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l-S·R-daurisoline protects cultured hippocampal neurons against glutamate neurotoxicity by reducing nitric oxide production[1]

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KEY WORDS daurisoline; cultured cells; hippocampus; nitric oxide; thoracic aorta; sodium glutamate; nitroprusside

ABSTRACT

AIM: To explore mechanisms of *l-S·R*-daurisoline (DS)-mediated protection of cultured hippocampal neurons from sodium glutamate (Glu) cytotoxicity. **METHODS**: Cultured neurons obtained from rat hippocampus were used to exanine the protective effect of DS against Glu neurotoxicity. Cell viability was estimated using trypan blue dye exclusion method and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Release of nitric oxide (NO) from the hippocampus was assayed using rat thoracic aorta *in vitro*. **RESULTS**: DS 0.01 - 10 $\mu mol \cdot L^{-1}$ concentration-dependently inhibited Glu cytotoxicity and increased cell viability with 50 % prevention of cell death 2.8 $\mu mol \cdot L^{-1}$ (95 % confidence limit 1.2 - 5.9 $\mu mol \cdot L^{-1}$). This protection was mostly attenuated by *L*-arginine (Arg) 1 $mmol \cdot L^{-1}$. DS 0.01 - 10 $\mu mol \cdot L^{-1}$ did not prevent sodium nitropusside (SNP) 500 $\mu mol \cdot L^{-1}$-induced cytotoxicity. DS 10 $\mu mol \cdot L^{-1}$ blocked Glu-elicited relaxation of the endothelium-denued rat aortic rings contracted by norepinephrine (NE) 10 $\mu mol \cdot L^{-1}$ in the presence of hippocampal tissue, but did not affect that induced by SNP. This indicated that DS inhibited Glu-triggered NO generation but did not prevent the effects of NO. **CONCLUSION**: DS prevented neurons from Glu neurotoxicity by inhibiting Glu-trigged NO generation.

INTRODUCTION

Glu cytotoxicity played an important role in pathogenesis of neuronal cell death during ischemia and anoxia. An important insight into Glu neurotoxicity has been provided by the demonstration that NO production was a key step in neuronal death induced by Glu. NO was known to be responsible for cultured neuron death during Glu toxicity[1] and anoxic injury[2] and cerebral infarction in animal models of focal stroke[3]. Inhibition of NO synthesis prevents toxic effects of Glu. NO was generated by NO synthase (NOS). NOS was a calmodulin-dependent enzyme[4]. Cytosolic Ca^{2+} has a crucial role in NO formation. NMDA receptor-mediated neurotoxicity could be attenuated by blocking Ca^{2+} influx or by inhibiting activation of NOS.

l-S·R-daurisoline (DS), a synthetic bis-benzyl-isoqunoline (BBI) alkaloid, was one of three optical isomers of DS which was isolated from the roots of *Menispermum dauricum*. The stereo-configuration of DS was different from that of *l-R·R*-DS. Some BBI alkaloids inhibited NO synthesis[5]. *l-R·R*-DS had Ca^{2+} antagonistic effects[6,7] and protected against acute cerebral ischemia in mice and focal cerebral ischemia in rats[8]. We demonstrated that DS was more

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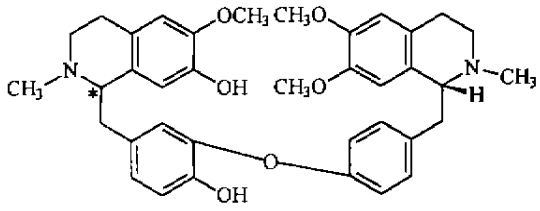
effective than *l-R* · *R*-DS in blockade of Ca^{2+} influx and prevention PC12 cells from ischemic injury[9,10]. We recently studied that DS also prevented cultured cortical neurons from Glu and NMDA-induced neurotoxicity (to be published). The present study was to investigate mechanims of DS-mediated protective effects against neurotoxicity induced by Glu in cultured neurons.



* H$^{\frown}$ = (*R,R*)-*l*-daurisoline; H$^{\frown}$ = (*S,R*)-*l*-daurisoline

MATERIALS AND METHODS

Cell culture Hippocampal cultures were prepared[11] with some modification. Briefly, The hippocampus were obtained from 1-d-old Sprague-Dawley rats. The tissue was minced, incubated at 37 ℃ in 0.25 % trypsin for 20 min, and further dissociated by gentle passage through the tip of flame-narrowed Pasteur pipette. The dissociated cells were placed in polylysine-coated 24-well plates and glass coverslips which were placed in Falcon 60-mm dishes at a density of $3 \times 10^6 - 4.5 \times 10^6$ cells/dish or well and cultured at 37 ℃ in 5 % CO_2 + 95 % air. Medium contained 90 % DMEM, 10 % heat-inactivated fetal bovine serum, and glutamine 2 mmol · L^{-1}. After 4 d, the cells were treated with cytosine arabinoside 10 μmol · L^{-1} to prevent proliferation of nonneuronal cells. The medium was changed twice weekly. Only mature cultures (2 – 3 wk) were used in the study.

