

Sevoflurane downregulates IGF-1 via microRNA-98

JUE JIANG, ZHIFENG CHEN, YAQIONG YANG, JIA YAN and HONG JIANG

Department of Anesthesiology, Shanghai Ninth People's Hospital,
Shanghai Jiao Tong University School of Medicine, Shanghai 200011, P.R. China

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Abstract. Insulin-like growth factor (IGF)-1 functions as a neuroprotective hormone and may protect against cognitive impairment, which may occur as a result of sevoflurane exposure. The aim of the present study was to assess the effect of sevoflurane on the production of IGF-1 and investigate the molecular mechanisms underlying this regulation. The BRL rat hepatocyte cell line and adult mice were exposed to 1 or 2 minimal alveolar concentrations sevoflurane for 4 or 8 h. IGF-1 and microRNA-98 levels were quantified using an enzyme-linked immunosorbent assay, western blotting and reverse transcription-quantitative polymerase chain reaction analyses. The importance of microRNA-98 in the regulation of IGF-1 by sevoflurane was investigated using a microRNA-98 inhibitor. Sevoflurane treatment reduced IGF-1 levels and simultaneously upregulated microRNA-98 expression levels in rat hepatocytes and adult mice. Inhibition of microRNA-98 attenuated this effect. Therefore, sevoflurane may reduce the synthesis of IGF-1 by upregulating microRNA-98 expression.

Introduction

Alzheimer's disease (AD) is one of the most common chronic neurodegenerative disorders. The pathology of AD is associated with the formation of amyloid- β (A β) plaques and the hyperphosphorylation of tau proteins, which may lead to the formation of intracellular neurofibrillary tangles (1). Postoperative cognition dysfunction (POCD) may occur partly due to A β accumulation and tau hyperphosphorylation induced by volatile anesthetics (2).

Insulin-like growth factor (IGF)-1 protects neurons against the toxic effects of A β (3). Circulating IGF-1 may traverse the blood-brain barrier (4) to increase A β clearance from the brain and reduce tau phosphorylation via inhibiting glycogen

synthase kinase 3 β (GSK3 β) (5-7). Previous population-based studies involving elderly individuals have revealed that low circulating IGF-1 levels contribute to cognitive decline (8,9). A previous study demonstrated that circulating IGF-1 levels were reduced in patients anesthetized with sevoflurane, which negatively correlated with POCD (10).

Halogenated inhalational anesthetics, including halothane, enflurane, isoflurane, methoxyflurane, desflurane and sevoflurane enter and primarily leave the body through the respiratory system. However, due to the lipophilic properties of the drugs, they may be absorbed and require cellular metabolism for detoxification and excretion (11,12). This process occurs primarily in the liver; therefore, volatile anesthetics may disproportionately affect hepatic function following surgery (13).

The aim of the present study was to investigate the effect of sevoflurane exposure on the production of IGF-1 in the liver, which is the primary site of IGF-1 production (14,15). Sevoflurane was selected for investigation in the current study, as it is currently the most frequently used inhalational anesthetic.

Materials and methods

Cell line and reagents. The BRL rat hepatocyte cell line was obtained from the Institute of Biomedical Sciences, Fudan University (Shanghai, China). BRL hepatocytes were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Bio-Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotic-antimycotic mixture containing 100 U/ml penicillin and 100 mg/ml streptomycin (Cellgro; Corning, Inc., Corning, NY, USA). Cells were maintained at 5% CO₂ and 37°C and subcultured to a density of 1x10⁵/ml every 2 days. All experiments were performed using cells in the logarithmic phase of growth. Sevoflurane was purchased from Shanghai Hengrui Pharmaceutical Co., Ltd. (Shanghai, China) ThemicRNA-98 inhibitor and inhibitor control were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Animals. A total of 10 C57BL/6J mice in each group (age, 18 months; weight, 23.9-40.1 g; female: Male, 1:1) were provided by the Laboratory Animal Center of Shanghai Tenth People's Hospital (Shanghai, China). The housing and treatment of the animals were in accordance with institutional

Correspondence to: Professor Hong Jiang, Department of Anesthesiology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, P.R. China
E-mail: dr_jianghong@163.com

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guidelines and were approved by the Institutional Animal Care and Use Committee of Shanghai Ninth People's Hospital (Shanghai, China): Room temperature 22-26°C, 12-h light dark cycle with free access to food and water. Estimates of sample size were based on previous reports (16,17). A sample size of 10 should enable the detection of >15% difference with 80% power and 5% significance level, with an assumed standard deviation of 12.5% (18). Therefore, the present study used a sample size of 10 mice.

