

# Sinomenine activation of Nrf2 signaling prevents inflammation and cerebral injury in a mouse model of ischemic stroke

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**Abstract.** Sinomenine (SINO), which is used clinically to treat rheumatoid arthritis and neuralgia, is derived from the root and stems of *Sinomenium acutum*. SINO has been reported to exert analgesic, sedative and anti-inflammatory effects, and provides a protective role against shock and organ damage. Studies have suggested that SINO primarily exerts its anti-inflammatory function by inhibiting NF- $\kappa$ B signaling. There is also evidence to indicate that SINO may regulate inflammation Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling. The present study aimed to investigate whether the anti-inflammatory and cerebral protective effects of SINO were induced through Nrf2 both *in vitro* and *in vivo*. The results revealed that SINO significantly upregulated Nrf2 protein expression levels, increased Nrf2 nuclear translocation and the upregulated the protein expression levels of downstream factors. The treatment of a middle cerebral artery occlusion model mice with SINO effectively reduced cerebral damage and inflammation, and restored the balance in cerebral oxidative stress. In addition, SINO treatment also promoted Nrf2-dependent

microglia M1/M2 polarization and inhibited the phosphorylation of I $\kappa$ B $\alpha$  as well as NF- $\kappa$ B nuclear translocation. This revealed an important upstream event that contributed to its anti-inflammatory and cerebral tissue protective effects. In conclusion, the findings of the present study identified a novel pathway through which SINO may exert its anti-inflammatory and cerebral protective functions, and provided a molecular basis for the potential applications of SINO in the treatment of cerebral inflammatory disorders.

## Introduction

Ischemic stroke is a common clinical cerebrovascular disease that is accompanied by significant disability and mortality, with survivors often suffering from various degrees of neurological dysfunction (1). The mechanism of cerebral ischemic injury involves a number of pathophysiological processes, with accumulating evidence suggesting that inflammation is involved (2,3). Therefore, the regulation of inflammatory signaling may represent a potential treatment strategy for ischemic stroke.

Microglia are a type of glial cell, which are the resident macrophages of the brain (4). Microglia are the first and most important line of defense in the central nervous system (CNS). As the immune effector cells of the CNS, microglia have been demonstrated to serve an important role in the processes behind cerebral injury (5). For example, activated microglia have been found in the surrounding lesions of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, muscular amyotrophic lateral sclerosis and multiple sclerosis (6). Activated microglia can release a number of molecules, including cytotoxic substances such as nitric oxide (NO), oxygen free radicals and proteolytic enzymes, as well as inflammatory factors such as IL-1, TNF- $\alpha$  and IFN- $\gamma$ , which are essential for the pathological process of cerebral ischemic injury (7). Microglia exist in a dynamic equilibrium between the pro-inflammatory (M1) and anti-inflammatory (M2) types, and the polarization state is associated with the local microenvironment (8). *In vitro*, M0 type microglia have been revealed to be induced to the M1 type through lipopolysaccharide or IFN- $\gamma$ , or induced to the M2 type through IL-4, IL-10 and TGF- $\alpha$  (8). The excessive activation of M1 type microglia

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**Abbreviations:** SINO, sinomenine; MCAO, middle cerebral artery occlusion; OGD, oxygen and glucose deprivation; Nrf2, nuclear factor-erythroid 2-related factor; HO-1, heme oxygenase-1; NQO1, NAD(P)H: Quinoneoxidoreductase 1; Arg-1, arginase-1; NOS2, nitric oxide synthase 2; SOD, superoxide dismutase; GPx, glutathione peroxidase

**Key words:** sinomenine, nuclear factor-erythroid 2-related factor, microglia, cerebral protection, inflammation

leads to the secretion of large amounts of inflammatory factors and free radicals, such as macrophage colony-stimulatory factor, TNF- $\alpha$ , IL-1 and IL-6, which subsequently promote a major inflammatory response (5). Therefore, it remains necessary to avoid the occurrence of neuroinflammatory reactions by regulating the balance of the polarization state of M1 and M2 microglia.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a major regulator of the cell defense response against chemical or oxidative stress (9). Nrf2 modulation is achieved by various proteins and signaling pathways at both the cytoplasmic and nuclear level (10). The pharmacological activation of Nrf2 has been suggested as a promising therapeutic approach for several chronic diseases, including Alzheimer's and Parkinson's diseases (11). Interestingly, a previous study revealed that sinomenine (SINO) provided protection against ischemic/reperfusion-associated liver damage in a heme oxygenase-1 (HO-1) dependent manner (12), indicating that SINO may exert its protective effects through the Nrf2 signaling pathway, since HO-1 is mainly regulated by Nrf2 signaling (13).

SINO is an active alkaloid extracted from the Chinese medical plant, *Sinomenium acutum* (14). Previous studies have reported that SINO possesses anti-inflammatory and immunoregulatory properties, as well as exhibiting significant therapeutic efficacy for rheumatoid arthritis (15,16). In addition, SINO is protective against various autoimmune and inflammation-associated diseases (17,18). SINO has been found to modulate immune responses mainly through down-regulating the expression of inflammation-related molecules, including NO, TNF- $\alpha$ , IL-1 $\beta$  and prostaglandin E3, both *in vitro* and *in vivo* (19-21). Other studies have demonstrated that SINO exerts its anti-inflammatory role by inhibiting I $\kappa$ B phosphorylation and subsequent NF- $\kappa$ B transcription (22,23). However, the underlying mechanism of action behind the protective effect of SINO in cerebral ischemic injury remains unclear. Therefore, the present study aimed to investigate the role of SINO and its potential mechanism of action in cerebral ischemic injury. The results indicated that SINO may inhibit neuroinflammation by targeting the microglia Nrf2 signaling pathway, both *in vivo* and *in vitro*, which provided a novel target for the treatment of ischemic stroke.

