ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Feb; 20 (2): 185 - 187
http://www.summ.ac.cn; www.chinainfo.gov.cn/periodical

Prophylactic effect of methylene blue against neurotoxicity of sodium nitroprusside

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KEY WORDS nitroprusside; methylene blue; nitric oxide; cyclic GMP; cerebellum; neurons; cultured cells

ABSTRACT

AIM: To examine the effect of methylene blue (MB) on cytotoxicities of sodium nitroprusside (SNP) and cyclic guanosine monophosphate (cGMP) in cultivated cerebellar neurons. **METHODS**: The cytotoxicities of xenobiotic SNP and cGMP on cultivated murine cerebellar neurons were examined according to Dessi's method. Toxicity of SNP icv to mice and the prophylactic effect of icv MB were investigated with respect to the incidence of seizure and the mortality of mice within 24 h. **RESULTS**: Ten min treatment of SNP 1 mmol·L^{-1} decreased the survival rate of murine cerebellar nerve cells from 92 % of normal control to 35 %. Incubation with cGMP 0.1 mmol·L^{-1} for 1 h declined the survival rate from 94 % of normal to 40 %. Injection icv SNP 20 nmol killed one tenth of the mice in 24 h, and SNP 30 nmol killed 11/13 of the mice. MB (100 nmol) icv injection protected 11/13 of the mice against seizure and death caused by SNP (30 nmol, icv), and completely eliminated the toxicity of SNP 20 nmol. **CONCLUSION**: SNP and cGMP inhibit the vitality of murine neurons *in vitro*. MB injection icv markedly antagonizes the dose-dependent neuron-toxic effect of SNP in respect of convulsion and mortality of mice.

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Received 1997-08-11 Accepted 1998-07-17

INTRODUCTION

Nitric oxide is a novel messenger in nervous system involving neurotoxicities of lots of xenobiotics[1,2]. In view of that sodium nitroprusside (SNP) causes damage of neurons[3], SNP slowly delivers NO *in vivo*[4], NO produced from SNP stimulates soluble guanylate cyclase (sGC) to increase the level of cyclic guanosine monophosphate (cGMP) in cells[5], injection icv of NO solution immediately set on seizure in mice[6], it is accepted that NO-sGC-cGMP mechanism is involved in the onset of seizure and death.

In this paper the cytotoxicity of SNP and cGMP to murine cerebellar neurons, and the toxicity of icv SNP as well as prophylatic effect of methylene blue (MB) in mice were invesitgated.

MATERIALS

Mice (Kunming species, weighing (18 ± 22) g, Grade Ⅱ, Certificate № 01-3023, male and female matched) and suckling mice of 8 d were obtained from the Animal Breeding Center affiliated to Academy of Military Medical Sciences. *L*-Nitro-arginine methyl ester (*L*-NAME), cGMP, trypsin, and polylysine were purchased from Sigma. DMEM was from Gibco and 24-well microplates for cell cultivation were the products of Costar. Other reagents used were all domastic products of AR grade.

METHODS AND RESULTS

Cytotoxicity of SNP to cultured murine

ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Feb; 20 (2)
E-mail aps@server.shenc.ac.cn Phn/Fax 86-21-6474-2629

cerebellar nerve cells Microplates (24-well) were pretreated with polylysine 50 mg · L^{-1} 0.3 mL per well for 12 h, then washed twice with H_2O. Cerebella of suckling mice (8 d) were dissected under sterilized condition and digested with 0.25 % trypsin for 12 min at ambient temperature and then translocated into the inoculating medium (DMEM, glucose 30, KCl 25, and Gln 2 mmol · L^{-1}, fetal bovine albumin 10 %, equine serum 10 %). Cells were separated by gentle blowing with a pipette, filtered via a sieve (200 mesh), inoculated onto the polylysine-treated microplate (10^6 cells/well), incubated at 37℃ under 8 % CO_2 for 20 h, and continuously incubated for 48 h after adding cytosine arabinoside 5 mg · L^{-1}. The cells were transferred in the feeding medium (DMEM, glucose 30, KCl 25 and Gln 2 mmol · L^{-1}, fetal bovine albumin 5 %, equine serum 5 %). Changing half volume of the medium twice a wk. The cultivated cells of d 18 – 25 were used in the cytotoxicity tests[7].

After sucking out the supernatant in each well, various concentrations of SNP preparing in feeding medium without serum were added and incubated at 37 ℃ for 10 min. Washed once with the feeding medium and continuously cultivated in the feeding medium for 16 – 20 h. Cells survived were counted under microscope in the trypane-blue exclusion test. The results showed that SNP 1 mmol · L^{-1} caused a marked cytotoxicity to the murine cerebellar cells, and the survival rate decreased from 92 % of the normal control to 35 % (Fig 1).

Cytotoxicity of cGMP to cultivated murine cerebellar nerve cells Murine cerebellar cells were used and the manipulation procedures were alike as above-mentioned except that different concentrations of cGMP were added to cells at 37 ℃ for 1 h instead. The results showed that the cytotoxicity increased along with the increase of concentration of cGMP. The survival rate decreased from 94 % of the normal control to 40 % in the presence of cGMP 0.1 mmol · L^{-1} (Fig 1).

