Sevoflurane promotes the proliferation of HUVECs by activating VEGF signaling

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Abstract. The vascular endothelium plays an essential role in vascular disease and cardiovascular diseases. The effects and underlying mechanisms of sevoflurane on vascular endothelial growth factor (VEGF) in human endothelial cells have not been elucidated. The MTT colorimetric assay was used to determine HUVEC activity at different concentrations (1 and 3%, respectively) of sevoflurane for different time-points (12, 24 and 48 h, respectively). The regulation of sevoflurane on the mRNA levels of VEGFa, VEGFb, VEGFc and VEGFR1,2,3 was analyzed by real-time PCR. When VEGFR2 was inhibited by axitinib, VEGFR2 protein expression was determined by western blotting, and the cell viability was assessed by MTT analysis. The results revealed that sevoflurane increased cell viability in a dose- and time-dependent manner. Sevoflurane significantly upregulated VEGFA mRNA expression only. In addition, sevoflurane increased the expression of VEGFR2 at the mRNA and protein levels, whereas sevoflurane did not modulate the mRNA expression of VEGFR1 and VEGFR3. Furthermore, sevoflurane failed to increase the mRNA and protein expression of VEGFR2 when VEGFR2 was inhibited by axitinib, an inhibitor of VEGF receptors. In conclusion, sevoflurane may be a promising agent against endothelium dysfunction-caused vascular disease by activating the VEGF-A/VEGFR2 signaling pathway.

Introduction

The endothelium presents a single-cell lining on the internal surface of blood vessels, cardiac valves, and numerous body cavities. The vascular endothelium has been considered as a multifunctional organ, which protects the vessel wall from the vascular tone, vessel wall inflammation, and thrombosis resistance (1), and the endothelium participates in new vessel formation (2). Thus, the typical vascular function needs to keep the integrity of the vascular endothelium and a well-balanced release of numerous vasoactive substances (3). Endothelial dysfunction underlies the pathogenesis of vascular disease and cardiovascular diseases, such as coronary artery disease, coronary artery spasm, and atherosclerosis (3-5). Endothelial dysfunction has always been caused by reduced levels and adhesive function of circulating endothelial progenitor cells, which accelerates re-endothelialization (6,7). Previous studies have revealed that angiogenesis is a physiological process involving the growth of new blood vessels either from endothelial cell precursors or from the pre-existing vasculature, and the processes are regulated by various angiogenic growth factors, such as vascular endothelial growth factor (VEGF) (8). By binding to 1 of 3 cognate receptor tyrosine kinases (VEGF receptor 1-3), VEGF has been regarded as the most vital cytokine in enhancing endothelial cell growth. The VEGF-mediated signaling pathway exhibits a vital role in maintaining the structure and function of the vascular endothelium by promoting endothelial cell proliferation (9,10).

Sevoflurane is a general anesthetic, and it has been commonly used in the anesthesia of young children and infants (11). Sevoflurane has exhibited activity against oxidative stress, inflammation, and it has been revealed to protect organs against stress-caused injury (12-14). Sevoflurane pretreatment significantly inhibited TNF-α-induced permeability and p38 MAPK activation in rat pulmonary microvascular endothelial cells by decreasing ICAM-1 levels (15). Sevoflurane appears to offer a more stable heart rate profile compared with either isoflurane or desflurane (16). Notably, sevoflurane increases HUVEC proliferation and adhesion, in addition to the incorporation of tubular structures into endothelial progenitor cells (17). However, the effects and underlying mechanisms of sevoflurane on VEGF in human endothelial cells have not been elucidated. In the present study, the effects and molecular mechanisms of sevoflurane on the proliferation of human umbilical vein endothelial cells (HUVECs) were investigated.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Gibco; Thermo Fisher Scientific, Inc.

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(cat. no. C0155C). Cells were cultured in Medium 200 (cat. no. M200500) supplemented with LSGS (cat. no. S00310; both from Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. HUVECs were digested with Trypsin/EDTA at the appropriate confluency (~70-80%). Cells were cultured in an incubator under normal conditions or with sevoflurane treatment (1 and 3%). Treatment with sevoflurane was performed according to a previously reported method (18) and was achieved by connecting the incubator with the sevoflurane vaporizer (Abbott Laboratories) attached to the anesthetic machine (Dräger). The infrared gas analyzer (Puritan-Bennett) was used to monitor the sevoflurane concentration at the inflow and outflow connectors.

