Propofol inhibits T-helper cell type-2 differentiation by inducing apoptosis via activating gamma-aminobutyric acid receptor

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ABSTRACT

Background: Propofol has been shown to attenuate airway hyperresponsiveness in asthma patients. Our previous study showed that it may alleviate lung inflammation in a mouse model of asthma. Given the critical role of T-helper cell type-2 (Th2) differentiation in asthma pathology and the immunomodulatory role of the gamma-aminobutyric acid type A (GABA_A) receptor, we hypothesized that propofol could alleviate asthma inflammation by inhibiting Th2 cell differentiation via the GABA receptor.

Methods: For in vivo testing, chicken ovalbumin-sensitized and challenged asthmatic mice were used to determine the effect of propofol on Th2-type asthma inflammation. For in vitro testing, Th2-type cytokines as well as the cell proliferation and apoptosis were measured to assess the effects of propofol on Th2 cell differentiation and determine the underlying mechanisms.

Results: We found that propofol significantly decreased inflammatory cell counts and interleukin-4 and inflammation score in vivo. Propofol, but not intralipid, significantly reduced the Th2-type cytokine interleukin-5 secretion and caused Th2 cell apoptosis without obvious inhibition of proliferation in vitro. A GABA receptor agonist simulated the effect of propofol, whereas pretreatment with an antagonist reversed this effect.

Conclusions: This study demonstrates that the antiinflammatory effects of propofol on Th2-type asthma inflammation in mice are mediated by inducing apoptosis without compromising proliferation during Th2 cell differentiation via activation of the GABA receptor.

Introduction

Asthma is generally characterized by airway inflammation, wall remodeling, and hyperreactivity of the bronchial tree, which results in inappropriate, variable, inducible and reversible bronchoconstriction with excessive and abnormally viscous mucus secretion. The incidence of acute perioperative bronchospasm in asthmatic patients undergoing...
general anesthesia is approximately 10.2%. Severe asthma attacks may be induced by tracheal intubation, surgical manipulation, and some drugs, causing hypoxia or even death. Anesthetic agents that will not irritate the airway or even possess airway protective properties will be preferable for asthmatic patients.

Propofol is one of the most commonly used intravenous anesthetics. Several studies have shown that propofol is preferable for sedation and anesthesia in severe asthmatic patients because of its bronchodilatory effects and low incidence of inducing bronchospasm. Furthermore, propofol has also been shown to have anti-inflammatory effects in many cell and animal models. Our previous work also demonstrated that propofol may alleviate lung inflammation in asthmatic mice. However, it remains unclear how propofol mitigates asthmatic airway inflammation.

T-helper cell type-2 (Th2)-type asthma inflammation is characterized by increased infiltration of inflammatory cells into the lungs along with prominent Th2 cell differentiation. The Th2 cell differentiation process begins with the activation of T cells by interleukin (IL)-4 cytokine signaling, followed by continuous cell proliferation and apoptosis, and ultimately results in secretion of downstream cytokines. Typical Th2-type cytokines, such as IL-4, IL-5, and IL-13, play critical roles in asthma pathogenesis. The two opposing cellular processes, proliferation and apoptosis, regulate the balance between cell death and survival, thus influencing the cytokine production, which indicates the level of differentiation.

It has been shown that propofol exerts the hypnotic and anesthetic effect by acting on the gamma-aminobutyric acid type A (GABA<sub>A</sub>) receptor in the central nervous system. In addition to the central nervous system, the GABA receptor also exists on the peripheral immune cells and is involved in immune suppression.

Given the critical role of Th2 cell differentiation in asthma pathogenesis and the immunoinhibitory role of the GABA receptor in the peripheral immune system, especially on T cells, the present study aimed to determine the effect and mechanism of propofol on Th2 cell–mediated asthmatic inflammation, as well as the role of the GABA receptor. We hypothesized that propofol alleviates asthmatic inflammation via the GABA receptor by inhibiting Th2 cell differentiation through inducing Th2 cell apoptosis and/or inhibiting proliferation.

