S-Nitrosoglutathione Mimics the Beneficial Activity of Endothelial Nitric Oxide Synthase-Derived Nitric Oxide in a Mouse Model of Stroke

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Background: The nitric oxide (NO)-producing activity of endothelial nitric oxide synthase (eNOS) plays a significant role in maintaining endothelial function and protecting against the stroke injury. However, the activity of the eNOS enzyme and the metabolism of major NO metabolite S-nitrosoglutathione (GSNO) are dysregulated after stroke, causing endothelial dysfunction. We investigated whether an administration of exogenous of GSNO or enhancing the level of endogenous GSNO protects against neurovascular injury in wild-type (WT) and eNOS-null (endothelial dysfunction) mouse models of cerebral ischemia-reperfusion (IR). *Methods:* Transient cerebral ischemic injury was induced by middle cerebral artery occlusion (MCAO) for 60 minutes in male adult WT and eNOS null mice. GSNO (0.1 mg/kg body weight, intravenously) or N6022 (GSNO reductase inhibitor, 5.0 mg/kg body weight, intravenously) was administered 30 minutes before MCAO in preinjury and at the reperfusion in postinjury studies. Brain infarctions, edema, and neurobehavioral functions were evaluated at 24 hours after the reperfusion. *Results:* eNOS-null mice had a higher degree ($P < .05$) of injury than WT. Pre- or postinjury treatment with either GSNO or N6022 significantly reduced infarct volume, improved neurological and sensorimotor function in both WT and eNOS-null mice. *Conclusion:* Reduced brain infarctions and edema, and improved neurobehavioral functions by pre- or postinjury GSNO treatment of eNOS knock out mice indicate that GSNO can attenuate IR injury, likely by mimicking the eNOS-derived NO-dependent anti-ischemic and anti-inflammatory functions. Neurovascular protection by GSNO/N6022 in both pre- and postischemic injury groups support GSNO as a promising drug candidate for the prevention and treatment of stroke injury.

Key Words: S-nitrosoglutathione—eNOS—NO—GSNO—N6022—stroke—neuroprotection—cerebral ischemia

Introduction

Stroke is a complex syndrome and the leading cause of disability worldwide. The stroke injury is also associated with significant morbidity and mortality and a great financial burden to the country and family.[1] Unfortunately, the mechanisms of both the cause and the consequence of stroke are not well understood. Therefore,

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neither stroke preventive strategy nor proven effective stroke therapy for neuroprotection and functional recovery is currently available.[2] The FDA-approved endovascular thrombectomy or thrombolysis is effective only in the early acute phase of stroke thus the neuroprotective strategies are needed for a better outcome and functional recovery.[3,4]

Stroke is defined as rapidly developing clinical signs of disturbance of cerebral functions with no apparent cause other than that of vascular origin. Endothelial dysfunction is among the major risk factors of stroke, and stroke injury itself causes endothelial dysfunction. Although prevention from stroke involves homeostasis of nitric oxide (NO) metabolome, this mechanism is less understood. NO, especially produced by endothelial nitric oxide synthase (eNOS), is the major player to maintain endothelial function. Aberrant eNOS activity and the consequent disturbed NO metabolome, as observed in eNOS knock out mice following ischemia-reperfusion (IR), are mainly responsible for prolonged and sustained endothelial dysfunction.[5,6] Accordingly, eNOS-knock out (KO) mice compared with wild-type (WT) mice show a greater degree of injury due to the absence of beneficial effects from endothelial NO during ischemia.[5] Endothelial pathology associated with reduced NO bioavailability following stroke hinders the mechanisms of neuroprotection and the process of neurorepair. eNOS/NO activity is associated with the regulation of hypertension, endothelial function, and blood-brain barrier (BBB) integrity. The absence of eNOS in experimental stroke mice has been implicated in cognitive dysfunction.[7] Low levels of NO in plasma are also associated with severity and poor outcomes in stroke patients.[8,9] However, conventional NO donors including glycerol trinitrate do not alter clinical outcomes.[10] Preclinical studies have shown that the major NO metabolite S-nitrosoglutathione (GSNO) plays a significant protective role against endothelial dysfunction-associated pathology.[11-13]

The efficacy of GSNO therapy in a number of animal models of CNS injury including stroke, traumatic brain injury, and contusion spinal cord injury has been documented from our[13-17] and other laboratories.[18-21] The efficacy of GSNO has also been reported in other neuro- and cardiac diseases.[11,22-26] The objective of this study was to investigate whether pre- or postinjury GSNO treatment of cerebral IR mitigates injury and confers neuroprotection especially in the stroke-prone population who suffer from abnormal NO metabolome-mediated endothelial dysfunction. NO is essential for the activation of guanylyl cyclase[27] leading to cyclic guanosine monophosphate production, and protein S-nitrosylation (a covalent attachment of NO to cysteine of target proteins) for the regulation of signaling events.[28,29] In a stroke, endothelial NO synthase (eNOS)-derived NO and the mechanism of GSNO-dependent S-nitroslyation are reported to play a neuroprotective and function restorative role.[5,11,13,30]

