Desflurane preconditioning protects human umbilical vein endothelial cells against anoxia/reoxygenation by upregulating NLRP12 and inhibiting non-canonical nuclear factor-**k**B signaling

ZHIRONG SUN¹, JIANING LV², YUN ZHU¹, DONGLI SONG³, BIAO ZHU⁴ and CHANGHONG MIAO⁴

¹Department of Anesthesiology, Fudan University Shanghai Cancer Center; ²Department of Neurology, Fudan University Shanghai Zhongshan Hospital; ³Biomedical Research Center, Fudan University Zhongshan Hospital; ⁴Department of Anesthesiology and Critical Care Unit, Fudan University Shanghai Cancer Center, Shanghai, P.R. China

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Abstract. Volatile anesthetics modulate endothelial cell apoptosis and inhibit nuclear factor- κB (NF- κB) signaling. In this study, we aimed to assess whether desflurane preconditioning protects human umbilical vein endothelial cells (HUVECs) agaist anoxia/reoxygenation (A/R) injury. HUVECs were preconditioned with desflurane (1.0 MAC) for 30 min, followed by a 15-min washout, then exposed to 60 min anoxia and 60 min reoxygenation (A/R), and incubated with 10 ng/ml tumor necrosis factor (TNF)- α for 60 min. HUVEC viability and apoptosis were measured by MTT assay and Annexin V staining, and immunoblot analysis was used to measure the levels of Smac and cellular inhibitor of apoptosis 1 (cIAP1). NF-KB activation was assessed using the NF-KB signaling pathway real-time PCR array, and the levels of NF-KB inducing kinase (NIK), p52, IkB kinase (IKK)a, p100, RelB and NLR family, pyrin domain containing 12 (NLRP12) were assessed by immunoblot analysis. Desflurane preconditioning attenuated the effects of A/R and/or A/R plus TNF- α on cell viability, decreasing the levels of Smac and enhancing the levels of of cIAP1 (P<0.05). Preconditioning with desflurane also enhanced the mRNA levels of interleukin (IL)-10 and NLRP12 in the cells exposed to A/R by 2.40- and 2.16-fold, respectively. The HUVECs exposed to A/R had greater levels of NIK and p100 and reduced levels of p52 and IKKa. Desflurance preconditioning further increased p100 levels, decreased the level of NIK, further decreased p52 levels and further reduced IKKa levels. A/R in combination with TNF- α increased the NIK, IKK α , p100 and RelB levels, and this increase was significantly attenuated by desflurance preconditioning (all P<0.05).

Desflurane preconditioning enhanced HUVEC survival and protected the cells against A/R injury, and our results suggested that this process involved the upregulation of NLRP12 and the inhibition of non-canonical NF-κB signaling.

Introduction

Ischemia-reperfusion injury involves cellular responses to anoxia/reoxygenation (A/R), which initiate a cascade of cellular processes and molecular events that cause endothelial cell apoptosis (1-7). Volatile anesthetics have been demonstrated to induce caspase-dependent, mitochondrial-mediated apoptosis *in vitro* (8). However, preconditioning with volatile anesthetics, including desflurane, halothane, isoflurane and sevoflurane has been reported to protect against A/R injury both *in vivo* and *in vitro* (9-11). Preconditioning with volatile anesthetics has been reported to affect inflammation (12-16), and we have previously reported that desflurane preconditioning protects human umbilical vein endothelial cells (HUVECs) against A/R injury through a process involving nuclear factor- κ B (NF- κ B) (17,18).

The Rel-NF-KB family of transcription factors has been implicated in a variety of biological functions, including cellular proliferation and apoptosis, and in the initiation and propagation of innate and adaptive immune responses (19-22). Rel-NF-κB activates two distinct NF-kB activation pathways: the canonical and non-canonical NF- κ B pathways (23,24). The canonical NF-kB pathway is activated by the most stressful stimuli, and results in the IkB kinase (IKK) complex-mediated degradation of IkB and the rapid nuclear accumulation of p50-RelA and p50-cRel NF-KB complexes (25). By contrast, the non-canonical NF-kB pathway is activated by a group of tumor necrosis factor (TNF) receptors, such as CD40 (26), lymphotoxin β receptor (LT β R) (27) and BAFF receptor (BAFF-R) (28). The activation of the non-canonical NF-kB pathway results in the degradation of the C-terminus of p100 into p52 and the translocation of p52 into the nucleus. In the nucleus, p52 combines with RelB, producing the NF-KB complex (29).

