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1. 研究テーマ

軽度前脳虚血による C57BL/6 マウスの虚血耐性発現に関する研究

2. 本年度の研究業績

(1) 学会・研究会等における発表 (有) ・ 無 (学会名・演題)

第 5 4 回新潟麻酔懇話会
軽度前脳虚血による C57BL/6 マウスの虚血耐性発現に関する研究
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(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

1. Chaoran Wu, Ren-zhi Zhan, Sihua Qi, Hideyoshi Fujihara, Kiichiro Taga, Koki Shimoji. A forebrain ischemic preconditioning model established in C57Black/Crj6 strain mice. J Neuroscience Methods 107 (2001) 101-106.
2. Renzhi Zhan, Chaoran Wu, Hideyoshi Fujihara, Kiichiro Taga, Sihua Qi, Makoto Naito, Koki Shimoji. Both caspase-dependent and independent pathways are involved in hippocampal CA1 neuronal death due to loss of cytochrome c from mitochondria in a rat forebrain ischemia model. J Cereb Blood Flow Metab 21:529-540.
3. Renzhi Zhan, Sihua Qi, Chaoran Wu, Hideyoshi Fujihara, Kiichiro Taga, Koki Shimoji. Intravenous anesthetics differentially reduce neurotransmission damage caused by oxygen-glucose deprivation in rat hippocampal slices in correlation with NMDA receptor inhibition. Crit Care Med. 2001.Vol.29.No.4(808-813).

3．今後の研究計画

脳虚血により起きる神経細胞死に対し、BDNF,FGF,EGF に代表される神経栄養因子がどのような細胞保護効果をもたらすか、そして、その効果が cytochrome c の放出と Bcl-2 とどのように関連しているかを探求する計画である。免疫組織化学や生化学的手法を駆使して、ミトコンドリアを介したアポトーシスを中心に評価する。加えて、BDNF 遺伝子、EGF 受容体遺伝子のノックアウトマウスを利用して、内在性のこれら神経栄養因子の役割についても考察したい。

4．研究指導者の意見

呉超然氏は１９９９年に来日し、本学医学部麻酔学教室に客員研究員として入局しました。翌年４月には本学医学研究科博士課程（外科系・麻酔学専攻）に入学し、麻酔学教室に大学院生として在籍しています。

現在、脳虚血に関する病態生理及びその蘇生について昼夜も問わず、研究に取り組んでおります。２年生として、すでに論文も英文誌に採用され、学会口演の経験もあります。本学に入学後の学習態度は真剣であり、強い向学心を有していることが窺えます。呉超然氏は大学院生としてのふさわしい学力に加えて、日本語に関しても、日常生活はもとより研究を行う上でも十分に高い語学力を有していると判断されます。

以上のように呉超然氏は今後優れた研究を行うことが期待される人物であります。

研究指導者氏名　馬場　洋　㊞

5．研究報告

別紙形式により、報告本文4000字以上(英文は2600語以上)で報告して下さい（枚数自由・ワープロ使用）

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研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

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軽度前脳虚血によるC57BL/6マウスの虚血耐性発現に関する研究

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要旨

脳虚血前処置分子機序の研究は、虚血性脳損傷のメカニズムの解明だけではなくて脳虚血治療法の開発にも役立つと思われる。今回、我々はまず遺伝子操作の分野でよく使われるC57BL/6マウスを用いて、マウス脳虚血前処置モデルを作成し、そして神経細胞の生と死と関連するBcl-2 familyタンパクの発見について経時・定量的に検索する。

雄性C57BL/6マウスを用いた。前脳虚血は、両側の総頚動脈を閉塞することによって行った。対照群（shamコントロール）、虚血群（１８分間虚血）と前処置群（６分間虚血後４８時間後１８分間虚血）に分けた。線条体損傷は凍結切片に行われるMAP-2染色によって評価した。Bcl-２タンパクを定量的に測定するために、６分間虚血後再潅流４時間、２４時間、４８時間、７２時間、１週間ごとに脳を取り出して、皮質、海馬、線条体をそれぞれ剥離し、western blotの方法を用いて、Bcl-２タンパクを定量的に測定し、バンドの光学密度を評価する。