**Ovalbumin sensitization and challenge**

Ovalbumin sensitization and challenge is a classic method for establishing an asthma mouse model. Except for the control group, mice were sensitized on days 0, 7, and 14, with intraperitoneal injections of 50-μg grade V chicken ovalbumin (OVA; Sigma–Aldrich, St. Louis, MO) emulsified in 2-mg Inject Alum (Pierce, Rockford, IL) in a total volume of 200 μL. Mice in the control group received normal saline only. Subsequently, OVA-sensitized mice were intranasally challenged with 100-μg OVA (100 μg dissolved in 40 μL normal saline) on days 21, 22, and 23, whereas mice from the control group received normal saline alone under light isoflurane anesthesia (1.5%-2% inhalation). Five groups of mice were studied: (1) the normal saline-treated control group (control, n = 20); (2) the OVA-sensitized and OVA-challenged asthma group (asthma, n = 20); (3) the OVA-sensitized, OVA-challenged, and propofol-treated group (PF, n = 20) (Diprivan, 100 mg/kg administration 8 min before challenge); (4) the OVA-sensitized, challenged, and GABA<sub>A</sub> receptor agonist-treated group (MUS, n = 20; muscimol, 1 mg/kg, 30 min before OVA challenge); and (5) the OVA-sensitized, challenged, and propofol-treated group with GABA<sub>A</sub> receptor antagonist pretreatment (PIC + PFF, n = 20; picrotoxin, 10 mg/kg, 9 min before the administration of propofol 100 mg/kg injection). The concentration of propofol in vivo was determined based on our pilot study, which indicated that dosages greater than 100 mg/kg would cause high mouse mortality.

**Bronchoalveolar lavage**

All mice were deeply anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg, Merck, Germany) 24 h after the last challenge. Bronchoalveolar lavage was performed with injection of 0.5-mL ice-cold normal saline into the trachea through a cannula. Then, the lungs were rinsed and gently massaged for approximately 1 min before the liquid was suctioned. After three repetitions, 1.2-1.3 mL of bronchoalveolar fluid (BALF) was harvested from each animal. The BALF supernatants were stored at −80°C until cytokine measurement. The sediment was used for total and differential cell counting with an automated analyzer.

**Histological assessment**

For evaluation of peribronchial and perivascular inflammatory changes, lung tissues were harvested, fixed in 10% formalin overnight and embedded in paraffin. Five micrometer sections were affixed to slides and stained with hematoxylin-eosin (H&E). Images of H&E-stained slides were obtained on a Leica DM3000 microscope using a ×20 objective. The images were blindly scored by an experienced pathologist, and the scores were recorded by another independent observer. The scoring is based on a subjective scale of 0-5 points according to the method reported in a previous study. Briefly, inflammatory changes were graded using a semiquantitative scale of 0-5 for perivascular eosinophilia, bronchial eosinophilia, epithelial damage, and edema. The scoring is as follows: 0, normal; 1, low grade; 2, low to

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**Materials and methods**

**Animals**

A total of 100 female BALB/c mice (6-8 wk old) were purchased from the animal experimental center of Peking Union Medical College and Chinese Academy of Medical Sciences. Female mice were used because they display more severe Th2-type cytokine infiltration compared with male mice. They were maintained in a specific pathogen-free room at temperatures between 22°C-24°C and humidity between 50%-70%. The mice were provided with sterilized tap water and standard rodent chow. Before the study, the mice were acclimatized in this environment for 1 wk. This experiment was approved by the Chinese Institute of Animal Care and Use Committee.
moderate grade; 3, moderate grade; 4, moderate to high grade; and 5, high grade.

**Enzyme-linked immunosorbent assay (ELISA)**

The concentration of IL-4 and IL-5 was measured by ELISA kits according to the manufacturer’s instructions (eBioscience, San Diego, CA). Samples and standards were prepared and added to the 96-well plate and then incubated overnight at 4°C. Then, the samples were incubated with a detection antibody at room temperature for 1 h. Subsequently, 100-μL avidin horseradish peroxidase was added and incubated for 30 min. After the plate was washed five times, 100-μL tetramethylbenzidine was added to each well. The plate was incubated for 15 min in the dark, and then, 50-μL stop solution was added to each well. The absorbance was determined at 450 nm with 540 nm as the wavelength correction using a microplate ELISA reader (Bio-Rad Laboratories, CA). The samples were assessed in triplicate.