Experimental treatments Cells were exposed to Glu 500 μmol · L^{-1} or SNP 500 μmol · L^{-1}[11]. Before exposure, the cells were washed thrice with Tris-buffered control salt solution (CSS) containing NaCl 120, KCl 5.4, $CaCl_2$ 1.8, Tris 25, glucose 15 mmol · L^{-1}, pH 7.4 at 25 ℃ with brief exposure to Glu (30 min) and SNP (10 min). The exposure solution was replaced by DMEM with glucose 21 mmol · L^{-1}, then the cells were incubated for 20 – 24 h. In experiments involving NOS manipulation, either N^{ω}-nitro-arginine (*N*-Arg) or Arg was added directly to the cultures immediately prior to exposure to Glu or SNP. DS was added to the cultures for 30 min before exposure to Glu or SNP.

Assessment of neuronal death The medium was replaced by CSS containing 1.5 % trypan blue. The cells were stained at 25 ℃ for 5 min, fixed with isotonic formaline (pH 7.0 at 2 – 4 ℃), then rinsed with physiologic saline, cells stained with the dye were regarded as dead. Over 200 cells were counted with phase-contrast microscope (Japan Nikon) to determine the viability of the cell cultures. In each experiment, 6 – 8 coverglasses were used to obtain $\bar{x} \pm s$ of the cell viability. Drug protection against Glu and SNP-induced neurotoxicity was calculated[12]. In some experiments, overall neuronal injury was also assessed by MTT assay[13].

Measurement of NO released from the hippocampus Formation of NO from the hippocampus was measured[14] with some modifications. Rat thoracic aortic ring and hippocampal tissues were prepared[12]. The endothelium of aortic ring was removed by rubbing the inner surface with a wooden applicator stick for 30 – 60 s. The absence of endothelium in these rings were confirmed by the lack of relaxation to ACh after submaximal contraction to an agonist. Aortic rings were mounted using stainless steel wires under a tension of 1 g and were incubated with minced brain preparation in a 20-mL organ bath. In some experiments, brain tissues were not added to the bath. The organ bath contained warmed

(37 ℃), oxygenated (95 % O_2, 5 % CO_2) Krebs' solution containing (mmol·L^{-1}): NaCl 116.4, $NaHCO_3$ 6.2, KCl 5.4, NaH_2PO_4 0.92, $MgSO_4$ 1.3, $CaCl_2$ 2.5, and glucose 11.0 (pH 7.2 – 7.4). Aortic rings were allowed to equilibrate for at least 90 min (with several changes of bath solution) before being precontracted with NE 10 μmol·L^{-1}. Isometric tension was recorded and drugs were added in 20 μL volumes. Results are expressed in % of relaxation of NE-induced tension.

Chemicals DS was obtained from the Department of Pharmacochemistry, China Pharmaceutical University; monosodium glutamate, *L*-arginine, N^{ω}-nitro-arginine, sodium nitroprusside, acetylcholine chloride, norepinephrine hydrochloride, and hemoglobin were purchased from Sigma. Dulbecco's modified Eagles medium (DMEM) was purchased from Gibco. MTT was obtained from Fluka.

Statistic analysis Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Effects of DS on glutamate-induced neurotoxicity A marked reduction of the cell viability was observed when the cultures were exposed to Glu 500 μmol·L^{-1} for 30 min following by incubation in Glu-free medium for 24 h. The cell viability was decreased from 90.1 % ± 2.9 % in control to 26.9 % ± 2.1 % in Glu-treated cells. DS prevented hippocampal neurons from Glu toxicity and increased the survival rate of cells in a dose-dependent manner (Tab 1).

IC_{50} value of DS for inhibition of Glu cytotoxicity was 2.8 μmol·L^{-1} (95 % confidence limit 1.2 – 5.9 μmol·L^{-1}) obtained from data of MTT assay. N^{ω}-nitro-*L*-arginine, a potent NOS inhibitor, was also found to increase hippocampal neuronal cell survival in a dose-dependent manner with 50 % prevention of cell death 32.3 μmol·L^{-1} (95 % confidence limit 18.6 – 56.8 μmol·L^{-1}).

Tab 1. Effects of *l-S* · *R*-daurisoline (DS) on neurotoxicity induced by glutamate (Glu) in cultured hippocampal neurons. $x \pm s$ **of triplicate determinations from 5 experiments.**
$^{b}P < 0.05$, $^{c}P < 0.01$ *vs* Glu group.

