies using pharmacological inhibition of iNOS and eNOS knockout show protective effects against IR injury in the heart (6, 21). However, possible involvement of the neuronal NOS (nNOS) in cardiac IR 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 (12, 19, 35).

Stress-activated protein kinases (SAPKs),

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including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38 MAPK, play a crucial role in IR (14, 15, 27). Among them, p38 MAPK has been extensively studied as an effector. While ischemia reperfusion-mediated activation of p38 MAPK causes cellular injury and apoptotic cell death (14, 15), increasing number of studies have demonstrated that NO is involved in the activation of SAPKs, which mediate cardiac IR injury (25, 36). However, possible involvement of nNOS in activation of SAPKs during IR is unclear.

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

Materials and Methods

Animal Preparation for IR

Ten to twelve weeks-old male *nNOS KO* mice (Jackson Laboratories, Bar Harbor, ME, USA) and their littermate C57BL/6 mice (Animal Center of China Medical University, ShenYang, China) were used in this study. Mouse cardiac IR models were followed as previously described with modifications (3). In brief, animals were anesthetized by pentobarbital sodium (50 mg/kg, 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 2 mm from the tip of the left atrium using a 7/0 nylon suture. Successful LAD coronary artery occlusion was confirmed under the microscope. After 45 min of ischemia, the slipknot was released, and the myocardium was reperfused for 3 h. Sham-operated mice underwent the same surgery minus the coronary artery ligation. At the end of the reperfusion, the heart was removed and embedded in Optimal Tissue Compound to measure caspases activities and MAPK and NOS isoform protein expression. Plasma was collected for creatine kinase (CK) and lactate dehydrogenase (LDH) measurements. All surgical and experimental procedures were performed according to the guidelines for the care and use of animals established by China Medical University and conformed with the guide for the care and use of laboratory animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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, USA) 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 (16). Tissues from the area at risk were fixed in optimal tissue compound, 4- μ m thick slices were stained using the *in situ* cell death detection kit (Promega Technology, Madison, WI, USA) according to the manufacturer's instructions. Three sections from each myocardial sample were randomly selected and ten microscopic fields per section were evaluated by two independent observers. 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 and NOS Isoform Expression by Western Blotting

Ischemic myocardium tissue samples were lysed with lysis buffer. Tissue protein concentrations were determined by protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amount of protein (50 μ g) from tissue homogenates was separated on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% skim milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated with primary antibodies against phospho-p38, phospho-ERK, phospho-JNK MAPK (Cell Signaling Technology, Beverly, MA, USA), phospho-nNOS, phospho-eNOS and iNOS (Santa Cruz, Inc., Santa Cruz, CA, USA) overnight. Membranes were 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.) and nNOS, eNOS and actin (Santa Cruz, Inc.) 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, JNK, nNOS, eNOS and actin expression.

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

GROUP	CK (U/L)	LDH (U/L)
WT SHAM	1340 ± 310	1020 ± 280
WT IR	5480 ± 810 [#]	3970 ± 480 [#]
KO SHAM	1210 ± 280	1050 ± 290
KO IR	3890 ± 590 ^{*Δ}	2780 ± 310 ^{*Δ}

* $P < 0.05$ (KO IR vs. WT IR). [#] $P < 0.05$ (WT IR vs. WT SHAM). ^Δ $P < 0.05$ (KO IR vs. KO SHAM).

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.

Results

Mortality

No animals died in the sham group, 3 animals died in the wild type (WT) IR group with a mortality rate of 27.3% (3/11), and 4 animals died in knockout (KO) IR group and the mortality rate was 28.6% (4/14).

Plasma Creatine Kinase and Lactate Dehydrogenase Levels

IR induced increases in CK and LDH levels both in WT and in KO mice ($P < 0.05$). However, increase of CK and LDH levels in the KO mice was significant lower than in the WT mice ($P < 0.05$) (Table 1).

IR-Induced Apoptosis

IR induced increase of TUNEL staining both in WT and in KO mice ($P < 0.05$). However, increase of TUNEL staining in the KO mice was significant lower than in the WT mice ($P < 0.05$). Activities of caspase-3, -8 and -9 were not different between WT sham and KO sham groups, and increased both in WT and in KO mice after IR ($P < 0.05$), but the KO mice showed markedly reduced caspases activities compared with the WT IR group ($P < 0.05$) (Figs. 1 and 2).

