Research Paper

Ischemia-induced release of cytochrome c from mitochondria and up-regulation of Bcl-2 expression in rat hippocampus

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Abstract: To evaluate the effects of different antagonists on the release of cytochrome c from mitochondria to cytosol and the expression of Bcl-2 in mitochondria after ischemia, we examined Bcl-2 and cytochrome c expression by immunoblotting using 4-vessel occlusion (4-VO) as brain ischemia model. The results showed that after 24 h ischemia/reperfusion (I/R) cytochrome c decreased markedly in mitochondria, which was correspondingly increased in the cytosolic fraction. Bcl-2 expression was time-dependent, reaching its peak level after 6 h I/R. In all those samples, there were no alterations in the subcellular distribution of cytochrome oxidase, a mitochondrial respiratory chain protein. The decreases in Bcl-2 and cytochrome c in mitochondria were restored by pretreatment with non-competitive NMDA receptor antagonist ketamine or L-type voltage-gated Ca\(^{2+}\) channel (L-VGCC) antagonist nifedipine at 20 min prior to ischemia. The results demonstrate that the release of cytochrome c from mitochondria to cytosol and the up-regulation of Bcl-2 are possibly mediated by NMDA receptors or L-VGCC following brain ischemia. Cytochrome c release may be injurious while Bcl-2 up-regulation may be protective to ischemic hippocampus.

Key words: brain ischemia; cytochrome c; Bcl-2; N-methyl-D-aspartate receptor; L-type voltage-gated Ca\(^{2+}\) channel

大鼠脑缺血诱导的细胞色素c的释放和Bcl-2表达的上调

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摘要: 利用全脑缺血模型, 采用免疫印迹和免疫沉淀方法, 探讨N-甲基-D-天冬氨酸受体和L-型电压门控钙通道拮抗剂对细胞色素c从线粒体中的释放和Bcl-2的表达变化影响。缺血/复灌后24 h, 线粒体中细胞色素c明显降低而胞浆中细胞色素c的成分相应增加。Bcl-2的表达呈时间依赖性, 其表达在缺血/复灌后6 h达到最大。在所有样品中, 线粒体呼吸链蛋白细胞色素氧化酶没有变化, 表明线粒体的制备方法是可靠的。线粒体中Bcl-2的表达减少和细胞色素c的释放可以被NMDA受体拮抗剂氨酚酮和L-型电压门控钙通道拮抗剂地尔硫卓抑制。结果表明, N-甲基-D-天冬氨酸受体和L-型电压门控钙通道可能介导脑缺血后细胞色素c从线粒体中的释放和Bcl-2的上调表达。细胞色素c的释放具有损伤作用而Bcl-2的上调表达则对脑缺血具有一定的保护作用。

关键词: 脑缺血; 细胞色素c; Bcl-2; N-甲基-D-天冬氨酸受体; L-型电压门控钙通道

In recent years attention has been paid to the participation of mitochondria in the mechanism of cell death or apoptosis. Cytochrome c, a water-soluble peripheral membrane protein of the mitochondria, is known to be an essential component of the mitochondrial respiratory chain\(^1\). Early in the apoptosis, mitochondria releases cytochrome c from the mitochondrial intermembrane space to cytosol and forms a complex with another molecule, apoptotic protease-activating factor-1 (Apaf-1)\(^2\)\(^-\)\(^4\), an inactive preform of caspase-9. In the presence of dATP or ATP, this complex processes and activates other caspases, in particular, caspase-3,-6,-7\(^5\)\(^-\)\(^8\). The Bcl-2 gene encodes
a 26-kD membrane-associated protein that is located in multiple subcellular sites, including mitochondria, endoplasmic reticulum, and nuclear membrane[7]. Overexpression of Bcl-2, an apoptosis suppressor, blocks cytochrome c efflux in mitochondria induced by a variety of stimuli. The expression of Bcl-2 acts to inhibit cytochrome c, thereby blocking caspase-3 activation and apoptotic process, not only in a cell-free system, but also in intact cells[8,9]. Moreover, the Bcl-2 protein renders cells less susceptible to apoptotic stimuli, so the expression of Bcl-2 in neural cell lines markedly inhibits cell death induced by L-glutamate, free radicals, Ca$^{2+}$-ionophores, hypoglycemia or glutathione depletion[10,11].

