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Protective Role of S-Nitrosoglutathione (GSNO) Against Cognitive Impairment in Rat Model of Chronic Cerebral Hypoperfusion

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Abstract

Chronic cerebral hypoperfusion (CCH), featuring in most of the Alzheimer's disease spectrum, plays a detrimental role in brain amyloid- β (A β) homeostasis, cerebrovascular morbidity, and cognitive decline; therefore, early management of cerebrovascular pathology is considered to be important for intervention in the impending cognitive decline. S-nitrosoglutathione (GSNO) is an endogenous nitric oxide carrier modulating endothelial function, inflammation, and neurotransmission. Therefore, the effect of GSNO treatment on CCH-associated neurocognitive pathologies was determined *in vivo* by using rats with permanent bilateral common carotid artery occlusion (BCCAO), a rat model of chronic cerebral hypoperfusion. We observed that rats subjected to permanent BCCAO showed a significant decrease in learning/memory performance and increases in brain levels of A β and vascular inflammatory markers. GSNO treatment (50 μ g/kg/day for 2 months) significantly improved learning and memory performance of BCCAO rats and reduced the A β levels and ICAM-1/VCAM-1 expression in the brain. Further, *in vitro* cell culture studies, GSNO treatment also decreased the cytokine-induced proinflammatory responses, such as activations of NF κ B and STAT3 and expression of ICAM-1 and VCAM-1 in endothelial cells. In addition, GSNO treatment increased the endothelial and microglial A β uptake. Additionally, GSNO treatment inhibited the β -secretase activity in primary rat neuron cell culture, thus reducing secretion of A β , suggesting GSNO mediated mechanisms in anti-inflammatory and anti-amyloidogenic activities. Taken together, these data document that systemic GSNO treatment is beneficial for improvement of cognitive decline under the conditions of chronic cerebral hypoperfusion and suggests a potential therapeutic use of GSNO for cerebral hypoperfusion associated mild cognitive impairment in Alzheimer's disease.

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Keywords

Alzheimer's disease; amyloid- β ; bilateral common carotid artery occlusion; cerebral hypoperfusion; inflammation; S-nitrosylation; S-nitrosoglutathione

Introduction

Alzheimer's disease (AD) is the leading cause of irreversible, progressive dementia. It is characterized by a gradual loss of memory and cognitive skills. Based on age of onset, the disease is divided into two subtypes, early-onset AD and late-onset AD. Early-onset AD accounts for approximately 1% to 6% of all cases and age of onset ranges roughly from 30 years to 60 or 65 years. However, late-onset AD, which is the more common form of AD, is defined as AD with an age at onset later than 60 or 65 years. Late-onset AD is a complex and multifactorial disease with the possible involvement of several genetic and environmental risk factors. Recent findings concerning the overlap between risk factors for cardiovascular disease and late-onset AD, such as high blood pressure, diabetes, and high cholesterol, have indicated that there may be a strong connection between vascular functional status and development of late-onset AD. In addition, the observed regional cerebral hypoperfusion and microvascular abnormalities and hypometabolism in subjects with AD prior to cognitive decline suggest that early management of cerebrovascular changes and cerebral blood flow is considered one of the critical factors to delay, slow down, or prevent the impending development of late-onset AD (see reviews [1–3]).

Endothelium-derived nitric oxide (NO) is an important determinant of endothelium-dependent vasodilation and vascular homeostasis [4], and therefore its deficiency directly contributes to development of endothelial dysfunction and various vascular disorders [4, 5]. Recent studies have reported the possible implication of endothelium-derived NO deficiency in development and progression of late-onset AD [6–10]. In addition, studies also demonstrated that amyloid- β (A β) peptide, a component of senile plaques found in AD brain, could inhibit NO-mediated cGMP generation that regulates blood flow to the brain [11]. These studies thereby support the hypothesis that normalization of vascular NO bioavailability and/or NO-mediated signal transduction system may be effective in attenuation of cerebrovascular complications and thus the impending development of late-onset AD.

To compensate for or make up the low NO bioavailability under various physiopathological conditions, presently several NO donors have been used clinically. These include NO gas for inhalation, sodium nitroprusside, glyceryl trinitrate, isosorbide mononitrate, isosorbide dinitrate, sydnonimine derivative molsidomine, and pentaerythrityl nitrate (see review [12]). Studies have demonstrated that inflammation and oxidative stress in endothelium decreases synthesis of endothelium-derived NO by inhibiting expression and activity of endothelial NO synthase (eNOS) [13, 14]. However, the therapeutic efficacies of these NO donors on the pathologies associated with inflammation and oxidative stress are not promising because the increased oxidative stress can directly lower the NO bioavailability by reaction between NO and superoxide (O_2^-) to form peroxynitrite [15], one of the strongest oxidant in the

biological system [16]. Indeed, we previously reported that free NO donors, such as SNP, methyamine hexamethylene methyamine NONOate, propylamine propylamine NONOate, and 3-morpholinosydnonimine, had no significant beneficial effect on cerebrovascular pathology involved in experimental stroke [17, 18]. These data, therefore, suggest that the compounds that release free NO may not be suitable for treatment of any neurological disease involving inflammation and oxidative stress. Rather than the free NO donors, we found that *S*-nitrosothiol (*S*-NO) donors including *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl penicillamine (SNAP) provide efficient protection [17–19]. It is interesting to note that GSNO is an endogenous NO carrier which is able to induce both the classical NO-mediated signaling pathway (cGMP pathway) [20] and redox signaling pathway involving protein *S*-nitrosylation [21]. Also, GSNO is a potential anti-inflammatory and antioxidant agent able to inhibit inflammatory gene expression and neutralize oxidative stress induced by multiple insults [22, 23]. Most cases of late-onset AD involve chronic inflammation and oxidative stress in the brain [24–26]. Therefore, these studies suggest the therapeutic potential of GSNO in management of cerebrovascular complications associated with late-onset AD.

Extracellular deposition of A β peptides, called senile plaques or amyloid plaques, and intraneuronal formation of paired helical filaments by aggregation of hyperphosphorylated tau protein, called neurofibrillary tangles, are the most important neuropathological hallmarks of AD. A β peptides are proteolytic cleavage products of the amyloid- β protein precursor (A β PP) sequentially processed by β - and γ -secretases [27]. Recently, Kwak and colleagues have reported that the gene expression and activity of BACE1, one of β -secretases, is regulated by NO-dependent mechanisms [28]. At low concentration, NO inhibits BACE1 transcription possibly through upregulation of its transcriptional regulator PGC-1 α . At high concentration, NO inhibits BACE1 enzyme activity through *S*-nitrosylation of BACE1 at Cys⁴⁶¹ and Cys⁴⁷⁷ [28], thereby suggesting that *S*-nitrosylation may be a potential anti-amyloidogenic cell signaling mechanism in neurons.

Based on the above-mentioned beneficial properties of GSNO in vascular protection as well as inhibition of BACE1, we hypothesized that GSNO may be a promising NO-based drug for treatment of neuro and vascular complications associated with mild cognitive impairment and late-onset AD. Consistent with chronic cerebrohypoperfusion and associated vascular dysfunction as risk factors for late-onset AD [1–3], the animal model of cerebrohypoperfusion with permanent bilateral common carotid artery occlusion (BCCAO) developed A β pathology and cerebrovascular decline [8,29–31]. Therefore, in these studies, we investigated the therapeutic potential of GSNO in improving cognitive function, reduced A β accumulation, and inhibiting vascular inflammation under experimental chronic cerebral hypoperfusion conditions using a rat permanent BCCAO model. Mechanistically using an *in vitro* cell culture model, we demonstrate that GSNO may exert its neuroprotective effects by modulating inflammatory signaling (i.e., NF κ B and STAT1/3) and endothelial expression of ICAM-1/VCAM-1. Moreover, GSNO treatment inhibited β -secretase activity and the generation of A β in neurons, and it also enhanced A β clearance by increasing A β endocytosis in microglia and endothelial cells.