## Materials and methods

**Chemicals and reagents.** The following antibodies were used in the present study: SINO (MedChemExpress); ML385 (MedChemExpress); Nrf2, Keap1, NAD(P)H: Quinoneoxidoreductase (NQO1), Lamin B and HO-1 antibodies (Abcam);  $\beta$ -actin antibodies (Bioworld Technology, Inc.); NF- $\kappa$ B p65, Phosphorylated (p)-I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$  antibodies (Cell Signaling Technology, Inc.); HRP-conjugated secondary antibodies (Bioworld Technology, Inc.); and Dylight488 conjugated goat anti-rabbit IgG secondary antibodies (Bioworld Technology, Inc.).

**Cell culture and treatment.** BV2 cells (Shanghai Kang Lang Biological Technology Co. Ltd.) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and maintained

at 37°C in a 5% CO<sub>2</sub> incubator (Thermo Fisher Scientific, Inc.). Oxygen glucose deprivation (OGD) was induced according to a previously described method (24). BV2 cells were cultured at 37°C with deoxygenated DMEM without glucose and FBS, in the presence of a premixed gas (1% O<sub>2</sub>, 94% N<sub>2</sub> and 5% CO<sub>2</sub>) for 4 h. The cells were subsequently cultured in normal DMEM supplemented with 10% FBS and maintained at 37°C in a 5% CO<sub>2</sub> incubator. Cells in the control group were cultured with normal DMEM and 10% FBS throughout.

**Cell viability analysis.** BV2 cells were cultured on a 96-well plate and treated with increasing concentrations (0-200  $\mu$ M) of SINO for 24 h. Cell viability was then measured using a Cell Counting Kit-8 (ProteinTech Group, Inc.) according to the manufacturer's instructions. In total, 1 h was the duration of the incubation with the CCK-8 reagent at 37°C.

**Establishment of middle cerebral artery occlusion (MCAO).** The present study was approved by the Ethics Committee of the Medical College of Xi'an Peihua University. A total of 48 male C57BL/6 mice (age, 12 weeks; weight, 25 $\pm$ 2 g) were obtained from Xi'an Jiaotong University Experimental Animal Center. They were housed in the specific pathogen-free conditions with standard temperature (22 $\pm$ 1°C), humidity (50-60%) and light conditions (12 h light/dark cycle), with access to food and water *ad libitum*.

MCAO surgery was performed as previously described (25). Mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate (400 mg/kg). A silicone-coated 6-0 suture (Covidien; Medtronic, Ltd.) was slowly inserted from the exposed external carotid artery to the internal carotid artery and wedged into the circle of Willis to obstruct the opening of the middle cerebral artery. The distance from the bifurcation of the internal/external carotid artery to the middle cerebral artery was 9 $\pm$ 1.0 mm. The suture was withdrawn following 60 min of obstruction. Mice were divided into four groups (n=6) as follows: i) The sham group that was identical with MCAO but did not include the occlusion of the middle cerebral artery; ii) SINO group that received SINO at a dose of 20 mg/kg daily for 3 days; iii) MCAO group; and iv) SINO/MCAO group in which SINO was subsequently injected intraperitoneally into mice 6 h after MCAO surgery at a dose of 20 mg/kg daily for 3 days. Notably, 20 mg/kg SINO used were performed as previously described (26,27). The sham group received the same volume of saline intraperitoneally. After completion of the experiment, mice were sacrificed by exsanguination under deep anesthesia (sodium pentobarbital intraperitoneal injection, 50 mg/kg). Death was confirmed by cessation of the heartbeat.

**Histological analysis.** For histological analysis, brain tissues were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Paraffin-embedded tissues were cut into 4- $\mu$ m-thick sections and stained with H&E using standard procedures (28). Stained sections from 6 animals were visualized in 10 randomly selected fields of view using a light microscope with x20 magnification (Nikon Corporation).

**Brain water content measurement.** Mice were deeply anesthetized and decapitated 3 days following MCAO surgery. Brain

edema was analyzed according to a previously described method (29). Wet brains were weighed and then immediately dried at 95°C overnight. The brain water content was calculated using the following formula: [(Wet tissue weight-dry tissue weight)/wet tissue weight] x100%.

**Measurement of cerebral antioxidant enzyme activities.** The activities of cerebral enzymes, glutathione (GSH) peroxidase (GPx) and superoxide dismutase (SOD), were measured using their respective colorimetric assay kits (Abcam), according to the manufacturers' protocols. Briefly, for the GPx assay, the tissue homogenates were incubated with GSH reductase (GR), GSH, tert-butyl hydroperoxide and NADPH at 25°C for 3 min and the absorbance was measured at wavelength of 340 nm. In this assay, the generated GSH disulfide was reduced to GSH following the consumption of NADPH by GR. GPx activity was proportional to the decrease of NADPH (which is measured at 340 nm). For the SOD assay, tissue homogenates were treated with water-soluble tetrazolium at 37°C for 30 min and the absorbance was measured at a wavelength of 560 nm. In this assay, the water-soluble tetrazolium was converted by the superoxide radical anion to a formazan dye. SOD in the tissue lysates reduced O<sub>2</sub>-levels, thereby decreasing the dye formation.

