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Sevoflurane pretreatment attenuates TNF-α-induced human endothelial cell dysfunction through activating eNOS/NO pathway

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ABSTRACT

Endothelial dysfunction induced by oxidative stress and inflammation plays a critical role in the pathogenesis of cardiovascular diseases. The anesthetic sevoflurane confers cytoprotective effects through its anti-inflammatory properties in various pathologies such as systemic inflammatory response syndrome and ischemic-reperfusion injury but mechanism is unclear. We hypothesized that sevoflurane can protect against tumor necrosis factor (TNF)-α-induced endothelial dysfunction through promoting the production of endothelium-dependent nitric oxide (NO). Primary cultured human umbilical vein endothelial cells (HUVECs) were pretreated with different concentrations (0.5, 1.5 and 2.5 minimum alveolar concentration, MAC) of sevoflurane for 30 min before TNF-α (10 ng/mL) stimulation for 4 h. Sevoflurane pretreatment significantly reduced TNF-α-induced VCAM-1, ICAM-1, IκBα, and NF-κB activation, and blocked leukocytes adhesion to HUVECs. Meanwhile, sevoflurane (1.5 and 2.5 MAC) significantly induced endothelial nitric oxide synthase (eNOS) phosphorylation and enhanced NO levels both intracellularly and in the cell culture medium. All these cytoprotective effects of sevoflurane were abrogated by NG-nitro-L-arginine methyl ester (L-NAME), a non-specific nitric oxide synthase inhibitor. Collectively, these data indicate that sevoflurane protects against TNF-α -induced vascular endothelium dysfunction through activation of eNOS/NO pathway and inhibition of NF-κB.

1. Introduction

Endothelial cell activation and dysfunction play an important role in various pathological inflammatory states such as systemic inflammatory response syndrome (SIRS), ischemia-reperfusion injury and rejection of graft tissue [1–3]. In these conditions, pro-inflammatory cytokines (e.g. TNF-α) are released, and the normally "quiescent" endothelium becomes "activated", leading to expressions of multiple families of cell adhesion molecules (CAMs) [4]. Excessive leukocytes accumulation can contribute to the development of inflammatory injury. Many studies [5,6] aimed at protection of endothelial cells against inflammatory injury have shown a dramatic reduction in tissue injury and provided further evidence of an important role of endothelial cells in determining oxidative stress and attenuating infection.

Sevoflurane, one of the most commonly used volatile anesthetics, has been proven to be effective in combating oxidative stress, inflammation, and protecting organs against stress-induced injury in various conditions [7–9]. Sevoflurane preconditioning significantly attenuated TNF-α-induced permeability and activation of p38 MAPK in rat pulmonary microvascular endothelial cells by reducing ICAM-1 expression [10]. However, the underlying mechanism governing the cytoprotective effects of sevoflurane against cytokine-induced toxicity in human endothelial cells has not been fully defined.

The endothelial isoform of nitric oxide synthase (eNOS) is a key source of vascular nitric oxide (NO), a cell protective molecular with

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an anti-inflammatory property [11]. This inability to produce NO is critical in the development and progression of tissue inflammatory injury [12]. Volatile anesthetics have recently been shown to exert protective effects on NO production [13–15]. Whether eNOS plays a role in sevoflurane mediated anti-inflammation effects in endothelial cells has not been well characterized. The present study thus tested the hypotheses that anti-inflammatory effect of sevoflurane is NO dependent in TNF-α-induced endothelial cells injury and investigated the potential signaling cascades.

2. Material and methods

This investigation conformed to the principles outlined in the Declaration of Helsinki for the use of human umbilical cord. The informed consent was obtained from the donors, and the protocol was approved by the Ethics Committee of Experimental Research, Xiangya Medical College, Central South University.

2.1. Materials

Sources of materials were as follows: anti-VCAM-1, anti-ICAM-1 and anti-β-actin (Santa Cruz Biotechnology, CA); anti-IκBα, anti-NF-κB, anti-iNOS, anti-nNOS, anti-p-eNOS, and anti-total eNOS (Cell Signaling Technology, Boston, MA); sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan); tumor necrosis factor-α (TNF-α), NOS inhibitor L-NAME and collagenase type II (Sigma Aldrich, St. Louis, MO, U.S.).

2.2. Endothelial cell isolation and treatment

Primary cultures of HUVECs were performed as previously described [16]. Briefly, the cord was severed from the placenta soon after birth, cannulated and perfused with 0.1% collagenase type II in the umbilical vein. After incubation, the collagenase solution was collected, sedimented, and resuspended, and the endothelial cells were cultured in endothelial cell culture medium (ScienCell, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Studies with HUVECs were conducted with cells from passage two to five. The experimental protocol is illustrated in Figure S1, HUVECs were pretreated with or without L-NAME 1 mM for 1 h before exposure to sevoflurane or air for 30 min, after which the cell growth medium was replaced with phenol red-free and growth factor-free medium and then stimulated with TNF-α for 4 h.

