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Sevoflurane Combined with ATP Activates Caspase-1 and Triggers Caspase-1-Dependent Pyroptosis in Murine J774 Macrophages

Yue Jin,[1] Hui Li,[1] Guohao Xie,[1] Shuzhen Chen,[2] Shuijin Wu,[1] and Xiangming Fang[1,3]

Abstract—Sevoflurane is one of the most commonly used volatile anesthetics. Recent studies have shown that sevoflurane plays an important role in modulation of inflammation and immunity. However, little is known about the related molecular mechanisms. This study was designed to investigate the effects and mechanisms of sevoflurane on inflammatory cell death pyroptosis in the murine macrophage cell line J774 cells. Sevoflurane combined with ATP could increase the level of activated caspase-1, pyroptosis, and reactive oxygen species (ROS). Furthermore, treatment of cells with the caspase-1 inhibitor Ac-YVAD-CMK dramatically decreased the percentage of pyroptosis. In addition, inhibition of ROS with *N*-acetyl-L-cysteine or diphenyleneiodonium significantly reduced the activated levels of caspase-1. These results demonstrated that sevoflurane combined with ATP could activate caspase-1 and trigger caspase-1-dependent pyroptosis through the modulation of ROS production.

KEY WORDS: sevoflurane; caspase-1; pyroptosis; ROS.

INTRODUCTION

Sevoflurane, one of the most common volatile anesthetics, is widely used in surgical procedures not only because of its high-speed and non-irritation but also of its anti-inflammatory, organ protection features and its immune modulating properties [1–3]. Previous studies have demonstrated that both sevoflurane pre- and postconditioning have protective effects and inflammatory and immune regulation characteristics [4–6]. In recent years, the modulation of sevoflurane on inflammation and immunity has been extensively studied. However, the specific molecular mechanism remains unclear.

Caspase-1, one of the cysteinyl aspartate-specific proteases, is synthesized as an inactive pro-caspase-1, P45. The cleavage and activation of P45 are triggered by the assembly of inflammasome, the NOD-like receptor (NLR)/caspase-1 complex [7, 8], and then forming the activated subunits which are composed of P10 and P20 [9]. The activated caspase-1 can further trigger pyroptosis [10–12]. This newly discovered form of cell death is characterized by a unique morphological and biochemical features. It can produce specific membrane pores between 1.1 and 2.4 nm in diameter and dissipate ionic gradients leading to water uptake and cell swelling; ultimately, cells undergo osmotic lysis with release of intracellular contents [11]. Caspase-1-deficient mice are more susceptible to intracellular pathogens, for example *Francisella tularensis*, *Salmonella typhimurium*, *etc.* [12]. Caspase-1-dependent pyroptosis contributes to eliminate the intracellular bacteria [13, 14]. Additionally, it has been identified that caspase-1 activation plays an important role in the pathogenesis of chronic inflammatory and autoimmune diseases [15, 16].

Up to now, caspase-1 and caspase-1-dependent pyroptosis are important in inflammatory and immune regulation. However, whether sevoflurane affects these

Yue Jin and Hui Li contributed equally to this work.

[1] Department of Anesthesiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, QingChun Road 79, 310003, Hangzhou, China
[2] Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[3] To whom correspondence should be addressed at Department of Anesthesiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, QingChun Road 79, 310003, Hangzhou, China. E-mail: xiangming_fang@163.com

processes is still unknown. Herein, we investigated the effect of sevoflurane on the activation of caspase-1 and the extent of caspase-1-dependent pyroptosis in mouse macrophage cell line.

MATERIALS AND METHODS

Cell Culture

The murine J774 macrophage cell line was cultured in DMEM medium (Gibco, Grand Island, NY) containing 10 % fetal bovine serum (Invitrogen, Carlsbad, CA) and 1 % penicillin–streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a humidified incubator (5 % CO_2 and 95 % air; Thermo Electron, Waltham, MA). Before experiment, J774 cells were seeded in 24-well plates at 1×10^6 cells/well and incubated for 24 h.

