Overexpression of NLRC3 enhanced inhibition effect of sevoflurane on inflammation in an ischaemia reperfusion cell model

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Abstract

Brain ischaemia is one of the leading causes of mortality and disability worldwide, and the damage caused by ischaemia not only induces primary damage but also that induced by ischaemia-reperfusion (I/R) injury. Multiple processes including inflammation and oxidative stress response play important roles in the development of brain ischaemia injury. Sevoflurane is a well-known volatile anaesthetic, and a recent study discovered the role of sevoflurane in suppression of the inflammation response process via inhibition of inflammatory infiltrates and production, maintaining the balance of cytokine responses, although the possible mechanism was not fully clear. NLRC3 is a member of the nucleotide-binding domain and leucine-rich repeat containing (NLR) family, and it has been regarded as a regulator of the inflammation process via the regulation of inflammasome formation, which is an initiator of inflammatory events. In the present study, we found that overexpression of NLRC3 reduced the apoptosis in a cellular model of ischaemia reperfusion, and the expression of pro-inflammatory cytokines was also decreased. Further study found that these effects might be mediated by the TRAF6/TLR4/NF-κB signalling pathway. Thus, we speculate that overexpression might enhance the effect of sevoflurane in inhibiting the inflammatory response process in an ischaemia reperfusion model, which might be a new therapeutic strategy.

Key words: ischaemia reperfusion, NLRC3, sevoflurane, inflammation.

Introduction

Cerebral ischaemia is one of the leading causes of death and disability around the world. Early restoration of blood flow is important for the treatment of cerebral ischaemia, but the apoptosis of neuronal cells still occurs after the restoration of blood flow. These processes are thought to be mediated by activation of pro-inflammation factors including cytokines, nitric oxide, and free radicals [28]. However, the effective treatment for cerebral ischaemia is still lacking because multiple signalling pathways are only partially effective [41]. A recent study revealed that some anaesthetics possess a neuroprotective role, including isoflurane, sevoflurane, and desflurane. Among them, sevoflurane presented short-term (72 to 96 h) and long-term (28 days) neuroprotective effects in focal or global cerebral ischaemia [34]. A previous study found that sevoflurane could pre-

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vent the deficit in cognitive function induced by ischaemia when compared with a fentanyl treatment group [8]. However, the detailed mechanism was not clear. NOD-like receptor family CARD domain containing 3 (NLRC3) is an important suppressor of the inflammatory response in multiple species [45], and a recent study noted that NLRC3 is also an important regulator of the inflammatory response in a model of ischaemia reperfusion, because overexpression of NLRC3 alleviated the inflammation process induced by ischaemia reperfusion [24]. Thus, we speculated that overexpression of NLRC3 might also contribute to the effects of sevoflurane in the treatment of cerebral ischaemia. Herein, we established the an NLRC3 overexpression and knockdown model in PC12 cells, and we found that overexpression of NLRC3 reduced the apoptosis process in PC12 cells, while further study found that these processes might be mediated by inhibition of the expression of pro-inflammatory factors. Finally, we found that overexpression of NLRC3 promotes the survival of PC12 cells *via* regulation of the TRAF6/TLR4/NF-κB signalling pathway and its downstream molecules.

Material and methods

Cell culture and grouping

PC12 cells (CRL-1721) were purchased from ATCC. Cells were cultured under 37°C temperature with 5% CO_2 in RPMI-1640 medium supplied with 10% fetal bovine serum (FBS). In order to construct an *in vitro* ischaemia reperfusion model in PC12 cells, cells were cultured in glucose-free Earl's balanced salt solution with a 95% N_2 and 5% CO_2 atmosphere, and after 4 h of incubation, the medium was replaced with RPMI-1640, and cells were cultured under normal conditions for 24 h [46]. Then, cells were divided into four groups: a model group (M), single sevoflurane treatment group (S), sevoflurane treatment combined with NLRC3 overexpression group (O), and a sevoflurane treatment combined with NLRC3 inhibition group (I). In the sevoflurane treatment group, cells were treated with 2.4% (v/v) sevoflurane for 20 min at the end of ischaemia reperfusion model construction [14].

