

Effect of dopamine depletion on DARPP-32 protein in ischemic rat striatum¹

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KEY WORDS dopamine; corpus striatum; brain ischemia; immunohistochemistry; *in situ* hybridization

ABSTRACT

AIM: To study the effects of dopamine depletion on the phosphorylation level, intracellular distribution, and mRNA expression of DARPP-32 in the ischemic striatum and to elucidate the mechanisms underlying the ischemic injury. **METHODS:** A complex model of SN lesioning with 6-OHDA to deplete dopamine and four vessels occlusion for inducing forebrain ischemia was constructed in rats. DARPP-32 was investigated with autoradiogram, immunohistochemistry and *in situ* hybridization. **RESULTS:** The [³²P]phosphate incorporation of DARPP-32 was reduced *in vitro* following ischemia. However, the [³²P] phosphate incorporation, the numbers of positive neurons, and mRNA expression of DARPP-32 were increased in SN lesioning plus ischemic rats with denervated striatum. **CONCLUSION:** Dopamine depletion reduced the DARPP-32 phosphorylation *in vivo* following ischemia, and protected DARPP-32 immunoreactivity and mRNA expression level against the reduction induced by ischemia.

INTRODUCTION

The striatum is one of the most vulnerable regions in the central nervous system subject to transient cerebral ischemia. The striatum is innervated by nigrostriatal dopaminergic and corticostriatal glutaminergic projec-

tions. The excessive release of dopamine (DA) and glutamate (Glu) are involved in the development of striatal neuronal ischemic injury^(1,2). It has also been reported that DA depletion by injecting 6-OHDA into the substantia nigra could reduce neuronal death in the striatum after ischemia⁽³⁾.

DARPP-32 is selectively enriched in the medium-sized dense spiny neurons of the striatum and plays an important role in intracellular signaling cascade of striatum. In the present study, we evaluated the effect of DA depletion on phosphorylation level, protein content and mRNA expression, and distribution of DARPP-32 during striatal ischemia and reperfusion to get a better knowledge of the biochemical mechanism of ischemic striatal injury.

MATERIALS AND METHODS

Chemicals 6-Hydroxydopamine hydrochloride, desipramine hydrochloride (RBI, Sonoma, USA), catalytic subunit of PKA, pepstatin A, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), phenyl methyl sulphonyl fluoride (PMSF), adenosine 5'-triphosphate (ATP) and Kodak films (Sigma, Missouri, USA), DIG Oligonucleotide Tailing kit (Boehringer Mannheim, Germany) were used. Monoclonal antibody to bovine DARPP-32 was a gift from Dr HEMMINGS HC (University of Cornell, School of Medical College, USA). Nitrocellulose membranes (NC) was ordered from Amersham, USA. [³²P]ATP (185 PBq·mol⁻¹) was provided by Beijing Yahui Biomedical Co, China. All other reagents were of AR.

Animals Sprague-Dawley rats (♂, weighing 270 g ± s 20 g) were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade II, certification No 005).

Preparation of 6-OHDA-lesioned rats Rats were priority injected desipramine-HCl (25 mg·kg⁻¹, ip)

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to prevent the uptake of 6-OHDA into the noradrenergic neurons. After 30 min, 6-OHDA was microinjected automatically into the left medial forebrain bundle (Coordinate: AP-4.0 mm, ML 1.5 mm, DV 7.6 mm below the surface of brain) in 4 μL saline solution (containing 20 μg 6-OHDA and 1 μg ascorbic acid) at the rate of 0.5 $\mu\text{L} \cdot \text{min}^{-1}$. The needle was withdrawn after 10 min. The rats were tested with injected apomorphine (0.2 $\text{mg} \cdot \text{kg}^{-1}$, ip) in a bowl 4 weeks after lesioning. Only the rats showing contralateral rotation at a speed of more than 150 turns in 30 min were used in the subsequent experiments.

Four-vessel occlusion for forebrain ischemia

The lesioned rats were randomly assigned to four groups and were subjected to forebrain ischemia of 30 min by four vessels occlusion followed by reperfusion for 6 h, 12 h, 24 h, and a group of rats was sham-operated. The fifth group was only subjected to forebrain ischemia without any SN lesioning. At least six rats were assigned to each experimental group. Ten lesioned rats were used for DARPP-32 back-phosphorylation experiment. Transient forebrain ischemia was induced using the four vessels occlusion method following the procedure of Pulsinelli and Brierley^[4] with some modifications.