2.3. Exposure of cultured cells to sevoflurane

Anesthetic-enriched medium was prepared as described in previous study [17] with modification. The HUVECs were placed in an air-tight, humidified specifically modified container which was

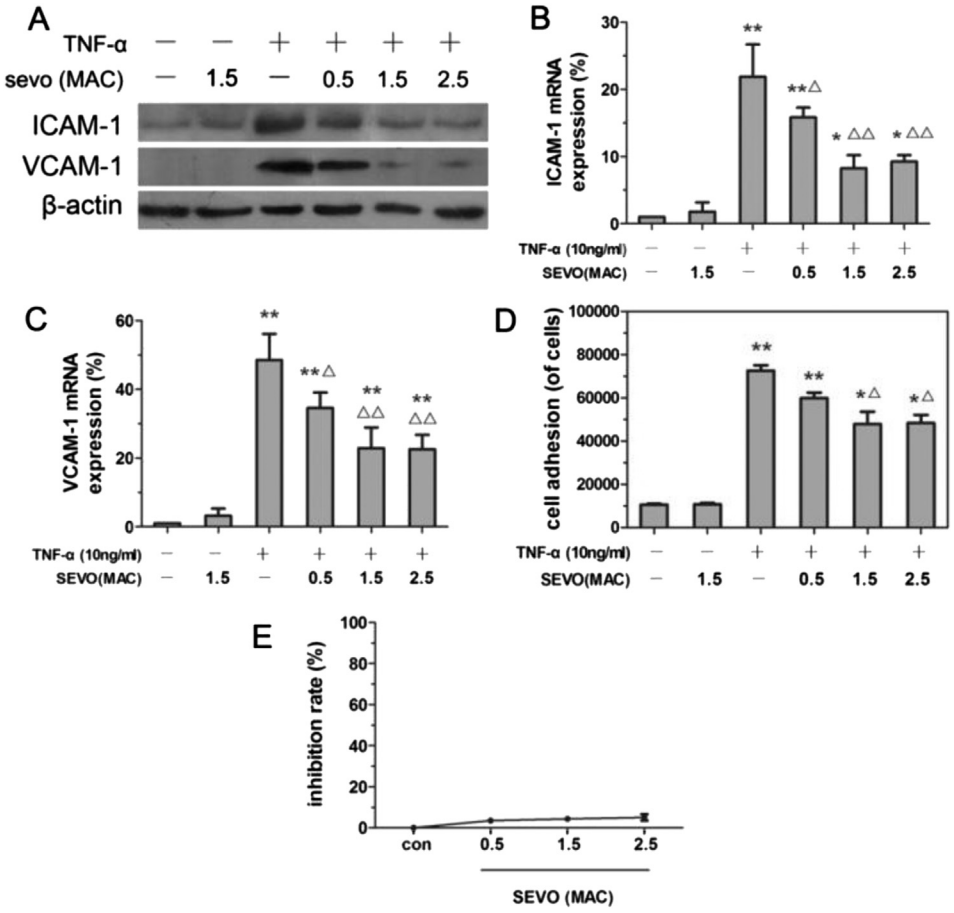


Fig. 1. Sevoflurane inhibited TNF-α-induced expression of ICAM-1, VCAM-1 and leukocytes adhesion to HUVECs. Cells were pre-incubated with various concentrations of sevoflurane for 30 min followed by TNF-α stimulus. Results of western blot (A) and real-time PCR (B, C) are shown for ICAM-1 and VCAM-1 expression in HUVECs. (D) HRP-labeled leukocytes were added to stimulated HUVECs, adherent cells were lysed and assayed by a spectrophotometer at A_{450}. (E) Cell viability was observed after 30 min incubation with sevoflurane. The data were expressed as mean ± SD of the results from five separate experiments. * indicates a $p < 0.01$, ** indicates a $p < 0.001$ compared with untreated cells; Δ indicates $p < 0.01$, ΔΔ indicates a $p < 0.001$ compared with TNF-α-treated cells.

matched with inflow and outflow connectors. The inlet port was connected to the sevoflurane vaporizer (Blease Medical Equipment Ltd, Chesham, UK) to make up for the volatilization loss of sevoflurane from the anesthetic-enriched medium. The outlet port was connected to a Datex-Ohmeda 5250 RGM gas analyzer (Drager, Lubeck, Germany) which measured sevoflurane concentration (1.1%, 3.3%, and 5.5% refer to 0.5 MAC, 1.5 MAC, and 2.5 MAC respectively). After 10 min continuous sevoflurane vapor balancing, air-tight container was sealed to incubate for 0.5 h at 37 °C.

2.4. Western blot

Cells were lysed and the protein samples were analyzed by the BCA method and the equal amounts of protein samples were resolved on a SDS/PAGE. Gels were transferred on to PVDF and the membranes were probed with antibodies. Membranes were further incubated with secondary antibodies conjugated to HRP which were detected by ECL reagent.