Materials and Methods

Rat model of chronic cerebral hypoperfusion and GSNO treatment

As an animal model for human chronic cerebral hypoperfusion featuring mild cognitive impairment [8, 29], rats (250~270g body weight) received permanent occlusion of bilateral common carotid arteries (BCCAO). It is well established that the BCCAO of rats causes reduction of hippocampal blood flow (22~30%) and thus declines learning memory functions [8, 29–31]. For permanent BCCAO or sham operation, the rats were anesthetized with vaporized isoflurane (induction 5%, maintenance 2%). Following a ventral midline incision, the common carotid arteries were bilaterally separated from the carotid sheath and vagus nerve and rigidly occluded with 3–0 silk sutures. The rats assigned to sham surgery were submitted to the same procedure without occlusion of the arteries. For post-procedural pain relief, the rats were administered buprenorphine (0.05 mg/kg, subcutaneous) twice daily for 3 days. Following surgery, the rats were fed *ad libitum* for 4 months to induce chronic hypoperfusion mediated changes and then treated daily with GSNO (50 µg/kg, i.p.) or saline for 2 months.

Morris water maze test

This task was adapted for rats from the paradigm originally described by Morris [32]. The water maze is a circular pool (183 cm diameter), filled with water ($26\pm1^{\circ}\text{C}$) and made opaque with white dye. The pool is divided into four quadrants. An escape platform was placed in the middle of one quadrant, 2.0 cm below the water surface, equidistant from the sidewall and middle of the pool. The platform providing the only escape from the water was located in the same quadrant on every trial. Three different starting points for rats were placed around the perimeter of the pool. On each of the four training days, all three start points were used once each in a pseudo-random sequence, so the starting point was different in every session. All assessments were performed using a CRE camera that was suspended over the center of the pool. The swimming activity of each rat was recorded using an automated tracking system coupled to a personal computer. The water maze was always located in a large room and the investigator always sat at the same position. All experiments were carried out between 10:00 h and 16:00 h.

Escape acquisition

A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. Each animal was allowed 120 s to escape onto the platform. If the animal failed to escape within this time, it was guided to the platform. Whether or not the animal had escaped or been guided to the platform, it remained there for 15 s before being removed and placed in the home cage for a 10 min inter-trial interval. Three escape trails were given to all rats per day for 6 consecutive days. The escape latency(s) to reach the platform was recorded during these trials. Following the trials, the data were analyzed with respect to acquisition (averages for day 1–3 of training), consolidation (averages for day 4–6 of training), and combined (overall) performance (average distance and latency for days 1–6).

Spatial memory test

Twenty four hours after the last training trial in the escape acquisition test, rats were submitted to the probe trial in which the platform was removed. In the 60-s probe trial, the time in the target quadrant (s; the quadrant in which the platform was located in the training sessions) was considered as a measure for spatial memory.

Immunofluorescence staining of paraffin-embedded sections

Paraffin-embedded sections from the formalin-fixed brain tissues were stained using antibodies against ICAM-1 (intercellular adhesion molecule 1; Santa Cruz Biotech., Santa Cruz, CA, USA) and VCAM-1 (vascular cell adhesion molecule 1; Santa Cruz Biotech.). Secondary anti-rabbit and mouse IgG, conjugated with Alexa Fluor 568 and 488, respectively, were used for visualizing the sections. Three areas of each immunostained section were digitized using an BX60 Olympus microscope equipped for epifluorescence and DP-70 camera (Olympus, Tokyo, Japan) and controller software. Images were captured and processed using Adobe Photoshop 7.0. The intensities of fluorescence were quantified by Image-Pro Plus (MediaCybernetics, Bethesda, MD, USA).

Cell culture and drug treatment

The bEND3 murine brain endothelial cell line and BV2 murine microglial cell line were purchase from American Type Culture Collection (ATCC) and maintained in Dulbecco's-Modified-Eagles-Medium (DMEM) containing 10% fetal bovine serum, and 1% penicillin/streptomycin under 37°C, 5% CO₂ incubator. At 80% confluency, the cells were switched with serum free DMEM and treated with GSNO two times (15 h before and 3 h before harvest or second drug treatment). Lipopolysaccharide (Sigma-Aldrich, St. Louis, MO, USA; 100ng/ml) or cytokine mix, tumor necrosis factor- α (TNF- α ; 10 ng/ml), interleukin-1 β (IL-1 β ; 10ng/ml), and interferon- γ (IFN- γ ; 10 ng/ml), were treated thereafter.

For neuronal cell culture, rat brain cortices derived from embryonic day 17 rats (Sprague Dawley) were treated with 0.125% trypsin, and the dissociated cells were cultured on poly-D-lysine coated culture plates or dishes in Neurobasal medium (Invitrogen, San Diego, CA, USA) containing 2% B27 supplement (Invitrogen), L-glutamine (0.5 mM; Sigma-Aldrich), L-glutamate (25 μ M; Sigma-Aldrich), and penicillin/streptomycin mixture (Invitrogen). The cultures were maintained in 5% CO₂ at 37°C for 7 days and exchanged with B27 free Neurobasal medium for drug treatment.

Western immunoblot assay

Western immunoblot analysis was performed as described previously [33] using antibodies against p65 (Santa Cruz Biotech.), I κ B (Santa Cruz Biotech.), β -actin (Santa Cruz Biotech.), pan- and phospho-STAT-1 (Cell Signaling Tech., Inc., Panvers, MA, USA), pan- and phospho-STAT3 (Cell Signaling Inc.), ICAM-1 (Santa Cruz Biotech.), VCAM-1 (Santa Cruz Biotech.), dynamin-2 (Abcam, Cambridge, MA, USA), biotin (Cell Signaling Inc.), and BACE1 (Chemicon-Millipore, Temecula, CA, USA).

Real time PCR assay

Real time reverse transcriptase polymerase chain reaction (RT-RT-PCR) was performed according to standard protocol as described previously [34]. Total cellular RNA from bEND3 cells were isolated using TRIzol reagent (Invitrogen) per the manufacturer's protocol. cDNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. RT-PCR was performed with SYBR Green PCR master mix (Bio-Rad) and Bio-Rad iCycler iQ PCR using the following primers: ICAM-1 (forward: 5'-GTC CAA TTC ACA CTG AAT GCC AGC-3' and backward: 5'-TTA AAC AGG AAC TTT CCC GCC ACC-3'), VCAM (forward: 5'-ACT CCT TGC ACT CTA CTG C-3' and backward: 5'-TCC ACC AGA CTG TAC GAT C-3'), and GAPDH (forward: 5'-ATG ACA TCA AGA AGG TGG TG-3' and backward: 5'-CAT ACC AGG AAA TGA GCT TG-3').

Electrophoretic mobility-shift analysis (EMSA)

Nuclear extract from bEND3 cells were prepared using previously published method [35]. The DNA probe sequences were 5'-ACA TGT TAT GCA TAT TCC TGT AAG TG-3' for STAT1, 5'-AGA TCC TTC TGG GAA TTC CTA GAT C-3' for STAT3, and 5'-AGT TGA GGG GAC TTT CCC AGG C-3' for NFκB. The complementary oligonucleotides were labeled with digoxigenin (DIG)-11-ddUTP (Roche, Mannheim, Germany) using terminal transferase (Roche). Following the reaction with nuclear proteins and resolution on 5% polyacrylamide gel containing 50 mM Tris, pH 7.5, 0.38 M glycine, and 2 mM EDTA, the DIG-labeled oligonucleotides were transferred onto positively charged nylon membrane (Amersham).

