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Research article

Rotenone decreases ischemia-induced injury by inhibiting mitochondrial permeability transition in mature brains

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HIGHLIGHTS

- Rotenone, an inhibitor of CI of the mitochondrial electron transport system, blocks mPTP in adult rat brain mitochondria.
- Single intravenous dose of rotenone prevents ischemia-induced mPTP, ROS and cell death in rat cortex and cerebellum.
- CI of mitochondrial electron transport system has a key role in mPTP regulation in rat brains.

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ABSTRACT

The mitochondrial permeability transition pore (mPTP) is thought to be implicated in brain ischemia-induced cell death. Here we sought to determine whether complex I (CI) of the mitochondrial electron transfer system may be involved in regulation of mPTP opening during ischemia and whether a specific inhibitor of this complex – rotenone can protect against ischemia-induced cell death in an experimental model of total ischemia in adult rat brains. Anesthetized Wistar rats were administered a single injection of rotenone (0.01 mg/kg) to the tail vein and brains were removed and subjected to 120 min ischemia. We found that intravenous injection of rotenone 20 min before ischemia increased resistance to Ca^{2+}-induced mPTP opening and decreased production of reactive oxygen species (ROS) in mitochondria isolated from ischemia-damaged cortex and cerebellum. Rotenone administration before ischemia decreased infarct size in both brain regions (cortex and cerebellum). Rotenone added directly to normal, non-ischemic cortical or cerebellar mitochondria increased their resistance to Ca^{2+}-induced mPTP opening at concentration which fully inhibited NAD-dependent mitochondrial respiration. Our data demonstrate that rotenone used intravenously may be protective against acute brain ischemia-induced injuries by inhibition of mPTP opening and ROS production. These findings suggest that CI of mitochondrial electron transfer system plays a role in mPTP regulation during cerebral ischemia in mature brains and that agents acting on CI activity may be clinically useful for stroke therapy.

1. Introduction

Ischemic brain pathologies and stroke are among leading causes of human deaths, disability and dementia worldwide (World Health organization). Nevertheless, immediate treatments for stroke are limited and usually consist of usage of thrombolytic compounds (such as tissue plasminogen activator) or endovascular mechanical devices to restore blood flow in the brain. In clinical settings, there is a need for new, effective neuroprotective drugs, however, development of such drugs is limited by the lack of full understanding of molecular mechanisms of neuronal damages during ischemia. Accumulating experimental evidence suggests that mitochondria mediate development of ischemic brain injury due to opening of the mPTP [1,2]. This prompted studies on mPTP as a potential therapeutic target for ischemic stroke. Cyclosporine A (CsA) is a well-known, specific and powerful inhibitor of the mPTP [3,4], however, the limitation of the effectiveness of CsA in brain mitochondria [5], restricted transport of CsA into the brain [6] and neurological side effects of CsA [7] limited the use of CsA as neuroprotective agent in clinical practise [8]. Recently, it has been

Abbreviations: mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; CsA, cyclosporine A; CRC, calcium retention capacity; V_{Leak}, mitochondrial leak respiration; V_{ADP}, mitochondrial ADP-stimulated phosphorylating respiration; V_{Atr}, mitochondrial atractyloside inhibited respiration; TTC, 2,3,5-triphenyltetrazolium chloride; CI, complex I; CII, complex II.

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demonstrated that suppression of mPTP opening by rotenone, an inhibitor of the mitochondrial respiratory chain CI, at concentrations which caused respiratory inhibition was more effective than CsA in mouse liver mitochondria and in several cell lines (astroglial, leukemic, fibroblast) [9]. In addition, synergistic protective effect of rotenone and CsA against hypoxia-reoxygenation was observed in cardiomyocytes [10]. Moreover, CI inhibitors, metformin [11] and amobarbital [12], inhibited mPTP, ROS generation and cell death in cardiomyocytes after acute ischemia/reperfusion injury. Batandier and co-authors demonstrated that rats submitted to acute stress showed inhibited mitochondrial CI and delayed opening of mPTP [13]. Consequently, these facts suggest that CI inhibition may modulate opening of mPTP and can be crucial for cell functions and survival. On the other hand, efficiency of CI inhibitors in regulation of mPTP opening varies in different tissues which raises an idea that CI may be involved in mPTP only in certain cell types. Up to now there are no data on involvement of CI in regulation of mPTP in ischemic mature brains.

Rotenone, a classical and most potent inhibitor of the mitochondrial CI [14], is highly hydrophobic and may easily cross biological membranes, therefore it is well suited to produce systemic effects on mitochondria throughout the body. It has been shown that rotenone infusion supressed CI selectively and uniformly in the whole brain [15].

