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Targeting the nNOS/peroxynitrite/calpain system to confer neuroprotection and aid functional recovery in a mouse model of TBI

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Abstract

Traumatic brain injury (TBI) derails nitric oxide (NO)-based anti-inflammatory and anti-excitotoxicity mechanisms. NO is consumed by superoxide to form peroxynitrite, leading to decreased NO bioavailability for S-nitrosoglutathione (GSNO) synthesis and regulation of neuroprotective pathways. Neuronal peroxynitrite is implicated in neuronal loss and functional deficits following TBI. Using a contusion mouse model of TBI, we investigated mechanisms for the opposed roles of GSNO versus peroxynitrite for neuroprotection and functional recovery. TBI was induced by controlled cortical impact (CCI) in adult male mice. GSNO treatment at 2 h after CCI decreased the expression levels of phospho neuronal nitric oxide synthase (pNOS), alpha II spectrin degraded products, and 3-NT, while also decreasing the activities of nNOS and calpains. Treatment of TBI with FeTPPS, a peroxynitrite scavenger, had effects similar to GSNO treatment. GSNO treatment of TBI also reduced neuronal degeneration and improved neurobehavioral function in a two-week TBI study. In a cell free system, SIN-1 (a peroxynitrite donor and 3-

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Conflict of interests

The author(s) declare that they have no competing interests.

Authors' Contributions

This study is based on an original idea of MK, AKS and IS. MK wrote the manuscript and all authors reviewed the manuscript. TS Dhammu, TS Dhindsa, BA and FM carried out animal and biochemical studies. MK, AKS, TS Dhammu, BA, IS, and TS Dhindsa critically examined biochemical studies. TS Dhammu, TS Dhindsa and FM performed histological, immunoblotting and IHC studies. All authors have approved the manuscript.

nitrotyrosinating agent) increased whereas GSNO (an S-nitrosylating agent) decreased calpain activity, and these activities were reversed by, respectively, FeTPPS and mercuric chloride, a cysteine-NO bond cleaving agent. These data indicate that peroxynitrite-mediated activation and GSNO-mediated inhibition of the deleterious nNOS/calpain system play critical roles in the pathobiology of neuronal protection and functional recovery in TBI disease. Given GSNO's safety record in other diseases, its neuroprotective efficacy and promotion of functional recovery in this TBI study make low-dose GSNO a potential candidate for preclinical evaluation.

Keywords

TBI; GSNO; Calpain; peroxynitrite; nNOS; neurodegeneration

1. Introduction

A major medical and socio-economic problem, TBI is the leading cause of death in aged adults due to falls and in young adults due to war and accident from motor vehicles and sports (Chauhan, 2014). Other than critical care management, no currently approved therapies exist for TBI (Haddad and Arabi, 2012). Mechanistically, TBI is characterized by sustained neuronal nitroxidative stress (Cherian et al., 2004; Prasad and Bondy, 2015), due mainly to calcium-induced aberrant activity of neuronal nitric oxide synthase (nNOS). Aberrant nNOS activity results in reduced bioavailability of nitric oxide (NO) and formation of large amounts of peroxynitrite for prolonged periods. Neuronal peroxynitrite production in TBI causes activation of calpain and thus axonal and neuronal cell injury. Because NO is consumed by superoxide to form peroxynitrite (Deng-Bryant et al., 2008; Deng et al., 2007), biosynthesis of GSNO, a reaction product of NO and glutathione, is anticipated to be reduced. We observed in animal models of TBI that the deleterious activity of peroxynitrite can be blocked by exogenously administered GSNO, an endogenous molecule with S-nitrosylating activity (Hess and Stamler, 2012), leading to reduced neuroinflammation and decreased neuronal cell death (Khan et al., 2009; Khan et al., 2011). Therefore, we investigated whether GSNO-mediated reversible mechanisms of S-nitrosylation inhibit "aberrant" activities of nNOS and calpains, leading to decreased peroxynitrite production, reduced neurodegeneration and improved neurobehavioral functions.

NO executes its major biological roles by its conversion to secondary metabolites, such as GSNO and peroxynitrite. GSNO invokes anti-inflammatory, anti-oxidant, anti-neurodegeneration, and vascular protection effects, likely via S-nitrosylation (Chiueh and Rauhala, 1999; Hess and Stamler, 2012; Khan et al., 2012; Kim et al., 2013; Prasad et al., 2007). Reduced levels of NO (Malinski et al., 1993), GSH (Khan et al., 2006) or both, combined with increased degradation of GSNO by the inflammation-induced enzyme activity of GSNO reductase (Que et al., 2005) may contribute to the reduced levels of GSNO following TBI. Reduced S-nitrosylation is observed in an animal model of cerebral ischemia reperfusion (Khan et al., 2012) and deficient S-nitrosylation is considered a general neurodegenerative mechanism (Ju et al., 2005; Rauhala et al., 1996; Schonhoff et al., 2006). On the other hand, peroxynitrite has been shown to be involved in neurodegeneration, especially under oxidative pathological conditions (Hall et al., 2004; Khan et al., 2011;