Detection of S-nitrosylated proteins

S-Nitrosylation of proteins was detected using the biotin-switch method as described previously [19]. In brief, cells were lysed in HEN buffer [250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 20 μM methyl methanethiosulfonate (MMTS), 80 μM carmustine, protease inhibitor mixture (Sigma)], then mixed with an equal volume of MMTS buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μM neocuproine, 5% SDS, 20 μM MMTS), and incubated at 50°C for 20min with frequent vortexing. Following the removal of free MMTS by acetone precipitation, the precipitates were resuspended in HENS buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μM neocuproine, 1% SDS). Following the addition of two volumes of neutralization buffer (20 mM HEPES, pH 7.7, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100), the samples were precleared with neutravidin-agarose (Pierce, IL, USA). The S-nitrosylated proteins were then modified with biotin in HPDP buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 1% SDS, 10 μM neocuproine, 10mM ascorbic acid sodium salt, and 0.2 mM N-[6-(biotinamido)hexyl]-30-(20-pyridyldithio) propionamide (biotin-HPDP, Pierce). Following the removal of free biotin-HPDP by acetone precipitation, the biotinylated proteins were absorbed to neutravidin-agarose. Neutravidin absorbates were eluted by β-mercaptoethanol (100 mM) and separated by SDS-PAGE followed by western blot assay.

A β uptake assay

Endothelial or microglial A β uptake assay was performed with C-terminal fluorescence amidated A β_{40} (FAM-A β_{40} ; Anaspec, San Jose, CA) or FAM-A β_{42} . Before the uptake assay, FAM-A β peptides were preincubated with basal media (fresh DMEM) or astroglial conditioned media (CM), which include lipidated ApoE (see Supplementary Data 1; available online: <http://www.j-alz.com/issues/34/vol34-3.html#supplementarydata01>). To examine the involvement of dynamin-2 in A β uptake, bEND3 cells were transiently transfected with human dynamin-2 cDNA or its mock control (pcDNA3 only). To examine whether CM-mediated A β uptake is mediated through LRP1, the A β uptake assay was performed following the treatment of cells with LRP1 inhibitor, receptor-associated protein (Rap). To examine whether GSNO enhances BM or CM-mediated A β uptake, the cells were pretreated with GSNO (250 μ M/3 h) prior to performance of A β uptake assay. Following the incubation with FAM-A β (200ng/ml) for 5min, the cells were washed with ice cold phosphate buffered saline (PBS), and then lysed with PBS containing 0.1% Triton X-100. The fluorescence of resulting lysates was measured by SpectraMax Gemini XS (Molecular Devices) with excitation (495 nm) and emission (520 nm) or visualized under a fluorescent microscope.

Analysis of β -secretase activity and A β_{40} /A β_{42} levels in primary neuron culture media and rat brains

The activities of β -secretases in post nuclear cell extracts were measured using fluorogenic assay kits purchased from R&D Systems Inc. (Minneapolis, MN, USA). The activities were measured by SPECTRAmax[®] Gemini XS[®] fluorimeter with SOFT-max PRO[®] software (Molecular Devices, Chicago, IL, USA) with excitation at 345 nm and emission detection at 500 nm. For quantification of A β levels in media, culture media was centrifuged (1000 $\times$ g for 10min at 4°C) and 100 μ l supernatant was used for colorimetric ELISA assay by using human A β (1-40; A β_{40}) and (1-42; A β_{42}) assay kits purchased from IBL Co., Ltd. (Japan) or Wako Chemicals (Japan) which are fully compatible with rat A β_{40} or A β_{42} . For quantification of brain levels of A β , the isolated rat brain cortices were homogenized in 70% formic acid. Following neutralization with 1M Tris, the homogenates were subjected to ELISA assay for quantification of A β as described above.

Results

A potential role for GSNO treatment in improvement of cognitive function in the rat model of chronic cerebral hypoperfusion (permanent BCCAO)

Permanent BCCAO in rat model is used to investigate the effects of chronic cerebral hypoperfusion on cognitive dysfunction and associated neurodegenerative processes [8, 30, 31]. Based on the observed efficacy of GSNO on vascular functions in the neurodegenerative disease model [17–19, 22, 23], we evaluated the therapeutic potential of S-nitrosylating agent (GSNO) on neurocognitive impairments in a rat model of chronic cerebral hypoperfusion. Following GSNO treatment, the rats were tested for spatial learning and memory performance by the water maze test as described in the method section. On the first day with the hidden platform, no significant differences between the sham nonBCCAO group and BCCAO group were observed (Fig. 1A-i). On the third day, however, the rats in

the sham nonBCCAO group showed a significant reduction in the time spent finding the hidden platform (escape latency or learning performance), compared to the rats in the BCCAO group. GSNO treated BCCAO rats initially showed no difference with BCCAO rats in the escape latency until the third day. However, there was progressive decline throughout the remaining test phase with significant shortening of the escape latency at sixth day of test (Fig. 1A-i). GSNO treatment had no significant effect on impaired learning performance of BCCAO rats in the acquisition phase of training (day 1–3 of training) (Fig. 1A-ii). However, GSNO treatment significantly improved learning performance in the consolidation phase (day 4–6 of training) (Fig. 1A-iii). Overall, GSNO treatment was beneficial in improving learning deficits in BCCAO rats (day 1–6 of training) (Fig. 1A-iv).

Following the test for learning performance, the rats were placed in the water maze without the platform to assess memory performance (permanence time in the target quadrant). Figure 1A-v shows that GSNO treated animals had improved memory performance as compared to untreated BCCAO rats, indicating that GSNO treatment is effective against cognitive impairment in the rat model of chronic cerebral hypoperfusion.

Chronic cerebral hypoperfusion caused by BCCAO results in heavy amyloid deposition in hippocampal capillaries and perivascular region [8]. Therefore, we next assessed whether the observed improvement in learning and memory performances by GSNO treatment correlates with decreased A β deposition. Brains from the above experiment and control groups were also analyzed for A $\beta_{40/42}$ levels. We observed that both A β_{40} and A β_{42} levels in cerebral cortices were increased in the rats of BCCAO group (Fig. 1B). On the other hand, GSNO treatment of BCCAO rats showed the significantly reduced A β_{40} and A β_{42} levels to near the control levels, suggesting for anti-amyloidogenic activity and/or endothelial/microglial A β clearance-promoting activity of GSNO.

A potential anti-inflammatory activity of GSNO

There is considerable evidence to suggest that neurodegeneration elicited by chronic cerebral hypoperfusion is associated with microvascular abnormalities [36] and inflammation [37–40]. Although modest inflammation plays a role in neuroprotection in acute insults, chronic inflammation is detrimental to neurogenesis [41]. Previously, we reported that GSNO protects the brain against injuries from ischemia/reperfusion [17, 18], traumatic brain injury [42, 43], and experimental autoimmune encephalomyelitis [19, 44] by inhibiting neural and vascular inflammation. Based on these studies, we investigated whether the GSNO-mediated protective mechanisms involve anti-inflammatory effects in animals with BCCAO. We observed that the chronic cerebral hypoperfusion induced by BCCAO resulted in increased mRNA expression of vascular inflammatory markers, such as ICAM-1 and VCAM-1, and that these increases were significantly decreased by GSNO treatment (Fig. 2A-i). Immunofluorescence staining of brain tissue sections also shows that the increased expressions of ICAM-1 and VCAM-1 proteins in the brains of BCCAO treated rats are prominent in threadlike capillaries (Fig. 2A-ii), suggesting for the presence of vascular inflammation in the brain of BCCAO rats. In accordance with mRNA expression, the elevated protein expression of ICAM-1 and VCAM-1 in capillaries was attenuated/inhibited by GSNO treatment (Fig. 2A-ii).