Treatment of BRL hepatocytes and adult mice with sevoflurane. The minimal alveolar concentration (MAC), which leads 50% patients to anesthesia, was used to assess the efficacy of inhalational anesthetics. Previous studies have employed concentrations of between 1.5 and 4.1%, with an exposure time of between 2 and 6 h (16,18-22). In the present study, hepatocytes and mice were treated with 1MAC or 2MAC sevoflurane (1 MAC=1.5% sevoflurane in adult mice) for 4 h or 8 h prior to quantification of IGF-1 expression level.

Cultured hepatocytes were treated with sevoflurane as previously described (2). CO₂, O₂ and sevoflurane levels were continuously monitored. O₂ (21%), 5% CO₂ and 1MAC or 2MAC sevoflurane were delivered using an anesthesia machine (Avance CS2; GE Healthcare Life Sciences, Chalfont, UK) to BRL hepatocytes cultured in 6-well plates in a sealed plastic box at 37°C. Cells were seeded at a density of 1x10⁶ cells/well and cultured in 1 ml cell culture media. Control BRL hepatocytes were exposed to 5% CO₂ plus 21% O₂. The effect of microRNA-98 on sevoflurane-mediated regulation of IGF-1 inhibition was investigated using 100 nM microRNA-98 inhibitor according to the manufacturer's protocol (RiboBio Co., Ltd.). Cells were treated with the same concentration of the inhibitor or inhibitor control 48 h prior to sevoflurane exposure.

The anesthesia of adult mice with sevoflurane was performed as previously described (23). Briefly, the adult mice were exposed to 1MAC or 2MAC anesthetics and 100% oxygen delivered by an anesthesia machine in individual, environmentally controlled chambers.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Mice were sacrificed by decapitation immediately following sevoflurane anesthesia. The liver was removed rapidly and frozen in liquid nitrogen for subsequent processing. Total RNA from treated cells (2x10⁶) or liver samples was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (24). A total of 1 µg RNA was reverse transcribed. cDNA was synthesized using random hexamer primers (Invitrogen; Thermo Fisher Scientific, Inc.) and the SuperScript II reverse transcriptase enzyme (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Sequences are as follows: IGF-1-F, CTGGACCAGAGACCCCTTTC; IGF-1-R, GGACGGGGA CTTCTGAGTCTT; GAPDH-F, AGGTCGGTGTGAACG GATTTG; GAPDH-R, TGTAGACCATGTAGTTGAGGT CA. The thermal cycling parameters are as follows: 95°C for 30 sec, 95°C for 5 sec for 40 cycles, 60°C for 30-34 sec. microRNAs were prepared using a microRNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). The expression of microRNA-98 was normalized to that of U6 small nuclear

RNA. The expression level of IGF-1 was normalized to that of GAPDH. The data were analyzed using MxPro-Mx3000P software (version, 4.00; Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) and the 2^{-ΔΔC_q} method (25).

Enzyme-linked immunosorbent assay (ELISA). IGF-1 levels in the culture medium were quantified using a rat IGF-1 enzyme immunoassay kit (cat. no.: FRK0032; Assay Designs, Inc., Ann Arbor, MI, USA) at an absorbance of 450 nm. The IGF-1 level (ng/ml) was calculated according to the manufacturer's protocol.

Blood samples were collected from the tail vein of mice prior to and following exposure to sevoflurane. A total of 500 µl blood was centrifuged at 4,000 x g for 20 min at 4°C, and the serum samples were stored at -80°C until required for downstream analysis. Serum IGF-1 concentrations were quantified using IGF-1 ELISA kit (cat. no. MG-100; R&D Systems Europe, Ltd., Abingdon, UK) at an absorbance of 450 nm.