Vector construction

In order to construct the NLRC3 overexpression vector, cDNA of NLRC3 was acquired with the following primers: Forward: 5'-CTGCAGCAATGACTCAAG-

GAT-3', Reverse: 5'-CTGTGTGAAGTCGTGTTCCCT-3'. pcDNA3.1-HA and cDNA of NLRC3 were digested with BamHI (R0136S, NEB) and EcoRI (R0101S, NEB) at 37°C for 4 h, followed by incubation with T4 DNA ligase (M0202S, NEB) at 4°C overnight. Then, the vector was transfected into PC12 cells using Lipofectamine 3000 transfection reagent (L3000075, Thermo), and stable expression cells were screened using 800 µg/ml G418 (G8160, Solarbio). The NLRC3 knockdown vector was constructed according to a previous study [13]. Pairs of oligos were obtained using the following primers: Forward: 5'-CACCGTCAGACTTCTGTTGACCAAG-3', Reverse: 5'-AACCTTGGTCAACAGAAGTCTGAC-3', and oligos were incubated with T4 PNK (M0201S, NEB) at 37°C for 30 min and 95°C for 5 min. Vector and oligos were digested with BsmBI (R0580S, NEB) overnight, and linked with Quick Ligase (M2200S, NEB) to construct the NLRC3 knockdown vector. Then the vector was transfected into 293T cells (CRL-11268) to construct the NLRC3 knockdown vector lentivirus, and stable expression cells were screened using 2 ng/ml puromycin (P8230, Solarbio).

MTT assay

Cells were seeded into a 96-well plate at a concentration of 1×10^5, then cells were grouped and treated as described above. The, cells were incubated with Cell Counting Kit-8 (CCK-8) reagent for 3 h. After incubation, the absorbance value at 450 nm was measured using a CMax Plus microplate reader. The viability rate was calculated with the following formula:

Viability rate = $(OD_{experiment} - OD_{blank})/(OD_{control} - OD_{blank})$.

Flow cytometry

Apoptotic cells were detected using an annexin V/PI apoptosis detection kit (CA1020, Solarbio). Briefly, cells were grouped and treated as described above, and then cells were diluted at 10^6/ml using binding buffer. Cells were incubated with annexin V for 10 min followed by incubation with PI for 5 min. Apoptotic cells were detected using NovoCyte.

RNA extraction

RNA extraction was performed as recommended by the manufacture's protocol (R1200, Solarbio). Cells were firstly grouped and treated as described above and lysed with lysis buffer, followed by incubation at room temperature for 5 min and incubation with

chloroform for 5 min. The water phase was collected after centrifugation at 12,000 rpm for 10 min. After washing with washing buffer, RNA samples were eluted with elution buffer. The concentration of the RNA sample was detected using a Nanodrop. RNA samples were stored at −80°C until the following experiment was performed.

Reverse transcription and quantitative polymerase chain reaction

Reverse transcription and quantitative polymerase chain reaction (qPCR) were performed as recommended by the manufacture's protocol (T2240, Solarbio). The reaction mixture was made up as recommended, and the reaction was performed with the following steps: reverse transcription: 50°C for 15 min, denaturation: 95°C for 5 min, with the following steps repeated for 45 cycles: denaturation: 95°C for 20 s, annealing: 57°C for 25 s. The qPCR experiment was performed with the following primers: IL-1β: Forward: 5'-AGAAGTACCTGAGCTCGCCA-3', Reverse: 5'-CTGGAAGGAGCACTTCATCTGT-3'; IL-6: Forward: 5'-ACTCACCTCTTCAGAACGAATTG-3', Reverse: 5'-CCATCTTTGGAAGGTTCAGGTTG-3'; TNF-α: Forward: 5'-GCTGCACTTTGGAGTGATCG-3', Reverse: 5'-GAGGGTTTGCTACAACATGGG-3'; IFN-γ: Forward: 5'-AGCTGATTCAAATTCCGGTGG-3', Reverse: 5'-TCTCCGGCCTCGAAAGAGAT-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control, and data were analysed using the $2^{-\Delta\Delta Ct}$ method.

Protein extraction and western blotting

Cells were firstly grouped and treated as described above. Then, cells were lysed with lysis buffer (RIPA supplied with protein inhibitor cocktail), and protein samples were collected after centrifugation at 12,000 rpm for 10 min. 60 µg of protein sample was separated with 10% SDS-PAGE electrophoresis and then transferred onto a PVDF membrane. Membranes were firstly blocked with 5% skim milk, and then incubated with primary antibodies at 4°C overnight and incubated with secondary antibody at room temperature for 4 h. The expression of each target protein was detected using a chemiluminescent immunoassay.

Enzyme-linked immunosorbent assay

Cells were firstly grouped and treated as described above, and then cultured medium was collected to perform the enzyme-linked immunosorbent assay (ELISA) assay. Briefly, samples and standards were added into a 96-well plate and incubated at room temperature for 1 h. After washing with washing buffer, samples were incubated with TMB solution for 10 min away from light. The absorbance value at 450 nm was measured using a CMax Plus microplate reader.

