Effects of anesthetic regimes on inflammatory responses in a rat model of acute lung injury

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Abstract

Background—Gamma amino butyric acid (GABA) is the major inhibitory neurotransmitter through activation of GABA receptors. Volatile anesthetics activate type A (GABA_A) receptors resulting in inhibition of synaptic transmission. Lung epithelial cells have been recently found to express GABA_A receptors that exert anti-inflammatory properties. We hypothesized that the volatile anesthetic sevoflurane (SEVO) attenuates lung inflammation through activation of lung epithelial GABA_A receptors.

Methods—Sprague-Dawley rats were anesthetized with SEVO or ketamine/xylazine (KX). Acute lung inflammation was induced by intratracheal instillation of endotoxin, followed by mechanical ventilation for 4 h at a tidal volume of 15 mL/kg without positive end-expiratory pressure (two-hit lung injury model). To examine the specific effects of GABA, healthy human bronchial epithelial cells (BEAS-2B) were challenged with endotoxin in the presence and absence of GABA with and without addition of the GABA_A receptor antagonist picrotoxin.

Results—Anesthesia with SEVO improved oxygenation and reduced pulmonary cytokine responses compared to KX. This phenomenon was associated with increased expression of the π subunit of GABA_A receptors and glutamic acid decarboxylase (GAD). The endotoxin-induced

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cytokine release from BEAS-2B cells was attenuated by the treatment with GABA, which was reversed by the administration of picrotoxin.

Conclusion—Anesthesia with SEVO suppresses pulmonary inflammation thus protects the lung from the two-hit injury. The anti-inflammatory effect of SEVO is likely due to activation of pulmonary GABA_A signaling pathways.

Keywords

volatile anesthetics; mechanical ventilation; inflammation

Introduction

Acute lung injury (ALI) is a major challenge in critical illness that is associated with high mortality [1]. The majority of patients suffering from ALI require mechanical ventilation for life support [2, 3]. However, mechanical ventilation per se bears the risk of inducing and worsening pulmonary dysfunction described as ventilator induced lung injury (VILI) [4]. An important approach to manage patients with ALI is to reduce pulmonary and systemic inflammatory responses that may have played an important role inducing multiple distal organ dysfunction in the context of VILI [1, 2].

Low tidal volume ventilation has been considered as a protective ventilator strategy [2], but it may not suit to all patients with respiratory failure [5]. Volatile anesthetics have been recently shown to exert anti-inflammatory effects in several experimental and clinical settings [6–8], which may be beneficial in the context of VILI. The use of sevoflurane (SEVO) can increase expression of I κ B while decreasing NF- κ B nuclear translocation following challenge with tumor necrosis factor α (TNF- α) in human monocytes [8]. The administration of SEVO improved gas exchange and attenuated lung injury after endotracheal installation of endotoxin in rats [6]. Anesthesia with SEVO has been shown to decrease the production of inflammatory cytokines including TNF- α , interleukin-6 (IL-6) and IL-8 in lung lavage fluids and reduced postoperative adverse events in patients under thoracic surgery [7]. However, the mechanisms by which SEVO exerts the anti-inflammatory effects remain unclear.

It is known that volatile anesthetics such as SEVO and isoflurane activate type-A gamma-aminobutyric acid (GABA_A) receptor resulting in inhibition of synaptic transmission in neurons [9, 10]. GABA, synthesized from glutamate by decarboxylation *via* the enzymatic activity of glutamic acid decarboxylase (GAD), produces fast synaptic inhibition in neurons through activating GABA_A receptor, a GABA-gated anion channel. In addition to its conventional role in synaptic transmission, GABA_A receptors also exert novel anti-inflammatory properties in the central nervous system [11] and in peripheral immune cells [12].

It is interesting that recent studies have demonstrated that GABA_A receptors are also expressed in lung airway and alveolar epithelial cells [13–16]. However, their local role in response to inhalation of SEVO remains to be investigated in the context of ALI. We thus hypothesized that anesthesia with SEVO attenuates pulmonary inflammatory response

through activation of GABA_A receptor in lung epithelial cells. To test this hypothesis we examined the effects of SEVO on the expression of GABA_A receptor and cytokine responses in a rat model of ALI, and the specific effects of GABA on cytokine responses *in vitro* in human lung epithelial cells.