The Nod-like receptor (NLR) family contributes either directly or indirectly to a variety of hallmarks associated with cancer, including inflammation, cell death, tumor growth, angiogenesis, invasion and metastasis (30-34). NLRs have

Correspondence to: Dr Changhong Miao or Dr Biao Zhu, Department of Anesthesiology and Critical Care Unit, Fudan University Shanghai Cancer Center, 270 Dong An Road, Shanghai 200032, P.R. China E-mail: miaochanghonganes@126.com E-mail: biaozhuzs@sohu.com

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been traditionally considered as pattern-recognition receptors (PRRs), as they are activated in response to conserved structural motifs found in microbes or pathogen-associated molecular patterns (PAMPs). There is a subgroup of NLRs that negatively regulate inflammation (35-37), currently including three NLR family members, NLRP12, NLRX1 and NLRC3. NLRP12 (previously known as Monarch-1, PYPAF7, or CLR19.3) is one of the first to be well described and is the most well characterized member of this subgroup. It has been demonstrated in vitro that the overexpression of NLRP12 induces the transcription of an NF-κB reporter construct (38), suggesting that it is an inflammasome-forming NLR and a positive regulator of NF-kB signaling. However, under physiological conditions and in the context of human disease, the ability of NLRP12 to form a functional inflammasome appears to occur only under highly specific conditions (39,40). In fact, several studies have evaluated NLRP12 inflammasome formation and have directly shown that NLRP12 does not regulate IL-1β/IL-18 maturation (41-46). Studies on NLRP12 have indicated that it functions as a negative regulator of inflammation by modulating canonical and non-canonical NF-kB signaling (37,42,44-46). NLRP12 negatively regulates non-canonical NF-KB signaling through its association with TRAF3 and NF-κB-inducing kinase (NIK) (37,42).

In the present study, we investigated whether the protective effects of desflurane preconditiong against A/R injury are mediated by the downregulation of the non-canonical NF- κ B signaling pathway.

Materials and methods

Primary culture of HUVECs. HUVECs were isolated from the human umbilical vein vascular wall using collagenase (Roche Diagnostics, Indianapolis, IN, USA) digestion, as previously described (47), and cultured in a humidified atmosphere containing 95% O₂ and 5% CO₂ at 37°C in endothelial cell culture medium (ECM; Sciencell Research Laboratories, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Gibco-Life Technologies, Grand Island, NY, USA), 1% endothelial cell growth supplement (ECGS), 100 U/ml penicillin and 100 μ g/ml streptomycin sulfate (all from Sciencell Research Laboratories). Cells were passaged 3-6 times before being used in the experiments. Ethics approval for the isolation of the HUVECs was obtained from the Ethics Committee of Fudan University Shanghai Cancer Center, Shanghai, China.

Immunofluorescence staining. Confluent endothelial cells in monolayer were fixed with 95% cold ethanol for 5 min (the cells were grown on sterile glass cover slides overnight at 37°C. The slides were briefly washed 3 times for 5 min in PBS, and then fixed with 95% cold ethanol and air dried). A drop of diluted anti-rabbit human factor VIII antibody (Abcam, Cambridge, UK) was added (1:10 dilution), allowed to react for 30 min in a moisture chamber, and then washed 3 times for 5 min in PBS. The slide was then incubated for 45 min at 37°C with FITCconjugated goat anti-rabbit globulin (Cwbiotech, Shanghai, China) at a 1:50 dilution, and the washing procedure was then repeated. A drop of mounting fluid consisting of 10% glycerol, 90% PBS and 0.25 mg/ml propidium iodide (PI; Cwbiotech) for counterstaining the nuclei was added. The slides were examined on a coverslip under an epifluorescence microscope (Olympus, Tokyo, Japan) (Fig. 1), as previously described (48).

Desflurane preconditioning and the A/R regimen. An in vitro model of A/R, which has been previously described (49), was used in the present study. The HUVECs were subjected to a period of anoxia, during which the cells were incubated in 95% N₂ and 5% CO₂ for 60 min, followed by 60 min reoxygenation, during which time the cells were incubated in 95% O₂ and 5% CO₂. Prior to exposure to A/R, the cells were incubated in the presence or absence of desflurane (1.0 MAC) for 30 min, and then allowed to rest for 15 min. Immediately after the A/R protocol, the cells were incubated in the presence or absence of 10 ng/ml recombitant human TNF- α (rhTNF- α) (ProSpec, Ness Ziona, Israel) for 60 min (Fig. 2).

