Carbon monoxide protects Neural Stem Cells against iron overload by modulating the crosstalk between Nrf2 and NF- κ B Signaling

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Research

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Abstract

Background: It is well known CO is an important gas transmitter. Although accumulating evidences have demonstrated pro-survival effects of CO against various insults, the precise mechanism explaining how neural stem cell is protected by CO also remains largely unknown.

Methods: After NSCs were challenged by iron overload with or without CO pretreatment, apoptosis of NSCs was tested by Flow cytometry and the level of antioxidant protein was also detected. ROS of NSCs was demonstrated via the fluorescent probe. We applied siRNA to demonstrate role of the protective effect of CO. In vivo experiment, NSCs with or without CO pretreatment were implanted into intracranial hemorrhagic mouse and the survival of NSCs were detected. The significance of the difference among mean values was determined by the Student–Newman–Keuls test or two-way analysis of variance followed by the Bonferroni post hoc test.

Results: CO effect on pro-survival was comparable to that obtained with pharmacological inhibitors of ROS. Its effect on pro-survival was accompanied by the inhibition of ROS and subsequent inhibition of NF- κ B. Such effect is believed to be mediated through Nrf2, in that siRNA-mediated inhibition of Nrf2 revealed that Nrf2 is required for the pro-survival effect of CO. CO also activated NQO-1, a downstream target of Nrf2, which was inhibited by down-regulating Nrf2. CO-mediated precondition results in Nrf2 stimulation and NF- κ B inhibition, suggesting that these two pathways act in an inverse manner to maintain redox homeostasis. The pro-survival effect of CO was also further endorsed by the evidences in mouse hemorrhagic stroke models.

Conclusions: Results, by showing that CO attenuates iron overload induced apoptosis by suppressing the NF- κ B signaling via Nrf2-mediated anti-oxidant effect, contribute to the understanding of essential cellular pathways. Our findings reveal CO preconditioning as a promising treatment strategy to improve efficacy of NSCs transplantation after HS.

Introduction

Neural stem cell (NSC) is considered to be a highly promising candidate for the therapy of hemorrhagic stroke (HS) because of its self-renew and multi-lineage differentiation characteristics. However, massive loss of donor cells post-engraftment becomes a major impediment that lessens the effectiveness of stem cells therapy. Transplanted cells in the deleterious microenvironment are subjected to various insults [1,2]. In some studies, only 1–3% grafted cells survived after grafting [3,4]. Oxidative stress mediated damage in stem cells has gained more attention as one of the vital mechanisms implicated in loss of implanted cells [5, 6]. Growing evidences indicate that there exists an oxidative component in HS-induced brain injury [7]. Indeed, accumulation of reactive oxygen species (ROS) generated by iron overload after HS, leads to ruining the cellular redox-balance. This event becomes a leading cause of brain damage and cell death after HS. Therefore, targeting iron overload-induced oxidative stress is a promising strategy to improve the clinical efficacy of NSCs therapy after HS.

Endogenous CO is a major product in heme degradation catalyzed by heme oxygenase 1 (HO-1) and it is considered to play a pivotal role in the protection effect of HO-1. CO has been recognized as an important gas-transmitter in the heart, lung, liver, and the central nervous system (CNS) [8, 9]. In the CNS, CO regulates various signaling pathways in neurons, astrocytes, and stem cells, resulting in neuroprotective and anti-inflammatory effects after acute or chronic CNS injuries [10, 11, 12]. The development of CO-releasing molecules (CORMs) provides a safe and specific solution for CO deliver [13]. Previously, we showed CO decreased expression of cleaved caspase 3 via inhibiting NF- κ B activation, which accounted for the protection against iron overload-induced damage in NSCs [14]. Nevertheless, the molecular mechanisms explaining how the NF- κ B pathway is modulated by CO also remain largely unknown.