GSNO is an endogenous neuroprotective agent and its exogenous administration or increasing its endogenous levels by pharmacologically inhibiting GSNO metabolizing enzyme GSNO reductase (GSNOR) may potentially protect against IR injuries following experimental stroke. Supporting this notion, pharmacological inhibition of GSNOR has also been shown to improve endothelial functions,[12] indicating a protective role of GSNO in neurovascular dysfunction-related diseases. To increase the levels of endogenous GSNO, we used a specific GSNOR inhibitor N6022.[31] It is a nontoxic drug[32] and has been used in animal and human studies.[33,34]

This study is designed to evaluate 2 aspects of GSNO/S-nitrosylation therapy. (1) Whether administration of GSNO to eNOS KO as well as WT mice protects against ischemic/hypoxic injury. If so, low dose GSNO may be used as a stroke preventive strategy in the stroke-prone population. After a stroke, the same may be used for neuroprotective intervention. (2) Whether exogenous GSNO has similar effects as endogenous GSNO (by inhibiting GSNOR enzyme using GSNOR specific inhibitor N6022). The data indicate that both exogenous and endogenous GSNO have similar efficacy when administered pre- or post-IR injury in reducing brain infarctions, decreasing edema and improving neurological functions in both WT and eNOS knock out mice. As recommended by the stroke therapy academic industry roundtable,[35] we evaluated the efficacy of GSNO/N6022 only in healthy young adult animals in this first study. In later studies, the efficacy will be determined in female and aged animals.

Materials and Methods

Reagents

GSNO was purchased from World Precision Instruments (Sarasota, FL) and N6022 (3-(5-(4-(1H-imidazol-1-yl)phenyl)-1-(4-carbamoyl-2-methylphenyl)-1H-pyrrol-2-yl)propanoic acid) was from Axon Medchem (Reston, VA). Evans blue and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Animals

Adult WT (*C57BL/6J, item# 000664)* and *C57BL/6J (B6.129P2, Nos3<tm1Unc>/J, item # 002684)* eNOS knock out (eNOS KO) mice were used in this study. Both WT and eNOS KO mice were purchased from Jackson Laboratory, Bar Harbor, ME. All animal procedures were approved by the Medical University of South Carolina Animal Review Committee and received humane care in compliance with the Medical University of South Carolina's experimental guidelines and the National Research Council's criteria for humane care (*Guide for the Care and Use of Laboratory Animals*).

Focal Cerebral Ischemia Model

Mice were anesthetized with an intraperitoneal injection of xylazine and ketamine hydrochloride. A rectal temperature probe was introduced, and the body temperature was maintained at 37 ± 0.5°C. IR injury was induced as described previously from our laboratory for rats[16] with modification of the method described by Belayev et al.[36] Briefly, focal cerebral ischemia was induced by introducing a silicone-coated (coating length 5 mm and 0.23-0.25 mm coating diameter) specialized occluding suture for MCA occlusion (from Doccol Corporation, Redlands, CA; Cat#602156 or 602356, as per the weight of the animal) into the internal carotid artery via an external carotid artery stump until the suture is wedged and the tip occluded the proximal stem of MCA, approximately internalizing 20 mm of total length. Sixty minutes postocclusion, the filament was withdrawn to allow reperfusion. During MCA occlusion (MCAO), the animals were left untouched under constant conditions. To make sure of the ischemia (obstruction to blood flow), regional blood flow (rCBF) was monitored during the occlusion and early reperfusion.[16] The surgical procedure was completed in 20 minutes and it did not involve significant blood loss.

Drug (GSNO or N6022) Treatment

In the preinjury treatment groups, GSNO (0.1 mg/kg body weight in 100 μl saline, iv) or N6022 (5.0 mg/kg body weight in 50% 100 μl DMSO/saline, iv) was slowly infused by tail vein. Similarly, in the postinjury groups, the same dose of GSNO or N6022 was administered at the reperfusion after 60 minutes MCAO by tail vein. Sham and IR animals were administered saline in GSNO group experiments whereas 50% DMSO in saline was given to IR animals in N6022 group experiments. The effective dose of GSNO and N6022 was determined by the dose curve as reported previously from our laboratory[34,16] and others.[37,38]

