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(16)マウス前脳虚血モデルにおいてカスパーゼ3活性化に対する虚血前処置の抑制効果

マウス前脳虚血モデルにおいてカスパーゼ−3活性化に対する虚血前処置の抑制効果

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要旨

あらかじめ脳に非致死的虚血を負荷した後には、後続する長い虚血による障害が軽減できることを虚血耐性という。虚血耐性機序の解明は将来、脳虚血治療方法の開発に役が立つと思われる。カスパーゼファミリー蛋白質はアポトーシスの発生に深く関与し脳虚血による神経細胞死にも重要な役割を演じると考えられる。この一年間にわたって、当研究者はマウス前脳虚血モデルを用いてこの虚血前処置による保護効果がカスパーゼ−3 活性の変化と関連するか否かのを検索しを行った。

18−22g の雄性 C57/BL マウスをハロセン麻酔下に、両総頚動脈を遮断、虚血を負荷した。動物はコントロール、6 分間虚血、18 分間虚血と 6-18 分間虚血の４群にわけた。カスパーゼ−3 活性は、再潅流後４、24、72 時間の時点に Ac-DEVD-AMC を substrate として蛍光光度法によって測定した。組織的変化について、再潅流後 7 日目に PLP による脳潅流固定を行い、16μm の凍結切片を作製し蛍光免疫組織化学染色行い、線条体領域の残存神経細胞数を算出した。カスパーゼ−3 活性は、コントロールと 6 分間虚血の両群間に有意差がないのに対して、18 分間虚血群と比べて 6-18 分間虚血群は著しく抑制された。また、6-18 分間虚血群の残存神経細胞数は 18 分間虚血群より有意に多かった。
以上の結果から、一過性脳虚血によるニューロン死に対して、虚血前処置はニューロン保護作用があり、カスパーゼ−3 活性の抑制が虚血前処置の神経保護作用に深く関与していることが解った。

Key word: C57BL/6 mice; Ischemic preconditioning; Striatum; Caspase-3; Neuro-specific nuclear protein

Introduction

Present findings indicate that caspases are key mediators of apoptosis initiating complex proteolytic reactions leading to cell assembly and eventually cell death [1,2]. Caspase-3 is an established member of the caspases family known to be involved in final execution phase of apoptosis [2]. There is now evidence in support of activation of caspase-3 in the brain after ischemia in newborn animals. The caspase-3-like activity increases progressively between 1 and 24 h of reperfusion and inactive (32 kDa proform) is cleaved to active forms (e.g. 17 kDa) along with increased immunoreactivity of active caspase-3 in tissue sections [3,4]. Furthermore, treatment with caspase-3 inhibitors reduces brain injury even if applied several hours after the insult [3]. These data suggest that caspase-3 is an important effector for development of brain injury after ischemia.

This phenomenon of ischemic preconditioning that was discovered in the myocardium in 1986 by Murray et al. [5] has been subsequently documented in a variety of tissues including the brain [6,7], skeletal muscle [8], spinal cord [9] and liver [10]. The hope that this fascinating phenomenon may find new therapeutic applications has provided a great impetus to identify the underlying mechanisms. Although a multitude of mediators have been investigated in various tissues and different experimental conditions, including adenosine [11], nitric oxide [12] and heat shock protein [13], the mechanisms by which ischemic preconditioning can protect the cells from a subsequent lethal insult remain to be elucidated.

Taken together, we postulated that attenuation of caspase-3 activation might be a pathway through which ischemic

preconditioning provide neuron protection. To test our hypothesis, we evaluate the extent to which caspase-3-like activity and neuronal cell damage after lethal ischemia are affected by ischemic preconditioning.

Materials and methods

This study was approved by the Committee on the Guidelines for Animal Experimentation of Niigata University.

