Pharmacology, Biochemistry and Behavior 108 (2013) 66–73

Contents lists available at SciVerse ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Short and long-term motor and behavioral effects of diazoxide and dimethyl sulfoxide administration in the mouse after traumatic brain injury

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ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form 2 April 2013
Accepted 6 April 2013
Available online 11 April 2013

Keywords:
Diazoxide
TBI
DMSO
Open field
Morris water maze

ABSTRACT

Traumatic brain injury (TBI) is a worldwide phenomenon that affects all ages and socioeconomic classes and results in varying degrees of immediate and delayed motor, cognitive, and emotional deficiencies. A plethora of pharmacologic interventions that target recognized initiators and propagators of pathology are being investigated in an attempt to ameliorate secondary injury processes that follow primary injury. Diazoxide (DZ), a K_{ATP} channel activator, has been shown to provide short- and long-term protective effects in a variety of *in vitro* and *in vivo* cerebral ischemia models. However, the effects of DZ on behavioral outcome following TBI have not been investigated. TBI was induced in male C57BL/6J mice by controlled cortical impact (CCI) and followed by intraperitoneal administration of either normal saline, dimethyl sulfoxide (DMSO), or 2.5 mg/kg DZ in DMSO, 30 min post-injury and daily for three days. Open field and beam walk performances were used to assess motor and behavioral function 1, 7, and 14 days following injury. Spatial learning and memory were assessed three weeks following injury using the Morris water maze. Injured mice were significantly impaired on the beam-walk and Morris water maze tasks, and were hyperactive and anxious in an open field environment. On post-injury days 1 and 14, mice treated with DMSO exhibited an increase in the amount of time required to perform the beam walk task. In addition, animals exposed to DMSO or DZ + DMSO exhibited slower swimming speed in the Morris water maze on the final day of testing. There was no therapeutic effect, however, of the treatment or vehicle on open field behavior or learning and memory function in the Morris water maze. In summary, CCI produced significant long-term impairment of motor, memory, and behavioral performance measures, and DZ administration, under the conditions used, provided no functional benefits following injury.

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1. Introduction

Due to significant heterogeneity of the primary injury among TBI victims and the complexity of the secondary injury cascades that follow the primary event, promising preclinical drug therapy results have thus far been inconclusive or even harmful when evaluated in clinical trials (Lei et al., 2012). One theme that has emerged from both preclinical and clinical trials is the need to evaluate either combinatorial pharmacologic therapy and/or assess the ability of agents that possess multiple mechanisms of action in order to counter the progression of early and late neuropathology resulting from TBI (Maas et al., 2010). A survey of research in the field of ischemic brain injury reveals a possible candidate: diazoxide (DZ).

Diazoxide, a benzothiadiazine derivative, has been used clinically for over 30 years as a therapy for symptomatic hypoglycemia or clinical hypertension (Arnoux et al., 2011; He et al., 2008) and as a result, the chemical, safety, and side-effect profile is well established. Regarding potential neuroprotective attributes, the primary mechanism of action appears to be the ability of DZ to act as a putative mitochondrial ATP-sensitive potassium channel (mK_{ATP}) opener. Preclinical efforts have demonstrated significant *in vivo* and *in vitro* neuroprotective effects in brain tissue when administered before and after exposure to oxygen-glucose deprivation conditions (Abe et al., 2010; Adamczyk et al., 2010; Domoki et al., 2005; Farkas et al., 2004, 2005a, 2006; Garcia de Arriba et al., 1999; Kis et al., 2003; Liu et al., 2002; Nagy et al., 2004; O'Sullivan et al., 2007; Rajapakse et al., 2003; Robin et al., 2011; Shake et al., 2001; Shimizu et al., 2002; Zhang et al., 2010). Because DZ does not dissolve well when mixed with inorganic solvents, dimethyl sulfoxide (DMSO) can be used as a solvent for drug administration. Dimethyl sulfoxide [$(CH_3)_2SO$] is an amphipathic solvent that was discovered in the 19th century and is widely used as a reagent in bioscience research and therapeutically in human and veterinary medicine (Santos et al., 2003). With over 10,000 articles dedicated to investigating the biological actions of DMSO (Jacob and de la Torre, 2009), it has been referred to as "a relatively simple compound that has

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0091-3057/$ – see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.pbb.2013.04.001

stimulated much scientific controversy" (Ali, 2001). A wide range of DMSO concentrations have demonstrated neuroprotective effects in human and animal subjects in the face of assorted insults, including various TBI models (see Jacob and de la Torre, 2009 for review). However, recent experimental data have revealed that not only does DMSO possess several favorable attributes but that it may also project far-reaching effects on metabolism and cell dynamics that could produce undesired or deleterious effects on experimental outcome (Nasrallah et al., 2008).

The objective was to evaluate the short- and long-term effects of post-TBI DZ administration on behavioral outcomes in mice that received a moderate controlled cortical impact (CCI) injury. For the purposes of this study, moderate injury was defined as a lack of noticeable hemorrhage or significant physical disruption in the underlying hippocampus when examined at 24 h and 3 weeks, respectively, after injury. To the best of our knowledge, prior assessment of the effects of DZ administration on behavioral outcomes following TBI has not been undertaken. An interesting and unexpected finding regarding the effect of DMSO on behavioral outcome may serve as evidence that a drug vehicle may produce undesirable effects on test subject outcome.