Ischemic insults may result in overstimulation of glutamate receptors and L-type voltage-gated Ca$^{2+}$ channel (L-VGCC) which then mediate different kinds of downstream protein expressions[12]. Modification of the NMDA receptors during hypoxia/ischemia leads to increased intracellular calcium and is associated with increased generation of oxygen free radicals[13,14]. Mitochondria, which undergo harmful Ca$^{2+}$-loading after NMDA receptor activation, has an important signaling function in apoptosis[8,9,15]. Glutamate receptor antagonists, calcium-stabilizing agents and antioxidants have been proven effective in reversing ischemic damage to neuron[16]. Ketamine is a voltage-dependent antagonist of NMDA receptor ion-channels that can attenuate the ischemia-induced increase in intracellular calcium influx, while nifedipine can directly inhibit intracellular calcium influx.

Our present studies suggest that global ischemia induces the release of cytochrome c and the up-regulation of Bcl-2 protein. Ketamine and nifedipine inhibit the release of cytochrome c, but up-regulate Bcl-2 expression in the ischemic hippocampus. These results suggest that cytochrome c release and Bcl-2 up-regulation may be mediated by NMDA receptors or L-VGCC and that pharmacological modulation of cytochrome c release or Bcl-2 expression may become a new strategy to interfere with neuronal damage.

1 MATERIALS AND METHODS

1.1 Induction of ischemia. Adult male Sprague Dawley (SD) rats (purchased from Sippr-BK Experimental Animal Ltd Co, Shanghai, Grade¢ò, Certificate No D52) weighing 250~300 g were subjected to transient brain ischemia by 4-VO[17]. The rats were anesthetized with chloral hydrate and both vertebral arteries were occluded permanently by electrocoagulation. On the next day, both carotid arteries were occluded with aneurysm clips for 15 min. The rats that lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light were selected for the experiments. Electroencephalogram (EEG) was monitored to ensure isoelectricity. Rectal temperature was continuously monitored and kept at 37~37.5ºC using a heating pad and a temperature-regulated heating lamp. After 15 min occlusion, blood flow was restored by loosing aneurysm clips. A sham operation was performed in additional animals using the same anesthesia and surgical exposure procedures except that the arteries were not occluded. Ketamine, nifedipine (Sigma, St. Louis, USA) or solvent were administered to the rats by abdominal injection 20 min before occlusion.

1.2 Brain tissues preparation and drug treatment. At different times of reperfusion, animals were killed by decapitation and hippocampi were removed quickly. Protein extraction of both mitochondrial and cytosolic fractions was performed as described with some modifications[18]. Tissues were homogenized in ice cold suspension buffer (pH 7.5) which contained: (in mmol/L) HEPES-KOH 20, sucrose 250, KCl 1.5, EDTA 1, EGTA 1, dithiothreitol 1, phenylmethylsulfonyl fluoride (PMSF) 0.1; (in µg/ml) aprotinin 2, leupeptin 10, pepstatin A 5 and N-acetyl-leu-leu-norleucinal 12.5. The homogenates were first centrifuged at 800 g for 10 min, and then at 14 000 g for 20 min at 4ºC. Pellets were used as the mitochondrial fraction, the supernatant was further centrifuged at 100 000 g for 1 h. Protein concentrations were determined by the method of Lowry et al[18].

