The role of peroxynitrite in chemical preconditioning with 3-nitropropionic acid in rat hearts

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Abstract

Objectives: 3-Nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase, has been shown to protect against ischemic injury in the brain and in the heart via a preconditioning-like effect; however, the cellular mechanism is not known. The aim of the present study was to investigate if 3-NP pretreatment reduces infarct size and if altered metabolism of nitric oxide and reactive oxygen species are involved.

Methods: Hearts were assigned into 3 groups: 3 intermittent cycles of 5 min no-flow ischemia separated by 5 min aerobic perfusion protocol were used to induce ischemic preconditioning as a positive control; a time-matched non-preconditioning group served as control; and 3-NP (20 mg/kg, i.p.) was injected 3 h before the perfusion protocol to induce pharmacological preconditioning. Hearts from all groups were then subjected to 30 min global ischemia followed by 120 min reperfusion.

Results: Infarct size and lactate dehydrogenase release were significantly reduced after ischemia/reperfusion. While cardiac nitric oxide (NO) was increased, superoxide formation, nitrotyrosine level, and cardiac NADH oxidase and xanthine oxidase (XO) activities were markedly reduced by 3-NP administration. Cardiac activities of NO synthase and superoxide dismutase were not changed by 3-NP.

Conclusion: This is the first demonstration in the rat myocardium that 3-NP induces pharmacological preconditioning, thereby limiting infarct size, and that this effect is associated with increased NO bioavailability and reduced peroxynitrite formation due to inhibition of superoxide formation by XO and NADH oxidase.

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1. Introduction

Short ischemic episodes increase tolerance against subsequent severe ischemia in the heart and in other tissues. This phenomenon is termed ischemic preconditioning [1], (see Refs. [2,3] for reviews). Ischemic preconditioning limits ischemia/reperfusion injury by decreasing necrotic tissue mass after ischemia. It confers a remarkable protection in a variety of organs including either heart or brain. The exact mechanisms of ischemic preconditioning are not completely understood, although several mechanisms have been proposed to mediate increased tolerance against ischemia including altered metabolism of nitric oxide (NO) and reactive oxygen species such as peroxynitrite (ONOO−) formation [4–7], (see Ref. [3] for review).

Chemical preconditioning is a novel and practical strategy of cardioprotection and neuroprotection [8–10]. The term chemical preconditioning was used first by Riepe and
Ludolph [8] for the induction of hypoxic tolerance by using 3-nitropropionic acid (3-NP) in the brain. Other studies also suggested that the application of 3-NP in a single dose up to 20 mg/kg is a suitable strategy to induce chemical preconditioning with an early onset and long duration in the brain [8,11], (see Ref. [12] for review). 3-NP, a plant mycotoxin, is an irreversible inhibitor of succinate dehydrogenase which is an important enzyme of the Krebs cycle in complex II of the mitochondrial electron transport chain [13,14]. Subtoxic chemical inhibition of oxidative phosphorylation can induce preconditioning [15]. It has to be noted, however, that 3-NP given systematically in sufficiently low dose over a period of weeks results in neuronal death predominantly in striatum, therefore, 3-NP is widely used in animal models of neurodegenerative disorders such as Huntington’s disease (see Ref.[16] for review).

The action mechanism of 3-NP strongly relates to the generation of reactive oxygen species [17–19]. Superoxide combines with nitric oxide at a reaction rate that is only limited by diffusion to form ONOO⁻, a highly reactive molecule with cytotoxic effects (see Ref.[3] for review). Schulz et al. [20] described that after systemic 3-NP treatment in rats an increased production of ONOO⁻ was observed in brain. We have previously shown that ONOO⁻ triggers ischemic preconditioning in the heart and that ischemic preconditioning in turn attenuates the overproduction of NO, superoxide, and ONOO⁻ during a subsequent episode of ischemia and reperfusion, thereby confers cardioprotection [6,7], (see Ref. [3] for review).

Although 3-NP administration is a well-established method to induce chemical preconditioning in the brain, little is known on the effect of 3-NP induced chemical preconditioning in the myocardium. Only one study [9] showed that 3-NP is able to induce chemical preconditioning in rabbit heart.