Evaluation of Ischemic Infarct by TTC Staining and Image Acquisition

Infarct volume was evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Briefly, after an overdose of ketamine and xylazine, the animals were sacrificed by decapitation after 24 hours of reperfusion. The brain was quickly removed and placed in ice-cold saline for 5 minutes and then cut into four 2-mm coronal brain slices by Brain Matrix (Brain Tree Scientific). Sections were then be incubated in 2% 2,3,5-triphenyltetrazolium chloride; TTC (Sigma, MO, USA); in saline for 15 minutes at 37°C as described earlier.[39] Coronal sections (caudal side) were placed on a flat-bed color-scanner (HP scan jet 5400 C) connected to a computer. The image was acquired in Photoshop 7.0 (Adobe Systems) and quantified using image-analysis software Scion Image (Scion Corporation, Frederick, Maryland).[40] The volume of infarctions in each animal is obtained from the product of average slice thickness (2 mm) and the sum of infarction area in all brain slices or as mentioned. The area of infarction is expressed as a percent area of the whole coronal section. The volume and area of infarction were calculated for the total as well as for cortex and striatum separately.

Physiologic Parameters

The physiologic parameters were measured in WT normal animals at 30 minutes after the treatment with GSNO (0.1 mg/kg body weight, iv) or N6022 (5.0 mg/kg body weight, iv) as described in Table 1. Mean blood pressure and heart rate (HR) and blood pH were measured without anesthesia. During the procedure, the rectal temperature was monitored and maintained at about 37°C-37.8°C. Body temperature was monitored by a probe maintained at about 37 ± 0.5°C by a homeothermic blanket control unit (Harvard Apparatus, Holliston, MA). Mean blood pressure and HR were measured using an NIBP system (Kent Scientific, Torrington, CT). It is a noninvasive computerized tail-cuff system and uses an automated inflation/deflation pump. Regional CBF was monitored using laser Doppler flowmeter (Perimed Inc., Sweden and Oxford Optronix Ltd., Oxford, UK).

Evaluation of BBB Disruption by Evans Blue Dye Extravasation and Measurement of Edema

BBB leakage was assessed by the method of Weismann and Stewart[41] with slight modification. The mice received 100 μl of a 5% solution of EB in saline administered intravenously at 4 hours after N6022 treatment. At the completion of 24 hours reperfusion time, cardiac perfusion was performed under deep anesthesia with 100 ml of saline to clear the cerebral circulation of Evans blue dye. The brain was removed, sliced, and photographed. The 2 hemispheres were isolated and mechanically homogenized in 750 μl of N, N-dimethylformamide. The suspension obtained was kept at room temperature in the dark for 72 hours. It was centrifuged at 10,000 X g for 25 minutes, and the supernatant was spectrofluorimetrically analyzed (λ_{ex} 620 nm, λ_{em} 680 nm).

Table 1. *Physiological parameters*

Parameters	Saline-treated	GSNO-treated	N6022-treated
Rectal temp (°C)	37.2 ± 0.2	36.8 ± 0.1	37.1 ± 0.3
MABP (mm Hg)	165.11 ± 9.8	158.6 ± 14.5	161.6 ± 15.3
HR (beat/min)	742.0 ± 178.2	736.4 ± 231.9	745.0 ± 189.9

Abbreviations: GSNO, S-nitrosoglutathione; HR, heart rate; MABP, mean arterial blood pressure; N6022, GSNO reductase inhibitor.

Measurements were performed at 30 minutes after the treatment with saline, GSNO (0.1 mg/kg body weight, iv) or N6022 (5.0 mg/kg body weight, iv). Data are presented as means ± SD for n = 8. No significant differences were observed among the groups.

Measurement of Edema (Brain Water Content)

At 24 hours following MCAO, animals were euthanized to determine brain water content (edema) as previously described from our laboratory.[42] The cortices, excluding the cerebellum, were quickly removed and the contralateral and the ipsilateral hemispheres separately weighed. Each hemisphere was dried at 60°C for 72 hours and the dry weight was determined. Water content was calculated in the ipsilateral hemisphere as water content (%) = (wet weight−dry weight)/wet weight x 100.

Neurological Score Evaluation

A 4-point score test (Bederson scale) was used to assess the global neurologic examination.[43] An observer blinded to the identity of the groups assessed neurological deficit at 4 hours and 24 hours of reperfusion and score was assigned as described by,[44] 0; no observable neurological deficit (normal); 1, failure to extend left forepaw on lifting the whole body by tail (mild); 2, circling to the contralateral side (moderate); 3, no spontaneous motor activity with depressed levels of consciousness (severe).