MAPK Activation

To investigate the relationship between activation of nNOS and MAPKs during cardiac IR, the

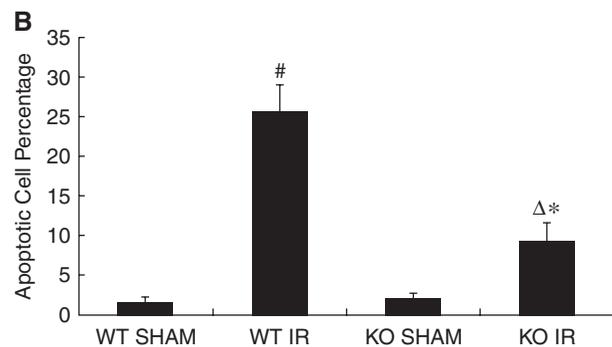
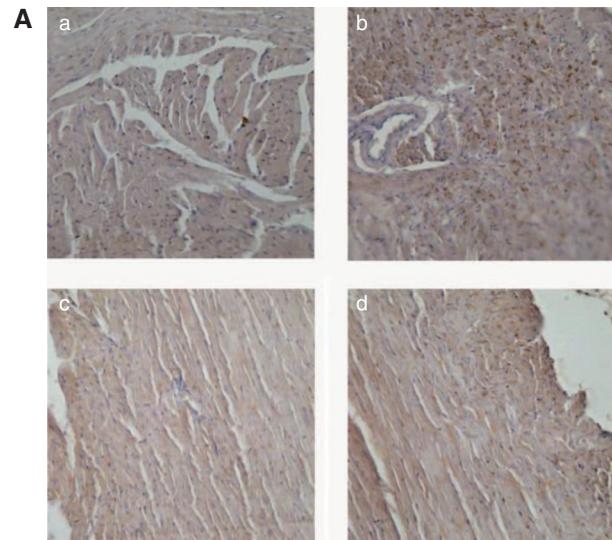


Fig. 1. TUNEL staining to show the tissue apoptosis. Staining was done at 3 h of reperfusion after 45 min of ischemia. Percentage of positive apoptosis nuclei and total nuclei were expressed as mean ± SE (n = 6 for each group). WT and KO indicate wild-type and KO mice, respectively; SHAM and IR represent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

phospho-p38, -ERK and JNK MAPKs were examined. As shown in Fig. 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 ($P < 0.05$), but there is no reduction of phospho-ERK and JNK compared with the WT IR group.

NOS Isoform Activation

To investigate the activation of three NOS

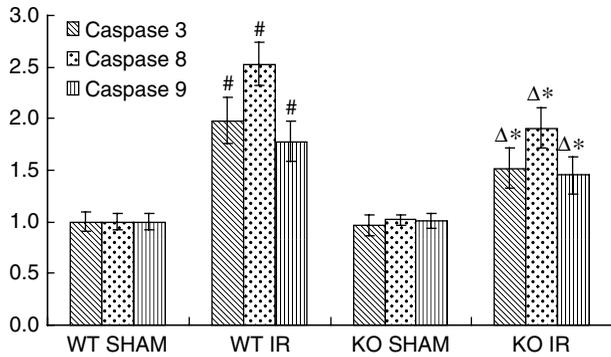


Fig. 2. Caspases-3, -8 and -9 activities measured by spectrophotometry. Results were expressed as fold increase compared with the wild-type sham group. Data were shown as mean \pm SE ($n = 6$ for each group). WT and KO indicate wild and KO mice, respectively; SHAM and IR represent sham and ischemia reperfusion groups, respectively. * $P < 0.05$ (KO IR vs. WT IR). [#] $P < 0.05$ (WT IR vs. WT SHAM). ^Δ $P < 0.05$ (KO IR vs. KO SHAM).

isoforms during cardiac IR, the phospho-nNOS, phospho-eNOS and iNOS were examined. IR increased phospho-nNOS, phospho-eNOS and iNOS as compared with the sham group in WT mice ($P < 0.05$) (Fig. 4). IR also induced increase of phospho-eNOS and iNOS compared with sham mice in the KO mice ($P < 0.05$), and the phosphorylation of eNOS was significantly higher compared with the WT IR group ($P < 0.05$).