**Reverse transcription-semi-quantitative PCR (RT-sqPCR).** Total RNA was extracted from the tissues or BV2 cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using a Reverse Transcriptase-kit (Qiagen, Inc.), according to the manufacturer's protocol. The RT-sqPCR was performed using a 2x Taq Plus MasterMix (Cwbiotech, Inc.) and products were analyzed on 1% agarose gel and the intensity of each band was quantified by ImageJ software version 1.8.0.112 (National Institutes of Health). qPCR was subsequently performed using a SuperReal PreMix Plus (SYBR-Green) kit (Qiagen, Inc.) on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The thermocycling conditions for the sqPCR reaction included an initial step of 15 min at 95°C to activate the chemically modified hot-start Taq DNA polymerase, followed by 40 cycles of a 15-sec denaturation at 95°C and then 30 sec annealing and extension at 60°C. GAPDH was used as the internal reference control. Primer sequences were as follows (28,30): TNF $\alpha$  forward, 5'-CATCTTCTCAAATTCGAGT GAC-3' and reverse, 5'-TGGGAGTAGACAAGGTACAACCC-3'; IL-1 forward, 5'-TGGAAGCGGTTTGTCTTC-3' and reverse, 5'-TACCAGTTGGGGAAGTCTGC-3'; NO synthase 2 (NOS2) forward, 5'-CAGCTGGGCTGTACAAACCT T-3' and reverse, 5'-CATTGAAGTGAAGCGTTTCG-3'; IL-6 forward, 5'-GCTGG-TGACAACCACGGCCT-3' and reverse, 5'-AGCCTCGACTTGTGA AGTGGT-3'; IL-10 forward, 5'-GCTCTTACTGACTGGCATGAG-3' and reverse, 5'-CGC AGCTTAGGAGCATGTG-3'; arginase-1 (Arg-1) forwards, 5'-GTGAAGAACCACGGTCTGT-3' and reverse, 5'-GCC AGAGATGCTTCCAAGT-3'; GAPDH forwards, 5'-GGC CCGGTGCTGAGTATGTC-3' and reverse, 5'-TGCCTG CTTACACACCTTCT-3'. Relative expression levels were analyzed using the 2<sup>- $\Delta\Delta C_q$</sup>  method (31).

**Western blotting.** Protein was extracted with RIPA Lysis Buffer (Beyotime Institute of Biotechnology) from the tissues and cells, and quantified using a BCA protein assay kit (Thermo Scientific, Inc.). The cytoplasmic/nuclear fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's manual. Proteins (40  $\mu$ g) were separated via 12% SDS-PAGE and transferred to PVDF membranes, which were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. The membranes were then incubated at 4°C overnight with the following primary antibodies: Anti- $\beta$ -actin (cat. no. AP0060, 1:5,000), anti-Nrf2 (cat. no. ab92946, 1:1,000), anti-NQO1 (cat. no. ab80588, 1:1,000), anti-Lamin B (cat. no. ab194109, 1:1,000), anti-HO-1 (cat. no. ab68477, 1:2,000), anti-NF- $\kappa$ B p65 (cat. no. 8242 1:2,000), anti-p-I $\kappa$ B $\alpha$  (cat. no. 5209, 1:2,000), anti-I $\kappa$ B $\alpha$  (cat. no. 4812 1:1,000). Following the primary antibody incubation, the membranes were washed with TBST (0.1% Tween 20) and incubated with a HRP-conjugated secondary antibody (cat. no. BS13278, 1:5,000) for 1 h at room temperature. Protein bands were visualized using an ECL Plus Western Blotting Detection system (Bio-Rad Laboratories, Inc.).

**Immunofluorescence staining.** BV2 cells were cultured at 37°C in a 24-well chamber and treated with SINO and/or OGD for 4 h. Immunofluorescence staining of NF- $\kappa$ B (cat. no. 8242, 1:500; Abcam) and subsequent nuclear staining with DAPI (2  $\mu$ g/ml; Sigma-Aldrich; Merck KGaA) were performed as previously described (32). Briefly, the cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized in PBS containing 2% Triton X-100 for 5 min at room temperature. The cells were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature and subsequently incubated with the anti-NF- $\kappa$ B antibody at 4°C overnight and a DyLight 594-conjugated AffiniPure donkey anti-rabbit IgG secondary antibody (cat. no. BS10030, 1:100) at 1 h room temperature. The cells were also stained with DAPI for 5 min and observed under a confocal microscope with x40 magnification.

**Statistical analysis.** All data are presented as the mean  $\pm$  SEM and statistical analysis was performed using SPSS Version 18.0 (SPSS, Inc.). Each experiment was repeated in triplicate and statistical differences between 2 groups were analyzed using a two-tailed Student's t-test, while statistical differences between >2 groups were determined using one-way ANOVA followed by Tukey's post-hoc tests. P<0.05 was considered to indicate a statistically significant difference.

