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Antagonistic effect of *l*-stepholidine on striatal ischemic injury in rat[1]

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KEY WORDS dopamine; stepholidine; corpus striatum; cerebral ischemia; Ca^{2+}-calmodulin dependent protein kinase; lactate dehydrogenase

ABSTRACT

AIM: To elucidate the protection of *l*-stepholidine (SPD) on neuronal morphology and function against the striatal ischemic injury in rat. **METHODS**: The forebrain ishemia to Sprague Dawley rats was induced with four-vessel occlusion. Histological examination was performed on the dorsolateral striatum with cresyl-violet stain. In striatal slices of rat as an *in vitro* ischemic model, the activity of calcium/calmodulin-dependent protein kinase Ⅱ (CCDPK) and lactate dehydrogenase (LDH) was examined by the method of ^{32}P-incorporation and colorimetry, respectively. **RESULTS**: In the SPD-treated groups, most of the neurons in the striatum kept the normal morphological appearance after 30-min ischemia followed by 6-h or 12-h reperfusion. The number of neurons was much more in SPD groups than that in vehicle group. The sparse and abnormal neurons were observed in the vehicle group. SPD attenuated the ischemic effect on the CCDPK activity in striatal slices. In addition, SPD inhibited the leakage of LDH from neurons induced by ischemia in incubated striatal slices. **CONCLUSION**: SPD protected striatal neurons against ischemic injury and antagonized the inhibitory action on CCDPK activity induced by ischemia. SPD reduced the leakage of LDH from striatal neurons induced by ischemia.

INTRODUCTION

The striatum is one of the most sensitive regions in the central nervous system to transient cerebral ischemia[1]. The striatum is heavily innervated by nigrostriatal dopaminergic and corticostriatal glutaminergic projections. The excessive release of dopamine (DA) and glutamate (Glu) is involved in the development of ischemic striatal neuronal damage[2]. DA might play a pivatol role in the hypoxic-ischemic brain damage, but the potential mechanisms underlying dopaminergic neurotoxicity are remained unclear[3].

l-Stepholidine (SPD), one of the tetrahydroprotoberberines, possesses the affinity for D_1 and D_2 receptors[4], and shows a D_1 agonistic-D_2 antagonistic dual action in behavioral experiments[5]. Our previous works indicated that SPD had an antagonistic effect on hydroxyl free radicals (OH·) (to be submitted). In the present study, the authors intend to study the protection of SPD from striatal ischemic damage.

Calcium/calmodulin-dependent protein kinase Ⅱ (CCDPK) is highly specific for the nervous system and greatly concentrated in the striatum[6]. The overload of intracellular Ca^{2+} is thought to be a pivotal factor in ischemic cerebral injury. Elevation of intracellular Ca^{2+} concentration leads to activation of CCDPK and results in cellular damage. This enzyme activity is very sensitive to the ischemic cerebra injury[7]. Therefore, research of CCDPK activity would provide insight into the molecular mechanism of dopaminergic neurotoxicity in striatal ischemic injury. In addition, lactate dehydrogenase (LDH) was released after

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ischemia and was considered as an indicator of injury[8], in the present work, the effects of SPD on CCDPK activity and the LDH activity during ischemia were studied.

MATERIALS AND METHODS

Chemicals SPD (Shanghai Institute of Materia Medica, Chinese Academy of Sciences), mp 161 – 162 ℃, $[\alpha]_D$-440 ℃ in pyridine, was dissolved in a small amount of H_2SO_4 0.1 mol/L, then diluted with distilled water and adjusted with NaOH 0.1 mol/L to pH 5.0 – 5.5. Phenyl methyl sulphonyl fluoride (PMSF), histone, sodium metabisulphite, and ATP were purchased from Sigma, USA. [γ-^{32}P] ATP (185 PBq · mol^{-1}) was provided by Beijing Yahui Biomedical Co, China. Other reagents were of AR.

Sprague-Dawley rats (♂, weighing 220 g ± *s* 20 g) were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade Ⅱ, Certificate № 005).

Transient forebrain ischemia Transient ischemia was induced using the four-vessel occlusion method[9]. In brief, rats were subjected to forebrain ischemia for 30 min by the occlusion of four vessels. One day prior to ischemia, bilateral vertebral arteries of rat were electrocoagulated under anesthesia with chloral hydrate (300 mg · kg^{-1}, ip) and bilateral common carotid arteries (BCCA) were exposed and an occlusion device was placed loosely around each artery. After 12 – 24 h, the BCCA were occluded by two small carotid clips for 30 min. Then the two clips were released and recirculation was established. After that, the wounds were sutured. During the experiment, rectal temperature was kept over 37.5 ℃.