Material and Methods

Anesthesia and mechanical ventilation

The study was approved by the institutional Animal Care Committee (ACC948) of St. Michael's Hospital. Thirty-four Sprague-Dawley rats (250 – 350 g) were randomly assigned to receive anesthesia with either ketamine + xylazine (KX, n = 17) or sevoflurane (SEVO, n = 17). In the KX group, anesthesia was induced using 80 mg/kg ketamine and 8 mg/kg xylazine intraperitoneally, followed by continuous intravenous infusion of ketamine at 15 mg/kg/h and xylazine at 3 mg/kg/h. In the SEVO group, anesthesia was induced by 3.5 Vol. % SEVO in a closed chamber and maintained with 2.6 Vol.%. These doses of anesthetics were chosen based on pilot experiments targeting similar mean arterial blood pressure (MAP) and comparable depth of anesthesia defined by no reaction to toe pinches prior to neuromuscular blockade. Tracheotomy was performed followed by intratracheal intubation with a 14G angiocatheter. Neuromuscular blockade was achieved by i.v. infusion of pancuronium at 0.5 mg/kg/h.

The initial ventilator settings were as follows by using a rodent ventilator (Harvard 683): inspired oxygen fraction (F_IO₂) of 0.40, positive end-expiratory pressure (PEEP) of 5 cmH₂O, tidal volume (V_T) of 6 mL/kg and respiratory rate (RR) adjusted to keep arterial partial pressure of CO₂ (PaCO₂) between 35 and 45 mmHg. The right carotid artery was cannulated (0.58 mm ID polyethylene tube) for continuous blood pressure monitoring. Intravenous infusion of lactated ringer solution at 3 mL/kg/h was administered through the tail vein in all animals. Rectal temperature was maintained at 37°C ± 1°C using a heating blanket.

Two-hit model of ALI

All animals received intratracheal instillation of endotoxin at 5 mg/kg (LPS, Escherichia coli serotype 055:B5, Sigma Aldrich). Thirty minutes later, mechanical ventilation was switched to high V_T at 15 mL/kg and PEEP = 0 cmH₂O for 4 h.

Measurements

MAP was monitored in a real time fashion. Arterial blood gases (PaO₂, PaCO₂) were recorded hourly throughout the experiments.

Bronchoalveolar lavage (BAL) was performed in the left lung by using cold normal saline upon completion of the experiments. The BAL fluid was centrifuged at 4°C and 1,200 g for 10 min and the supernatant was stored at –80°C for further analysis.

Lung wet/dry ratio (WD ratio) was obtained after excision of the right lung and drying at 60°C for 72h.

The concentrations of glucose and lactate in blood were measured (Radiometer ABL 700 blood gas analyzer) at the end of the study.

Levels of intercellular adhesion molecule 1 (ICAM-1) and multiple cytokines including interleukin 1- β (IL-1 β), IL-2, IL-4, IL-10, IL-12, keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1 α) and tumor necrosis factor-alpha (TNF- α) were measured using species specific multiplex cytokine assays (Bio-Rad) as previously described [17].

Basal expression of glutamic acid decarboxylase (GAD, GAD65/67 antibody, Abcam, Inc, Cambridge, MA), and GABA_A receptor (GABA A Receptor alpha 2 antibody, Abcam, Inc.) was detected by immunohistochemistry in rats sacrificed by cervical dislocation without undergoing the experimental protocol to serve as healthy controls. The methods have been previously described by the authors [15]. GAD expression was also assessed by Western blot (GAD65/67 antibody, Abcam, Inc.) analysis as previously described [15].

Cell culture and treatment

Human bronchial epithelial cells (BEAS-2B, ATTC) were cultured in full confluence and incubated with GABA (100 nM) alone or GABA + picrotoxin, a GABA_A receptor antagonist (PTX, 50 nM) for 10 min, followed by stimulation with LPS at 100 ng/mL (*Escherichia coli* serotype 055:B5, Sigma Aldrich) for 4h. This dose of LPS has been previously used for induction of inflammatory responses [18].

