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Research report

Neuroprotective effects of sodium orthovanadate after hypoxic-ischemic brain injury in neonatal rats

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Abstract

Sodium orthovanadate (SOV), a competitive inhibitor of protein tyrosine phosphatases, is neuroprotective in adult animals following an ischemic event. The present study evaluated whether SOV might be protective in a rat pup hypoxic-ischemic (HI) model. Seven-day-old rat pups had the right carotid artery permanently ligated followed by 140 min of hypoxia (8% oxygen). SOV 1.15, 2.3, 4.6, 9.2 or 18.4 mg/kg and vehicle were administered by i.p. injection at 5 min after reoxygenation. Brain damage was evaluated by weight loss of the right hemisphere at 22 days after hypoxia and by gross and microscopic morphology. SOV lowered blood glucose at doses of 1.15, 2.3 and 4.6 mg/kg and induced toxic effects at 9.2 mg/kg. The doses of 2.3 and 4.6 mg/kg of SOV significantly reduced brain weight loss ($p < 0.05$), but treatment with 1.15 or 9.2 mg/kg did not. SOV 4.6 mg/kg also improved the histopathologic score and diminished the HI induced reduction of Akt and ERK-1/2 phosphorylation in the cortex ($p < 0.05$) and increased the density of BrdU-positive cells in the subventricular zone ($p < 0.01$). In conclusion, SOV has neuroprotective effects in the neonatal rat HI model partially mediated by activating Akt and ERK-1/2 pathways.
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Keywords: Sodium orthovanadate; Neuroprotection; Akt/PKB; Apoptosis; Newborn rat

1. Introduction

Hypoxic-ischemic (HI) brain injury is an important cause of death and disability in human newborn infants [7]. The developmental stage of the brain of the 7-day-old rat pup resembles that of term infants [21] and the neonatal rat HI model at this age has been well characterized [23] and used extensively to assess the efficacy of neuroprotective agents [19]. Therefore, investigation of neuroprotective agents in the neonatal rat HI model may provide important information pertinent to the development of treatment for perinatal HI brain damage.

HI brain injury induces cell death in neurons by the mechanisms of apoptosis and necrosis [19]. The serine-threonine kinase, Akt, also known as protein kinase B, is one of the major downstream pathways of neurotrophin signaling and plays a critical role in controlling the balance between survival and apoptosis [10]. Activation of Akt has been shown to promote neuronal survival after ischemia [4,11,15,29].

Vanadium, a transition metal found in relative abundance in nature, has widely varied biologic and physiologic significance [3,8]. Vanadium compounds mimic many of the physiological actions of insulin [3]. Sodium orthovanadate (Na_3VO_4, SOV) affects phosphorylation levels of intracellular proteins via competitive inhibition of protein tyrosine phosphatases [8]. It has been reported that SOV treatment ameliorates ischemic neuronal cell injury via the activation of both Akt and extracellular signal-regulated kinase (ERK) [15]. SOV also potently enhances proliferation of progenitor cells in the adult rat subventricular zone (SVZ) after focal cerebral ischemia [17] and accelerates angiogenesis in a rat model of hindlimb ischemia [27]. SOV has been shown to delay death of gerbil hippocampal CA1 neurons after transient forebrain ischemia and protect against neuronal injury in adult rats with transient middle cerebral artery occlusion (MCAO) [13–15]. SOV manifests a therapeutic time window of at least 90 min in an adult rat MCAO model [14]. However, little is known about the role of SOV in neonatal brain

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injury. SOV has a variety of side-effects [26]; safety and effective doses of SOV should also be determined in the newborn animals. The purpose of the present study was to evaluate the effects of SOV on brain injury and Akt and ERK-1/2 pathways in the neonatal rat HI model.

2. Materials and methods

2.1. Animal protocol

This protocol was approved by the University of Mississippi Institutional Committee on animal use. Rats were cared for in accordance with National Institutes of Health Guidelines. The neonatal rat HI procedure was performed as described by Rice et al. [23]. Because, evidence suggests that sex is not a factor affecting brain damage in the neonatal rat HI brain injury model [9], we chose 7-day-old Sprague–Dawley rat pups of either sex, weighing between 12 and 16 g (Charles River Laboratories, Wilmington, MA) for our experiments. The rat pups were anesthetized with isoflurane. The right common carotid artery was exposed, isolated and permanently doubly ligated. After surgery, the rat pups were returned to their dams for 2–3 h recovery. Hypoxic exposure was achieved by placing the rat pups in 1.5 L sealed jars immersed 5.5 cm deep in a 37 °C water bath and subjected to a warmed, humidified mixture of 8% oxygen/92% nitrogen bubbled through 37 °C water and delivered at 4 L/min for 140 min. After this hypoxic exposure, the pups were returned to their dams and allowed to recover and grow for the following experiments.

2.2. Drug treatment

Pups from each litter were randomly assigned to the control group or treatment with SOV (Sigma–Aldrich Co., St. Louis, MO) group. SOV in doses of 1.15, 2.3, 4.6, 9.2 or 18.4 mg/kg was dissolved in 10 μL of saline per gram of body weight and administered by i.p. injection at 5 min after reoxygenation (n = 21–25 in each). These doses were chosen from previous studies in adult animals [14]. The vehicle groups were given the same volume of saline (n = 21–24 in each). The sham-operated pups (n = 30) were treated similarly to the operated ones, except that the carotid artery was not ligated and they were not exposed to hypoxia.