Pacher et al., 2007). In the injured brain, the observed increased 3-nitrotyrosination, a peroxynitrite adduct of tyrosine residue, suggests the pathological role of peroxynitrite (Khan et al., 2011). The deleterious role of neuronal peroxynitrite is further substantiated by peroxynitrite-mediated increased calpain activity (Deng-Bryant et al., 2008; Deng et al., 2007). In contrast, GSNO-mediated mechanisms are reported to reduce the activity of calpains, likely via S-nitrosylation (Annamalai et al., 2015).

nNOS is primarily expressed in neurons and its products play critical roles in neuronal loss following neurotrauma (Wei et al., 1999). Inhibition of nNOS activity has been shown to be neuroprotective in TBI (Mesenge et al., 1996), indicating a deleterious role of nNOS activity in TBI. However, irreversible inhibition of nNOS by its active-site directed inhibitors results in the loss of the deleterious effects of peroxynitrite as well as the beneficial roles of NO/GSNO, thus limited efficacy. Recent reports document that the S-nitrosylation of nNOS, in addition to phosphorylation/dephosphorylation, regulates its activity reversibly (Khan et al., 2012; Qu et al., 2012; Rameau et al., 2007). Similarly, the activity of calpain is also regulated by NO-mediated S-nitrosylation of cysteine at the active site of calpain, in addition to binding to the endogenous inhibitor calpastatin or autoproteolysis (Koh and Tidball, 2000; Samengo et al., 2012). In contrast, Peroxynitrite has been shown to activate calpain via the mechanisms of 3-nitrotyrosination (Deng et al., 2007), thus maintaining a deleterious nNOS/peroxynitrite/calpain system.

Calpains are calcium-activated neutral proteases that play a critical role in regulation of physiological and pathological activities (Goll et al., 2003). Two major calpain isoforms are present in the brain; μ -calpain (calpain-1; activated by μM Ca^{2+} concentration) and m-calpain (calpain-2; activated by mM Ca^{2+} concentration). Both calpains participate in neuronal excitotoxic stress and neurodegeneration mediated by proteolysis of prominent calpain substrates, including cytoskeletal neuron-specific alpha II spectrin (Bevers and Neumar, 2008; Deng et al., 2007; Saatman et al., 2010), neurofilament protein NF200 (Schumacher et al., 2000), and myelin basic protein (MBP) (Liu et al., 2006). Recently, we reported that, under Alzheimer disease conditions, glutamate (Glu) and $\text{A}\beta$ -induced calpain activation is inhibited by GSNO treatment (Annamalai et al., 2015), suggesting a potential regulatory role of NO/GSNO in calpain inactivation. Therefore, the goal of this study was to investigate whether exogenous GSNO administration provides neuroprotection by inhibiting the deleterious nNOS/peroxynitrite/calpain system. To determine the clinical significance of this hypothesized mechanism, we also examined whether GSNO treatment improves neurological functions.

2. Results

2.1 GSNO treatment of TBI for 4 hours inhibited nNOS (Ser¹⁴¹²) phosphorylation and nNOS activity in a CCI mouse model of TBI

In the traumatic penumbra region, we observed TBI-induced increased expression levels of phosphorylated Ser¹⁴¹² at 4 h after the injury (Fig 1A), increasing both nNOS enzymatic activity (Fig 1B) and peroxynitrite production, as previously described (Khan et al., 2011). GSNO treatment of TBI decreased the levels of TBI-induced phosphorylation of Ser¹⁴¹² (Fig 1A–a), thus resulting in reduced nNOS enzymatic activity (Fig 1B), which may keep

the levels of peroxynitrite low (as measured by the expression levels of 3-NT), as shown in Fig 2D–d.

2.2 GSNO or FeTPPS treatment of TBI for 24 hours inhibited calpain activity and expression levels of 3-NT in a CCI mouse model of TBI

The regulation of calpain activity by GSNO versus peroxynitrite at 24 h was examined in the traumatic penumbra region. A peroxynitrite scavenger FeTPPS was used to further support the causative effects of peroxynitrite. The expression of calpain 1 and 2 remained unaltered (Fig 2A). However, calpains' activity increased significantly in the TBI group, measured as cleaved alpha II spectrin breakdown products (SBDP 150 kDa and SBDP 145 kDa; indicated by arrows) (Fig 2B–b) and through fluorescence substrate assay (Fig 2C). The levels of 3-NT in TBI were also increased significantly (Fig 2D–d). GSNO or FeTPPS treatment of TBI (Fig 2Bb–Dd) inhibited calpain activity as well as reduced the expression levels of 3-NT ($p<0.001$ vs. Normal, Sham, TBI+GSNO and TBI+FeTPPS). Normal animals had no significant difference compared to sham animals in terms of the calpain activity and 3-NT levels.

