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Propofol specifically reduces PMA-induced neutrophil extracellular trap formation through inhibition of p-ERK and HOCl

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ABSTRACT

Neutrophil extracellular traps (NETs) are net-like chromatin fibers that can trap and kill microorganisms. Although several anti-inflammatory effects of intravenous anesthetics have been reported, it has not been investigated whether intravenous anesthetics influence NET formation.

Aims: To compare the effects of four intravenous anesthetics (propofol, thiamylal sodium, midazolam, and ketamine) on phorbol myristate acetate (PMA)-induced NET formation and analyze the associated signaling pathways.

Materials and methods: PMA-stimulated NETs formed in the absence or presence of intravenous anesthetics were stained with SYTOX Green and then quantified. Inhibitors were applied to investigate the related mechanism, which was confirmed by western blotting, and ROS were detected.

Key findings: The neutrophils incubated with propofol showed the lowest degree of NET formation compared with those incubated with the other intravenous anesthetics. Propofol significantly reduced the level of myeloperoxidase (MPO)-derived HOCl but not that of superoxide. Aminopyrine, an MPO inhibitor, markedly decreased the number of PMA-induced NETs, indicating the involvement of HOCl in the inhibitory effect of propofol on NET formation. According to western blotting results, the level of p-ERK was reduced by propofol during PMA-induced NET formation. The ERK inhibitor PD98059 decreased NET formation but did not inhibit PMA-induced HOCl generation, and aminopyrine did not reduce ERK phosphorylation.

Significance: Through this study, we define a new anti-inflammatory effect of intravenous anesthetics. Of the four intravenous anesthetics tested, propofol was the most potent inhibitor of NET formation. Moreover, propofol resulted in a decrease in PMA-induced NET formation by two independent mechanisms: inhibition of HOCl and p-ERK.

1. Introduction

Neutrophils are innate immune cells of primary importance for protecting against infections by microorganisms [1,2] through various antimicrobial abilities, including microorganism phagocytosis and antimicrobial agent secretion [1]. Furthermore, a specific mechanism of neutrophils is the release of extracellular traps to ensnare and kill microorganisms [3,4]. These neutrophil extracellular traps (NETs) are composed of net-like chromatin fibers with attached antimicrobial proteins such as histones, elastase, cathepsin G, proteinase 3, lactoferrin, myeloperoxidase, and calprotectin and cytoplasmic proteins [3,5]. All of these factors can inhibit or kill pathogens by disrupting virulence factors or the integrity of the pathogen's cell membrane [6]. In addition to their antimicrobial properties, NETs appear to trap microorganisms to prevent them from spreading, and NETs maintain a high concentration of antimicrobial agents at the infection site to degrade virulence factors and pathogens [3].

The induction of NET production in neutrophils by phorbol myristate acetate (PMA) is a well-characterized NETosis model. PMA is an activator of protein kinase C (PKC) that can trigger NET formation though a reactive oxygen species (ROS)-dependent pathway [7,8]. Inhibition of NADPH oxidase with diphenyleneiodonium (DPI) notably decreases PMA-induced NET formation [9], and phosphorylation of p38 MAPK and ERK1/2 has been demonstrated to be involved in the underlying mechanism. However, it remains unclear whether MAPK and ROS are upstream of the signaling pathway [10,11].

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Intravenous anesthetics are extensively used for general anesthesia and sedation. They have also been shown to have immunomodulatory effects, such as inhibiting phagocytic function, chemotaxis, cytokine secretion and ROS generation [12–15]. Propofol and midazolam are commonly used for sedation in the intensive care unit (ICU) [16]; the most common cause of ICU admission is sepsis, and increased NET formation during sepsis is critical for host survival [17,18]. As mentioned above, NETs are a unique means by which neutrophils control infections [3,4], although it remains unknown whether intravenous anesthetics have effects on NET formation. Thus, we compared the effects of four intravenous anesthetics (propofol lipid emulsion, thiamylal sodium, midazolam, and ketamine) on NET formation and analyzed the signaling pathway involved in the modulation of NET formation by these anesthetics.

