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Research report

GSNO promotes functional recovery in experimental TBI by stabilizing HIF-1α

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HIGHLIGHTS

- GSNO aids functional recovery in TBI animals by stimulating neurorepair.
- GSNO invokes neurorepair through S-nitrosylation of HIF-1α.
- S-nitrosylation stabilizes HIF-α and thus increases its activity.
- Inhibiting HIF-1α blocks GSNO-mediated neurorepair mechanisms and functional recovery.

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ABSTRACT

Traumatic brain injury (TBI) causes sustained disability due to compromised neurorepair mechanisms. Crucial to neurorepair and functional recovery following both TBI and stroke is hypoxia-inducible factor-1 alpha (HIF-1α). Based on reports that HIF-1α could be stabilized via S-nitrosylation, we tested the hypothesis that the S-nitrosylating agent S-nitrosoglutathione (GSNO) would stabilize HIF-1α, thereby stimulating neurorepair mechanisms and aiding in functional recovery. TBI was induced by controlled cortical impact (CCI) in adult rats. GSNO (0.05 mg/kg) was administered at two hours after CCI. The treatment was repeated daily until the 14th day after CCI. Functional recovery was assessed by motor and cognitive functions, and the recovery was compared with the expression of HIF-1α. The mechanisms of GSNO-mediated S-nitrosylation of HIF-1α were determined using brain endothelial cells. While non-treated TBI animals showed sustained neurobehavioral deficits, GSNO treatment of TBI improved neurobehavioral functions. GSNO also increased the expression of HIF-1α and VEGF. The beneficial effects of GSNO on neurobehavioral functions in TBI animals were blocked by treatment with the HIF-1α inhibitor 2-methoxyestradiol (2-ME). The stimulatory effect of GSNO on VEGF was reversed not only by 2-ME but also by the denitrosylating agent dithiothreitol, confirming our hypothesis that GSNO's benefits are mediated by the stabilization of HIF-1α via S-nitrosylation. GSNO's S-nitrosylation of HIF-1α was further confirmed using a biotin switch assay in endothelial cells. The data provide evidence that GSNO treatment of TBI aids functional recovery through stabilizing HIF-1α via S-nitrosylation. GSNO is a natural component of the human brain/body, and its exogenous administration has not shown adverse effects in humans. Therefore, the translational potential of GSNO therapy in TBI is high.

1. Introduction

Traumatic brain injury (TBI) is the leading cause of long-term disability worldwide [1,2]. About 3 million patients with TBI receive

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medical care annually, and more than 5.3 million Americans live with neurological deficits caused by TBI, a heavy burden for individuals and societies [3]. In spite of TBI's disabling consequences and significant financial burdens, no currently approved therapy exists due to limited understanding of TBI's neuroprotection/neurorepair mechanisms and functional recovery processes [4]. For functional recovery, clinical trials in human TBI show that neuroprotective drugs fail due to a lack of efficacy in the chronic phase. Therefore, an ideal therapy must ameliorate acute as well as chronic phases of the injury by well-understood mechanisms [4]. Previously, we documented that an S-nitrosylating agent S-nitrosoglutathione (GSNO) confers neuroprotection and protects against endothelial dysfunction in TBI's acute phase in animal models [5,6]. Using an animal model of controlled cortical impact (CCI) to assess the chronic phase of TBI, we focused on delineating the mechanisms of GSNO-mediated neurorepair leading to improvements in neurobehavioral functions.

GSNO, a biologically active member of the nitric oxide (NO) metabolome and a mediator of S-nitrosylation, is present in the brain and other organs [7]. GSNO is formed by a reaction between glutathione and NO in the presence of oxygen [8]; however, its synthesis is also influenced by redox [9]. Pharmacokinetic studies indicate that the half-life of GSNO varies in human [10] and rodent [11]. It is anticipated that GSNO/S-nitrosothiols levels are decreased in brain trauma due to an instantaneous reaction between nitric oxide synthase (NOS)-derived NO and superoxide, forming peroxynitrite. This pathological peroxynitrite formation hampers the biosynthesis of GSNO/nitrosothiols, a reaction product of NO and glutathione (GSH)/protein thiols (PSH). GSNO, via S-nitrosylation, regulates the activities of key enzymes involved in TBI such as NF-κB, STAT3, COX-2, caspase-3, nitric oxide synthases (NOS) [12–16]. An exogenous administration of GSNO protects against cardiac ischemic injury via the mechanism of S-nitrosylation [17], supporting the notion that, when channeled adequately into S-nitrosylation, GSNO shows therapeutic potential [18]. A recent stroke study showed that S-nitrosylation of PTEN inhibits its activity, leading to the activation of Akt [19]. Akt activation results in hypoxia-inducible factor-1 alpha (HIF-1α) stabilization, which, in turn, induces vascular endothelial growth factor (VEGF) and angiogenesis/neurogenesis and thus stimulates the neurorepair process [20].

