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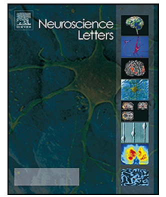

Plenary Article

Neuroprotective effects of nitric oxide donor NOC-18 against brain ischemia-induced mitochondrial damages: role of PKG and PKC

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HIGHLIGHTS

- Brain ischemia causes early inhibition of mitochondrial respiration at complex I.
- There is no release of cytochrome *c* from mitochondria during brain ischemia.
- Brain ischemia causes opening of mitochondrial permeability transition pore (MPTP).
- Injection of NOC-18 in rats protects against ischemia-induced MPTP and necrosis.
- NOC-18-induced protective mechanism is mediated by PKG and PKC.

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ABSTRACT

In this study we sought to determine whether NO donor NOC-18 can protect brain mitochondria against ischemia-induced dysfunction, particularly opening of mitochondrial permeability transition pore (MPTP), and cell death. We found that inhibition of respiration with NAD-dependent substrates, but not with succinate, was observed after 30 min ischemia indicating that complex I of the mitochondrial respiratory chain is the primary site affected by ischemia. There was no loss of mitochondrial cytochrome *c* during 30–120 min of brain ischemia. Prolonged, 90 min ischemia substantially decreased calcium retention capacity of brain mitochondria suggesting sensitization of mitochondria to Ca^{2+}-induced MPTP opening, and this was prevented by NOC-18 infusion prior to ischemia. NOC-18 did not prevent ischemia-induced inhibition of mitochondrial respiration, however, it partially protected against ischemia-induced necrosis. Protective effects of NOC-18 were abolished in the presence of selective inhibitors of protein kinase G (PKG) and protein kinase C (PKC). These results indicate that pre-treatment with NOC-18 protected brain mitochondria against ischemia-induced MPTP opening by decreasing mitochondrial sensitivity to calcium and partly protected brain cells against necrotic death in PKG- and PKC-depending manner.

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1. Introduction

Ischemic stroke is one of the most common disorders which lead to disability and mortality worldwide. In most cases, brain ischemia results from the suppression and blockage of blood flow to neurons that require continuous supply of oxygen and glucose to maintain normal brain function and viability [1]. Low levels of oxygen in the brain primarily affect mitochondria, the organelles involved in energy transformation and signaling pathways leading to cell death or survival. Under ischemic conditions mitochondrial Ca^{2+} uptake results in opening of MPTP leading to dysfunction of the organelle and release of pro-apoptotic proteins followed by cell death [2,3]. There is strong evidence that MPTP is implicated in ischemic heart damage [4–6], however, the role of MPTP in ischemic brain injury is relatively less investigated. Nevertheless, it has been reported that cyclosporine A, an inhibitor of MPTP, exerts some neuroprotective effects *in vitro* and *in vivo* models of brain ischemia [7,8]. However, brain mitochondria exhibit somewhat different features of MPTP such as higher resistance to Ca^{2+} and relative insensitivity to cyclosporine A compared to other tissues [9], which may be related to differences in content of proteins involved in MPTP

Abbreviations: CRC, calcium retention capacity; LDH, lactate dehydrogenase; MPTP, mitochondrial permeability transition pore; NOC-18, NO donor 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene synonims 2,2′-(hydroxynitrosohydrazino)bis-ethanamine; NOS, nitric oxide synthase; PKG, protein kinase G; PKCε, protein kinase C epsilon.

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formation such as cyclophiline D [10] or its interaction with other inner mitochondrial membrane proteins [11]. The main trigger for MPTP opening is Ca^{2+}, however MPTP can be regulated by multiple molecular effectors such as adenine nucleotides, inorganic phosphate, reactive oxygen and nitrogen species, including NO. These factors may have different effects in different tissues.

NO is involved in regulation of various physiological processes in the brain [12,13]. In cells, NO is synthesized by three isoenzymes: endothelial, neuronal and inducible NO synthases (eNOS, nNOS and iNOS) [14]. NO has been thought to be implicated in protection against ischemic brain damage [15], however some controversies still exist, particularly concerning the role of various NOS isoforms. It has been reported that eNOS-deficient mice showed reduced neuronal recovery after ischemia [16], whereas flavanoid-induced eNOS overexpression exerted protective effects [17]. In contrast, higher survival rate and less neuronal damage was observed in nNOS-deficient mice comparing to wild type [18]. Similarly, selective inhibition of nNOS in neuronal cultures resulted in reduced neuronal death after hypoxia [19]. The inhibition of nNOS and iNOS at the transient brain ischemia reduced infarct zone size [20]. Expression of iNOS, which produces high levels of NO, is usually related to neuroinflammation and activated phagocytosis [21]. Elevated NO synthesis may also cause inhibition of mitochondrial functions leading to neuronal death [22], but low concentrations of NO acting *via* activation of soluble guanylate cyclase and PKG may have protective effects [23].