Cell viability assay. Cell viability was performed by MTT assay (cat. no. KA1606; Abnova). HUVECs were seeded in a 96-well plate at 2,000 cells/well under different conditions for 12, 24 48, and 72 h. Reagent medium (15/80 μl per well) was added followed by incubation for 4 h at 37°C. For the treatment with the VEGFA antibody, the cells were incubated with the antibody (20 μM; cat. no. AF-493-NA; R&D Systems) to chelate the effects of VEGFA in the culture medium and the corresponding control antibody (20 μM; cat. no. AB-108-C; R&D Systems) was applied as a control during the exposure of sevoflurane. Then 100 μl of the solubilizer was added to each well. OD570 nm was measured for each well on an absorbance plate reader. The cell viability was calculated by the ratio of OD570 at each time-point to OD570 at 0 h of each well and presented as the percentage of the ratio.

Quantitative RT-PCR. Total RNA was extracted from HUVECs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). For mRNA analysis, 2 μg total RNA was reverse-transcribed to cDNA using the PrimeScript® reagent kit (Takara Bio, Inc.). Quantitative PCR for mRNA was performed using SYBR-Green (Takara Bio, Inc.) with an ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min; followed by 40 cycles of 95°C for 10 sec, 60°C for 5 sec and final extension at 72°C for 10 sec. Quantification of the mRNA expression was normalized to β-actin. The fold-change of the mRNA levels relative to the control cells was calculated using the $2^{-\Delta\Delta Cq}$ method (19). The sequences of the human-specific primers were as follows: VEGFa forward, 5'-CGAAGAGAAGAGACACATTG-3' and reverse, 5'-GGA TGGAGGAAGGTCAAC-3'; VEGFb forward, 5'-ACAGGA CAGAGTTGGAAGA-3' and reverse, 5'-GGAAGAGCC AGTTGTAAGAT-3'; VEGFc forward, 5'-TGTGTCCAGTGT AGATGAAC-3' and reverse, 5'-TCTTCTGTCCTTGAGTTG AG-3'; VEGFR1 forward, 5'-ACTCGTGGCTACTCGTTA-3' and reverse, 5'-ACCTTGCTTCGGAATGATT-3'; VEGFR2 forward, 5'-ACTGTCATCCTTACCAATCC-3' and reverse, 5'-CCTCCAACTGCCAATACC-3' and VEGFR3 forward, 5'-ATGCGAATACCTGTCCTAC-3' and reverse: 5'-GTT GCCGATGTGAATGAG-3'.

siRNA transfection. Cells subjected to siRNA transfection were firstly cultured in half the volume of the culture medium and transfected with 3 ng/ml siRNA (sense, 5'-GCAGCGACAAGG
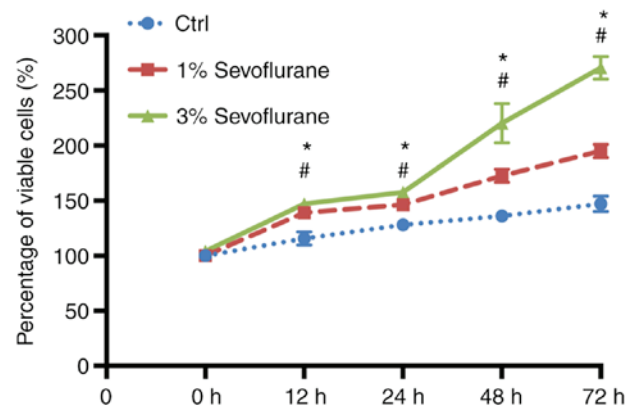

Figure 1. Sevoflurane increases the viability of HUVECs in a dose- and time-dependent manner. HUVECs were incubated in the absence or presence of 1, and 3% concentrations of sevoflurane for various time-points and cell viability was determined by MTT assay. Values represent the means ± SD of triplicate measurements. *P<0.05 of 1% sevoflurane vs. the control group; #P<0.05 of 3% sevoflurane vs. the control group. Data represent three independent experiments. HUVEC, human umbilical cord endothelial cells.