## Results

**SINO treatment relieves MCAO-induced cerebral injuries.** The cerebral protective role of SINO in a MCAO model mice was analyzed. Briefly, 20 mg/kg SINO was injected intraperitoneally into mice for 3 days following MCAO surgery, after which the cerebral histological changes and the brain water content were determined. H&E staining revealed that mice in the sham and SINO treatment groups did not have altered cerebral morphology or brain water content, while the brains of MCAO model mice exhibited a marked reduction

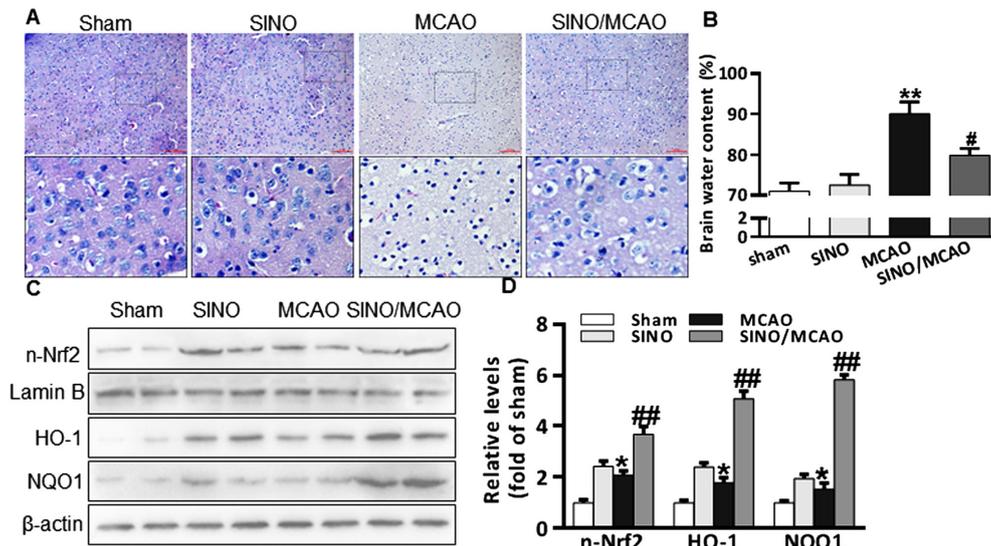


Figure 1. SINO treatment relieves MCAO-induced cerebral injuries. (A) Representative micrographs of H&E staining of brain sections (x20 magnification; scale bar, 50  $\mu$ m). (B) Brain water content analysis in brain tissues from Sham (sham-operated, n=6), SINO (treatment only, n=6), MCAO (MCAO-operated, n=6) and SINO/MCAO (MCAO operated plus SINO treatment, n=6). (C) Western blot analysis of n-Nrf2, HO-1 and NQO1 protein expression levels from brain tissue. The samples from two randomly selected brains in each group were presented. (D) Quantification of brain protein expression levels. All the experiments were repeated at least three times. The data are presented as the mean  $\pm$  SEM. \* $P$ <0.05 and \*\* $P$ <0.01 vs. Sham; # $P$ <0.05 and ## $P$ <0.01 vs. MCAO. HO-1, heme oxygenase-1; MCAO, middle cerebral artery occlusion; n-Nrf2, nuclear-nuclear factor-erythroid 2-related factor; NQO1, NAD(P)H: Quinoneoxidoreductase 1; SINO, sinomenine.

in the number of nerve cells, as well as an increased brain water content (Fig. 1A and B). Notably, treatment with SINO following MCAO reduced the degree of pathological damage in the cerebral tissue, as well as the brain water content (Fig. 1A and B). A previous study reported that SINO-induced HO-1 serves an important role in attenuating cold ischemia/reperfusion injury in rats (12) and that HO-1 is an Nrf2 target gene (33). As such, the translocation of nuclear of Nrf2 and the expression levels of HO-1 and NQO1 were investigated. Compared with the sham group, Nrf2 translocation and HO-1 and NQO1 expression levels were increased following MCAO surgery. This result was and further enhanced by SINO treatment (Fig. 1C and D). These results indicated that SINO may protect against cerebral injury in MCAO model mice and the protective effect may be related to the activation of the Nrf2 signaling pathway.

*SINO treatment activates the Nrf2 signaling pathway.* To investigate the effect of SINO treatment on Nrf2 signaling, microglia BV2 cells were treated with 50-200  $\mu$ M SINO for various durations (2-24 h) and the levels of HO-1 and NQO1 were analyzed using western blotting. As presented in Fig. 2A and B, SINO treatment significantly upregulated HO-1 and NQO1 expression levels in a concentration- and time-dependent manner. Nrf2 nuclear translocation is a necessary step for Nrf2 activation (30). SINO treatment markedly induced Nrf2 nuclear accumulation after 4 h (Fig. 2C). SINO treatment also upregulated the mRNA expression of HO-1 and NQO1 in a dose-dependent manner (Fig. 2D), suggesting that SINO may upregulate HO-1 and NQO1 at the transcriptional or post-transcription level. The cytotoxic effects of SINO were analyzed using a Cell Counting Kit 8 assay, which revealed that treatment with  $\leq$ 200  $\mu$ M SINO for 24 h was unable to induce cell death, with no obvious changes in the amount of cell death following treatment with between 0-200  $\mu$ M SINO

(Fig. 2E). Taken together, the results suggested that SINO may be an activator of the Nrf2 signaling pathway in microglial BV2 cells.