To further support the observed anti-inflammatory effects of GSNO, cultured bEND3 murine brain endothelial cells were pre-treated with GSNO (250 μ M) for 3 h and then stimulated with a cytokine mixture (Cyto; TNF α , IL1 β , and IFN γ ; 25 ng/ml each) for 0.5 h, and the activities of two key inflammatory transcription factors, nuclear factor κ -B (NF κ B) and signal transducer and activator of transcription 1/3 (STAT1/3), were analyzed. Treatment of bEND3 cells with cytokine mixture induced NF κ B activation as demonstrated by the increased nuclear localization of p65, decreased levels of I κ B in cytoplasm, and increased the DNA binding activity of NF κ B (Fig. 2B-i). Cytokine treatment also induced STAT1 and STAT3 activation as demonstrated by the increased phosphorylation of STAT1 (Tyr701) and STAT3 (Tyr705) and their DNA binding activities (Fig. 2B-ii). The activation of NF κ B and STAT1/3 activities in cytokine treated bEND3 cells was completely abolished by pretreatment with GSNO. Accordingly, the increased mRNA and protein expression for ICAM-1 and VCAM-1 induced by cytokine treatment was also reduced by pretreatment with GSNO in bEND3 cell culture (Fig. 2C-i and ii). These *in vivo* studies with the BCCAO model and *in vitro* studies with bEND3 cell culture model document GSNO as a potent antiinflammatory signaling molecule in the vasculature.

A potential regulatory role of GSNO in A β clearance

Inefficient clearance of A β from brain is now recognized as the major cause of excessive accumulation of A β in brain with AD [45]. Both A β efflux mediated by microvascular endothelial cells and A β degradation mediated by microglia are now recognized as major A β clearance mechanisms in the brain [46, 47]. At the molecular level, both the endothelial and the microglial A β clearance mechanisms are largely dependent on A β interaction with A β carrier molecules, such as apolipoprotein E (ApoE), and their uptake or endocytosis mediated by apolipoprotein receptors, LDL receptor related protein 1 (LRP1) [46, 47]. Recent studies have described that S-nitrosylation and activation of dynamin-2 protein participates in membrane trafficking and endothelial functional activities [48–51]. Dynamin-2, a GTPase, is involved in endocytic vesicle formation and its activation by S-nitrosylation on specific cysteine residues (86 and 607) facilitates the cellular endocytosis activity [50, 51]. Using bEND3 cell cultures, we observed that the over-expression of wild type dynamin-2 (*w*tDNM-2) markedly increased A β ₄₂ uptake as compared to control cells treated with empty vector (Fig. 3A-i), indicating that dynamin-2 is involved in upregulation of endothelial A β endocytosis. To examine whether GSNO increases S-nitrosylation of dynamin-2 in bEND3 endothelial cells, the cells were treated with GSNO and S-nitrosylation of dynamin-2 was analyzed by biotin switch method. Figure 3A-ii shows that treatment of bEND3 cells with GSNO for 3 h increased S-nitrosylation of a variety of cellular proteins. Following the pull down of S-biotinylated (S-nitrosylated) proteins with avidin-conjugated agarose beads, the levels of co-precipitated dynamin-2 levels were analyzed to quantify the levels of S-nitrosylated dynamin-2. GSNO treatment increased the S-nitrosylation of dynamin-2 protein without altering the total dynamin-2 levels (Fig. 3A-ii). To assess the involvement of S-nitrosylation mechanisms in endothelial A β uptake mediated by dynamin-2, the bEND3 endothelial cells were transfected with vector constructs encoding the wild type or C86A/C607A mutant of dynamin-2. In bEND3 cells transfected with the vector construct encoding C86A or C607A, the S-nitrosylation of dynamin-2 was greatly reduced as compared to the cells transfected with wild type dynamin-2 (Fig. 3A-iii).

Accordingly, the uptake of A β ₄₂ by bEND3 cells was also significantly reduced by transfection of the cells with C86A/C607A mutant, suggesting the functional involvement of S-nitrosylation mechanism in dynamin-2 mediated endothelial A β uptake.

Formation of ApoE and A β complexes and their endocytosis by LRP1 have been shown to play a critical role in endothelial A β efflux and microglial A β degradation [46, 47]. To examine the effect of GSNO treatment on ApoE/LRP1-mediated A β endocytosis, A β ₄₂ peptide was pre-incubated with conditioned media (CM) generated by liver X-receptor (LXR)-agonist (TO901317) treated astroglial culture. As reported previously [52] and shown in Supplementary Data 1, the treatment of primary cultured rat astroglia with LXR agonist increased the secretion of HDL-like ApoE complex that preferentially interacted with the FAM-A β ₄₂ peptide (Supplementary Data 1). Based on these observations, we preincubated green fluorescence labeled A β ₄₂ (FAM-A β ₄₂) with CM which was then incubated with bEND3 endothelial cell culture for A β uptake analysis. The data presented in Fig. 3B-i show that the preincubation of FAM-A β ₄₂ peptide with CM significantly increased the endothelial FAM-A β ₄₂ uptake as compared to the preincubation of FAM-A β ₄₂ with basal media (BM; fresh serum free DMEM without incubation with astrocytes), and this increase was completely inhibited by LRP1 antagonist, receptor-associated protein (Rap), indicating the role of LRP1-mediated endocytosis pathway in the observed endothelial A β ₄₂ uptake. To assess the role of GSNO in endothelial A β -uptake, the bEND3 cells were preincubated with vehicle (VHC) or GSNO for 2h prior to treatment with FAM-A β ₄₂ peptide which was pre-incubated with BM or CM. Figure 3B-ii shows that GSNO treatment slightly increased the endothelial uptake of FAM-A β ₄₂ pre-incubated with BM. However, a marked increase was observed in the uptake of FAM-A β ₄₂ by GSNO treatment of bEND3 cells incubated with CM, documenting that GSNO efficiently increases A β ₄₂ uptake via LRP1-mediated endocytosis activity. Similar to the effects on A β ₄₂ uptake, GSNO treatment also increased the uptake of A β ₄₀ peptide preincubated with CM extracts (Fig. 3B-iii and iv). These observations document that GSNO treatment enhances the uptake of both A β ₄₀ and A β ₄₂. Since the microglial A β uptake also plays an important role in A β degradation in the brain [47], the A β uptake assay was also performed in BV2 microglial cells. We observed that GSNO treatment also increased the uptake of both A β ₄₀ and A β ₄₂ peptides in microglial cells (Fig. 3C), suggesting that GSNO-mediated mechanisms may play a functional role in A β clearance from brain.

The potential role of GSNO-mediated mechanisms in inhibition of neuronal A β generation

Recent studies have reported that neuronal A β generation is modulated by S-nitrosylation-dependent inhibition of BACE1 β -secretase activity [28]. Therefore, we assessed the potential role for GSNO in inhibition of neuronal A β generation. In primary cultured rat cortical neurons, GSNO treatment decreased the secretions of both A β ₄₀ and A β ₄₂ peptides in a dose dependent manner (Fig. 4A-i and ii). Similar to the effect of GSNO, A β secretion was also decreased by treatment of neurons with S-nitroso-N-acetylpenicillamine (SNAP), another S-nitroso donor, but glutathione ethylester (GSHEE; a cell permeable GSH) had no effect (Fig. 4A-iii), suggesting that GSNO may reduce A β secretion via S-nitrosylation mediated mechanisms. While the neuronal A β secretion was significantly decreased by

GSNO and SNAP treatments, these S-NO donors did not show any significant cytotoxicity as demonstrated by LDH and MTT assays (Fig. 4A-iv and v).