Treatment/μmol·L^{-1}	Viability/%	Protection/%
Control	90.1 ± 2.9	
Glu (500)	26.9 ± 2.1	
Glu + DS (10)	72.6 ± 2.4^{c}	72.3 ± 3.4
Glu + DS (1)	60.5 ± 3.6^{c}	53.2 ± 5.6
Glu + DS (0.1)	46.8 ± 1.9^{b}	31.5 ± 2.8
Glu + DS (0.01)	33.9 ± 1.6	11.1 ± 1.9

Attenuation of protective effects of DS by *L*-arginine Arg 1 mmol·L^{-1}, a substrate of NOS and a precursor for NO production, was added to the exposure solution, the neuroprotective effects of DS were mostly reduced (Tab 2). Addition of Arg 1 mmol·L^{-1} also completely reduced the effects of *N*-Arg.

Tab 2. Effects of *L*-arginine (Arg, 1 mmol·L^{-1}) on neuroprotective effect by DS in cultured hippocampal neurons. $\bar{x} \pm s$ **of triplicate determinations from 5 experiments.**
$^{c}P < 0.01$ *vs* Glu + DS group.

Treatment/μmol·L^{-1}		Protection/%
Glu + DS (10)		77.3 ± 3.8
Glu + DS (10)	+ Arg	31.2 ± 3.4
Glu + DS (1)		52.9 ± 6.5
Glu + DS (1)	+ Arg	17.8 ± 3.4
Glu + DS (0.1)		35.2 ± 5.6
Glu + DS (0.1)	+ Arg	8.4 ± 2.4^{c}
Glu + DS (0.01)		14.8 ± 2.9
Glu + DS (0.01)	+ Arg	1.8 ± 0.1^{c}

Effects of DS on sodium nitroprusside (SNP)-induced neurotoxicity Hippocampal neurons were exposed to NO generator SNP 500 μmol·L^{-1} for 10 min. The viability of cultures was markedly reduced by a brief exposure of the

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cells to SNP. DS had not pronounced protection against SNP toxicity. Hemoglobin 10 μmol·L^{-1} prevented the cytotoxicity induced by SNP (Tab 3).

Tab 3. Effects of DS on neurotoxicity induced by sodium nitroprusside (SNP) in cultured hippocampal neurons. $\bar{x} \pm s$ of triplicate determinations from 3 experiments. $^cP<0.01$ *vs* SNP groups. Hb: hemoglobin

Treatment/μmol·L^{-1}	Viability/%	Protection/%
Control	91.4±5.8	
SNP(500)	31.6±4.6	
SNP+DS(10)	50.4±8.2	31.4±5.6
SNP+DS(1)	41.4±4.3	16.4±3.6
SNP+DS(0.1)	36.4±5.2	8.0±2.5
SNP+Hb(10)	82.8±7.8^c	85.6±8.1

Effects of DS on the formation of NO stimulated by glutamate The endothelium-denuded aortic ring was contracted by NE 10 μmol·L^{-1}, the aortic ring precontracted by NE was not relaxed by ACh (up to 100 μmol·L^{-1}) but was concentration-dependently relaxed by the addition of SNP 1 – 1000 μmol·L^{-1}. DS 10 μmol·L^{-1} failed to inhibit SNP-induced relaxation of aortic ring contrated by NE (Fig 1).

DS 10 μmol·L^{-1} itself did not affect the NE-induced contraction of the aortic ring but elicited slight increase of the resting tension. When endothelium-denuded aortic ring was maintained in the absence of the rat hippocampal tissues, Glu 10 μmol·L^{-1} did not elicit relaxation of aortic ring precontrated by NE although subsequent addition of SNP 500 μmol·L^{-1} elicited a prompt relaxation. NO released from the hippocampus was assessed by incubating the endothelium-denuded aortic ring with minced brain tissue. Precontracted aortic ring was relaxed by addition of Glu at concentration 1 – 100 mmol·L^{-1} in the presence of the brain tissue. DS added prior to Glu application markedly inhibited Glu-induced relaxation of aortic ring (Fig 2). SNP 500 μmol·L^{-1} added following DS elicited a prompt relaxation.

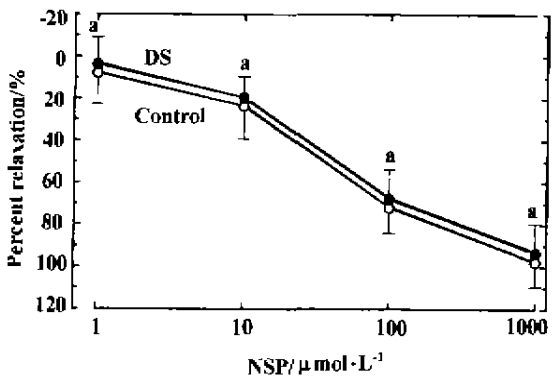

Fig 1. Effects of DS 10 μmol·L^{-1} on SNP-induced relaxation in endothelium-denuded rat thoracic aortic ring in the absence of hippocampal tissues. $n=4$ rats. $\bar{x} \pm s$. $^aP>0.05$ *vs* control.