Immunoblot analysis. A total of 2x10⁶ BRL hepatocytes and liver tissues were harvested at the end of each experiment and were placed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) on ice. Homogenates were then centrifuged at 12,000 x g for 15 min at 4°C, and the protein concentrations were quantified using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 20 µg protein for each sample were separated using 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1.5 h at room temperature, before they were incubated overnight at 4°C with anti-β-actin (cat. no. ab3280; dilution, 1:1,000; Abcam, Cambridge, UK) and anti-IGF-1 primary antibodies (cat. no. ab9572; dilution, 1:1,000; Abcam, Cambridge, UK). The immune complexes were detected by incubating of the membranes in a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (IgG; cat. no. 33101 ES60; dilution, 1:1,000; Shanghai Yeasen Biological Technology Co., Ltd., Shanghai, China) for 1 h at room temperature. Labeled protein bands were detected by enhanced chemiluminescence (EMD Millipore).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version, 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as mean ± standard deviation. Statistical significance was determined using unpaired Student's *t*-tests to compare a sevoflurane exposure group with the control group, or to compare the two groups with different doses of sevoflurane. P<0.05 was considered to indicate a statistically significant difference.

Results

Sevoflurane downregulates IGF-1 and upregulates microRNA-98 in BRL hepatocytes. IGF-1 levels were quantified following sevoflurane exposure in BRL hepatocytes. ELISA analysis revealed that sevoflurane significantly downregulated IGF-1 protein levels in BRL hepatocytes in a dose- and time-dependent manner when compared with untreated controls (Fig. 1A). Western blot analysis confirmed these results (Fig. 1B). No significant alterations in IGF-1

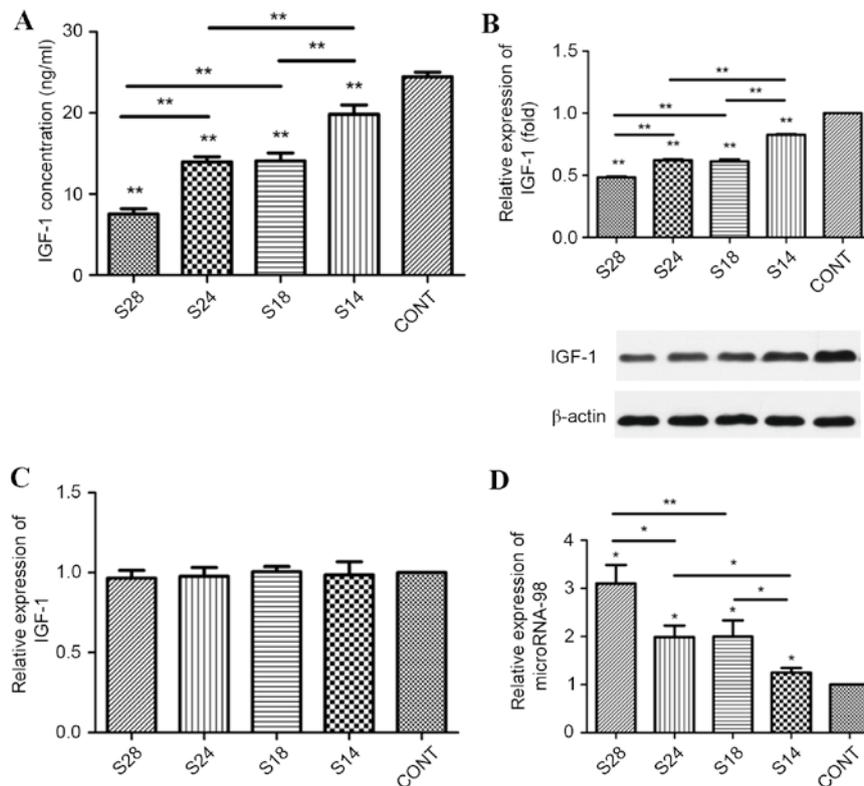


Figure 1. Sevoflurane affects IGF-1 production and microRNA-98 levels in rat BRL hepatocytes *in vitro*. (A) IGF-1 concentration in the culture medium as determined by ELISA. (B) Quantification of IGF-1 protein expression levels, as determined by western blot analysis. Quantification of (C) IGF-1 mRNA and (D) microRNA-98 expression levels by reverse transcription-quantitative polymerase chain reaction analysis. * $P < 0.05$ and ** $P < 0.01$ vs. CONT or as indicated. IGF-1, insulin-like growth factor 1; S28, 2MAC sevoflurane for 8 h; S24, 2MAC sevoflurane for 4 h; S18, 1MAC sevoflurane for 8 h; S14, 1MAC sevoflurane for 4 h; CONT, control group; MAC, minimal alveolar concentration.