Acid extract of DARPP-32 protein The rats were decapitated and both sides of striatum and hippocampus were dissected and rapidly homogenized in ice-cold buffer (in $\text{mmol} \cdot \text{L}^{-1}$: Tris-HCl 10, edetic acid 2, NaF 2.5, PMSF 0.1, pH 7.4) with Glas-Col homogenizer on a high setting. Immediately after homogenization, proteins were precipitated by adding ice-cold ZnAc 5 $\text{mmol} \cdot \text{L}^{-1}$ to 5 mL of the above and were centrifuged at 4000 $\times g$ for 5 min. The pellet was resuspended in 0.5 mL of citric acid buffer 10 $\text{mmol} \cdot \text{L}^{-1}$ (pH 2.8) containing 0.1 % Triton X-100, pepstatin A 2 $\text{mg} \cdot \text{L}^{-1}$ and centrifuged at 28 000 $\times g$ for 15 min. The supernatant was adjusted to pH 6.5 with Na_2HPO_4 0.5 $\text{mol} \cdot \text{L}^{-1}$ and left on ice for 10 min. The final supernatant (15 000 $\times g$, 15 min) was collected for further use.

Back-phosphorylation assay of phospho-DARPP-32 protein Phosphorylation reaction was carried out for 60 min at 30 $^{\circ}\text{C}$ in a final volume of 50 μL containing ($\text{mmol} \cdot \text{L}^{-1}$): HEPES 50 (pH 7.4), MgCl_2 10, egtazic acid 2, DTT 2, NaF 5, cAMP 10, catalytic subunit of PKA 40 $\text{g} \cdot \text{L}^{-1}$, [$\gamma\text{-}^{32}\text{P}$] ATP 1 $\text{mmol} \cdot \text{L}^{-1}$ (1 TBq $\cdot \text{mol}^{-1}$), and 20 μg of striatum protein. The reaction was initiated by the addition of [$\gamma\text{-}^{32}\text{P}$] ATP and stopped by 18 μL of SDS sample buffer.

SDS-PAGE (12.5 %) was carried out according to

the method of Laemmli^[5]. The gel was stained with coomassie brilliant blue, destained, dried, and autoradiographed on Kodak LS-7300 films at -80 $^{\circ}\text{C}$ for 48 h.

Immunoblotting The purified proteins (20 μg) after acid extraction were treated with SDS buffer and boiled for 2 min. Aliquots were then subjected to SDS-PAGE (12.5 %) and electrophoretically transferred to nitrocellulose membranes (0.45 μm) according to Hemmings and Greengard^[6]. The blots were quenched in Tris-buffer saline (TBS) containing 0.05 % sodium azide and 1 % BSA at 45 $^{\circ}\text{C}$ for 8-12 h. The membranes were then incubated at room temperature with DARPP-32 monoclonal antibody diluted at 1:2000 in TBS containing 0.1 % BSA for 2 h. After being washed for 20 min in TBS containing 0.05 % Tween-20, 2 \times 20 min in TBS, the membranes were then incubated with goat anti-rabbit IgG-HRP conjugated antibody (1:50) for 2 h. At last, the membranes were washed (3 \times 20 min) with TBS containing 0.05 % Tween 20 ending with a single wash with TBS alone. The protein band was detected with the ECL immunoblotting detection system.

Tissue preparation The experimental rats were anaesthetized with chloral hydrate (400 $\text{mg} \cdot \text{kg}^{-1}$, ip) and then perfused through the ascending aorta with saline followed by 4 % ice-cold paraformaldehyde in phosphate buffer 0.1 $\text{mol} \cdot \text{L}^{-1}$ (pH 7.4). Brains were quickly removed and post-fixed for 8 h at 4 $^{\circ}\text{C}$ before being transferred into phosphate buffer 0.1 $\text{mol} \cdot \text{L}^{-1}$ containing 30 % sucrose. The brains were kept in the buffer for 2-3 d followed by serial coronal sectioning (30 μm) with a freezing microtome and were collected for successive experiments for DARPP-32 immunohistochemistry and for hybridization of DARPP-32 mRNA.

