Abstract. In the present study, the mechanisms underlying the protective effects of microRNA-132 (miRNA-132) on sevoflurane-induced neuronal apoptosis were investigated. Reverse transcription-quantitative polymerase chain reaction and gene microarray hybridization were used to analyze alterations in microRNA levels. Cell viability, apoptosis and caspase-3/9 activity were measured using MTT, flow cytometry and caspase-3/9 activity kits. Immunofluorescence staining and western blot analysis were used to measure protein expression of phosphoinositide 3-kinase (PI3K) and phosphorylated (p-)AKT, forkhead box O3a (FOXO3a). In sevoflurane-induced rats, the expression of miRNA-132 was downregulated, compared with that in negative control rats. The downregulation of miRNA-132 increased neuronal apoptosis and the upregulation of miRNA-132 inhibited neuronal apoptosis in the sevoflurane-induced in vitro model. The downregulation of miRNA-132 suppressed the protein expression of PI3K and p-AKT, and suppressed the protein expression of FOXO3a in the sevoflurane-induced in vitro model. The PI3K inhibitor increased the effects of anti-miRNA-132 on neuronal apoptosis through the AKT/FOXO3a pathway in the sevoflurane-induced in vitro model. The promotion of FOXO3a inhibited the effects of anti-miRNA-132 on neuronal apoptosis through the AKT/FOXO3a pathway in the sevoflurane-induced in vitro model. These data suggested that miRNA-132 caused sevoflurane-induced neuronal apoptosis via suppression of the PI3K/AKT/FOXO3a pathway.

Introduction

Surgery has developed rapidly in recent years, which has led to a gradual increase in the proportion of patients receiving general anesthesia (1). General anesthesia has been applied for almost two centuries. However, the precise mechanism of action remains to be fully elucidated (2). Sevoflurane has the advantages of rapid induction and resuscitation. In addition, it has a small influence on liver and renal function, and hemodynamics (2). Therefore, it is applied extensively clinically. It is considered that intravenous and inhaled drugs can induce the apoptosis of central nervous system cells (2). In particular, anesthetic drugs affect the construction of the central nervous network at a critical stage of brain tissue development (2). In addition, they can impair learning and memory function in the future (1). Preclinical experiments have indicated that the neurotoxicity of sevoflurane in general anesthesia on the developing brain is associated with neuronal apoptosis and subsequent cognitive impairment (3). Consequently, treatment against neuronal apoptosis may alleviate sevoflurane-induced neurocognitive impairment (4).

Cell apoptosis, the most commonly observed sevoflurane-induced injury, is associated with complicated and diverse mechanisms (5). The phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway is the most important among numerous signal transduction pathways in the cell apoptotic mechanism (5). Its mechanism of inhibiting cell apoptosis may be realized through regulating apoptin and apoptotic genes (6). Alternatively, it can be achieved through affecting the corresponding changes of mitochondrion and endoplasmic reticulum. Neurotrophin can activate the PI3K/Akt signal transduction pathway (7). The nuclear transcription factor forkhead box O3a (FOXO3a) is a downstream substrate of the PI3K/Akt signaling pathway (8). It is closely associated with cell proliferation, aging, apoptosis, differentiation and tumor genesis (8).

MicroRNAs (miRNAs) are highly conserved, non-coding, single-stranded RNAs, which are only 21-24 nucleotides in length (9). They can inhibit target gene expression at the transcriptional level (10). In addition, they are involved in regulating almost all physiopathological processes, including growth, development, inflammation, swelling and neuro-
degenerative disease (10). The expression of miRNAs is time- and space-specific. The expression level of a specific miRNA may be different under various developmental stages. Alternatively, it may be different under different histological or pathological conditions (9). Wang et al showed that miR-132 affects glioma cell growth (11). Leinders et al also reported that the expression of miR-132-3p is associated with chronic neuropathic pain (12). Yao et al suggested that the hypoxia-induced upregulation of miR-132 promoted Schwann cell migration and facilitated peripheral nerve regeneration (13). Therefore, miR-32 has become a focus of investigations in the medical community. In the present study, the mechanisms underlying the protective effects of miRNA-132 on sevoflurane-induced neuronal apoptosis were investigated.