Measurement of rCBF

rCBF was measured as described previously. A flexiprobe (Periflux System 5000, Perimed, Inc., Sweden) was placed over the skull to monitor the regional blood flow (rCBF) by laser Doppler flowmetry. After obtaining a baseline of preischemic rCBF, the MCA was occluded and the rCBF monitored during the time of occlusion with a criterion of less than 25% of baseline blood flow remaining after MCAO. The rCBF was monitored continuously up to 60 minutes after MCAO.

Sensorimotor Deficit Behavioral Test Using the Adhesive Tape-Removal Test

The sensorimotor deficit was assessed by investigators blinded to the study conditions by the adhesive tape-removal test before IR and days after. Small adhesive tape strips (0.3 × 0.4 cm) were used as bilateral tactile stimuli attached to the distal-radial region on each forelimb. The latency was recorded (the time to remove each stimulus from the forelimbs) during 3 trials per day for each forepaw as described.[45] Before the injury, mice were trained for 3 days. The test has been described in mouse models.[46,47]

Statistical Evaluation

Statistical analysis was performed as described[13] using software Graph Pad Prism 5.01. Unless otherwise stated, all values are expressed as mean ± SD of n determinations. The results were examined by unpaired Student t-test. Multiple comparisons were performed by 1-way ANOVA, followed by the post hoc Tukey test. A *P*value less than .05 was considered significant.

Results

A total of 120 male adult (~6 months old) mice were used in the present study. The number of mice used in each group is described in figure and table legends. Mice showing no neurological deficits at 4 hours after MCAO were excluded from the study.

Treatment With GSNO or N6022 did not Change Physiologic Parameters

The selected dose (100 μg/kg body weight of GSNO or 5 mg/kg body weight of N6022) administered via iv route did not alter physiologic parameters including mean arterial blood pressure, HR and rectal temperature in control normal WT mice. The parameters were measured at 30 minutes following the treatment with GSNO or N6022 (Table 1).

eNOS KO IR Mice Show More Infarctions and Greater Edema Than IR WT Mice, Which Were Attenuated by Pre- or PostIschemia Treatment With GSNO

To examine the injury-reducing and the neurovascular-protective effects of preischemia GSNO treatment, both WT and eNOS KO mice were treated with GSNO 30 minutes before MCAO (Fig 1). Pharmacokinetic studies indicate that half-life of GSNO significantly varies in human and rodent blood/tissue.[48-50] However, it is always less than an hour. Therefore, we opted 30 minutes before ischemia for the GSNO treatment. The treatment with GSNO reduced brain infarctions (Fig 1A, measured by TTC staining) quantified as infarct volume (Fig 1B) and decreased edema (Fig 1C, measured as brain water content). Edema was observed mainly in ipsilateral (ipsi) compared with the contralateral (contra) side of the brain (Fig 1C). Both the infarct volume (Fig 1B) and the edema levels (Fig 1C) were significantly greater in eNOS KO IR group ($P < .05$) than WT IR group.

The neurological functional deficit was evaluated using 2 different tests. A 4-point score test (Bederson scale[43]) was used to assess global neurological deficits (Fig 2A). The neurological score was severely compromised in eNOS (KO) IR mice (score 3) compared with WT IR (score 2). Treatment with GSNO improved neurological score in both groups; however, the eNOS (KO) IR + GSNO had score 2 compared with score 1 in the WT IR + GSNO. Sensory dysfunction was assessed using the tactile adhesive removal test,[45,47,51] which was significantly improved in both WT IR + GSNO and eNOS (KO) IR + GSNO groups compared to the corresponding IR groups as shown in Figure 2B. However, the deficit was still greater in eNOS (KO) IR + GSNO than WT IR + GSNO group.

To determine the efficacy of GSNO after ischemic injury, both WT and eNOS KO mice were treated with the same dose of GSNO as used in preischemia injury treatment at the time of reperfusion (Table 2). We opted for the treatment