Here, we aimed to elucidate whether and how the cytoprotection by CO preconditioning is the result of a cross-talk between Nrf2 and NF- κ B signaling pathways that leads to anti-oxidative and pro-survival effects. Furthermore, we provided in vivo evidences to support the pro-survival effects of CO on NSCs.

Materials And Methods

CORM-2 (288144) and N-acetyl-cysteine (NAC) (A7250-10G) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CORM-2 was dissolved in dimethyl sulfoxide, and then diluted in culture media to achieve the required concentrations. Inactivated CORM-2 (iCORM-2) was prepared by dissolving CORM-2 under the same conditions for 3 days at room temperature to liberate all CO from the molecule [14]. NSC C17.2, a stable, fully characterized, mouse stem cell line [15], was kindly provided by Prof. Jin WL (Institute of Nano Biomedicine and Engineering, Shanghai Jiao Tong University, Shanghai, China). Ferrous chloride (FeCl_2) was purchased from Sinopharm Chemical Reagent (Shanghai, China). Annexin V-FITC Apoptosis Detection Kit (C1062), Diamidino-2-phenylindole dihydrochloride (DAPI) (C10005) and Reactive Oxygen Species Assay Kit (S0033) were from Beyotime Biotechnology (Shanghai, China). The primary antibodies Nrf2 (C-20) (SC-722) and NF- κ B P65 (SC-372) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NQO1 antibody (ab2346) was from Abcam (Cambridge, UK). β -Actin (4970), Tubulin (2148) and H3 (12167), were from Cell Signaling Technology (USA).

In vitro experiment

Cell Culture And Treatment

C17.2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, USA) containing 10 % (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA), 5 % (v/v) horse serum (Gibco), and 2 mM L-glutamine (Gibco) at 37°C in a humidified incubator supplemented with 5 % (v/v) CO₂. C17.2 cells were pretreated with or without 50 μ M CORM-2 / iCORM-2 for 6 h prior to stimulation with 500 μ M FeCl_2 for 24 h [14].

Western blot analysis

Western blotting was performed using standard techniques. Briefly, C17.2 cells were grown in 6-well cell culture plates (2×10^5 cells/mL). After treatment, Protein was loaded on 8 % (w/v) or 10 % (w/v) SDS-

polyacrylamide gel and protein levels determined by Western blotting using Nrf2 (1:1000), NF- κ B p65 (1:1000), NQO-1 (1:1000) and HRP-conjugated secondary antibody (1:5000). H3 (1:5000) antibodies, and Tubulin (1:5000) antibodies were used as marker proteins for nuclear and cytosolic extracts respectively.

RNA interference by small interfering RNA (siRNA) of Nrf2

Pre-designed siRNAs against mouse Nrf2 and nontargeting control-pool siRNA were purchased from GenePharma (Shanghai, China). For Nrf2-siRNA transfection, C17.2 cells were grown in 6-well plates (2×10^5 cells per well) until the confluence of cells reached approximately 50%. The cells were then subjected to transient transfection with Nrf2-negative control siRNA, Nrf2-siRNA using the siRNA transfection reagent Lipofectamine™ 2000, following the manufacturer's protocol. After 48 h, the transfected cells were exposed to CORM-2/ iCORM-2 for 6 h, followed by lysis buffer for western blot analysis. Nontargeting siRNA construct (NC) was used as negative control.

Intracellular reactive oxygen species (ROS) measurement

Detection of intracellular oxidative states was performed by using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) [16]. Briefly, C17.2 cells were grown in 6-well cell culture plates (2×10^5 cells/mL) and pretreated with CORM-2 (50 μ M) or iCORM-2 for 6 h prior to stimulation with 500 μ M FeCl₂ for 24 h. The DCFH-DA solution (10 μ M) was added to the suspension of the cells. After incubation with DCFH-DA for 20 min at 37°C, the fluorescence of DCF was quantified using fluorescence microscopy at excitation and emission wavelengths of 488 and 525 nm, respectively. ROS production was expressed as a percentage of the control. The fold-increase of ROS generation was compared with the control cells, which were arbitrarily considered as 1-fold.