Therefore, the aim of this study was to investigate whether 3-NP induces chemical preconditioning in the isolated rat heart and whether alterations of metabolism of NO, superoxide, and ONOO⁻ are involved in the cardioprotective effect of 3-NP.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by local ethics committee of the University of Szeged.

2.1. Animals and heart perfusion

Male Wistar rats (300–350 g) were housed in a room maintained at 12 h light–dark cycles and a constant temperature of 22±2 °C. To isolate the hearts, rats were anesthetized with diethyl ether. After i.v. administration of heparin (500 IU/kg), the chests were opened and the hearts were rapidly excised and mounted on a non-recirculating Langendorff perfusion apparatus perfused at 37 °C with Krebs–Henseleit bicarbonate buffer containing (in mM) NaCl 118.4, KCl 4.1, CaCl₂ 2.5, NaHCO₃ 25, KH₂PO₄ 1.17, MgCl₂ 1.46 and glucose 11.1; gassed with 95% O₂ and 5% CO₂, pH 7.4.

2.2. Experimental groups

Animals were divided into three groups (Fig. 1): In the preconditioned group, served as positive control, after 10 min stabilization period, preconditioning was induced by three intermittent cycles of 5 min no-flow ischemia, separated by 5 min aerobic perfusion, which was followed by 30 min global ischemia and 120 min reperfusion. In the control group, solvent for 3-NP was applied intraperitoneally 3 h before isolation of the hearts. After isolation of the hearts 10 min stabilization period followed by 30 min normal perfusion was applied, then hearts were subjected to 30 min global ischemia followed by 120 min of reperfusion. In the 3-NP treated group, animals were pretreated in vivo by a single injection of 3-NP 3 h before the perfusion protocol. 3-NP (Sigma, St Louis, MO) was dissolved in distilled water at 1 mg/ml (pH 7.4, adjusted with NaOH). A single subtoxic dose of 3-NP (20 mg/kg, [8]) was applied intraperitoneally as described previously [11]. After isolation of the hearts, 10 min stabilization period, 30 min aerobic perfusion, 30 min global ischemia, and 120 min reperfusion were applied as in the non-treated control group.

2.3. Evaluation of myocardial infarct size

To measure infarct size, at the end of reperfusion hearts from all groups were perfused with 1% triphenyltetrazolium-chloride (Sigma) dissolved in phosphate buffer and incubated for 5 min at 37 °C. Hearts were then sectioned.
(2 mm) and fixed in 4% formaldehyde. Infarct size was determined from images of the sections by planimetry.

2.4. Measurement of cardiac lactate dehydrogenase (LDH) release

Coronary effluents were collected for 5 min before and after global ischemia in all groups. LDH levels were measured immediately after collection of the effluent and evaluated spectrophotometrically at 340 nm wavelength using a kit (Diagnosticum Rt, Budapest, Hungary).

2.5. Measurement of cardiac NO and superoxide

To examine the role of NO in chemical preconditioning, in separate experiments, NO content of ventricular tissue from control and 3-NP groups was measured using electron spin resonance (ESR) spectroscopy after in vivo spin trapping with the NO-specific Fe²⁺-diethyldithiocarbamate (DETC) as described [21]. The spin-trap for NO was prepared freshly before each experiment. Two hundred mg/kg DETC, 50 mg/kg FeSO₄, and 200 mg/kg sodium-citrate were slowly administered to the non-treated control and treated animals 3 h after 3-NP injection intravenously into femoral vein under ether anesthesia, respectively. DETC was dissolved in distilled water and injected separately from FeSO₄ and sodium-citrate in 0.5 ml volume to avoid precipitation of Fe²⁺-(DETC)₂. FeSO₄ and sodium-citrate were dissolved in distilled water (pH 7.4). Five minutes after DETC, FeSO₄, and citrate treatment, hearts were isolated and perfused in Langendorff mode for 1 min to eliminate blood. Tissue samples from the apex of the heart (approximately 150 mg) were placed into quartz ESR tubes and frozen in liquid nitrogen. ESR spectra of NO–Fe²⁺-(MGD)₂ adducts were recorded with a Bruker ECS106 spectrometer (Rheinstetten, Germany; ESR parameters: X band, 100 kHz modulation frequency, 160 K temperature, 10 mW microwave power, 2.85 G modulation amplitude, 3356 G central field) and analyzed for NO signal intensity as described [6].

Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in separate experiments as described [21]. Hearts from control and treated animals were isolated and perfused with Krebs–Henseleit solution to eliminate blood. Approximately 100 mg of the apex of the right ventricle was placed in 1 ml air-equilibrated Krebs–Henseleit solution containing 10 mmol/l HEPES (pH 7.4) and 5 µmol/l lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter using a single active photomultiplier positioned in out-of-coincidence mode in the presence or absence of the superoxide scavenger nitroblue tetrazolum (NBT, 200 µmol/l). NBT-inhibitable chemiluminescence was considered an index of myocardial superoxide generation. It should be noted that NBT, like other superoxide scavengers, is not entirely specific for superoxide.

2.6. Measurement of cardiac NO synthase, xanthine oxidoreductase (XOR), superoxide dismutase (SOD), and NAD(P)H oxidase activities

To estimate endogenous enzymatic NO production, Ca²⁺-dependent and Ca²⁺-independent NO synthases activities in ventricular homogenates were measured by the conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline as we previously described [21]. Powdered frozen ventricular tissue from control and 3-NP groups was placed in four volumes of ice-cold homogenization buffer [22] and homogenized with an UltraTurrax disperser. The homogenate was centrifuged (1000 × g for 10 min at 4 °C) and the supernatant was kept on ice until enzyme activity assays. Protein concentration was measured from the supernatant using a Lowry–Folin method. Samples were incubated for 25 min at 37 °C in the presence or absence of either EGTA (1 mM) or EGTA plus N⁵-monomethyl-L-arginine (1 mM) to determine the level of Ca²⁺-dependent and Ca²⁺-independent NO synthase activities, respectively.

Activity of XOR (xanthine oxidase (XO) and xanthine dehydrogenase), one of the dominant sources of superoxide in rat hearts, was determined from ventricular homogenates by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR activity) and absence (XO activity) of the electron acceptor methylene blue as described [21,23]. Ventricular homogenates were prepared as for the measurement of NO synthase activity.

Total activity of SOD was measured by a spectrophotometric assay using a kit (Randox Laboratories Ltd, Crumlin UK). Approximately 100 mg ventricular tissue was homogenized in 10 volumes of ice-cold phosphate buffer (0.01 M, pH 7.0). Total SOD activity in homogenates was determined by the inhibition of formazan dye formation due to superoxide generated by xanthine and XO.

NAD(P)H-stimulated superoxide production in freshly prepared ventricular homogenates was assessed by a lucigenin-enhanced chemiluminescence method. To estimate background level of luminescence 30 µL of the ventricular homogenate was added to 1 mL Krebs–Henseleit buffer (room temperature) containing 10 mmol/L HEPES–NaOH (pH 7.4) and 5 µmol/L lucigenin (Sigma). Chemiluminescence was measured with a liquid scintillation counter (Packard, Meriden, CT) every 15 s for 5 min and the last 6 readings were averaged. To measure NAD(P)H-stimulated superoxide generation, either 100 µmol/L NADH or 100 µmol/L NADPH (Sigma) was added to the tube following background measurements and changes in luminescence were recorded. The background luminescence was subtracted from the readings with NADH or NADPH. Values were standardized to the amount of protein present and expressed as counts per minute/mg protein.

2.7. Measurement of nitrotyrosine, a marker of ONOO⁻

To investigate the role of ONOO⁻ in chemical preconditioning, we measured free nitrotyrosine content as a marker for ONOO⁻.
of ONOO\(^{-}\) formation by enzyme-linked immunosorbent assay (ELISA) in serum of the control and 3-NP treated groups 3 h after 3-NP injection, at the time of isolation of hearts. ONOO\(^{-}\) promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of stable product, nitrotyrosine. Briefly, 220 \(\mu\)L serum samples were added to 4 \(\times\) volume of ethanol at 4 °C, vortexed and centrifuged at 3000 \(\times\)g for 10 min. Supernatant was evaporated under nitrogen and redissolved in 105 \(\mu\)L of ultra-pure water. Samples were then incubated with nitrotyrosine acetylcholinesterase tracer in precoated (mouse antirabbit IgG) microplates followed by development with Ellman’s reagent as we described previously [21]. Reagents were provided by Cayman Chemicals (Ann Arbor, MI, USA) and the detailed protocol to conduct the ELISA measurement is available at the website of Cayman Chemicals. Serum nitrotyrosine concentration is expressed as nM/L.