Statistical analysis

Data from each experiment were presented as mean ±SD. Each experiment was repeated for three times independently. The difference between groups was analysed using one-way ANOVA analysis, and $p < 0.05$ was set as a statistical difference.

Results

Effect of sevoflurane on proliferation and apoptosis of cells

The viability rate in the M, S, O, and I groups was 100.0 ±6.3, 116.2 ±7.6, 141.1 ±9.8, and 104.5 ±6.7, respectively (Fig. 1A). The viability rate was significantly increased in the S and O group compared with the M group ($p < 0.05$), and it was significantly increased in the O group compared with the S group ($p < 0.05$). The percentage of apoptotic cells in these groups was 31.3 ±4.2, 24.1 ±3.5, 14.5 ±1.9, and 28.6 ±3.7, respectively (Fig. 1B). The percentage of apoptotic cells was significantly decreased in the O group compared with the M and S groups ($p < 0.05$).

Expression of pro-inflammatory factors

The expression of interleukin 1β (IL-1β) in the M, S, O, and I groups was 1.21 ±0.13, 1.05 ±0.11, 0.72 ±0.07, and 1.37 ±0.15, respectively. The expression of IL-1β was significantly decreased in the O group compared with the M and S groups ($p < 0.05$) and was significantly increased in the I group compared with the S group ($p < 0.05$). The expression of interleukin 6 (IL-6) in these groups was 1.34 ±0.17, 1.19 ±0.14, 0.85 ±0.09, and 1.38 ±0.16, respectively. The expression of IL-6 was significantly decreased in the O group compared with the M and S groups ($p < 0.05$). The expression of tumour necrosis factor α (TNF-α) in these groups was 1.62 ±0.18, 1.35 ±0.15, 0.94 ±0.09, and 1.58 ±0.17, respectively. The expression of TNF-α was significantly decreased in the O group compared with the M and S groups ($p < 0.05$). The expression of interferon γ (IFN-γ) in these groups was 1.29 ±0.13, 1.10 ±0.10, 0.64 ±0.06,

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and 1.42 ±0.15, respectively. The expression of IFN-γ was significantly decreased in the O group compared with the N group ($p < 0.05$), and it was significantly decreased in the O group and significantly increased in the I group compared with the S group ($p < 0.05$) (Fig. 2).

Activation of the TRAF6/TLR4/NF-κB signalling pathway

The expression of TRAF6 in the M, S, O, and I groups was 0.64 ±0.05, 0.41 ±0.03, 0.17 ±0.01, and 0.56 ±0.05, respectively. Expression of TRAF6 was
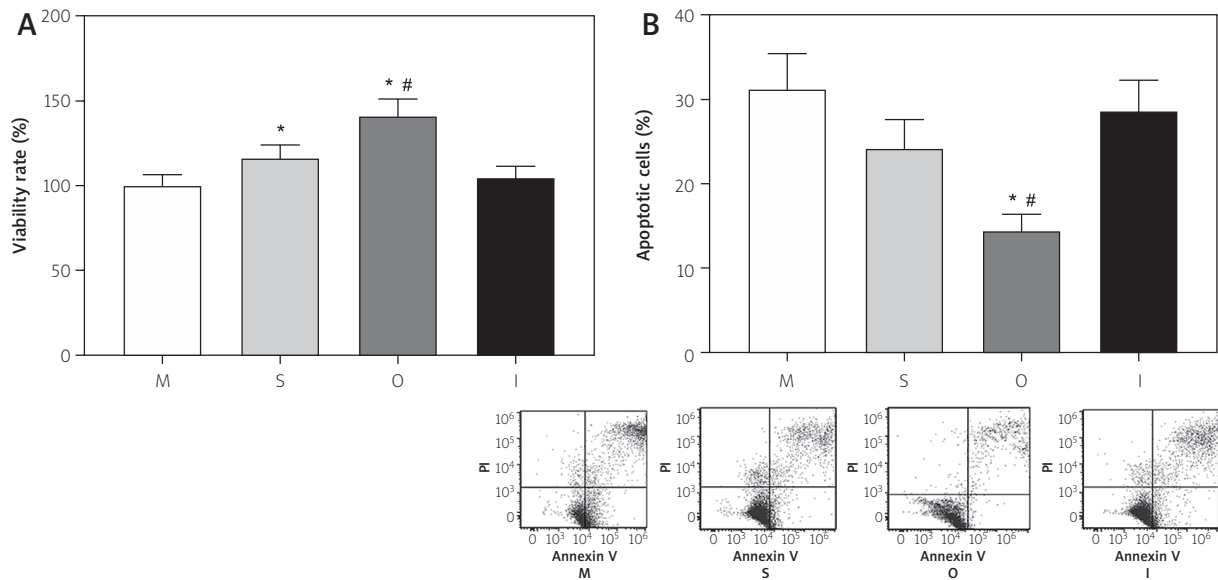