2.5. Real-time PCR

Total RNA was isolated using Qiagen RNeasy Mini Kit. qRT-PCR was performed on complementary DNA generated from 100 ng of total RNA using the protocol from a qRT-PCR mRNA detection kit (Roche, Indianapolis, IN, USA). -actin was used as an internal control. The relative expression level between treatments was then calculated using the following equation: relative gene expression $= 2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$. Primer sets for qPCR were as follows: ICAM-1, 5′-CGT GGG GAG AAG GAG CTG AA-3′ (forward) and 5′- CAG TGC GGC ACG AGA AAT TG-3′ (reverse); and VCAM-1, 5′-TGG GCT GTG AAT CCC CAT CT-3′ (forward) and 5′-GGG TCA GCG CGT GGA ATT GGT C-3′ (reverse); β-actin forward primer 5′-CGC AAA GAC CTG TAC GCC AAC-3′ (forward) and 5′- CAC GGA GTA CTT GCG CTC AGG-3′ (reverse).

2.6. Evaluation of nitric oxide production

The intracellular NO level was measured using a NO-sensitive fluorescence probe DAF-FM DA (Calbiochem, San Diego, CA, USA) by an inverted florescence microscope (Nikon, Tallahassee, Florida, USA). Images were processed with image analysis software (NIS-Elements BR 3.0, Nikon, Tallahassee, Florida, USA). The average fluorescent density of intracellular areas was measured to index the NO level. The nitrite concentrations of cell culture medium was analyzed by using the Nitric Oxide Assay Kit. Absorbance of the samples was measured at 540 nm using a microplate reader.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was carried out by following the manufacturer's instruction with minor modifications (Millipore). Briefly, we added cell with L-NAME or PBS before

