ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Mar; 22 (3): 249 - 252
http://www.chinaphar.com

A phospholipase C inhibitor, phenylmethylsulfonyl fluoride, ameliorates ischemic injury to brain mitochondria in rats[1]

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KEY WORDS phospholipase C; brain ischemia; mitochondria; phospholipids; membrane fluidity

ABSTRACT

AIM: To study the effect of phenylmethylsulfonyl fluoride (PMSF), a phospholipase C inhibitor, on ischemic injury to brain mitochondria in rats. **METHODS**: The phospholipid level, membrane fluidity, and respiratory control ratio of brain mitochondria were measured. The effect of phenylmethylsulfonyl fluoride was tested. **RESULTS**: The phospholipid level, membrane fluidity, and respiratory control ratio of brain mitochondria in ischemia group decreased but increased in the PMSF treatment group ($P < 0.05$). **CONCLUSION**: PMSF ameliorated ischemic injury to brain mitochondria in rats.

INTRODUCTION

It is well known that ischemic injury to brain mitochondria takes place during cerebral ischemia. Factors that have been proposed to account for ischemic injury to brain mitochondria during ischemia and reperfusion include intercellular acidosis, Ca^{2+}-induced membrane damage, and free-radical-dependent membrane lipid peroxidation. Measures can be taken to manipulate these factors so that ischemic injury to brain mitochondria may be minimized and cell viability be optimized during resuscitation.

During brain ischemia, many kinds of neurotransmitters are released from synaptomes in large amounts by membrane depolarization. Some neurotransmitter receptors including muscarinic cholinergic, adrenergic, histaminergic, and serotonergic receptors activate phospholipase C (PLC) by a receptor-mediated mechanism coupled to a G protein and linked to PI turnover. Recently it has become evident that glutamate, which may play an important role in neurotoxicity in ischemia, has such a receptor activating phosphatidylinositol (PI) turnover[1]. The activation of PLC may lead, through IP_3-mediated Ca^{2+} mobilization from intracellular stores to increase cytosolic calcium concentration. It therefore seems possible that the activation of PLC by the excessive release of neurotransmitters in ischemia is related to neuronal damage[2].

It was reported that the release of free fatty acid in the early period of ischemia can be attributed mostly to the activation of PLC, and that the activation of PLC further influenced the release of free fatty acid by phospholipase A_2 (PLA_2) in the subsequent course. This effect seemed to be related to IP_3-mediated Ca^{2+} release. The activation of PLC seems to trigger some specific events, such as the activation of PLA_2 in ischemia[3].

The present study examined the effect of phenylmethylsulfonyl fluoride (PMSF), a PLC inhibitor, on brain mitochondrial damage in rats. The role of PLC and PLA_2 on ischemic injury to brain mitochondria is then discussed.

MATERIALS AND METHODS

Drugs PMSF was obtained from Sigma Chemical Co. It was mixed with 1 mL of 4 % gum acacia suspension before use. In the treatment group, rats were treated with PMSF 100 mg/kg ip 60 min before ischemia. In the vehicle treatment group, rats were treated with the vehicle (1 mL of 4 % gum acacia suspension) ip 60 min before ischemia.

Animal model Twenty-eight male Wistar rats (weighing 250-300 g, Certificate № 24301050, Grade Ⅱ, from Experimental Animal Center of Third Military Medical Universtiy) were equally divided into 4 groups: 1) control group, 2) ischemia group, 3) PMSF treatment

[1] Project supported by National 973 Project, № G1999054007.
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Received 2000-06-15 Accepted 2000-12-22

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Mar; 22 (3)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

group, 4) vehicle treatment group. Under 2 % halothane anesthesia, both vertebral arteries of each rat were electrocoagulated. On the following day both common carotid arteries were gently exposed under 2 % halothane anesthesia. Three minutes after the halothane was discontinued both common carotid arteries were occluded with aneurysmal clips for 20 min[2] to induce complete brain ischemia.