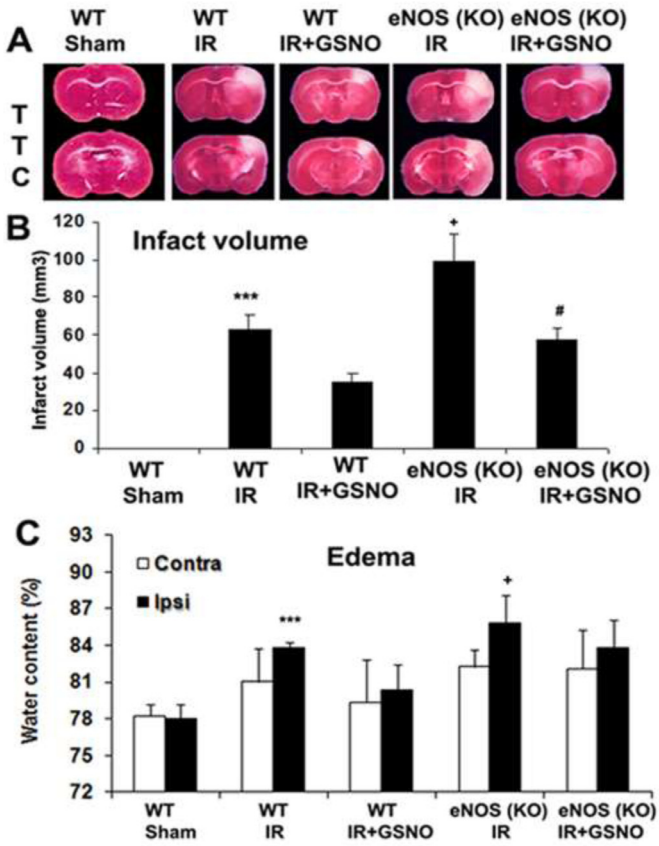

Figure 1. *Representative TTC stained brain sections (# 2 and 3 out of four consecutive sections from cranial to caudate region) (A), infarct volume (B) and water content (edema) (C) at 24 hours of reperfusion following 60 minutes MCAO. GSNO (0.1 mg/kg body weight, iv) was administered 30 mininutes before MCAO. Data are presented as mean± SD (n = 8). ***P < .001 vs. Sham and WT IR+GSNO, $^{+}$P < .05 versus WT IR, #P < .05 versus eNOS (KO) IR.*

of GSNO at reperfusion to correlate with the treatment after successful thrombolysis or endovascular thrombectomy. Like preischemic GSNO treatment, GSNO significantly reduced infarct volume and improved neurological score in both WT-IR and eNOS (KO)-IR, indicating that GSNO is effective in both pre- and post-IR settings (Table 2).

Effect of Endogenous GSNO (by Inhibition of GSNOR Using GSNOR Specific Inhibitor N6022) on Brain Infarctions, Neurological Score, BBB Leakage, and Edema

Like pre- or postischemia GSNO treatment of IR animals (Figs 1, 2; Table 2), N6022 was administered to IR animals. This compound was given in 50% DMSO in saline. IR animals were also treated with 50% DMSO in saline. Like GSNO, N6022 also significantly reduced brain infarctions (Fig 3A, TTC) measured as infarct volume (Fig 3B) in both pre- and postinjury treatment groups. The treatment with N6022 also decreased BBB leakage measured as Evans blue extravasation (Fig 3D), quantified as fluorescence (Fig 3E) in the brain from both groups. Edema was also significantly lower in the N6022 treated groups (Fig 3F). These data support that, like GSNO, N6022 also shows stroke injury-reducing as well as neurovascular protective efficacy.

Discussion

For the first time, this study provides evidence that the principal metabolite of NO metabolome GSNO (a reaction product of NO, glutathione (GSH), and oxygen)[52,53] targets the beneficial functions of eNOS derived-NO following IR injury. GSNO is always in equilibrium with S-nitrosylated proteins. In stroke pathology, the levels of GSNO/S-nitrosylated proteins are believed to decrease due to four major events. (i) Excessive superoxide formed during reperfusion instantaneously reacts with NOS-derived NO to form peroxynitrite that reduces NO bioavailability for GSNO biosynthesis and NO-based signalings. (ii) Biosynthesis of GSNO is decreased as a result of reduced levels of GSH (redox imbalance) in stroke conditions.[54] Furthermore, NO reacts slowly with GSH as compared with superoxide. (iii) Oxygen is required for GSNO synthesis and decreased oxygen supply in ischemic/hypoxic tissue reduces GSNO biosynthesis. (iv) In an inflammatory environment, the expression of GSNO degrading enzyme GSNO reductase (GSNOR) is increased,[11,55] resulting in reduced levels of GSNO. Endothelial dysfunction due to reduced eNOS-derived NO bioavailability is associated with major stroke risk factors. The significance of vascular NO is highlighted by its association with vascular dementia and poor outcomes after stroke.[7,56]