Flow cytometry

Apoptosis was determined by using Annexin V-FITC apoptosis kits (Beyotime, China). The assays were performed according to the manufacturer's instructions. Briefly, the cells were pretreated with CORM-2 (50 μ M) or iCORM-2 for 6 h prior to stimulation with 500 μ M FeCl₂ for 24 h. After treatment, single cell suspension was incubated with 5 μ L of Annexin V-FITC and 10 μ L of PI in 195 μ L of binding buffer for 15 min at room temperature in the dark. Then, the rates of apoptosis were analyzed by a flow cytometry (BD Biosciences, San Jose, CA). A minimum of 10,000 events were acquired for each sample.

Immunofluorescent staining

NSCs cultured on eight-well chamber slides poly-D-lysine-coated glass slips in 24-well dishes were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Then, cells were incubated for 1 h in blocking solution (PBS containing 3% bovine serum albumin and 0.3% Triton X-100). Cells were then incubated with antibodies against Nrf2 (1:200), NF- κ B p65 (1:200) at 4 °C overnight. After three washes, samples were incubated with donkey anti-rabbit IgG (A21207; Alexa Fluor 594, 1:500; Invitrogen) or goat

anti-rabbit IgG (A11008; Alexa Fluor 488, 1:500; Invitrogen) at 37 °C for 2 h. Samples were then washed with PBS and cover slipped with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlington, ON, Canada) nuclear counterstain. Samples were mounted onto slides with anti-fade solution and examined under a confocal laser-scanning microscope.

In vivo experiments

Animals

All animal procedures were carried out in accordance with the guideline of the institutional animal care and use committee of The Affiliated Wuxi No.2 Peoples' Hospital of Nanjing Medical University, Wuxi, China. Donate NSCs were harvested from homozygous red fluorescent protein transgenic (RFP Tg) mice (C57BL/6- RFP Tg; Model Animal Research Center of NanJing University, Nanjing, China). Wild-type C57BL/6 mice (Slac laboratory animal CO.LTD, Shanghai, China) were for HS models. All animals were housed on a 12: 12-h light/dark cycle with environmental temperatures at 18-22°C. Food and water were freely available.

Isolation and culture of fetal neural stem cells

NSCs were isolated from the subventricular zones of RFP Tg fetal mice at 14 days of gestation, as described previously [17]. In brief, bilateral subventricular zones were dissected and mechanically dissociated. After collection, the cells were resuspended in Neuro-basal-A medium (Invitrogen, Carlsbad, CA, USA) containing B-27 supplement (Invitrogen), L-glutamine (Invitrogen), 20ng/ml mouse fibroblast growth factor-basic (PeproTech, Rocky Hill, NJ, USA), and 10 ng/ml mouse epidermal growth factor (PeproTech, Rocky Hill, NJ, USA). Cells were grown as suspending neurosphere. The medium was half-changed every 2 days and cells were passaged weekly. Cells that had been passaged 5 to 10 times were used for the experiments.

HS model and experiment group

We used an experimental HS procedure, described previously [2]. Briefly, male C57BL/6 mice (8 weeks old, 20to25g) were anesthetized with ketamine/xylazine (100 mg/10 mg/kg, Sigma, St. Louis, MO), and positioned in the stereotactic frame. After a midline scalp incision, a hole was drilled in the right side of the skull (0.0mm anterior and 2.5mm lateral to the bregma). Blood (20 µl) without any anticoagulant was collected from the tail tip for injection. A 30-gauge needle attached to a 50-ml Hamilton micro-syringe was inserted 3.5mm ventral from the surface of the skull. Ten microliters of blood was injected over 10 minutes. The remaining 10 ml of blood was injected using the same procedure. After infusion, the needle was left in place for 25 minutes and then slowly removed.