Isolation of brain mitochondria The brain mitochondria were isolated according to the procedure described by Michele *et al*[4]. Rats were decapitated and their forebrains were removed immediately and placed into an ice cold isotonic sucrose medium. This medium consisted of sucrose 150 mmol/L, HEPES 10 mmol/L (pH 7.4), bovine serum albumin 1 g/L , edetic acid 0.5 mmol/L . The tissues were homogenized with a Teflon pestel. The homogenate was immediately centrifuged at 2000 × *g* for 3 min. The supernatant was decanted and centrifuged at 12 000 × *g* for 8 min. The supernatant was discarded and the pellet was resuspended into the isotonic sucrose medium. Then the suspension was centrifuged at 12 000 × *g* for 10 min. The pellet was resuspended into sucrose 0.25 mol/L and centrifuged at 12 000 × *g* for 10 min. The mitochondrial pellets were then rinsed lightly with sucrose 0.25 mol/L and suspended into it to give approx concentration of 10 – 20 g protein/L.

Measurement of phospholipids in brain mitochondria Phospholipids in brain mitochondria were measured by HPLC (Shimadzu LC-9A Japan), using the method described by Tang *et al*[5]. The chromatographic column 150 mm × 6 mm ID was pre-packed with Bio-Sil HP-10 (10 μm). The guard column was Guard-PAKTM (RCSS Silica, Water Associates). Mobile phase was a solvent of acetonitrile : methanol : 85 % H_3PO_4 (φ = 250 : 7 : 2). The flow rate was 1 mL/min for 90 min. Phospholipids were obtained from Sigma Co.

Measurement of membrane fluidity in brain mitochondria Membrane fluidity (MF) of brain mitochondria was measured using the method described by Villacara *et al*[6]. MF was determined by a fluorescence polarization technique using 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) as a fluorescent probe. Samples were excited at 360 nm and emission intensity was measured at 435 nm. First, fluorescence anisotropy (P) was got. The MF was expressed by η. The η was calculated as follows: $\eta = 2P/(0.46 - P)$. The bigger η, the less the MF.

Measurement of respiratory control ratio in brain mitochondria Respiratory control ratio (RCR) of brain mitochondria was measured using the method described by Michele[4] *et al*. The reaction mixture consisted of sucrose 150 mmol/L, Tris-HCL 25 mmol/L, phosphate buffer 10 mmol/L (pH 7.4) and 0.7 – 1.0 mg mitochondrial protein. The final volume was 1.0 mL and the assay temperature was 30 ℃. RCR was calculated as follows: RCR = State 3/State 4.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

As shown in Fig 1, some major phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) in rat brain mitochondria were separated by HPLC.

The phospholipid levels of PC, PE, CL, and RCR

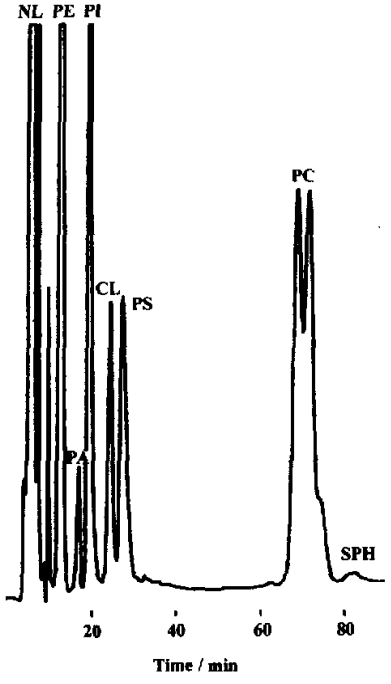

Fig 1. Separation of major phospholipids in rat brain mitochondria by HPLC. NL (nonhydroxylipids); PE (phosphatidylethanolamine); PI (phosphatidylinositol); PA (phosphatidic acids); CL (cardiolipin); PS (phosphatidylserine); PC (phosphatidylcholine); SPH (sphingomyelin).

in ischemia group were lower than control group but η was higher indicated that MF was decreased in ischemia group ($P<0.05$, Tab 1). PMSF increased the level of PC, PE, CL, and RCR and decreased η compared with ischemia group ($P<0.05$, Tab 1).

