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Possible mechanisms of Cyclosporin A ameliorated the ischemic microenvironment and inhibited mitochondria stress in tree shrews' hippocampus

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Abstract

Objective: The ischemic brain damage is always accompanied by the significant accumulation of glutamate and calcium ions (Ca^{2+}). Our objectives were to observe the effects of glutamate and Ca^{2+} overloading in tree shrew's hippocampal microenvironment on mitochondrial stress resulting in cytochrome *C* release and caspase apoptotic gene activation, and to explore the possible mechanism of Cyclosporin A (CsA) inhibiting mitochondrial stress. **Methods:** The thrombotic focal cerebral ischemia was induced by photochemical reaction in *tree shrews*. The extracellular contents of amino acidic neurotransmitters and Ca^{2+} were determined, respectively, with high performance liquid chromatography (HPLC) and atomic absorption spectrophotometry at 4, 24 and 72 h after cerebral ischemia. The glutamate–calcium chloride solutions were microperfused into hippocampus by a kind of single-pumped push–pull perfusion (SPPP) system under three-dimensional orientation instrument in tree shrews. At 24 h, the expression of cytochrome *C* was observed in perfused lateral hippocampus by immunochemistry. Also, the hippocampus was removed, then mitochondria and cytoplasmic fragment were divided by low temperature centrifugation and the distribution of cytochrome *C* was assessed through Western blot. Real time fluorescence polymerase chain reaction was used to evaluate the relative amounts of caspase-3 and caspase-9 mRNA. In the treated group, CsA (40 mg/kg) was intravenously injected at 6 h after the microperfuse or cerebral ischemia. The glutamate–calcium solutions were perfused into the hippocampus and inspected the above-mentioned items at 24 h. Data were compared between the two groups (ischemia group vs. sham group, or ischemia group vs. CsA group). **Results:** Thrombotic cerebral ischemia led to significant increase in extracellular glutamate and Ca^{2+} level of hippocampus ($P < 0.01$). The cerebral ischemia group and the microperfusion group, which cytochrome *C* immunoreactivity increased and Western blot analysis demonstrated that the cytochrome *C* content in the mitochondria of hippocampal cells decreased ($P < 0.01$), but the cytochrome *C* in the cytosol increased ($P < 0.01$). When CsA was intravenously injected at 6 h after the microperfusion or cerebral ischemia, the cytochrome *C* expression weakened and its release was diminished to a lesser extent. By real time PCR, in relation to the control group, the caspase-3 and caspase-9 mRNA was higher in the glutamate–calcium chloride solution perfused group. CsA treatment cut down the contents of caspase-3 mRNA and caspase-9 mRNA ($P < 0.01$). **Conclusions:** It is a primary factor that glutamate and Ca^{2+} accumulate in hippocampal microenvironment, which results in proapoptotic protein cytochrome *C* release from mitochondria into cytoplasm and caspase cascade activation, and finally mitochondria stress and neuronal secondary injury appear. The neuroprotection of CsA is in relation to inhibiting glutamate receptor overactivation and reducing the Ca^{2+} influx, which can decrease cytochrome *C* release and caspase mRNA transition.

Keywords: Photochemistry; Cerebral ischemia; Hippocampus microenvironment; Mitochondria stress; Cytochrome *C*; Caspase; Cyclosporin A; Tree shrew

When isolated mitochondria are exposed to high concentrations of calcium ions, a mitochondrial megachannel (MMC) opens in the inner mitochondrial membrane. This event, also named the mitochondrial permeability transition pore (mPTP), is considered to be an early event in apoptosis in some cells [1] and possibly a trigger of cell death in ischemia–reperfusion damage [2]. The current research suggests that mPTP and mitochondrial swelling is inhibited by Cyclosporin A (CsA, a mPTP inhibitor), which may also inhibit apoptosis in some cells. Heretofore, the microenvironment changes leading to mitochondrial stress and the neurons secondary injury after cerebral ischemia is still unclear. However, most of the experimental results

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were from stroke models on mechanical occlusion of brain arteries and therefore do not simulate the clinical condition of this disease and this implies that little has been known about the exact correlation between mPTP opening and caspase activation in abnormal microenvironment after cerebral ischemia or the microperfusion. Furthermore, caspase inhibitors have shown promising effect in attenuating brain injury after ischemia [2]. Moreover, information concerning the dysfunction of and glutamate accumulate in hippocampus induced by thrombotic cerebral ischemia is poorly understood. This study is focused on hippocampal CA1 area, where pyramidal neurons of hippocampus CA1 are susceptible to ischemic damage, and glutamate and Ca^{2+} accumulation often occurs in ischemic area where the regional cerebral blood flow decreases [3]. Hausenloy [2] found that preventing mPTP opening during the first few minutes of myocardial reperfusion using CsA improved post-ischemic contractile function in human atrial trabeculae harvested from patients undergoing cardiac surgery. CsA possesses cardioprotection effects by improving mitochondrial metabolism [4]. Further investigations should clarify whether treatment with CsA after photochemical lesioning can inhibit the release of cytochrome *C* and improve extracellular microecosystem of hippocampus.