Experimental groups were non-precondition group, transplantation of RFP-NSCs ($3 \times 10^5/2\mu\text{l}$; n=15); iCORM-2-precondition group, transplantation of iCORM-2 preconditioned RFP-NSCs ($3 \times 10^5/2\mu\text{l}$; n=15) and

CORM-2-precondition group, transplantation of CORM-2 preconditioned RFP-NSCs ($3 \times 10^5/2\mu\text{l}$; $n = 15$). All methods and assessments described below were carried out by individuals blinded to the groups.

CO preconditioning and Intracerebral transplantation

CORM-2/iCORM-2 dissolved in DMSO was added to the cell culture medium (final concentration: $50 \mu\text{M}$) for 6 h prior to transplantation, followed by drug washout before transplanting. NSCs were transplanted on the 3rd day after HS as described previously [2].

Assessment of survival of donate NSCs

HS animals were sacrificed on the thirtieth days after transplantation to detect the implanted NSCs. The survival of donate RFP Tg NSCs were assessed on 12 serial coronal sections per brain (0.25mm apart) using unbiased computational stereology.

Statistical analysis

Data were analyzed by using SPSS version 18 software. The significance of the difference among mean values was determined by the Student–Newman–Keuls test or two-way analysis of variance followed by the Bonferroni post hoc test. P values < 0.05 were accepted to be statistically significant.

Results

The ROS role in apoptosis of NSCs challenged by iron overload

The present investigation was aimed to determine the role of ROS in iron overload –induced apoptosis in neural stem cells. After 24 h of iron overload, flow cytometry analysis revealed a significant increase of apoptosis of NSCs up to $21.4 \pm 3.0\%$ VS control cells $1.9 \pm 0.4\%$ ($p < 0.05$). As the results showed in Fig1, co-treatment with ROS inhibitor N-acetylcysteine (NAC 1mM) protected NSCs against iron overload-induced cell apoptosis and the apoptosis rate decreased from $21.4 \pm 3.0\%$ to $7.5 \pm 0.8\%$ ($p < 0.05$).

Iron overload induced activation of NF- κ B signaling pathway via ROS

Our previous study demonstrated iron overload induced significant activation of NF κ B [14]. To confirm the role of ROS on iron overload-induced NF κ B activation, immunofluorescence was performed to detect the nuclear translocation of NF κ B (Fig.1E). We found that NF κ B was tethered in cytoplasm in un-stimulated control cells, but in those cells challenged by FeCl_2 , the nuclear translocation of NF κ B was significant. In NSCs co-treated with ROS inhibitor (NAC 1mM) the translocation was inhibited. For further confirmation of ROS role, next we performed Western blot and examined the changes in NF κ B. We found that iron overload -induced up-regulation of NF κ B in nucleus fraction was substantially suppressed by NAC (Fig.1G). These findings strongly demonstrated that ROS signaling cascades are involved in the regulation of iron overload -induced NF κ B activation.

CO inhibits iron overload-induced intracellular ROS accumulation

ROS are known to play a key role in the damage of neural cells after HS. Our in vitro studies demonstrated that iron overload-induced ROS accumulation in NSCs may mediate the increase of apoptosis of NSCs. As depicted in Fig. 2, NSCs subjected to FeCl_2 (500 μM) for 24h triggered the intracellular ROS production ($p \leq 0.001$). However, the aberrant accumulation of ROS was reduced by preconditioning with CORM-2 (50 μM) prior to iron overload stimulation ($p \leq 0.05$). Despite, cells incubated with iCORM-2 showed little effect on the increased ROS levels compared to CORM-2 pretreatment. This finding suggests that resistance to oxidative stress is one of the mechanisms of CO-mediated cyto-protection.