mRNA levels were observed in sevoflurane-treated BRL hepatocytes when compared with untreated controls, as determined by RT-qPCR analysis (Fig. 1C). These results suggest that sevoflurane may regulate IGF-1 expression in BRL hepatocytes at a post-transcriptional level. Analysis of microRNA-98 expression in BRL hepatocytes revealed that sevoflurane exposure significantly increased microRNA-98 expression in a time- and dose-dependent manner when compared with untreated controls (Fig. 1D).

Sevoflurane downregulates circulating IGF-1 levels in adult mice. To investigate the effect of sevoflurane on circulating IGF-1 levels in adult mice, the serum IGF-1 concentration was quantified using ELISA. It was determined that, administration of sevoflurane significantly reduced serum IGF-1 levels when compared with the same mice prior to treatment (Fig. 2A). This effect was dose- and time-dependent (Fig. 2B).

Sevoflurane treatment reduces IGF-1 and increases microRNA-98 expression in the liver of adult mice. As the majority of serum IGF-1 is produced in the liver (14,15), the levels of IGF-1 protein in liver samples were quantified using RT-qPCR and western blot analyses in the present study. No significant differences in IGF-1 mRNA expression levels in mouse liver tissues were identified among the sevoflurane treatment groups (Fig. 3A). However, it was evident that the expression level of microRNA-98 in the liver of adult mice was significantly increased in sevoflurane-treated groups in a

dose- and time-dependent manner ($P < 0.01$; Fig. 3B). Similar to the mRNA expression levels, exposure to sevoflurane significantly reduced the protein expression levels of IGF-1 in liver tissues derived from sevoflurane-treated mice when compared with untreated controls ($P < 0.01$; Fig. 3C).

Sevoflurane inhibits IGF-1 translation by upregulating of microRNA-98 expression. In order to determine whether sevoflurane inhibited IGF-1 translation by upregulating microRNA-98, BRL hepatocytes were pretreated with 100 nM microRNA-98 inhibitor and inhibitor control at 48 h prior to exposure to sevoflurane. The microRNA-98 inhibitor significantly reduced microRNA-98 expression when compared with those treated with the microRNA-98 inhibitor control ($P < 0.01$; Fig. 4A). No significant alterations in IGF-1 mRNA expression levels among all treatment groups were observed (Fig. 4B). However, microRNA-98 inhibitor treatment significantly increased the IGF-1 protein expression levels when compared with the sevoflurane-only treated group ($P < 0.01$; Fig. 4C).

Discussion

Sevoflurane may affect spatial memory by increasing the level of A β (26) and tau phosphorylation (22). IGF-1 has been demonstrated to protect neurons against the toxic effects of A β (3) and reduce tau phosphorylation by inhibiting GSK3 β (5,6). In addition, low concentrations of circulating IGF-1 may contribute to cognitive decline (8,9). This suggests that sevoflurane may