**CD4+ T cell purification, in vitro differentiation, and treatment**

Naive spleen cells were isolated aseptically from the spleens of 6- to 8-week-old female BALB/c mice. CD4+ T cells were selected with anti-CD4 MACS beads (Miltenyi Biotec, Auburn, CA) through positive magnetic cell selection. With this protocol, CD4+ T cells with 95% purity can be routinely acquired. The cells (2 × 10^5 cells/well) were cultured in complete medium (Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum [FBS], glutamine [2 mM], penicillin [100 U/mL], and streptomycin [100 μg/mL]). T cells were stimulated with Dynabeads Mouse T-activator CD3/CD28 (2 μL per well; Life Technologies, Carlsbad, CA), anti-interferon gamma antibody (10 μg/mL, XMG1.2; eBioscience), IL-4 (20 ng/mL; PeproTech, Rocky Hill, NJ), IL-2 (20 ng/mL; Peprotech), propofol (30 μg/mL and 3 μg/mL, named PPF 1 and PPF 0.1, respectively, AstraZeneca, London, UK), intralipid (diluted in accordance with the propofol protocol, named LIP 1 and LIP 0.1; Huarui, China), muscimol (100 μmol, Sigma Aldrich), and picrotoxin (30 μmol, 30 min before propofol addition) plus propofol (30 μg/mL, Sigma Aldrich) were added to the culture separately. The concentration of 30 μg/mL and 3 μg/mL were determined based on one previous research which reported that the blood concentration of propofol can reach up to 170 μM (approximately 30 μg/mL) after 100 mg/kg administration. Cells were cultured for 5 consecutive days. The Th2 cell differentiation was examined by measuring IL-5 levels. The cell-free supernatants were analyzed for IL-5, and the sediment was prepared for cell apoptosis and proliferation assays.

**Apoptosis assay**

Assessment of apoptosis in CD4+ T cells was determined with Annexin V and propidium iodide (PI) staining on days 0, 1, 3, and 5. The Annexin V/PI protocol is a commonly used approach for studying apoptotic cells. The cells were collected, washed twice with cold 10 mM 1 × phosphate-buffered saline, and resuspended in 1 × Annexin V binding buffer (BD Biosciences, San Jose, CA). Then, a 100-μL sample was transferred to test tubes, and 2.5 μL allopurinol-labeled Annexin V as well as 5-μL PI solution was added. The samples were vortexed and incubated for 15 min at room temperature in the dark. After adding 200-μL 1× binding buffer to each tube, the samples were analyzed using an Accuri C6 Flow Cytometer. Annexin V-allophycocyanin and PI emissions were detected in the FL4 and FL2 channel. The data in the left lower quadrant of the scatter plot represents the percentage of viable cells, and the data in the right quadrant (both upper and lower) demonstrate the percentage of apoptotic cells.

**Proliferation assay**

Labeling cells with carboxyfluorescein succinimidyld ester (CFSE) is currently one of the most informative experimental techniques for studying cell proliferation in immunology. The selected CD4+ cells were washed twice with 10% FBS and resuspended in 1-mL Roswell Park Memorial Institute 1640 medium. Then cells were labeled with 1-mL 5-mM stock CFSE for a final concentration of 5 μM. The cells were incubated for 15 min at 37°C in the dark and vortexed three times gently. Then, 5-mL FBS was immediately added to terminate the reaction. The cells were washed twice with 10% FBS again, and the drugs same as in the apoptosis assay were added to the culture system. After 5 days of culturing, the cells were collected and washed twice with phosphate-buffered saline. CFSE staining was assessed with the FL1 detector of the Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA). The histograms of the CFSE intensity for original and proliferating cell populations were obtained by fluorescence-activated cell sorting on days 0 and 5, providing quantitative data for kinetic analysis of cell division.

**Statistical analysis**

Statistical analysis of all data was performed using GraphPad Prism 6 for Mac OS X (GraphPad Software). All continuous data were expressed as the mean ± standard deviation and were analyzed by one-way analysis of variance followed by Holm-Sidak’s multiple-comparison test as indicated. A P value < 0.05 was considered statistically significant.

**Results**

**Propofol treatment decreased the lung inflammation score in asthmatic mice**

To confirm the antiinflammatory effects of propofol, H&E staining was conducted first to observe the general inflammatory changes. Compared with the control group, lungs from the asthma group showed more severe infiltration of inflammatory cells into the alveoli, congested blood capillaries, and thickened alveolar septa after H&E staining, indicating that the asthmatic mouse model was successfully established. The lung inflammation score of the PPF group (2.8 ± 0.8) was significantly lower (P < 0.05) than that of the asthma group (4.2 ± 0.5). Muscimol treatment showed a similar decrease (1.7 ± 0.5), and picrotoxin pretreatment (3.5 ± 0.8) reversed the effect of propofol and muscimol (Fig. 1).
Propofol decreased total, eosinophil, and lymphocyte cell counts in the BALF of asthmatic mice