CO triggers the nuclear translocation of Nrf2 and up-regulates the level of Nrf2 in nucleus

Nrf2 is an important transcription factor, which mediate antioxidant responses upon stimulation. Here we examined whether CO suppressed the iron overload-induced ROS accumulation via activation of Nrf2. Nrf2 is normally located in the cytosol, bound and inhibited by Keap1. Following activation, Nrf2 is released from this inhibition, and translocates to the nucleus and activates transcription of downstream antioxidant genes. To verify this, immunofluorescence was performed to detect the nuclear translocation of Nrf2. We found that Nrf2 was tethered in cytoplasm in un-stimulated control cells, but in those cells preconditioned by CORM-2 such translocation is significant (Fig.3A). For further confirmation of CO antioxidant effects, next we performed Western blot and examined the changes in nuclear and cytosolic fractions of NSCs. Interestingly, Consistent with nuclear translocation of Nrf2 induced by CO, a significantly increase of the level of Nrf2 in nucleus fractions about 2-3 fold was determined by western blot (Fig.3C).

CO protective effects are associated with enhanced antioxidant genes

We next investigated whether CO could stimulate Nrf2 mechanism, with its transcriptional activity being related to phase II antioxidant enzyme genes such as NQO-1 which are responsible for scavenging ROS [18]. We hypothesized that protective effect of CO may be due to induction of antioxidant genes, NQO-1 through the typical Nrf2/ ARE-signaling cascades. To confirm this, first we measured the time dependent changes in proteins levels of NQO-1 after CO preconditioning. As expected, CO preconditioning significantly increased NQO-1 protein expressions in a time dependent manner (Fig.4A). The significantly increased NQO-1 protein after CO preconditioning started after treatment for 6h (Fig.4A). CO preconditioning increased NQO-1 significantly in NSC challenged by FeCl_2 (Fig.4B). Next we down-regulate Nrf2 to confirm whether CO could promote antioxidant genes via Nrf2/ARE signaling pathway. Results showed Nrf2-siRNA significantly down-regulated CO-mediated protein expression of Nrf2, NQO1 (Fig.4C).

CO suppresses NFkB activation through up-regulation of Nrf2

It has been reported that CO pretreatment can suppress the activation of NFkB. To delineate the role of Nrf2 signaling cascade on the fate of NFkB, we examined nucleus fraction protein level of NFkB in NSCs

transfected by siRNA Nrf2. We found that NFκB increased significantly and the inhibition of NFκB by CO was abolished in nucleus fraction after Nrf2 was knocked down (Fig.4D). These findings illustrate that CO-mediated suppression of iron overload-induced NFκB activation was regulated by activation of Nrf2.

Nrf2 knockdown diminishes the protective effects of CO in iron overload stimulated NSCs

To reveal the importance of Nrf2 activation behind CO-induced protection against iron overload, we developed an Nrf2 knockdown C17.2 cells using siRNA transfection. The apoptosis of NSCs was determined by flow cytometry. CO decreased the apoptosis of NSCs induced by iron overload from $24.4 \pm 4.4\%$ to $7.5 \pm 1.0\%$. However, CO-mediated protection was abolished in the cells transfected by siRNA Nrf2 with an apoptosis even up to $38.6 \pm 3.1\%$ (Fig.5A). The protective effect was exclusively for CO because pretreatment with iCORM-2 did not cause any additional effect, i.e. the apoptosis of neural stem cells were unmodified. Concomitant with the apoptosis of NSCs, CO-induced up-regulation of Nrf2 and NQO-1 proteins was barely observed in Nrf2 knockdown cells (Fig.4C). This data suggest that induction of NQO-1 by CO requires Nrf2 activation, which seems to be essential behind CO antioxidant effects against iron overload.