Histological examination Six or twelve hours after recirculation, the rats were anesthetized and killed by intracardiac perfusion with 100 mL saline followed by 300 mL 4 % ice-cold paraformaldehyde in phosphate buffer (PB) 0.1 mol · L^{-1}. The brain was removed from the skull and postfixed in the same fixative overnight. Blocks of striatum were dissected and immersed into 30 % sucrose in PB 0.1 mol · L^{-1} until they sank. The serial coronal sections of striatum were cut (15-μm thick) in a cryostat corresponding to the plane of Bregma 0.7 mm according to the Paxinos and Watson atlas. The sections were stained with cresyl violet and examined by light microscopy.

Striatal slices preparation and drug application Male Sprague-Dawley rats weighing 200 g – 220 g were decapitated and the brains were placed in ice-cold Krebs-Ringer solution (in mmol · L^{-1}: NaCl 122, KCl 3.1, KH_2PO_4 0.4, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25, glucose 10; pH 7.4) equilibrated with 95 % O_2 + 5 % CO_2. Striatal slices (350 μm) were prepared with a McILWAIN tissue chopper and preincubated in normal Krebs-Ringer solution and incubated with or without drugs for 20 min. During the course of preincubation and drug application, incubation medium was continuously bubbled with 95 % O_2 + 5 % CO_2. SPD was added 20 min before exposure to free-glucose and 95 % CO_2 + 5 % O_2 solution, an ischemic model *in vitro*. After experiments, the slices were quickly collected and frozen in liquid nitrogen until used.

Assay for CCDPK activity The slices were homogenized in a glass homogenizer on the ice bath with ice-cold homogenization buffer (in mmol · L^{-1}: Tris-HCl 20; egtazic acid 2, NaF 2, PMSF 0.5, β-ME 10; pH 7.5). The homogenate was spun at 10 000 × *g* at 4 ℃ for 10 min, and the supernatant was assayed for CCDPK activity by the previous method[7]. The radioactivity was measured by a liquid scintillation spectrometer (LS-6500, Beckman). Protein concentration of the supernatant was determined by the Lowry method with BSA as standard. The CCDPK activity was expressed in the amount of ^{32}P-incorporation (nmol · min^{-1} · g^{-1}).

Assay for LDH activity In a second set of experiment, the release of LDH was measured. Slices were incubated with Krebs-Ringer solution at 37 ℃ under 95 % O_2 + 5 % CO_2. Following preincubation for 90 min, slices were transferred to glucose-free Krebs-Ringer solution containing SPD or vehicle. After 20 min, hypoxia was induced by switching to 95 % CO_2 + 5 % O_2 for 30 min. The Enzymatic activity of LDH in the supernatant was measured using the colorimetry[10] and expressed as King's unit of total protein content.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and the statistical analysis was performed with ANOVA followed by the Duncan's new multiple range method. $P < 0.05$ indicates that the difference was statistically significant.

RESULTS

Effect of SPD on striatal neurons after ischemia Histological examination was performed in six serial sections of the striatum. Remarkable ischemic damage occurred in the striatal neurons at 6 h or 12 h after 30-min ischemia. The sparse and abnormal neurons were observed in the vehicle group. Particularly, neurons in the dorsolateral part of striatum were more heavily damaged with irregular shapes and dense staining, and many neuronal loss (Fig 1C, D). However, in the SPD group (30 $mg \cdot kg^{-1}$, ip × 3 d, prior to ischemia) the numbers of surviving neurons in striatum were significantly increased and the most of neurons in the striatum had round morphological appearance (Fig 1A, B).

SPD antagonized the inhibition of CCDPK activity induced by ischemia After hypoxia for 30 min, the activity of CCDPK in striatal slices was significantly decreased ($P < 0.01$). The normal value of the CCDPK activity was (83.5 ± 9.4) $nmol \cdot min^{-1} \cdot g^{-1}$, but the activity of CCDPK was decreased to (39.0 ± 6.9) $nmol \cdot min^{-1} \cdot g^{-1}$. After incubation for 20 min with SPD ($0.1 - 50$ $\mu mol \cdot L^{-1}$) followed by 30 min of ischemia, the CCDPK activity gradually increased. At SPD 10 $\mu mol \cdot L^{-1}$, the CCDPK activity was raised to (66.3 ± 5.2) $nmol \cdot min^{-1} \cdot g^{-1}$, which showed that SPD could antagonize the inhibitory effect of ischemia on CCDPK activity (Fig 2).