2.3 GSNO or FeTPPS treatment of active calpain-1 peptide inhibited calpain activity in a cell free system

A cell free system with and without active calpain peptide was used to determine the effects of GSNO (as an S-nitrosylating agent) and SIN-1 (as a peroxynitrite-producing agent) on the activity of calpain and to correlate the results with animal studies as described in Fig 2. While GSNO significantly decreased ($p<0.001$), SIN-1 significantly increased ($p<0.001$) the basal activity of active calpain peptide in a dose dependent manner (Fig 3A). Results from animal studies (Fig 2) that GSNO decreases calpain activity were further confirmed using HgCl_2 (S-nitrosothiol decomposing agent (Cook et al., 1996) and SIN-1 in the presence of GSNO or a peroxynitrite scavenging agent FeTPPS (Thiyagarajan et al., 2004) (Fig 3B). In a cell free system, the activity of active calpain was significantly increased following SIN-1 treatment, which was reversed to basal activity by pretreatment with the peroxynitrite scavenger FeTPPS or with GSNO (Fig 3B). The inhibitory effect of GSNO on calpain peptide activity was blocked by S-NO bond cleaving agent HgCl_2 (Fig 3B).

2.4 GSNO treatment of TBI for 14 days inhibited calpain activity and expression levels of 3-NT in a CCI mouse model of TBI

To determine that the activation of the peroxynitrite/calpain system is prolonged and sustained in TBI and that GSNO maintains its inhibitory effect for an extended period, a 14-day TBI study was performed. Like in the acute phase of TBI (Fig 2), GSNO inhibited calpain activity as indicated by reduced levels of the spectrin breakdown products SBDP 145/150 (Fig 4Aii–a). The decreased levels of the spectrin breakdown products correlated with decreased levels of the expression levels of 3-NT (Fig 4B–b), indicating that GSNO inhibits calpain activity by inhibiting the production of peroxynitrite. GSNO treatment did not alter the expression of calpain as evidenced by calpain-1 immunoblot (Fig 4Ai).

2.5 GSNO treatment of TBI for 14 days improved neurological function and reduced neuronal loss/neurodegeneration in a CCI mouse model of TBI

The efficacy of GSNO in improving motor, subtle behavior, and non-spatial memory functions was evaluated for 14 days after TBI. GSNO treatment significantly improved beam walking speed (beam walk task, Fig 5A) compared with the TBI group, measured until 14 days after TBI. The treatment with GSNO significantly decreased the number of foot faults (Fig 5B) and remarkably reduced the time to remove adhesive tape (Fig 5C) at all the recorded time points after TBI. A preference for novel objects assessed (at 14th day after TBI) through the NOR test (Huang et al., 2014) was severely compromised in TBI animals, and GSNO treatment of TBI remarkably improved the degree of preference for novel objects (Fig 5D). Neurodegeneration and neuronal loss were evaluated by cresyl violet (Nissl, Fig 5E–e) and neuronal nuclear protein (NeuN, Fig 5F–f) stainings, respectively. The stainings highlight neuronal population (NeuN) and important structural features of neurons (Nissl). Condensed fragmented Nissl staining (Fig 5E) in TBI shows neuronal degeneration. In contrast, GSNO and Sham had cells that contained Nissl substance, indicating viable neurons and neuroprotection (Fig 5E).

1. Discussion

Brain imaging, biochemical markers and neurobehavioral studies support that TBI causes a neurodegenerative disease which, if untreated, results in profound loss of neurological functions. The results presented here suggest that a high level of the 3-nitrotyrosinating (3-NT-forming) agent peroxynitrite is formed for a prolonged period, which induces neurodegeneration and causes functional deficits in a mouse model of TBI. In contrast, administering the S-nitrosylating (S-NO-forming) agent GSNO following TBI inhibits peroxynitrite formation, confers neuroprotection, and promotes functional recovery. Therefore, the focus of the present study was to investigate the opposed mechanisms of peroxynitrite-mediated neurodegeneration versus GSNO-mediated neuroprotection in TBI.

Previously, we have shown that GSNO protects against neuroinflammation and BBB disruption by reducing the levels of peroxynitrite in animal models of TBI (Khan et al., 2009; Khan et al., 2011) and stroke (Khan et al., 2006; Khan et al., 2012). Several other studies also confirm the deleterious role of peroxynitrite in TBI (Deng-Bryant et al., 2008; Deng et al., 2007; Pacher et al., 2007). Neuronal peroxynitrite originates mainly from TBI-induced calcium dysregulation-mediated aberrant enzymatic activity of nNOS. Therefore, nNOS activation and resultant neuronal peroxynitrite formation, leading to neurodegeneration, are considered the hallmarks of TBI disease. In spite of the activation of nNOS following TBI, the levels of NO are decreased due to the diffusion-limited instantaneous reaction between NO and superoxide, forming peroxynitrite. Decreased NO bioavailability may also result in reduced formation of GSNO because GSNO is synthesized by the reaction of NO with GSH (Singh et al., 1996). Taken together, these observations indicate a disturbed NO metabolome in TBI. This disturbed NO metabolome in TBI is supported by previous studies from our laboratory in TBI (Khan et al., 2011) and stroke (Khan et al., 2012; Khan et al., 2015b) and the current results, which show increased enzymatic nNOS activity (Fig 1B) and sustained high levels of peroxynitrite for a prolonged

