

Minocycline attenuates sevoflurane-induced cell injury via activation of Nrf2

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Abstract. Minocycline has been demonstrated to exert neuroprotective effects in various experimental models. In the present study, we investigated the mechanisms underlying the protective effects of minocycline on cell injury induced by the inhalation of the anesthetic, sevoflurane. In our *in vivo* experiments using rats, minocycline attenuated sevoflurane-induced neuronal degeneration and apoptosis in the rat hippocampus, and this effect was associated with the minocycline-mediated suppression of oxidative stress in the hippocampus. In *in vitro* experiments, minocycline inhibited sevoflurane-induced apoptosis and the production of reactive oxygen species (ROS) in H4 human neuroglioma cells. In addition, minocycline suppressed the sevoflurane-induced upregulation of interleukin (IL)-6 and the activation of the nuclear factor- κ B (NF- κ B) signaling pathway in H4 cells. Furthermore, we found that nuclear factor E2-related factor 2 (Nrf2), an activator of the stress response, was upregulated and activated upon sevoflurane treatment both in the rat hippocampus and in H4 cells. In addition, minocycline further augmented the upregulation and activation of Nrf2 when used in conjunction with sevoflurane. Moreover, the knockdown of Nrf2 in H4 cells by small interfering RNA (siRNA) diminished the cytoprotective effect of minocycline, and attenuated the inhibitory effect of minocycline on ROS production, IL-6 upregulation and the activation of the NF- κ B signaling pathway. On the whole, our findings indicate that minocycline may exert protective effects against sevoflurane-induced cell injury via the Nrf2-modulated antioxidant response and the inhibition of the activation of the NF- κ B signaling pathway.

Introduction

There is accumulating evidence to suggest that the inhalation of anaesthetics can induce nervous system injury which is characterized by cognitive dysfunction, a lack of concentration and an impaired ability to process information (1). Sevoflurane, for example, which is one of the most commonly used inhaled anaesthetics, has been demonstrated to impair learning and memory abilities in aged rats and to induce the apoptosis of rat hippocampal neurons (2). In addition, sevoflurane has been demonstrated to induce apoptosis, to increase interleukin (IL)-6 expression and to induce the activation of the nuclear factor- κ B (NF- κ B) signaling pathway in human neuroglioma cells (3). Therefore, more attention should be paid to the use of inhaled anaesthetics, such as sevoflurane in elderly patients, while finding an agent that can counteract the nervous system injury induced by the inhalation of anaesthetics would be of great significance for reducing the potential adverse effects of anaesthetics.

Minocycline, a second generation semi-synthetic derivative of tetracycline antibiotics, is mainly used in the treatment of infectious diseases in clinical practice. Minocycline can easily pass through the blood-brain barrier (BBB) (4), and has been demonstrated to attenuate neuronal cell death and improve the symptoms of neurodegenerative disorders in animal models (5,6). Recent studies have demonstrated that minocycline exerts neuroprotective effects by suppressing inflammation (7,8) and combating oxidative stress in the brain (9). Our group recently reported that minocycline attenuated sevoflurane-induced cognitive impairment in aged rats (10), suggesting that minocycline may be a promising agent which may be used to counteract anaesthetic-induced neurotoxicity. Hence, in this study, we aimed to validate the cytoprotective effects of minocycline and to investigate the molecular mechanisms underlying the protective effects of minocycline against anaesthetic-induced cytotoxicity. Hopefully, our study will shed some light on the discovery of more neuroprotective agents for the prevention and treatment of anaesthetic-induced nervous system injury.

In our previous study, we identified that the NF- κ B signaling pathway was activated in sevoflurane-induced neuronal injury in aged rats, and the excessive activation of NF- κ B signaling was inhibited by minocycline treatment (10). Yet, the exact mechanisms underlying the sevoflurane-

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induced activation of NF- κ B and its inhibition by minocycline remain unclear. Nuclear factor E2-related factor 2 (Nrf2) is an important stress response transcription factor. In response to various stimulators, Nrf2 translocates from the cytosol to the nucleus, where it binds to the antioxidant response element (ARE) located in the promoter region of a spectrum of oxidative stress-inducible genes (11). A crosstalk between the NF- κ B and Nrf2 pathways under conditions of oxidative stress and during inflammation has been proposed in several studies (12-14). In this study, the involvement of Nrf2 in the minocycline-implemented protective effects against sevoflurane-induced cell injury was investigated. Furthermore, the interaction between the Nrf2 and NF- κ B pathways during this process was characterized via a small interfering RNA (siRNA)-mediated knockdown approach.

Materials and methods

Animal model of sevoflurane-induced hippocampal neuronal injury and treatment with minocycline. Hippocampal neuronal injury in aged rats was induced by the inhalation of sevoflurane as previously described (10). Briefly, 24 male, 20-month-old Sprague-Dawley rats (n=6 per group) were purchased from the Laboratory Animal Center of China Medical University (Shenyang, China). The rats were either pre-treated or not with 45 mg/kg minocycline (Dalian Meilun Biology Technology Co., Ltd., Dalian, China) by intraperitoneal injection before being subjected to the inhalation of 2% sevoflurane (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) for 5 h, and the histology of the hippocampus was analyzed 24 h later after the rats were sacrificed and the brains were removed. All animal experiments and protocols were approved by the Animal Ethics Committee of China Medical University, Shenyang, China.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The histology of the hippocampus and the apoptotic status were examined by hematoxylin staining and by TUNEL assay using the *in situ* Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland). Specifically, the brains were fixed in 4% paraformaldehyde, paraffin-embedded and cut into 5 μ m-thick sections. The sections were then dewaxed, permeabilized with 0.1% Triton X-100 (Beyotime Institute of Biotechnology, Haimen, China) and subjected to TUNEL assay according to the manufacturer's instructions. Hematoxylin was used to stain the nuclei. The sections were examined under an optical microscopy at magnification, x400.

Malondialdehyde (MDA) assay. The hippocampal tissue was excised from the mouse brains, homogenized and centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was collected, and the protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). The level of MDA in the supernatant was measured using the MDA assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Cell culture, transfection and treatment. The H4 human neuroglioma cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The

cells were cultured in RPMI-1640 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

The H4 cells were transfected with Nrf2 siRNA (5'-GCACCUUAUAUCUCGAAGUTT-3') or non-targeting scramble (NS) oligonucleotides (5'-UUCUCCGAACGUG UCACGUTT-3') (both were synthesized by GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. The cells were subjected to treatment 24 h after transfection.