Vulnerability following oxygen deprivation within different brain subregions varies [16], and possibly may correlate with different sensitivity of mPTP to Ca^{2+} and to other modulators of its activity in different types of cells [17]. In the present study, we aimed to investigate the effects of rotenone, administrated intravenously before rat brain ischemia, on cell viability and mitochondrial functions in two brain regions, cortex and cerebellum. These regions differ in their cellular composition: cortex is relatively rich in glial cells whereas neurons are the main type of cells present in cerebellum [18]. We found that despite different cellular composition of cortex and cerebellum, rotenone infusion exerted similar protective effects on brain cell viability after ischemia in both regions possibly via blockage of ischemia-induced opening of mPTP.

2. Materials and methods

2.1. Animals and experimental protocols of rotenone treatment and brain ischemia

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals and approved by the National Ethical Committee for Animal Care (Licences No0217, No0228). The rats were maintained and handled at Lithuanian University of Health Sciences animal house in agreement with the Guide for the Care and Use of Laboratory Rats. Mature Wistar male rats (8–12 weeks, 200–250 g) were randomly assigned to four experimental groups, as below: control, rotenone control, 120 min ischemia, rotenone plus 120 min ischemia. Rats in all groups were exposed to increasing concentrations of CO_2 in air. In order to evaluate the rotenone effects, vehicle (ethanol/0.9%NaCl, 1:5) or rotenone at a dose of 0.01 mg/kg in 0.2 ml of vehicle was slowly infused into rats through the tail vein and after 20 min the rats were killed by cervical dislocation. Brains were removed and immediately used for mitochondrial isolation and preparation of brain slices. In 120 min ischemia ± rotenone groups, the removed brains were exposed to deep hypoxia (93%N_2, 5%CO_2, 2%O_2) for 120 min keeping isolated brain in glucose-free medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH7.4). The temperature during ischemia was maintained at 37.0–37.5 °C. Reagents are described in [19].

2.2. Isolation of mitochondria, measurements of CRC, respiration, activities of complexes of electron transfer system and H_2O_2 generation

Mitochondria were isolated from the cortex and cerebellum structures separately by differential centrifugation as described in [19]. Ca^{2+}-induced mPTP opening referred as calcium retention capacity (CRC) of isolated brain mitochondria was determined fluorimetrically using 100 nM Calcium Green 5N as described in [19]. Mitochondrial respiration was measured with OROBOROS Oxygraph–2 k as described in [19]. Activities of mitochondrial complexes CI and CII were determined spectrophotometrically as described in [19]. H_2O_2 generation in isolated mitochondria was estimated fluorimetrically with Amplex Red and horseradish peroxidase as described in [19].

2.3. Preparation of rat brain tissue slices and assessment of infarct area

Infarct area was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Rat brains were immersed in ice cold Hanks' balanced salt solution buffer with 13 mM glucose. The cerebrum (whole brain except cerebellum) and cerebellum were sectioned to coronal slices of 1 mm thickness with a 1000 Plus Sectioning System (TPI Vibratome), immediately immersed in 2% TTC for 15 min at 37 °C in the dark and then transferred to 4% buffered formalin solution for 15 min. Following fixation the brain slices were photographed with digital camera Nikon D3100. TTC stained normal viable areas of brain deep red but did not stain infarcted tissue (white areas). Pictures of each sample were analysed quantitatively with ImageJ software, version 1.47. The area of infarction in the slices of cortex and cerebellum was expressed as the percent of the whole coronal section.

2.4. Statistical analysis

Statistical analysis was performed using One Way ANOVA followed by Tukey or Fisher LSD post hoc tests, Sigma Plot 13 Statistics software. Results are expressed as mean ± standard errors.

3. Results

3.1. Direct effect of rotenone on CRC and respiration in control cortex and cerebellum mitochondria

We conducted the series of experiments to investigate direct effects of rotenone on Ca^{2+}-induced opening of mPTP in isolated normal (non-ischemic) cortical and cerebellar mitochondria. Rotenone at 1000 nM concentration significantly increased CRC of cortex mitochondria by 52% compared to control (see Fig. 1A in [19]). Lower rotenone concentrations (50–500 nM) had no significant effect on CRC of cortical mitochondria. In cerebellar mitochondria (Fig. 1B in [19]), 100 nM and 1000 nM rotenone increased resistance to mPTP opening by 38% and 78% respectively. Importantly, rotenone at concentration as low as 100 nM totally inhibited V_{ADP} of mitochondria energized with pyruvate/malate in both, cortex (53 ± 8 versus 851.0 ± 45 pmolO/s/mg protein) and cerebellum mitochondria (54 ± 11 versus 857.1 ± 40 pmolO/s/mg protein). Direct addition of 1000 nM rotenone did not inhibit V_{ADP} with succinate of isolated cortical and cerebellar mitochondria: respiratory rates were as high as in the absence of rotenone (data not shown).