periods (Figs 2D–d and 4B–b). We investigated the mechanisms of the efficacy of GSNO for TBI therapy because it corrected not only the disturbed NO metabolome (Fig 1) and NO/S-nitrosylation-based cellular functions (Khan et al., 2005; Khan et al., 2011; Khan et al., 2015a; Sakakima et al., 2012) but also provided neuroprotection and improved neurological functions, as shown in Fig 5.

The deleterious role of nNOS activation has been supported by the observation that the inhibition of nNOS activity following TBI provides neuroprotection (Mesenge et al., 1996) and nNOS KO mice show reduced infarct volume in stroke animal models (Eliasson et al., 1999; Hara et al., 1996; Huang et al., 1994; Samdani et al., 1997; Wei et al., 1999). The role of endothelial NOS (eNOS) is to some extent limited to blood-brain barrier (BBB) pathology and cerebral blood flow (CBF), whereas the role of inducible NOS (iNOS) is not clear in TBI (Cherian et al., 2004). Nevertheless, GSNO inhibits the induction of iNOS in TBI (Khan et al., 2009) and decreases the activities of aberrant eNOS following neurotrauma (Khan et al., 2012). Because the focus of this study was to investigate the direct role of neuronal peroxynitrite in calpain activation and neurodegeneration, the effect of GSNO on eNOS/iNOS was not investigated. In resting neurons, nNOS activity is regulated by reversible S-nitrosylation of Cys³³¹ (Qu et al., 2012). Under pathological conditions, nNOS turns aberrant/uncoupled due to loss of homeostasis of nNOS Ser¹⁴¹² phosphorylation (Rameau et al., 2007) versus Cys³³¹ S-nitrosylation in addition to decreased levels of its cofactor (tetrahydrobiopterin; BH₄) and/or substrate (L-Arginine). Aberrant nNOS begins to generate superoxide and NO in the same compartment, thus forming excessive peroxynitrite and causing neuronal cell death (Hall et al., 2012). TBI-induced neuronal cytoskeleton degradation leading to neurodegeneration is mediated prominently by calpain activities (Deng-Bryant et al., 2008; Deng et al., 2007). Therefore, we investigated whether neuronal peroxynitrite invokes its neurodegenerative effects via the activation of calpains, and GSNO extends its neuroprotective efficacy via the inhibitions of calpain activities.

In TBI settings, calpain inhibitors are shown to provide some degree of neuroprotection (Saatman et al., 2010) and calpain-1 knock out mice show reduced cortical degeneration (Yamada et al., 2012), indicating calpain's deleterious role. The activity of calpain is inhibited by NO/S-nitrosylation of cysteine at the active site of calpain (Koh and Tidball, 2000; Samengo et al., 2012). Inhibition of calpains results in reduced levels of calpain-specific alpha II spectrin breakdown product (SBDP)145 kDa fragment (Yoon et al., 2011). Recently, we also observed that glutamate (Glu) and Aβ-induced calpain activation is inhibited by GSNO treatment (Annamalai et al., 2015), suggesting a potential regulatory role of NO/GSNO in calpain inactivation. In contrast, neuronal peroxynitrite was found to increase the activity of calpain as it increased the ratio of calpain-degraded p25 to p35 (Annamalai et al., 2015), indicating a calpain-activating role of peroxynitrite.

Following TBI, peroxynitrite (3-NT) levels were significantly increased as early as 4 h (Khan et al., 2011) and the levels remained high at 24 h (Fig 2D–d), as well as at 14 days (Fig 4B–b), indicating sustained prolonged production of peroxynitrite. Increased peroxynitrite correlated with increased calpain activity (Fig 2C) and levels of the calpain-specific breakdown product of alpha spectrin (SBDP 145 kDa) at 24 h (Fig 2B–b) as well as

14 days (Fig 4A–a), indicating that a neuronal peroxynitrite-mediated mechanism is involved in calpain-mediated neurodegeneration and functional deficits (Fig 5). GSNO-mediated inhibition of TBI-induced peroxynitrite accumulation and calpain activation (Figs 2 and 4) correlated with neuroprotection (Fig 5E–F) and significantly improved motor, somatosensory and cognition functions (Fig 5A–D). These findings support that GSNO invokes its effect via the inhibition of activities of nNOS and calpains. Neither TBI nor GSNO treatment of TBI altered the protein expression levels of calpains (Fig 2A and Fig 4A), indicating that both peroxynitrite and GSNO regulate calpain activities via secondary modification.