SPD reduced the leakage of LDH from striatal slices induced by ischemia Ischemia could increase the level of LDH from the striatal slices

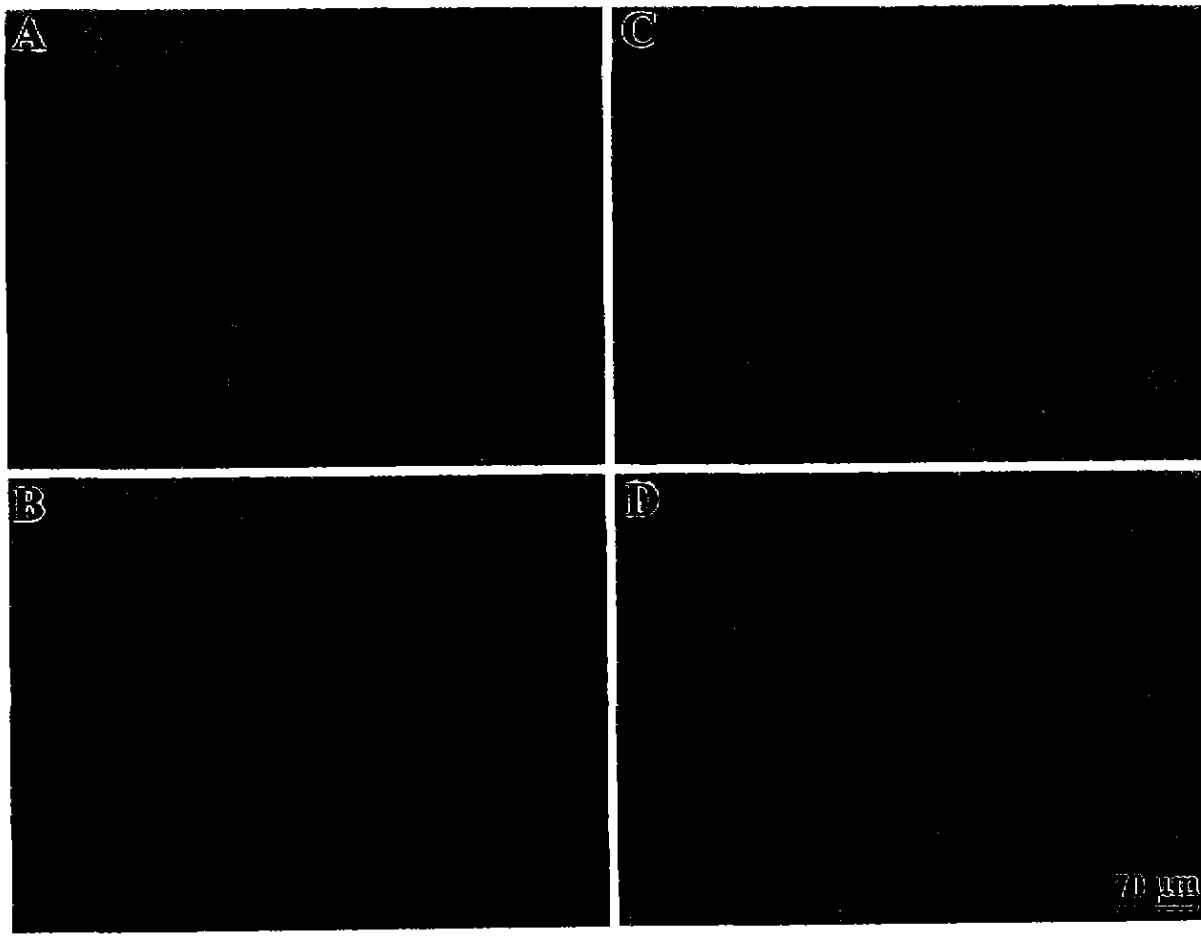

Fig 1. Photographs showing cresyl violet stained sections from rat dorsolateral striatum with ischemia for 30 min and reperfusion for 6 h (A, C) and 12 h (B, D). Obvious protective actions of SPD aganist ischemic-induced neuronal loss were shown. A, B: SPD (30 $mg \cdot kg^{-1}$, × 3 d); C, D: vehicle.