虚血後の残存神経細胞数は虚血群に比較して前処置群で有意に多かった。虚血群においては、MAP-2免疫染色はほぼ完全に脱落した。前処置群では対照群に比べてMAP-2免疫染色は若干減弱したが、かなり正常に近い状態で染色された。６分間虚血後、線状体においてBcl-２タンパクの量が増える一方で４８時間の時点でピークに達した。

C57BL/6マウスにおいて、軽度前脳虚血はその後の致死性脳虚血に対して保護作用があることが示唆された。この保護作用は短期間虚血によるBcl-２タンパクの増加と関連があると示唆する。

Key word: C57BL/6 mice; Bcl-2; Ischemic preconditioning; Striatum; Sublethal ischemia

Introduction

The bcl-2 proto-oncogene is first discovered in b-cell lymphoma and a variety of other cancers (Tsujimoto et al., 1985). It is also observed in the central nervous system (CNS) during embryonic development (LeBrun et al., 1993; Novack and Korsmeyer, 1994), but levels are greatly downregulated in the majority of postmitotic CNS neurons of adult brain (Merry et al., 1994). The bcl-2 family of oncogenes encodes specific proteins that regulate apoptosis induced by a variety of stimuli (Vaux et al., 1988; Davies 1995). Among these related proteins, Bcl-2 and Bcl-xl suppress apoptotic cell death, whereas Bax promote it (Oltvai et al., 1993). Changes in the bcl-2 family genes levels have been observed as early as 6 to 8 hours after ischemia (Krajewski et al., 1995; Chen et al., 1996; Antonawich et al., 1996). Therefore, it has attracted the most attention that the counterregulated expression of Bcl-2 family proteins determines whether neurons toward survival or apoptosis (Yang E et al., 1996, Kroemer G., 1997).

Most evidence indicated that apoptotic cell death may occur after transient cerebral ischemia and that dysregulation of Bcl-2 and related proteins accelerates ischemic neuronal injury. Furthermore, a forebrain ischemic preconditioning model in C57Black/Crj6 (C57BL/6) mice developed recently in our laboratory has shown that striatal neuronal injury after transient bilateral common carotid artery occlusion (BCCAO) can be strongly reduced by a sublethal ischemic episode (Wu CR et al., 2001). To address the question of whether these Bcl-2 family proteins participate in the occurrence of ischemic preconditioning protection induced by sublethal forebrain ischemia, we examined Bcl-2, Bcl-xl, and Bax expression by immunohistochemistry and immunoblot assays in the normal striatum and following 6 min of sublethal forebrain ischemia in C57BL/6 mice.

Objective

We developed an ischemic preconditioning model in C57BL/6 mice. And then aimed to address the question of whether Bcl-2 family proteins participate in the induction of ischemic tolerance. As a preliminary experiment, we examined if ischemic preconditioning alters expression of apoptosis inhibitor Bcl-2, Bcl-xl, and apoptosis promoter Bax by using western blot.

Materials and Methods

Induction of forebrain ischemia

Experiments were carried out in adult male C57BL/6 mice weighing 19 to 23 g (8-10 weeks old). Animals were allowed free access to food and water and housed in a climate-controlled environment (25℃). Forebrain ischemia was induced by bilateral common carotid artery occlusion (BCCAO) for 18 min under 1.0% halothane/oxygen anesthesia via a face mask. During the procedure, the rectal temperature was maintained between 36.7 and 37.5℃. After reperfusion, the animals were cared in a warm, humidified chamber (32-33℃) for another 3 hours before being returned to their cages. For preconditioning, animals were subjected to a 6-min BCCAO 48 h before the 18-min ischemia. The animals underwent exposure of bilateral common carotid arteries without BCCAO were used as sham-controls.

