

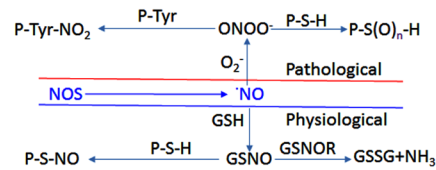
● PERSPECTIVE

# Therapeutic exploitation of the S-nitrosoglutathione/S-nitrosylation mechanism for the treatment of contusion spinal cord injury

Contusion spinal cord injury (SCI) is a major medical and socio-economic problem globally. The incidence of SCI is highest among young adults due to motor vehicle accidents, military or sports injuries, and violence (Selvarajah et al., 2014). The elderly and children are also at risk due to falls and accidents. SCI causes neurodegeneration, with profound loss of locomotor and sensory functions (Siddiqui et al., 2015). Pain and depression are also prevalent in a majority of SCI patients. Expenses for severe SCI are high: initial hospitalization, rehabilitation, and most likely the continuing need for a caregiver and medical care. SCI survivors with less severe injuries usually face lower but still hefty medical bills. However, people  $\geq 50$  years old with severe SCI may face medical expenses of over \$1.8 million during their lifetimes. These injuries also affect spouses and family members, emotionally and financially, and most injuries jeopardize employment for those affected. In sum, SCI has a lifelong effect on many people; it represents a major challenge for successful health care management.

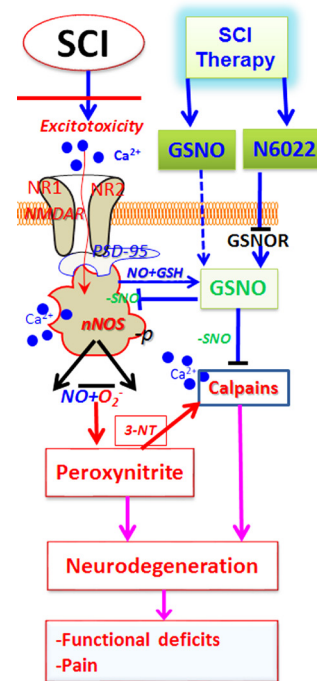
Other than critical care management and rehabilitation, no current Food and Drug Administration-approved drug therapy exists for traumatic SCI (Siddiqui et al., 2015). Several pharmacological therapies, including methylprednisolone, have been investigated in SCI, without therapeutic success. SCI is divided into two distinct phases (primary and secondary). The primary injury (immediately after SCI) is the physical damage caused by a traumatic event. It cannot be restored. Secondary spinal cord injury is a response to the initial physical insult and results from mechanistic crosstalk between several deleterious pathways: neuroinflammation, redox, and excitotoxicity (Siddiqui et al., 2015). Secondary injury is therefore amenable to reversal and treatment. Spinal cord contusion at thoracic or cervical levels shows similar molecular mechanisms and responds to similar treatment strategies. Functional deficits in rats after SCI are evaluated mainly by "Basso Beattie Bresnahan" locomotor rating scale. The Basso Beattie Bresnahan rating scale includes with a 21-point scale to measure hind limb function at various time points after injury. The scale assesses several different categories, including limb movement and tail position, as described in our publications (Chou et al., 2011; Khan et al., 2018).

A critical examination of SCI pathobiology, its signaling mechanisms, and results from our initial studies indicate a disturbed nitric oxide (NO) metabolome as shown in **Figure 1**. Under physiological condition, nitric oxide synthase (NOS)-derived NO reacts with Glutathione in presence of oxygen and forms S-nitrosoglutathione (GSNO). After SCI (pathological condition), calcium dysregulation-induced excitotoxicity mechanisms are responsible for producing deleterious neuronal nitric oxide synthase (nNOS)-dependent peroxynitrite and activating calpain (**Figures 1 and 2**). These mechanisms cause lesions, neurodegeneration, pain sensitivity and functional deficits following SCI (Chou et al., 2011; Khan et al., 2018). Our publications (Chou et al., 2011; Khan et al., 2016, 2018) in SCI/traumatic brain injury show that these deleterious mechanisms are effectively down regulated by GSNO (an endogenous component of the human brain/body), leading to neuroprotection, improved pain sensitivity and functional recovery after SCI (Chou et al., 2011; Khan et al., 2018) and traumatic brain injury (Khan et al., 2016). GSNO invokes its effect mainly *via* S-nitrosylation, a reversible secondary modification of cysteines, leading to altered activity of S-nitrosylated enzymes such as NOS, calpains, nuclear factor-kappa B, hypoxia-inducible factor 1 $\alpha$ , signal transducer and activator of transcription 3, and others. The degree of S-nitrosylation is reduced in many of neurodegenerative diseases due to increased peroxynitrite formation and thus decreased bio-