*SINO treatment mitigates MCAO-associated inflammation and oxidative stress.* Cerebral inflammation and oxidative stress are critical pathological processes associated with cerebral pathogenesis in MCAO model mice (34). MCAO model mice had a markedly upregulated expression of TNF- $\alpha$  and IL-1 $\beta$  compared with the sham group. However, SINO treatment significantly inhibited the production of both TNF- $\alpha$  and IL-1 $\beta$  (Fig. 3A). NOS2 is not only considered to be a pro-inflammatory mediator, but also the key enzyme that increases peroxynitrite production, a major reactive oxygen species (35). The brains of MCAO model mice had significantly upregulated mRNA expression levels of NOS2 (Fig. 3B). SOD and GPx are two important antioxidant enzymes (36). The enzymatic activities of SOD and GPx in the brains of MCAO model mice were found to be decreased (Fig. 3C and D); however, treatment with SINO significantly reversed the upregulation of NOS2 mRNA expression levels (Fig. 3B) and the reduction in SOD and GPx enzyme activities (Fig. 3C and D). Collectively, these results indicated that SINO may exert strong anti-inflammatory and anti-oxidative stress functions in MCAO model mice.

*SINO treatment regulates microglia polarization and inflammation in an Nrf2-dependent manner.* Although SINO exerts strong anti-inflammatory properties, its regulatory role in microglial polarization has not been fully determined. Thus, whether SINO affected the expression levels of M1 markers (NOS2 and IL-6) and M2 markers (Arg-1 and IL-10) in BV2 cells was investigated. The results demonstrated that OGD upregulated the expression levels of M1 markers, while SINO treatment significantly inhibited the OGD-induced increases

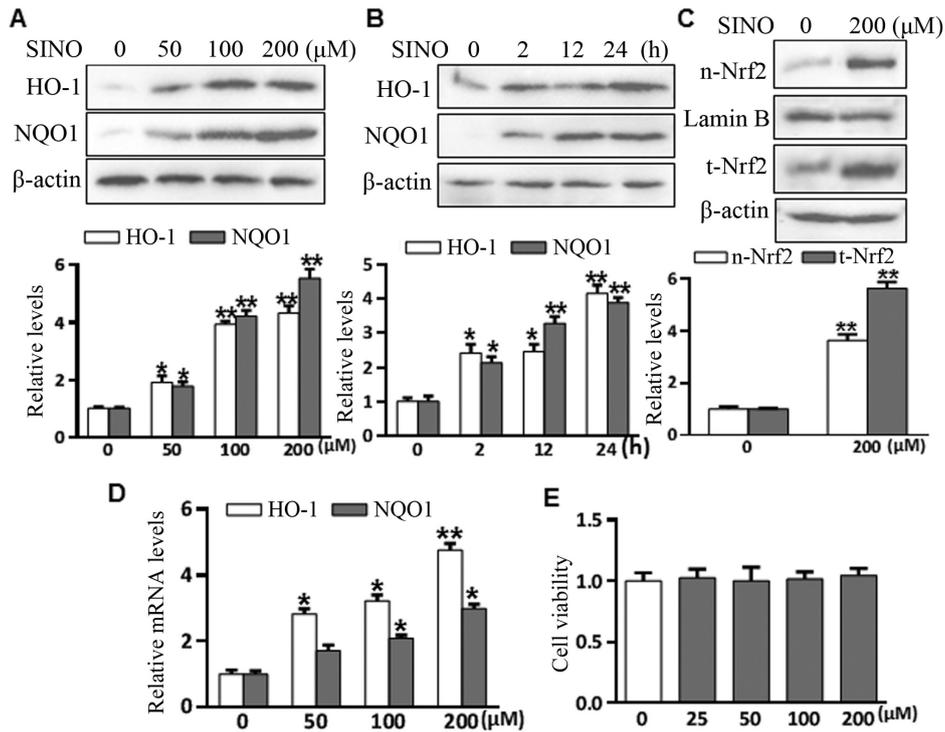


Figure 2. SINO treatment activates the Nrf2 signaling pathway. (A) BV2 cells were treated with a serial of doses of SINO for 24 h. HO-1 and NQO1 protein expression levels were assayed using western blotting. (B) BV2 cells were treated with SINO (200  $\mu$ M) for various durations. HO-1 and NQO1 protein expression levels were assayed using western blotting. (C) BV2 cells were treated with SINO (200  $\mu$ M) for 4 h. n-Nrf2 and t-Nrf2 protein expression levels were assayed using western blotting. Nuclear protein Lamin B and  $\beta$ -actin were used as controls. (D) BV2 cells were treated with serial doses of SINO for 24 h. The mRNA expression levels of HO-1 and NQO1 were measured using reverse transcription-semi-quantitative PCR. (E) Cell viability assay. BV2 cells were treated with various doses of SINO for 24 h. The cell viability was assayed by a CCK-8 kit. All the experiments were repeated at least three times. \* $P < 0.05$  and \*\* $P < 0.01$  vs. Control HO-1, heme oxygenase-1; NQO1, NAD(P)H: Quinoneoxidoreductase 1; n-, nuclear; Nrf2; nuclear factor-erythroid 2-related factor; SINO, sinomenine; t-, total.