Assessment of cell viability. An MTT assay was used, as tetrazolium salts are cleaved to form a formazan dye only by metabolically active cells and are particularly useful for quantifying the number of viable cells. The HUVECs were seeded in 96-well plates (3x10⁴ cells/well) and incubated overnight for complete cell adhesion. On the second day, desflurane preconditioning and A/R exposure were carried out as described above. At the end point of the experiment, MTT (50 μ l/well; Beyotime Institute of Biotechnology, Haimen, China) was added to the medium followed by incubation at 37°C for a further 4 h. The medium was removed from all wells, and the insoluble formazan product was dissolved in 150 μ l of DMSO for 10 min at room temperature. The optical density (OD) of each culture/ well was measured using a spectrophotometer (UV-2450/2550, Shimadzu Corp., Tokyo, Japan) at 550 nm. The OD of the cells in the control group represented 100% viability.

Flow cytometric analysis. Cell apoptosis was detected by flow cytometry. Cells were double-stained with Annexin V-FITC and PI (Beyotime Institute of Biotechnology) according to the manufacturer's instructions, and cell fluorescence was analyzed on a FACSan flow cytometer. Annexin V-FITC-positive cells reflected the relative proportion of apoptotic cells.

PCR array. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The expression of inflammatory genes was examined by real-time PCR, utilizing the NF-κB signaling pathway RT² RNA QC PCR array (PAHS-025; Qiagen, Inc., Valencia, CA, USA), which profiled the expression of 84 key genes related to NF-κB-mediated signal transduction. The expression of the genes of interest (84 key genes related to NF-κB-mediated signal transduction) was compared between the treated and untreated cells. The fold change in expression for each gene between the treated and untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method (Shanghai Kangcheng Biological Engineering Co., Ltd. Shanghai, China).

Immunoblot analysis. Cells were harvested and homogenized using RIPA lysis buffer (Beyotime Institute of Biotechnology). Proteins were separated on 8% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in TBST (containing 0.05% Tween-20), and incubated overnight at 4°C with the following primary anti-



Figure 1. Expression of factor VIII in isolated human umbilical vein endothelial cells (HUVECs) (original magnification, x40). HUVECs were isolated from human umbilical vein vascular wall by collagenase digestion, and cultured *ex vivo*. HUVEC phynotype was confirmed by factor VIII staining (green), and propidium iodide (PI) staining (blue), and analyzed by immunofluorescence.



Figure 2. Desflurane preconditioning and anoxia/reoxygenation exposure protocol. As described in the Materials and methods, after 3 to 6 passages *ex vivo*, the HUVECs were incubated in the presence or absence of 1.0 MAC desflurane for 30 min, followed by a 15-min washout period before A/R, and in the presence or absence of 10 ng/ml tumor necrosis factor (TNF)- α for 60 min after A/R. CON, control; A/R, anoxia/reoxygenation; DES+A/R, desflurane preconditioning and anoxia/reoxygenation; A/R + TNF- α , anoxia/reoxygenation and tumor necrosis factor- α (10 ng/ml); DES + A/R + TNF- α , desflurane preconditioning, anoxia/reoxygenation and tumor necrosis factor- α (10 ng/ml).

bodies: NLR family, pyrin domain containing 12 (NLRP12; sc-99175), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Smac (2952), cellular inhibitor of apoptosis 1 (c-IAP1; 4592), NIK, IKKa, p100/p52, RelB (included in the Non-Canonical Pathway Antibody Sampler kit) (all from Cell Signaling Technology, Danvers, MA, USA), GAPDH (AG019; Beyotime Institute of Biotechnology) and β -actin (A2668; Sigma, St. Louis, MO, USA). The blots were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactivity was enhanced with a chemiluminescence kit (Millipore) and exposed to film. GAPDH (Beyotime Institute of Biotechnology) or β -actin (Sigma) were used as internal controls. The density of the bands on the blots was quantified using a Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Data are expressed as the means \pm SD, and were analyzed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. A P-value <0.05 was considered to represent a statistically significant difference. All analyses were conducted using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Effect of desflurane preconditioning on A/R-induced damage to HUVECs

Desflurane preconditioning promotes HUVEC survival during A/R. As previously established, exposure to A/R and/or TNF- α reduces HUVEC viability (50). In this study, we detected HUVEC viability by MTT assay, and found that our A/R protocol significantly reduced HUVEC viability (P<0.05), and