Fig. 1. Effect of sevoflurane on proliferation of cells. **A**) Detection of cellular viability rate using MTT assay. **B**) Detection of apoptotic cells using flow cytometry. *$p < 0.05$ vs. M group; #$p < 0.05$ vs. S group. Data presented as mean ±SD. Each experiment was repeated three times independently.
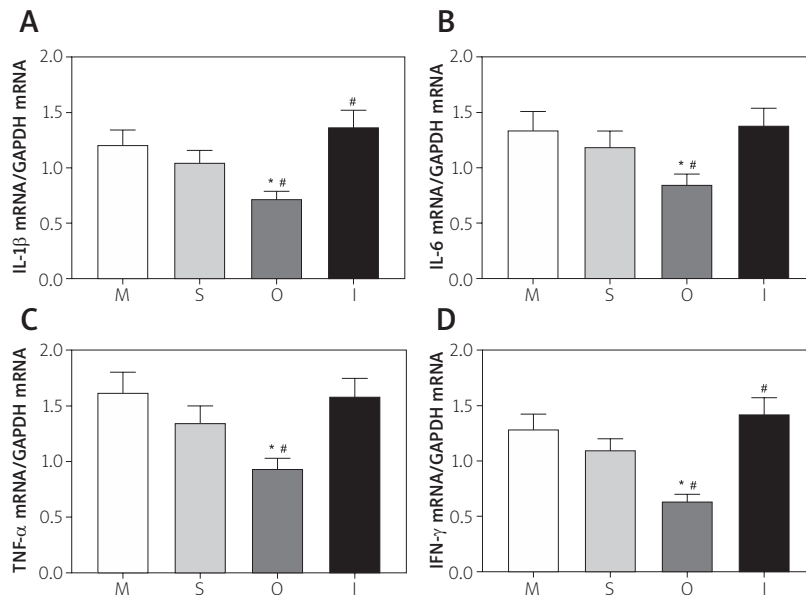

Fig. 2. Detection of **A**) interleukin 1β (IL-1β), **B**) interleukin 6 (IL-6), **C**) tumour necrosis factor α (TNF-α) and **D**) interferon γ (IFN-γ) mRNA expression. *$p < 0.05$ vs. M group; #$p < 0.05$ vs. S group. Data presented as mean ±SD. Each experiment was repeated three times independently.

significantly decreased in the S and O group compared with the M group ($p < 0.05$), and the expression was significantly decreased in the O group and significantly increased in the I group compared with the S group ($p < 0.05$). The expression of TLR4 in these groups was 0.84 ±0.07, 0.74 ±0.06, 0.34 ±0.03, and 0.65 ±0.05, respectively. Expression of TLR4 was significantly decreased in the O and I groups compared with the M group ($p < 0.05$), and it was significantly decreased in the O group compared with the S group ($p < 0.05$). The expression of TIRAP in these groups was 1.54 ±0.13, 1.41 ±0.12, 0.47 ±0.04, and 0.79 ±0.07, respectively. Expression of TIRAP was significantly decreased in the O and I groups compared with the M and S groups ($p < 0.05$). The expression of MyD88 in these groups was 1.08 ±0.09, 1.10 ±0.09, 0.57 ±0.05, and 0.99 ±0.08, respectively. Expression of MyD88 was significantly decreased in the O group

compared with the M and S groups ($p < 0.05$). The ratio of p-NF-κB/NF-κB in these groups was 0.50 ±0.04, 1.10 ±0.09, 1.57 ±0.13, and 0.91 ±0.07, respectively. The ratio of p-NF-κB/NF-κB was significantly increased in all treatment groups compared with the M group ($p < 0.05$), and it was significantly increased in the O group and significantly decreased in the I group compared with the S group ($p < 0.05$) (Fig. 3).