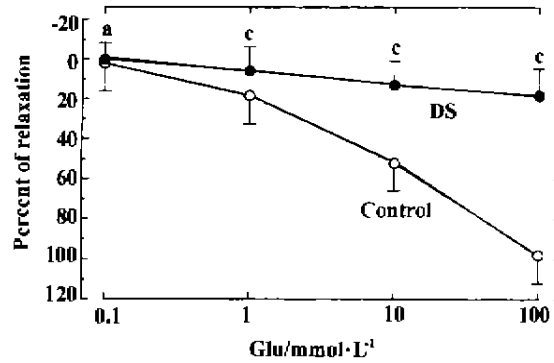

Fig 2. Effects of DS 10 μmol·L^{-1} on glutamate-induced relaxation in denuded rat thoracic ring in the presence of hippocampal tissues. $n=5$ rats. $\bar{x} \pm s$. $^aP>0.05$, $^cP<0.01$ *vs* control.

DISCUSSION

The present study demonstrated that DS significantly inhibited Glu-induced neurotoxicity in cultured hippocampal neurons. This result further supports our previous studies. *N*-Arg also potently inhibited Glu-induced neurotoxicity in the hippocampal cultures used in this study, this indicated that NO mediated cytotoxicity of Glu in the hippocampal cultures maintained in our laboratory. Arg, a precursor for NO produc-

tion, mostly attenuated the effects of DS, suggesting that DS protected neurons against Glu toxicity either by preventing NO generation or by protecting neurons against the deleterious effects of NO. Whereas, DS failed to protect neurons from the NO generator SNP-induced cytoxicity, indicating that DS did not directly prevent the deleterious effects of NO. These results led us to assume that the neuroprotective effects of DS might be caused by its inhibitory action on intracellular response-linked with NO-formation, but not directly by the prevention of NO cytotoxicity. To confirm this speculation, NO released from hippocampus was assessed using the isolated aorta as a biological sensor of NO. Garthwaite *et al* demonstrated that NMDA caused a relaxation of the aorta contracted with phenylephrine when the aorta was incubated with suspension of single cells dissociated from 8 – 9-d rat cerebella, the present study showed that Glu also elicited a relaxation of the aorta contracted with NE when the aorta was incubated with minced hippocampal tissues dissected from adults rats. Glu did not affect muscle tension when applied in the absence of the hippocampal tissues, suggesting that Glu directly acted upon the brain tissue but not upon the smooth muscle, the relaxation of aorta was caused by NO released from hippocampal tissues stimulated by Glu. DS inhibited Glu-induced relaxation of endothelium-denuded aorta in the presence of hippocampus, suggesting that it inhibited Glu-stimulated NO-formation in hippocampus. Since daurisoline has been shown to have Ca^{2+} and calmodulin antagonistic activity[15], it is speculated that DS may have an inhibitory effect on NOS but further studies would be needed to confirm such speculation.

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l-S·R-蝙蝠葛苏林碱通过减少一氧化氮的产生保护培养的海马神经元对抗谷氨酸神经毒性

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关键词 蝙蝠葛苏林碱；培养的细胞；海马；一氧化氮；胸主动脉；谷氨酸钠；硝普钠

目的：探讨 *l-S·R*-蝙蝠葛苏林碱（DS）保护神经元的作用机制. **方法**：原代培养大鼠海马神经细胞，用台盼蓝染色和 MTT 法检测神经细胞损伤，以去内皮的大鼠胸主动脉作为分析 NO 含量的生物检测器. **结果**：DS 0.01 – 10 $\mu mol \cdot L^{-1}$ 剂量依赖性地抑制谷氨酸（Glu）神经毒性，IC_{50} 为 2.8 $\mu mol \cdot L^{-1}$（95 % 可信限为 1.2 – 5.9 $\mu mol \cdot L^{-1}$），DS 10 $\mu mol \cdot L^{-1}$ 能够抑制 Glu 引起的主动脉条舒张，但对亚硝基铁氰化钠（SNP）的动脉条舒张和神经毒性无明显的影响. 表明 DS 不能直接对抗 NO 的毒性，但能抑制 Glu 刺激 NO 的产生. **结论**：DS 对抗 Glu 引起的神经毒性的作用与抑制 Glu 刺激 NO 的合成有关.

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