2.8. Statistics

Data were expressed as mean\(\pm\)SEM. Analysis were done by one-way ANOVA followed by Tukey’s test and student \(t\)-test as appropriate, \(p<0.05\) was accepted as statistically significant difference.

3. Results

3.1. Infarct size and cardiac LDH release

In control hearts, test ischemia/reperfusion resulted in a large infarction (Fig. 2A) and LDH release (Fig. 2B). When ischemic preconditioning was applied before test ischemia (positive control), both infarct size and LDH release significantly decreased showing the protective effect of ischemic preconditioning against acute ischemia/reperfusion injury. Administration of 3-NP 3 h before the perfusion protocol showed an infarct size limiting effect similar to that of ischemic preconditioning. LDH release was significantly decreased by 3-NP pretreatment when compared to controls, however, LDH release remained significantly higher than that observed in the ischemic preconditioned group.

3.2. NO content and cardiac NO synthase

Myocardial NO content was significantly increased 3 h after 3-NP treatment as measured by ESR spectroscopy after in vivo spin trapping of NO compared to the non-treated control group (Fig. 3A). To test enzymatic synthesis...
of NO in the heart, we also measured cardiac activities of NO synthases. Endogenous enzymatic sources of NO, Ca²⁺-dependent and Ca²⁺-independent NO synthases activities in the myocardium were not affected by 3-NP treatment (Fig. 3B).

3.3. Cardiac superoxide and XOR, NAD(P)H oxidase, and SOD activity

To test if 3-NP treatment influences cardiac superoxide generation, we performed a lucigenin-enhanced chemiluminescence assay in freshly minced cardiac tissue. Cardiac superoxide generation was significantly decreased due to 3-NP treatment as compared to controls (Fig. 4A). To test possible changes in the enzymatic synthesis of superoxide, we measured activity of XOR, one of the major enzymatic source of superoxide in rat hearts. XOR and XO enzyme activity was significantly decreased in the 3-NP treated group (Fig. 4B). Other main sources of superoxide generation in the myocardium are the NADH and NADPH oxidases. NADPH oxidase activity was minimal in the heart tissue and was not influenced by 3-NP treatment (data not shown), however, NADH oxidase activity showed significant decrease when compared to controls (Fig. 4C). We also assayed total activity of SOD in the myocardium, the enzyme responsible for detoxification of superoxide. SOD activity was not changed in 3-NP-treated hearts when compared to controls (Fig. 4D).

3.4. ONOO⁻

To study the role of ONOO⁻ in 3-NP-induced cardioprotection serum-free nitrotyrosine concentration was measured in non-treated control and 3-nitropropionic acid treated (3-NP) groups 3 h after 3-NP administration. Values are mean±SEM, *p<0.05 (n=7–8).

Fig. 5. Serum-free nitrotyrosine concentration as an indicator of peroxynitrite formation in control and 3-nitropropionic acid treated (3-NP) groups 3 h after 3-NP administration. Values are mean±SEM, *p<0.05 (n=7–8).
tyrosine was decreased approximately two-fold in 3-NP treated rats as compared to controls (Fig. 5).

4. Discussion

We have shown here that 3-NP pretreatment, similarly to ischemic preconditioning, markedly reduced infarct size and LDH release in isolated rat hearts subjected to global ischemia/reperfusion. Cardiac superoxide content and serum nitrotyrosine level were also decreased 3 h after 3-NP treatment due to decreased activities of XOR and NADH oxidase, main sources of superoxide generation in the heart, whereas SOD activity was not changed. We have also shown that 3-NP pretreatment increased cardiac NO content whereas activities of NOS were not changed. These results provide evidence for the first time in the rat heart that 3-NP shown that 3-NP pretreatment increased cardiac NO bioavailability and reduced ONOO\textsuperscript{−} and superoxide formation via inhibition of cardiac XO and NADH oxidase activities.