DARPP-32 immunohistochemistry Coronal sections at the coordinate Bregma 0.7 $\text{mm}^{[7]}$ were first blocked in the blocking solution at 37 $^{\circ}\text{C}$ for 1 h. Then, the sections were transferred to PBS containing 1:5000 anti-DARPP-32 and 0.3 % Triton-X 100 at 37 $^{\circ}\text{C}$ for 1 h and subsequently 4 $^{\circ}\text{C}$ for 48 h. The sections were washed with PBS and then incubated separately in 1:200 biotinylated secondary IgG and 1:200 avidin-biotin complex at room temperature for 2 h. After incubating in 0.05 % diaminobenzidine (DAB) and 0.003 % H_2O_2 , the sections were mounted on gelatin coated slides, dehydrated, cleared and coverslipped.

In situ hybridization for DARPP-32 mRNA

Sections were first dealt with proteinase K 2 $\text{mg} \cdot \text{L}^{-1}$ at 37 $^{\circ}\text{C}$ for 30 min. After incubated in 0.25 % acetate

anhydride and triethanolamine $0.1 \text{ mol} \cdot \text{L}^{-1}$ for 10 min, the sections were immersed in prehybridization solution (50 % formamide, $4 \times \text{SSC}$) and then hybridized in the solution containing digoxigenin-labelled antisense oligodeoxynucleotide probe ($300 \text{ g} \cdot \text{L}^{-1}$) over night at 37°C . Then, sections were washed in $0.1 \times \text{SSC}$ at 42°C for 30 min. After incubated with alkaline phosphatase labelled anti-digoxigenin Fab fragment (1:1000, Boehringer) at 4°C overnight, the sections were rinsed in Buffer I and in Buffer III and visualized by incubating with NBT/BCIP in Buffer II. Finally, the sections were mounted, dehydrated, and coverslipped with neutral balsam.

Quantification and statistical analysis The amount of [^{32}P]phosphate incorporated into DARPP-32 was revealed by densitometry using UVP Image analyzer software (USA), and the number of DARPP-32 positive neurons was counted with MD-20 (German) Image analyzer. Data ($\bar{x} \pm s$) were analyzed by ANOVA followed by Duncan's new multiple range method.

RESULTS

DARPP-32 phosphorylation After ischemia, there was a difference of the DARPP-32 phosphorylation levels between the denervated and intact striatum. The [^{32}P]phosphate incorporation in the intact striatum reduced by 30 % and 50 % at 10 min and 30 min ischemia, respectively. But in the denervated striatum, there was no difference in [^{32}P]phosphate incorporation after 10 min ischemia, and [^{32}P]phosphate incorporation was only reduced to 84 % of the control after 30 min ischemia (Fig 1).

No obvious variation in the content of DARPP-32 was revealed by quantitative immunoblotting in either normal or ischemia striatum. And there was also no difference in the content of DARPP-32 between intact and denervated striatum after 10 min and 30 min ischemia (the result not shown).

DARPP-32 immunohistochemistry An apparent protection of DARPP-32 immunoreactivity (IR) against ischemia and reperfusion was found in denervated striatum. Cerebral ischemia and reperfusion resulted in decrease of DARPP-32 IR in striatum. In intact striatum, DARPP-32 IR distinctly decreased after 30 min ischemia followed with 6 h and 12 h reperfusion, and nearly vanished after 24 h reperfusion. The number of DARPP-32 positive neurons was increased in denervated striatum than in the intact striatum after ischemia ($P < 0.05$, Fig 2). The DARPP-32 IR were also stronger in

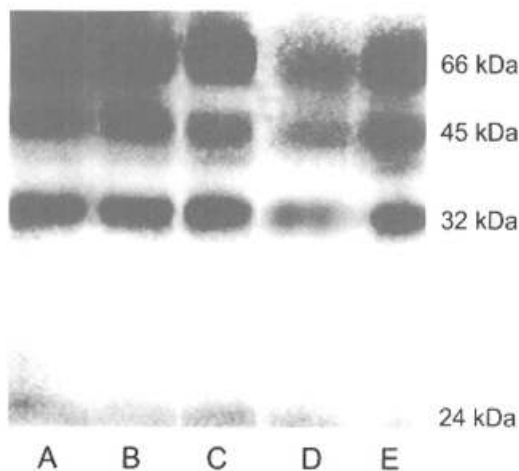


Fig 1. Representative autoradiography of SDS-PAGE back-phosphorylation of proteins in acid extracts prepared from both denervated and intact striatum. A: sham-operated animals; B: intact striatum plus ischemia of 10 min; C: denervated striatum plus ischemia of 10 min; D: intact striatum plus ischemia of 30 min; E: denervated striatum plus ischemia of 30 min.