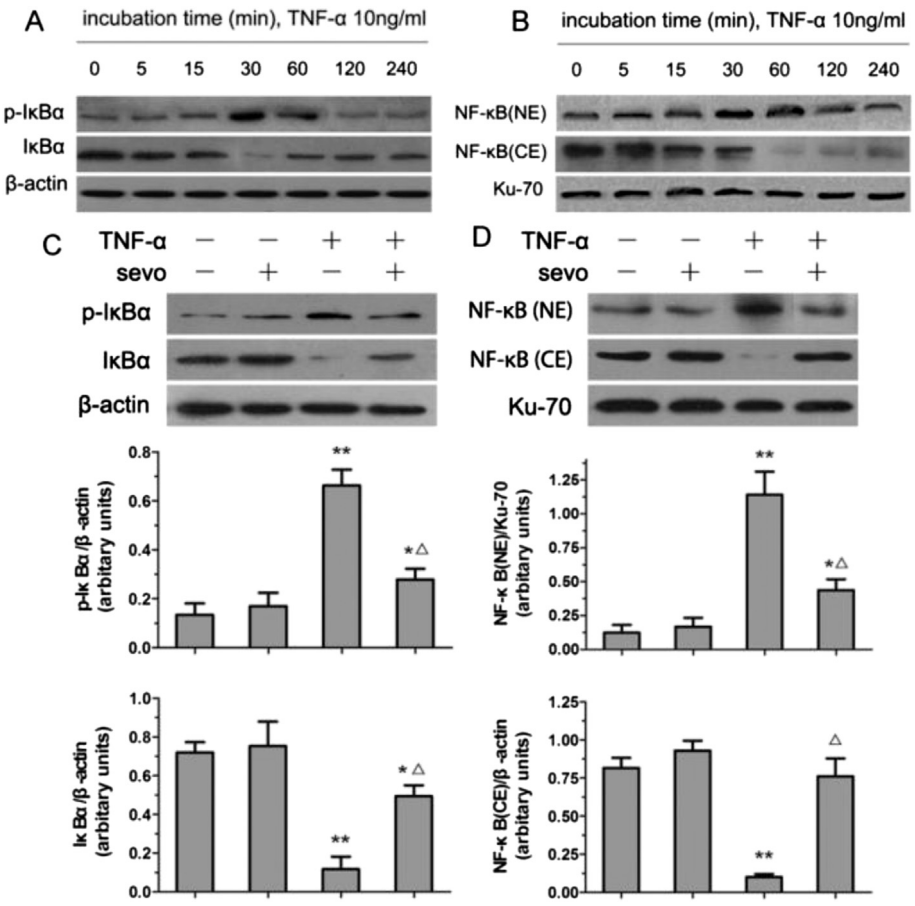
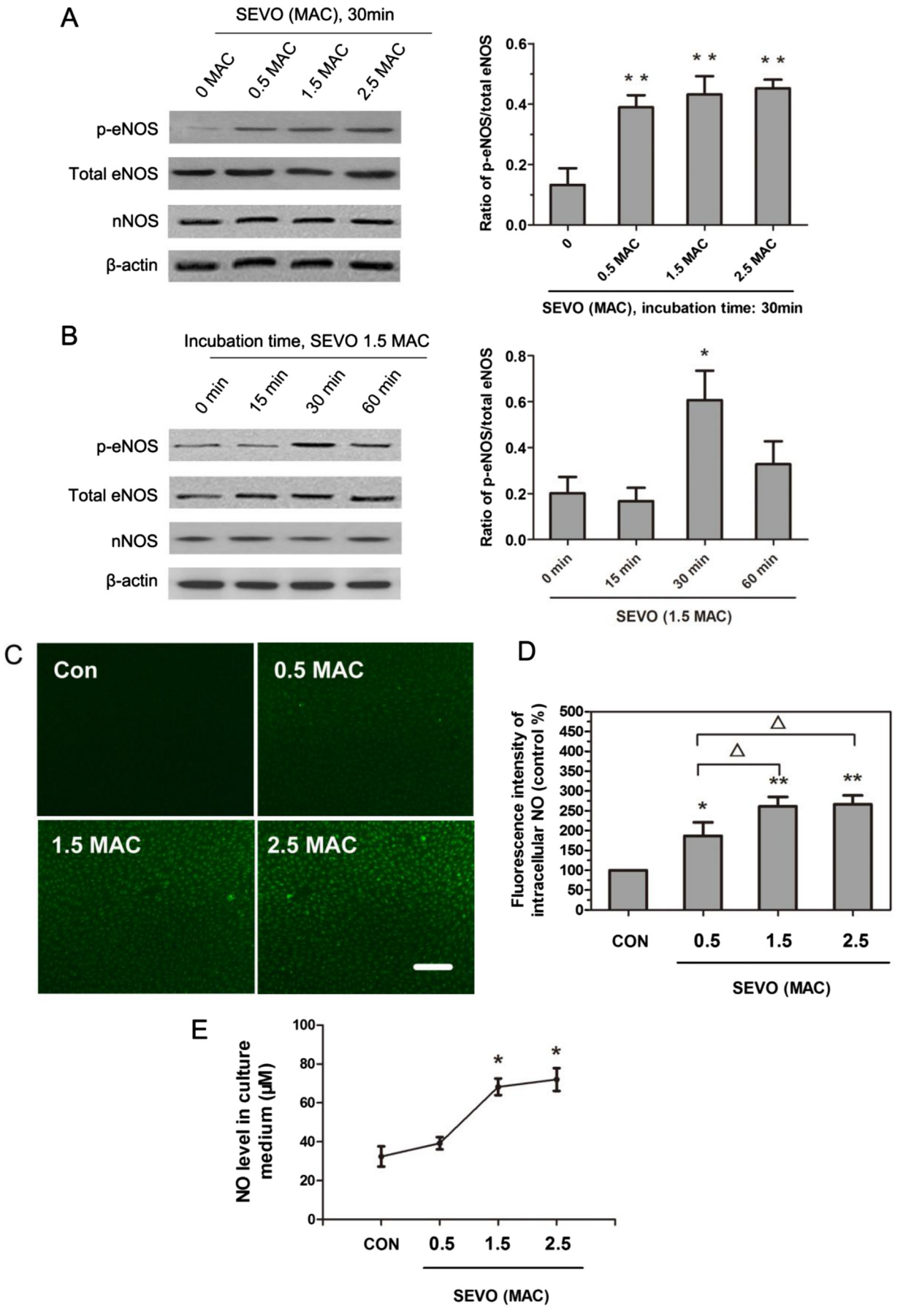


Fig. 2. Sevoflurane pretreatment suppresses TNF-α-stimulated NF-κB activation in HUVECs. Cells were treated with 10 ng/mL TNF-α for different time courses, the phosphorylation and degradation of IκBα (A) and the translocation of NF-κB p65 (B) were measured to examine the time course of TNF-α-induced NF-κB signaling pathway activation in HUVECs. Cells were pre-incubated with 1.5 MAC sevoflurane for 30 min followed by TNF-α (10 ng/mL) treatment for 4 h. Results of western blot are shown for phosphorylation and degradation of IκBα in the cytoplasm (C) and cytoplasmic localization of NF-κB p65 (D). The data were expressed as mean ± SD of the results from three separate experiments. * indicates a $p < 0.01$, ** indicates a $p < 0.001$ compared with untreated cells; Δ indicates $p < 0.01$ compared with TNF-α-treated cells. (NE: nuclear extracts, CE: cytosolic extracts).