The H4 cells were assigned to 4 experimental groups as follows: i) the control group; ii) the minocycline group; iii) the sevoflurane group; and iv) the minocycline + sevoflurane group. Minocycline was dissolved in DMSO to yield a stock solution (40.5 mM), which was added to the culture medium to a final concentration of 200 μ M for treatment. An equal volume of DMSO was added to the culture medium of the cells in the control and sevoflurane groups. An ambient 4.1% sevoflurane *in vitro*, equivalent to 2 minimum alveolar concentration (MAC) in humans (15), has been previously shown to increase IL-6 levels and to activate NF- κ B signaling pathway in H4 cells (3). The cells were incubated with or without 200 μ M minocycline for 6 h in the presence or absence of 4.1% sevoflurane in the atmosphere consisting of 21% O₂, 5% CO₂ and the remainder, N₂.

Hoechst staining. The Hoechst staining kit (Beyotime Institute of Biotechnology) was used to detect apoptotic cells according to the manufacturer's instructions. The cells were seeded in 12-well microplates and allowed to adhere. After treatment, the cells were fixed, washed with PBS and stained with 0.5 ml Hoechst staining solution for 5 min. Thereafter, the cells were washed, observed under a fluorescence microscopy (BX53; Olympus, Tokyo, Japan) and photographed at a magnification of x400.

Apoptosis assay by flow cytometry. Cell apoptosis was determined by flow cytometry using an Annexin V/propidium iodide (PI) apoptosis detection kit (Beyotime Institute of Biotechnology). The cells were harvested and resuspended in 500 μ l binding buffer. Anti-Annexin V-FITC antibody (5 μ l) and PI (5 μ l) were added to the cell suspension, and the cells were then incubated for 15 min at room temperature, followed by flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using a RNAsimple total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China), and reversed transcribed into cDNA by Super M-MLV reverse transcriptase (BioTeke Corporation, Beijing, China). Quantitative PCR (qPCR) was performed using the cDNA template, SYBR-Green Master Mix (Solarbio, Beijing, China) and the following primers: homo Nrf2 forward, 5'-CAGTCAGC GACGGAAAGAGTA-3' and reverse, 5'-TGTGGGCAA CCTGGGAGTAG-3'; homo β -actin forward, 5'-CTTAGT TGC GTTACACCCTTTCTTG-3' and reverse, 5'-CTGT CACCTTCACCGTTCCAGTTT-3'; rat Nrf2 forward, 5'-CCC

ATTGAGGGCTGTGATCT-3' and reverse, 5'-GTTGGC TGTGCTTTAGGTCC-3'; rat β -actin forward, 5'-GGAGA TTAAGTCCCTGGCTCCTAGC-3' and reverse, 5'-GGCCGG ACTCATCGTACTCCTGCTT-3'. The fluorescence signals were detected using the Exicycler™ 96 quantitative fluorescence analyzer (Bioneer, Daejeon, Korea).

Western blot analysis. NP-40 cell lysis buffer (Beyotime Institute of Biotechnology) was used to extract total proteins from the H4 cells, and RIPA lysis buffer (Beyotime Institute of Biotechnology) was used to extract total proteins from the hippocampal tissues. Nuclear and cytosolic proteins were extracted using the nuclear and cytosolic protein extraction kit (Beyotime Institute of Biotechnology) in accordance with the manufacturer's instructions. BCA protein assays (Beyotime Institute of Biotechnology) were used to determine protein concentrations. Proteins (40 μ g) from each sample were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk at room temperature for 1 h, the membrane was incubated with the following specific primary antibodies: anti-p-I κ B α (1:500; Cat. no. bs-5515R; BIOSS, Beijing, China), anti-Nrf2 (1:400; Cat. no. BA3790; Boster, Wuhan, China), anti-caspase-3 (Cat. no. sc-56053), anti-Bax (Cat. no. sc-7480), anti-Bcl-2 (Cat. no. sc-7382), anti-IL-6 (Cat. no. sc-130326) and anti-NF- κ B (Cat. no. sc-8008) (all 1:1000; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. Subsequently, the membrane was incubated with corresponding horseradish peroxidase (HRP)-labeled secondary antibodies (Cat. nos. A0208 and A0216; Beyotime Institute of Biotechnology) at 37°C for 45 min. The signals of the proteins were visualized using the ECL reagents (7SeaPharmTech, Shanghai, China). The membranes blotted for p-I κ B α was stripped and re-blotted for I κ B α with an anti-I κ B α antibody (Cat. no. sc-1643, Santa Cruz Biotechnology, Inc.). All membranes were stripped and re-probed with anti- β -actin (Cat. no. sc-69879; Santa Cruz Biotechnology, Inc.), anti-histone H3 (Cat. no. bsm-33042M; BIOSS) or anti-lamin A (Cat. no. sc-56137; Santa Cruz Biotechnology, Inc.) to verify equal loading and transfer of protein samples, wherein β -actin and lamin A/histone H3 were used as the corresponding antibodies (Santa Cruz Biotechnology, Inc.) as the internal controls for cytosolic and nuclear proteins, respectively. Following exposure, the films were scanned and analyzed by Gel-Pro analyzer software to determine the densitometric values of the target bands.

Enzyme-linked immunosorbent assay (ELISA). A human IL-6 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) was used to detect the level of IL-6 in the H4 culture medium. The cells were seeded in 12-well microplates and cultured to 80% confluence. After the indicated treatments, the culture medium was collected and tested for the level of IL-6. The absorbance at 450 nm was measured using the ELX-800 absorbance microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The concentration of IL-6 was calculated based on the standard curve generated with the BSA standards in the kit.

Reactive oxygen species (ROS) assay. Intracellular ROS production was determined using the ROS assay kit (Beyotime

Institute of Biotechnology) according to the manufacturer's instructions. After being subjected to the indicated treatments, the cells were incubated in serum-free medium containing 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) for 20 min at 37°C. The cells were then washed with serum-free medium 3 times, resuspended in 500 μ l PBS, and analyzed by flow cytometry.