Quantification of survival of the transplanted red fluorescent protein-positive neural stem cells in vivo

The transplanted RFP-positive cells were counted on 12 serial coronal sections per brain as described [2]. CO preconditioning NSCs showed a significant improvement of survival in HS mice (Fig.6B) while pretreatment with iCORM-2 did not cause any additional effect (Fig.6C)

Discussion

For the first time we provide in vitro and in vivo evidences to demonstrate that CO protects implanted NSCs against iron overload –induced redox imbalance in HS. Our results show that CO effectively enhances the survival of NSCs after HS which is likely associated with reestablishing the redox balance via modulation of the crosstalk between Nrf2 and NF-κB signaling pathway.

It has been well established that iron overload was responsible for the adverse outcome in stem cell [19,20]. In our study, when NSCs were treated with FeCl_2 for 24 h, we found a marked cellular toxic effect consistent with a sustained increase of ROS. Under these experimental conditions, nuclear protein level of NF-κB was increased concomitant with significant translocation. The activation of the NF-κB signaling pathway seems to be responsive to excessive ROS generation, thus affecting the redox sensitive NF-κB signaling pathway [21]. Indeed, considerable demonstrations established the link between oxidative stress and NF-κB which was mainly from the inhibition of NF-κB activation by cellular antioxidants [22,23]. Further evidence in our study showed that iron overload-induced NF-κB activation was inhibited by N-acetylcysteine (NAC) (pharmacological inhibitors of ROS), which implies that iron overload -mediated NF-κB activation in NSC was ROS dependent. A growing body of evidence has demonstrated the deleterious role of the activation of NF-κB induced by iron overload [24,25]. Such deleterious role of the activation of NF-κB has also been shown in PC12 cell and hippocampal neuron cell challenged by other oxidant reagent

[26]. The neuroprotective effect of inhibition of NF- κ B was found in NSCs in our research and reproduced in other study [27]. Our previous data indicated pretreatment with CO could inhibit the activation of NF- κ B induced by iron overload. Such inhibition was reasoned by suppression of ROS as evidenced by suppressed ROS in NSCs upon the pretreatment with CO and suppressed NF- κ B activation upon treatment via inhibition of ROS with NAC. In this regard, CO protective effects are similar to these of aspirin, which has been shown to attenuate H₂O₂-induced apoptosis in Hela cell through NF- κ B inactivation [28]. The present findings further emphasized that CO-mediated suppression of NF- κ B activation is associated with the inhibition of iron overload-induced ROS accumulation in NSCs.

The Nrf2 signaling pathway is the main pathway responsible for cell defense against oxidative damage and maintaining the cellular redox balance [29]. Disruption of Nrf2-mediated Nrf2/ARE-signaling pathway could exacerbate the oxidative damage[30]. There are several examples that CO acts in CNS by activation of a preconditioning state via Nrf2 signaling [31,32]. In our study, the activation of Nrf2 was evidenced by the significant upregulation of the protein level of Nrf2 and the nuclear translocation of Nrf2. Elevated antioxidant enzymes levels by CO through Nrf2/ARE cascades may play a frontline of defense against iron overload-induced ROS. The expressing of NQO-1 in NSC exhibited in a time-dependent manner after CO preconditioning. The pivot role of NQO-1 induced by Nrf2 in NSC against oxidative stress has been well established [33]. Nrf2 knockdown (siNrf2) studies confirmed this phenomenon by detecting the unchanged NQO-1 level and significant cell apoptosis even in the presence of CO.