2. Materials and methods

2.1. Neutrophil isolation

Peripheral blood was obtained from healthy volunteers using BD Vacutainer® Blood Collection Tubes containing EDTA. All participants provided informed written consent for the collection of samples and subsequent analysis, as approved by the Institutional Review Board of the Ditmanson Medical Foundation Chia-Yi Christian Hospital, Taiwan. Peripheral blood polymorphonuclear cells (PMNs) were isolated by Ficoll Hypaque gradient centrifugation followed by ammonium chloride potassium lysis of red blood cells. An EasySep™ Human Neutrophil Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) was then used to negatively select neutrophils. The eluted cells were stained with an anti-CD66b antibody (Ab), and the neutrophil content was evaluated by flow cytometry, reaching > 90% of the cell population in each isolation. Because eosinophils also express CD66b, we further applied an anti-CD16 Ab (BioLegend, Inc. San Diego, CA, USA) to identify the neutrophil content (CD16 + CD66b+), which ranged from 98 to 99%. CD16 + CD66b + cells stained with Hoechst 33342 displayed a lobulated nucleus (Fig. 1A). Both results confirmed that the cells isolated were neutrophils.

2.2. Reagents

Four intravenous anesthetics (propofol lipid emulsion, thiamylal sodium, midazolam, and ketamine) were purchased from Fresenius Kabi Austria GmbH (Graz, Austria), Shinlin Sinseng Pharmaceutical Co., Ltd. (Taoyuan, Taiwan), Nang Kuang (Tainan, Taiwan) and Taiwan Pfizer Inc. (New Taipei, Taiwan), respectively. The concentrations of intravenous anesthetics used in this study were according to the clinical plasma concentrations of propofol (6 μg/ml), thiamylal sodium (30 μg/ml), midazolam (1.5 μg/ml), and ketamine (2 μg/ml), which are equal to the typical therapeutic doses administered by intravenous injection to adult patients. The NADPH oxidase inhibitor DPI, obtained from Abcam (ab141310, Cambridge, UK), was used to inhibit ROS production, and PD98059 (TargetMol, Boston, MA, USA) was applied to inhibit ERK1/2 activation. Aminopyrine, an inhibitor of myeloperoxidase (MPO), was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.3. Viability assay

Neutrophil viability was evaluated by a Cell Counting Kit-8 assay (Sigma-Aldrich, Inc., St. Louis, MO, USA). Neutrophils (1×10^5 cells/well) suspended in the recommended medium (1× PBS containing 2% FBS) were seeded in the wells of a 96-well microplate for 4 h. After adhering, the neutrophils were treated with or without intravenous anesthetics for 120 min and washed 3 times with 1× PBS. Metabolically active cells were detected by adding cell medium containing a WST-8 solution for 2 h and then quantifying using a Model 680 microplate reader (absorbance at 450 nm; reference at 655 nm) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Neutrophil viability was calculated using the formula $A_{treated}/A_{control}$.

2.4. Visualization and quantification of NETs

NET formation was visualized by fluorescence microscopy and quantified by spectrophotometry after the addition of SYTOX Green dye, a cell-impermeable DNA-binding dye that can be used to stain and detect extracellular DNA. Neutrophils (5×10^5 cells/well) were allowed to adhere to a Nunc™ 177437 Lab-Tek Chamber Slide System (1.8 cm^2/well), and the effects of intravenous anesthetics on NET formation induced by PMA (10 nM) were investigated after incubating the neutrophils with the anesthetics for 120 min. During the incubation, SYTOX Green (1 μM) (Life Technologies, Darmstadt, Germany) was added to stain the NETs, followed by fluorescence microscopy (Olympus Corporation, Tokyo, Japan). Unstimulated cells treated with or without intravenous anesthetics served as controls. After fluorescence microscopy, micrococcal nuclease (1 unit/ml) was added to partially digest any released NETs at 37 °C for 10 min, which was stopped with EDTA (5 mM). The suspension was centrifuged at 1800 ×*g* for 10 min, and 100 μl of the supernatant was plated in a 96-well black plate. NET DNA fragments were quantified using a BioTek Synergy H1 microplate reader at 485 nm excitation and 525 nm emission (BioTek, Winooski, USA). The data were recorded as arbitrary fluorescent units (AFU).