Immunofluorescence staining. The cells were fixed in 4% paraformaldehyde for 15 min, briefly rinsed with PBS, and permeabilized with 0.1% Triton X-100. After blocking with goat serum for 15 min, the cells were incubated with 1:100 diluted anti-NF- κ B p65 antibody (1:100; Cat. no. sc-8008; Santa Cruz Biotechnology) overnight at 4°C, and then with 1:200 diluted FITC-labeled goat anti-rabbit secondary antibody (Cat. no. A0562; Beyotime Institute of Biotechnology) for 60 min at room temperature. Thereafter, the cell nuclei were counter-stained with DAPI, and the cells were observed under a fluorescence microscopy (BX53; Olympus) and photographed at a magnification of \times 400.

Statistical analysis. GraphPad Prism 5 software was used for data processing and plotting. Values are expressed as the means \pm standard deviation. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test for comparisons between 2 groups. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Minocycline attenuates sevoflurane-induced neuronal injury and oxidative stress in the rat hippocampus. The effect of minocycline on hippocampal neuronal injury in sevoflurane-exposed rats was assessed by TUNEL assay and histological examination. As shown in Fig. 1A, sevoflurane induced the degeneration of rat hippocampal neurons, as evidenced by condensation of the cell nuclei, TUNEL-labeled chromosomal fragmentation, as well as by the sparse and irregular arrangement of the neurons. By contrast, minocycline significantly inhibited sevoflurane-induced neuronal apoptosis and prevented neuronal degeneration in the hippocampus.

ROS is known to be generated during cell metabolic processes and can induce apoptosis through oxidative reactions (16,17). Thus, in this study, we assessed oxidative stress in the rat hippocampus by measuring the level of MDA, a final product of lipid peroxidation. The results revealed that sevoflurane markedly increased the MDA content in the hippocampus, whereas minocycline significantly suppressed the sevoflurane-induced elevation of MDA (Fig. 1B), suggesting that the neuroprotective effects of minocycline are associated with its antioxidant properties.

Minocycline inhibits the sevoflurane-induced apoptosis of and ROS generation in H4 cells. To investigate the mechanisms underlying the protective effects of minocycline against anesthesia-induced toxicity, we employed an *in vitro* model using the H4 cell line. Hoechst staining and flow cytometric analysis were performed to assess the apoptotic status of the H4 cells subjected to the different treatments (exposure to

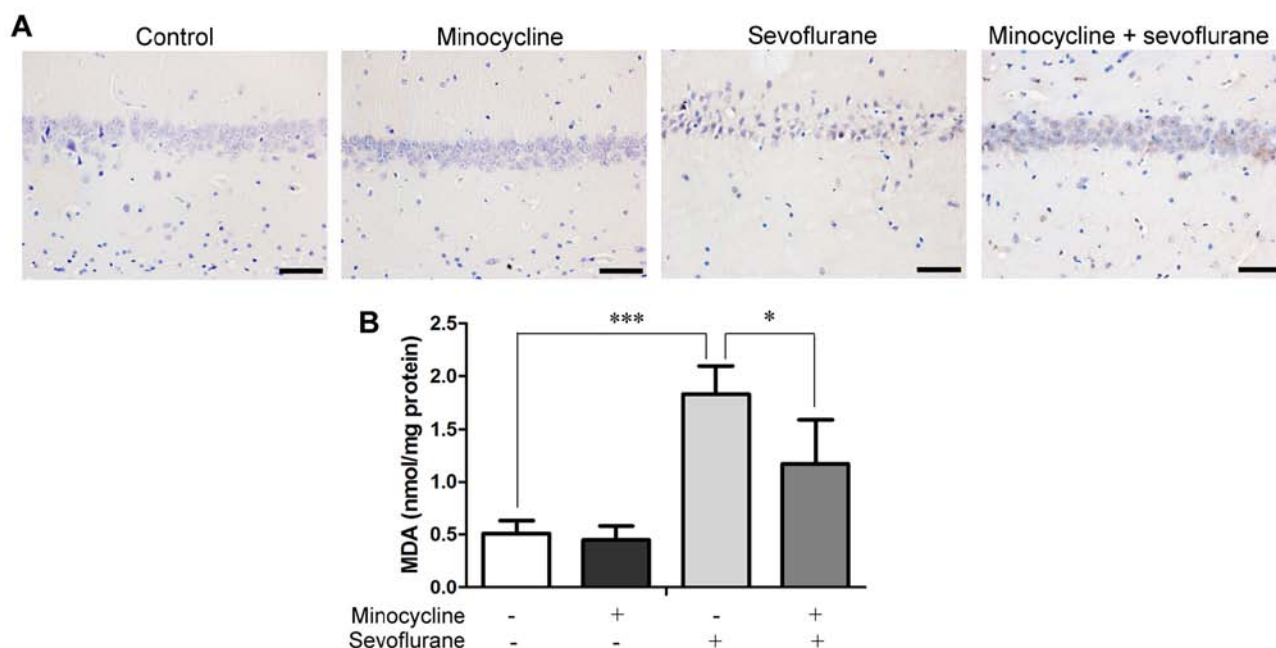


Figure 1. Minocycline alleviates sevoflurane-induced neuronal injury and oxidative stress in the rat hippocampus. Rats were exposed to 2% sevoflurane for 5 h with or without minocycline pre-treatment. (A) The rat brains were fixed, paraffin-embedded, sectioned, and subjected to terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay. The apoptotic cells were labeled and turned brownish ($\times 400$ magnification; scale bar, 50 μm). (B) The level of malondialdehyde (MDA) in rat hippocampal tissues was determined using a respective kit. The values are presented as the means \pm standard deviation ($n=6$ per group). * $P<0.05$ and *** $P<0.001$.

