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.

ARTICLE INFO

Article history:
Received 23 July 2014
Received in revised form 1 September 2014
Accepted 3 September 2014
Available online 16 September 2014

Keywords:
Ischemia
Nitric oxide
Mitochondria
Protein kinases

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 Ca2+-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 Ca2+ 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 Ca2+ 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(aminomethyl)-1-hydroxy-2-oxo-1-triazene synonyms 2,2′-(hydroxynitroso-hydrazino)bis-ethanamine; NOS, nitric oxide synthase; PKG, protein kinase G; PKCε, protein kinase C epsilon.

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http://dx.doi.org/10.1016/j.neulet.2014.09.012
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formulation such as cyclophiline D [10] or its interaction with other inner mitochondrial membrane proteins [11]. The main trigger for MPTP opening is Ca2+, 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 flavonoid-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 supply 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 intraparenchymal 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% N2, 5% CO2, 2% O2; 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 mM 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

Crtc 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-Cl, 1 mM KH2PO4, 10 μM EGTA, 0.3 mM pyruvate plus 0.3 mM maltate, pH 7.4. Experiments were started by the addition of 0.05 mg/ml of mitochondrial protein and measurement started with 125 mM CaCl2 pulses were added at 2 min intervals until opening of MPTP occurred as a large increase in fluorescence due to release of intramitochondrial Ca2+.

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 MgCl2, 1 mM EGTA, 1 mM KH2PO4, 20 mM HEPES pH 7.2, 1 mM pyruvate plus 1 mM maltate, 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 Quantikinin 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. We 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
3.2. Effect of ischemia and NOC-18 on mitochondrial CRC

Next we investigated whether prolonged brain ischemia may cause opening of MPTP by measuring mitochondrial CRC. In these experiments, added Ca$^{2+}$ was rapidly taken up by the mitochondria, and the concentration at which mitochondria started to abruptly release accumulated Ca$^{2+}$ was taken as indicator of MPTP opening. We found that 30 min ischemia caused negligible (by 13%) decrease in CRC compared to control mitochondria (data not shown). However, CRC of mitochondria isolated from 90 min ischemia-damaged brains decreases by 44% compared with control (Fig. 2B) suggesting that ischemia sensitizes brain mitochondria to Ca$^{2+}$-induced MPTP. Pre-ischemic treatment with NOC-18 increased CRC up to the level of control mitochondria. The protective effect of NOC-18 was abolished by inhibitors of PKG (KT5823) or PKC/6925 (Ro-32-0432) in both these groups CRC was similar to ischemia-damaged mitochondria (Fig. 2B). This suggests that NOC-18-induced desensitization of ischemic mitochondria to Ca$^{2+}$-induced MPTP may be mediated by PKG and PKC.

Despite the fact that NO donor increased CRC of ischemic mitochondria, treatment with NOC-18 did not prevent ischemia-induced inhibition of mitochondrial respiration. As shown in Fig. 2A, phosphorylating respiration with pyruvate plus malate as
well as with succinate was similarly inhibited in ischemia + NOC-18 group and ischemic group compared to control. This suggests that ischemia-induced inhibition of brain mitochondrial respiration was caused by factors other than by MPTP.

3.3. Effect of NOC-18 on necrosis

To evaluate brain cell death during ischemia we measured the activity of LDH released into the incubation medium. Pre-ischemic treatment of brains with NOC-18 reduced the release of LDH by 33% compared to ischemic group (Fig. 2C). KT5823 and Ro-32-0432 applied together with NOC-18 caused the level of necrosis to come back to the ischemic level, indicating that PKG and PKC mediated the protective action of NO donor.

4. Discussion

The main findings of the study are that pre-treatment of rats with 50 μM NOC-18 induced sustained protection of brain mitochondria against ischemia-induced MPTP opening and partially protected against ischemia-induced necrosis. This protection was inhibited by inhibitors of PKG and PKCe. NOC-18 did not exert any protective effect against ischemia-induced inhibition of mitochondrial respiration. This may explain why NOC-18 had only partial protection against necrosis in the brain: protracted inhibition of mitochondrial oxidative phosphorylation may cause ATP depletion in cells leading to excitotoxic neuronal death.

We also showed that brain ischemia caused time-dependent inhibition of mitochondrial respiration which was observed after 30 min ischemia and was related to inhibition of complex I of the respiratory chain. This conclusion is based on the findings that after 30 min ischemia, leak and phosphorylating respiration with NAD-dependent substrates, but not with succinate, were suppressed. Longer periods of ischemia caused more pronounced inhibition of phosphorylating respiration with both NAD-dependent substrates and succinate. Importantly, inhibition of respiration was not reversed in the presence of exogenous cytochrome c suggesting that mitochondrial outer membrane remained intact at least during 30–120 min ischemia and that inhibition was not due to the loss of cytochrome c from mitochondria. Indeed, during development of ischemia content of brain mitochondrial cytochrome c remained constant at the same level as in non-ischemic
References

[19] A. Jekabsone, J.J. Neher, V. Borutaite, G.C. Brown, Nitric oxide from neuronal nitric oxide synthase mediates neuroprotection against ischemia-induced loss of CRC and reduction of necrosis. Similar mechanism of NO-induced PKG- and PKC-dependent protection against ischemia-induced MPTP and cell death has been reported to occur after 90 min ischemia followed by 4 h reperfusion or 60 min ischemia and 6 h reperfusion indicating that it may be the late sequel of brain dysfunction.

The effects of NO in ischemic brains are dependent on concentration: high doses of NO may be neurotoxic whereas low doses may activate pro-survival signaling cascades. It has been demonstrated that low physiological concentrations of NO protected PC12 cells from apoptosis via activation of cGMP/PKG pathway. Activated PKG has been shown to improve hippocampal neuronal functions after ischemia in rat brain slices. Activation of PKC has been observed during ischemic preconditioning of the brain exerting beneficial effects on neuronal mitochondrial functions and reducing ischemic zone in rat brains, protecting neurons against necrosis in cell lines or animal models. In line with that, we showed that systemic application of NO donor in whole experimental animal resulted in PKG- and PKC-dependent protection of brain mitochondria against ischemia-induced loss of CRC and reduction of necrosis. Similar mechanism of NO-induced PKG- and PKC-dependent protection against ischemia-induced MPTP and cell death has been reported to operate in the heart. PKG has been shown to transmit the protective signal from cytosol to mitochondria in a PKC-dependent manner resulting in activation of mitochondrial ATP sensitive potassium channels. This leads to influx of potassium into the matrix and the resulting intramitochondrial signaling that inhibits MPTP and prevents cell death.

Our data are in general agreement with such mechanism.

Acknowledgement

This research was supported by European Social Fund under the Global grant measure: project No. VPI-3.1-SMM-07-K-01-130.

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