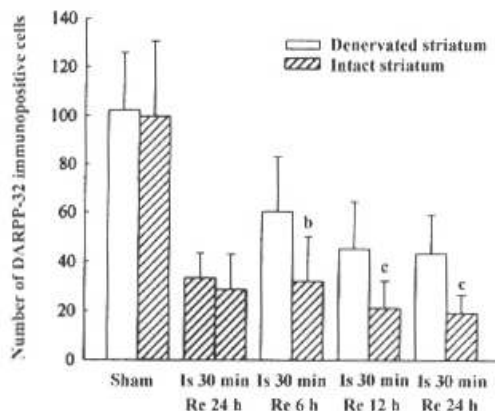


Fig 2. The number of DARPP-32 immunopositive cells in the intact and denervated striatum after ischemia of 30 min following different periods of reperfusion (Bregma level 0.7 mm, dorsolateral subregion). $n = 3$. $x \pm s$. $^b P < 0.05$, $^c P < 0.01$ vs Denervated striatum. (Is: ischemia, Re: reperfusion).

denervated striatum (Fig 3).

DARPP-32 mRNA expression The specificity of the DARPP-32 probe has been demonstrated previously with *in situ* hybridization. The DARPP-32 mRNA expression was mainly observed over the dorsal lateral part of striatum after 30 min ischemia followed by different

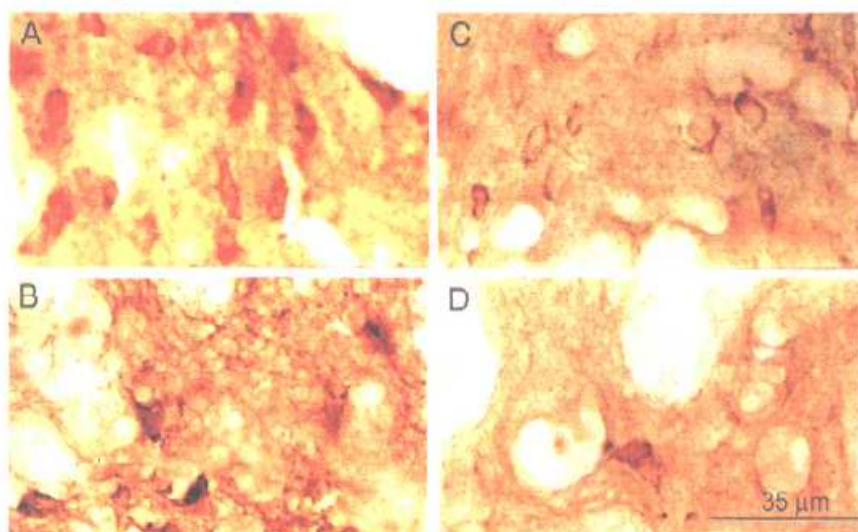


Fig 3. Immunohistochemical reaction of DARPP-32 in the denervated and intact striatum of rat brain after ischemia of 30 min with 12 h (A, C) and 24 h (B, D) of reperfusion. A, B: denervated striatum; C, D: intact striatum.

durations of reperfusion. The positive hybridization was observed as brown or blue black blots. The number of positive DARPP-32 mRNA expression neurons showed a significant difference between denervated and intact striatum. The hybridization density over denervated striatum

was higher than that over intact striatum. And the hybridization density was the highest over denervated striatum after 12 h reperfusion following a 30 min ischemia (Fig 4).