Exposure of Cultured Cells to Sevoflurane

Anesthetic-enriched medium was prepared as described in a previous study [17]. Continuous sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) infusion was used to reduce the volatilization loss of sevoflurane from the anesthetic-enriched medium. Additionally, the J774 cells were exposed to different volume percent of sevoflurane. High concentration (≥6 %) of sevoflurane presented a significant activation of caspase-1 and caspase-1-dependent pyroptosis (data not shown), which is in accordance with previous studies [18].

After incubation of 24 h, the J774 cells were stimulated with or without 1 μg/mL LPS (Sigma, St. Louis, MO) for 4 h. The old medium was aspirated and the anesthetic-enriched medium was added, simultaneously stimulated with or without 5 mM ATP (Roche Diagnostics, Indianapolis, IN). Meanwhile, the J774 cells were placed in an air-tight, humidified specifically modified incubator with inflow and outflow connectors for 0.5 h at 37 °C. The inlet port was connected to the sevoflurane vaporizer (Blease Medical Equipment Ltd, Chesham, UK) to deliver 6 % sevoflurane at 3 L/min. The outlet port was connected to a Datex-Ohmeda 5250 RGM gas analyzer (Dräger, Lübeck, Germany) which measured sevoflurane concentration. Non-sevoflurane group cells were seeded in the anesthetic-free medium then exposed to 95 % air and 5 % CO_2 in a specifically modified incubator. After 0.5 h, the cells were harvested for further analysis.

Additionally, for pharmacological assessments, cells were pretreated with or without 10 mM *N*-acetyl-L-cysteine (NAC; Sigma, St. Louis, MO) or 12.5 μM diphenyleneiodonium (DPI; Sigma, St. Louis, MO) for 15 min before ATP stimulation, after that the cells were exposed to ATP and 6 % sevoflurane for 0.5 h.

Western Blotting Analysis

Cells were lysed in lysis buffer containing protease inhibitors phenylmethylsulfonyl fluoride, followed by incubation on ice for 45 min. The cell lysates were clarified by centrifugation for 15 min. 20 μg of each clarified lysate were separated on 12 % SDS polyacrylamide gels, transferred to nitrocellulose membranes. Membranes were blocked in TBS-T containing 5 % BSA for 1 h. Membranes were washed three times in TBS-T for 10 min and incubated with rabbit polyclonal caspase-1 antibody SC-514 (Santa Cruz, CA) or anti-mouse β-actin antibody overnight. Membranes were washed three times with TBS-T, followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody for 2 h. Thereafter, membranes were washed three times in TBS-T and detected by chemiluminescence.

Ethidium Bromide and EthD-2 Staining

After being washed twice with PBS, cells of each group were stained with the membrane permeable dye 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probe, Eugene, OR) for 10 min and caspase-1 inhibitor Ac-YVAD-CMK (Merck Chemicals, Kilsyth, Victoria, Australia), the red membrane impermeant dyes, EtBr (MW394 Da; Molecular Probe, Eugene, OR) or EthD-2 (MW 1,293 Da; Molecular Probe, Eugene, OR) according to the manufacturer's instructions. Coverslips were analyzed using Olympus IX81 fluorescence microscope (Tokyo, Japan). The percentage of positive cells was derived from counting a minimum of 300 cells/sample under each experimental condition.

ROS Detection

Intracellular ROS was determined with the ROS-specific fluorescent probe CM-H2DCFDA (Molecular Probe, Eugene, OR) [19]. Cells of each group were loaded for 15–30 min with 10 mM CM-H2DCFDA, washed twice with OPTI-MEM (Gibco, Grand Island, NY). The level of fluorescence was determined by flow cytometry (BD LSRII System; BD Biosciences, San Jose, CA).

Cell Viability Assay

Cell viability was detected using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). J774 cells were

pated at a density of 5,000 cells/well in 96-well plates. Pre-incubate the plate for 24 h in the incubator. Then the cells were exposed to 6 % sevoflurane/95 % air and 5 % CO_2 with or without 5 mM ATP. After that, 10 μL of CCK-8 solution was added to each well of the plate and incubated for 2 h. After incubation, the optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Richmond, CA).