Statistical analysis

Data are presented as mean and standard deviation. Student's t-test for unpaired samples was used to compare differences among groups at time 0 and for post-mortem analysis. Repeated measurements over time were analyzed with two-way analysis of variance for repeated measurements and adjusted with the Bonferroni post-test. Statistical significance was considered if $p < 0.05$.

Results

Bodyweight were similar in all animals. The amount of fluid infused was not different in both groups (11.3 ± 0.7 mL in SEVO vs. 12.5 ± 1.4 mL in KX group). Although the glucose level was slightly higher in the SEVO than in the KX group (8.59 ± 0.57 mmol/L vs. 7.83 ± 0.18 mmol/L) the difference did not reach statistical significance. However, a lower level of lactate in blood was observed in the SEVO group than the KX group (2.26 ± 0.23 mmol/L vs. 3.35 ± 0.45 mmol/L, $p < 0.05$).

Hemodynamics and gas exchange

MAP and PaCO₂ showed a similar time course in all animals (Figure 1A and 1B, respectively). PaO₂ decreased significantly starting 2h after high V_t ventilation in the KX group as compared to the SEVO (Figure 1C). The decreased level of PaO₂ was associated with an increase in lung wet/dry ration suggesting enhanced lung permeability in the KX group (Figure 1D).

Pulmonary inflammatory response

The total leukocyte count in BALF was significant lower in the SEVO group compared to the KX group ($0.85 \pm 0.04 \times 10^6$ vs. $1.03 \pm 0.05 \times 10^6$, $p < 0.05$). Although there was a trend of decrease in the percentage of polymorphonuclear cells out of the total leukocytes ($55 \pm 5\%$ vs. $69 \pm 6\%$), the difference did not reach statistical significance. Cytokine profile in BAL fluid showed decreased levels of TNF- α , MIP-1 α , IL-1 β , KC, MCP-1 and ICAM-1 under anesthesia with SEVO compared to that with KX (Figure 2).

Immunohistochemistry

Immunohistochemistry showed a basal expression of GAD and GABA_AR alpha unit colocalized with lung epithelium stained with surfactant protein C of healthy control rats (Figure 3A). Western blot assay showed a higher protein level of GAD in the SEVO group than in the KX group (Figure 3B).

In vitro BEAS-2B cell experiments

To investigate the specific effects of GABA in lung epithelial cells, human BEAS-2B cells were stimulated with LPS followed by treatment with GABA, PTX or GABA+PTX. We first confirmed that the dose of PTX used did not induce cytotoxicity as reflected by a constant LDH level before and after PTX administration [19], thus cell viability was not affected (data not shown). IL-1 β was significantly reduced with GABA and GABA+PTX compared to Control and PTX alone group (Figure 4A). IL-2 was significantly decreased with GABA and PTX compared to GABA+PTX (Figure 4B). The concentrations of IL-8 and IL-10 decreased significantly with GABA, which was reversed by administration of PTX (Figure 4C and 4D, respectively).

Discussion

We demonstrate that anesthesia with SEVO improves oxygenation and reduced pulmonary cytokine responses associated with increased expression of GABA_A as compared to anesthesia with KX. These results suggest that the activation of GABA_A receptors may play a role in the lung protective effect seen with SEVO.

An established two-hit model was chosen for its clinical relevance that primary lung inflammation/injury was followed by mechanical ventilation as a treatment [6]. Since a single drug of either ketamine or xylazine is not recommended, a regime of the combination is commonly used for animal anesthesia [20]. We examined two regimes of anesthesia by choosing SEVO and KX in the present study. To avoid possible interference and/or crossover effects between the SEVO and KX, the same anesthetic regime was used for both induction and maintenance. The two anesthetic regimes are functioning through distinct mechanisms of action that would help understand the possible signaling pathways responsible for their effects. SEVO acts mainly via agonistic effects on GABA_A receptors. Ketamine interacts mainly with the NMDA-subtype glutamate receptor but at high, fully anesthetic level doses, it binds to opioid μ receptors and sigma receptors [21–23]. In comparison with SEVO, ketamine has minor effects by selectively acting on $\alpha 6$ and δ subunits of GABA receptors [22]. A majority of literature suggested that ketamine exerts antagonistic action at