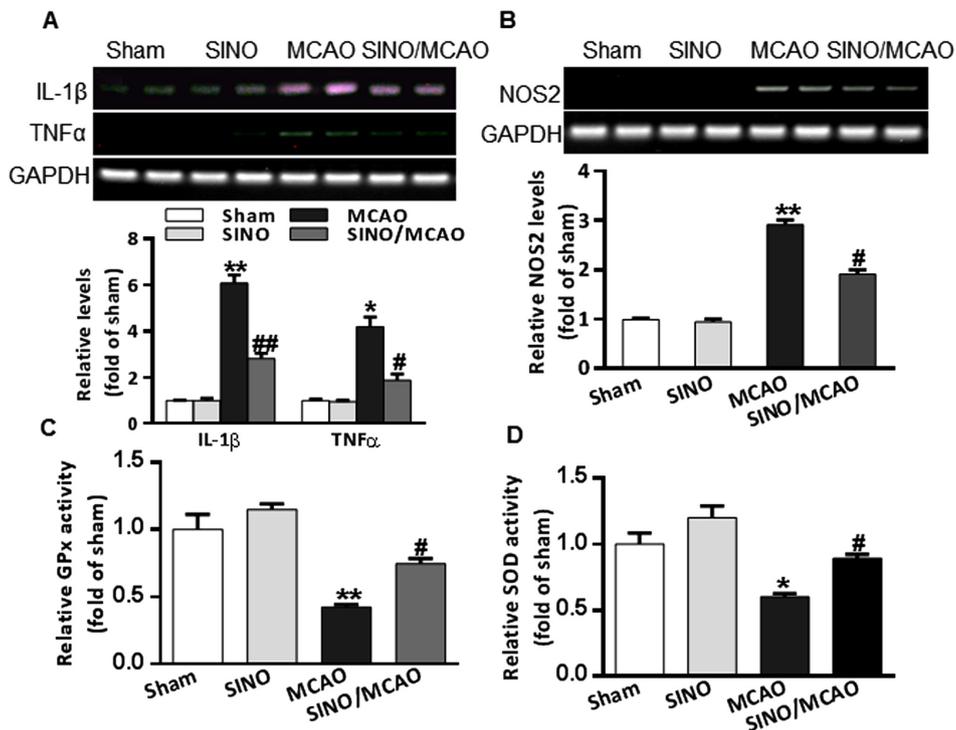


Figure 3. SINO treatment mitigates MCAO-associated inflammation and oxidative stress. (A) RT-sqPCR analysis of TNF- $\alpha$  and IL-1 $\beta$  mRNA expression levels in brain tissues. The results of two samples from each group were presented. (B) RT-sqPCR analysis of NOS2 mRNA expression levels in brain tissues. (C) The relative enzyme activities of GPx from the brain tissue of the four groups. (D) The relative enzyme activities of SOD from the brain tissue of four groups. All experiments were repeated at least three times. The data are presented as the mean  $\pm$  SEM. \* $P < 0.05$  and \*\* $P < 0.01$  vs. Sham; # $P < 0.05$  and ## $P < 0.01$  vs. MCAO. GPx, glutathione peroxidase; MCAO, middle cerebral artery occlusion; NOS2, nitric oxide synthase 2; RT-sqPCR, reverse transcription-semi-quantitative PCR; SINO, sinomenine; SOD, superoxide dismutase.