Discussion

In the present study, we found that nNOS exacerbated IR-induced cardiac injury and that the effects of nNOS in IR might be involved in p38 MAPK activation. There is increasing evidence on the role of NOS in cardiac IR injury. Studies using NOS inhibitors suggested the protective role of NOS on vascular function and cardioprotection during IR (11, 31). NOS inhibitor L-NAME was shown to increase cardiac dysfunction during IR and NO donor improved function (20); another NOS inhibitor L-NNA was demonstrated to worsen stunning after IR (8). Using genetic knockout mice models, several studies have shown the protective role of eNOS in cardiac IR (25, 33), and an adaptive induction of iNOS in eNOS knockout mice, but there was no significant role of iNOS in early myocardial IR in iNOS knockout mice (30). Although most of the literature supported the protective role of NOS in IR-induced injuries, however, numerous evidences also showed that cardiac IR induced increase of NO and superoxide generation resulting in the formation of peroxynitrite, which has been demonstrated to be

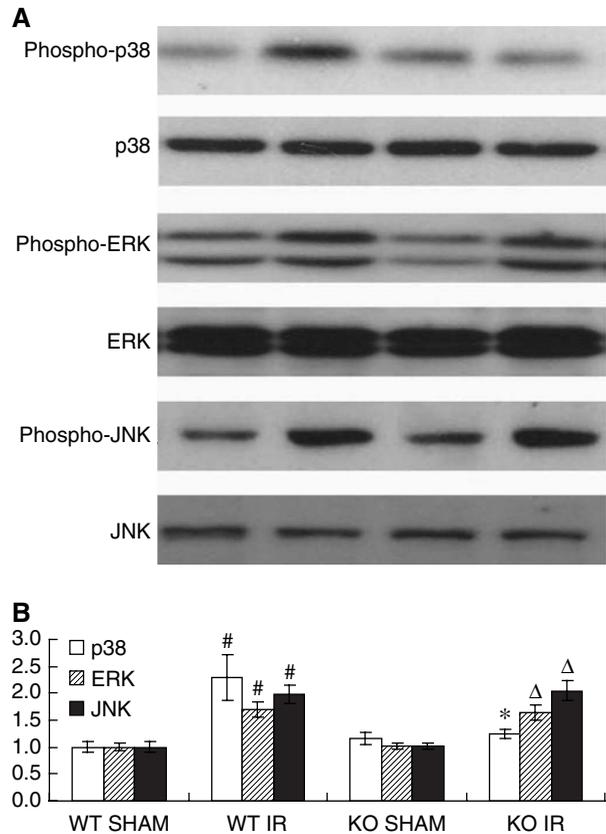


Fig. 3. Analysis of MAPK activation. The phosphorylated-p38, -ERK and JNK in the ischemic area of the heart after 45 min ischemia followed by 3 h of reperfusion were analyzed. (A) Western blot bands of phosphorylated-p38, -ERK, JNK and their total protein at each group. (B) Densitometric values normalized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

detrimental during acute IR (5, 22). To date, although it is still controversial, but, in general, lower amounts of NO production have been regarded to be beneficial, but high amounts of NO are considered harmful. The balance between NO and oxygen free radicals is crucial in modulating the outcome after an ischemic insult.

In the present study, we demonstrated the deleterious role of nNOS in cardiac IR, and the following mechanisms may involved: first, the mitochondrial NOS (mtNOS) has been molecularly cloned and confirmed to be nNOS, hence, activation of mitochondrially localized nNOS may lead to transient inhibition of mitochondrial ATP production (4, 13), which may, in turn, modulate myocardial