A
SEVO (MAC), 30min
0 MAC
0.5 MAC
1.5 MAC
2.5 MAC
p-eNOS
Total eNOS
nNOS
β-actin
Ratio of p-eNOS/total eNOS
0.6
0.4
0.2
0.0
* *
* *
* *
0
0.5 MAC
1.5 MAC
2.5 MAC
SEVO (MAC), incubation time: 30min
B
Incubation time, SEVO 1.5 MAC
0 min
15 min
30 min
60 min
p-eNOS
Total eNOS
nNOS
β-actin
Ratio of p-eNOS/total eNOS
0.8
0.6
0.4
0.2
0.0
*
0 min
15 min
30 min
60 min
SEVO (1.5 MAC)
C
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0.5 MAC
1.5 MAC
2.5 MAC
D
Fluorescence intensity of intracellular NO (control %)
500
450
400
350
300
250
200
150
100
50
0
△
△
*
**
**
CON
0.5
1.5
2.5
SEVO (MAC)
E
NO level in culture medium (μM)
100
80
60
40
20
0
*
*
CON
0.5
1.5
2.5
SEVO (MAC)

incubated with sevoflurane for 30 min, then followed by 10 ng/mL of TNF-α treatment for 4 h. Cells were then incubated with 1% formaldehyde solution for the crosslinking followed by l0 × glycine to quench unreacted formaldehyde. Cells were collected and only the nuclear fractions were collected for the sonication. After sonication, samples were incubated with NF-κB antibody and protein A magnetic beads overnight at 4 °C. Input samples without antibody binding were saved for control. After washing steps, protein and DNA complexes were eluted and reverse cross-linked. Purified DNA samples were subjected to real-time PCR with primers targeting VCAM-1 promoter region as follow, VCAM-1 forward primer 5′-AAA TCA ATT CAC ATG GCA TA-3′, VCAM-1 reverse primer 5′-AAG GGT CTT GTT GCA GAG A-3′.

2.8. Evaluation of HL-60 cell binding to HUVEC

HL-60 (ATCC, USA) were labeled with HRP and incubated with TNF-α-stimulated HUVEC at 37 °C for 45 min. The culture wells were washed several times with serum-free media and lysed with 1% Triton solution. 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma, St Louis, MO, USA) substrate was added, and the concentration of HRP was measured at A_{450}. To quantitate the precise number of adherent HL-60 cells, a standard curve was constructed by using known amounts of HRP-labeled HL-60 cells.

2.9. Statistical analysis

All data are presented as means ± SD. Student's two-tailed unpaired t-tests and ANOVA were used for statistical evaluation of the data. Sigmastat statistical analysis program was used for data analysis. P values < 0.05 were considered significant.

3. Results

3.1. Sevoflurane inhibits TNF-α-induced expression of ICAM-1, VCAM-1 and leukocytes adhesion

As shown in Figure S2, HUVEC were treated with various concentrations of TNF-α for different time courses, exposure of HUVEC to 10 ng/mL TNF-α for 4 h resulted in a significant increase of the protein and mRNA expression of ICAM-1 and VCAM-1. As shown in Fig. 1, incubation of endothelial cells with sevoflurane (0.5 MAC, 1.5 MAC, 2.5 MAC) alone did not significantly alter basal adhesion molecule expression and did not alter leukocytes adhesion in cultured HUVEC (for data with 1.5 MAC, other values are not shown). Pretreatment of cells with sevoflurane (0.5 MAC, 1.5 MAC, 2.5 MAC) for 30 min reduced TNF-α-induced expression of ICAM-1 and VCAM-1 in a concentration-dependent manner, with the most significant reduction observed at a concentration of 2.5 MAC, the highest concentration ever tested (Fig. 1A–C). Compared with control cells, TNF-α increased leukocytes binding by almost 7-fold. Sevoflurane inhibited the adhesion induced by TNF-α independently from the concentration used (approximate 4-fold increase compared with control cells), but its inhibitory effect was barely stronger with higher concentrations of sevoflurane (1.5 MAC and 2.5 MAC) (Fig. 1D).

3.2. Sevoflurane suppresses TNF-α-stimulated NF-κB activation in HUVECs

As shown in Fig. 2, TNF-α rapidly led to phosphorylation of IκBα and then to degradation of IκBα expression in a time-dependent manner in the cytoplasm, with a maximal peak at 30 min (Fig. 2A) and increased NF-κB (p65 sub-unit) nuclear translocation compared to normal cells with a maximal peak at 60 min (Fig. 2B). Pretreatment with sevoflurane (1.5 MAC) for 30 min reduced the TNF-α-induced phosphorylation of IκBα and degradation of IκBα (Fig. 2C). Pretreatment with sevoflurane (1.5 MAC) for 30 min resulted in significant cytoplasmic localization of NF-κB p65 (Fig. 2D).