1.3 Western blot. The proteins in mitochondria and cytosol were separated by 15% SDS-PAGE and were electrotransferred onto nitrocellulose membrane (Amersham Pharmacia) by the method of Sambrook et al[19] and ours[20]. Membrane was probed with the indicated antibodies at 4ºC overnight. The primary antibodies were either a 1:1 000 dilution of rabbit cytochrome c and Bcl-2 polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) or 1 µg/ml cytochrome oxidase subunit I (COX I, 20E8C12) monoclonal (Molecular Probes, Eugene, OR, USA). Detections were carried out by alkaline phosphatase conjugated sheep anti-rabbit IgG or sheep anti-mouse IgG (Sigma, 1:10000) and developed using NBT/BCIP color substrate (Promega, Madison, USA). After immunoblot, the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP upland, CA).
1.4 Statistical analysis. All data are expressed as mean±SD from three independent animals. Statistical analysis of the results was carried out by one-way analysis of variance (ANOVA) followed by the Duncan’s new multiple range method or Newman-Keuls test. The difference was considered to be significant when the P value was less than 0.05.

2 RESULTS

2.1 Ischemia-induced release of cytochrome c from mitochondria to cytosol in rat hippocampus after ischemia/reperfusion

We examined cytochrome c at protein levels in normal and ischemic brains, focusing on the hippocampus where cells are particularly vulnerable to transient global ischemia. As shown in Fig.1A and B, cytochrome c immunoreactivity was evident as a single 15 kD band, detected by Western blot analysis. No cytochrome c was detected in the cytosolic fraction of the hippocampal region in sham control group. In the ischemia group, a cytochrome c-specific band was observed in the cytosolic fraction from 24 h after 15 min ischemia, which was correspondingly decreased after I/R in the mitochondrial fraction. COX IV was strongly expressed in the mitochondrial fraction and did not decrease after I/R (Fig. 1C), and virtually no band was seen in the cytosolic fraction in both control and ischemic hippocampus (Fig. 1D).

2.2 Ketamine and nifedipine effect on ischemia-induced release of cytochrome c

To determine the effects of ketamine and nifedipine on the release of cytochrome c in rat hippocampus after transient global ischemia, ketamine (12.5~50 mg/kg) or nifedipine (10~30 mg/kg) was intraperitoneally injected at 20 min prior to ischemia. As shown in Fig.2A and B, the

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Fig. 1. Western blot analysis of mitochondrial cytochrome c and cytochrome oxidase subunit IV (COX IV). A: Western blot analysis of mitochondrial cytochrome c in rat hippocampus following 24 h ischemia/reperfusion (I/R) after 15 min ischemia. B: Western blot analysis of cytosolic cytochrome c. C: Western blot analysis of mitochondrial COX IV. D: Western blot analysis of cytosolic COX IV. From the left, the control and I/R for 24 h in rat hippocampus are shown respectively.

Fig. 2. Antagonists of NMDA receptors and L-VGCC effect on release of cytochrome c. A, B: Western blot analysis of mitochondrial cytochrome c in the hippocampus after 24 h I/R. Different concentrations of ketamine (A) or nifedipine (B) were administered to rats by abdominal injection 20 min before ischemia and hippocampi were removed at 24 h of reperfusion. Dissolvents (0.9% NaCl or DMSO) were also given in the same way as control. C, D: Western blot analysis of mitochondrial and cytosolic COX IV. From the left, hippocampus of the control, 24 h, vehicle (0.9% NaCl or DMSO), KT (12.5, 25, 50 mg/kg) or ND (5, 10, 20 mg/kg). E, F: Data are expressed as mean±SD from three independent animals (n=3). aP<0.05 vs control, bP<0.05 vs 24 h I/R+vehicle, cP<0.05 vs KT12.5 or ND5.
release of cytochrome c from mitochondria was suppressed in the ketamine or nifedipine-pretreated animals. COX IV was strongly expressed in the mitochondrial fraction and had no changes after I/R (Fig. 2C, D).

2.3 Ketamine and nifedipine effect on decreased expression of Bcl-2 after ischemia

As shown in Fig. 3A, Bcl-2 expression in hippocampus exhibited a low expression in non-ischemic brain, but its expression was induced by cerebral ischemia and its level increased at 6 h (4.4-fold) after 15 min of ischemia, then decreased in a time-dependent manner. At 72 h after ischemia, Bcl-2 protein expression was barely detectable.