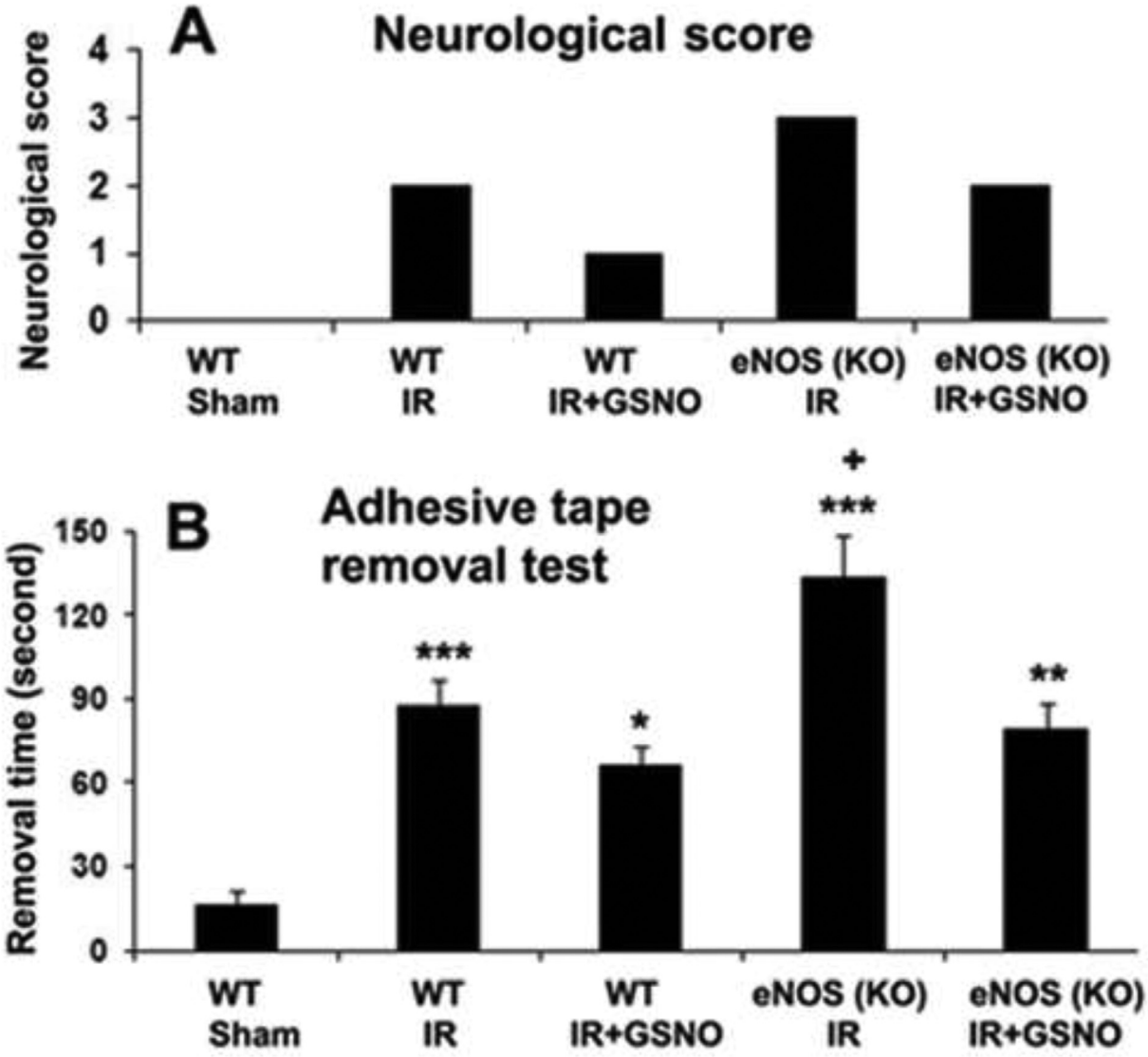

Figure 2. *GSNO treatment of wild-type (WT) and eNOS KO IR mice, as described in Fig 1, reduced neurological deficit score (A). The treatment with GSNO also reduced time to remove stimuli (adhesive tape) from limbs (adhesive tape test for tactile strength) (B). Data are presented as mean ± SD (n = 8). ***P < .001 versus sham, **P < .01 versus eNOS (KO) IR, *P < .05 versus WT IR.*

In this context, we have previously documented that reduced NO bioavailability-mediated stroke injury is ameliorated by GSNO.[15,16]

Based on GSNO's potential in preclinical studies, the efficacy of GSNO was investigated in a number of human diseases using different doses and routes. Recently, a number of studies on the use of GSNO in patients, mainly associated with coronary and carotid diseases as well as in normal humans have been summarized.[57] GSNO's antiplatelet[58,59] and embolization-reducing activities[19,60] are well established in human clinical studies. None of these studies report any adverse effects associated with the use of GSNO. In our studies, GSNO and N6022 treatment had no effect on physiological parameters (Table 1). GSNO was also well tolerated in patients with cystic fibrosis without showing any adverse effect.[61] Furthermore, the effect of GSNO has also been reported as clinically relevant to the therapeutic potential for women with pre-eclampsia.[62] The safety and pharmacology of N6022 have also been evaluated in animal and human studies.[31,32,38] However, human stroke studies using GSNO/N6022 are lacking due to perhaps a lesser understanding of the complex nature of the NO metabolome in the brain. Favorable outcomes from this and other animal stroke studies may be helpful to design GSNO-based human stroke protective/preventive studies. eNOS KO mice were used in this study because NO levels in eNOS KO mice are decreased by almost 50%[63] and studies in eNOS KO mice show exacerbations of IR injury.[5] Supporting previous reports, our data show a significant increase in brain infarctions and edema in eNOS KO IR compared with WT IR mice brain (Fig 1). Preinjury GSNO treatment of IR animals not only mitigated brain infarctions and reduced edema but also improved neurological (Fig 2A)