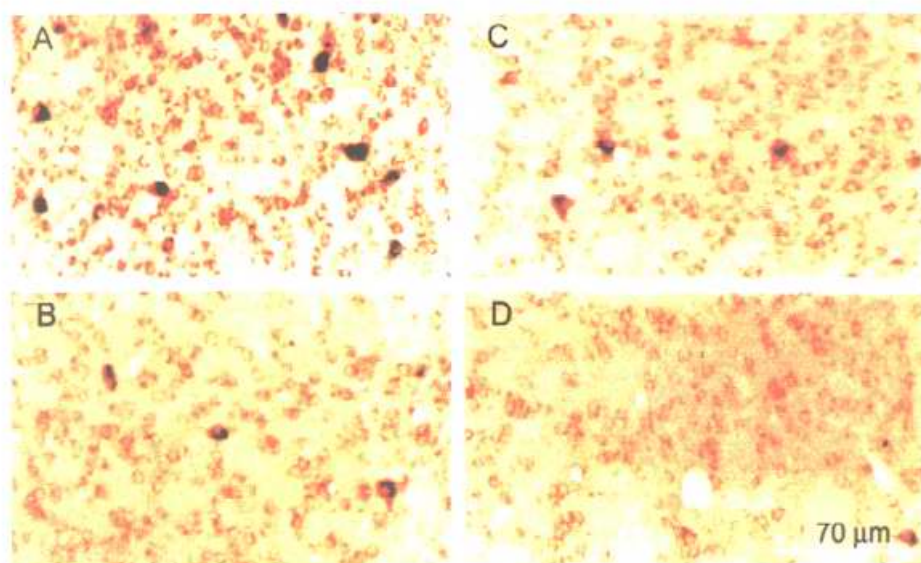


Fig 4. DARPP-32 mRNA expression in the denervated and intact striatum of rat brain after ischemia of 30 min with 12 h (A, C) and 24 h (B, D) of reperfusion. A, B: denervated striatum; C, D: intact striatum.

DISCUSSION

According to current views, DA activates adenylate cyclase through D_1 receptor^[8]. The increased cAMP levels stimulate the activity of cAMP-dependent protein kinase (PKA) which phosphorylate DARPP-32, a dopamine and cyclic AMP-regulated phosphoprotein ($M_r = 32\ 000$). When phosphorylated, DARPP-32 is a potent inhibitor of the protein phosphatase 1 (PP-1)^[9]. Dopamine innervated brain regions are rich in DARPP-32. It is now widely accepted that the interaction between DA and Glu in striatum is associated with regulating the level of DARPP-32 phosphorylation^[10].

Both dopamine and glutamate extensively increased in the extracellular fluid during forebrain ischemia^[2]. The activation of D_1 receptors by DA may increase DARPP-32 phosphorylation in striatum. The phospho-DARPP-32 inhibits protein phosphatase-1 and the subsequent phosphorylation of intracellular proteins. Activation of the NMDA receptor by Glu leads to Ca^{2+} influx and activation of the calcineurin; then the calcineurin dephosphorylates the DARPP-32^[11]. Increase in phospho-DARPP-32 *in vivo* during forebrain ischemia was observed in our experiment. But in lesioned rats, DA had been depleted, hence the increase of phospho-DARPP-32 during forebrain ischemia was inhibited, and the ability of phospho-DARPP-32 to inhibit PP-1 was abolished, and the phosphorylation level of subsequent intracellular proteins was decreased. The decrease of phosphorylation level reduced the neuronal injury of ischemia^[12]. Our experiment indicated that with 6-OHDA there was a neuroprotective effect on SN lesioning.

At the same time, the number of DARPP-32 positive neurons was increased in the denervated striatum of rats after SN lesioning at 30 min ischemia and reperfusion, and the DARPP-32 mRNA expression determined by *in situ* hybridization was also increased in denervated striatum after ischemia and reperfusion. This result coincides with the results of previous studies^[13]. The study demonstrated that depletion of DA protected DARPP-32 positive neurons and its mRNA expression against reduction after ischemic injury.

This study has revealed that DARPP-32 directly interacts at the level of the dendritic spine between dopamine and glutamate and plays an important role in

intracellular signaling cascade of striatum. The alterations of functional states and mRNA expression of DARPP-32 during ischemia elucidate dopamine's effects on the cerebral injury induced by ischemia.

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多巴胺耗竭对大鼠纹状体缺血 DARPP-32 蛋白的影响¹

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关键词 多巴胺; 纹状体; 脑缺血; 免疫组织化学; 原位杂交

目的:研究多巴胺(DA)耗竭对纹状体缺血后 DARPP-32 蛋白磷酸化水平、细胞内分布和 mRNA 表达的影响, 探讨 DA 在缺血性纹状体损伤中的作用机制。

方法:采用 6-羟基多巴胺损毁大鼠黑质和四血管阻断全脑缺血模型, 用放射自显影、免疫组化和原位杂交的方法观察 DARPP-32 的含量、磷酸化水平及 mRNA 表达情况的变化。 **结果:**缺血后体外测定 DARPP-32 [³²P] 的掺入量降低。黑质损毁后再缺血, 损毁侧纹状体 DARPP-32 [λ -³²P] 掺入量有明显升高, 且 DARPP-32 免疫反应阳性神经元及 mRNA 表达均明显强于对照侧。 **结论:**纹状体缺血时 DARPP-32 体内磷酸化增加, 而耗竭 DA 可抑制这种作用。黑质损毁可升高纹状体缺血时 DARPP-32 免疫反应性及 DARPP-32 mRNA 的表达水平。

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