Expression of target molecules of the TRAF6/TLR4/NF-κB signalling pathway

The expression of PARP-1 in the M, S, O, and I groups was 1.17 ±0.10, 1.05 ±0.08, 0.75 ±0.06, and 1.54 ±0.13, respectively. Expression of PARP-1 was significantly decreased in the O group and significantly increased in the I group ($p < 0.05$) compared with the M and S groups. The expression of SIRT1 in these
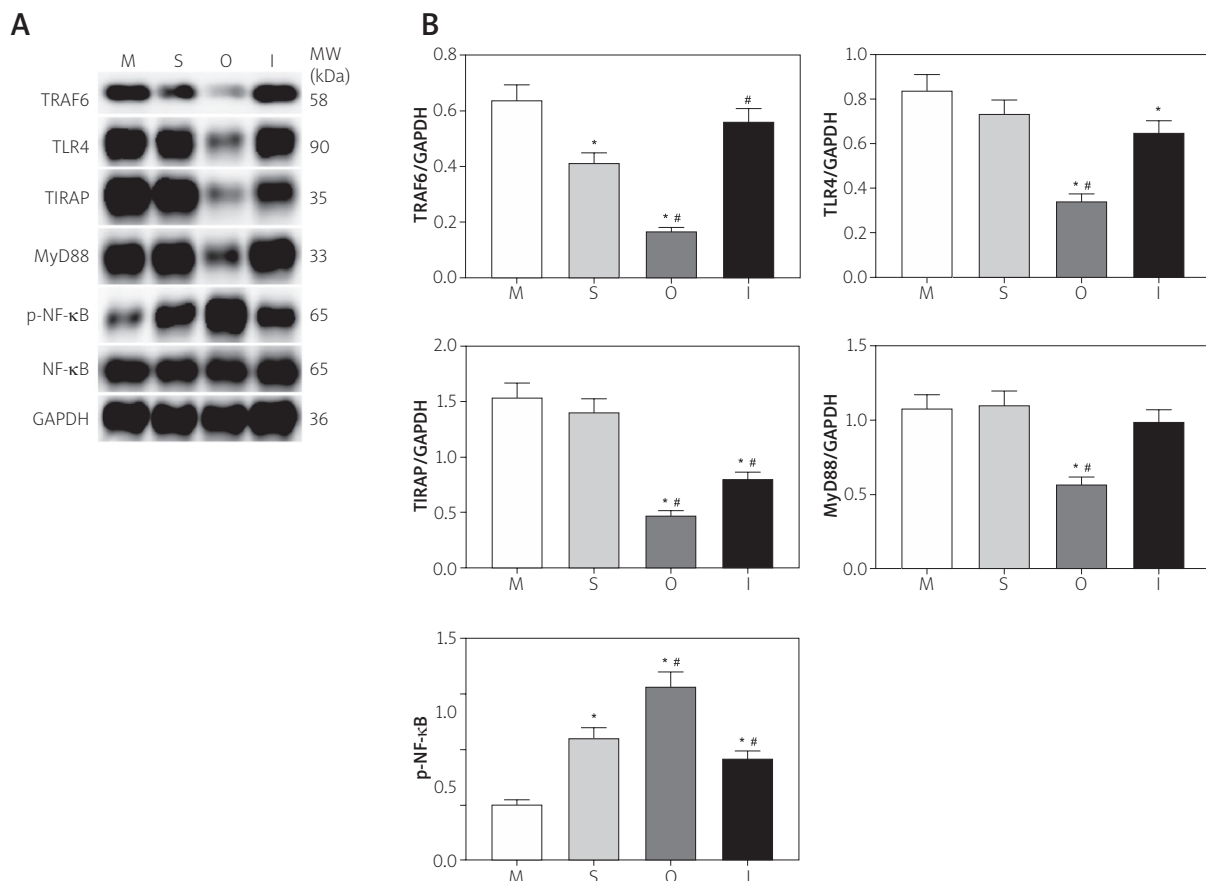

Fig. 3. Expression of TRAF6/TLR4 signalling pathway. **A**) Western blotting analysis of TRAF6, TLR4, TIRAP, MyD88, p-NF-κB, and NF-κB. **B**) Quantitative analysis of each target protein. *$p < 0.05$ vs. M group; #$p < 0.05$ vs. S group. Data presented as mean ±SD. Each experiment was repeated three times independently.

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groups was 0.38 ±0.03, 0.76 ±0.06, 1.04 ±0.09, and 0.60 ±0.05, respectively. Expression of SIRT1 was significantly increased in all treatment groups ($p < 0.05$) and was significantly increased in the O group and significantly decreased in the I group ($p < 0.05$) compared with the S group. The expression of ROCK2 in these groups was 0.93 ±0.08, 0.84 ±0.07, 0.37 ±0.03, and 0.92 ±0.08, respectively. Expression of ROCK2 was significantly decreased in the O group ($p < 0.05$) compared with the M and S groups. The expression of eNOS in these groups was 0.68 ±0.06, 0.90 ±0.08, 1.29 ±0.10, and 0.84 ±0.07, respectively. Expression of eNOS was significantly increased in all treatment groups ($p < 0.05$) and was significantly increased in the O group ($p < 0.05$) compared with the S group (Fig. 4).