**Figure 1** Schematic showing metabolism of NOS-derived NO under physiological vs. pathological condition.

NOS-derived NO slowly reacts with GSH in presence of oxygen and forms GSNO under physiological condition. GSNO invokes its signaling *via* the mechanism of S-nitrosylation (adding NO moiety to sulfhydryl group) of proteins/enzymes in a regulated fashion. In contrast, in oxidative (pathological) environment, NO instantaneously reacts with superoxide forming peroxynitrite. Peroxynitrite, *via* nitration of tyrosine residue, forms 3-nitrotyrosine adduct of proteins/enzymes thus altering their functions. Peroxynitrite also oxidizes cysteine residue of proteins/enzymes rendering them inactive. NOS: Nitric oxide synthase; NO: nitric oxide; O<sub>2</sub><sup>-</sup>: superoxide; GSH: glutathione; GSNO: S-nitrosoglutathione; GSSG: oxidized glutathione; NH<sub>3</sub>: ammonia; GSNOR: S-nitrosoglutathione reductase; P-S-H: cysteine-protein; P-S-NO: S-nitrosoprotein; ONOO<sup>-</sup>: peroxynitrite; P-Tyr: tyrosine-protein; P-Tyr-NO<sub>2</sub>: nitrated-tyrosine protein; P-S(O)<sub>n</sub>-H: oxidized protein (where n represents number of oxygen atom ranging from 1 to 3).



**Figure 2** Schematic showing events involved in nNOS/peroxynitrite/calpain system vs. GSNO-mediated mechanisms under SCI.

NO released from nNOS mediates physiological signaling events by reacting with GSH to generate GSNO. nNOS activation is mediated by NMDAR-induced Ca<sup>2+</sup> influx and activation *via* phosphorylation of Serine<sup>1412</sup>, and denitrosylation of cysteine<sup>331</sup>. SCI induces sustained Ca<sup>2+</sup> influx, activation of NMDAR, and dysregulation of nNOS. Consequent increase in superoxide (O<sub>2</sub><sup>-</sup>) and depletion of GSH result in decreased GSNO biosynthesis and increased peroxynitrite formation, thus causing a sustained and prolonged activation of nNOS and calpains, leading to sustained nitro-oxidative stress, neurodegeneration, pain pathology and functional deficits. This deleterious nNOS/peroxynitrite/calpain system is down regulated by exogenously-treated or endogenously-produced (inhibiting GSNOR by N6022) GSNO *via* S-nitrosylation of specific cysteines of nNOS and calpains, leading to neuroprotection, improved pain sensitivity and optimal functional recovery. While arrows indicate increasing the activity, expression or levels, horizontal (cross) lines indicate inhibiting or decreasing the activity, expression or levels. NO: Nitric oxide; nNOS: neuronal nitric oxide synthase; GSH: glutathione; GSNO: S-nitrosoglutathione; NMDAR: N-methyl-D-aspartate receptor; Ca<sup>2+</sup>: calcium ion; SCI: spinal cord injury; O<sub>2</sub><sup>-</sup>: superoxide; GSNOR: S-nitrosoglutathione reductase; -SNO: S-nitroso; N6022: GSNO reductase inhibitor(3-(5-(4-(1H-imidazol-1-yl) phenyl)-1-(4-carbamoyl-2-methylphenyl)-1H-pyrrol-2-yl) propanoic acid)); NR1: Anti-NMDA Receptor 1; NR2: Anti-NMDA Receptor 2; PSD-95: postsynaptic density 95; 3-NT: 3-nitrotyrosine.

availability of NO for GSNO biosynthesis.