sevoflurane and/or treatment with minocycline). The increased membrane permeability of apoptotic cells allows the uptake of the Hoechst stain by the cells, such that the Hoechst stain binds to the chromosomal DNA and emits fluorescence under a fluorescence microscope. As shown in Fig. 2A, the H4 cells that were co-treated with minocycline and sevoflurane exhibited lower numbers of Hoechst-positive cells, as compared with those treated with sevoflurane alone, suggesting that minocycline attenuated the sevoflurane-induced apoptosis of H4 cells. Consistently, the results of flow cytometric analysis revealed that the apoptotic ratio in the sevoflurane-treated cells ($19.14 \pm 1.97\%$) was significantly increased compared with that in the control cells ($6.96 \pm 0.84\%$) (Fig. 2B and C; $P<0.001$), whereas minocycline co-treatment markedly reduced the apoptotic ratio ($11.33 \pm 1.39\%$) compared with the sevoflurane-exposed cells (Fig. 2B and C; $P<0.001$). These results confirmed the protective effects of minocycline against sevoflurane-induced cell apoptosis. In addition, the results of western blot analysis indicated that exposure to sevoflurane led to the downregulation of Bcl-2 (Fig. 2D and E; $P<0.01$), the upregulation of Bax and to the elevated level of cleaved caspase-3, as compared with the control cells (Fig. 2D, F and G; $P<0.001$ and $P<0.01$, respectively). By contrast, the sevoflurane-induced alterations in the protein levels of Bcl-2, Bax and cleaved caspase-3 were reversed by co-treatment with minocycline. Taken together, the above-mentioned results indicated that minocycline inhibited the sevoflurane-induced apoptosis of H4 cells.

H_2DCFDA assay was performed to investigate whether sevoflurane-induced cell injury and apoptosis are attributed to intracellular ROS production. The ratio of cells generating moderate to high levels of ROS (H_2DCFDA -positive) was

significantly increased in the sevoflurane-exposed cells as compared with the control cells ($P<0.001$), whereas minocycline reduced the number of ROS-producing cells induced by sevoflurane ($P<0.01$) (Fig. 2H and I). Thus, minocycline suppressed sevoflurane-induced ROS production in H4 cells.

Minocycline suppresses the sevoflurane-induced upregulation of IL-6 expression and the activation of the NF- κB signaling pathway. Our previous study demonstrated that minocycline inhibited the sevoflurane-induced upregulation of IL-6 and the activation of NF- κB in the rat hippocampus (10). In this study, in the H4 cells, minocycline significantly reduced the sevoflurane-induced elevation of secreted and intracellular IL-6 (Fig. 3A and B). In addition, compared with the control cells, the levels of the proteolytic and phosphorylated form of I $\kappa\text{B}\alpha$ (i.e., p-I $\kappa\text{B}\alpha$) and nuclear NF- κB p65 were increased in the sevoflurane-exposed cells, resulting in reduced levels of full-length I $\kappa\text{B}\alpha$ and cytosolic NF- κB p65. Sevoflurane-stimulated I $\kappa\text{B}\alpha$ phosphorylation and NF- κB nuclear translocation were significantly inhibited by minocycline co-treatment (Fig. 3C-E). Moreover, immunofluorescence staining also revealed the nuclear translocation of NF- κB p65 upon exposure to sevoflurane, which was blocked by minocycline (Fig. 3F). These results indicated that minocycline inhibited the sevoflurane-induced activation of NF- κB signaling and the production of IL-6 in H4 cells.

Minocycline further augments the upregulation and activation of Nrf2 in the hippocampus of sevoflurane-treated rats and in H4 cells. In our rat model, the prolonged inhalation of sevoflurane led to increased mRNA and protein levels of Nrf2 and Nrf2 in the hippocampus (Fig. 4A and B), implying