To assess the effect of GSNO on A β PP processing enzyme activities, we next examined the effect of GSNO on neuronal activities of α - and β -secretases. As shown in Figs. 4B-i and ii, GSNO treatment of neuronal cultures had no effect on the activities of α -secretase up to 200 μ M but it inhibited β -secretase activity in a dose dependent manner. Consistent with the S-nitrosylating activity of SNAP, treatment of neurons with SNAP also decreased β -secretase activity (Fig. 4B-iii). On the other hand, decomposed GSNO (dc-GSNO; decomposed under light for 1 week) or GSNO metabolite related compounds, such as sodium nitrite and oxidized glutathione (GSSG), had no effect on neuronal β -secretase activity, suggesting the involvement of an S-nitrosylation dependent mechanism in GSNO-induced inhibition of neuronal β -secretase activity. These conclusions were further supported by detection of S-nitrosylated BACE1 in GSNO treated cultured neurons. Figure 4C shows that the GSNO treatment increased S-nitrosylation of a variety of proteins in neurons in a dose dependent manner as shown by Western analysis with anti-biotin antibody. Following the pulldown of S-biotin labeled proteins with avidin-agarose beads, the levels of co-precipitated BACE1 were examined by western analysis with antibody against BACE1 for examination of S-nitrosylated BACE1 levels (for assay details, please see Materials and Methods section). GSNO treatment dose-dependently increased the BACE1 S-nitrosylation as levels of S-nitrosylated BACE1 protein (Fig. 4C). Taken together, these data document that GSNO may be a potent anti-amyloidogenic agent that can modulate β -secretase activity via S-nitrosylation-dependent mechanism.

Discussion

Recent evidence has suggested that early treatment and management of cerebrovascular abnormalities are essential for the prevention of and intervention in the impending neurocognitive decline associated with mild cognitive impairment and late-onset AD [1–3]. AD patients exhibit disturbed brain metabolism of tetrahydrobiopterin, an essential cofactor for NOS [12, 53], and lower steady levels of NO in the plasma [54], thereby suggesting the contribution of low bioavailability of NO in reduced cerebral blood flow under those neurodegenerative conditions. In support, de la Torre and colleagues previously reported (using rat model of permanent BCCAO) that vascular NO derived from eNOS plays a critical role in spatial cognitive function by keeping cerebral perfusion optimal through its regulation of microvessel tone and cerebral blood flow [8], in turn suggesting that the restoration of NO bioavailability or NO-mediated vascular function may be beneficial for alleviating cognitive decline associated with conditions of chronic cerebral hypoperfusion.

GSNO has been identified as a potential carrier, storage, and transport vehicle of NO in the body. In addition, GSNO is a useful NO donor which slowly releases NO or donates a S-nitroso group to other protein thiols under physiological conditions [55]. GSNO was reported to provide protection in the animal model of cerebral hypoxia-reperfusion [17, 18], traumatic brain injury [42, 43], and multiple sclerosis [19, 44] possibly via inhibition of NF κ B and STAT3 activities. Various studies have also described the vascular activities of GSNO, such as potassium channel-mediated vaso-relaxation [56], endothelial proliferation

[57], anti-platelet/anti-thrombotic activity [58, 59], and cardio protection [60]. Moreover, the reported roles of GSNO in activation of cell survival signaling cascades mediated by phosphatidylinositol 3-kinase, extracellular signal-regulated kinase, and Bcl-2, and in inhibition of proapoptotic caspase activation [61, 62] further suggest that GSNO is a potent vaso-protective agent and thus may be helpful in improving neurocognitive functions under conditions of chronic cerebral hypoperfusion. Based on this background, we examined the effect of chronic GSNO treatment on spatial learning and memory performance in rats subjected to cerebral hypoperfusion for 4 months by permanent BCCAO operation followed by GSNO treatment for 2 months. GSNO treatment significantly reduced cerebral hypoperfusion-induced decline in spatial learning and memory performance in rats with permanent BCCAO. The permanent BCCAO rat model was established to investigate the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes [29]. Therefore, our data suggest a potential benefit of GSNO treatment in cognitive activity during chronic cerebral hypoperfusion.

Late onset AD involves multiple pathogenic mechanisms including A β deposition, neurofilament tangle formation, inflammation, and oxidative stress, which must be intervened for protection against neuronal damage and cognitive impairment. Although GSNO has been well recognized as a vasoregulatory and vaso-protective agent, studies from our laboratory and others have also documented that GSNO is a potential antiinflammatory and antioxidant agent [17–19, 22, 23, 43, 55]. Therefore, GSNO-mediated mechanisms are expected to improve cognitive function by reducing neuro- and cerebrovascular-inflammatory responses. In these studies, we observed that chronic cerebral hypoperfusion in rats treated with permanent BCCAO caused vascular inflammation as evident by increased gene expression of ICAM-1 and VCAM-1 (Fig. 2A); their expression was effectively reduced by GSNO treatment. Accordingly we also observed in cell culture studies that GSNO treatment inhibited the gene expression for proinflammatory mediators in endothelial cells as well as in microglia (Fig. 2C and Supplementary Data 2). In addition to the well-established efficacies of GSNO in vasoregulation and vasoprotection [56–60], these data further report the therapeutic potential of GSNO in modulation of inflammatory responses under conditions of chronic cerebral hypoperfusion.

Progressive accumulation of A β leading to the formation of A β oligomers and fibrils, principal components of A β plaque, is a critical pathological feature in AD. The abnormal accumulation of A β in AD brain results from an imbalance between A β production and clearance [27, 46, 47]. Therefore, modulation of A β production and clearance in the brain has been a major therapeutic target for treatment of AD. Previously, de la Torre et al. reported heavy A β deposition in hippocampal capillaries and perivascular regions in rats with permanent (surgical) BCCAO [8], suggesting an imbalance between A β production and clearance in the brain undergoing chronic cerebral hypoperfusion. Accordingly, we also observed increased levels of A β ₄₀ and A β ₄₂ in brains of rats with permanent BCCAO (Fig. 1B) and that GSNO treatment reduced A β levels, suggesting that GSNO mediated mechanisms may regulate the extracellular A β homeostasis by reduction of A β production or promotion of A β clearance.

A β is produced by serial cleavage of its precursor protein A β PP by two enzymes called β - and γ -secretase. β -secretase is the rate limiting enzyme in the production of A β and has been considered as one of the most important therapeutic targets for treating AD [63]. Recent studies on *in vitro* cell cultures reported that S-nitrocysteine treatment inhibits neuronal A β production by inhibiting gene expression and enzyme activity of BACE1 β -secretase [28]. Consistent with these studies, we also observed that GSNO treatment of cultured neurons inhibits β -secretase activity and that this inhibition of BACE1 is mediated by its S-nitrosylation (Fig. 4B, C). Consequently, GSNO treatment decreased neuronal A β production to within the concentration range considered non-toxic. These studies, therefore, suggest a potential role for GSNO in inhibition of the neuronal amyloidogenic pathway. Although at present it is not known whether GSNO treatment reduces neuronal A β production in the brain of rats with permanent BCCAO, evidence supporting the low NO bioavailability [12, 53, 54] and the decreased BACE1 S-nitrosylation in brains of patients with AD [28] suggest a beneficial role in GSNO treatment in reducing the levels of A β under the disease conditions involving chronic cerebral hypoperfusion and AD.

Clearance of A β from the brain is mediated by multiple diverse processes. These include drainage along perivascular basement membranes, possibly to cervical lymph nodes and into the cerebrospinal fluid, transport across the blood-brain barrier into the circulation (transcytosis) by LRP1 or the P-glycoprotein efflux pump, the sequestration of A β by LRP1 in the circulation to promote the efflux of soluble A β out of the CNS, microglial phagocytosis and its degradation [46]. Among these pathways, A β transcytosis and microglial A β phagocytosis are of particular interest because both pathways require LRP1-mediated A β endocytosis [46, 47]. Recent studies have reported that NO-mediated mechanisms regulate endocytosis by S-nitrosylation of dynamin-2 [50]. In the present study, we observed that GSNO treatment promoted the endothelial and microglial A β endocytosis with increasing dynamin-2 S-nitrosylation on specific cysteine residues (Cys 86 and 607). Since the A β endocytosis is the first step in endothelial A β transcytosis and microglial A β degradation, the increased A β endocytosis in GSNO treated cells suggests the potential role of GSNO-mediated mechanisms in promoting A β clearance. However, its effect on the brain A β clearance under the conditions of chronic cerebral hypoperfusion or AD remains to be further investigated.