CAGACUAUU-3' and antisense, 5'-UAGUCUGCCUUGUCG CUGCGU-3') or control siRNA (sense, 5'-UUCUCCGAACGU GUCACGUU-3' and antisense, 5'-ACGUGACACGUUCGG AGAATT-3') (Shanghai GenePharma Co., Ltd.) by RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) for 8 h according to the product protocol. After 8 h of incubation, the other half of the culture medium was added, and cells were incubated for another 40 h so that the total interference lasted for 48 h. After 48 h of incubation, the culture medium was changed to normal conditions without siRNA and subjected to further experiments.

Western blotting. Cells from each group were scraped in RIPA lysis buffer (Thermo Fisher Scientific, Inc.) with serine protease inhibitor. Proteins (50 μg) were separated on 10% SDS-polyacrylamide gels and electroblotted onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore). After incubating with blocking buffer, the membranes were incubated overnight at 4°C with the primary antibodies: Anti-VEGFR2 (dilution, 1:2,000; product code ab2349), anti-phosphorylated (p)-VEGFR2 (phosphorylated at Tyr 1175; dilution, 1:2,000; product code ab194806) and anti-beta Actin (dilution, 1:2,000; product code ab8226; all obtained from Abcam). Next, the membranes were washed and incubated with appropriate anti-rabbit HRP-conjugated secondary antibodies (dilution, 1:3,000; product code ab6728, Abcam) at 37°C for 30 min. Protein bands were detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). The integrated optical density of the detected protein band was normalized to the control band for quantitative comparison by ImageJ (v. d1.47; National Institutes of Health).

VEGFR inhibitor. Axitinib, a VEGFR inhibitor, was purchased from Selleck Chemicals and was dissolved in DMSO at a stock concentration of 10 mM. The inhibitor was diluted to an appropriate final concentration in the culture medium.

Statistical analysis. All experiments were performed at least three times with data expressed as the mean ± SEM or as otherwise stated. Statistical analyses were performed with
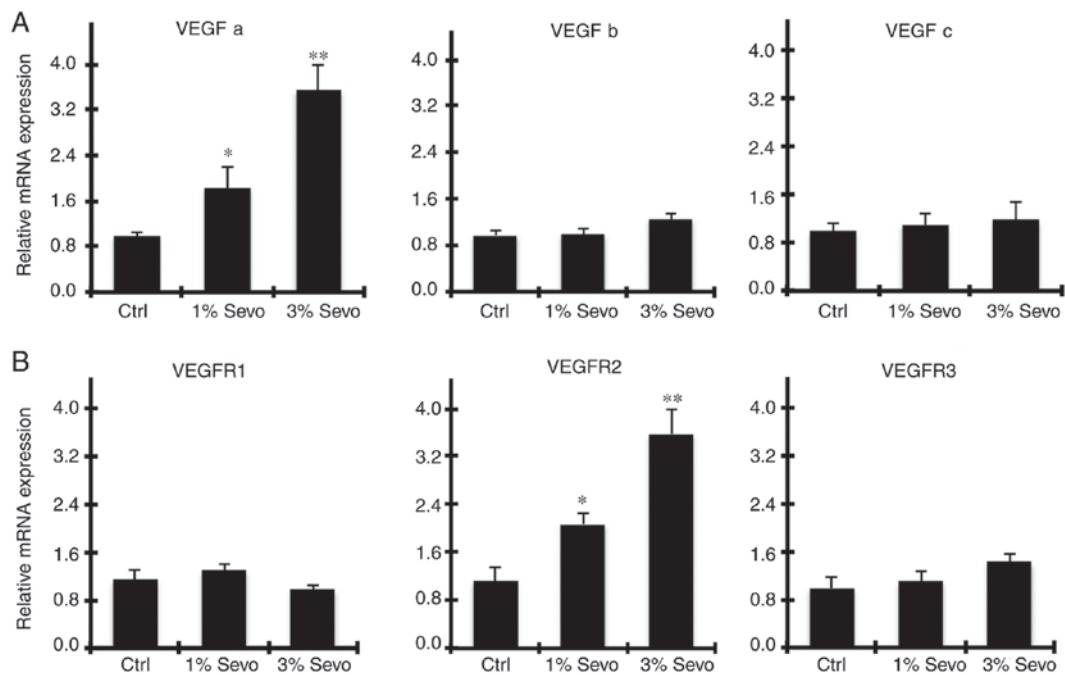
Figure 2. Sevoflurane induces the expression of VEGFa and VEGFR2. Reverse transcription-quantitative PCR analysis of (A) VEGFa, b, c and (B) VEGFR1, 2, 3 expression in HUVECs exposed to the absence or presence of 1 and 3% concentrations of sevoflurane. Only VEGFa and VEGFR2 levels were induced by sevoflurane. Data represent the means ± SD (n=3 for each group, *P<0.05, **P<0.01). Data represent three independent experiments. VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HUVEC, human umbilical cord endothelial cells.