HIF-1, a nuclear transcription factor, is characterized as the master regulator of cellular oxygen homeostasis. HIF-1 is a combination of the HIF-1α (120 kDa) and HIF-1β (91–94 kDa) subunits. There are three HIF-α isoforms (HIF-1α, HIF-2α and HIF-3α). The beta class includes HIF-1β. The HIF-1β subunit is a constitutively expressed protein, but the expression of the HIF-1α subunit (a cytosolic protein) is largely dependent on oxygen levels. HIF-1α is rapidly up regulated in response to hypoxia and is rapidly degraded upon reoxygenation/reperfusion. Admittedly, it is directly involved in both pathological (hypoxia) and neurorepair (normoxia) pathways following brain trauma [21]. The HIF-1α stabilizers/inducers, such as desferrioxamine (an iron chelator approved for haemochromatosis treatment), promote a number of survival pathways, including neuroprotection, angiogenesis and neurotrophins. They also reduce brain infarctions when administered pre- or post-stroke [21]. HIF-1α hydroxylating enzyme (prolyl-4-hydroxylase domain proteins; PHDs) inhibitors, such as FG-4539, are presently in a phase II anemia trial because of their activity to stabilize HIF-1α by preventing degradation with the ubiquitin proteasome system [22]. However, early inhibition of HIF-1α in the acute phase of stroke and TBI has also been reported to be neuroprotective [22–24]. Under normoxic conditions, studies are lacking on direct stabilization of HIF-1α by secondary modification and the induction of consequent protective genes. An S-nitrosylation reaction has been shown to stabilize HIF-1 protein expression and thus enhance its activity in endothelial cells [25]. Later, it was found that, while GSNO stabilizes HIF-1α by S-nitrosylation, reactive oxygen species (peroxynitrite, superoxide) destabilize HIF-1α, likely via thiol oxidation [26]. Furthermore, GSNO attenuates PHD activity during normoxia, thus inhibiting proteasomal degradation of HIF-1α [27]. S-nitrosylation-mediated stabilization of HIF-1α has been shown to protect against myocardial injury via the VEGF/angiogenesis pathway in GSNO reductase (GSNOR) knockout mice [28], indicating that HIF-1 is one of the key players in tissue repair processes. Using a rat model of ischemia reperfusion (IR), we observed that stabilization of HIF-1α by GSNO results in enhanced angiogenesis and the stimulation of neurorepair processes, leading to functional recovery [29]. The data presented in this study support that the GSNO-mediated neurorepair process and functional recovery in TBI are invoked by the stabilization of HIF-1α via S-nitrosylation.

2. Methods

2.1. Reagents

GSNO was obtained from World Precision Instruments (Sarasota, FL) and recrystallized before use. 2-Methoxyestradiol (2-ME), dithiothreitol (DDT), and all other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

2.2. Animals

Sprague-Dawley (SD) male rats approximately three months (250–300 g) old were obtained from Harlan Laboratory (Wilmington, MA). TBI (controlled cortical impact; CCI) surgery was performed in animals weighing 260–300 g. All animals received humane care in compliance with the Medical University of South Carolina's (MUSC) guidance and the National Research Council's criteria for humane care. Animal procedures were approved by the institutional animal care and use committee of MUSC. The animals were allowed to acclimatize for at least 3–5 days before the experiments. They were randomly divided into 4 groups: 1) sham-operated control without treatment (Sham) for 14 days, 2) TBI for 14 days (TBI), 3) TBI + GSNO for 14 days (GSNO), 4) TBI + GSNO + 2-ME (GSNO + 2-ME) for 14 days. At least 7 animals per group were used, and the number of animals used in each experiment is indicated in the figure legends.

2.3. Controlled cortical impact (CCI) rat model of focal TBI

Ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight) as surgical anesthesia were administered intraperitoneally (ip) [30]. Analgesic buprenorphine was administered pre-emptively to alleviate pain following surgery. Utilizing aseptic techniques, a midline scalp incision was made, and the skin and fascia were reflected to expose the skull. A bone flap (5 mm, diameter) was removed from the right side. A 3-mm diameter craniotomy (centered at the +3.0 mm posterior and 2.7 mm lateral to the bregma) was made with a handheld Michele trephine [31]. The craniotomy was enlarged further with cranial rongeurs. This process does not cause rupture or significant bleeding acutely. CCI injury was produced as previously described in the extensive literature [32–34]. A cortical contusion was produced on the exposed cortex using a controlled impactor device reported by Bilgen [35] and described in our TBI studies [5,6]. Briefly, the impacting shaft was extended, and the impact tip was centered and lowered over the craniotomy site until it touched the dura mater. Then, the rod was retracted and the impact tip was advanced to produce a brain injury of moderate severity (tip diameter, 4 mm; cortical contusion depth, 3 mm; impact velocity, 1.5 m/s) [6]. The impact tip was wiped clean with sterile alcohol after each

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