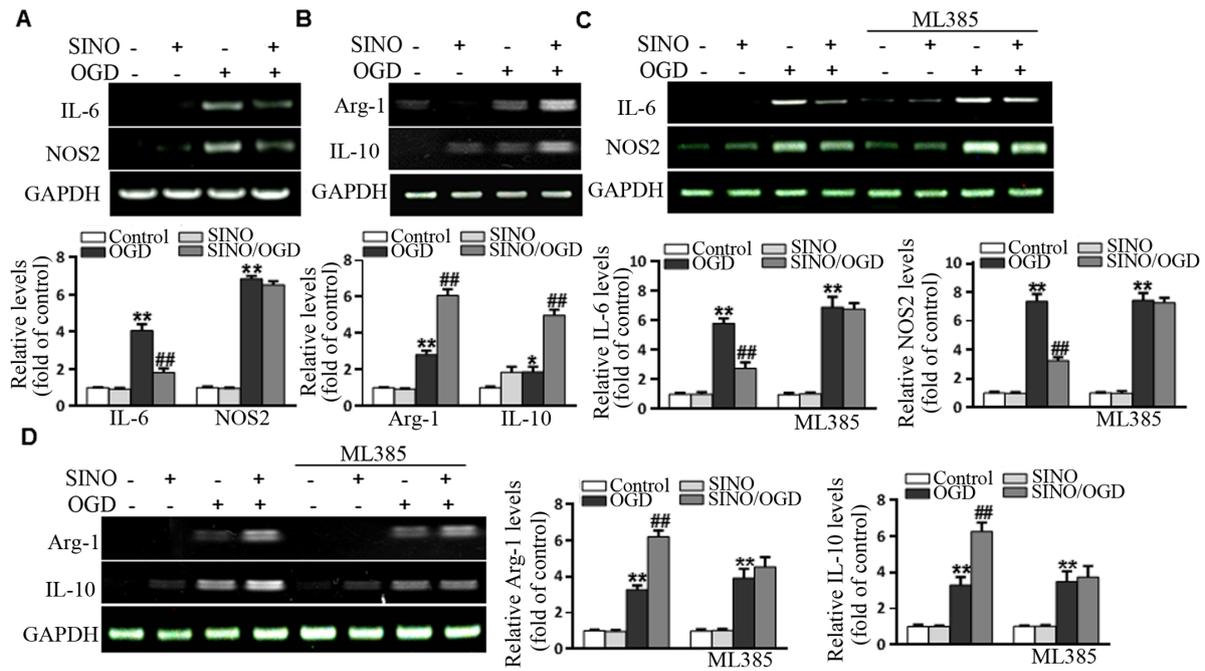


Figure 4. SINO treatment regulates microglia polarization and inflammation in an Nrf2-dependent manner. BV2 cells were stimulated with OGD for 4 h followed by treatment with SINO (200  $\mu$ M) for 12 h. The mRNA expression levels of (A) M1 markers (IL-6/NOS2) and (B) M2 markers (IL-10/Arg-1) were determined using RT-sqPCR. BV2 cells were pretreated with ML385 (5  $\mu$ M) for 48 h to inhibit Nrf2 expression. Cells were then stimulated with OGD for 4 h followed by treatment with SINO (200  $\mu$ M) for 12 h. The mRNA expression levels of (C) M1 and (D) M2 markers were measured using RT-sqPCR. The data are presented as the mean  $\pm$  SEM of three independent experiments. \* $P$ <0.05 and \*\* $P$ <0.01 vs. Control; ## $P$ <0.01 vs. OGD. Arg-1, arginase-1; NOS2, nitric oxide synthase 2; OGD, oxygen and glucose deprivation; SINO, sinomenine; RT-sqPCR, reverse transcription-semi-quantitative PCR.

in IL-6 and NOS2 levels (Fig. 4A). In addition, OGD upregulated the expression levels of M2 markers, while SINO treatment markedly enhanced the OGD-induced expression of Arg-1 and IL-10 compared with the control group (Fig. 4B), suggesting that SINO may have dual functions in regulating microglial polarization. To further determine whether SINO exerted its effects through Nrf2, BV2 cells were treated with 5  $\mu$ M of the Nrf2 inhibitor, ML385, for 48 h. As shown in Fig. 4C and D, the effect of SINO treatment on the OGD-induced upregulation of NOS2, IL-6, Arg-1 and IL-10 expression levels was markedly reduced following the inhibition of Nrf2. In addition, the SINO-induced inhibition of OGD-induced I $\kappa$ B $\alpha$  phosphorylation (Fig. 5A) and NF- $\kappa$ B p65 nuclear translocation (Fig. 5B) was also markedly reduced following the inhibition of Nrf2, suggesting that Nrf2 signaling may be a crucial upstream event involved in mediating the SINO-induced inhibition of NF- $\kappa$ B signaling and inflammatory responses.

## Discussion

The present study used MCAO model mice to establish an *in vivo* model of ischemic stroke to investigate the role of Nrf2 signaling in SINO-induced cerebral protection. The results of the present study revealed that SINO served dual functions in regulating inflammatory responses in microglia, in that SINO downregulated NOS2 and IL-6 expression levels and promoted Arg-1 and IL-10 expression levels. This subsequently suppressed NF- $\kappa$ B signaling, suggesting that the SINO-induced activation of Nrf2 may act upstream of its inhibition of NF- $\kappa$ B signaling.

Stroke is the main cause of disability in adults worldwide (37). Ischemic stroke accounts for 85% of strokes (38,39) and is a clinical syndrome which leads to neurological deficits due to the ischemic and anoxic necrosis of the local tissue (40). Inflammation is an important response in the pathological process of ischemic stroke. Previous studies have reported that inflammation is the main factor that determines the outcome and long-term prognosis of patients with ischemic strokes (41). Microglia are macrophages that are widespread throughout the CNS and participate in the cellular immune process (42). At present, therapies that inhibit the M1 polarization of microglia cells through physical and pharmaceutical intervention, to promote anti-inflammatory functions, have been applied in the clinic (43). Physical methods to inhibit M1 polarization include electro-acupuncture guidance and hyperbaric oxygen treatment. Pharmaceutical interventions include exenatide acetate and minocycline. Exenatide acetate has been found to promote the change of microglia from the M1 to M2 type (44,45). Minocycline has been found to inhibit the polarization of microglia to the M1 type, but has no effect on the M2 type (46-48). Kata *et al* (49) reported that rosuvastatin inhibits the proliferation and adhesion of microglia and upregulates the expression levels of certain anti-inflammatory genes, including C-X-C motif chemokine ligand 1, C-C motif chemokine ligand 5 and mannose binding lectin 2. Concurrently, rosuvastatin also downregulated the expression levels of the pro-inflammatory cytokines, IL-1 $\beta$  and TNF- $\alpha$ , upregulates the expression levels of the anti-inflammatory factor, IL-10, and promotes the polarization of microglia to the M2 type. The results of the present study confirmed that SINO treatment effectively inhibited the OGD-induced