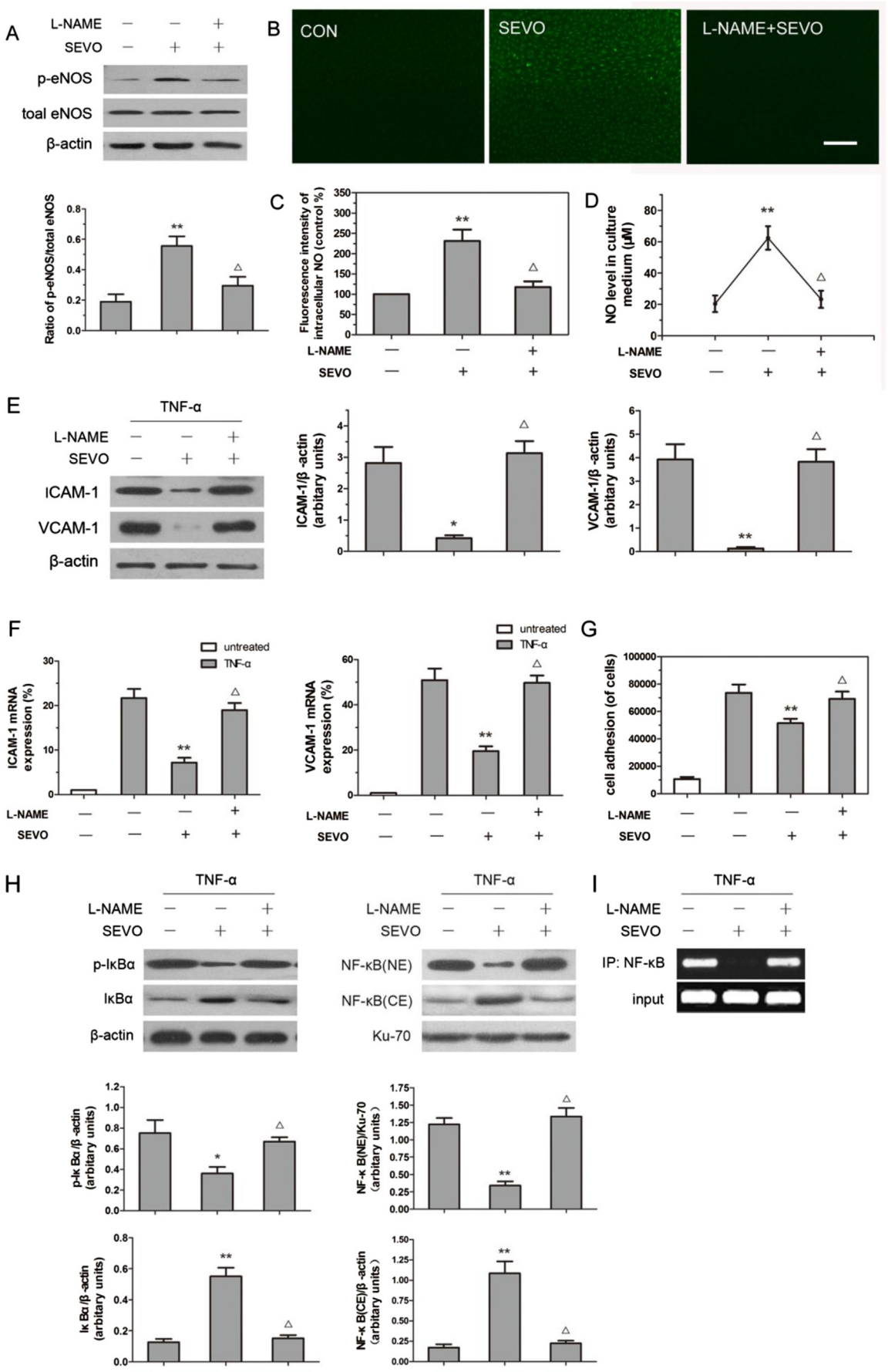
3.3. Sevoflurane promotes eNOS phosphorylation and NO production

As shown in Fig. 3, eNOS phosphorylation was significantly enhanced by sevoflurane in a dose dependent manner (Fig. 3A). When HUVECs were incubated with 1.5 MAC sevoflurane for 15, 30 or 60 min eNOS phosphorylation levels peaked after 30 min of treatment and decreased after 60 min (Fig. 3B). While, the expression levels of total eNOS and neuronal nitric oxide synthase (nNOS) remained unchanged. And no inducible nitric oxide synthase (iNOS) was detected (data not shown). Incubation with sevoflurane (0.5, 1.5 or 2.5 MAC) for 30 min increased intercellular NO production evidenced as the increases in fluorescence intensity, which were 170.0 ± 6.1%, 255.9 ± 4.1% and 264.2 ± 5.9% respectively, as compared to the vehicle group (Fig. 3C and D). Accordingly, sevoflurane (1.5 or 2.5 MAC) incubation triggered a comparable increase of NO production in cell culture medium in a concentration-dependent manner (Fig. 3E).