Materials and methods

Animals and anesthesia treatment. The study protocol was approved by the Animal Use and Care Committee for Research and Education of Shandong University (Shandong, China). Sprague-Dawley rats (male, 220–250 g, 8–10 weeks) were purchased from Shandong Animal Laboratory (Shandong, China) and housed at 22±1°C under a 12-h light/dark cycle (7:00 am-7:00 pm). A total of 12 rats were randomly assigned into two groups: Normal control group (n=6) and sevoflurane-induced model group (n=6). In the sevoflurane-induced model group, the rats were placed into a chamber and exposed to 2% sevoflurane for 6 h. After 24 h, the hippocampus of rats in all groups was analyzed following sacrifice of the rats.

Hematoxylin and eosin staining. The histology of the hippocampus and the apoptotic status were examined using hematoxylin staining. The brains were fixed in 4% paraformaldehyde for 15 min at room temperature followed by 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Beyotime Institute of Biotechnology) at 405 nm. The cells were stained with 1:100 diluted anti-nuclear factor (NF)-κB p65 antibody and resuspended in 500 µl binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with annexin V-FITC antibody (5 µl) and PI (5 µl) for 15 min at room temperature in the dark. The apoptotic rates were measured using flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences). The cells were washed with PBS and resuspended in 500 µl binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with anti-Annexin V-FITC antibody (5 µl) and PI (5 µl) for 15 min at room temperature in the dark. The apoptotic rates were measured using flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences).

Analysis of apoptosis by flow cytometry and caspase-3/9 activity. The cells (1x10^5 cell/well) were washed with PBS and resuspended in 500 µl binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with anti-Annexin V-FITC antibody (5 µl) and PI (5 µl) for 15 min at room temperature in the dark. The apoptotic rates were measured using flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences).

Immunofluorescence staining. The cells were fixed using 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 (Beyotime Institute of Biotechnology) for 15 min at room temperature. The cells (1x10^4 cell/well) were then incubated with 1:100 diluted anti-nuclear factor (NF)-κB p65 antibody
Western blot analysis. Western blot analysis was performed to determine the protein levels of Bcl-2-associated X protein (Bax), PI3K, phosphorylated (p-)AKT, FOXO3a, and GAPDH. The proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat milk and incubated with specific primary antibodies followed by corresponding horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using Enhanced Chemiluminescence reagents and quantified using Image Lab 3.0.

Statistical analysis. The statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). All data were analyzed using Student’s t-test or one-way analysis of variance with Bonferroni’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-132 in sevoflurane-induced rats. Western blot analysis was used to determine the protein levels of Bcl-2-associated X protein (Bax), PI3K, phosphorylated (p-)AKT, FOXO3a, and GAPDH. The expression levels of miR-132 in the H4 cells transfected with miR-132, anti-miR-132 mimic or mimic control were determined by RT-qPCR analysis. As shown in Fig. 2A and B, miR-132 and anti-miR-132 increased and inhibited the expression level of miR-132 in the sevoflurane-induced cells, respectively, compared with the mimic control group. The overexpression of miR-132 and anti-miR-132 mimic increased and inhibited the expression level of miR-132 in the sevoflurane-induced cells, respectively, compared with the mimic control group. The overexpression of miR-132 and anti-miR-132 mimic increased and inhibited the expression level of miR-132 in the sevoflurane-induced cells, respectively, compared with the mimic control group.

miR-132 affects neuronal cell growth in a sevoflurane-induced in vitro model. The expression levels of miR-132 in the H4 cells transfected with miR-132, anti-miR-132 mimic or mimic control were determined by RT-qPCR analysis. As shown in Fig. 2A and B, miR-132 and anti-miR-132 mimic increased and inhibited the expression level of miR-132 in the sevoflurane-induced cells, respectively, compared with the mimic control group. The overexpression of miR-132 and anti-miR-132 mimic increased and inhibited the expression level of miR-132 in the sevoflurane-induced cells, respectively, compared with the mimic control group.