Statistical Analysis

Statistical comparisons between different treatments were performed by Student's *t* test (two-tailed) or analysis of variance with Tukey's post hoc pairwise comparisons using SPSS16.0 for Windows (SPSS, Inc., Chicago, IL). Values were reported as mean±SD from three independent experiments. Differences were considered significant for $P<0.05$.

RESULTS

LPS and ATP Activate Caspase-1 in Murine J774 Macrophages

LPS (1 μg/mL) treatment prior to 5 mM ATP pulse induced caspase-1 activation (Fig. 1) in murine J774 macrophages but not sevoflurane or ATP alone.

Sevoflurane Combined with ATP Activates Caspase-1

In the level of activated caspase-1, P10 was markedly increased in the sevoflurane-ATP group compared with that in the non-sevoflurane ATP group (220.4±75.6 vs. 44.9±24.1 Int/mm^2, $P=0.019$) (Fig. 2a) while, sevoflurane alone or sevoflurane combined with LPS could not activate caspase-1 (Fig. 2a). Sevoflurane did not significantly affect the viability of the cells in the presence and absence of ATP (Fig. 2b).

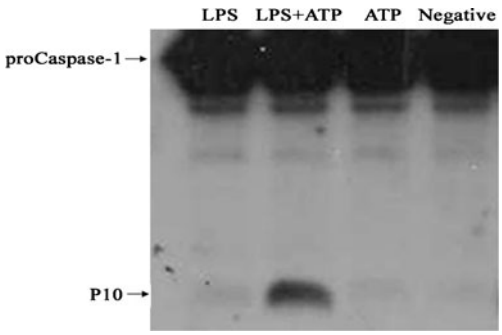

Fig. 1. LPS and ATP activate caspase-1 in murine J774 macrophages. The J774 cells were incubated with 1 μg/mL LPS for 4 h prior to 5 mM ATP pulse, then procaspase-1 and activated caspase-1 (*P10*) were analyzed by western blot of cell extracts. *Negative* stimulated with PBS.

Sevoflurane Combined with ATP Triggers Caspase-1-Dependent Pyroptosis

The membrane impermeant dye EtBr, EthD-2 and the membrane permeable dye DAPI were used to examine membrane pore formation during pyroptosis [11]. Consis-