The observed mechanistic results were further supported by cell-free system experiments using calpain-1 active peptide (available from AbCam Inc., Cambridge, MA, USA) as a positive control for calpain activity as previously described (Annamalai et al., 2015). The advantage of the cell-free experiment is that it provides a direct evidence of 3-nitrotyrosination and S-nitrosylation of calpain peptide. In this cell free system, a treatment with SIN-1, a peroxynitrite-producing agent (Khan et al., 2006; Pacher et al., 2007), significantly and dose dependently increased whereas GSNO significantly and dose dependently decreased calpain activity (Fig 3A). Reversal of the SIN-1-mediated enhanced calpain activity by FeTPPS, a peroxynitrite scavenger (Khan et al., 2015b; Thiyagarajan et al., 2004), supports the role of peroxynitrite for increasing calpain activity, likely via the mechanism of 3-nitrotyrosination (Fig 3B). Blocking the GSNO-mediated reduced calpain activity by HgCl₂, a Cys-NO bond cleaving agent (Cook et al., 1996), supports the role of GSNO in decreasing calpain activity, likely via the mechanisms of S-nitrosylation (Fig 3B). These observations support the mechanism of animal studies that TBI-induced nNOS-dependent peroxynitrite increases the activity of calpains, causing neurodegeneration and that GSNO provides neuroprotection by inhibiting nNOS/calpain activity and thus decreasing peroxynitrite production as described in Fig 6.

Conclusions

Conclusively, these data show that the NO metabolites GSNO and peroxynitrite play opposing roles in the pathobiology of TBI. Produced by nNOS under excitatory/oxidative conditions as observed in TBI, NO is consumed by superoxide to form excessive amounts of peroxynitrite. Increased levels of peroxynitrite cause a sustained upregulation of the deleterious nNOS/peroxynitrite/calpain system. Under such pathological conditions, S-nitrosylation (GSNO)-mediated biological regulation (inhibition) of the activity of nNOS and calpain is lost due to reduced bioavailability, and thus levels of NO/GSNO. Optimization of cellular GSNO homeostasis with supplementation of exogenous GSNO provides neuroprotection through biological regulation and thus the inhibition of TBI-induced deleterious activities of the nNOS/calpain system, leading to improved neurobehavioral functions in TBI animals.

2. Experimental Procedure

4.1 Reagents

GSNO was purchased from World Precision Instruments (Sarasota, FL, USA) and 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) (FeTPPS) and 3-morpholino sydnonimine (SIN-1) from Calbiochem, (La Jolla, CA, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. All other chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

4.2 Animals

Animals were young adult male (~3–4 months old) wild type C57BL/6) weighing between 25 and 30 g at the time of surgery. 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.

4.3 Experimental groups, drugs and dose

The animals (n=84) were randomly divided into five groups: 1) TBI animals treated with vehicle (TBI; n=21), 2) TBI+GSNO treatment (TBI+GSNO; n=21), 3) TBI+FeTPPS treatment (TBI+FeTPPS; n=7), 4) sham-operated treated with vehicle (Sham; 21), 5) normal control (Normal; n=14). The number of animals used in each experiment is indicated in figure legends. In the TBI+GSNO treatment group, the mice were administered freshly prepared GSNO (0.05 mg/kg body weight), which was dissolved in sterile saline (~25 µl) and administered iv 2 h after CCI. FeTPPS (3 mg/kg, iv) dissolved in sterile saline (~25 µl) was also administered 2 h after CCI. The same dose of GSNO (orally) and FeTPPS (iv) was administered daily thereafter. The dose of GSNO treatment was based on our previously reported dose response curve study in a rat model of TBI and SCI (Chou et al., 2011; Khan et al., 2009; Khan et al., 2011). The effective dose of FeTPPS was determined based on the reported dose in a stroke IR model (Khan et al., 2015b; Thiagarajan et al., 2004). The selected dose of both GSNO and FeTPPS did not alter physiologic parameters, including BP, HR, and temperature, measured at 1 hour following GSNO or FeTPPS treatment. Details of the study on physiologic parameters in rats have been previously reported (Khan et al., 2006; Khan et al., 2011).

4.4 Controlled cortical impact (CCI) mouse model of TBI

Surgical anesthesia was induced by ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight) administered intraperitoneally (ip). Analgesic buprenorphine was administered pre-emptively to alleviate pain following surgery. The animal was then placed onto a heated pad and core body temperature was maintained at 37.0±1 °C. The animals were secured in a stereotaxic frame and ventilated mechanically. A dab of sterile ophthalmic ointment was placed on each eye to compensate for the decrease in lacrimation during anesthesia. A midline scalp incision was made, and the skin and fascia were reflected to expose the skull. A craniotomy (4 mm) was made in the right hemisphere encompassing bregma and lambda and between the sagittal suture and the coronal ridge with a handheld