Secretion of pro-inflammatory factors in culture medium

The concentration of IL-1β in the M, S, O, and I groups was 82.3 ±9.7, 67.5 ±6.8, 42.8 ±5.2, and 86.4 ±10.3 pg/ml, respectively. The concentration of IL-1β was significantly decreased in the O group compared with the M and S groups ($p < 0.05$). The concentration of IL-6 in these groups was 224.3 ±17.3, 184.6 ±15.4, 135.7 ±12.6, and 213.7 ±16.8 pg/ml, respectively. The concentration of IL-6 was significantly decreased

in the S and O groups compared with the M group ($p < 0.05$), and it was significantly decreased in the O group compared with the S group ($p < 0.05$). The concentration of TNF-α in these groups was 382.1 ±27.6, 321.5 ±21.4, 248.6 ±16.9, and 379.8 ±26.5 pg/ml, respectively. The concentration of TNF-α was significantly decreased in the S and O groups compared with the M group ($p < 0.05$), and it was significantly decreased in the O group and significantly increased in the I group compared with the S group ($p < 0.05$). The concentration of IFN-γ in these groups was 21.3 ±3.8, 17.0 ±2.9, 11.6 ±2.1, and 22.5 ±3.6 ng/ml, respectively. The concentration of IFN-γ was significantly decreased in the O group compared with the M and S groups ($p < 0.05$) (Fig. 5).

Discussion

Acute ischaemic stroke, accounts for more than 80% of stroke cases and is one of the major causes of mortality and disability around the world [9]. Sevoflurane was firstly discovered to exert a protective role in ischaemic myocardium in 2013 [47], and a recent study found that sevoflurane treatment enhanced the tolerance of cells to cerebral ischaemic damage, as well as a reduction in neurologic deficit scores, infarction volume, and oxidative stress levels [23]. However, the detailed mechanism was
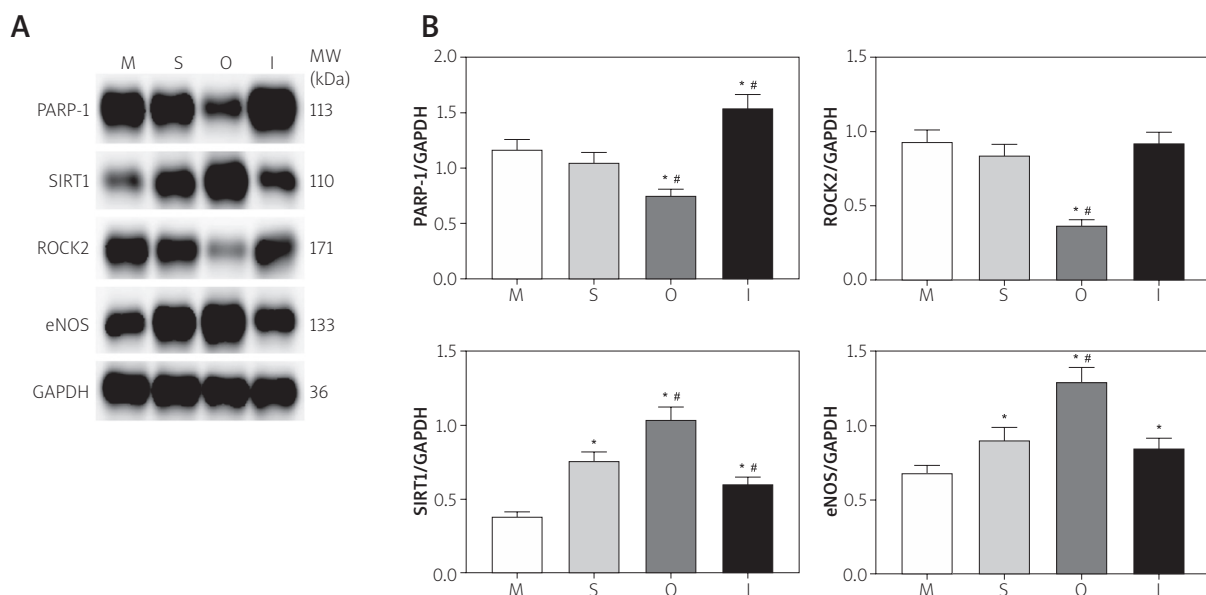