Figure 1. Expression of miRNA-132 in sevoflurane-induced rats. Expression of miRNAs in the (A) control group and (B) sevoflurane-induced rat group were evaluated using a gene chip assay. Each rat was labeled as sample 1-6. Expression of miRNA-132 was determined using (C) reverse transcription-quantitative polymerase chain reaction analysis and (D) in the hippocampus using a hematoxylin and eosin staining assay (magnification, x100). Values are expressed as the mean ± standard deviation (n=6). **P<0.01, compared with the control group. Control, normal rat; sevoflurane, sevoflurane-induced rat. miRNA, microRNA.
downregulation of miR-132 promoted neuronal cell growth and inhibited neuronal cell growth in the sevoflurane-induced in vitro model, respectively, compared with the mimic control group (Fig. 2C and D).

**miR-132 affects neuronal cell apoptosis in a sevoflurane-induced in vitro model.** The overexpression of miR-132 and downregulation of miRNA-132 reduced and increased the apoptotic rate in the sevoflurane-induced in vitro model, respectively, compared with the mimic control group (Fig. 3A and B). DAPI was used to stain cells, and it was also found that the overexpression of miRNA-132 and downregulation of miRNA-132 inhibited and increased cell apoptosis in the sevoflurane-induced in vitro model, respectively, compared with the mimic control group (Fig. 3C and D). The activity of LDH in the cells transfected with miR-132 was reduced and that transfected with anti-miR-132 mimic was increased, compared with that in the mimic control group (Fig. 3E and F).

**miRNA-132 affects neuronal cell growth in a sevoflurane-induced in vitro model.** Expression of miRNA-132 determined using reverse transcription-quantitative polymerase chain reaction following transfection with (A) miRNA-132 and (B) anti-miRNA-132 mimics. Cell proliferation in cells transfected with (C) miRNA-132 and (D) anti-miRNA-132 mimics. Values are expressed as the mean ± standard deviation (n=3). "##P<0.01, compared with the control group. miRNA, microRNA; control, negative control; miRNA-132, overexpression of miRNA-132; anti-miRNA-132, downregulated expression of miR-132.

**miRNA-132 affects the expression of Bax and caspase-3/9 in the sevoflurane-induced in vitro model.** The overexpression of miRNA-132 suppressed the expression levels of Bax and caspase-3/9, and the downregulation of miRNA-132 induced the expression of Bax and caspase-3/9 in the sevoflurane-induced in vitro model, respectively, compared with that in the mimic control group (Fig. 4A-F).
miRNA-132 affects the PI3K/AKT/FOXO3a pathway in the sevoflurane-induced in vitro model. To investigate whether miRNA-132 exerts its cytoprotective effect via the PI3K/AKT/FOXO3a pathway, the protein expression levels of PI3K, p-AKT and FOXO3a were measured in the sevoflurane-induced in vitro model. The overexpression of miRNA-132 induced the PI3K/AKT/FOXO3a pathway in the sevoflurane-induced in vitro model (Fig. 5A-D). The down-regulation of miRNA-132 inhibited the PI3K/AKT/FOXO3a pathway in the sevoflurane-induced in vitro model (Fig. 5E-H), as shown in the immunofluorescence staining (Fig. 5I and J).

PI3K inhibitor increases the effects of anti-miRNA-132 on the AKT/FOXO3a pathway in a sevoflurane-induced in vitro model. The present study also examined whether PI3K inhibitor increased the effects of anti-miRNA-132 on the PI3K/FOXO3a pathway by using the PI3K inhibitor, LY294002 (100 nM), to reduce the PI3K/AKT/FOXO3a pathway in the sevoflurane-induced in vitro model following anti-miRNA-132. As shown in Fig. 6A-D, the PI3K inhibitor suppressed the PI3K/AKT/FOXO3a pathway in the sevoflurane-induced in vitro model following anti-miRNA-132 treatment, compared with the anti-miRNA-132 only-treated group.

PI3K inhibitor increases the effects of anti-miRNA-132 on neuronal apoptosis in the sevoflurane-induced in vitro model. The PI3K inhibitor increased the effects of anti-miRNA-132 on the inhibition of neuronal cell growth, activation of cell apoptosis, activity of LDH, and induction of the expression of Bax and caspase-3/9 in the sevoflurane-induced in vitro model, compared with the effects in the anti-miRNA-132 group (Figs. 7A-D and 8A-C).