Michele trephine. Care was taken to avoid injury to the underlying dura mater, which was continuously bathed in sterile normal saline warmed to 37.5 °C as described (Fox et al., 1998). The resulting bone flap was removed, and the craniotomy enlarged further with cranial rongeurs. CCI injury was produced in mice as previously described (Fox et al., 1998; Smith et al., 1995; Wang et al., 2013). A cortical contusion was produced on the exposed cortex using a controlled impactor device described by Bilgen (Bilgen, 2005) and used in our laboratory (Shunmugavel et al., 2010) or TBI-0310 TBI Model system (Precision Systems and Instrumentation, LLC, Fairfax Station, VA) as described in our TBI study (Khan et al., 2009). 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 farther to produce a brain injury of moderate severity for mice (tip diameter, 1 mm; cortical contusion depth, 1 mm; impact duration 85 msec; impact velocity, 1.5 m/sec). The impact tip was wiped clean with sterile alcohol after each impact and cleaned/disinfected further with cidex after surgery. Core temperature was maintained at $37 \pm 0.1^{\circ}\text{C}$ with a heating pad during surgery and recorded with a rectal probe. Immediately after injury, the skin incision was closed with nylon sutures, and 2% lidocaine jelly was applied to the lesion site to minimize discomfort. Sham animals underwent the same procedure, excepting the cortical impact.

4.5 Histology and immunohistochemistry

The degree of neuronal damage (loss of viable neurons) was evaluated by cresyl violet (Nissl) staining. The Nissl staining highlights important structural features of neurons. The NeuN immunostaining indicates number of neurons. Paraffin-embedded brain sections from the formalin-fixed brain tissues, processed at 14 days after CCI, were used to stain with Nissl and the staining was performed according to classical histology methods as previously described from our laboratory (Khan et al., 2015a; Sakakima et al., 2012). Cells that contained Nissl substance were considered to be viable neurons. Condensed fragmented staining shows neuronal degeneration.

Paraffin-embedded brain sections from the formalin-fixed brain tissues, processed at 14 days after CCI, were used to immunostain with NeuN and the staining was performed using NeuN specific antibody as described (Liu et al., 2009). Percent of NeuN positive area were determined as previously described from our laboratory (Sakakima et al., 2012) and viable neurons from Nissl staining were quantified by manually counting cells as previously described from our and other laboratories (Khan et al., 2015a; Zhu et al., 2014) in the traumatic penumbra area using an Olympus microscope. Histological and immunohistological analysis was performed by an observer blinded to the identity of groups as previously described from our laboratory (Khan et al., 2015a; Sakakima et al., 2012).

4.6 NOS activity assay

NOS activity in the traumatic brain penumbra area was determined by the conversion of L-[4,5-³H] arginine (American Radiolabeled, Inc., St. Louis, MO) to L-[4,5-³H] citrulline using an NOS assay kit from Cayman Chemicals (Ann Arbor, MI). Total cNOS activity was determined by subtracting calcium independent NOS activity. The nNOS inhibitor, 7-NI (50 mg/kg), and the NOS inhibitor, L-NAME (30 mg/kg), were used to differentiate between

endothelial nitric oxide synthase and nNOS activity (Coert et al., 2003). The activity was expressed as pmol/mg protein/min.

4.7 Calpain activity assay

Analysis of calpain activity was performed using a calpain activity assay kit from Abcam Inc., Cambridge, MA, USA, according to the manufacturer's directions and as described (Annamalai et al., 2015). Briefly, equal amounts of brain homogenates from the traumatic penumbra area or active calpain-1 were incubated with calpain substrate (Ac-LLY-AFC) and reaction buffer. Calpain-mediated breakdown of substrate product (AFC) was analyzed by fluorometric analysis. In the cell free assay, the dose curve of GSNO and SIN-1 (10–100 μ M) was determined 1 h after the incubation. To investigate the effect of SIN-1, FeTPPS (100 μ M) and GSNO (100 μ M) were treated 10 min before SIN-1 (100 μ M). HgCl₂ (100 μ M) was treated 10 min before GSNO treatment (100 μ M) to determine the effect of GSNO.

4.8 Western blot analysis

In the traumatic penumbra area from the ipsilateral injured brain tissue, Western blot was performed using antibodies against calpain 1, calpain 2 (Cell signaling Technology, Danvers, MA), alpha spectrin (Santa Cruz Biotechnology, Santa Cruz, CA), 3-NT, nNOS, phospho nNOS Ser¹⁴¹⁷, equivalent to human Ser¹⁴¹² (Abcam Inc., Cambridge, MA) and β -actin, as described earlier (Khan et al., 2012; Khan et al., 2015b). Protein concentrations were determined using protein assay dye from Bio-Rad Laboratories (Hercules, CA). Densitometry of protein expression was performed using a GS800 calibrated densitometer from Bio-Rad laboratories (Hercules, CA).

4.9 Evaluation of motor behavior, sensory function and novel object recognition

Animals in each group were evaluated for beam walking, foot faults, adhesive tape removal time, and novel object recognition before and after CCI. All the tasks described below were performed by investigators blinded to the study groups and conditions.