3.4. Inhibition of eNOS abolishes sevoflurane cytoprotection in TNF-α-stimulated HUVECs

Exposure of endothelial cells to L-NAME (1 mmol/L) for 1 h had no effect on the expression of CAMs and leukocytes adhesion in cultured HUVECs (data not shown). Sevoflurane-induced eNOS phosphorylation were abolished by L-NAME without affecting protein expression of total eNOS (Fig. 4A), sevoflurane-induced NO release was completely inhibited by L-NAME both in intracellular level and in cell culture medium (Fig. 4B–D). L-NAME almost completely blocked sevoflurane-induced inhibitory effect on the expression of ICAM-1 and VCAM-1 and the subsequent HL60 binding in TNF-α-treated cells. (Fig. 4E–G). Compared with sevoflurane group, L-NAME significantly reversed phosphorylation of IκBα, increased the amount of nuclear NF-κB and decreased the amount of cytosolic NF-κB in HUVECs (Fig. 4H). With L-NAME treatment, a higher level of VCAM-1 was detected in the ChIP assay in TNF-α-treated cells, in shape contrast with sevoflurane pretreated cells where no VCAM-1 can be detected (Fig. 4I).

Fig. 3. Sevoflurane induced eNOS phosphorylation and NO production in HUVECs. Cells were incubated with (A) various concentrations of sevoflurane (0, 0.5, 1.5, or 2.5 MAC) for 30 min and (B) 1.5 MAC of sevoflurane for the indicated times (0, 15, 30, or 60 min), phosphorylated-eNOS (p-eNOS), total eNOS and nNOS were analyzed by Western blotting respectively. (C) Representative fluorimetric dye DAF-FM DA-stained cell photomicrographs. The content of intracellular NO production was assayed by measuring the intensity of DAF-FM DA fluorescence 60 min after incubating with sevoflurane (0, 0.5, 1.5 or 2.5 MAC). Scale bar represents 100 μm. (D) Quantitative analysis of average fluorescent density of intracellular areas in HUVECs. Each bar represents fold changes in fluorescent density in sevoflurane-treated HUVECs relative to vehicle control. (E) Secreted NO measured by Griess in cell culture medium. The data were expressed as mean ± SD of the results from five separate experiments. * indicates a $p < 0.01$, ** indicates a $p < 0.001$ compared with untreated cells. Δ indicates a $p < 0.01$ compared with 0.5 MAC sevoflurane group.


A
L-NAME
SEVO
p-eNOS
toal eNOS
β-actin
Ratio of p-eNOS/total eNOS
B
CON
SEVO
L-NAME+SEVO
C
Fluorescence intensity of intracellular NO (control %)
D
NO level in culture medium (μM)
E
TNF-α
ICAM-1
VCAM-1
ICAM-1/β -actin (arbitary units)
VCAM-1/β -actin (arbitary units)
F
untreated
TNF-α
ICAM-1 mRNA expression (%)
VCAM-1 mRNA expression (%)
G
cell adhesion (of cells)
H
p-IκBα
IκBα
NF-κB(NE)
NF-κB(CE)
Ku-70
p-Iκ Bα /β -actin (arbitary units)
NF-κ B(NE)/Ku-70 (arbitary units)
Iκ Bα /β -actin (arbitary units)
NF-κ B(CE)/β -actin (arbitary units)
I
IP: NF-κB
input

4. Discussion

The results of our present studies clearly suggested that eNOS activation plays a critical role in sevoflurane mediated anti-inflammatory response by inhibiting the expression of CAMs. Specifically, we found that the down-regulation of adhesion molecules ICAM-1 and VCAM-1 by sevoflurane were abolished by NOS inhibition and eNOS may directly regulate NF-κB, the upstream transcription factors of these adhesion molecules. In addition, we found that sevoflurane-induced eNOS activation blocked the phosphorylation of IκBα, which, in turn, inhibited TNF-α mediated NF-κB activation. These effects of sevoflurane change the expression profiles of ICAM-1 and VCAM-1, molecules affecting leukocyte adhesion to endothelial cells, a hallmark of inflammatory response in many pathophysiologic status. Our study provide, for the first time, a direct link between eNOS activation and the down-regulation of CAMs expression in anesthetic sevoflurane-induced anti-inflammatory effects.

Vascular endothelial dysfunction plays a pivotal role in the development and maintenance of inflammatory states in various diseases such as SIRS, ischemia-reperfusion injury and atherosclerosis. The expression of adhesion molecules in endothelial cells is regulated by activated NF-κB, which can be upregulated in conditions with inflammatory mediators induced oxidative stress. NF-κB also exists in an inactive form when it binds to the inhibitor of nuclear factor kappa B (IκB) in the cytosol. The proinflammatory cytokine TNF-α causes oxidative stress of vascular endothelial cells and induces phosphorylation and degradation of IκB, allowing the activation of NF-κB [18]. In an animal model of acute myocardial ischemia-reperfusion injury, volatile anesthetic preconditioning has been shown to attenuate NF-κB activation and subsequent down-regulation of NF-κB-dependent inflammatory cytokines [19,20]. However, it remains unclear whether sevoflurane has the similar anti-inflammatory effects as other volatile anesthetics in endothelial cells. In our experiments, pretreatment with sevoflurane inhibited the subsequent TNF-α-induced phosphorylation of IκBα and degradation of IκBα, and inhibited NF-κB p65 translocation into the nucleus.