Recently, substantial efforts have been put on the issue that when Nrf2 talks who is listening. It is worthwhile to note that suppression of NF- κ B accumulation by CO was accompanied by activation of Nrf2. Evidences have also been accumulating that suggest that activation of Nrf2 antioxidant signaling occurs concomitantly with suppression of NF- κ B response. [34,35]. Furthermore, Nrf2^{-/-} mice has been demonstrated increased NF- κ B activation when compared with wild-type after traumatic brain injury[36]. These findings further emphasize the crosstalk between NF κ B and Nrf2 transcription factors [37]. The relationship between Nrf2 and NF- κ B signaling pathway is now suggested as an important cross-talk mechanism by which Nrf2 can overcome apoptosis and provide protection against various deleterious stresses [38, 39]. Several lines of evidence suggest that Nrf2 regulates NF- κ B activation through maintenance of redox homeostasis. In the presence of CO, Nrf2 protein expression was up-regulated concomitant with increase of nuclear translocation, which leads to the induction of antioxidant enzymes NQO1 with simultaneous inhibition of iron overload-induced ROS production and a subsequent suppression of the NF- κ B. Nrf2-mediated expression of NQO1 has been demonstrated efficiently to neutralize reactive oxygen species (ROS) and to cause the reduction of ROS-mediated NF- κ B activation [40]. Notably, siRNA-mediated suppression of Nrf2 expression in NSCs also witnessed that iron overload-induced elevation of NF- κ B levels remains higher and more significant cell apoptosis occurs. Since Nrf2-regulated genes are responsible for increased cellular oxidative stress and ROS is involved in iron overload-induced activation of NF- κ B, we postulate that Nrf2 activation by CO may, at least in part, down-regulate the iron overload-induced NF- κ B through the modulation of redox status. In accordance with our findings, Minellia et al have shown that regulation of the crosstalk between Nrf2 and NF- κ B by CO is highly neuroprotective by reducing oxidative stress in PC12 and murine microglial BV2 cells [41]. These

findings underlined the possibility that CO-mediated neuro-protective effect may be achieved by the modulation of the crosstalk between Nrf2 and NF- κ B via reestablishing the redox balance in NSC.

On particular interest of our in vitro findings, we conducted in vivo studies to demonstrate the therapeutic potentials of CO pretreatment in HS mouse model. The pro-survival effect of CO was further endorsed by in vivo evidences. In our experiment, survival of more CO preconditioned RFP-NSCs ascertains the protective role of CO. Also, day 3 after HS is an important time point for hematoma evacuation operation in clinic, and NSCs could be implanted at the same time of hematoma evacuation. SO, day 3 after HS is an interesting time point for investigation of the survival of donor NSCs and it has potential implication for clinical therapy [42].

Discussion

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Conclusion

Our findings demonstrated that CO preconditioning reprograms NSCs to tolerate oxidative stress induced by iron overload after HS. The anti-oxidant effects of CO are mediated through the activation of Nrf2 and subsequent up-regulation of antioxidant genes NQO-1. Our data directly implies that CO protects NSCs against iron overload by modulating the crosstalk between Nrf2 and NF- κ B via reestablishing intracellular redox balance. Through this better understanding of CO-mediated signal transduction and gene regulations involved in redox status, our findings may provide an opportunity to improve the efficacy of NSCs therapy after HS.

Declarations

Ethics approval and consent to participate: The study was approved by the Ethical committee of The Affiliated Wuxi No.2 Peoples' Hospital of Nanjing Medical University, Wuxi, China

Consent for publication: All authors consent for publication

Availability of data and material: Y

Competing interests: All authors declare no conflicts of interests

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Authors' contributions: Designs and funding (Xie Zhengxing); Cell culture and treatment, Western blot analysis(Wang Qing); RNA interference and Flow cytometry(Xie Zhengxing, Hu Aiying); Immunofluorescent staining, Isolation and culture of fetal neural stem cells, HS model and Intracerebral transplantation(Xie Zhengxing, Hu Aiying); Statistical analysis(Xie Zhengxing,Miao Zengli)