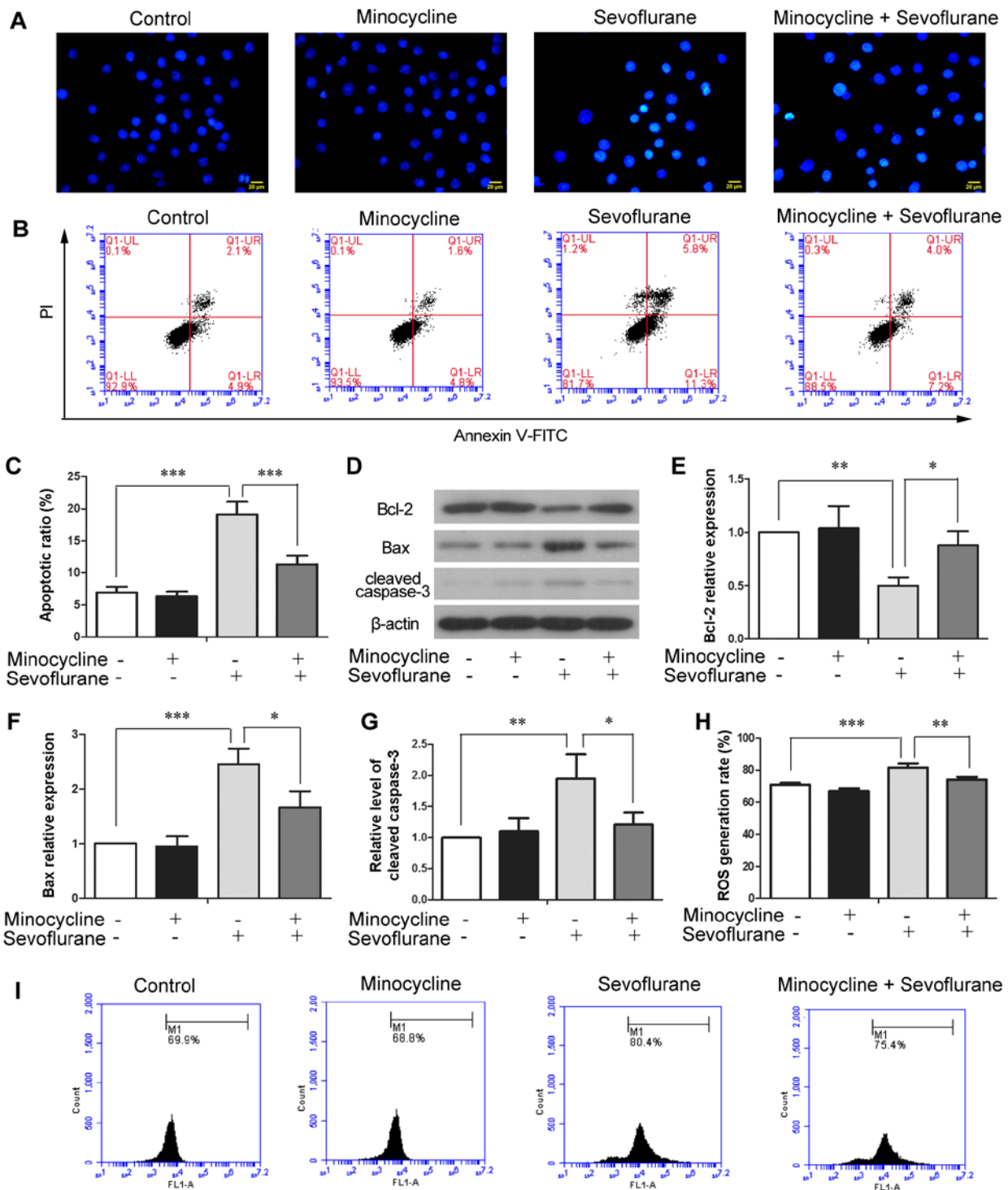


Figure 2. Minocycline inhibits sevoflurane-induced apoptosis and reactive oxygen species (ROS) generation in H4 cells. H4 cells were treated with minocycline, sevoflurane or both for 6 h, and analyzed for apoptosis and ROS production. (A) Hoechst assay (x400 magnification; scale bar, 20 μ m) and (B) FACS analysis were performed to examine cell apoptosis. The cells falling in the lower right and upper right quadrants in the FACS charts were considered as early and late apoptotic cells, and were (C) statistically analyzed. (D-G) Western blot analysis was performed to determine the levels of Bcl-2, Bax and cleaved caspase-3 in H4 cells after treatment. β -actin was used as an internal control for grayscale analysis. (H and I) 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA) stain was used to label intracellular ROS, and the ratio of positively stained cells was analyzed by flow cytometry. This figure shows the representative images of 3 independent experiments, and the values are presented as the means \pm standard deviation. *P<0.05, **P<0.01 and ***P<0.001.

that exposure to sevoflurane led to the upregulated expression and activation/nuclear translocation of Nrf2. Of note, minocycline alone did not affect Nrf2 expression or activation in the rat hippocampus, but further augmented Nrf2 expression and

activation when used in conjunction with sevoflurane (Fig. 4A and B). Similar observations were noted in the H4 cells; minocycline further enhanced the sevoflurane-induced upregulation and activation of Nrf2 (Fig. 4C and D).

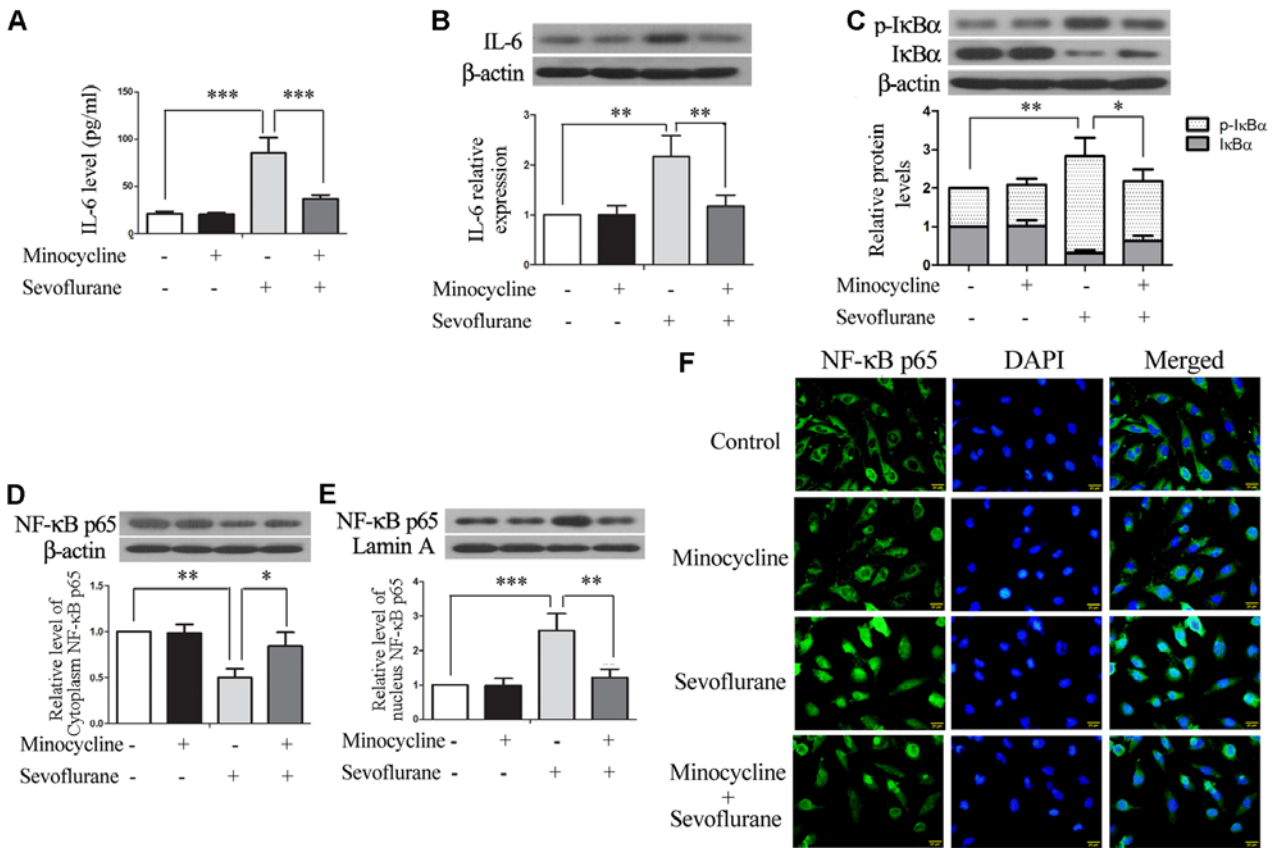


Figure 3. Minocycline inhibits interleukin (IL)-6 expression and activation of nuclear factor-κB (NF-κB) signaling pathway induced by sevoflurane. (A) Enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels of IL-6 in the culture medium of H4 cells after the indicated treatment. Western blot analysis was performed to examine the intracellular levels of (B) IL-6, (C) p-IκBα and IκBα in H4 cells, as well as (D) cytosolic NF-κB p65 and (E) nuclear NF-κB p65 after treatment. (F) The distribution of NF-κB p65 in the cytoplasm and the nucleus was detected by immunofluorescence staining (x400 magnification; scale bar, 20 μm). The figure shows representative images from 3 independent experiments, and the values are presented as the means ± standard deviation. *P<0.05, **P<0.01, ***P<0.001.