Under physiological conditions, NO is an important effective molecule in the cardiovascular system. Endothelial basal NO formation catalyzed by eNOS plays a critical role in vascular homeostasis affecting vasomotor tone as well as platelet aggregation and expression of adhesion molecules [11,21]. It has been shown that eNOS and NO production play critical roles in isoflurane preconditioning mediated cardiovascular protection [17]. Until now, it is not clear whether or not NOS system is directly involved in sevoflurane induced anti-inflammatory effects in response to inflammation in endothelial cells. In our study, we found that sevoflurane promoted phosphorylation of eNOS and increased the production of NO without altering the expression level of total eNOS and nNOS isoforms. In agreement with our data, Frädorf et al. also confirmed that sevoflurane preconditioning induced a profound increase in eNOS phosphorylation in the rat heart *in vivo* [22]. Furthermore, in the current study, sevoflurane induced increase in eNOS phosphorylation was blunted by the NOS-inhibitor L-NAME, which also abolished the effects of sevoflurane on the down-regulation of ICAM-1 and VCAM-1 expression, and on the inhibition of NF-κB activation, thus abrogated sevoflurane mediated attenuation of leukocytes adhesion. Our results indicate that the sevoflurane induced increased eNOS phosphorylation is functionally related to the anti-inflammatory effects. Indeed, NO is a potent inhibitor of leukocyte adhesion, at least in part through decreased expression of adhesion molecules [23]. We could speculate that the prevention of organ damage is a consequence of a more controlled immune reaction that prevents host organs from being attacked by leukocytes.

There are three NOS isoforms in mammals: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [11]. While eNOS plays a protective role and its release is impaired after ischemia-reperfusion injury [24,25], iNOS derived NO is implicated in the pathophysiology of the inflammatory state inducing vasodilation and increased vascular permeability. As already shown [12], an increase in NO bioavailability may lead to the down regulation of iNOS expression or even prevent iNOS induction, thereby limiting or preventing the toxic consequences of the inflammatory response afterward. In the light of this new vision on the key role played by the cross-talk between n/eNOS and iNOS, it is believed that, new pharmacological pretreatment, such as sevoflurane, aimed at appropriately generating small amounts of NO before noxious stimulus can represent a more efficient strategy to reduce organ functional impairment and tissue damage in the subsequent inflammatory injury, as also indicated by the present study.

In conclusion, this is the first report to show that eNOS plays an important role in sevoflurane mediated anti-inflammatory response in endothelial cells. Specifically, we found that the down-regulation of adhesion molecules ICAM-1 and VCAM-1 by sevoflurane treatment can be abolished by eNOS phosphorylation inhibition and eNOS may directly regulate the upstream transcription factors NF-κB. We found that eNOS blocked the phosphorylation of IκB, which, in turn, inhibited NF-κB activation incited by TNF-α in endothelial cells. Therefore, our results suggest that sevoflurane induced eNOS regulates phosphorylation of IκBα and translocation of NF-κB into nucleus, thereby changing the expression of ICAM-1 and VCAM-1, and affecting leukocytes adhesion to endothelial cells.

Conflict of interest

None.

Acknowledgments

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Fig. 4. L-NAME abrogates the anti-inflammatory effects of sevoflurane in TNF-α-induced endothelial cells. HUVECs were incubated with 1 mmol/L L-NAME or PBS for 1 h, with or without 1.5 MAC sevoflurane treatment for 30 min, followed by TNF-α treatment for 4 h, and then they were incubated with HRP-labeled HL-60 leukocytes for quantifying the degree of binding. phosphorylated-eNOS (p-eNOS) and total eNOS (A) were analyzed by Western blotting respectively. The content of intracellular NO production (B, C) was assayed by measuring the intensity of DAF-FM DA fluorescence 30 min after incubating with sevoflurane (1.5 MAC) for 30 min. Effects of 1.5 MAC sevoflurane on secreted NO (D) were measured by Griess in cell culture medium. The data were expressed as mean ± SD of the results from five separate experiments. ** indicates a $p < 0.001$ compared with untreated cells, Δ indicates a $p < 0.01$ compared with 1.5 MAC sevoflurane group. Results of western blot (E) and real-time PCR (F) are shown for ICAM-1 and VCAM-1 expression in TNF-α-stimulated HUVECs. HRP-labeled leukocytes were added to stimulated HUVEC, and then adherent cells were lysed and assayed by a spectrophotometer at A_{450} (G). Phosphorylation and degradation of IκBα in the cytoplasm and cytoplasmic localization of NF-κB p65 were analyzed by Western blotting respectively (H). ChIP analysis in which immunoprecipitated samples by NF-κB antibody were subjected to PCR amplification by using primers for the human VCAM-1 promoter region that NF-κB binds (I). The data were expressed as mean ± SD of the results from five separate experiments. * indicates a $p < 0.01$, ** indicates a $p < 0.001$ compared with TNF-α-treated cells. Δ indicates a $p < 0.01$ compared with 1.5 MAC sevoflurane group.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.126.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.126.

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