Then, the cells and supernatants in the BALF were analyzed to determine the effect of propofol on specific inflammatory cells and cytokines. In regard to total and differential cell counts, including lymphocytes and eosinophils, the asthma group showed significant increases (total: $100.3 \pm 17.0 \times 10^4$, lymphocytes: $6.9 \pm 4.6 \times 10^4$, and eosinophils: $8.9 \pm 0.9 \times 10^4$) compared with those of the control group (total: $10.2 \pm 7.1 \times 10^4$, lymphocytes: $1.3 \pm 0.5 \times 10^4$, and eosinophils: $0.3 \pm 0.2 \times 10^4$). After treatment with propofol, the inflammatory cell count in the BALF (total: $55.6 \pm 19.4 \times 10^4$, lymphocytes: $5.6 \pm 1.8 \times 10^4$, and eosinophils: $5.8 \pm 1.7 \times 10^4$) was substantially decreased ($P < 0.05$). Muscimol treatment simulated the effect of propofol (total: $36.7 \pm 9.6 \times 10^4$, lymphocytes: $3.5 \pm 1.1 \times 10^4$, and eosinophils: $4.1 \pm 0.6 \times 10^4$), whereas picrotoxin pretreatment reversed this effect (total: $99.9 \pm 21.6 \times 10^4$, lymphocytes: $9.9 \pm 2.9 \times 10^4$, and eosinophils: $9.4 \pm 1.8 \times 10^4$; Fig. 2).

Propofol downregulated pulmonary Th2-type cytokine IL-4 production in vivo

The expression of Th2-type cytokines was detected by ELISA of the BALF supernatant. The IL-4 level in the asthma group
(356.0 ± 81.4 pg/mL) was significantly increased after OVA sensitization and challenge compared with that of the control group (4.6 ± 1.6 pg/mL), but this elevation was suppressed after propofol (156.7 ± 128.2 pg/mL) and muscimol treatment (113.1 ± 107.0 pg/mL, P < 0.05, Fig. 3). Picrotoxin pretreatment partly reversed the effects of propofol on IL-4 (190.3 ± 90.1 pg/mL).

Propofol decreased IL-5 levels in vitro

Cell culture experiments were conducted to further investigate the effects of propofol on Th2 differentiation. After 5 days of culture, the IL-5 level of the positive control group (21.8 ± 7.1 pg/mL) was significantly higher than that of the negative control group (0.56 ± 0.08 pg/mL), suggesting successful differentiation of Th2 cells. Propofol reduced the level of IL-5 in a dose-dependent manner (3.5 ± 2.2 pg/mL in PPF 1 and 4.4 ± 1.6 pg/mL in PPF 0.1 group). Muscimol showed similar reduction (10.48 ± 3.9 pg/mL), while picrotoxin pretreatment significantly reversed this decrease (11.21 ± 4.3 pg/mL, Fig. 4).

Propofol-attenuated cell viability and enhanced Th2 cell apoptosis during cell development

After determining the effect of propofol on Th2-type cytokines in vitro, we conducted apoptosis and proliferation assays to identify possible mechanisms. The viability of cells in all groups on day 0 and day 1 was approximately 75%. However, the percentage of apoptotic cells in the propofol-treated group began to increase on day 3 compared with that of the positive control group (data not shown) and peaked on day 5 in a dose-dependent manner (47.3 ± 1.9 and 40.9 ± 3.9 in PPF 1 and PPF 0.1 compared with positive control 38.7 ± 4.8, Fig. 5). Muscimol showed similar changes (54.8 ± 4.4) as PPF 1, whereas picrotoxin pretreatment (48.5 ± 3.2) significantly reversed the increase induced by the GABA receptor agonist. Although the intralipid-treated groups showed no significant changes (37.2 ± 1.1 and 38.4 ± 4.7 in LIP 1 and LIP 0.1) compared with the positive control, they demonstrated statistical differences compared with PPF 1 and MUS group.

Propofol had no effect on proliferation during cell development

CFSE staining was used to measure proliferation. The CFSE histograms showed the proliferative responses of cultured CD4+ T cells on days 0 and 5. The histogram on the right shows the original state on day 0. The four histograms on the left show vigorous proliferation up to day 5 (except for the negative control group). The mean fluorescence intensity revealed no obvious difference between the propofol group, positive control group, and intralipid group (33.0 ± 0.7, 31.2 ± 2.2, 33.7 ± 1.7 respectively, Fig. 6), indicating that neither propofol nor intralipid influences the proliferation process significantly during Th2 cell development.