Figure 3. Desflurane preconditioning (DES) attenuates anoxia/reoxygenation (A/R) injury in human umbilical vein endothelial cells (HUVECs). (A) HUVECs were incubated with 1.0 MAC desflurane, 10 ng/ml tumor necrosis factor (TNF)- α and/or exposed to A/R as indicated in Fig. 2. Cellular viability was measured by MTT assay. (B and C) Apoptosis was assessed by propidium iodide (PI) and Annexin V staining, analyzed by flow cytometric analysis, and the data in (B) were derived from (C). Data represent the means ± SD of 3 independent experiments. *P<0.05 vs. control (CON), **P<0.05 vs. A/R, ***P<0.05 vs. A/R + TNF- α .

when A/R was followed by incubation with TNF- α , HUVEC viability was further reduced (P<0.05; Fig. 3A). However, desflurane (1.0 MAC) preconditioning significantly attenuated the effects of A/R or A/R and TNF- α on HUVEC viability (P<0.05; Fig. 3A), suggesting that desflurane preconditioning promotes HUVEC survival under certain conditions of cellular stress.

Desflurane preconditioning decreases the apoptosis of HUVECs exposed to A/R. The rate of apoptosis was determined by Annexin V and PI staining, and analyzed by flow cytometry. Spontaneous apoptosis was low in the HUVECs in the control group, whereas exposure to A/R increased apoptosis (P<0.05), and when A/R was followed by incubation with TNF- α , HUVEC apoptosis increased even further (P<0.05). Pre-treatment with desflurane (1.0 MAC) attenuated the effects of A/R or A/R and THF- α on HUVEC apoptosis (P<0.05) (Fig. 3B and C), suggesting that desflurane preconditioning protects HUVECs against A/R induced apoptosis.

Desflurane preconditioning increases the expression of cIAP1 and decreases the expression of Smac. In order to further elucidate the effects of desflurane preconditioning on A/R-induced HUVEC apoptosis, we examined Smac activation and cIAP1 inhibition, as these are processes unique to apoptosis, which do not occur in other forms of cell death. We detected increased levels of Smac and cIAP1 in the HUVECs exposed to A/R and A/R plus TNF- α (P<0.05). Desflurance preconditioning increased c-IAP1 levels (P<0.05) and decreased the levels of Smac (P<0.05; Fig. 4), suggesting that desflurane preconditioning protects HUVECs against A/R-induced apoptosis through a mechanism involving the inhibition of Smac and the activation of cIAP1.

Desflurane preconditioning affects the expression of inflammatory genes. In the HUVECs exposed to A/R or A/R plus TNF- α , elevated protein levels of NLRP12 (a regulator of

Gene symbol	Gene name	GenBank accession no.	Description	Upregulation (fold change)	Downregulation (fold change)
IL-10	CSIF/IL-10/IL-10A/ MGC126450/ MGC126451/TGIF	NM_000572	Interleukin 10	2.40	
NLRP12	CLR19.3/FCAS2/ NALP12/PAN6/ PYPAF7/RNO/RNO2	NM_033297	NLR family, pyrin domain containing 12	2.16	

Table I. IL-10 and NLRP12 mRNA expression in HUVECs in response to A/R and desflurane preconditioning.

HUVECs, human umbilical vein endothelial cells; A/R, anoxia/reoxygenation.



Figure 4. Desflurane preconditioning (DES) attenuates Smac and increases cellular inhibitor of apoptosis 1 (cIAP1) expression in human umbilical vein endothelial cells (HUVECs). Protein levels of (A) Smac and (B) cIAP1 in HUVECS treated as described in Fig. 2, determined by immunoblot analysis. Data are presented as the means \pm SD (n=3 per group). [#]P<0.05 vs. control (CON), ^{##}P<0.05 vs. anoxia/reoxygenation (A/R), ^{###}P<0.05 vs. A/R + tumor necrosis factor (TNF)- α .

inflammation), were detected by immunoblot analysis (Fig. 5). In addition, in the HUVECs exposed to A/R or A/R plus TNF- α elevated mRNA levels of interleukin (IL)-10 and NLRP12 were detected by PCR array. Preconditioning with desflurane increased the mRNA level of IL-10 and NLRP12 in the cells exposed to A/R by 2.40- and 2.16-fold, respectively (Table I), and enhanced the protein level of NLRP12 (P=0.0013). In the cells exposed to A/R or A/R plus TNF- α higher levels of NLRP12 were noted, and desflurane preconditioning further increased the NLRP12 protein levels (P=0.0118; Fig. 5). These results suggest that the NLRP12 and IL-10 genes associated with inflammation are invoved in the effects of desflurane preconditioning.