Fig. 4. Expression of PARP-1, SIRT1, ROCK2 and eNOS in each group. *$p < 0.05$ vs. M group; #$p < 0.05$ vs. S group. Data presented as mean ±SD. Each experiment was repeated three times independently.
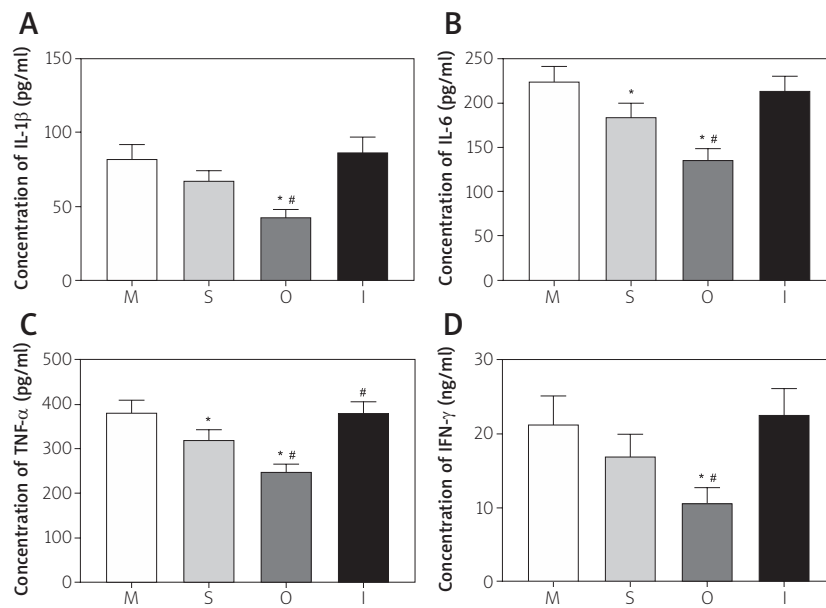
Fig. 5. Detection of **A**) interleukin 1β (IL-1β), **B**) interleukin 6 (IL-6), **C**) tumour necrosis factor α (TNF-α) and **D**) interferon γ (IFN-γ) concentration. *p < 0.05 vs. M group; #p < 0.05 vs. S group. Data was presented as mean ±SD. Each experiment was repeated three times independently.

not fully understood. NLRC3 is the most commonly described members of the NLR family, and it has been proven to regulate the activation of the NF-κB signalling pathway *via* the degradation of IκBα [37]. A recent study found that NLRC3 plays an important role in regulating the innate immune response of the host [27]. Thus, we speculated that NLRC3 might contribute to the therapeutic effect of sevoflurane.

The recovery of blood flow after cerebral ischaemic injury leads to the activation of inflammatory cascades, such as the infiltration of inflammatory cells and release of inflammatory mediators, further leading to the death of neuron cells [38]. Here, we detected the expression of IL-1β, IL-6, IFN-γ, and TNF-α in each group of cells.

Interleukin 1β is constitutively expressed in central nervous system (CNS) and regulates the activity of neurotrophic factor and ion channels [42]. A previous study found that the expression of IL-1β was elevated after ischaemic stroke and reached the peak at 12-24 h [7]. Using gene knockout mice, researchers found that IL-1β aggravates the pathology of ischaemia, while knockout of IL-1β would reduce the infarct volumes [5], and a further study noticed that administration of IL-1β before MCAO would worsen the outcome in rodents [31]. Interleukin 6 is also expressed in normal CNS and regulates homeosta-

sis *via* the classical signalling pathway. Significant elevation of IL-6 was also observed after ischaemic damage, and differing from IL-1β, IL-6 elevation occurred from 6 to 12 h after injury [11]. Another study found that the concentration of IL-6 in serum was increased in the first 24 h with increasing infarct size, and this process not only occurred in the acute phase but also at later time points [33]. The concentration of TNF-α remains at a low level in CNS under normal conditions and regulates transmission and synaptic plasticity [39]. TNF-α is secreted by microglia in the early phase after ischaemia and by macrophages in the late phase [19], and after ischaemia the concentration of TNF-α is significantly elevated at 12-24 h and remains at a high level for days [20]. Interferon γ is another regulator of the inflammatory response process in the CNS; however, the role of IFN-γ in cerebral disease such as ischaemia and trauma is not clear. A previous study found that there is no relevant correlation with IFN-γ and focal ischaemia in mice [21], arguing against IFN-γ as the key regulator of injury after focal ischaemia. T cells were considered the main effector of the antigen-independent innate inflammatory response after the acute phase of ischaemic inflammation, which is mainly regulated by IFN-γ. Interferon γ is regarded as a neurotoxic factor because IFN-γ directly induces

neuronal cell death [25]. Additionally, another study found that regulatory T cells might present a neuroprotective effect *via* suppression of the neurotoxic function of IFN-γ through expression of IL-10 [25]. Here, we found that the expression of these factors was decreased after application of NLRC3 overexpression, indicating a protective role in an ischaemia model.