In summary, our data for the first time documents that systemic GSNO administration reduces vascular inflammatory response (expression of ICAM-1 and VCAM-1) and the A β burden and protects against cognition decline in an animal model of chronic cerebral hypoperfusion, a model for mild cognitive impairment associated with development of late-onset AD. Studies using cell culture models, primary neurons, microglia (BV2) and endothelial cell (bEND3) lines also document that GSNO mediated mechanisms inhibit β -secretase activity and thus A β generation in neurons and A β endocytosis in microglia and endothelial cells as an index of A β clearance from brain. Taken together, these findings document that GSNO mediated mechanisms may prove to have therapeutic potential in chronic cerebral hypoperfusion associated with mild cognitive impairment and development of late-onset AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. de la Torre JC. Cerebrovascular and cardiovascular pathology in Alzheimer's disease. *Int Rev Neurobiol.* 2009; 84:35–48. [PubMed: 19501712]
2. de la Torre JC. Alzheimer's disease is a vasocognopathy: A new term to describe its nature. *Neurol Res.* 2004; 26:517–524. [PubMed: 15265269]
3. de la Torre JC. Alzheimer disease as a vascular disorder: Nosological evidence. *Stroke.* 2002; 33:1152–1162. [PubMed: 11935076]
4. Landmesser U, Hornig B, Drexler H. Endothelial function: A critical determinant in atherosclerosis? *Circulation.* 2004; 109:II27–II33. [PubMed: 15173060]
5. Braam B, Verhaar MC. Understanding eNOS for pharmacological modulation of endothelial function: A translational view. *Curr Pharm Des.* 2007; 13:1727–1740. [PubMed: 17584103]
6. de la Torre JC, Stefano GB. Evidence that Alzheimer's disease is a microvascular disorder: The role of constitutive nitric oxide. *Brain Res Brain Res Rev.* 2000; 34:119–136. [PubMed: 11113503]
7. de la Torre JC, Pappas BA, Prevot V, Emmerling MR, Mantione K, Fortin T, Watson MD, Stefano GB. Hippocampal nitric oxide upregulation precedes memory loss and A beta 1-40 accumulation after chronic brain hypoperfusion in rats. *Neurol Res.* 2003; 25:635–641. [PubMed: 14503018]
8. de la Torre JC, Aliev G. Inhibition of vascular nitric oxide after rat chronic brain hypoperfusion: Spatial memory and immunocytochemical changes. *J Cereb Blood Flow Metab.* 2005; 25:663–672. [PubMed: 15703700]
9. McCarty MF. Vascular nitric oxide may lessen Alzheimer's risk. *Med Hypotheses.* 1998; 51:465–476. [PubMed: 10052865]
10. Moreno-Lopez B, Gonzalez-Forero D. Nitric oxide and synaptic dynamics in the adult brain: Physiopathological aspects. *Rev Neurosci.* 2006; 17:309–357. [PubMed: 16878402]
11. Miller TW, Isenberg JS, Shih HB, Wang Y, Roberts DD. Amyloid-beta inhibits No-cGMP signaling in a CD36- and CD47-dependent manner. *PLoS One.* 2010; 5:e15686. [PubMed: 21203512]
12. Schade D, Kotthaus J, Clement B. Modulating the NO generating system from a medicinal chemistry perspective: Current trends and therapeutic options in cardiovascular disease. *Pharmacol Ther.* 2010; 126:279–300. [PubMed: 20226211]
13. Tai SC, Robb GB, Marsden PA. Endothelial nitric oxide synthase: A new paradigm for gene regulation in the injured blood vessel. *Arterioscler Thromb Vasc Biol.* 2004; 24:405–412. [PubMed: 14656742]
14. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation.* 2006; 113:1708–1714. [PubMed: 16585403]
15. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol.* 1996; 271:C1424–C1437. [PubMed: 8944624]
16. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H. Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A.* 1996; 93:2448–2453. [PubMed: 8637894]
17. Khan M, Sekhon B, Giri S, Jatana M, Gilg AG, Ayasolla K, Elango C, Singh AK, Singh I. S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke. *J Cereb Blood Flow Metab.* 2005; 25:177–192. [PubMed: 15647746]

18. Khan M, Jatana M, Elango C, Paintlia AS, Singh AK, Singh I. Cerebrovascular protection by various nitric oxide donors in rats after experimental stroke. *Nitric Oxide*. 2006; 15:114–124. [PubMed: 16524750]
19. Prasad R, Giri S, Nath N, Singh I, Singh AK. GSNO attenuates EAE disease by S-nitrosylation-mediated modulation of endothelial-monocyte interactions. *Glia*. 2007; 55:65–77. [PubMed: 17019693]
20. Gaston B, Reilly J, Drazen JM, Fackler J, Ramdev P, Arnelle D, Mullins ME, Sugarbaker DJ, Chee C, Singel DJ, et al. Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc Natl Acad Sci U S A*. 1993; 90:10957–10961. [PubMed: 8248198]
21. Sun J, Steenbergen C, Murphy E. S-nitrosylation: NO-related redox signaling to protect against oxidative stress. *Antioxid Redox Signal*. 2006; 8:1693–1705. [PubMed: 16987022]
22. Rauhala P, Mohanakumar KP, Sziraki I, Lin AM, Chiueh CC. S-nitrosothiols and nitric oxide, but not sodium nitroprusside, protect nigrostriatal dopamine neurons against iron-induced oxidative stress *in vivo*. *Synapse*. 1996; 23:58–60. [PubMed: 8723136]
23. Rauhala P, Lin AM, Chiueh CC. Neuroprotection by S-nitrosoglutathione of brain dopamine neurons from oxidative stress. *FASEB J*. 1998; 12:165–173. [PubMed: 9472981]
24. Sastre M, Klockgether T, Heneka MT. Contribution of inflammatory processes to Alzheimer's disease: Molecular mechanisms. *Int J Dev Neurosci*. 2006; 24:167–176. [PubMed: 16472958]
25. Parihar MS, Hemnani T. Alzheimer's disease pathogenesis and therapeutic interventions. *J Clin Neurosci*. 2004; 11:456–467. [PubMed: 15177383]
26. Durany N, Munch G, Michel T, Riederer P. Investigations on oxidative stress and therapeutical implications in dementia. *Eur Arch Psychiatry Clin Neurosci*. 1999; 249(Suppl 3):68–73. [PubMed: 10654103]
27. Selkoe DJ. Alzheimer's disease: Genes, proteins, and therapy. *Physiol Rev*. 2001; 81:741–766. [PubMed: 11274343]
28. Kwak YD, Wang R, Li JJ, Zhang YW, Xu H, Liao FF. Differential regulation of BACE1 expression by oxidative and nitrosative signals. *Mol Neurodegener*. 2011; 6:17. [PubMed: 21371311]
29. Farkas E, Luiten PG, Bari F. Permanent, bilateral common carotid artery occlusion in the rat: A model for chronic cerebral hypoperfusion-related neurodegenerative diseases. *Brain Res Rev*. 2007; 54:162–180. [PubMed: 17296232]
30. de la Torre JC, Butler K, Kozlowski P, Fortin T, Saunders JK. Correlates between nuclear magnetic resonance spectroscopy, diffusion weighted imaging, and CA1 morphometry following chronic brain ischemia. *J Neurosci Res*. 1995; 41:238–245. [PubMed: 7650759]
31. Tsuchiya M, Sako K, Yura S, Yonemasu Y. Cerebral blood flow and histopathological changes following permanent bilateral carotid artery ligation in Wistar rats. *Exp Brain Res*. 1992; 89:87–92. [PubMed: 1601104]
32. Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*. 1984; 11:47–60. [PubMed: 6471907]
33. Won JS, Im YB, Khan M, Contreras M, Singh AK, Singh I. Lovastatin inhibits amyloid precursor protein (APP) beta-cleavage through reduction of APP distribution in Lubrol WX extractable low density lipid rafts. *J Neurochem*. 2008; 105:1536–1549. [PubMed: 18266936]
34. Nath N, Khan M, Paintlia MK, Singh I, Hoda MN, Giri S. Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis. *J Immunol*. 2009; 182:8005–8014. [PubMed: 19494326]
35. Won JS, Im YB, Key L, Singh I, Singh AK. The involvement of glucose metabolism in the regulation of inducible nitric oxide synthase gene expression in glial cells: Possible role of glucose-6-phosphate dehydrogenase and CCAAT/enhancing binding protein. *J Neurosci*. 2003; 23:7470–7478. [PubMed: 12930785]
36. De Jong GI, Farkas E, Stienstra CM, Plass JR, Keijser JN, de la Torre JC, Luiten PG. Cerebral hypoperfusion yields capillary damage in the hippocampal CA1 area that correlates with spatial memory impairment. *Neuroscience*. 1999; 91:203–210. [PubMed: 10336071]