GraphPad Prism software (version 7; GraphPad Software, Inc.). Group comparisons were performed using one-way ANOVA with post hoc test using Dunnett's test or Sidak's multiple comparisons test. The two-column comparison was performed using Student's t-test. P<0.05 or P<0.01 were considered to indicate a statistically significant difference (*P<0.05 and **P<0.01, as indicated in the figures and legends).

Results

Sevoflurane promotes the proliferation of human umbilical vein endothelial cells (HUVECs). To investigate whether sevoflurane was able to stimulate proliferation of HUVECs, the HUVECs were cultured in different concentrations of sevoflurane. The MTT colorimetric assay was used to determine HUVEC activity at different concentrations (1 and 3%, respectively) of sevoflurane at different time-points (12, 24, 48 and 72 h, respectively). The results revealed that sevoflurane increased cell viability in a dose- and time-dependent manner (Fig. 1).

Sevoflurane increases VEGF signaling systems in HUVECs. The VEGF pathway has been demonstrated to serve essential roles in promoting HUVEC proliferation (5). The most well-known VEGFs and their receptors (VEGFRs) include VEGFa, b, and VEGFR-1, 2, 3. Thus qPCR was used to examine the expression levels of VEGFs and VEGFRs of HUVECs treated with 1, and 3% sevoflurane, respectively. As revealed in Fig. 2A, compared with the control group, only the expression level of VEGFa was increased after exposure to sevoflurane for 48 h, and the increase was in a concentration-dependent manner (P<0.05; Fig. 2A). Similarly, VEGFR2 expression was significantly increased after sevoflurane

exposure (P<0.05; Fig. 2B). These data indicated that sevoflurane induced the expression of VEGFa, which may regulate VEGFR2 and activation of the following signaling pathway.

VEGFα inhibition protects the proliferation of HUVECs exposed to sevoflurane. To explore whether VEGFa secreted by HUVECs is involved in regulating the cell proliferation under sevoflurane exposure, HUVECs in the presence of 1 and 3% sevoflurane were transfected with VEGFa siRNA. The interference efficiency is presented in Fig. S1. The data revealed that silenced expression of VEGFa resulted in decreased Ki67-positive HUVECs (Fig. 3A and B). Moreover, the decreased expression of VEGFa exhibited a similar effect on the viability of cells (Fig. 3C and D). To further assess whether VEGFa was sufficient to target sevoflurane stimuli, a VEGFa chelation experiment was performed in cell culture using anti-VEGFa antibodies. As revealed in Fig. 3E and F the proliferation of HUVECs under either 1 or 3% sevoflurane medium was significantly blocked by the addition of anti-VEGFa while the group receiving control IgG antibodies exhibited high proliferation. These data indicated that the enhanced proliferation of HUVECs due to the effect of sevoflurane was regulated by the expression level VEGFa.