Increased levels of neuronal peroxynitrite are involved in SCI secondary injury, causing nitro-oxidative stress and neuronal cell death (Bao and Liu, 2003). Peroxynitrite is formed by a diffusion limited reaction between NO and superoxide when they are simultaneously and instantaneously produced in the same compartment by aberrant nNOS activity. Interestingly, peroxynitrite is also responsible for the aberrant activity of nNOS because peroxynitrite oxidizes cysteines of the zinc thiolate cluster of NOS. Peroxynitrite also oxidizes and depletes tetrahydrobiopterin, a cofactor required for NOS activity, which also contributes to the aberrant activity and uncoupling of nNOS. Furthermore, Peroxynitrite causes a sustained activation of calpains via 3-nitrotyrosine (3-NT) formation (Khan et al., 2016), leading to neurodegeneration and functional deficits (Khan et al., 2011, 2016). In SCI, the observed increased levels of 3-NT, a peroxynitrite adduct of tyrosine residue, suggest its pathological role in SCI (Khan et al., 2018). Inhibition of nNOS activity following SCI has been shown to provide some degree of neuroprotection, and nNOS KO mice show improved recovery after SCI (Farooque et al., 2001), indicating a deleterious role of nNOS activity in SCI and that nNOS-based therapy for SCI is a logical approach.

Furthermore, 3-NT positive neurons are more sensitive to increased calpain activity and neuronal cell death (Rameau et al., 2003). Increased calpain activity was indicated by specific SBDP145 kDa alpha II spectrin degradation product, without changes in expression levels of either calpains in SCI. These results indicate that increased peroxynitrite enhances calpain activity. As with GSNO, calpain inhibitors inhibit calpain activity. However, calpain inhibitors had no effect on the activation of nNOS and the production of peroxynitrite, supporting our hypothesis that GSNO invokes its effects via the inhibition of both nNOS and calpain, thus decreasing peroxynitrite, reducing neurodegeneration and showing greater and accelerated efficacy. Reversible down regulation of nNOS as well as calpain activity, such as via GSNO/S-nitrosylation, is preferred because it maintains the required physiological activity of calpains/nNOS. The pathological roles of other NOS enzymes (inducible and endothelial) in contusion SCI's neurodegeneration and pain are not well understood.

In addition to GSNO's anti-neurodegenerative, anti-inflammatory and anti-oxidant effects, it also protects against endothelial dysfunction and increases vessel density via angiogenesis (Khan et al., 2015). In clinical settings of SCI, GSNO is of great relevance due to its vasodilatory, cerebral blood flow-enhancing and blood-brain barrier-protecting activities (Khan et al., 2011). However, the mechanism of GSNO's effect on pain is not clearly understood because the mechanisms underlying neuropathic pain are multifactorial and complex. Among them, peroxynitrite originating from N-Methyl-D-aspartic acid receptor activation and NOS activity (Tanabe et al., 2009) are prominent causes of neuropathic pain following nerve injury. N-Methyl-D-aspartic acid receptor activation and calcium dysregulation are also associated with nNOS activation and peroxynitrite formation in SCI.

Recently, the mechanism of S-nitrosylation/GSNO has been reported to reduce pain sensitivity (Shunmugavel et al., 2012). Therefore, S-nitrosylation/GSNO-mediated inhibition of peroxynitrite production, leading to reduced neurodegeneration and demyelination, may be responsible for alleviating pain sensitivity. GSNO is a natural component of the human body, and its exogenous increase by administration or by inhibiting GSNO reductase is not known to be associated with any adverse effects. GSNO reductase is the major GSNO-degrading enzyme and thus GSNO reductase KO mice store GSNO in excess.

Conclusively, GSNO blocked the production of peroxynitrite by inhibiting aberrant nNOS activity, reduced calpain activity, improved locomotor rating scale (Basso Beattie Bresnahan), and mitigated mechanical allodynia (Figure 2). These findings support the efficacy of GSNO in ameliorating SCI and provide a rationale to evaluate GSNO-mediated signaling events in SCI. Furthermore, the therapeutic efficacy of GSNO may be amenable to manipulation by exogenous vs. endogenous (using N6022) and single dose vs. multiple doses. GSNO also shows similar efficacy in an animal model

of lumbar spinal stenosis/compression (Shunmugavel et al., 2012), supporting its therapeutic potential for SCI therapy. Due to its translational potential, clinical relevance, and non-toxic nature, testing the efficacy of low dose GSNO may lead to human SCI therapy.