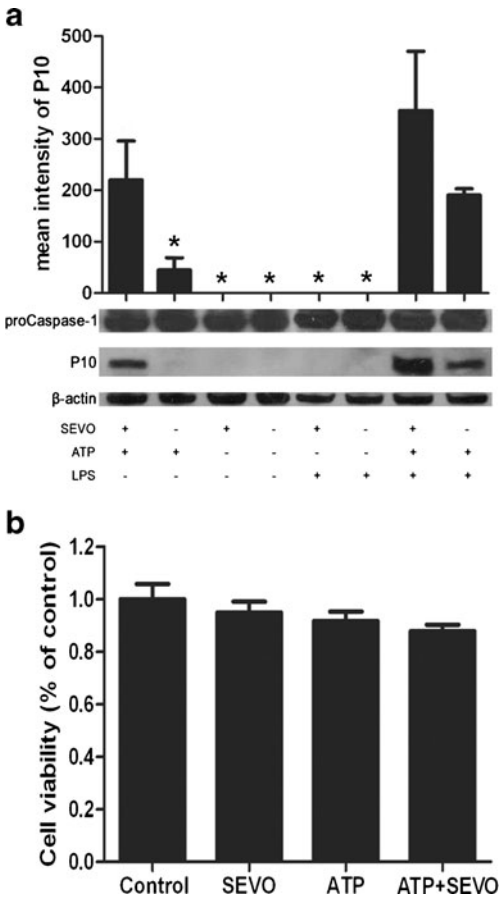

Fig. 2. Sevoflurane combined with ATP activates caspase-1. The J774 cells were seeded in 24-well plates at 1×10^6 cells/well and incubated for 24 h. After that, the cells were stimulated with LPS (1 μg/mL, 4 h), ATP (5 mM, 0.5 h), and/or sevoflurane (6 %, 0.5 h). The negative control was exposed to 95 % air and 5 % CO_2 for 0.5 h. **a** Expression of P10 under stimulation with sevoflurane, LPS, or ATP. Results are mean±SD from three independent experiments. *$P<0.05$ versus ATP+SEVO sample. **b** The effect of sevoflurane on J744 cell viability. J774 cells were pated at 5,000 cells/well in 96-well plates and incubated for 24 h. Then the cells were exposed to 6 % sevoflurane/95 % air and 5 % CO_2 with or without 5 mM ATP, followed by a CCK-8 assay performed to quantitatively assess the cell viability using adsorption of $OD_{450\ nm}$.

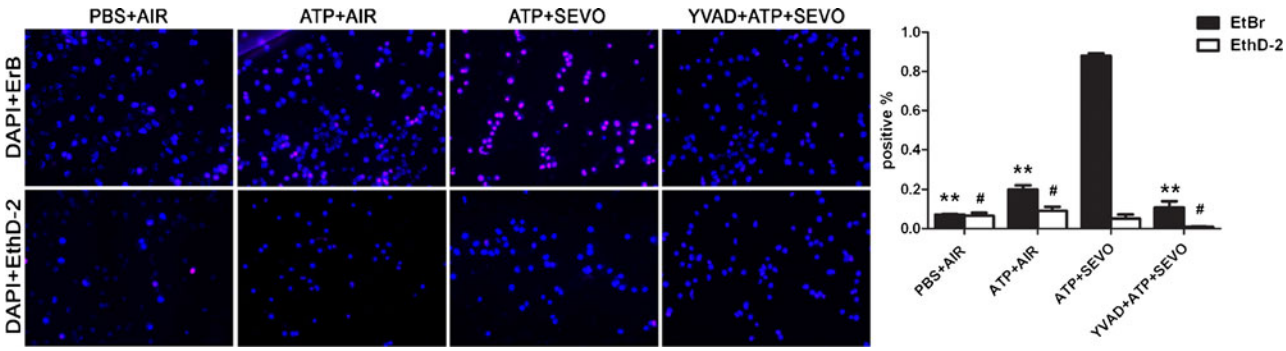

Fig. 3. Sevoflurane combined with ATP triggers caspase-1-dependent pyroptosis. Each group of cells after being exposed to sevoflurane or 95 % air and 5 % CO_2 for 0.5 h, were washed twice with PBS, then cells were stained with the membrane permeable dye DAPI for 10 min and the red membrane impermeant dyes EtBr or EthD-2 as directed by the manufacturer's instructions. The percentage of positive cells was derived from counting a minimum of 300 cells per sample for each experimental condition. Results are mean±SD from three independent experiments. $^{**}P<0.001$ versus ATP+SEVO sample; *number sign* no significant difference versus ATP+SEVO sample.

tent with results shown in Fig. 2a, the percentage of EtBr uptake was significantly increased in the sevoflurane group (87.9±1.3 vs. 20.0±2.2 %, $P<0.001$) and can be inhibited by the caspase-1 inhibitor Ac-YVAD-CMK (87.9±1.3 vs. 10.7±3.3 %, $P<0.001$) (Fig. 3). However, the EthD-2 uptake was similar between the two groups (5.0±2.2 vs. 9.1 ±2.1 %, $P=0.10$) (Fig. 3).