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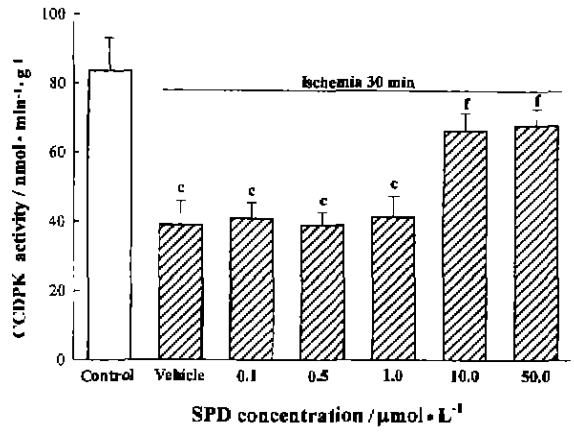

Fig 2. Protection of SPD against inhibition of CCDPK activity induced by ischemia in rat striatal slices. The slices were incubated with SPD for 20 min followed by 30-min ischemia. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* vehicle.

mediated via the leakage of cell membrane. Following the hypoxic time extended, the activity of LDH in superfusate was gradually increased. After hypoxia for 10 min, 20 min, and 30 min, the LDH activities were increased by 13 %, 30 %, and 60 %, respectively. When the incubation with SPD ($0.5 - 50$ μmol·L^{-1}) for 20 min followed by ischemia for 30 min, this leakage effect induced by ischemia was significantly inhibited. SPD at 50 μmol·L^{-1} could abolish the ischemia-induced leakage of LDH (Fig 3).

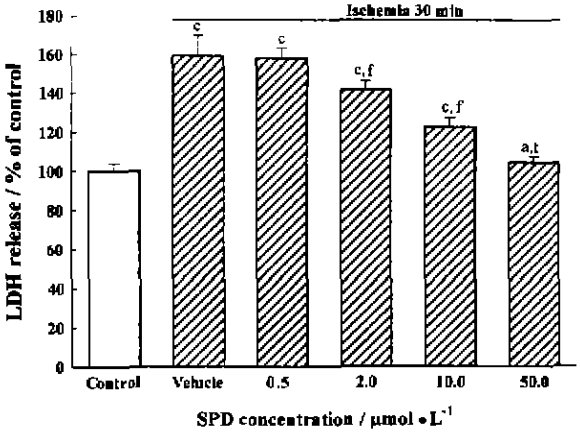

Fig 3. Effects of different concentrations of SPD on ischemia-induced release of LDH from rat striatal slices. The slices were incubated with SPD for 20 min followed by 30-min ischemia. $n = 6$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* vehicle. The control value: (62.40 ± 5.33) King unit/g protein.

DISCUSSION

From both of morphological and biochemical findings in the present paper, SPD exhibited the protective effect on striatal ischemic injury *in vivo* and *in vitro*.

The four-vessel occlusion model was used to produce complete forebrain ischemia with reperfusion in rat. The cardiovascular and respiratory centers in the brainstem still receive enough blood flow from spinal arteries to remain vital functions while forebrain structures are rendered severe ischemia[11]. In this experiment, ischemia was maintained for 30 min before the carotid clamps were released and reperfusion began. This characteristic damage is very reliable. The ischemic duration for the cell death is various in different brain areas. Striatal cell loss is maximal at 6 h – 24 h whereas CA1 cell loss of the hippocampus requires 72 h to reach its maximal level. Our experiment found that striatal cell injury was very obvious at 30 min of ischemia followed by 6 h of reperfusion. Under the same ischemic and reperfusional conditions, SPD could protect striatal cells against this ischemic injury observed from morphological examination.