Experiments were performed in C57Black/Crj6 mice. The methods for induction of cerebral ischemia and ischemic tolerance were described previously [14]. Briefly, mice weighting 18 to 22 g (8-9 weeks olds) were given free access to food and water and housed in a climate-controlled environment before surgery. Animals were divided into four groups (n = 6 for each). Forebrain ischemia was induced by occlusion of bilateral common carotid arteries for 18 min. In conditioning ischemic group, animals were treated with two episodes of 6-min bilateral common carotid artery occlusion (BCCAO) at an interval of 24 h. In ischemic preconditioning group, animals were treated with two episodes of 6-min ischemia BCCAO 48 h before the 18-min BCCAO. In sham group, bilateral common carotid arteries were exposed for two times at an interval of 48 hours. During the operation, the rectal temperature was maintained at 36.8 to 37.2°C. After recovery from anesthesia, the animals were cared in a warm, humidified chamber (32-33°C) for 3 hours before being returned to their cages.

essment of neuronal damage

On the 7th day after reperfusion, animals were reanesthetized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg) and transcardiacally perfused with 0.1 M PBS containing heparin (4 units/ml), followed by 0.01 M periodate-0.075 M lysine-2% paraformaldehyde (PLP) in 0.0375 M phosphate-buffered solution (pH 6.3). The whole brain was removed from the skull, and post-fixed in the same fixative for 4 h. Thereafter, the brains were washed in gradually increasing concentrations of sucrose in 0.1 M PBS (10% for 4 h, 15% and 20% for 12h each) and then rapidly frozen in 2-methylbutane chilled at -80°C. Consecutive coronal sections (12 μm) were prepared on a microtome and used for assessing neuronal damage. Neurons in the striatum were visualized by neuro-specific nuclear (NeuN) protein immunofluorescent staining. After the sections were washed four times with 0.1 M PBS, they were incubated with 5% goat serum in 0.1 M PBS containing 1.5% BSA and 0.3% Triton X-100 (blocking solution) for 120 minutes at room temperature. The sections were then incubated with mouse anti-NeuN monoclonal antibody (Chemicon Inc. Temecula, CA, USA) diluted with the blocking solution at a concentration of 5 μg/ml at 4°C overnight. After washing, the sections were incubated with Cy3-labeled goat anti-mouse IgG (Chemicon Inc. Temecula, CA, USA) diluted in the ' ' cking solution for 6 h at room temperature. Sections were examined with a Nikon fluorescence microscope (Optiphot-2, ...on, Tokyo, Japan). The number of NeuN–positive cells was counted in five assigned subregions (250 × 125 μm) as described previously [14].

Fluorometric assay of caspase-3-like activity

Caspase-3-like activity was measured in extracted cytosolic fraction, as described [15] with modifications. At 8, 24 and 72 h after reperfusion and 24 h after sham operation, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg) and decapitated. Brains were quickly removed, and the bilateral striatums were dissected. Striatal protein extracts were prepared on ice by Dounce homogenization of tissues in a lysis buffer containing 15 mM Tris-HCl (pH 7.5), 250 mM sucrose, 2mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 50 mM NaF, 100 mM Na_3VO_4, 2 mM sodium pyrophosphate, 100 μg/ml of PMSF and 10 μg/ml each of pepstain, leupeptin, and aprotinin (all obtained from Sigma, St. Louis, MO, USA). Cell lysates were centrifuged at 14000 g for 60 min, and the supernatants were stored at -80°C until use. Protein concentrations were determined using the method of Lowry with BSA as a standard. For each reaction, 100 μg of the striatal protein extracts and 5 μl (1 μg/μl) of reconstituted Ac-DEVD-AMC were mixed with the reaction buffer [20 mM HEPES (pH

7.5), 10% glycerol, 2 mM DTT] in a total volume of 150 μl. Ac-DEVD-AMC [*N*-acetyl-Asp-Glu-Val-Asp-AMP (7-amino-4-methylcoumarin)] (PharMingen, component no.) was used to determine caspase-3-like activity. Ac-DEVD-AMC has been reported to have linear Michaelis-Menton kinetics with a Michaelis constant of 10 μmol/L for caspase-3. Caspase-3 cleaves the tetrapeptide between D and AMC, releasing the fluorescent AMC, which can be quantified by microscopic fluorospectrometer (JASCO CAM-500, Japan Spectroscopic Co., Tokyo, Japan), using an excitation wavelength of 380 nm and emission wavelength of 440 nm. The change of caspase-3-like activity was expressed by the net change in fluorescence.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) and analysed using Student's t test or one-way ANOVA as appropriate. ANOVA was followed by Dunnett's *post hoc* test for multiple comparisons. We considered significant differences at less than 5% level of p value.