Table 2. *Effect of postinjury GSNO treatment on infarct volume and neurological score*

Group	Infarct volume (% change)	Neurological score
WT-IR	100 + 13.5	2
WT-IR+GSNO	58.3 ± 6.5*	1
eNOS (KO)-IR	133.0 ± 11.2†	3
eNOS (KO)-IR+GSNO	91.6 ± 9.1‡	2

Abbreviations: GSNO, S-nitrosoglutathione; WT-IR, wild-type ischemia-reperfusion.

Development of infarct volume at 24 hours of reperfusion after 60 minutes MCA occlusion in the mouse brain. Neurological score was also evaluated at 24 hours after MCA occlusion. The animals were administered with GSNO (0.10 mg/kg body weight, iv) at the reperfusion. Data are presented as mean ± SD (n = 8).

*$P < .001$ versus WT-IR.

†$P < .01$ versus WT-IR.

‡$P < .001$ versus eNOS (KO)-IR.

and sensorimotor functions (Fig 2B), indicating a neurovascular protective efficacy of GSNO in animals which are lacking vascular NO (Figs. 1, 2). Increasing GSNO levels before the injury in WT mice also protects against IR injury indicating that low dose GSNO may be used as a stroke preventive strategy. Like our previous postinjury treatment of IR rats with GSNO,[13,30] its treatment of either WT or eNOS KO IR mice reduced infarct volume and improved neurological function (Table 2), supporting the notion that GSNO is effective irrespective of species and stroke pathology.

From the pharmacology point of view, both GSNO and N6022 are attractive candidates because both are stable, solid, nontoxic, and easy to administer compared with other NO-modulating drugs such inhaled NO (iNO)[64,65] and conventional NO donors.[16] Another NO-releasing agent nitrite has also been reported to protect neurons against hypoxic damage.[66] NO-releasing agents invoke their effects partly via free NO and partly via S-nitrosylation. However, the mechanisms of GSNO/N6022 versus iNO or free NO donors are