37. Pappas BA, de la Torre JC, Davidson CM, Keyes MT, Fortin T. Chronic reduction of cerebral blood flow in the adult rat: Late-emerging CA1 cell loss and memory dysfunction. *Brain Res.* 1996; 708:50–58. [PubMed: 8720859]
38. Farkas E, Luiten PG. Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog Neurobiol.* 2001; 64:575–611. [PubMed: 11311463]
39. Farkas E, Institoris A, Domoki F, Mihaly A, Luiten PG, Bari F. Diazoxide and dimethyl sulphoxide prevent cerebral hypoperfusion-related learning dysfunction and brain damage after carotid artery occlusion. *Brain Res.* 2004; 1008:252–260. [PubMed: 15145763]
40. Liu J, Jin DZ, Xiao L, Zhu XZ. Paeoniflorin attenuates chronic cerebral hypoperfusion-induced learning dysfunction and brain damage in rats. *Brain Res.* 2006; 1089:162–170. [PubMed: 16678139]
41. Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science.* 2003; 302:1760–1765. [PubMed: 14615545]
42. Khan M, Sakakima H, Dhammu TS, Shunmugavel A, Im YB, Gilg AG, Singh AK, Singh I. S-nitrosoglutathione reduces oxidative injury and promotes mechanisms of neurorepair following traumatic brain injury in rats. *J Neuroinflammation.* 2011; 8:78. [PubMed: 21733162]
43. Khan M, Im YB, Shunmugavel A, Gilg AG, Dhindsa RK, Singh AK, Singh I. Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. *J Neuroinflammation.* 2009; 6:32. [PubMed: 19889224]
44. Nath N, Morinaga O, Singh I. S-nitrosoglutathione a physiologic nitric oxide carrier attenuates experimental autoimmune encephalomyelitis. *J Neuroimmune Pharmacol.* 2010; 5:240–251. [PubMed: 20091246]
45. Kim J, Basak JM, Holtzman DM. The role of apolipoprotein E in Alzheimer's disease. *Neuron.* 2009; 63:287–303. [PubMed: 19679070]
46. Wang YJ, Zhou HD, Zhou XF. Clearance of amyloid-beta in Alzheimer's disease: Progress, problems and perspectives. *Drug Discov Today.* 2006; 11:931–938. [PubMed: 16997144]
47. Lee CY, Landreth GE. The role of microglia in amyloid clearance from the AD brain. *J Neural Transm.* 2010; 117:949–960. [PubMed: 20552234]
48. Kang-Decker N, Cao S, Chatterjee S, Yao J, Egan LJ, Semela D, Mukhopadhyay D, Shah V. Nitric oxide promotes endothelial cell survival signaling through S-nitrosylation and activation of dynamin-2. *J Cell Sci.* 2007; 120:492–501. [PubMed: 17251380]
49. Lowenstein CJ. Nitric oxide regulation of protein trafficking in the cardiovascular system. *Cardiovasc Res.* 2007; 75:240–246. [PubMed: 17490627]
50. Wang G, Moniri NH, Ozawa K, Stamler JS, Daaka Y. Nitric oxide regulates endocytosis by S-nitrosylation of dynamin. *Proc Natl Acad Sci U S A.* 2006; 103:1295–1300. [PubMed: 16432212]
51. Praefcke GJ, McMahon HT. The dynamin super family: Universal membrane tubulation and fission molecules? *Nat Rev Mol Cell Biol.* 2004; 5:133–147. [PubMed: 15040446]
52. Abildayeva K, Jansen PJ, Hirsch-Reinshagen V, Bloks VW, Bakker AH, Ramaekers FC, de Vente J, Groen AK, Wellington CL, Kuipers F, Mulder M. 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. *J Biol Chem.* 2006; 281:12799–12808. [PubMed: 16524875]
53. Aziz AA, Leeming RJ, Blair JA. Tetrahydrobiopterin metabolism in senile dementia of Alzheimer type. *J Neurol Neurosurg Psychiatry.* 1983; 46:410–413. [PubMed: 6152915]
54. Selley ML. Increased concentrations of homocysteine and asymmetric dimethylarginine and decreased concentrations of nitric oxide in the plasma of patients with Alzheimer's disease. *Neurobiol Aging.* 2003; 24:903–907. [PubMed: 12928048]
55. Chiueh CC. Neuroprotective properties of nitric oxide. *Ann N Y Acad Sci.* 1999; 890:301–311. [PubMed: 10668435]
56. Ceron PI, Cremonez DC, Bendhack LM, Tedesco AC. The relaxation induced by S-nitroso-glutathione and S-nitroso-N-acetylcysteine in rat aorta is not related to nitric oxide production. *J Pharmacol Exp Ther.* 2001; 298:686–694. [PubMed: 11454932]
57. Sarkar R, Webb RC, Stanley JC. Nitric oxide inhibition of endothelial cell mitogenesis and proliferation. *Surgery.* 1995; 118:274–279. [PubMed: 7638744]

58. de Belder AJ, MacAllister R, Radomski MW, Moncada S, Vallance PJ. Effects of S-nitroso-glutathione in the human forearm circulation: Evidence for selective inhibition of platelet activation. *Cardiovasc Res.* 1994; 28:691–694. [PubMed: 8025915]
59. Langford EJ, Brown AS, Wainwright RJ, de Belder AJ, Thomas MR, Smith RE, Radomski MW, Martin JF, Moncada S. Inhibition of platelet activity by S-nitrosoglutathione during coronary angioplasty. *Lancet.* 1994; 344:1458–1460. [PubMed: 7526102]
60. Konorev EA, Tarpey MM, Joseph J, Baker JE, Kalyanaraman B. S-nitrosoglutathione improves functional recovery in the isolated rat heart after cardioplegic ischemic arrest-evidence for a cardioprotective effect of nitric oxide. *J Pharmacol Exp Ther.* 1995; 274:200–206. [PubMed: 7616400]
61. Ju TC, Chen SD, Liu CC, Yang DI. Protective effects of S-nitrosoglutathione against amyloid beta-peptide neuro-toxicity. *Free Radic Biol Med.* 2005; 38:938–949. [PubMed: 15749390]
62. Zeigler MM, Doseff AI, Galloway MF, Opalek JM, Nowicki PT, Zweier JL, Sen CK, Marsh CB. Presentation of nitric oxide regulates monocyte survival through effects on caspase-9 and caspase-3 activation. *J Biol Chem.* 2003; 278:12894–12902. [PubMed: 12566444]
63. Ghosh AK, Brindisi M, Tang J. Developing beta-secretase inhibitors for treatment of Alzheimer's disease. *J Neurochem.* 2012; 120(Suppl 1):71–83. [PubMed: 22122681]

Fig. 1.