Transcardiac perfusion and brain tissue preparation

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 12 h each) and then rapidly frozen in 2-methylbutane chilled at -80℃. Consecutive coronal sections (16 μm) were prepared on a microtome and used for microtubule-associated protein-2 (MAP-2) immunohistochemistry staining. For in situ

detection of DNA fragmentation, another set of animals were perfused with 0.1 M PBS containing heparin (4 units/ml) followed by 20% sucrose in 0.1 M PBS at 72 h after reperfusion. The brains were then removed from the skull, and rapidly frozen in 2-methylbutane chilled at -80℃.

Assessment of neuronal damage

Ischemic neuronal damage was visualized by MAP-2 immunohistochemistry. Briefly, sections were post-fixed with 4% paraformaldehyde in 0.1 M PBS for 10 min and washed three times with 0.1 M PBS. Endogenous peroxidase was inactivated by incubating the sections with 30% methanol containing 0.1% H_2O_2 (v/v) for 45 min. Sections were then followed by sequential incubations in 1) 2.5% horse serum in 0.1 M PBS containing 0.2% Tween-20 and 1.5% BSA for 60 min, 2) anti-MAP-2 monoclonal antibody (Boehringer Mannheim, Mannheim, Germany) diluted in 0.1 M PBS at 4℃ overnight, 3) biotinylated horse anti-mouse IgG for 60 min, and 4) avidin-biotin-peroxidase complex in 0.1 M PBS for 60 min. The biotinylated secondary antibody and the avidin-biotin-peroxidase were purchased from Vector Laboratories (Burlingame, CA). The MAP-2 immunoreaction was visualized by incubating the sections with 0.05% 3,3'-diaminobenzidine and 0.002% H_2O_2.

In situ labeling of DNA fragmentation

By using an apoptosis in situ detection kit (Wako Pure Chemical Industries, Osaka, Japan), terminal deoxytransferase-mediated dUTP-nick end labeling (TUNEL) was carried out on non-fixed sections (16 μm). Sections were post-fixed with 4% paraformaldehyde in 0.1 M PBS for 15 min at room temperature, followed by ethanol/acetic acid (2:1) for 5 min at -20℃, and then penetrated with 3% Triton X-100 in 0.1 M PBS for 60 min at room temperature. The sections were then incubated in TUNEL reaction solution for 60 min at 37℃. After washing, sections were incubated in 30% H_2O_2 in 0.1 M PBS for 10 min to quench endogenous peroxidase and incubated with anti FITC-conjugated peroxidase for 60 min at 37℃. TUNEL-positive cells were visualized with diaminobenzidine. Identical to the assessment of intact neurons, TUNEL-positive cells were counted in five assigned subregions.

Western blot analysis

Immediately after decapitation at 4, 24, 48, 72 h, and 7 day following 6 min ischemia (n = 12 per group), mouse striatum was dissected, and homogenized in RIPA buffer containing protease inhibitors on ice. After centrifugation at 14,000 g for 30 minutes, the serum was collected. The protein levels were quantified by spectrophotometry, and the lysate was boiled at 95 in sodium dodecyl sulfate loading buffer (100 mmol/l Tris-HCl, 200mmol/l dithiourea, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, and 20% glycerol) for 5 minutes. Then, 40 μ g protein samples were loaded onto a 12% SDS-polyacrylamide gel. The transferred polyvinylidene difluride (PVDF) membrane was incubated in either mouse anti Bcl-2 (1:1000, Transduction Laboratories.) or rabbit anti Bcl-XL, Bax antibody (1:500, Santa Cruz Biotechnology Inc.) for 2 hours at room temperature. After washing three times in PBS containing 0.1% Tween-20, the appropriate secondary antibody was applied at a 1:3000 dilution for 1 h. The membrane was washed in PBS containing 0.1% Tween-20 three times over 30 min, then incubated in

ECL Plus western blotting detection reagents (Amersham Pharmacia Biotech) and exposed to Fuji RX film (Fuji, Tokyo, Japan). Autoradiogram signals were quantified by a gel densitometric scanning program. The relative protein levels were determined from the relative optical densities of the corresponding protein bands and were normalized to background values obtained on the same lane.