*This work was supported by the grants from South Carolina Spinal Cord Injury Research Fund (No.CIRF2017 I-01) and VA award (No. RX2090).*

**Mushfiquddin Khan\*, Inderjit Singh**

Department of Pediatrics, Children's Research Institute, Medical University of South Carolina, Charleston, SC, USA

\*Correspondence to: Mushfiquddin Khan, PhD, [khanm@muscu.edu](mailto:khanm@muscu.edu). orcid: 0000-0001-7945-3237 (Mushfiquddin Khan)

Received: October 24, 2018

Accepted: December 16, 2018

doi: 10.4103/1673-5374.250572

**Copyright license agreement:** The Copyright License Agreement has been signed by both authors before publication.

**Plagiarism check:** Checked twice by iThenticate.

**Peer review:** Externally peer reviewed.

**Open access statement:** This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

**Open peer reviewer:** Adriana Vizuete, Universidade Federal do Rio Grande do Sul Instituto de Ciencias Basicas da Saude, Brazil.

**Additional file:** Open peer review report 1.

## References

- Bao F, Liu D (2003) Peroxynitrite generated in the rat spinal cord induces apoptotic cell death and activates caspase-3. *Neuroscience* 116:59-70.
- Chou PC, Shunmugavel A, El Sayed H, Desouki MM, Nguyen SA, Khan M, Singh I, Bilgen M (2011) Preclinical use of longitudinal MRI for screening the efficacy of S-nitrosoglutathione in treating spinal cord injury. *J Magn Reson Imaging* 33:1301-1311.
- Farooque M, Isaksson J, Olsson Y (2001) Improved recovery after spinal cord injury in neuronal nitric oxide synthase-deficient mice but not in TNF-alpha-deficient mice. *J Neurotrauma* 18:105-114.
- Khan M, Dhammu TS, Matsuda F, Annamalai B, Dhindsa TS, Singh I, Singh AK (2016) Targeting the nNOS/peroxynitrite/calpain system to confer neuroprotection and aid functional recovery in a mouse model of TBI. *Brain Res* 1630:159-170.
- Khan M, Dhammu TS, Matsuda F, Baarine M, Dhindsa TS, Singh I, Singh AK (2015) Promoting endothelial function by S-nitrosoglutathione through the HIF-1 $\alpha$ /VEGF pathway stimulates neurorepair and functional recovery following experimental stroke in rats. *Drug Des Devel Ther* 9:2233-2247.
- Khan M, Dhammu TS, Singh I, Singh AK (2018) Amelioration of spinal cord injury in rats by blocking peroxynitrite/calpain activity. *BMC Neurosci* 19:50.
- Khan M, Sakakima H, Dhammu TS, Shunmugavel A, Im YB, Gilg AG, Singh AK, Singh I (2011) S-nitrosoglutathione reduces oxidative injury and promotes mechanisms of neurorepair following traumatic brain injury in rats. *J Neuroinflammation* 8:78.
- Rameau GA, Chiu LY, Ziff EB (2003) NMDA receptor regulation of nNOS phosphorylation and induction of neuron death. *Neurobiol Aging* 24:1123-1133.
- Selvarajah S, Hammond ER, Haider AH, Abularrage CJ, Becker D, Dhiman N, Hyder O, Gupta D, Black JH 3rd, Schneider EB (2014) The burden of acute traumatic spinal cord injury among adults in the united states: an update. *J Neurotrauma* 31:228-238.
- Shunmugavel A, Khan M, Martin MM, Copay AG, Subach BR, Schuler TC, Singh I (2012) S-Nitrosoglutathione administration ameliorates cauda equina compression injury in rats. *Neurosci Med* 3:294-305.
- Siddiqui AM, Khazaei M, Fehlings MG (2015) Translating mechanisms of neuroprotection, regeneration, and repair to treatment of spinal cord injury. *Prog Brain Res* 218:15-54.
- Tanabe M, Nagatani Y, Saitoh K, Takasu K, Ono H (2009) Pharmacological assessments of nitric oxide synthase isoforms and downstream diversity of NO signaling in the maintenance of thermal and mechanical hypersensitivity after peripheral nerve injury in mice. *Neuropharmacology* 56:702-708.

C-Editors: Zhao M, Liu WJ; T-Editor: Liu XL