Many studies have shown that ischaemia-reperfusion injury induced multiple pathological alterations, including metabolism abnormality, inflammatory reaction, and oxidative stress leading to the regeneration of neuron cells [48]. A previous study proved that the TLR signalling pathway plays an important role in the development of ischaemia-reperfusion injury, which is activated by DNA damage and protein degradation [2]. NF-κB could be activated by TRAF6 to regulate the inflammatory response process *via* the MyD88-dependent or -independent pathway. In an MCAO model, researchers noticed that brain oedema and TRAF6 expression were obviously observed, and after the application of TRAF6 inhibitor, the infarction size and the degree of oedema were reduced, as well as the expression of TRAF6 [26]. Further study found that the effect of TRAF6 in ischaemia-reperfusion injury was mainly mediated by the TLR4 signalling pathway [49]. Additionally, TRAF6 is the only member of the TRAF family that can regulate the TNF-α and IL-1β signalling pathway [16], and thus the expression of TRAF6 is closely related to the regulation of the cellular apoptosis process after ischaemia-reperfusion injury [32], and the reduction of TRAF6 expression leads to the inactivation of NF-κB signalling pathway as well as the reduction of cytokine production [10]. In order to activate downstream pathways, TLRs need to bind with a variety of adaptor proteins, including TIR-containing adaptor protein (TIRAP) and MyD88. Under oxidative stress, TRIAP binds with PIP2, leading to the activation of MyD88 and its downstream molecular, NF-κB [36]. A recent study proved that TIRAP could facilitate the interaction of TLR4 and MyD88, indicating that TRIAP is critical for the formation of endosomal compartments [6]. Another researcher found that atorvastatin inhibits the inflammatory response process due to its ability to inactivate caspase-1 and further inhibit the secretion of IL-1β, and these effects might be mediated by the TLR4/MyD88/NF-κB pathway [17], indicating that MyD88 is an important adaptor of the TLR4 signalling pathway [3].

NF-κB is the central regulator of the inflammatory response, which is regulated by multiple signalling pathways. Besides TLR4/TRAF6, poly(adenosine diphosphateribose) polymerase-1 (PARP-1)/sirtuin 1 (SIRT1) is another possible pathway involved in the regulation of NF-κB activity [40]. It is well-known that activity of PARP leads to the reduction of the intracellular concentration of NAD^+ [1], which is correlated with the reduction of SIRT1 activity because SIRT1 is a NAD^+-dependent protein deacetylase [12]. Further study found that inflammation-related diseases commonly occurred due to the overexpression of PARP-1 [29], and oxidative stress status induced by the inflammatory response leads to the activation of PARP, resulting in the strain of NAD^+ and further inhibition of SIRT1 activity. Reduction of SIRT1 activity further induces the dysfunction of mitochondria, a possible leading cause of chronic inflammatory and metabolic diseases [30]. Rho-associated protein kinase (ROCK) is a downstream target of Rho GTPases, a kind of serine-threonine protein kinase, and is divided into two subtypes: ROCK1 and ROCK2 [18]. ROCK is ubiquitously expressed and performs an important role in neurological diseases including cerebral injury [15]. A previous study found that ROCK2 is activated after ischaemia in a rat model of cerebral ischaemia, and another study found that myosin-binding subunit, a substrate of ROCK2, is elevated in cerebral ischaemia rats and reaches a peak at six weeks, presenting a similar trend to changes in ROCK2 expression [44]. Activation of ROCK2 leads to the degradation of many downstream effectors of the PI3K/AKT signalling pathway, including NF-κB [4]. More evidence has shown that endothelial nitric oxide synthase (eNOS), an important regulator of endothelial function, is regulated by the RhoA/ROCK pathway [43]. Inhibition of RhoA geranylgeranylation would decrease the activation of RhoA, leading to the reduction of ROCK activity and the upregulation of eNOS [22]. Additionally, direct inhibition of ROCK using ROCK inhibitor also increases the expression of eNOS [35]; because eNOS is a protector of the vasculature, these findings show that ROCK might be a protector of cerebral ischaemia. Based on these findings, we speculated that overexpression of NRLC3 might enhance the therapeutic effect of sevoflurane on ischaemia reperfusion through inhibition of the inflammatory response process *via* inhibition of the TRAF6/TLR4 signalling pathway.

Disclosure

The authors declare no conflict of interest.

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