Sevoflurane promotes the proliferation of HUVECs by activating the VEGF/VEGFR signaling pathway. To investigate whether VEGF signaling was the underlying mechanism of sevoflurane in enhancing HUVEC proliferation, a small molecular VEGFR inhibitor, axitinib, was used to block the VEGFR signaling pathway. Firstly, western blotting was used to assess the levels of VEGFR and p-VEGFR after exposure of HUVECs to different concentrations of Sevoflurane with VEGFR inhibitor for 48 h. As revealed in Fig. 4A and B, the
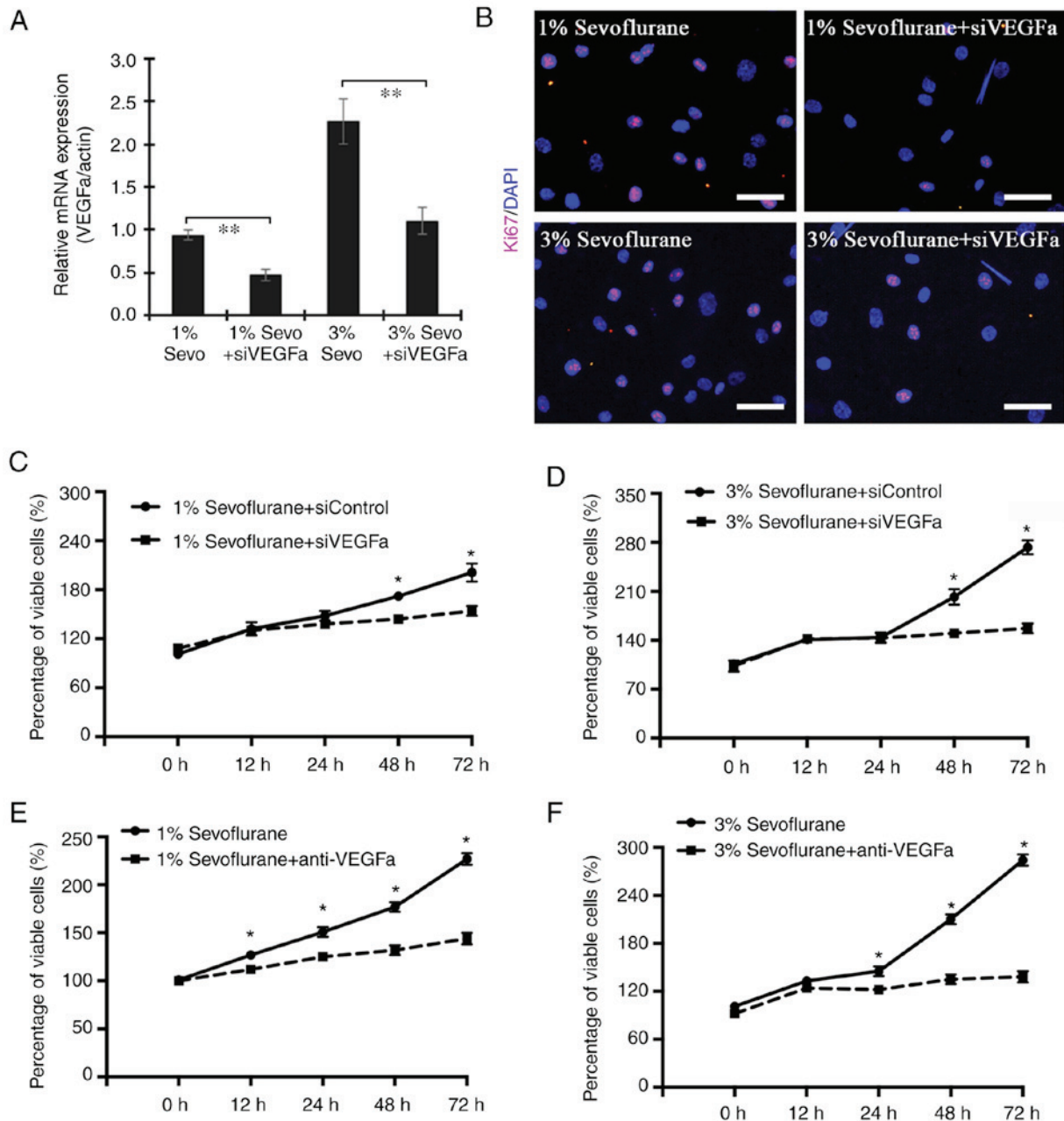
Figure 3. VEGFa inhibition protects the proliferation of HUVECs exposed to sevoflurane. HUVECs in the presence of 1 and 3% sevoflurane were transfected with VEGFa siRNA. (A and B) The data revealed that abrogated expression of VEGFa could result in the decreased Ki67-positive HUVECs. (C and D) Depletion of VEGFa revealed a similar effect on proliferation. (E and F) The increased proliferation of HUVECs incubated in the presence of 1 and 3% sevoflurane was chelated by anti-VEGFa at 0.1 μg/ml concentrations. Data represent three independent experiments. VEGF, vascular endothelial growth factor; HUVEC, human umbilical cord endothelial cells; siRNA, small interfering RNA.

significant decrease of the p-VEGFR level was observed in HUVECs exposed to axitinib and sevoflurane when compared with cells treated with sevoflurane alone. Notably, following the inhibition of the VEGFR pathway, there was significantly decreased cell proliferation in the cells treated with axitinib plus sevoflurane than with sevoflurane alone (Fig. 4C and D), implying that sevoflurane promotes the proliferation of HUVECs via activation of the VEGF/VEGFR pathway.