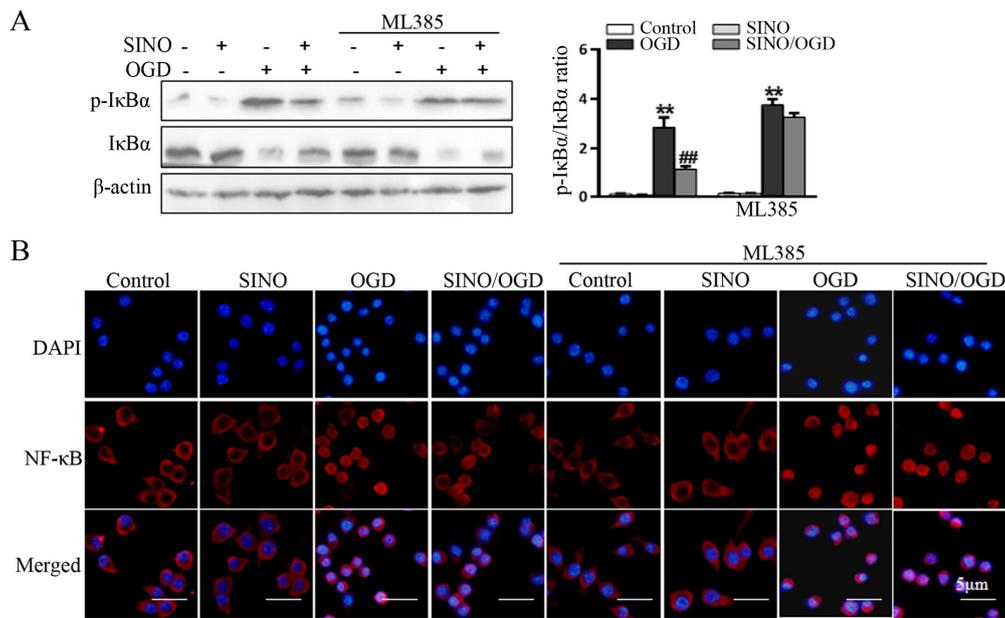


Figure 5. SINO treatment regulates microglia inflammation in an Nrf2-dependent manner. (A) BV2 cells were pretreated with ML385 (5 μM) for 48 h to inhibit Nrf2 expression. Cells were then stimulated with OGD for 4 h followed by treatment with SINO (200 μM) for 12 h. The protein expression levels of p-IκBα and total IκBα were analyzed using western blotting. (B) Immuno-fluorescent staining of cells was performed using an NF-κB p65 primary antibody and Dylight488 conjugated secondary antibody (middle row of panels). The cells were also stained with DAPI (top row of panels) and merged with NF-κB images (lower row of panels). The data are presented as the mean ± SEM of three independent experiments. \*\*P<0.01 vs. Control; ##P<0.01 vs. OGD. OGD, oxygen and glucose deprivation; p-, phosphorylated; SINO, sinomenine.

upregulation of the expression levels of M1 markers, IL-6 and NOS2, while it markedly upregulated the expression levels of the M2 markers, Arg-1 and IL-10, suggesting that SINO may have dual functions in modulating microglia polarization.

Accumulating evidence has revealed that Nrf2 serves a crucial role behind the processes of oxidative stress and inflammation (50,51). Therefore, Nrf2 may represent a promising target for stroke intervention (50). However, the present understanding of the functions of Nrf2 and its application for stroke-targeted therapy remains limited (50). In the present study, SINO was demonstrated to protect against cerebral injury in MCAO mice. The protective effects were associated with the ability of SINO to activate the Nrf2 signaling pathway. The functional importance of Nrf2 has been reported in several pathological conditions, most of which are from studies performed investigating Nrf2<sup>-/-</sup> mice (52,53). Protoganaxtriols, as a potent Nrf2 natural inducer extracted from the root of *Panax ginseng*, C.A. Mey. has been widely used in East Asia for thousands of years, exhibiting potent anti-inflammatory and antioxidative properties (54,55). Previous research has reported that Nrf2 deficiency causes a significant increase in infarct volume following MCAO for 3 days, which indicates that Nrf2 may exert a beneficial role in ischemic injury during the acute development and progression (56-58). The findings of the present study confirmed that SINO treatment effectively reversed the MCAO-associated induction of the inflammatory cytokines, TNF-α and IL-1β, and the activities of the antioxidant enzymes, GPx and SOD. In addition, the SINO-induced activation of Nrf2 signaling was found to be essential for SINO-induced cerebral protection, as the inhibition of Nrf2 eliminated the protective effects of SINO on MCAO-associated cerebral injury. In theory, the activation of Nrf2 should upregulate SOD; however, in the

current study, the enzymatic activity of SOD in the brains of MCAO model mice was found to be decreased and Nrf2 was induced to act against brain tissue damage in MCAO model mice. Therefore, the light upregulation of Nrf2 in MCAO model mice may not prevent the downregulation of SOD.

According to a previous study, the Nrf2-target genes were revealed to be preferentially activated in glial cells, which produces a more effective antioxidant effect than neurons (59). Over the past decade, previous findings have suggested that SINO may exert a significant potential for the treatment of strokes (27,60-62). Glial cells contribute to the expansion and resolution of infarctions, and affect the process of ischemic injury. However, the long-term effects of Nrf2 function and SINO neuroprotection on ischemic injury remain unclear (50). Previous studies have revealed the mechanism of Nrf2 pathway (63,64). The present study suggested that SINO-induced regulation of Nrf2 signaling may be an important pathway through which SINO protects against MCAO-associated cerebral injury, although the precise mechanism of action requires further investigation.

In conclusion, the findings of the present study suggested that the SINO-induced regulation of Nrf2 signaling may be an important pathway through which SINO protects against MCAO-associated cerebral injury. The results indicated that SINO treatment may activate Nrf2 in microglia and subsequently modulate microglia polarization. The SINO-induced inhibition of M1 polarization and promotion of M2 polarization in microglia contributed to its anti-inflammatory and cerebral protective properties. Since the current MCAO model shares its pathogenesis with numerous other cerebral diseases, these findings may provide novel insights into the potential application of SINO for the treatment of brain or other inflammatory diseases.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

FB, YZ and WL carried out the experimental work, as well as the data collection and interpretation. FB and WL participated in the design and coordination of experimental work and acquisition of data. WL and KX carried out the study design; the analysis and interpretation of data; and drafted the manuscript. WL and KX confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Medical College of Xi'an Peihua University.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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