Sevoflurane Combined with ATP Regulates Caspase-1 Activation by Modulating ROS Production

Sevoflurane upregulated caspase-1 activation and caspase-1-dependent pyroptosis. Since ROS is believed to be a common NLR/caspase-1 complex activator [20], we hypothesized that sevoflurane mediates caspase-1 activation via ROS production. The level of ROS

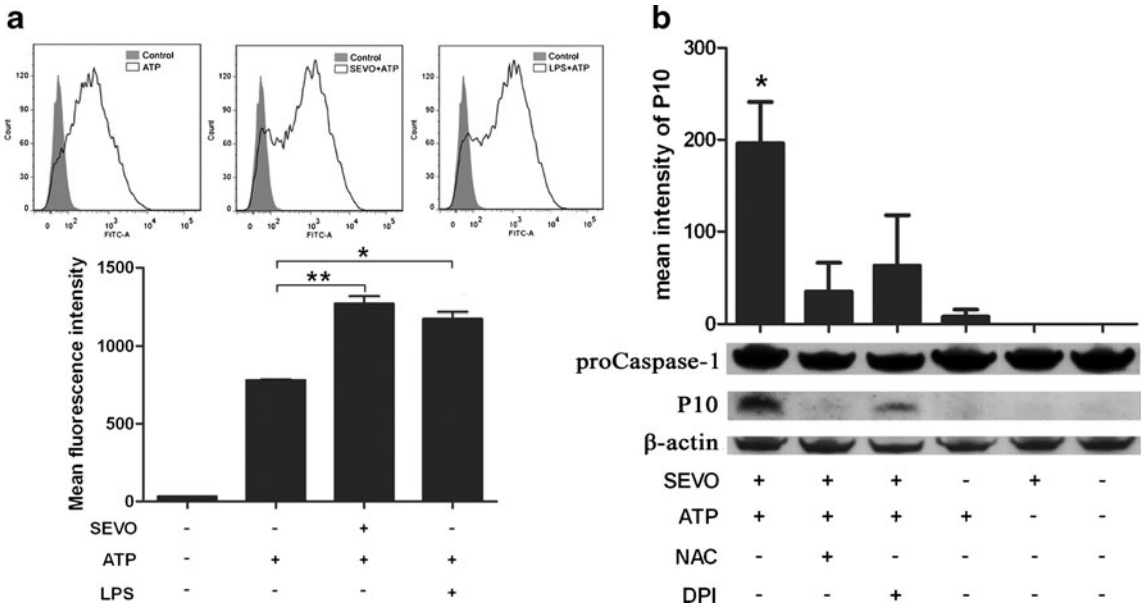

Fig. 4. Sevoflurane combined with ATP regulates caspase-1 activation by modulating ROS production. **a** Sevoflurane modulates ROS production. Each group of cells were exposed to 6 % sevoflurane or 95 % air and 5 % CO_2 for 0.5 h, and then loaded for 15–30 min with 10 mM CM-H2DCFDA. Results are mean±SD from three independent experiments. $^{*}P<0.05$; $^{**}P<0.001$. **b** ROS inhibitor reduced the activation of caspase-1. The J774 cells were seeded in 24-well plates at 1×10^6 cells/well and incubated for 24 h. Cells were pretreated with or without 10 mM NAC or 12.5 μM DPI for 15 min, and then the anesthetic-enriched medium was added, simultaneously stimulated with 5 mM ATP and/or 6 % sevoflurane for 0.5 h. *Negative* stimulated with PBS. Results are mean±SD from three independent experiments. $^{*}P<0.05$ compared with all other groups.

production in J774 cells was measured with CM-H2DCFDA. As predicted, the ROS fluorescence was significantly increased after sevoflurane plus ATP stimulation in J774 cells and the LPS plus ATP stimulation group than that in the non-sevoflurane ATP group (1,270.0±81.3 vs. 780.0±8.8 Int/mm^2, $P<0.001$; 1,175.7±70.6 vs. 780.0±8.8 Int/mm^2, $P=0.001$) (Fig. 4a). To further address whether ROS was involved in sevoflurane combined with ATP-regulated caspase-1 activation, J774 cells were treated with ROS inhibitor NAC or DPI before ATP stimulation. The activation of caspase-1 was reduced by both NAC (196.1±26.0 vs. 37.8±20.4, $P=0.013$) and DPI (196.1 ±26.0 vs. 62.8±31.8, $P=0.028$), which disrupted the ROS production (Fig. 4b).