1. Materials and methods

1.1. Group division

78 cerebral ischemic animals were divided in four groups: sham group ($n = 6$), ischemia 4 h group ($n = 24$), ischemia 24 h group ($n = 24$) and ischemia 72 h group ($n = 24$). 24 microdialytic animals were divided in three groups: sham group ($n = 8$), glutamate plus Ca^{2+} group ($n = 8$) and CsA group ($n = 8$). In CsA group tree shrews received iv CsA (40 mg/kg) at 6 h after photochemical reaction, while other ischemic group received only normal saline, the sham operation animals underwent the same surgical procedure and were either irradiated for 10 min following the injection of rose Bengal or not irradiated (iv normal saline, solvent of rose Bengal).

1.2. Thrombotic cerebral ischemia

The cerebral ischemia was induced by a photochemical reaction on 18 tree shrews (except 6 sham or control group) on the day of the experiment. Anesthesia was induced with 2.5% thiopentalum natricum (40 mg/kg) i.p. The animals received a lingual vein injection of 20 mg/kg of rose Bengal (Fluka) in 15 g/L saline solution. Their scalp was incised to expose the right skull and was irradiated with a spectrally filtered beam green light (centered at $\lambda = 560$ nm with a bandwidth of 60 nm from cerebral thrombotic apparatus, including xenon arc lamp, interference filter and thermal reflector) [5,6] and was passed through an interference filter on to the parietal bone for 15 min.

1.3. Surgical procedure and microdialysis

Animals were anesthesized with 2.5% thiopental sodium (40 mg/kg) and positioned in ventral recumbency on the stereotaxic apparatus while the head was positioned in a Frankfurt plane. A Φ1 mm small hole approximately 6.5 mm away from the midline was created in the left temporal bone. A small dural incision and the microdialysis probe was lowered into the hippocampus (upright 8.0 mm) using the stereotaxic carrier and the microdialysis dual syringe pumps [7]. When sampling was complete all microdialysis probes were removed, and the animal was recovered from anesthesia and gentamicin was administered for 3 days after the procedure. For the continuous infusion experiments, the infusion started before placement of the microdialysis probes in the brain to ensure that sampling was performed at steady state. The microdialysis probe perfusate (aCSF, maintained at 37 °C) flow rate was 10 μL/min and the microdialysis sampling began at least 60 min after probe insertion. Microdialysis samples were stored at 70 °C until analyzed. The concentrations of amino acidic neurotransmitters in hippocampus extracellular fluid was calculated by measuring the acreage of wave crest.

1.4. Measurement of extracellular amino acidic neurotransmitters and Ca^{2+}

The pathobiological changes in the abnormal microenvironment or extracellular ecosystem of hippocampus were confirmed by using high performance liquid chromatography (HPLC) at 4, 24 and 72 h after cerebral ischemia. Briefly, all the samples should be filtrated to make sure that there are no particles in the sample before making injections. The hippocampus perfusate was added to normal saline (0.5 mL) and albumen precipitator (1 mL) and centrifuged for 20 min at 2 °C, 10,000 rpm. The supernatant (50 μL) was used for the assay of GABA and glutamate by means of HPLC with ultraviolet detector. Practical measures were performed according to Tang et al.'s method [8] and extracellular Ca^{2+}, Cl^- contents were determined with atomic absorption spectrophotometry.

1.5. Measurements of MPT pore opening

Animals were decapitated, and the brain tissue was transferred to ice-cold isolation buffer (0.25 mol/L sucrose, 0.0005 mol/L EDTA-K_2, 0.01 mol/L Tris–HCl, pH 7.40). The neuronal mitochondria were isolated according to the Matsumoto's method [9]. The homogenates were centrifuged at $2000 \times g$ in 4 °C for 3 min, and the suspension was obtained and centrifuged at $12{,}500 \times g$ in 4 °C for 8 min. The sediment was in turn added to a gradient buffer A (0.12 mol/L mannitol, 0.03 mol/L sucrose, 0.0025 mmol/L EDTA-K_2, pH 7.4) and buffer B (0.24 mol/L mannitol, 0.06 mol/L sucrose, 50 μmol/L EDTA-K_2 pH 7.4) and centrifuged according to above authors' method. Mitochondria were activated by a