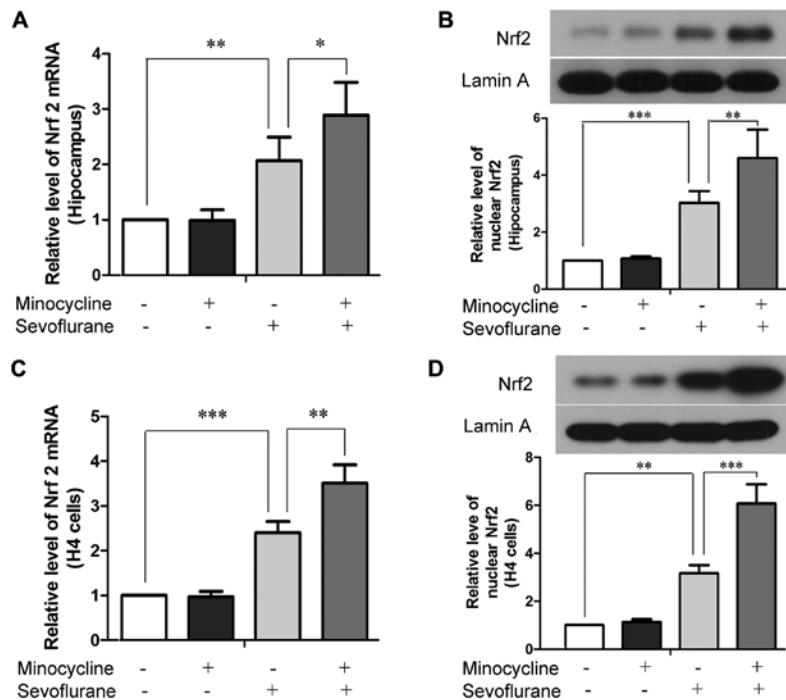


Figure 4. Expression and activation of nuclear factor E2-related factor 2 (Nrf2) in the rat hippocampus and H4 cells after sevoflurane and minocycline treatment. (A) RT-qPCR of Nrf2 mRNA expression and (B) western blot analysis of nuclear Nrf2 protein expression in the rat hippocampus after the indicated treatments (n=6 per group). (C) RT-qPCR of Nrf2 mRNA expression and (D) western blot analysis of nuclear Nrf2 protein expression in H4 cells after the indicated treatments. The figure shows representative images of 3 experiments, and the values are presented as the means ± standard deviation. *P<0.05, **P<0.01, ***P<0.001.