DICUSSION

The present study demonstrated that 6 % sevoflurane combined with 5 mM ATP could activate caspase-1, trigger caspase-1-dependent pyroptosis in murine J774 macrophages by modulating ROS production.

Caspase-1 plays an important role in both innate and acquired immune responses. Studies have shown that caspase-1-mediated innate immunity and the downstream proinflammatory response can protect host against many invading pathogens. Recently, Miao *et al*. indentified that caspase-1-mediated macrophage pyroptosis is an innate immune effector mechanism against intracellular bacteria. Indeed, this effect resulted in the intracellular pathogens releasing into the extracellular space. The latter then were uptaken and killed by neutrophils efficiently [14]. In addition, current studies have indicated that pyroptosis eliminates cellular niches for bacterial replication, recruits and activates immune effector cells to the site of infection and enhances the host defense against the pathogenic microorganisms [10, 11, 13].

Sevoflurane, one of the most frequently used volatile anesthetics, has been extensively studied due to its inflammatory and immune modulatory effect. The present study firstly reveals that sevoflurane plus ATP may regulate macrophages through activation of caspase-1 and triggering caspase-1-dependent pyroptosis. Virtually, many kinds of cells such as stimulated T cells, polymorphonuclear granulocytes and intact cells can release ATP during conditions of inflammation or hypoxia [21–23]. Considering the immune regulatory properties of caspase-1-mediated pyroptosis, our results provides preliminary evidences that perioperative sevoflurane application may enhance host immune defense function and benefit for preventing from postoperative infection. It might be particularly helpful for patients suffering from immune dysfunction, especially in sepsis patients with pro-caspase-1 and activated caspase-1 markedly inhibited [24]. Therefore, further exploration of the role of pyroptosis initiated by sevoflurane *in vivo* may lead to new therapies for both infectious and inflammatory diseases.

Activation of caspase-1 needs the formation of NLR/caspase-1 complex. It has also been demonstrated that K^+ efflux is mediated by P_2X_7, phagosomal destabilization and reactive oxygen species involve in the activation of NLR/caspase-1complex [25–27]. Studies have shown that lysosomal destabilization mainly participates in triggering the formation of NLR/caspase-1complex induced by small crystalline materials [20, 25]. Additionally, ATP could increase the level of ROS, and sevoflurane plays an important role in organ-protecting through ROS-dependent mechanism [28–30]. Therefore, we speculate that sevoflurane mediates caspase-1 activation through ROS production. As predicted, our results confirmed that ROS significantly increased after sevoflurane plus ATP stimulation in J774 cells for 0.5 h, which was even higher than the LPS plus ATP stimulation. These findings are consistent with previous studies. The speculation was further confirmed by the finding that ROS inhibitors NAC and DPI reduced the activation of caspase-1 by sevoflurane combined with ATP. The current study also demonstrated that promoting ROS production was the main mechanism in sevoflurane plus ATP induced activation of caspase-1 and caspase-1-dependent pyroptosis in murine macrophages.

There are potential limitations regarding the interpretation of our data. In this paper, we focused on the J774 macrophage cell line *in vitro* and presented a preliminary result on the immune regulation of sevoflurane. To our knowledge, cell lines might be different from primary cells in some of the biological activities. So, further *ex vivo* and *in vivo* study is warranted. However, based on the common characteristics shared by the cell line and primary cells, cell lines are routinely adopted models in pilot studies before in-depth investigations. On the other hand, differences in the molecular conformation and structure-activity relationships of halogenated anesthetic agents may lead to different biological functions. Therefore, the sevoflurane-mediated effects observed in this study might not be generalized to the other halogenated anesthetics.

In summary, sevoflurane combined with ATP can activate caspase-1 and trigger caspase-1-dependent pyroptosis. The possible mechanism is related to sevoflurane increased ROS production. These results indicate that sevoflurane might regulate immune response via activating caspase-1 and caspase-1-dependant pyroptosis of macrophages *in vivo*, which may be beneficial for better choice of clinical anesthesia and the witness of sevoflurane immune regulatory mechanism.

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Conflict of Interest. None.

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