the GABA_A-receptor complex [24–26]. In particular, ketamine has been reported to decrease the GAD67 isoform [27]. Our results are in agreement with these previous studies [24] [25–27]. It is noted that subanesthetic doses of ketamine was reportedly to inhibit tonic convulsions induced by the GABA_A receptor antagonist bicuculline, and the latter antagonized ketamine anesthesia. However, the suggested agonistic effects of ketamine on GABA_A was not examined directly in the study [28]. Xylazine is a clonidine analogue acting on presynaptic and postsynaptic receptors of the central and peripheral nervous systems as an α_2 -adrenergic agonist that is used primarily for sedation, aesthesia, analgesia and muscle relaxation in animal models [29]. Similar drugs such as clonidine and dexmedetomidine are increasingly used in clinical anesthesia and critical care [30]. The different pharmacological actions of the anesthetics used in this study may explain their distinct anti-inflammatory properties. In addition, paralysis was applied by administration of a given dose of pancuronium in all animals to reach synchrony between the animals and the ventilator in order to minimize signal to noise ratio and to enhance reproducibility of the experiments. It is therefore that the paralysis was part of the anesthesia regimes used in the present study.

Various inflammatory mediators including the cytokines TNF- α and IL-1 β , chemokines and ICAM-1 have been shown to act as effector molecules in the disruption of the epithelial and endothelial barrier during mechanical ventilation [31, 32]. We observe that the administration of SEVO attenuated the production of the inflammatory mediators in the lung and thus reduced lung permeability. Our results are in agreement with other studies showing anti-inflammatory effects of volatile anesthetics in a variety of *in vivo* and *in vitro* models. In an ischemia-reperfusion model of acute kidney injury, Lee et al. [33] reported direct anti-inflammatory and anti-necrotic effects of SEVO by activation of prosurvival kinases and increase in de-novo synthesis of heat shock protein 70. Boost et al. [8] demonstrated that isoflurane, acting also on GABA_A receptors [34], attenuates the release of IL-8 and heme oxygenase 1 in human monocytic THP-1 cells *in vitro* through a mechanism by which the volatile anesthetic stabilizes the NF- κ B inhibitory pathway.

IL-10 was originally discovered as the cytokine synthesis inhibiting factor based on its biological activity. It down-regulates the expression of pro-inflammatory cytokines such as IL-1 β , MHC class II antigens, and co-stimulatory molecules by upregulation of itself. In the present study, the administration of GABA attenuated the LPS-induced production of IL-1 β and IL-10 in BEAS-2B cells. This phenomenon is consistent with previous reports showing that concentrations of IL-1 β and IL-10 increased in response to LPS challenge to counterbalance the pro- and anti-inflammatory responses [35, 36].

A recent study by Faller and colleagues [37] who reported in a mouse model of VILI by using a tidal volume of 12 mL/kg for 6 hours that the administration of the volatile anesthetic isoflurane resulted in a reduction in lung damage, inflammation and stress protein expression. Our data are consistent with their observation despite that we use a two hit model of ALI followed mechanical ventilation under the anesthesia with SEVO. Taken together, our and the previous study [37] support the concept that volatile anesthetics exert anti-inflammatory effects protecting the lung from injury.

Volatile anesthetics produce anesthetic action primarily by binding to GABA_A receptor in neurons of the central nervous system (CNS) [15]. Specifically, SEVO activates GABA_A receptor in the CNS resulting in an inhibition of neuronal activities [9, 15]. We have previously discovered the existence of GAD and GABA receptors in human airway epithelial cells [15], and other investigators identified GABA receptors in alveolar epithelial type II cells [14]. In an ongoing study, we observed that volatile anesthetics including SEVO also enhance GABA_A receptor-mediated anion current in pulmonary epithelial cells. In the present study, we further confirmed the presence of GAD and GABA_A receptor both in rat airway and alveolar epithelial cells. We also demonstrate that anesthesia with SEVO results in an increase in expression of GAD and GABA_A receptor in the lung compared with KX. We speculate that an increase in GAD expression after SEVO administration may be due to a positive feedback mechanism as a result of the binding between SEVO and GABA_A receptors.