2.5. Luminol chemiluminescence assay

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was applied to detect the sum of intracellular and extracellular ROS. Neutrophils (1×10^5 cells/well) seeded in a 96-well white microplate were incubated with intravenous anesthetics, stained with 12.5 mg/ml luminol (Sigma-Aldrich, Inc., St. Louis, MO, USA) and stimulated with 50 nM PMA. The increasing chemiluminescence due to ROS production was immediately measured and continuously monitored every 5 min for a period of 1 h at 37 °C using a BioTek Synergy H1 microplate reader (BioTek, Winooski, VT, USA). Neutrophils not incubated with intravenous anesthetics but stimulated with PMA were used as the positive control. Treatment with each intravenous anesthetic alone but not PMA stimulation was applied as basal controls. The peak value of chemiluminescence at 15 min was utilized to calculate the effect of intravenous anesthetics on ROS production, which was normalized to the ROS production in the positive control.

2.6. Nitroblue tetrazolium assay

Nitroblue tetrazolium (NBT) is cell membrane permeable and is reduced to water-insoluble blue formazan particles by intracellular superoxide [11]. Neutrophils (1×10^5 cells/well) seeded in a 96-well microplate were preincubated with or without PD98059 (20 μM) for 30 min. Subsequently, 100 μl of NBT (0.5 mM) was added, and the neutrophils were stimulated with PMA (50 nM) in the absence or presence of propofol, PD98059 (20 μM) or DPI (10 μM) for 60 min at 37 °C. After washing twice with 1× PBS, the neutrophils were fixed with methanol and air-dried. The NBT deposited inside the cells was dissolved by adding 120 μl of 2 M potassium hydroxide (KOH) to solubilize the cell membranes and then adding 140 μl of dimethyl sulfoxide (DMSO) followed by gentle pipetting at room temperature to dissolve the blue formazan crystals. Absorbance was detected at 620 nm using a microplate reader.

2.7. Determination of hypochlorous acid (HOCl) production by neutrophils

The HOCl level was determined by measuring the formation of taurine-chloramine, which results from the reaction of HOCl with taurine, according to the method established by Dypbukt et al. [19].