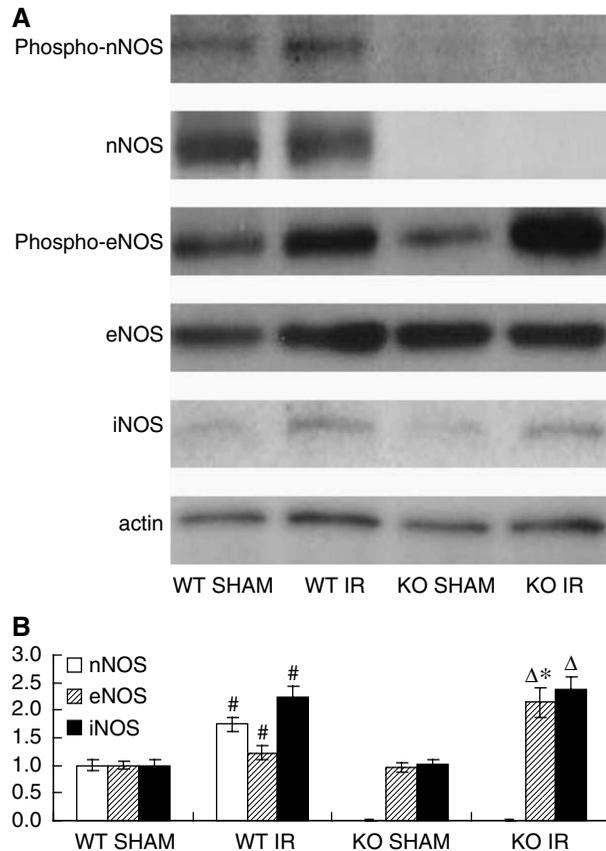


Fig. 4. Analysis of NOS isoform activation. The phospho-nNOS, phospho-eNOS and iNOS in the ischemic area of the heart after 45 min ischemia followed by 3 h of reperfusion were analyzed. (A) Western blot bands of phospho-nNOS, phospho-eNOS, iNOS and total nNOS, eNOS and actin in each group. (B) Densitometric values normalized by total nNOS, eNOS and actin, respectively. Results were expressed as fold increase compared with the wild-type sham group. Data were shown as mean \pm SE (n = 6 for each group). WT and KO indicate wild and KO mice, respectively; SHAM and IR represent sham group and ischemia reperfusion group respectively. * $P < 0.05$ (KO IR vs. WT IR). # $P < 0.05$ (WT IR vs. WT SHAM). $\Delta P < 0.05$ (KO IR vs. KO SHAM).

contractility. Second, recent studies have localized nNOS to the cardiac sarcoplasmic reticulum (32). Compartmentalized release of NO in this important subcellular organelle is believed to play a key role in regulating calcium kinetics in cardiac myocytes and, in turn, myocardial contractility. Lastly, nNOS in cardiac nerves may act to reduce heart rate and, in turn, oxygen consumption (2). The precise mechanisms of how nNOS plays a detrimental role in cardiac IR still need to be further investigated.

According to recent literature, we tend to explain the discrepancy by the different functions of NOS in varied situations. Normally, NOS can catalyze to generate NO in many physiological procedures, and

NO then plays a role in many biological functions. But under some conditions, NOS was activated but the product is not NO. For example, NOS can become “uncoupled” and preferentially synthesize superoxide anion radical which often has opposing effects to NO. It has been demonstrated that fully-coupled NOS catalyzes formation of NO, l-citrulline, NADP⁺ and water from l-arginine, NADPH and oxygen. Uncoupled or partially-coupled NOS catalyzes the synthesis of reactive oxygen species such as superoxide, hydrogen peroxide and peroxynitrite depending on the availability of cofactor tetrahydrobiopterin (BH4) and l-arginine during catalysis (29). Therefore, it raises the possibility that the exaggerated injury obtained in our results was due to the uncoupling of the nNOS and the following production of harmful free radicals especially under ischemia conditions. Furthermore, our results show that the phosphorylation of eNOS was significantly higher in nNOS KO mice than in wild-type mice after IR. This suggests that nNOS may result in the compensatory effects of eNOS and, therefore, an alternative explanation for our findings is that in the nNOS-deficient mouse, cardioprotection is mediated through an eNOS dependent pathway.

Mammalian cells respond to ischemia with activation of numerous cell signaling cascades that can lead eventually to irreversible damage of the myocardium and cell death (14, 15). It was found that ischemia and reperfusion induce distinct regulation of various MAPK cascades (27, 36). The ERK, p38 and JNK have been shown to be activated during ischemia reperfusion in *in vivo* models, in neonatal rat cardiomyocytes as well as in human hearts (1, 9, 26). The p38 MAPK pathway is investigated most frequently, but it is also the most controversial signaling pathway in myocardial responses to ischemic injury. Most studies have demonstrated that activation of p38 MAPK occurs either during ischemia alone, or persists throughout reperfusion, and other studies have reported a negative role of p38-MAPK during ischemia/reperfusion injury. Inhibition of p38 MAPK activation delayed the development of infarcts, increased cell survival, reduced myocardial apoptosis and improved post-ischemic recovery of cardiac function (18, 28). In contrast, other observations showed that the inhibition of p38-MAPK during lethal ischemia did not influence the extent of IR-induced injury or even increased it (23), suggesting a protective role of p38 MAPK activation during ischemia. These discrepancies suggest that, apart from species differences, specific p38 MAPK isoforms might play a role in the different outcomes. Recent studies revealed a different role for p38 α and β isoforms in apoptotic responses and cell survival: a negative role of p38 α and positive of p38 β (mediating hypertrophic

response) (14). It may be explained that ischemia activates p38 MAPK isoforms. Our results showed that the activation of p38 was decreased in KO mice after IR, which was accompanied by different manifestations, revealing that p38 MAPK is potentially involved in nNOS-mediated cardiac IR injury in mice.

In conclusion, our present data demonstrated that cardiac injury and phospho-p38 MAPK were markedly suppressed in nNOS KO mice, while there was an up-regulation of phosphorylated eNOS compared to WT mice. Our results suggest that nNOS plays a role in cardiac IR, which exacerbates IR-induced injury, maybe mediated by p38 MAPK.

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