Desflurane preconditioning inhibits the non-canonical NF- κ B signaling pathway. To determine whether desflurane preconditioning has an effect on the non-canonical NF- κ B signaling pathway, we measured the levels of NIK, IKK α , p52 and RelB in HUVECs exposed to A/R by immunoblot analysis, as illustrated in Fig. 6. The HUVECs exposed to A/R had greater levels of NIK and p100, and and reduced levels of p52 and IKK α . Desflurance preconditioning reduced the level of NIK below baseline levels, further increased p100 levels, and further reduced p52 and IKK α levels. A/R in combination with TNF- α increased the levels of NIK, IKK α , p100 and RelB. These changes were significantly attenuated by desflurance preconditioning (all P<0.05; Fig. 6).



Figure 5. Desflurane preconditioning (DES) increases NLR family, pyrin domain containing 12 (NLRP12) expression in human umbilical vein endothelial cells (HUVECs). Protein levels of NLRP12 in HUVECS were treated as described in Fig. 2, and determined by immunoblot analysis. Data are presented as the means \pm SD (n=3 per group). *P<0.05 vs. control (CON), **P<0.05 vs. anoxia/reoxygenation (A/R), ***P<0.05 vs. A/R + tumor necrosis factor (TNF)- α .



Figure 6. Desflurane preconditioning (DES) influences NF- κ B inducing kinase (NIK), I κ B kinase (IKK) α , p100, p52 and RelB expression in human umbilical vein endothelial cells (HUVECs). Protein levels of (A) NIK, (B) IKK α , (C and D) p100 and p52 and (E) RelB-B in HUVECs treated as described in Fig. 2, and determined by immunoblot analysis. Data are presented as the means \pm SD (n=3 per group). [#]P<0.05 vs. control (CON), ^{##}P<0.05 vs. anoxia/reoxygenation (A/R), ^{###}P<0.05 vs. A/R + tumor necrosis factor- α (TNF- α).

Discussion

A/R has previously been reported to activate NF- κ B in HUVECs, with parallel increases in oxidase stress, inflamma-

tory responses and apoptosis (23,51-53). Apoptosis is mediated by an increase in the permeability of the outer mitochondrial membrane (54), leading to the release of apoptogenic factors from the mitochondrial inter-membrane space into the cytosol. Apoptogenic factors include Smac, which in turn binds to and neutralizes caspase inhibitors of apoptosis proteins, such as IAPs (57), thereby activating caspases. A previous study indicated that desflurane induced A β production and caspase activation under hypoxic conditions (58). Our results, however, suggest that desflurane preconditioning protects endothelial cells by activating anti-apoptotic cIAP1 and decreasing the expression of Smac, thus resulting in decreased levels of apoptosis.

cIAP1 is an NF- κ B responsive gene (59,60). Desflurane preconditioning has previously been reported to activate the canonical NF- κ B pathway (17,18). However, whether volatile anesthetics protect HUVECs against A/R injury through the non-canonical NF- κ B signaling pathway or crosstalk with other pathways remains to be established. In the present study, with the use of a human NF- κ B signaling pathway array, we revealed that NLRP12 expression was upregulated by desflurane preconditioning.

It has previously been reported that NLRP12 suppresses the production of pro-inflammatory cytokines and chemokines (55). Lich and Ting (54) reported that NLRP12 suppressed 'non-canonical' NF-κB activation. Furthermore, this alternative pathway is activated downstream of TLRs in addition to the TNF family receptors (61,62). Unlike the canonical NF-κB signaling pathway, which can be activated by multiple upstream kinases, the non-canonical pathway is strictly dependent upon the kinase NIK (63). Upon activation, NIK recruits IKKa and NF-kB2/p100, which in turn leads to the processing of p100 into its active form, p52. In the present study, we found that desflurane preconditioning downregulated the expression of NIK, IKKa, p52 and upregulated the expression of p100 during A/R-induced injury. These results validated those of previous studies indicating that desflurane preconditioning inhibits the non-canonical NF-kB signaling pathway and thus protects cells against A/R induced cell injury (1,18).

In conclusion, in this study, we demonstrated that desflurane preconditioning attenuated HUVEC inflammatory responses to A/R. Desflurane preconditioning upregulated cIAP1 and NLRP12 expression, and downregulated Smac, NIK, IKK α and p52 expression, ameliorating cellular stress processes and apoptosis during A/R. As desflurane is increasingly applied in clinical settings, the role of desflurane in A/R injury needs to be further characterized, in an aim to obtain a deeper understanding of the cellular responses to desflurane, which may support the extension of this therapeutic strategy in the treatment of A/R-induced inflammatory responses.

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