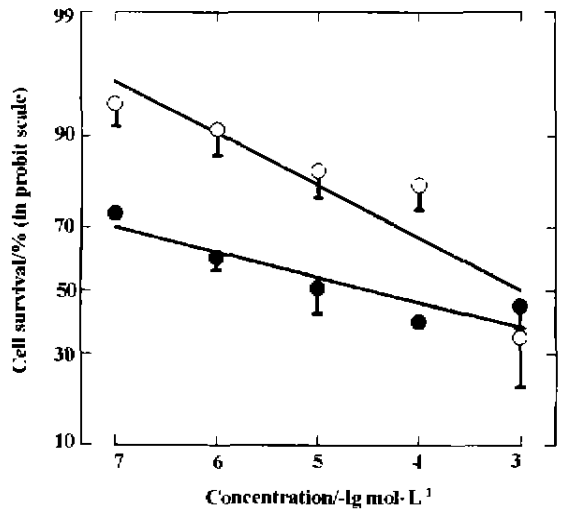

Fig 1. Cytotoxicity of SNP (○) and cGMP (●) to murine cerebellar nerve cells.
$n = 4$ experiments. $\bar{x} \pm s$.

Toxicity of SNP in mice and prophylactic effect of methylene blue Mice were fixed in prone position. A T-shape incision was made on the scalp for exposure of the skull. Needle was inserted 1.5 mm at a point 2 – 3 mm in front of the lambdoid suture and 2 mm aside of the sagittal suture. MB or SNP diluted in Alder's solution (NaCl 100, KCl 5.4, $CaCl_2$ 3.6, $MgCl_2$ 2.6, NaH_2PO_4 1.0, $NaHCO_3$ 41.8, glucose 7.6 mmol · L^{-1}, pH 7.4) was injected icv in a volume of 5 μL. In control mice Alder's solution 5 μL was used instead. Incidence of seizure and mortality of mice were examined within 24 h. The results showed that the incidence of convulsion and the death rate of mice increased along with the increase of the dosage of SNP exhibiting in a dose-dependent fashion ranging from 10 to 30 nmol. Injection (icv) of MB 5 min prior of SNP (icv) manifested obviously a protective effect against intoxication of SNP. MB 100 nmol decreased the incidence of convulsion and the mortality from 85 % in

control mice to 15 % of the SNP-intoxicated (30 nmol) mice. In cases of SNP 20 nmol intoxication, MB 100 nmol completely blocked the onset of seizure and kept all the intoxicated (SNP 20 nmol) mice alive within 24 h examined (Tab 1).

Tab 1. Toxicity of SNP in mice. MB was icv to mice 5 min before icv of SNP. $^{c}P<0.01$ *vs* SNP control (20 nmol).
$^{f}P<0.01$ *vs* SNP control (30 nmol).

	Dosage/nmol	Seizured/Total	Dead/Total
SNP	10	0/10	0/10
	20	6/10	1/10
	30	11/13	11/13
MB + SNP	100 + 20	0/10^{c}	0/10
	100 + 30	2/13^{f}	2/13^{f}

DISCUSSION

In this paper, SNP and cGMP have been demonstrated to be toxic to murine cerebellum neurons. The animal experiments showed that SNP was also toxic *in vivo*, and the mice could be fairly prevented from intoxication of SNP by MB. Though MB is known to be a potent inhibitor of NO synthase (NOS) and sGC[8], however, since SNP may deliver NO spontaneously in the body without the involvement of NOS catalysis[4], thus the prophylactic effect of MB appeared in our experiment is ascribed to the inhibition of sGC other than NOS.

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亚甲蓝对硝普钠神经毒性的预防作用

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关键词 硝普盐；亚甲蓝；一氧化氮；环鸟苷一磷酸；小脑；神经元；培养的细胞

目的：测定硝普钠（SNP）及环鸟苷酸（cGMP）对培养的小脑神经原的细胞毒性和亚甲蓝（MB）对其小鼠毒性的预防作用。**方法**：用 Dessi 法测定 SNP 和 cGMP 的小鼠小脑神经细胞毒性。测定 icv SNP 的小鼠惊厥发生率和 24 h 死亡率及 MB 对其毒性的预防作用。**结果**：给予 SNP 1 mmol·L^{-1} 10 min 细胞存活率由对照组 92 %降至 35 %。cGMP 0.1 mmol·L^{-1}作用 1 h，细胞存活率由94 %降至40 %。SNP 20 nmol icv 使 1/10 小鼠在 24 h 内死亡，30 nmol 使 11/13 小鼠死亡。MB 100 nmol icv 可预防 11/13 的 SNP 中毒（30 nmol，icv）小鼠发生惊厥及死亡，可完全消除 SNP 20 nmol 的中毒效应。**结论**：SNP 和 cGMP 对小鼠小脑神经细胞有细胞毒性。亚甲蓝明显对抗 icv SNP 小鼠的惊厥和死亡。

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