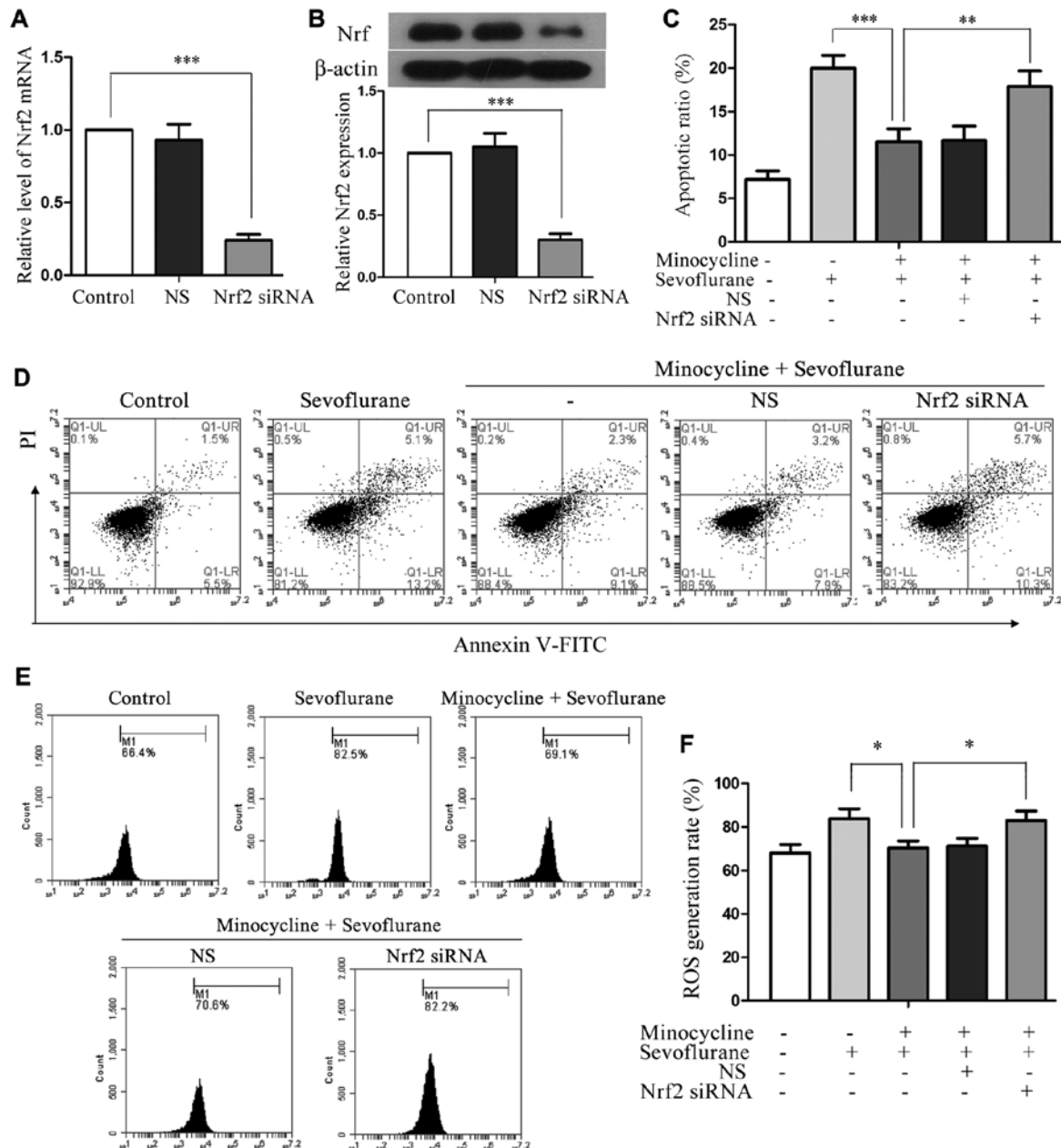


Figure 5. Knockdown of nuclear factor E2-related factor 2 (Nrf2) by small interfering RNA (siRNA) attenuated the cytoprotective effect of minocycline in sevoflurane-treated H4 cells. H4 cells were transfected with Nrf2 siRNA or NS control sequence, and the expression levels of (A) Nrf2 mRNA and (B) Nrf2 protein were examined by real-time polymerase chain reaction (PCR) and western blot analysis respectively. (C and D) FACS analysis of apoptosis after the indicated treatment. (E and F) reactive oxygen species (ROS) production was assessed by 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) staining, followed by flow cytometric analysis. This figure shows the representative images of 3 independent experiments, and the values are presented as the means ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001.

Silencing of Nrf2 attenuates the cytoprotective effects of minocycline in sevoflurane-treated H4 cells. To investigate whether minocycline exerts its cytoprotective effect via Nrf2, we used a siRNA-mediated knockdown approach. Nrf2 siRNA was transfected into H4 cells and achieved approximately 75% reduction in the expression of Nrf2 at both the mRNA and protein level, whereas the NS control sequence did not affect Nrf2 expression or activation (Fig. 5A and B). Compared with the cells co-treated with sevoflurane and minocycline, the Nrf2 siRNA-transfected cells displayed a significantly increased apoptotic ratio (P<0.01; Fig. 5C and D) and an increased number of ROS-producing cells following co-treat-

ment (P<0.05; Fig. 5E and F). These results demonstrated that the anti-apoptotic and anti-oxidative effects of minocycline in sevoflurane-induced cell injury were compromised when Nrf2 was silenced, suggesting that minocycline may exert its cytoprotective effects via the activation of Nrf2.

Silencing of Nrf2 attenuates the inhibitory effects of minocycline on the sevoflurane-induced upregulation of IL-6 and the activation of NF-κB. Compared with the H4 cells co-treated with sevoflurane and minocycline, the level of nuclear Nrf2 was profoundly reduced in the Nrf2 siRNA-transfected cells subjected to co-treatment (P<0.01), although Nrf2 siRNA did

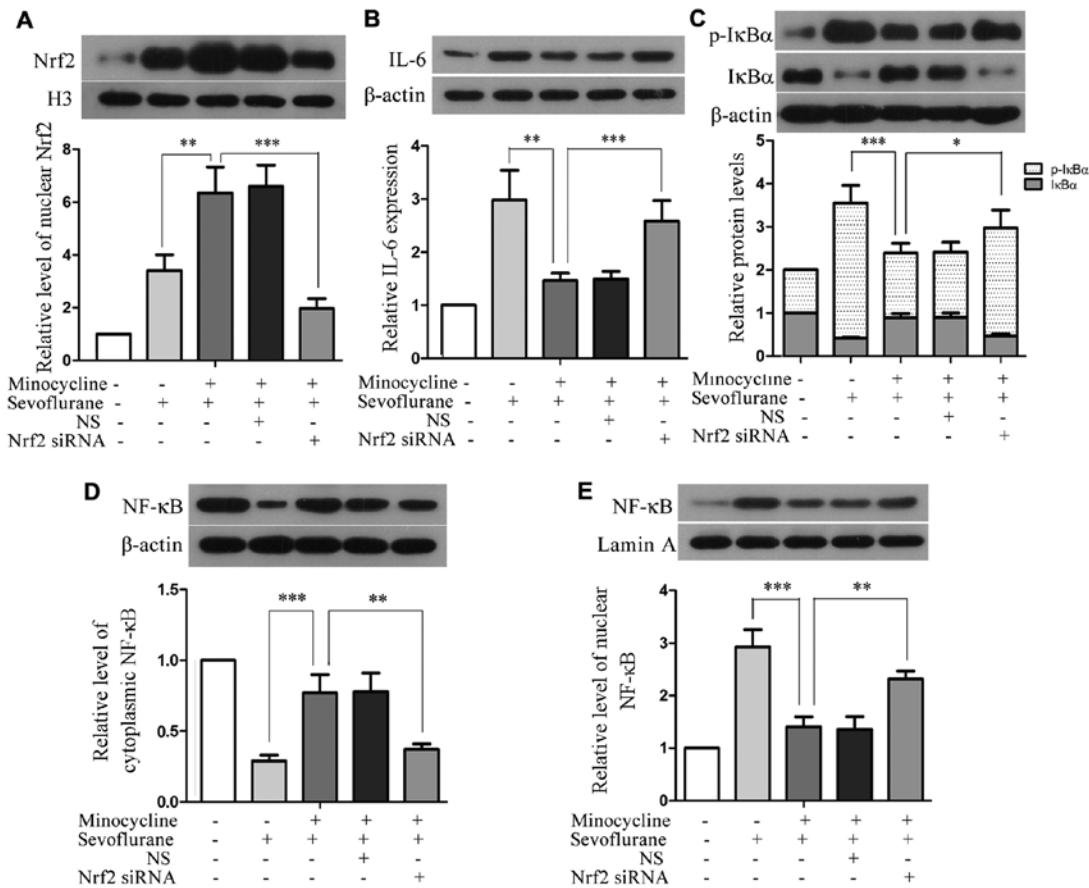


Figure 6. Silencing of nuclear factor E2-related factor 2 (Nrf2) attenuates the inhibitory effect of minocycline on sevoflurane-induced upregulation of interleukin (IL)-6 and activation of the nuclear factor- κ B (NF- κ B) signaling pathway. The levels of (A) Nrf2, (B) IL-6, (C) p-I κ B α and p-I κ B α , (D) cytoplasmic NF- κ B and (E) nuclear NF- κ B after the indicated treatments were examined by western blot analysis. β -actin was used as an internal control for total proteins, while histone H3 and lamin A were used as the control for nuclear proteins. This figure shows the representative images of 3 independent experiments, and the values are presented as the means \pm standard deviation. * P <0.05, ** P <0.01, *** P <0.001.