Discussion

The endothelium is involved in new vessel formation (2). The integrity of the vascular endothelium and an appropriate release of numerous vasoactive factors contribute to normal vascular physiology, and its dysfunction may incur the pathogenesis of vascular disease and cardiovascular diseases, such as coronary artery disease, coronary artery spasm, and atherosclerosis (3-5,20,21). One of the major therapeutic strategies for cardiovascular diseases is to maintain vessel function and promote vessel regeneration (4,5,20). In the present study, it was revealed that sevoflurane significantly increased cell viability and proliferation of HUVECs in a dose- and time-dependent manner, indicating that sevoflurane may increase new vessel formation resulting in attenuation of vascular disease.

The establishment and remodeling of the vascular system are regulated by several secreted signaling molecules, including
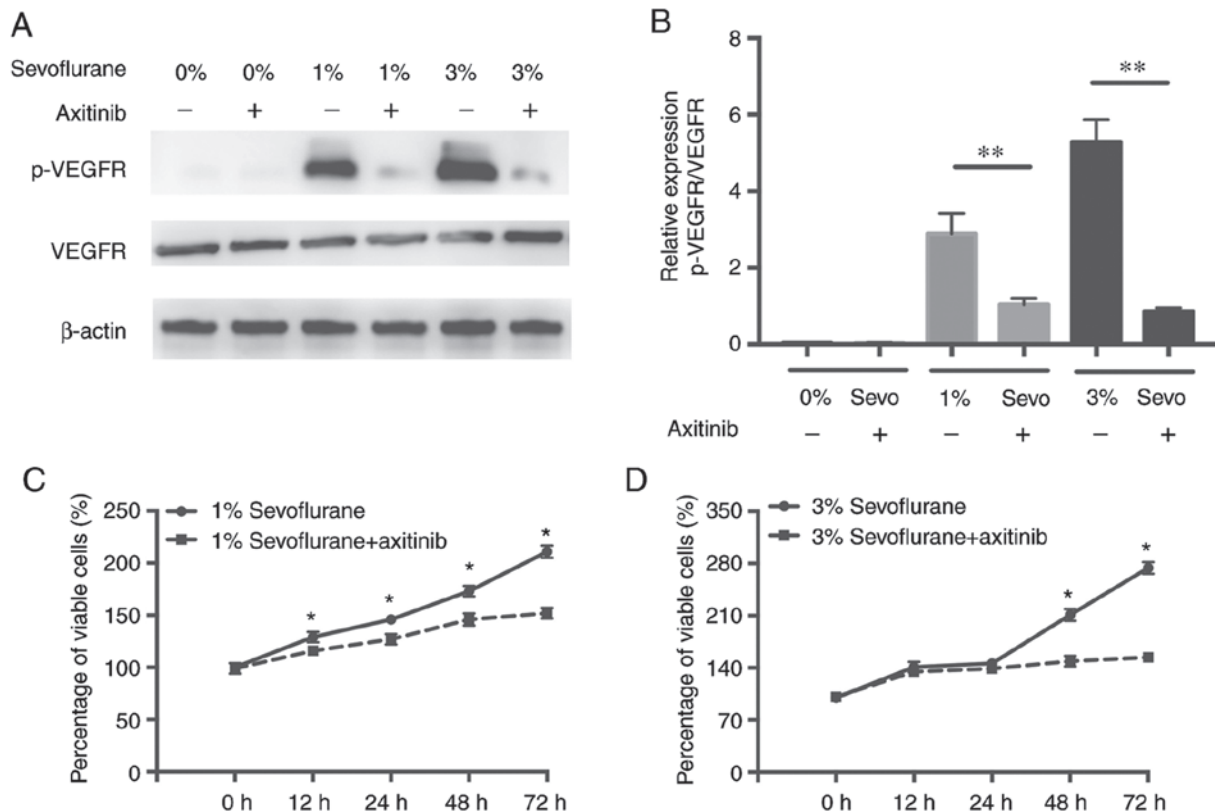
Figure 4. VEGF signaling is activated to promote the proliferation of HUVECs exposed to sevoflurane. (A) Representative western blots of VEGFR and p-VEGFR expression after exposure of HUVECs to 1 and 3% sevoflurane with and without the VEGFR inhibitor, axitinib. (B) The graph revealed the relative levels of phosphorylated VEGFR (p-VEGFR) normalized to VEGFR. Values without the inhibitor treatment were set as the baseline and considered equal to 1. (C and D) The MTT assay was used to detect cell viability of HUVECs incubated in (C) 1% and (D) 3% sevoflurane with and without the VEGFR inhibitor. Data are presented as the mean ± SD. $^{*}P<0.05$ vs. 1% sevoflurane and $^{**}P<0.01$. Data represent three independent experiments. VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HUVEC, human umbilical cord endothelial cells.