For determination of the effects of ketamine and nifedipine on expression of Bcl-2 in rat hippocampus after transient global ischemia, ketamine (12.5–50 mg/kg) and nifedipine (10–30 mg/kg) were injected intraperitoneally 20 min before ischemia. In the pretreated animals, Bcl-2 was clearly up-regulated in rat hippocampal mitochondria (Fig. 3B, D).

3 DISCUSSION

Attention has been paid to cytochrome c because of a recent cell-free study suggesting its critical role in apoptosis. It is reported that mitochondria are involved in apoptosis by releasing cytochrome c to the cytoplasm where it activates caspase-3, a molecule of the interleukin-1β-converting enzyme (ICE) family[2,6]. On the other hand, the proto-oncogene Bcl-2 plays a key role in regulating programmed cell death in neurons. Bcl-2 is altered in cerebral ischemia and traumatic brain and acts to inhibit cytochrome c translocation[8,9], prevents the activation of caspases, inhibits free radical formation, regulates calcium sequestration, and blocks the pro-apoptotic actions of other members of the Bcl-2 family such as Bax and Bad[21,22]. However, after cytochrome c is released, Bcl-2 overexpression can not prevent subsequent caspase activation and cell apoptosis, so Bcl-2 anti-apoptotic function acts primarily at the level of mitochondria[22].

Our studies show that a significant amount of cytochrome c in mitochondrial was detected in non-ischemic brain, which decreased in ischemic brain after 24 h I/R, while the cytosolic fractions from the same samples showed a significant increment. COX IV, a mitochondrial marker protein, was not detected in the cytosolic fraction, indicating that the detected cytosolic cytochrome c did not result from mitochondrial damage during preparation of the cytosolic fraction. Fifteen minutes of global ischemia induced a change in Bcl-2 protein expression at hippocampal region from 6 h to 72 h. Our studies also show that Bcl-2 protein is weakly expressed in non-ischemic hippocampus, which was induced by cerebral ischemia and reached its peak level as early as 6 h after I/R. When the neuronal damage was getting more and more severe with time, Bcl-2 expression decreased rapidly, which was rarely detected at 72 h after I/R.
studies have shown that Bcl-2 expression is induced primarily in living neurons after ischemia[23,24].

The biochemical and cellular events that lead to ischemic neuronal degeneration are beginning to be understood that include ATP depletion, oxyradical production, and disruption of cellular ion homeostasis[13]. Accordingly, glutamate receptor antagonists and calcium-stabilizing agents have proven effective in reducing ischemic damage to neurons [12,15], but the downstream mechanism is unclear. Previous studies have demonstrated that brain hypoxia/ischemia modifies the recognition and modulatory sites of NMDA receptor ion-channel complex, resulting in an increase in the NMDA receptor-mediated calcium influx. An excessive and prolonged glutamate release has been found to disrupt neuronal mitochondrial function, leading to activation of apoptotic pathways[25,26]. By immunoprecipitation of cytochrome c from cytosolic fractions, cytochrome c was detected as early as 3 h following NMDA receptor activation[27]. Cytochrome c release was found to be associated with permeability transition pore opening or occurring via the voltage-dependent anion channel. Ketamine is a voltage-dependent non-competitive antagonist of the NMDA receptors. When mitochondria are overloaded with Ca2+, that the release of cytochrome c from mitochondria to cytoplasm and the up-regulation of Bcl-2 in cytoplasm after brain ischemia. NMDA receptors and L-VGCC antagonists suppressed the release of cytochrome c, whereas they increased Bcl-2 expression in the ischemic hippocampus. It suggests that cytochrome c release or Bcl-2 up-regulation may be mediated by NMDA receptors or L-VGCC, and cytochrome c release may be injurious while Bcl-2 up-regulation may be protective to ischemic hippocampus.

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