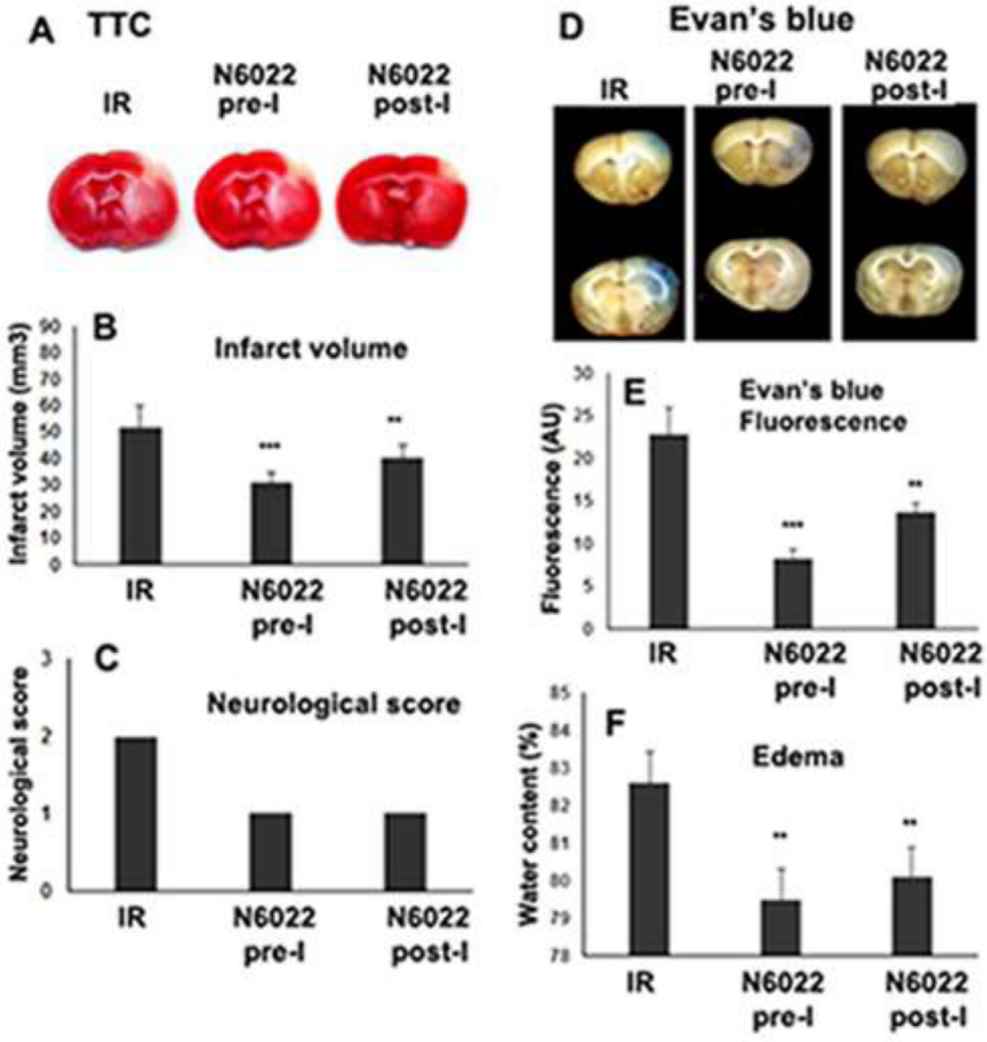

Figure 3. *N6022 (5.0 mg/kg body weight, iv) treatment (pre-injury; pre-I or post-injury; post-I) of IR mice reduced brain infarctions (A; TTC, B; infarct volume), improved neurological score (C), reduced BBB leakage (D; Evans blue extravasation, E; Evans blue fluorescence) and decreased edema (F). All measurements were performed at 24 hours after reperfusion. Data are presented as mean ± SD (n = 8). ***P< .001 versus IR, **P< .01 versus IR.*

different and distinct. In clinical settings, GSNO is a potent antiplatelet and vasodilator. Unlike other antiplatelet agents such as aspirin,[67] GSNO is not associated with undesirable side effects. Its activity is also not associated with tolerance as is the case with conventional NO donors including trinitroglycerine.[68] However, certain precautions are associated with the use of GSNO. Based on the structural configuration, it must be protected from light and high temperatures.[52] A slow infusion of GSNO is suggested to avoid its hypotensive effect.[23] In contrast to exogenous GSNO, increasing the levels of endogenous GSNO by the inhibition of GSNOR using its selective inhibitor N6022 is not associated with undesirable effects. In this study, N6022 treatment of either pre- or post-IR injury not only reduced brain infarct volume and improved neurological score but also protected against BBB leakage and edema (Fig 3). Taken together, the study shows that both exogenous and endogenous GSNO is effective to mitigate/prevent stroke injury.

We recognize several limitations of our study. First, this study is limited to single-sex (male) and thus may not represent a complete understanding of the role of GSNO/N6022. Second, the study is also restricted to the acute phase of stroke injury only. Third, it includes only a transient focal cerebral ischemia mouse model. Last, the role of neuronal NOS or inducible NOS-derived NO has not been taken into account.

Summary and Conclusion

GSNO (exogenous or endogenous) blocks endothelial dysfunction and improves functional outcomes after acute IR stroke injury. Neurovascular injury and functional improvements in pre- or post-IR injury GSNO treatment of eNOS$^{-/-}$ mice indicate stroke preventive and neuroprotective effects of GSNO, supporting the notion that low dose GSNO or N6022 may be used to prevent the stroke incident especially in a population with reduced vascular NO/GSNO. In clinical settings, GSNO is an attractive candidate to be evaluated in human stroke because GSNO/N6002 is readily available, nontoxic, inexpensive, and easy to administer. Furthermore, both GSNO and N6002 can be safely administered in the prehospital setting prior to brain scanning.

Clinical Implications

- GSNO-dependent mechanisms may reduce the severity of stroke injury and may provide neuroprotection/functional recovery after stroke injury.
- Strategies to inhibit GSNOR for increasing the levels of endogenous GSNO may help to confer neuroprotection and thus accelerated functional recovery from cerebral IR injury.
- GSNO and N6022 are inexpensive and nontoxic drugs, and thus their effective low doses may be used in stroke-prone population as a stroke-preventing and stroke injury-mitigating strategies.

Conflicts of interest

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

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Authors' Contributions

This study is based on an original idea of M.K., A.K.S., and I.S. MK wrote the manuscript and all authors reviewed the manuscript. T.SD., P.K., and F.Q. carried out animal and biochemical studies. M.K., A.K.S., I.S., T.S.D., and P.K. critically examined the animal and biochemical studies. All authors have approved the manuscript.

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