Statistical analysis

Quantitative data are expressed as mean ± standard deviation (SD) and analyzed by one-way ANOVA after normality of distribution was proved. ANOVA was followed by Dunnett's post hoc test for multiple comparisons. A p-value less than 0.05 is considered statistically different.

Result

On the seventh day after 18-min ischemia, neuronal loss could be found in the striatum, hippocampal CA1, CA2 and CA3 regions, and the cortex as well as other regions, as visualized by MAP-2 immunostaining. In the hippocampus, neuronal loss always occurred in the dorsal CA1 region, CA2 as well CA3 region. In 3 out of 6 animals, CA1 neuronal damage was asymmetric after 18-min ischemia. In contrast, striatal neuronal damage in the bilateral striatum was symmetric and more consistent.

Since neuronal damage in the striatum was more consistent after the 18-min ischemia, the extent of protection by ischemic preconditioning was examined in the striatum by MAP-2 immunohistochemistry staining. In the sections obtained from sham-control animals, both soma and dendrites were intensively stained. Ischemia for 6 min resulted in no detectable loss of MAP-2 immunoreactivity in the bilateral striatum. An 18-min ischemia caused near complete loss of MAP-2 immunoreactivity in 4 out of 6 animals. A 6-min ischemic episode performed 48 h before the 18-min ischemia strongly reduced the loss of MAP-2 immunoreactivity due to the 18-min ischemia.

There were no TUNEL-positive cells to occur in sections obtained from sham-control animals. TUNEL-positive cells intensely existed at 72 h after reperfusion in animals subjected to 18-min ischemia but not 6-min ischemia. Again, the 6-min ischemia performed 48 h before the 18-min ischemia significantly reduced the number of TUNEL-positive cells caused by the 18-min ischemia.

The levels of Bcl-2 and Bcl-xl proteins expression were increased from 4h to 7 days after 6 min of BCCAO with a maximum intensity occurred in the 48h time point. Bax immunoreactivity was very faint in the above mentioned time points.

Discussion

The major finding of the present study is that transient bilateral common carotid artery occlusion produces a consistent striatal injury that can be strongly attenuated by a sublethal ischemic episode. And also we demonstrate that the Bcl-2 family proteins participate in the process of this ischemic protective effect.

Currently, almost all available ischemic preconditioning models have been developed in rats or gerbils,

but this is not corresponding to that most genetic alterations have been performed in mice. Among different strains, the C57BL/6 strain has been shown to be most susceptible to transient ischemia induced by BCCAO (Barone et al., 1994; Fujii et al., 1997; Yang et al., 1997). Additionally, studies have shown that injury to the striatum in C57BL/6 mice is more consistent in comparison with injury to the hippocampal CA1 region (Terashima et al., 1998).

In preliminary experiments, we had examined neuronal damage induced by BCCAO for 3, 4, 6 and 8 min. Although there was no striatal and hippocampal neuronal damage to be found after 3 or 4 min ischemia, their protections against the subsequent 18-min ischemia were weaker. In contrast, ischemia for 8 min sometimes caused serious striatal and hippocampal neuronal damage. We thus chose a duration of 6 min as the conditioning ischemic insult. While the 6-min ischemia did not cause noticeable striatal neuronal damage as demonstrated by both MAP-2 immunohistochemistry staining, it rendered strong protection against ischemic neuronal damage induced by the subsequent 18-min ischemia. The extents of protection are comparable with that reported in hippocampal CA1 neurons in a gerbil ischemic preconditioning model (Kirino et al., 1991; Kitakawa et al., 1990). In addition, we also found that ischemia-induced DNA fragmentation was attenuated by the conditioned ischemia, indicating that anti-apoptotic actions may be induced by the conditioning ischemic episode.