Hypoxia suppresses NO synthesis by NOS as these enzymes require oxygen. In such conditions, application of NO donors supplying NO independently of oxygen may be more effective. It has been shown that NO donors improve functions of hippocampal neurons in rat brain slice cultures after ischemia [23], and intraperitonal injection of NO donor Rut-bpy prior to ischemia/reperfusion reduced brain infarct zone, improved viability of hippocampal neurons and inhibited NF-κB [15]. However, the exact mechanism of NO-induced protection against brain ischemic damages and particularly whether this involves suppression of MPTP in brain mitochondria is not clear yet.

In this study, we sought to investigate whether NO donor NOC-18 can protect against ischemia-induced mitochondrial dysfunction and cell death by inhibiting MPTP and whether this is mediated by PKG and PKC.

2. Methods

2.1. Procedure

Experimental procedures were carried on according to the EC Directive 86/609/EEC for animal experiments and the Republic of Lithuania law on the care, keeping and use of animals. Male Wistar rats were anesthetized using 40 mg/kg dolethal and 50 mg/kg ketamine. NO donor NOC-18 (50 μM) was infused into *vena cava* and 5 min later brains were removed and ischemia was induced by keeping isolated brain slices in hypoxic camera (93% N_2, 5% CO_2, 2% O_2; 37 °C) for 90 min. PKG inhibitor KT5823 (1 μM; Sigma) or PKC inhibitor Ro-32-0432 (5 μM; Calbiochem) were infused into *vena cava* 5 min before injection of NOC-18. As previously reported [24], 50 μM NOC-18 produced 281 ± 55 nM NO in the incubation buffer.

2.2. Isolation of mitochondria

Brain tissue was homogenized in glass-teflon homogenizer in the medium containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4. Mitochondria were isolated by differential centrifugation (5 min × 1000 × *g*, 10 min × 10,000 × *g*). Pellet of mitochondria was suspended in the isolation medium and total mitochondrial protein was determined by the modified Biuret method [25].

2.3. Measurement of mitochondrial calcium retention capacity

CRC was measured fluorimetrically using dye Calcium Green-5 N (excitation at 506 nm, emission at 535 nm) in a medium containing 200 mM sucrose, 10 mM Tris-HCl, 1 mM KH_2PO_4, 10 μM EGTA, 0.3 mM pyruvate plus 0.3 mM malate, pH 7.4. Experiments were started by the addition of 0.05 mg/ml of mitochondrial protein and measurement started with 125 nM $CaCl_2$ pulses were added at 2 min intervals until opening of MPTP occurred as a large increase in fluorescence due to release of intramitochondrial Ca^{2+}.

2.4. Measurement of mitochondrial respiration

Mitochondrial respiration rate was measured with OROBOROS high resolution respirometry system at 37 °C in the medium containing 125 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 20 mM HEPES pH 7.2. 1 mM pyruvate plus 1 mM malate, or 5 mM succinate (plus 1.6 mM amytal) were used as respiratory substrates. Mitochondrial state 3 respiration was achieved by adding 0.4 mM ADP. In some experiments, 30 μM exogenous cytochrome *c* was used.

2.5. Measurement of mitochondrial cytochrome c content

Cytochrome *c* content in isolated brain mitochondria was determined using Quantikinine M rat/mouse Immunoassay ELISA kit (R&D Systems). Mitochondria were dissolved in 0.5% Triton X-100 and further procedures were performed according to manufacturer's protocol.

2.6. Evaluation of necrosis

Necrosis was evaluated as the release of LDH into incubation medium of brain slices during ischemia. This method is rapid, sensitive and widely used in evaluation of necrosis in tissue slices and slice cultures [26,27]. Enzymatic activity of LDH was measured spectrophotometrically by monitoring the rate of decrease in NADH (at 340 nm) as pyruvate is converted to lactate in the buffer containing 0.1 M Tris–HCl, 0.1 mM NADH and 1 mM Na-pyruvate (pH 7.5). A unit of LDH was defined as the amount of enzyme necessary to catalyze oxidation of 1 μmol NADH per minute (IU). Activity of LDH after 90 min incubation of brain tissue on ice under normoxic conditions was taken as control.

2.7. Statistical analysis

SPSS Statistics 20 software was used for statistical analysis. Data were expressed as means ± S.E. from at least three separate experiments. Were used Kolmogorov–Smirnov and Shapiro–Wilk normality tests for evaluation of data and in all cases null hypothesis about normality of distribution retained $p > 0.05$. Statistical comparison between experimental groups was performed using ANOVA followed by Tukey or LSD tests. A value of $p < 0.05$ was considered as statistically significant result.

3. Results

3.1. Effect of ischemia on mitochondrial respiration and cytochrome c content

One of the consequences of ischemia-induced MPTP opening is the release of cytochrome *c* and subsequent inhibition of

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