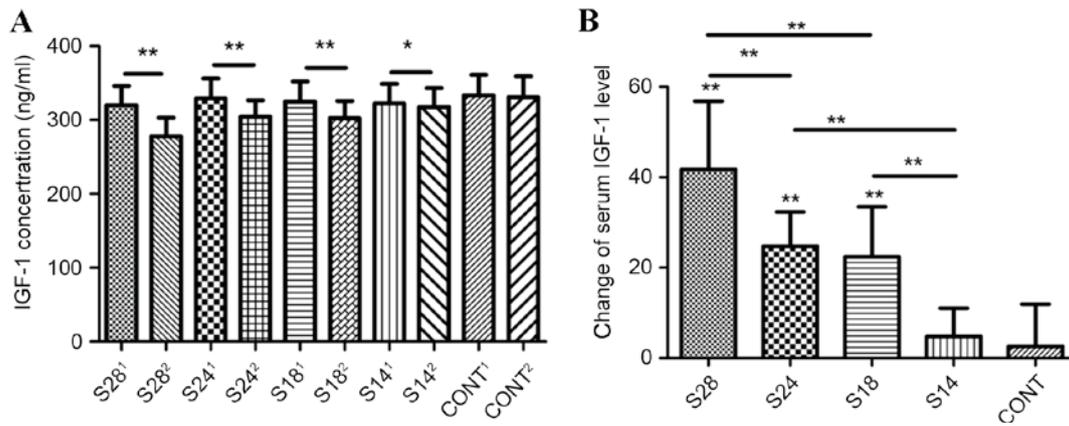


Figure 2. Sevoflurane influenced circulating IGF-1 levels in adult mice. (A) Circulating IGF-1 levels prior to and following sevofluranetreatment. ¹IGF-1 levels prior to sevoflurane exposure; ²IGF-1 levels following sevoflurane exposure. *P<0.05 and **P<0.01 as indicated. (B) Change in circulating IGF-1 levels between pre- and post-treatment groups. *P<0.05 and **P<0.01 vs. CONT or as indicated. IGF-1, insulin-like growth factor 1; S28, 2MAC sevoflurane for 8 h; S24, 2MAC sevoflurane for 4 h; S18, 1MAC sevoflurane for 8 h; S14, 1MAC sevoflurane for 4 h; CONT, control group; MAC, minimal alveolar concentration.

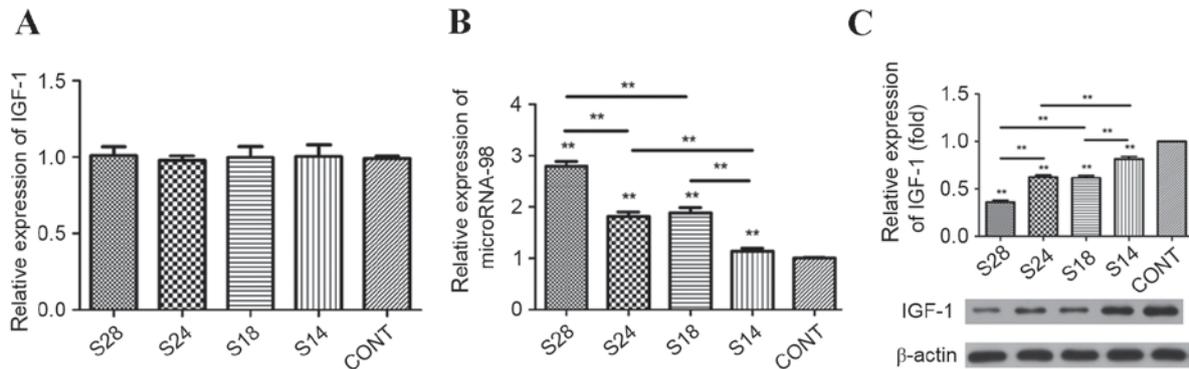


Figure 3. Effect of sevoflurane on IGF-1 production and microRNA-98 expression in mouse livers *in vivo*. Quantification of (A) IGF-1 mRNA and (B) microRNA-98 expression levels as determined by reverse transcription-polymerase chain reaction analysis. (C) Quantification of IGF-1 protein expression levels as determined by western blot analysis. *P<0.05 and **P<0.01 vs. CONT or as indicated. IGF-1, insulin-like growth factor 1; S28, 2MAC sevoflurane for 8 h; S24, 2MAC sevoflurane for 4 h; S18, 1MAC sevoflurane for 8 h; S14, 1MAC sevoflurane for 4 h; CONT, control group; MAC, minimal alveolar concentration.