There may be several mechanisms by which Bcl-2 and Bcl-xl exerts their protective effects on ischemic neurons. Bcl-2 plays a key role in regulating initiation of programmed cell death by preventing egress of cytochrome C into cytoplasm. Cytochrome C, in turn, complexes with apaf-1 activating caspase 9, an important triggering event in apoptosis. Bcl-2 may also play a role in stabilizing the mitochondria and maintaining its membrane potential, thus preventing the generation of free radicals. So that, the expression of Bcl-2 in neurons that are stressed may be an important event that ensures neuronal survival.

References

Antonawich FJ, Federoff HJ, Krajewski S, Reed JC, Davis JN (1996) Ischemic CA1 hippocampal neurons express bax and can be rescued by bcl-2 induction with a herpes simplex virus (HSV) amplicon (Abstract). Soc Neurosci Abstr 22:1179

Barone FC, Knudsen DJ, Nelson AH, Feuerstein GZ, Willette NR (1993) Mouse strain differences in susceptibility to cerebral ischemia and related to cerebral vascular anatomy. J Cereb Blood Flow Metab 13:683-92.

Chen J, Zhu RL, Nakayama M, Kawaguchi K, Jin KL, Stetler RA, Simon RP, Graham SH (1996) Expression of the apoptosis effector gene, bax, is up-regulated in vulnerable hippocampal CA1 neurons following global ischemia. J Neurochem 67:64-71

Davies JC (1995) The Bcl-2 family of proteins, and the regulation of cell survival. Trends Neurosci 18:355-358

Fujii M, Hara H, Meng W, Vonsattel JP, Huang Z, Moskowitz MA (1997) Strain-related differences in

susceptibility to transient forebrain ischemia in SV-129 and C57Black/6 mice. Stroke 28:1805-11.

Kirino T, Tsujita Y, Tamura A (1991) Induced tolerance to ischemia in gerbil hippocampal neurons. J Cereb Blood Flow Metab 11:299-307.

Kitakawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T (1990) "Ischemic tolerance" phenomenon found in the brain. Brain Res 528:21-24.

Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski M, Reed JC (1995) Upregulation of bax protein levels in neurons following cerebral ischemia. J Neurosci 15:6364-6376

Kroemer G (1997) The proto-oncogene bcl-2 and its role in regulating apoptosis. Nature Med 3:614-620

LeBrun DP, Warnke RA, Cleary ML (1993) Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. Am J Pathol 142:745-753

Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ (1994) Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development 130:301-311

Novack DV, Korsmeyers SJ (1994) Bcl-2 protein expression during murine development. Am J Pathol 145:61-73

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609-619

Terashima T, Namura S, Hoshimaru M, Uemura Y, Kikuchi H, Hashimoto N (1998) Consistent injury in the striatum of C57BL/6 mice after transient bilateral common carotid artery occlusion. Neurosurgery 43:900-08.

Tsujimoto Y, Cossman J, Jaffe E, Croce CM (1985) Involvement of the bcl-2 gene in human follicular lymphoma. Science 228:1440-3

Vaux DL, Cory S, Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335:440-442

Wu CR, Zhan R-Z, Qi SH, Fujihara H, Taga K, Shimoji K (2001) A forebrain ischemic preconditioning model established in C57Black/Crj6 mice. J Neurosci Meth 107:101-106

Yang E, Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the bcl-2 family and cell death. Blood 88: 386-401

Yang G, Kitakawa K, Matsushita K, Mabuchi T, Yagita Y, Yanagihara T, Matsumoto M (1997) C57BL/6 strain is most susceptible to cerebral ischemia following bilateral common carotid occlusion among seven mouse strains: selective neuronal death in the murine transient forebrain ischemia. Brain Res 752:209-18.