We selected CCDPK as a experimental target for the following reasons: (a) CCDPK is highly specific in the nervous system and greatly concentrated in the striatum; (b) CCDPK may be involved in Ca^{2+}-dependent reactions regulated by the intracellular Ca^{2+} during ischemia; and (c) DA via D_1 and D_2 receptors may influence NMDA receptor function and intracellular Ca^{2+} concentration, and in turn regulates the enzyme activity[12]. For example, the activation of D_1 receptor may increase PKA activity and enhance DARPP-32 phosphorylation. Phospho-DARPP-32 is a potent inhibitor of protein phosphatase 1 (PP-1) and reduces CCDPK dephosphorylation. D_2 receptor has a synergistic action with NMDA receptor in presynaptic terminal. DA may enhance the influx of extracellular Ca^{2+}, the release of intracellular Ca^{2+} storage by receptor, and post-receptor signal transduction mechanism. The antagonism of SPD against the decrease of CCDPK activity *in vitro* reflected the effect of SPD on anti-hypoxia mechanism, such as the decrease of intracellular Ca^{2+} concentration and the

enzyme autophosphorylation *in vivo*. The decrease of CCDPK activity measured *in vitro* in the present study as an indicator of increase of CCDPK autophosphorylation *in vivo*. The autophosphorylation converts the kinase from the Ca^{2+}/CaM dependent form to Ca^{2+}/CaM independent form, which results in the inhibition of Ca^{2+}/CaM-dependent activity.

In focal forebrain ischemia Hillered *et al* found a 70-fold and a 30-fold increase of striatal DA and *glutamate* (*Glu*) *concentration respectively in the* extracellular fluid[13]. Besides vascular reactivity, free radicals produced through excessive DA metabolism, and neurotoxicity of DA itself, the excessive stimulation of DA receptors by DA during ischemia and reperfusion played an important role on striatal injury. Hashimoto *et al* reported D_2 receptor activation would be involved in cytotoxic effect[14].

As one of the novel tetrahydroprotoberberine analogues, SPD possesses both of the D_2 receptor antagonistic action and potent anti-oxidative effect against free radical damage. Possibly, these two characteristics of SPD were involved in the protection of striatal neurons against ischemic injury.

Besides morphological approaches utilized, the LDH activity assay was used as a reliable biochemical method to detect neuronal membrane injury in ischemia. Membrane damage, as indicated by leakage of lactate dehydrogenase into superfusate, occurred coincidentally with Ca^{2+} influx and ATP loss during both hypoxia and ischemia. DA released during ischemia may be responsible for the production of oxidative free radicals, such as O_2^- and OH·. Hydroxyl radical is the most toxical radical and plays a critical role in many pathological processes[15,16]. In the experiment, SPD significantly decrease the activity of LDH after hypoxia, showing the evidence that SPD can decrease the injury induced by hypoxia. It may be presumed that this protection of SPD is related to its ability to scavenge hydroxyl radical.

In conclusion, SPD can protect striatal neurons from ischemic injury in morphology and antagonize the effect of ischemia on the CCDPK activity and LDH release. As a DA receptor antagonist and hydroxyl radical scavenger[17], SPD may be utilized as a cytoprotective drug to attenuate ischemic neuronal injury.

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左旋千金藤立定对大鼠纹状体缺血性损伤的拮抗作用[1]

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目的: 研究左旋千金藤立定(SPD)对大鼠纹状体缺血性损伤的拮抗作用. **方法**: 用四动脉结扎形成大鼠前脑缺血模型, 焦油紫染色从组织形态观察纹状体中神经元的变化; 以纹状体脑片孵育的缺糖缺氧为离体缺血模型, 用同位素^{32}P掺入法和比色法测定 CCDPK 和 LDH 活性的变化及 SPD 对它们的影响. **结果**: 缺血 30 min 复灌 6 h 和 12 h, 对照组大鼠纹状体大部分神经元丧失, 细胞形态异常; 而 SPD 给药组大鼠纹状体神经元未见明显减少, 形态基本正常. SPD 减轻缺血时纹状体脑片 CCDPK 活性的降低及 LDH 的漏出. 缺血 30 min 复灌 6 h 和 12 h, 对照组大鼠纹状体大部分神经元丧失, 细胞形态异常; 而 SPD 给药组大鼠纹状体神经元未见明显减少, 形态基本正常. SPD 减轻缺血时纹状体脑片 CCDPK 活性的降低及 LDH 的漏出. **结论**: SPD 对纹状体神经元缺血性损伤有拮抗作用, 并可拮抗缺血对纹状体神经元 CCDPK 活性的抑制及 LDH 的漏出.

关键词 多巴胺; 千金藤立定; 纹状体; 脑缺血; Ca^{2+} 钙调蛋白依赖的蛋白激酶; 乳酸脱氢酶

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