2.3. Measurements of rectal temperature and body weight

To evaluate whether neuroprotection by SOV was dependent on systemic hypothermia, rectal temperature was measured in 12 pups from 1 litter (6 from the vehicle group and 6 given 4.6 mg/kg of SOV) prior to hypoxia, and at 0 and 0.25, 0.5, 1, 1.5, 2, 2.5, 3 and 4 h after hypoxia. Since decreased body temperature both during and after the hypoxia can affect the outcome, it is essential that both the treated and control animals maintain similar temperatures [28].

Body weight was measured in the rat pups (vehicle, n = 89; SOV 1.15 mg/kg, n = 22; 2.3 mg/kg, n = 23; 4.6 mg/kg, n = 25; 9.2 mg/kg, n = 21) at 0, 1, 4, 7, 10, 14 and 22 days after injury.

2.4. Measurement of blood glucose concentration

SOV can decrease blood glucose [13,14]; therefore, plasma glucose was measured in rat pups treated with SOV (n = 29), or with vehicle (n = 6). Using the above neonatal HI procedure, the rat pups were treated with 1.15, 2.3, 4.6 or 9.2 mg/kg of SOV by i.p. injection at 5 min after hypoxia. The pups were anesthetized at 30 min after injection and blood was extracted from the heart. Plasma glucose was measured by the glucose oxidase technique.

2.5. Gross brain damage grading

Rat pups were decapitated 22 days after hypoxic exposure. Brains were scored normal, mild, moderate or severe by a blinded observer according to the method of Palmer et al. [21]. Normal is no reduction in the size of the right hemisphere, mild is visible reduction in right hemisphere size, moderate is large reduction in hemisphere size from a visible infarct in the right parietal area and severe is near total destruction of the hemisphere. After removing the cerebellum and brainstem, the brain was divided into two hemispheres and weighed. Results are presented as the percent loss of hemispheric weight of the right side relative to the left [(left − right)/left × 100]. This HI model results in brain damage only on the ipsilateral side [21,23]. There is a high degree of correspondence between the weight deficit of the injured hemisphere and histologically evaluated loss of brain tissue [2,9].

2.6. Microscopic brain damage grading

To verify that the gross changes were a reflection of the expected histopathologic changes, microscopic examination of the tissues was carried out in 24 rat pups from 2 litters: 12 pups treated with 4.6 mg/kg of SOV and 12 vehicle treated pups at 3 days after injury using the previously described method [9]. The cerebral cortex was scored by an observer blind to the treatment group of the animal from 0 to 5 according to the method of Cataltepe et al. [5], where “0” is normal, “1” is 1–5% of neurons damaged, “2” is 6–25% of neurons damaged, “3” is 26–50% of neurons damaged, “4” is 51–75% of neurons damaged and “5” is >75% of neurons damaged.

2.7. Western blot analysis for Akt and ERKs

Using the above neonatal HI procedure, the rat pups were treated with vehicle or 4.6 mg/kg of SOV by i.p. injection at 5 min after hypoxia. At 45, 90 min, 6 and 24 h (n = 5–8 in each group) after hypoxia, the pups were decapitated, the brains removed and cortexes in both lesioned and unlesioned hemispheres were separately dissected and were frozen at −80 °C. Akt and ERKs proteins were assessed as described by Hasegawa et al. [14]. Samples with 30 μg of protein were denatured and separated electrophoretically and transferred to a PVDF membrane. After incubation in 5% (w/v) non-fat-dried milk in TBS with 0.1% Tween-20, the membranes were washed and incubated at 4 °C overnight with the primary antibodies. Anti-Akt (1:1,000), anti-phospho-Ser-473 Akt (1:200) and anti-p44/42 MAP kinase (1:1000) antibodies were obtained from Cell Signaling Technology (Beverly, MA) and anti-phospho ERK-1/2 (1:500) antibody was obtained from Sigma–Aldrich (St. Louis, MO). After washes, the membranes were incubated with 1:3000 diluted secondary antibodies of horseradish peroxidase-conjugated to antirabbit antibody (Amersham Life Science Inc., Heights, IL) or antimouse antibody (Transduction Laboratories, Lexington, KY, USA) for 1 h at room temperature. The membranes were washed, then incubated in Amersham’s ECL™ Western blotting detection reagents and exposed to ECL™ Hyperfilm (Amersham, IL). Films were scanned using a Logitech Scanman densitometer (Logitech, Inc. Freemont, CA). Separate assays were done on each pup.

2.8. Bromodeoxyuridine (BrdU) labeling

BrdU, the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling. BrdU labeling and immunostaining were performed according to the protocols as previously described [17,20] with some modifications. For cumulative labeling of proliferating cells during the 7 days following HI, the rat pups were injected with 50 mg/kg of BrdU (Sigma–Aldrich, St. Louis, MO) i.p. twice daily for six consecutive days starting at 12 h after HI. The rat pups were sacrificed at 24 h after the last injection. The rat pups who were given the BrdU injection as described were also treated with either 4.6 mg/kg of SOV (n = 6) or saline (n = 5) at 5 min after hypoxia as the experimental groups. Sham operated (n = 5) and sham operated plus BrdU injection (n = 3) rat pups served as controls. Five-micrometer thick coronal sections of brain between bregma levels +3 and −1 mm were cut and then used for immunohistochemical study.

2.9. Immunohistochemistry and cell counting

Proliferating cells were identified by BrdU incorporation inside the nucleus using a kit from Calbiochem (La Jolla, CA) according to the manufacturer’s protocol. BrdU positive cells were displayed on a computer monitor for better visualization and counted at 40× magnifications in each field of the four regions