In the present study we showed that cardiac NO signal intensity significantly increased in response to the 3-NP administration, however, activities of endogenous enzymatic sources of NO, Cu\textsuperscript{2+}-dependent and Cu\textsuperscript{2+}-independent NO synthases, were not changed. In agreement with our results, von Arnim et al. [24] described that mRNA of neuronal, endothelial, and inducible NO synthases stayed at the control level 12, 24, 72 h after preconditioning with 3-NP (20 mg/kg) in mice hippocampal slices. According to these results, the increase in cardiac NO bioavailability cannot be explained by increased NO synthesis. However, it should be noted that a possible increase in NO synthase activity in the heart in vivo cannot be excluded due to the limitations of the in vitro NO synthase activity assay used in our present study. Nevertheless, it is plausible to speculate that reduced formation of superoxide played a major role in the increased cardiac NO level found in our present study. Indeed, we have also shown here that chemical preconditioning with 3-NP significantly decreased superoxide level, and activities of XOR and NAD(P)H oxidase enzymes, major sources of superoxide in the rat heart. SOD activity, which is responsible for dismutation of superoxide was not changed, therefore, it did not influence superoxide content after 3-NP administration. These findings are supported by Riepe et al. [25,26], who reported that chemical preconditioning induced by 3-NP delayed the hypoxia-induced increase in NADH oxidase activity in rat hippocampus. In contrast, others have shown that 3-NP treatment is associated with generation of reactive oxygen species in brain [17–19]. However, in these studies high concentration or repeated applications of 3-NP were applied not to induce chemical preconditioning but to examine the neurotoxic effect of 3-NP to study neurodegenerative disorders.

To investigate the role of ONOO\textsuperscript{−} in 3-NP-induced chemical preconditioning, we measured 3-nitrotyrosine, a ONOO\textsuperscript{−} marker concentration in the serum 3 h after 3-NP treatment, and we found that 3-NP administration significantly reduced formation of ONOO\textsuperscript{−}. Decreased cardiac XOR and NADH oxidase activities which resulted in a reduced superoxide production explain the reduced generation of ONOO\textsuperscript{−}. Some groups showed that at the repeated [19] or high doses [27] of 3-NP, nitrotyrosine generation could be induced in spheroid cultures from rats or in mouse brain. In this study we measured ONOO\textsuperscript{−} after 20 mg/kg single dose of 3-NP, 3 h after its intraperitoneal injection, and found a significant decrease in serum nitrotyrosine. In our previous studies [7], we have shown that although the first brief cycle of preconditioning-ischemia/reperfusion significantly enhanced ONOO\textsuperscript{−} formation, after the third cycle of ischemia/reperfusion ONOO\textsuperscript{−} formation was significantly reduced. This showed that ONOO\textsuperscript{−} might act as a trigger of preconditioning but preconditioning in turn decreases increased formation of ONOO\textsuperscript{−} upon ischemia/reperfusion and thereby confers cardioprotection. Taken together, acute 3-NP treatment may induce increased formation of reactive oxygen species, however, 3 h after treatment, a significant reduction in formation of reactive oxygen species including ONOO\textsuperscript{−} occurs, which results in cardioprotection. Nevertheless, our present results do not clarify the exact cellular mechanisms of 3-NP-induced chemical preconditioning. Besides ONOO\textsuperscript{−}, ATP-sensitive potassium channels and altered mitochondrial function may play a role in the chemical preconditioning induced by 3-NP [10,28,29]. Furthermore, the mechanism by which 3-NP leads to decreased activities of XOR and NADH oxidase needs further studies.

In conclusion, our results clearly show that chemical preconditioning with 3-NP markedly reduces infarct size via a mechanism that may involve increased bioavailability of NO and decreased ONOO\textsuperscript{−} formation due to decreased cardiac formation of superoxide by 3-NP-induced inhibition of XOR and NADH oxidase activities.

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References