VEGF, and their corresponding receptors. They mediate downstream signaling that can result in the regulation of endothelial cell number and function (8,22-24). The VEGF gene undergoes alternative splicing to form 6 isoforms, VEGFa, VEGFb, VEGFc, VEGFd, VEGFe, and VEGFf, where VEGFa is the most biologically active among them (25). VEGF receptors are classified into three groups, VEGFR1, VEGFR2, and VEGFR3 (26,27). VEGF-A and VEGF-B always bind to VEGFR1, VEGFa and VEGFe frequently bind to VEGFR2, whereas VEGFc and VEGFd mainly bind to VEGFR3 (28). Previous studies have revealed that VEGFR1 [also known as Fms-related tyrosine kinase 1 (FLT1)] plays an essential role in vessel formation (10,29,30). Depletion of VEGFR1 has been revealed to cause early embryonic lethality at embryonic day 8.5 to 9 by enhancing endothelial cell overgrowth and excessive VEGFR2 [also known as kinase insert domain receptor, (KDR)] activation, which results in increased hemangioblast commitment and disorganized vessel formation (10,29,30). This suggests that VEGFR1 negatively regulates VEGFR2 signaling by sequestering VEGF and preventing it from binding to VEGFR2, which is the primary receptor to modulating angiogenesis by elevating the cell proliferation/survival, migration, and differentiation of the endothelium (10,29-31). VEGFR2-null mice die at embryonic day 8.5 to 9 by increasing the defective blood-island formation and nearly absence of vasculature (32,33). In addition, VEGFR3 (FLT-4), which may be activated by VEGF-C, has been revealed to play a vital role in establishing and maintaining lymphatic endothelial cells (34), and VEGFR3-mediated endothelial Snail was revealed to regulate capillary branching morphogenesis in the retina (35,36). Furthermore, clinical studies demonstrated that VEGF inhibition was associated with cardiovascular disease (37,38). It has been reported that sevoflurane exhibits its protective effects on pulmonary microvascular endothelial cells by downregulating ICAM-1 expression and inhibiting TNF-α and p38 MAPK signaling (15). In the present study, it was revealed that VEGF signaling could be activated by sevoflurane at a dose- and time-dependent manner in HUVEC cells. This was consistent with a previous study in which sevoflurane increased proliferation, adhesion on HUVECs, and incorporation in tubular structures of endothelial progenitor cells (17). Moreover, sevoflurane significantly increased the mRNA expression of VEGFa, however, sevoflurane did not affect VEGFb and VEGFc mRNA expression. In addition, sevoflurane upregulated the expression of VEGFR2 at the mRNA and protein levels, however it did not change the mRNA expression of VEGFR1 and VEGFR3. Furthermore, sevoflurane failed to elevate the VEGFR2 mRNA and protein expression when VEGFR2 was inhibited by axitinib, an inhibitor of VEGF receptors. These data indicated that sevoflurane could promote the proliferation of HUVEC cells by activating the VEGFa/VEGFR2 signaling pathway.

In conclusion, sevoflurane may be a promising agent against endothelium dysfunction-induced vascular disease by activating the VEGFa/VEGFR2 signaling pathway.

Acknowledgements

Availability of data and materials

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

Authors' contributions

ZW and CW performed majority of the experiments and contributed to the manuscript preparation. MZ and AD assisted with the animal experiments. RN performed the statistical analysis. JZ supervised the experiments and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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