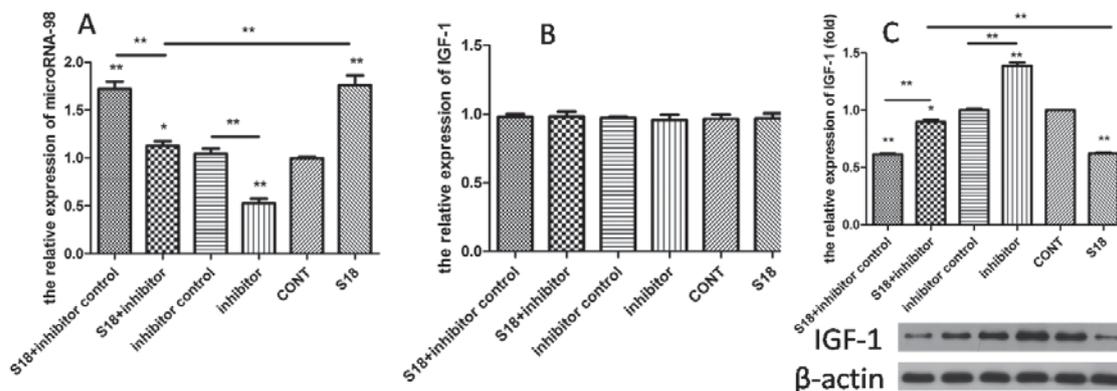


Figure 4. Treatment of rat BRL hepatocytes with a microRNA-98 inhibitor reduced the effect of sevoflurane exposure on IGF-1 expression *in vitro*. (A) Quantification of microRNA-98 and (B) IGF-1 mRNA expression levels as determined by reverse transcription-quantitative polymerase chain reaction. (C) Quantification of IGF-1 protein expression levels as determined by western blotting. *P<0.05 and **P<0.01 vs. CONT. IGF-1, insulin-like growth factor 1; S18, 1MAC sevoflurane for 8 h; CONT, control group; MAC, minimal alveolar concentration.

impair cognition by reducing the level of circulating IGF-1. To investigate this further, the present study quantified IGF-1 expression levels in rat BRL hepatocytes and mice following

sevoflurane treatment. Hu *et al* (27) combined the use of PicTar, miRBase and TargetScan software programs and determined that one binding site of microRNA-98 was located within the

3'-untranslated region (UTR) of IGF-1. Therefore, IGF-1 may be a direct target of microRNA-98, thereby inhibiting IGF-1 expression at a post-transcriptional level. As a result, the present study selected microRNA-98, in order to investigate the mechanisms underlying the effects of sevoflurane exposure on IGF-1 production. The results of current study demonstrated that sevoflurane reduced IGF-1 protein levels, whereas IGF-1 mRNA levels were unaffected. This was accompanied by an upregulation of microRNA-98 levels. Inhibition of microRNA-98 reduced the effect of sevoflurane exposure on IGF-1 protein expression levels, which suggests that sevoflurane may regulate IGF-1 expression at the translational level via microRNA-98.

microRNAs are non-coding RNAs of ~22 nucleotides, and are widely expressed in eukaryotic cells. microRNAs regulate gene expression by repressing translation or by base-pairing with the 3'-UTR of target mRNAs to induce degradation (28). Sevoflurane may influence microRNA-98 expression and has been demonstrated to ameliorate endotoxin-induced acute lung injury by inhibiting the expression of microRNAs that regulate inflammation (29), increasing the expression of rno-microRNA-339-3p, rno-microRNA-448 and rno-microRNA-466b-1FNx01 (30). Additionally, the upregulation of microRNA-101a and microRNA-34b has been demonstrated to be important for neuroprotection against sevoflurane (31). Furthermore, microRNA-1, microRNA-17, microRNA-133 and microRNA-205, associated with the protein kinase B/GSK/cyclin D1 signaling pathway, were significantly downregulated by sevoflurane in the liver (32).

The present study determined that sevoflurane significantly reduced IGF-1 expression and upregulated microRNA-98 expression in the liver, which is in agreement with a previous study that revealed that IGF-1 was a target of microRNA-98 (27). In addition, the expression patterns of IGF-1 and microRNA-98 in response to sevoflurane treatment were consistent with a previous report (27). Inhibition of microRNA-98 reduced the effect of sevoflurane on IGF-1 protein expression, suggesting that sevoflurane regulated IGF-1 production by enhancing microRNA-98 expression.

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