2. Materials and methods

2.1. Animals

A total of 122 six-to-nine-week old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) weighing 20–29 g at the time of surgical procedures were group-housed in an animal colony at a constant temperature (23 ± 2 °C) with a 12-hour light/dark cycle and food and water *ad libitum* for at least three days prior to base line behavioral testing. Mice were assigned to groups designated saline-treated Control, Sham, or CCI (n = 8, 16, 16, respectively), DMSO-treated Control, Sham, or CCI (n = 8, 16, 16), or DZ-treated Control, Sham, or CCI (n = 10, 16, 16). Before behavioral experiments commenced, a pilot study was performed to determine the optimal CCI parameters for producing the desired moderate injury. For this study, 20 animals were processed 24 h or three weeks after injury for assessment of hemorrhage and structural alterations using whole brain and coronal section analysis. All experimental procedures used in this investigation were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC).

2.2. Surgery

Anesthetic induction was performed *via* spontaneous ventilation using 3% isoflurane in 100% oxygen (1.0 L per minute flow rate) in a rodent volatile anesthesia box. After application of protective ointment (Lacri-Lube®) to the eyes, the head was shaved using electric clippers, placed in a standard rodent stereotaxic frame and positioned using atraumatic ear and incisor bars (Stoelting, Wood Dale, IL). The skin was prepped with 70% isopropyl alcohol and Betadine® ointment in sequence and 0.1 mL of 0.025% bupivacaine was injected subcutaneously into the planned incision site. An isothermal heating pad with feedback controller (Stoelting, Wood Dale, IL) was used to maintain rectal temperature at 37 °C and isoflurane (1.5–2%) during the procedure maintained an adequate level of anesthesia. Employing a previously described technique (Brody et al., 2007; Budinich et al., 2012), unilateral craniotomies and CCI to the left cerebral hemisphere were performed. The CCI device (Impact One™, Leica Microsystems, Buffalo Grove, IL) consisted of a computer-controlled, electromagnetically-driven impactor fitted with a 3.0-mm-diameter steel tip mounted on a stereotaxic micromanipulator. After positioning of the center of the impactor tip (3.0 mm anterior to lambda and 2.7 mm left of midline), moderate injury was produced with the following parameters: 1.0 mm depth of impact at 5 m/s with a dwell time of 0.1 s. Following injury, the skull fragment was carefully replaced, and the incision was closed with silk sutures. Sham-operated mice underwent all of the described surgical procedures but did not receive CCI. Control mice did not receive any surgical procedures or anesthesia. All surgical procedures were performed in an aseptic manner. At the conclusion of the procedure, all animals that underwent sham or CCI surgery received a subcutaneous injection of 0.5 mL of 37 °C 0.9% sodium chloride to combat dehydration. Mice were placed in a heated cage to maintain body temperature until fully awake and were then returned to their home cages.

2.3. Drugs

All mice were administered intraperitoneal (i.p.) normal saline (NS: sterile 0.9% sodium chloride, Hospira, Lake Forest, IL), 2.5 mg/kg DZ in 10–15% DMSO (Sigma Chemical Co., St. Louis, MO), or a 10–15% DMSO solution 30 min following craniotomy, CCI, or at a designated time for Controls and at 24-hour increments for three days using a standardized technique (Arras et al., 2001). To ensure that DZ remained in suspension, a fresh stock solution of DZ dissolved in full strength DMSO with a final concentration of 2.5 mg/mL was mixed at each scheduled dosing time. Due to syringe calibration limitations, administration volumes were calculated in two-gram increments (*i.e.*, 25 g mouse received 26 μL of the stock solution diluted to 260 μL with saline) to ensure that a 10–15% DMSO final solution was administered to the DZ and DMSO groups. The dose of 2.5 mg/kg DZ was chosen for our investigation based on prior studies that demonstrated *in vivo* neuroprotective effects associated with DZ doses ranging from 0.5 to 10 mg/kg administered in both ischemic preconditioning and postconditioning paradigms (Farkas et al., 2004, 2005b; Shake et al., 2001).

2.4. Histologic assessment of injury

At the conclusion of behavioral testing three weeks following injury, all mice were administered deep anesthesia (60 mg/kg ketamine with 60 mg/kg xylazine, i.p.) and were processed for immunohistochemical analysis as previously described (DiLeonardi et al., 2009). A frozen sliding microtome was used to acquire 30-μm-thick coronal sections from the olfactory bulbs to the rostral cerebellum, and all slices were stored in cryoprotectant at −20 °C until processed for assessment using a standard hematoxylin and eosin (H&E) staining technique.

2.5. Motor and behavioral training and evaluation

2.5.1. Beam walking assay

The method instituted to evaluate motor deficits resulting from injury and the response to drug therapy was a modification of a previously published protocol (Stanley et al., 2005). All mice were trained to complete the beam walk task over three days prior to sham or CCI procedures. Each mouse was placed on a start platform and trained to walk across a wooden beam (80 cm long, 6 mm wide, and 30 cm above a padded surface) toward a goal box containing bedding. A basal level of competence on this task was established on the final day of training. Acceptable performance was defined as the ability to take 50 steps with fewer than 10 foot-slips in less than 240 s. All mice were judged to be competent on the final training day and subsequently evaluated on post-operative days 1, 7, and 14 (PODs 1, 7, and 14).

2.5.2. Open field evaluation

Evaluation of anxiety, exploratory behavior, and spontaneous locomotor activity occurred in a 40 cm × 40 cm open field apparatus with opaque black plastic walls (Stoelting, Wood Dale, IL). Each mouse was placed in the arena for 10 min on Day 0 (baseline evaluation before TBI or sham procedures), and on PODs 1, 7, and 14. Mice were placed in the center of the apparatus and monitored *via* an overhead camera linked to ANY-maze behavioral tracking software (Stoelting, Wood Dale, IL). The following parameters were recorded: total distance traveled as a measure of exploratory behavior (Walsh and Cummins,