not completely abolish Nrf2 upregulation (Fig. 6A). Moreover, compared with the cells co-treated with sevoflurane and minocycline, the silencing of Nrf2 resulted in elevated levels of IL-6, p-I κ B α and nuclear NF- κ B, and reduced levels of I κ B α and cytoplasmic NF- κ B in the cells subjected to co-treatment (Fig. 6B-E), implying that the minocycline-mediated inhibition of IL-6 upregulation and the activation of the NF- κ B signaling pathway in sevoflurane-induced cell injury were attenuated upon the silencing of Nrf2. These results suggest that minocycline suppresses the sevoflurane-induced upregulation of IL-6 and the activation of NF- κ B through Nrf2.

Discussion

Minocycline, a broad-spectrum tetracycline antibiotic, has been shown to exhibit independent neuroprotective properties and therapeutic effects in various experimental models of neurodegenerative diseases (5-9); yet the underlying molecular mechanisms responsible for its neuroprotective function remain to be elucidated. Our group previously reported the neuroprotective effect of minocycline in counteracting sevoflurane-induced neurotoxicity in rats (10). In this study, we further characterized the molecular mechanisms responsible for the protective effects of minocycline against sevoflurane-induced neuronal injury. Our results demonstrated that minocycline

effectively inhibited sevoflurane-induced cell apoptosis, ROS production and the activation of the NF- κ B pathway, and simultaneously stimulated the activation of the Nrf2 antioxidant pathway. Our study provides evidences of the potential clinical value of minocycline in the prevention of anesthetic-induced neurotoxicity.

Cell apoptosis is regulated by multiple factors, including caspase family proteins, Bcl-2, Bax and NF- κ B signaling (18). Among these factors, Bax which belongs to the Bcl-2 gene family and caspase-3 have been demonstrated to accelerate apoptosis, while Bcl-2 plays an anti-apoptotic role in cells (19). Previous studies have shown that minocycline inhibits the activation of the caspase pathway (20), and exerts an anti-apoptotic effect by upregulating the apoptotic suppressor Bcl-2 and downregulating pro-apoptotic caspase-1 and caspase-3 (19,21). In this study, we demonstrated that minocycline counteracted the sevoflurane-induced downregulation of Bcl-2, while it significantly reduced the levels of Bax and cleaved caspase-3, which were elevated upon exposure to sevoflurane. These results indicate that minocycline exerts an anti-apoptotic effect in injured cells by suppressing alterations in apoptotic regulatory proteins.

ROS generation, as part of the normal physiological metabolic process, plays important roles in the regulation of cell survival and apoptosis (16,17). Minocycline has been shown

to play a protective role by reducing the production of ROS in 6-OHDA-induced PC12 cell injury and in primary rat oligodendroglial precursor cells upon oxygen-glucose deprivation (22,23). Consistently, our results also demonstrated that minocycline suppressed the elevation of ROS production in the injured H4 cells challenged with sevoflurane. Nrf2 is known to be activated in response to oxidative stress, and it in turn transactivates the expression of antioxidant response genes (11). In this study, Nrf2 was activated upon sevoflurane-induced oxidative stress both *in vivo* and *in vitro*. Intriguingly, minocycline alone had no effect on Nrf2 activation or ROS generation, but it further augmented the activation of Nrf2 when used in conjunction with sevoflurane, and suppressed the sevoflurane-induced elevation of ROS production. Furthermore, the antioxidant effects of minocycline were markedly attenuated when Nrf2 expression was downregulated by siRNA. These results suggest that minocycline exerts its antioxidant effects by activating the antioxidant machinery via Nrf2.

NF- κ B exists as a dimer composing of polypeptide chain p50 and p65. I κ B, an inhibitor of NF- κ B signaling, binds to the NF- κ B dimer to form a trimer in the cytoplasm, and undergoes proteolysis and phosphorylation in response to the activating stimuli of NF- κ B signaling, resulting in the dissociation of the trimer and NF- κ B nuclear translocation. Previous studies have shown that elevated cellular ROS levels can contribute to the prolonged activation of c-Jun N-terminal kinase (JNK) and other kinases, leading to the phosphorylation of I κ B by I κ B kinase (IKK) (24), and the nuclear translocation of NF- κ B. Hence, sevoflurane-induced ROS elevation and the counteracting action by minocycline in this study is probably the regulatory mechanism for the activation of the NF- κ B signaling pathway. In the nucleus, NF- κ B activates the transcription of downstream genes that are critical players in inflammation and apoptosis, such as IL-6 (25). Moreover, both Bcl-2 and Bax have been shown to be downstream targets of NF- κ B, and the transcriptional tuning of these two genes by NF- κ B for promoting or inhibiting apoptosis depends on the stimulus and the cell type (26). In addition, it is well recognized that the ROS-mediated crosstalk between NF- κ B and JNK signaling is critical for the regulation of the Bcl-2/Bax ratio and apoptosis (27). In the present study, our results suggested that the activation of NF- κ B signaling was involved in the sevoflurane-induced cytotoxicity and apoptosis. Moreover, the silencing of Nrf2 inhibited the activation of the NF- κ B signaling pathway, and this may be mediated via Nrf2-controlled intracellular oxidative stress. Therefore, alterations in cellular ROS levels, modulated by the Nrf2 pathway, signal the NF- κ B pathway and control cell fate.

In conclusion, our findings demonstrate that minocycline attenuates sevoflurane-induced cell injury by inhibiting apoptosis, reducing ROS production and blocking the activation of the NF- κ B signaling pathway. Its antioxidant and anti-apoptotic effects are likely to be mediated via the activation of Nrf2. Our study suggests a protective property of minocycline against anesthetic-induced cell injury, and provides insight into the mechanisms underlying the cytoprotective properties of minocycline. Thus, we propose further development and validation of minocycline as a cytoprotective agent for its potential clinical application in the prevention/treatment of anesthetic-induced nervous system injury.

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