Discussion

The present study aimed to evaluate the effect of propofol on Th2-mediated asthma inflammation in vivo and in vitro. The major findings of this report are as follows: (1) propofol treatment alleviated asthmatic lung inflammation and reduced Th2-type cytokine IL-4 production in vivo; (2) propofol decreased IL-5 levels, and this effect may be attributed to apoptosis induction without proliferation inhibition in vitro; and (3) a GABA receptor agonist simulated the effect of
propofol, and GABA antagonist pretreatment reversed the changes by the GABA receptor agonist, indicating that the GABA receptor is the binding site of propofol in the peripheral immune system.

Previous studies have reported the possible mechanisms of propofol in attenuating smooth muscle contraction in a variety of experimental and functional studies over the years. However, airway inflammation is another important contributor to asthma pathogenesis. In vivo results demonstrated that propofol alleviated general asthma inflammation. Further assessment of IL-4 indicated that propofol suppressed Th2 cell differentiation, which is attributable to general alleviation of inflammation.

Consistent with the localization of the binding site of propofol in the central nervous system, GABA receptor potentiation was also shown to mediate the effect of propofol in the peripheral immune system. This is supported by our data indicating that muscimol simulated the effect of propofol, and picrotoxin pretreatment antagonized this effect to a certain extent. These results not only confirmed the presence of the GABA receptor on developing T cells but also demonstrated the inhibitory role of the GABA receptor on Th2-mediated asthma inflammation. This corresponds with previous published studies, which suggested a role of the GABA receptor in peripheral immune suppression, such as inhibition of cytokine secretion, cell proliferation, phagocytic activity, and chemotaxis.

To further elucidate the mechanism of propofol on Th2 cell-mediated asthma inflammation, we differentiated Th2 cells in vitro with propofol administration for further investigation. From the relatively high level of IL-5 expression in the positive control group compared with the negative control
group, Th2 cells were shown to be successfully differentiated. Propofol decreased the production of IL-5 dose dependently, which not only corresponded with the inhibitory effects on Th2 cells in vivo but also further confirmed that the effect is due to suppression of the Th2 cell differentiation process. Because Th2 cell differentiation is a result of continuous apoptosis and proliferation, both apoptosis and proliferation assays were performed to determine the possible mechanism. Our data showed that the inhibitory effect of propofol on Th2 cell differentiation can be attributed to induction of apoptosis, without proliferation inhibition.

The antiinflammatory effect of propofol has been demonstrated in many studies, for example, on ischemia-reperfusion injury, neuroinflammation, and many inflammatory cells, including neutrophils, monocytes, macrophages, and T cells. Our research demonstrated the antiinflammatory effect on asthmatic inflammation. Regarding Th2-type inflammation, our results were consistent with those of a previous study, which suggested that propofol facilitates Th0 to Th1 cell differentiation, and thus, Th2 cell polarization is inhibited.

The apoptogenic action of propofol has been widely studied in developing neurons, epithelial ovarian cancer cells, and hepatocellular carcinoma cells. Pešić et al. found that propofol induced apoptotic neurodegeneration, and this effect is closely correlated with the timing of synaptogenesis. The study suggested that the developing cells are vulnerable to the proapoptotic effect of propofol, and the timing is critical. This correlates with our research that showed that differentiating Th2 cells, which are developing, sensitive, and vulnerable, underwent apoptosis after propofol treatment. The proapoptotic effect may be mediated by activating the GABA receptor, triggering Ca$^{2+}$ influx and the apoptosis pathways. Accordingly, our results suggested that propofol, rather than its intralipid counterpart, may inhibit Th2 cell differentiation by activating the GABA receptor and inducing developing Th2 cell apoptosis.

Our study has several limitations. First, the intralipid used in our study was not provided by AstraZeneca, so there may be possible differences in the components. Second, more specific approaches are underway to elucidate the relationship between the GABA receptor and propofol, such as GABA receptor knocked out or knocked down animals or cells in future studies.

**Conclusion**

In summary, the present study demonstrates that in asthmatic mice, the antiinflammatory effect of propofol on Th2-induced asthma inflammation is mediated by inhibition of Th2 cell differentiation, and the mechanism is attributed to induction of apoptosis via the GABA receptor during Th2 cell development without compromising proliferation.

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Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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