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Abbreviations

CNS central nervous system

CO carbon monoxide

CORMs CO-releasing molecules

DCFH-DA 2',7'-dichlorofluorescein diacetate

FeCl₂ Ferrous chloride

HS hemorrhagic stroke

NAC N-acetylcysteine

NF- κ B nuclear factor kappa-B

Nrf2 nuclear factor erythroid 2-related factor 2

NSC neural stem cell

RFP Tg fluorescent protein transgenic

ROS reactive oxygen species

siRNA RNA interference by small interfering RNA

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Figures

Figure 1

Role of ROS in NSCs challenged by iron overload in vitro. (A) apoptosis of NSCs without any insult; (B) apoptosis of NSCs challenged by 24h iron overload without NAC co-incubation. (C) Apoptosis of the NSCs challenged by 24h iron overload without NAC co-incubation. (*p<0.05 vs. the control group; #p<0.05 vs. FeCl₂ group). Data represent the mean ± SD (n = 3).(E) Representative images showing co-incubation with 10 μM NAC inhibited the translocation of NF-κB induced by iron overload. Double staining with DAPI (blue) and NF-κB (green). (F), (G) Representative bands and quantification of NF-κB in the cytoplasm and nuclear fraction (*p<0.05 vs. the FeCL2 group; #p<0.05 vs. FeCL2+NAC- group). Data represent the mean ± SD (n = 3). Bar25 μm

Figure 2

Effect of CO on ROS generation. Cells were challenged by 500μM FeCL2 with or without pretreatment with 50 μM CORM-2/ICORM-2 for the indicated time periods and followed by incubation with 10 μM ROS-

sensitive fluorophore H2DCFDA. Quantitative induction is shown as an increase in the treated cells relative to the control. (**p<0.001 vs. the control group; #p<0.05 vs. the FeCL2 group). Data represent the mean ± SD (n = 3). Bar 25 μm .

Figure 3

Effects of CO preconditioning on Nrf2 signaling pathway in NSC challenged by iron overload in vitro. (A) Representative images showing CO preconditioning (50 μM CORM-2) triggered activation of Nrf2. Double

staining with DAPI (blue) and Nrf2 (red). (B), (C) Representative bands and quantification of Nrf2 in the cytoplasm and nuclear fraction (#p<0.05 vs. the FeCl₂ group or iCORM-2 group). Data represent the mean ± SD (n = 3). Bar 25 μm .

Figure 4

Effects of CO on antioxidant protein level in NSCs in vitro. (A) Cells were pretreated with 50 μM CORM-2 for the indicated time periods. Representative bands and quantification revealed that CO preconditioning

induced higher expression of the Nrf2-regulated antioxidant genes NQO1 in a time dependant manner. (* $p < 0.05$ vs. the control(0h) group). (n = 3) (B) CO preconditioning upregulated the expression of NQO1 in NSC challenged by 24h iron overload. (# $p < 0.05$ ** $p < 0.001$ vs. the FeCl₂ group). (C) Knocking down Nrf2 by Nrf2 SiRNA reverses the upregulation of NQO1. (D) Representative bands in the nuclear fraction showing CO suppresses NF κ B activation through upregulation of Nrf2 (* $p < 0.05$; # $p < 0.05$ vs. NC group). Data represent the mean $\pm$ SD (n = 3).

Figure 5

Inhibition of Nrf2 abolished CO-induced cytoprotection in vitro. Apoptosis of the NSCs was analyzed by flow cytometry after 24h iron overload. Iron overload induced significant apoptosis in NSCs(A,B,F); CO preconditioning significantly attenuated the iron overload induced apoptosis(C,D,F); A significant increase of apoptosis in NSC knocked down Nrf2 with SiRNA after 24 h of iron overload(E,F). (* $p < 0.05$ vs. the FeCl₂ group or iCORM-2 group; # $p < 0.05$ vs. the control group; \$ $p < 0.01$ vs CORM-2 group.). Data represent the mean $\pm$ SD (n = 3).

Figure 6

Increased cell survival in vivo. Fluorescent staining with RFP (red) revealed that the grafted cells surrounded the HS lesion 30 days after transplantation (A) NSCs in non-preconditioned group; (B) more NSCs in CO preconditioned group; (C) NSCs in iCORM-2-preconditioned group. ($\#p \leq 0.05$ vs. non-preconditioned group or iCORM-2-preconditioned group)