In the motor function test, the mice were examined for fine motor function using a beam-walking task with an elevated narrow beam (80 cm long $\times$ 2.5 cm wide) raised to 10 cm above ground level. The time to traverse the beam was recorded and analyzed after three trials (60 seconds allotted time). The beam test was selected over other motor function tests to detect motor deficits because the test is suitable for aged as well as young rodents. Animals were pre-trained for 3 days before CCI. We routinely use this task, as published for IR and TBI studies (Khan et al., 2009; Khan et al., 2015a; Sakakima et al., 2012).

The forelimb foot faults or grid walking task, a motor impairment test of limb functioning, was used to determine forelimb movement dysfunction while walking. Mice were placed on elevated horizontal grids. Mice placed their paws on the wire while moving along the grid. With each step, the paw may fall or slip between wires. These events were recorded as foot faults. The total number of steps that the mouse used to cross the grid was counted, and the total number of foot faults was recorded as described (Zhang et al., 2002). Mice were trained for 3 days before CCI, and the number of foot faults following CCI at days 0, 3, 7, and 14 was recorded.

The somatosensory function deficits test is routinely used to assess the progress of recovery as described in mice (Venna et al., 2014). The test was performed by adhesive tape-removal before CCI and 3, 7, and 14 days after CCI. Small adhesive tape strips (0.3×0.4 cm) were used as bilateral tactile stimuli attached at the distal-radial region on each forelimb. The latency (the time to remove adhesive from the contralateral forelimbs) was recorded during three trials per day for each forepaw as described (Bouet et al., 2009). Before injury, mice were trained for 3 days as previously described (Khan et al., 2009). The time to contact the tape was not recorded as animals take little time (~1–10 seconds) to sense the tape in our CCI model.

In a separate set of experiments, we tested the animals for their handedness as described (Signore et al., 1991) and determined the effects of handedness on the outcome of adhesive tape test. Handedness had no significant effects on adhesive tape removal. This may be attributed to animals' use of mouth also to remove the adhesive tape as described (Bouet et al., 2009).

Non-spatial memory was determined by the novel object recognition (NOR) test as described (Huang et al., 2014). The NOR test evaluates the ability of the animal to recognize a novel object in the environment. The NOR test wooden box ($36\text{cm} \times 22\text{cm} \times 18$ cm) was located in an isolated and illuminated animal testing room. The animals were allowed to explore the test box for 15 min per day before the actual experiments. The NOR test box was devoid of any object during the habituation trial. The test was performed on day 14 following CCI, as described (Kruk-Slomka et al., 2014). During session I, two objects, A1 and A2, with identical texture, color, and size were presented to the test animal for 10 min. After a 24 h delay in the home cage, the mice were again exposed to the same area with one novel object of different texture, color, and size included (A1 and B) for 4 min. Both the objects and the box were cleaned with alcohol and dried after each trial to remove olfactory cues. Latency (% preference for a novel object) was recorded using a video camera. The advantage of the test is that, unlike the Morris water-maze task, it is independent of motor function (Dhawan et al., 2011).

4.10 Statistical evaluation

Statistical analysis was performed as described (Khan et al., 2015a) using software Graph pad Prism 5.01. Unless otherwise stated, all values are expressed as mean $\pm$ SD of n determinations or as mentioned. The results were examined by unpaired Student t -test. Multiple comparisons were performed by one-way ANOVA followed by the post-hoc Tukey test. A p value less than 0.05 was considered significant.

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Abbreviations

BH4	tetrahydrobiopterin
CCI	controlled cortical impact
FeTPPS	5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron (III)
HgCl₂	mercuric chloride
GSNO	S-nitrosoglutathione
MBP	myelin basic protein
NeuN	neuronal nuclear protein
7-NI	7-nitroindazole
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
3-NT	3-nitrotyrosine
ONOO⁻	peroxynitrite
Sham	sham-operated animals
SIN-1	3-morpholiniosyndonimine
TBI	traumatic brain injury

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Highlights

- TBI increases the activities of nNOS/calpain system and the levels of peroxynitrite.
- GSNO inhibits the activities of nNOS/calpain and reduces the levels of peroxynitrite.
- GSNO reduces TBI-induced neurodegeneration and aids in functional recovery.
- Mechanism of 3-nitrotyrosination of calpain is associated with its aberrant activity.
- Mechanism of S-nitrosylation of nNOS/calpain is associated with neuroprotection.

Figure 1.

Immunoblots of nNOS and phosphorylated nNOS (Ser¹⁴¹²) and the nNOS enzymatic activity in the cortical traumatic penumbra at 4 h after TBI. TBI increased the expression levels of phosphorylated nNOS (Ser¹⁴¹²) whereas GSNO treatment of TBI decreased the expression levels of the same (A–a). Expression of nNOS remained unchanged in all the three groups (A). Similar to nNOS (Ser¹⁴¹²) phosphorylation, TBI increased the enzymatic activity of nNOS, measured as the conversion of L-arginine to L-citrulline, and GSNO treatment of TBI decreased nNOS enzymatic activity (B). Data are presented as mean+

standard deviation (n=7). **p<0.01 vs. Sham and GSNO. Label a indicates densitometric analysis of immunoblot and labels Sham, TBI and TBI+GSNO represent respective groups.