DISCUSSION

In the present study, we found that the phospholipid level, membrane fluidity, and respiratory control ratio of brain mitochondria in ischemia group decreased significantly and that ischemic injury to brain mitochondria took place during cerebral ischemia. From our result, we might infer the mechanism of ischemic injury to brain mitochondria as follows: (1) Phospholipid hydrolysis on the mitochondrial membrane will change membrane permeability characteristics to Ca^{2+}, which cause "Ca^{2+} overload". The excessive mitochondrial Ca^{2+} loading led to an uncoupling of oxidative phosphorylation[7]. (2) The elevated cellular levels of polyunsaturated fatty acids (FFA) and lysophospholipids, resulting from PLA_2-induced phospholipid hydrolysis, also can be toxic to cells[8,9]. (3) The phospholipids of mitochondrial membrane are particularly good substrates for PLA_2 action. Activation of PLA_2 results in hydrolysis of cardiolipin, which is a critical component of the catalytic subunits of the eletron transport chain, ATP synthetase, and adenine nucleotide translocase[10,11]. (4) Lipid peroxidation causes the release of FFA from the mitochondrial membrane. The presence of fatty acids with longer chain was reduced. This change decreased mitochondrial membrane fluidity[7,11].

PMSF is a serine esterase inhibitor. It is known that PMSF can pass the blood-brain barrier freely. PMSF inhibited the increase of arachidonic and stearic acids that seemed to be released from PI during the early period of ischemia in rat neocortex[3]. Umemura[2] *et al* reported that PMSF pretreatment at any dose significantly ameliorated ischemic delayed neuronal damage in the rat hippocampal CA1 subfield. In the present study, we found that the phospholipid level, membrane fluidity and respiratory control ratio of brain mitochondria in the treatment group were significantly higher than that of the vehicle treatment group. PMSF can ameliorate ischemic injury to brain mitochondria in rats. From our result, we might infer the mechanism of PMSF treatment as follows: (1) PMSF treatment might inhibit some specific events triggered by the activation of PLC, such as the activation of PLA_2 in ischemia. (2) PMSF treatment might protect mitochondrial membrane from phospholipid hydrolysis. (3) PMSF treatment might stabilize mitochondrial membrane fluidity. (4) PMSF treatment might improve mitochondrial respiratory function.

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Tab 1. The effect of PMSF on the phospholipid level, η, and respiratory control ratio (RCR) of brain mitochondria. $^bP<0.05$ *vs* control group. $^cP<0.05$ *vs* ischemia group.

	Control group	Ischemia group	PMSF group	Vehicle group
PC/mg·g^{-1}(protein)	97 ± 8	61 ± 10^b	91 ± 7^c	68 ± 10
PE/mg·g^{-1}(protein)	56 ± 4	38 ± 5^b	51 ± 4^c	38 ± 5
CL/mg·g^{-1}(protein)	3.1 ± 0.4	2.0 ± 0.4^b	2.9 ± 0.4^c	1.9 ± 0.4
η	2.82 ± 0.28	4.7 ± 0.6^b	3.1 ± 0.5^c	4.5 ± 0.6
RCR	4.13 ± 0.28	1.69 ± 0.18^b	3.98 ± 0.20^c	1.71 ± 0.19

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Mar; 22 (3)
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磷脂酶 C 抑制剂苯甲磺酰氟改善脑缺血线粒体损伤[1]

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关键词 磷脂酶 C; 脑缺血; 线粒体; 磷脂类; 膜流动性

目的: 研究磷脂酶 C 抑制剂 PMSF 在缺血性脑线粒体损伤中的作用. **方法**: 采用全脑缺血模型, 观察 Wistar 大鼠脑缺血 20 分钟再灌流 1 小时脑线粒体磷脂含量、膜流动性、呼吸控制率, 研究磷脂酶 C 抑制剂 PMSF 对上述指标的影响. **结果**: (1)脑缺血 20 分钟再灌流 1 小时脑线粒体磷脂含量、膜流动性、呼吸控制率显著下降; (2)PMSF 治疗组脑线粒体磷脂含量、膜流动性、呼吸控制率显著高于治疗对照组. **结论**: PMSF 能改善缺血性脑线粒体损伤, 其机制与抑制磷脂酶 C 有关.

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