Promotion of FOXO3a inhibits the effects of anti-miRNA-132 on neuronal apoptosis through the FOXO3a pathway in a sevoflurane-induced in vitro model. To confirm the activation of the FOXO3a signaling pathway following anti-miRNA-132 treatment in the sevoflurane-induced in vitro model, western blot analysis was performed. The FOXO3 plasmid induced the protein expression of FOXO3a and suppressed the expression of Bax and caspase-3/9 in the sevoflurane-induced in vitro model induced by anti-miRNA-132, compared with the expression levels in the anti-miRNA-132 group (Fig. 9A-D).

Promotion of FOXO3a inhibits the effects of anti-miRNA-132 on neuronal apoptosis through the AKT/FOXO3a pathway in a sevoflurane-induced in vitro model. Finally, the promotion of FOXO3a inhibited the effects of anti-miRNA-132 on the inhibition of neuronal cell growth, activation of cell apoptosis and activity of caspase-3/9 in the sevoflurane-induced in vitro model, compared with the effects in the anti-miRNA-132 group (Fig. 10A-D).

Discussion

Sevoflurane is a common inhaled anesthetic gas (15). At present, the problem of whether sevoflurane anesthesia produces neuroprotective or neurotoxic effects is controversial (15). Numerous existing preclinical studies indicate that general inhaled anesthetics, including sevoflurane, induce extensive neuronal
Figure 5. miRNA-132 affects the PI3K/AKT/FOXO3a pathway in a sevoflurane-induced in vitro model. Protein expression levels of (A) PI3K, (B) p-AKT and (C) FOXO3a were determined using statistical analysis. Western blot analysis was used to determine effects in the (D) overexpression of miRNA-132 group. Protein expression levels of (E) PI3K, (F) p-Akt and (G) FOXO3a were determined using statistical analysis. (H) Western blot analysis following the downregulated expression of miRNA-132. Immunofluorescence staining (magnification, x40) of protein expression following (I and J) the downregulated expression of miRNA-132. Values are expressed as the mean ± standard deviation (n=3). ## P<0.01, compared with control group. miRNA, microRNA; control, negative control; miRNA-132, overexpression of miRNA-132; anti-miRNA-132, downregulated expression of miRNA-132.

Figure 6. PI3K inhibitor increases the effects of anti-miRNA-132 through the AKT/FOXO3a pathway in a sevoflurane-induced in vitro model. Protein expression of (A) PI3K, (B) p-Akt and (C) FOXO3a, determined via statistical analysis of (D) western blot assays. Values are expressed as the mean ± standard deviation (n=3). #### P<0.01, compared with control group; #### P<0.01, compared with the anti-miRNA-132 group. miRNA, microRNA; control, negative control; anti-miRNA-132, downregulated expression of miRNA-132; PI3K inhibitor, LY294002; PI3K, phosphoinositide 3-kinase; p-Akt, phosphorylated Akt; FOXO3a, forkhead box O3A.
Figure 7. PI3K inhibitor increases the effects of anti-miRNA-132 on neuronal apoptosis in a sevoflurane-induced in vitro model. (A) Cell proliferation and (B) apoptosis, determined using flow cytometry and (C) DAPI staining (magnification, x10). (D) LDH activity. Values are expressed as the mean ± standard deviation (n=3). ##P<0.01, compared with the control group; ###P<0.01, compared with the anti-miRNA-132 group. miRNA, microRNA; control, negative control; anti-miRNA-132, downregulated expression of miRNA-132; PI3K inhibitor, LY294002; LDH, lactate dehydrogenase. Arrows indicate apoptotic nuclei.

Figure 8. PI3K inhibitor increases the effects of anti-miRNA-132 on the expression of Bax and caspase-3/9 in a sevoflurane-induced in vitro model. Protein expression of Bax, determined via (A) statistical analysis of (B) western blot assays; (C) caspase-8/9 activity. Values are expressed as the mean ± standard deviation (n=3). ##P<0.01, compared with the control group; ###P<0.01, compared with the anti-miRNA-132 group. miRNA, microRNA; control, negative control; anti-miRNA-132, downregulated expression of miRNA-132; PI3K inhibitor, LY294002.