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Figure 2.

Immunoblots of calpain-1, calpain-2, alpha II spectrin and 3-NT, and total calpain activity in the cortical traumatic penumbra at 24 h after TBI. Neither TBI nor GSNO or FeTPPS treatment of TBI altered the expression of calpain 1 and calpain 2 (A). However, TBI increased alpha spectrin breakdown products (SBDP 145/150) (B–b) and total calpain activity measured by calpain fluorescent substrate assay (C). Both GSNO and FeTPPS decreased SBDP 145/150 (B–b) and calpain activity (C). Similarly, both GSNO and FeTPPS decreased TBI-mediated increased expression levels of 3-NT (D–d). Data are presented as

mean+ standard deviation (n=7). ***p<0.001 vs. normal, Sham, TBI+GSNO and TBI +FeTPPS. Labels b and d indicate densitometric analysis of immunoblots and ILabels Normal, Sham, TBI, TBI+GSNO and TBI+FeTPPS represent respective groups.

Figure 3.

Effect of GSNO, SIN-1, HgCl₂ and FeTPPS on calpain-1 activity in a cell free system. Calpain-1 active peptide (active peptide) activity was measured using a calpain substrate assay fluorescence kit in presence and absence of different concentrations of GSNO and SIN-1 (A) to determine their dose response curve. While GSNO decreased, SIN-1 increased calpain activity (A). While HgCl₂ (100 μ M) pre-treatment reversed the effect of GSNO, FeTPPS (100 μ M) pre-treatment reversed the effect of SIN-1 (100 μ M). Post-treatment of GSNO (100 μ M) also reversed the effect of SIN-1 (B). Data are presented as mean+

standard deviation (n=5). aaap<0.001 vs. active peptide, bbp<0.01 vs. vs 10 μ M GSNO or SIN-1, ***p<0.001 vs. GSNO, SIN-1, SIN-1+GSNO, ###p<0.001 vs. GSNO, SIN-1+GSNO, +++p<0.01 vs. SIN-1+GSNO, SIN-1+FeTPPS, \$\$\$p<0.001 vs. SIN-1+GSNO.

Figure 4. Immunoblots of calpain-1, alpha II spectrin and 3-NT in the cortical traumatic penumbra at 14 days after TBI. Neither TBI nor GSNO treatment of TBI changed the expression levels of calpain-1 (Ai). However, TBI showed increased alpha spectrin breakdown product expression (SBDP 145/150), which was decreased by GSNO treatment (Aii-a). Similarly, GSNO treatment of TBI decreased TBI-mediated increased expression levels of 3-NT (B-b). Data are presented as mean+ standard deviation (n=3 in each group). Labels a and b

indicate densitometric analysis of immunoblots and labels Normal, Sham, TBI and TBI +GSNO represent respective groups.

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Figure 5. Effect of GSNO on improvement of neurological functions and neuroprotection. Studies on beam walking speed (A), number of foot faults (B), removal time of adhesive tape (C) at days 0, 3, 7, and 14 days and novel object recognition (NOR; D) at 14th day, and tissue histology and immunohistochemistry (Nissl; E–e, NeuN; F–f) at 14th day in a mouse model of TBI. Animals pre-trained on behavioral tasks/tests were subjected to CCI. The animals were treated with GSNO (0.05 mg/kg, iv) at 2 h after the CCI, and the same dose was repeated orally every 24 h. The data in general indicate that GSNO group had reduced

neurobehavioral deficits as well as decreased neurodegeneration and reduced neuronal loss vs. TBI group. Thick black wavy arrows indicate neuronal degeneration Blue arrows indicate viable neurons on Nissl staining. Percent of viable neurons on Nissl (e) and percent of NeuN positive area on NeuN staining (f) were quantified. Data are presented as mean +SE. *** $p < 0.001$, ** $p < 0.00$ vs. Sham and TBI. (n=5 for neurobehavioral and n=3 for histology and immunohistochemistry studies).

Figure 6.

Schematic showing hypothesized mechanisms that excitotoxicity-mediated aberrant activity of nNOS results in prolonged and sustained production of peroxynitrite, which maintains sustained calpain activation via 3-nitrotyrosination mechanisms, leading to neurodegeneration. GSNO inhibits the deleterious nNOS/peroxynitrite/calpain system via the reversible mechanism of S-nitrosylation of both nNOS and calpain, thus reducing not only the levels of nNOS-derived peroxynitrite but also TBI-induced calpain activity, leading

to reduced neurodegeneration and improved neurological functions in a CCI mouse model of TBI.

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