Effect of GSNO treatment on spatial learning and memory deficits and Aβ accumulation in rat model with permanent bilateral common carotid artery occlusion (BCCAO). A) Time needed to reach the hidden platform (latency in seconds) in the Morris maze by vehicle or S-nitrosoglutathione (GSNO; 50 μg/kg, i.p. for 2 months)-treated rats ($n = 6$) (i). Data were also further analyzed with respect to acquisition (averages for day 1–3 of training) (ii), consolidation (averages for day 4–6 of training) (iii), and combined (overall) performance (average distance and latency for days 1–6) (iv). Following the training phase, the hidden platform in the target quadrant was removed and the time spent in the target quadrant (permanence time) was measured for analysis of spatial memory function (v). B) Rats were dissected for the extraction of brain cortices from above experimental and control animals for analysis of Aβ₄₀ (i) and Aβ₄₂ (ii) levels by sandwich ELISA assay. The data were analyzed for mean values and standard error for all experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for comparison to sham control group; # $p < 0.05$ for comparison to vehicle treated BCCAO group; NS for no-significant changes compared to vehicle treated BCCAO group.

Fig. 2. Anti-inflammatory activity of GSNO. A) The effect of GSNO treatment on the expression of vasoinflammatory markers (ICAM-1 and VCAM-1) in the brains of rats treated with permanent BCCAO was examined by real time quantitative PCR and by immunofluorescence staining of paraffin sections as described in method section. In the quantitative PCR analysis, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was quantified as an internal control. B) Further, to study the mechanism of GSNO in endothelial inflammatory signaling events, the cultured mouse brain endothelial cells (bEND3) were pretreated with GSNO (250 μ M) for 3 h and then treated with cytokine mix

(Cyto; TNF α , IL1 β , and IFN γ ; 25 ng/ml each). Following incubation for 0.5 h with cytokine mix, the activities of nuclear factor κ -B (NF κ B) were analyzed by western analysis for nuclear p65 and cytoplasmic I κ B and by gel-shift assay of NF κ B DNA binding activity (i). The activities of signal transducer and activator of transcription 1 (STAT1) and STAT3 were analyzed by western analysis of phospho-(Tyr701)-STAT1 and phospho-(Tyr705)-STAT3 (ii). C) Following GSNO cytokine treatment, the mRNA (i) and protein (ii) levels of ICAM-1 and VCAM-1 were analyzed. In these experiments, GSNO was pretreated for 3 h before treatment with cytokine mix. The mRNA and proteins were extracted 6 h and 12 h after cytokine treatment. The data was analyzed for mean values and standard error for all experiments; * p < 0.05 and ** p < 0.01 for comparison to vehicle (VHC) treated control (CTL) group; + p < 0.05 and ++ p < 0.01 for comparison to cytokine (Cyto) treated group.

Fig. 3.

Promotion of Aβ uptake by GSNO in endothelial and microglial cells. A) To assess the effect of GSNO on dynamin-2-mediated endothelial Aβ uptake, the mammalian expression vector expressing human dynamin-2 was transfected to bEND3 murine brain endothelial cells, and then the cellular levels of dynamin-2 and the endothelial uptake of fluorescence-labeled Aβ (FAM-Aβ) were analyzed (i). To examine whether GSNO is able to S-nitrosylate dynamin-2, the bEND3 cells were treated with GSNO (250 μM/3 h) and subjected to biotin-switch assay. The resulting S-biotinylated (S-nitrosylated) proteins in whole cell lysates were detected by western analysis using antibody specific to biotin (ii). To assess the involvement of S-nitrosylation mechanism on GSNO-induced Aβ uptake, the wild type, C86A mutant, or C607A mutant dynamin-2 was transfected to bEND3 cells and biotin-switch assay and Aβ uptake assay were performed following the GSNO treatment (250 μM/3 h). Following the pull-down of S-biotinylated proteins with avidin-conjugated agarose beads, the levels of co-precipitated dynamin-2 (DNM-2) were analyzed by western blot. The

levels of dynamin-2 in whole cell lysates were analyzed to examine the total dynamin-2 levels. The levels of β -actin in whole cell lysates were used for internal loading control. B) To examine the effect of GSNO on A β uptake, FAM-A β uptake assay was performed in bEND3 and BV2 microglial cells. Before the uptake assay, FAM-A β peptides were preincubated with basal media (fresh DMEM) or astroglial conditioned media (CM), which included lipidated ApoE (see Supplementary Data 1). To examine whether CM-mediated A β uptake is mediated through LRP1, the A β uptake assay was performed following the treatment of cells with LRP1 inhibitor, receptor-associated protein (Rap) (i). To examine whether GSNO enhances basal media (BM) or CM-mediated A β uptake, the cells were pretreated with GSNO (250 μ M/3 hrs) prior to performance of A β uptake assay (ii). To examine whether GSNO treatment enhances the endothelial uptake of A β_{40} as well as A β_{42} , the bEND3 cells were incubated with GSNO and then subjected to A β uptake assay using FAM-A β_{40} and FAM-A β_{42} peptides which were pre-incubated with CM (iii). A β uptake was also visualized under a fluorescent microscope (iv). C) In addition to bEND3 cells, the A β uptake assay was also performed on BV2 microglia under the same experimental conditions. The data were analyzed for mean values and standard error for all experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for comparison to mock control (empty), BM, or vehicle (VHC) treated group; + $p < 0.05$ for comparison to CM treated group.

Fig. 4.

Inhibitory effect of GSNO on neuronal $A\beta$ production. A) To examine the effect of GSNO treatment on neuronal $A\beta$ production, primary cultured rat cortical neurons were treated with increasing doses of GSNO for 24 h, and the levels of $A\beta_{40}$ (i) and $A\beta_{42}$ (ii) secreted into the media were determined by ELISA. In addition, the effects of glutathione ethylester (GSHEE), a cell permeable GSH analogue, and S-nitroso-N-acetylpenicillamine (SNAP), a S-nitroso donor, on $A\beta_{42}$ secretion (iii) and neuronal survival (iv and v) were compared with the equal concentrations of GSNO (200 μ M). For the assay of neuronal survival, lactate dehydrogenase (LDH) release assay (iv) and MTT assay (v) were performed. B) To examine the effect of GSNO treatment on the regulation of α - (i) or β -secretase (ii) activity, the neuron cells were treated with increasing doses of GSNO and α - or β -secretase activity assay was performed by method as described in materials and methods. Further, the effect of equimolar concentrations of GSNO, decomposed GSNO (dsGSNO), SNAP, GSHEE, and nitrite (200 μ M each) on neuronal β -secretase activity was assayed (iii). C) To examine whether GSNO is able to S-nitrosylate neuronal BACE1, the neuron cells treated with increasing concentrations of GSNO for 3 h were subjected to biotin-switch assay. The resulting S-biotinylated (S-nitrosylated) proteins in whole cell lysates were detected by western analysis using antibody specific to biotin. Following the pull-down of S-biotinylated proteins with avidin-conjugated agarose beads, the levels of co-precipitated BACE1 were analyzed by western blot. The levels of BACE1 in whole cell lysates were analyzed to examine the total BACE1 levels. The levels of β -actin in whole cell lysates were used for internal loading control. The data were analyzed for mean values and standard error for all experiments; * p < 0.05, ** p < 0.01, and *** p < 0.001 for comparison to vehicle (VHC) treated control (CON) group.