The concentrations of cytokines were lower and the GAD expression was higher in the SEVO group than in the KX group *in vivo* conditions. These observations suggest that SEVO exerts anti-inflammatory properties partially by upregulation of GABA receptors. This concept was further supported by the *in vitro* lung epithelial cell studies where LPS induced-cytokine responses were attenuated in the presence of GABA, which was reversed by the administration of picrotoxin. Furthermore, it has been reported that an increase in surface levels of GABA_A receptors requires the activity of phosphoinositide 3-kinase (PI3K) and protein kinase C [38]. This is in accord with a recent study reporting that a mechanism by which isoflurane reduced VILI was through increasing phosphorylation of Akt protein, since the inhibition of phosphoinositide 3-kinase/Akt signaling prior to mechanical ventilation completely reversed the lung-protective effects of isoflurane in mice [37].

It is worthy note that anesthesia with SEVO or KX may result in hyperglycemia in rats [39, 40]. Clinical and experimental data suggest that hyperglycemia is protective against the development of ALI/ARDS [41]. We observe that the blood glucose level was higher in the SEVO than in the KX groups although the difference did not reach statistically significant. However, whether a different effect on glycemia between SEVO and KX might provide an additional pathway by which SEVO exerts a protective effect remains to be further investigated.

There are several limitations in the study, 1) The experimental double-hit model used in this study may not reflect the complex clinical scenario seen in patients with ALI/ARDS. 2) The involvement of GABA_A receptor was the focus of the study, whether mechanisms other than GABA_A receptor are also responsible in the protective effects of SEVO remains to be elucidated. 3) The study design compares the modulation of pulmonary inflammatory responses during mechanical ventilation by two different anesthesia regimens, it is impossible to draw conclusion whether the difference is due to the protective effects of one or the detrimental effects of the other single agent. 4) We focused the investigation on SEVO in the present study, further studies using other volatile (i.e., desflurane or isoflurane) or intravenous (i.e., propofol or midazolame) anesthetics are warranted.

In summary, we demonstrate that anesthesia with sevoflurane can improve oxygenation and reduce pulmonary cytokine responses as compared to ketamine/xylazine. The protective effects of sevoflurane appeared to be associated with its agonistic effects at GABA_A receptors.

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Figure 1. Mean arterial pressure, gas exchange and lung permeability

Mean arterial blood pressure (MAP); arterial partial pressure of carbon dioxide (PaCO₂) and arterial partial pressure of oxygen (PaO₂) in animals anesthetized with ketamine/xylazine (KX) or sevoflurane (SEVO) over time. Lung wet/dry weight ratio was determined at the end of 4-h mechanical ventilation started 30 min after LPS administration. N=17 per group.

Figure 2. Inflammatory mediator responses in BAL fluid

Cytokine and ICAM-1 responses in animals anesthetized with ketamine/xylazine (KX) or sevoflurane (SEVO). Bronchoalveolar lavage (BAL) fluid was obtained at the end of 4-h mechanical ventilation started 30 min after LPS intratracheal administration. Tumor necrosis factor- α (TNF- α); macrophage inflammatory protein-1 alpha (MIP-1 α); interleukin-1 beta (IL-1 β); keratinocyte growth factor (KC); monocyte chemotactic protein-1 (MCP-1) and intracellular adhesion molecule-1 (ICAM-1). N=17 per group.

Figure 3. Lung expression of GAD and GABA_AR receptor

A. Immunohistochemistry staining for the epithelial marker surfactant protein C (green), and GAD (red), GABA_AR (red) and their overlay (orange) in healthy rat lungs. **B.** Western blot for detection of GAD in animals anesthetized with KX or SEVO at the end of 4-h mechanical ventilation started 30 min after LPS intratracheal administration. Average expression of GAD over β-actin was from 5 experiments.

Figure 4. Cytokine responses in BEAS-2B cells

Concentration of cytokines measured in cell culture supernatants of human bronchial epithelial cells (BEAS-2B) stimulated with LPS (Control) followed by incubation with gamma amino butyric acid (GABA), picrotoxin (PTX) or GABA+PTX for 8h. N = 5 experiments per group.