Results

A sublethal Ischemic preconditioning protects neurons against subsequent prolonged ischemia

The extent of neuroprotection achieved by 6-min ischemic preconditioning was similar to that reported previously [14]. There e no significant differences between sham group and 6-min ischemia group in the number of NeuN-positive neurons in the bilateral striatum. In contrast, an 18-min ischemia caused a near complete loss of NeuN-positive neurons in the bilateral striatum in 4 out of 6 animals. In ischemic preconditioning group, a 6-min ischemic episode performed 48 h before the 18-min ischemia strongly reduced the loss of NeuN-positive neurons due to the ischemia group in 5 out of 6 animals.

Ischemic preconditioning decreased caspase-3-like activity

In comparison with sham group (2.28 ± 0.47 arbitrary units), almost no changes in conditioning ischemic group (2.80 ± 0.75 arbitrary units) in the caspase-3-like activity of striatum extracts as assessed by monitoring the cleavage of the fluorogenic peptide substrate Ac-DEVE-AMC. In contrast, an 18-min ischemia caused significant increasing of caspase-3-like activity at 8 (17.68 ± 1.93 arbitrary units), 24 (14.03 ± 1.77 arbitrary units) and 72 h (9.96 ± 1.83 arbitrary units) after reperfusion, respectively. In ischemic preconditioning group, 6-min BCCAO significantly reduced the caspase-3-like activity induced by subsequent 18-min ischemia at 4 h (10.63 ± 1.57; $p < 0.05$, compared with 18-ischemia group) and 24 h (7.5 ± 0.76, $p < 0.05$), but not 72 h of reperfusion. The effect of ischemic preconditioning to caspase-3-like activity induced by subsequent lethal ischemia at 4 h, 24 h ' 72 h for reperfusion was shown in figure.

.scussion

Our major finding is that striatal neuroprotection achieved by ischemic preconditioning was associated with an attenuation of caspase-3-like activity in striatal extracts in C57Black/Crj6 mice.

There have been several lines of evidence indicating that activation of caspase-3 is involved in ischemic neuronal death [16, 17]. At the transcription level, a study has found that transient global forebrain ischemia upregulated expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons [18]. Additionally, many studies have shown that cerebral ischemia enhanced caspase-3 activity [19]. The involvement of caspase-3 in ischemic neuronal death is further supported by that inhibition of caspase-3 activity with chemically synthesized peptides enables to protect neurons against ischemia [20].

Recent studies have shown that attenuation of caspase-3 activation may be involved in ischemic tolerance. Studies on the heart [21] and the liver [22] have shown that ischemic preconditioning could inhibit caspase-3 activation. Our study confirmed that inhibition of caspase-3 activation by ischemic preconditioning occurred in the mouse brain also. Taken these results together, attenuation of caspase-3 activation may take part in the neuroprotection achieved by ischemic preconditioning.

Why ischemic preconditioning could inhibit caspase-3 activation induced by subsequent prolonged ischemia? It is known that caspase-3 exists as an inactive precursor (procaspase-3) in the mammalian cells. The procaspase-3 can be activated via both the extrinsic and intrinsic pathways. The extrinsic pathway involves in caspase-8 activation through tumor necrotic factor family of cytokine receptors. The intrinsic pathway needs the release of cytochrome c from mitochondria. Once in the cytosol, cytochrome c binds to Apaf-1 and enables this protein to activate procaspase-9, with the existing of dATP. The activated caspase-9 then cleaves pro-caspase-3 to the active form of caspse-3 [23]. There have been several studies showing that ischemic preconditioning enables to attenuate the release of cytochrome c from mitochondria [24]. So inhibition of cytochrome c release from mitochondria may take part in attenuating caspase-3 activation by ischemic preconditioning. Further study is necessary, however, to examine if other pathways are involved.

Conclusion

The ischemic preconditioning inhibited the caspase-3-like activity induced by subsequent lethal ischemia, suggesting that attenuation of caspase-3 activation by ischemic preconditioning may be an important step in protecting the brain against ischemia/reperfusion injury

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