Figure 9. Promotion of FOXO3a inhibits the effects of anti-miRNA-132 on neuronal apoptosis through the FOXO3a pathway in a sevoflurane-induced in vitro model. Statistical analysis of protein expression of (A) FOXO3a and (B) Bax from (C) western blot assays. (D) caspase-8/9 activity. Values are expressed as the mean ± standard deviation (n=3). ##P<0.01, compared with the control group; ###P<0.01, compared with the anti-miRNA-132 group. FOXO3a, forkhead box O31; Bax, B-cell lymphoma 2-associated X protein; miRNA, microRNA; control, negative control; anti-miRNA-132, downregulated expression of miRNA-132; FOXO3a, forkhead box O31; Anti-miRNA-132, downregulated expression of miRNA-132; Anti-miRNA-132+FOXO3a, anti-miRNA-132+FOXO3a plasmid.
apoptosis (3). In addition, they are accompanied with definite long-term cognitive and memory impairment (4). Neuronal apoptosis is considered a potential mechanism of cell injury. The activated caspase-3 can damage the corresponding cytoplasm and nuclear substrate during early apoptosis, thus inducing cell apoptosis. The results of the present study showed that the expression of miRNA-132 was downregulated in sevoflurane-induced rats, compared with that in the normal group. The downregulated expression of miRNA-132 induced neuronal cell apoptosis in the sevoflurane-induced in vitro model. There are numerous downstream effector molecules of PI3K. Of these, Akt is the central link in the pathway (16). The activation of Akt can act on multiple targets, including the downstream NF-kB family, Bcl-2 family and caspase protein family. It loses activity through phosphorylating the apoptotic protein, enhancing the transcription and expression of apoptotic genes (17). The decreased phosphorylation of Akt downregulates the expression of Bcl-2 and upregulates the expression of Bax in the cytoplasm. This was observed in Akt gene-knockout mice and further aggravated the degree of brain damage (17). The results of the present study indicated that the downregulation of miRNA-132 induced the expression of Bax and caspase-3/9, and promoted the PI3K/Akt pathway (24). The present study showed that the PI3K inhibitor increased the effects of anti-miRNA-132 on neuronal apoptosis through the PI3K/AKT/FOXO3a pathway in the sevoflurane-induced in vitro model. Qi and Zhang also showed that miRNA-132 regulates fluid shear stress-induced differentiation in periodontal ligament cells through the PI3K/AKT/mammalian target of rapamycin signaling pathway (21).

FoxO3a is an important downstream target of the PI3K/Akt pathway. It is important in the anti-apoptotic mechanism of insulin-like growth factor-1 (22). The dephosphorylation of FoxO3a can affect the Bcl-2 family genes, pre-apoptotic gene Bcl-2-interacting mediator of cell death and growth arrest. In addition, it can affect the DNA damage-inducible gene Gadd45α and non-specific cyclin-dependent kinase inhibitor P27kip1 (22). Therefore, it can induce cell apoptosis. Activation of the PI3K/Akt pathway can phosphorylate FoxO3a, which loses its transcription activity (20). Finally, it can reduce neuronal apoptosis. FoxO3a is a transcription factor, which is located in the cell nucleus. It can bind with the response elements on DNA and activate target genes (23). It is also involved in regulating cell proliferation, differentiation, metabolism and apoptosis (23). It has been suggested that FoxO3a can upregulate the expression of cell death-related genes, including P53, which indicates that FoxO3a is regulated by the PI3K/Akt pathway (24). The present study showed that the promotion of FoxO3a inhibited the effects of anti-miRNA-132 on neuronal apoptosis through the AKT/FOXO3a pathway in a sevoflurane-induced in vitro model (25). miRNA-132 may have a neuroprotective effect against sevoflurane-induced neuronal apoptosis through the PI3K/AKT/FOXO3a pathway.

In conclusion, the present study provided evidence that the expression of miRNA-132 was downregulated in sevoflurane-induced rats, compared with that in normal rats. The inhibition of miRNA-132 caused sevoflurane-induced neuronal apoptosis via the PI3K/AKT/FOXO3a pathway. The miRNA-132/PI3K/AKT/FOXO3a signaling pathway responsible for sevoflurane-induced neuronal apoptosis requires elucidation in vivo in future experiments.
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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

BY designed the experiments. PD, XD, JZ, DL and LL performed the experiments. BY and PD analyzed the data. BY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Animal Use and Care Committee for Research and Education of Shandong University (Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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