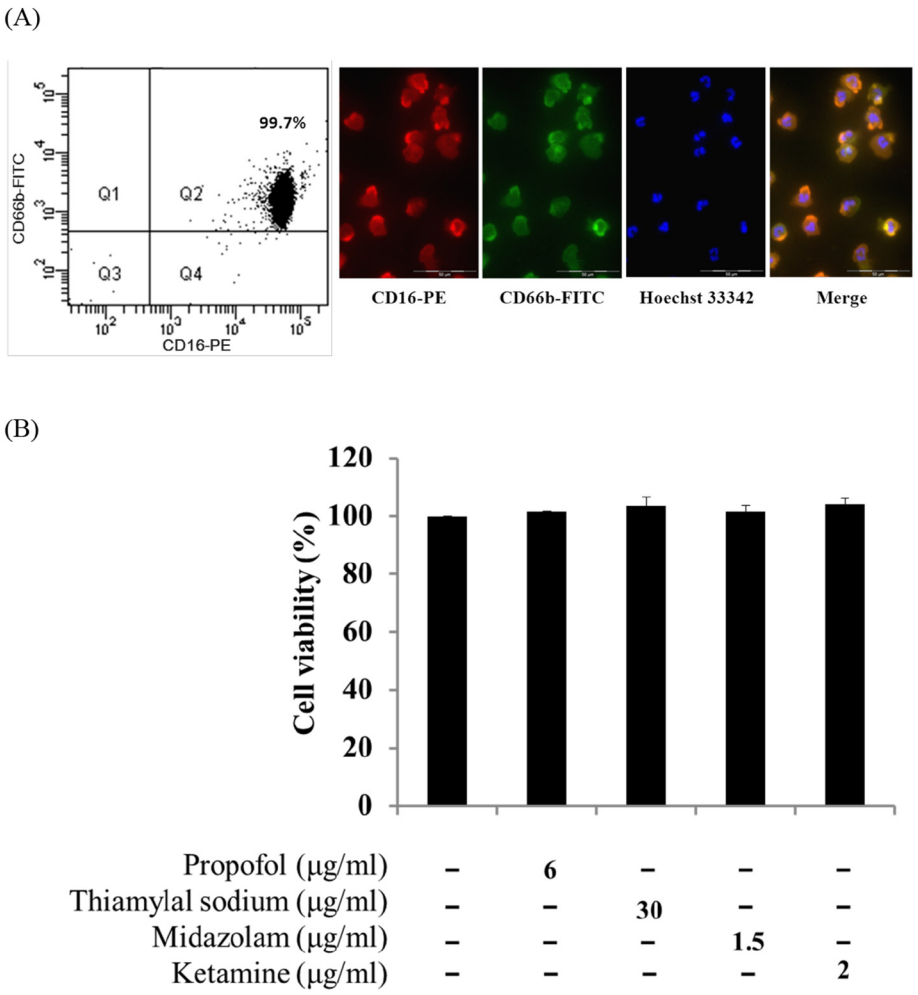


Fig. 1. Confirmation of the purity of isolated neutrophils and the viability of those treated with each of four intravenous anesthetics. (A) Neutrophil purity was detected with anti-CD16 (PE) and -CD66b (FITC) antibodies and analyzed by flow cytometry. The nucleus of CD16 + CD66b + cells appeared lobulated when stained with Hoechst 33342. The scale bar indicates 50 μm. (B) Viability of freshly isolated neutrophils from three individuals was assessed using a CCK-8 kit. The data are presented as the mean ± SD.

Neutrophils (1×10^6 cells) were preincubated with 15 mM taurine in 10 mM phosphate buffer (pH 7.4) containing 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mg/ml glucose for 10 min at 37 °C. The neutrophils were then stimulated with PMA (50 nM) in the absence or presence of inhibitors, DPI (10 μM), PD98059 (20 μM), propofol (6 μg/ml) and aminopyrine (200 μM) for 30 min at 37 °C. The reactions were performed in 1.5-ml Eppendorf tubes containing 200 μl of solution with gentle agitation. The reactions were stopped by adding catalase (100 μg/ml) for 10 min on ice, followed by centrifugation at 14,000 ×*g* for 5 min at 4 °C. The supernatants (200 μl) were harvested and mixed with 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) for 5 min to detect the formation of taurine-chloramine by spectrophotometry at 655 nm using a BioTek Synergy H1 microplate reader (BioTek, Winooski, VT, USA).

2.8. Myeloperoxidase activity assay

Neutrophils (2×10^6 cells/well) in 1.5-ml Eppendorf tubes were treated with PMA (50 nM) in the absence or presence of DPI (10 μM), PD98059 (20 μM), propofol (6 μg/ml) or aminopyrine (200 μM) for 30 min. MPO activity in the supernatant of each sample was assessed using a fluorometric assay kit purchased from Abcam (cat. no. ab111749, Cambridge, UK); the experimental procedures were performed according to the manufacturer's instructions. The increasing fluorescence due to MPO activity was kinetically measured at 2 min intervals for 30 min using a BioTek Synergy H1 microplate reader (BioTek, Winooski, VT, USA) with an excitation wavelength of 484 nm and an emission wavelength of 525 nm. MPO activity was calculated as μU/ml based on a standard curve.

2.9. Western blot analysis

Neutrophils (2×10^6 cells/well) pre-incubated for 30 min with or without PD98059 (20 μM) were stimulated with PMA (10 nM) and then incubated with or without propofol, PD98059 or DPI for 1 h. Unstimulated neutrophils treated with DMSO or drug alone served as controls. After detaching and pelleting the cells, the neutrophil were lysed with mammalian protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing 0.1% protease inhibitor cocktail. Equal amounts (10 μg) of cell lysate from each sample were separated by SDS-PAGE and transferred to PVDF membranes. Anti-ERK1/2, anti-phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies targeting specific proteins were detected using a secondary antibody conjugated with horseradish peroxidase (HRP). After developing the blots with Immobilon Western Chemiluminescent HRP substrate (EMD Millipore Corporation, Billerica, MA, USA), the signals were detected using a BioSpectrum Imaging System (UVP).

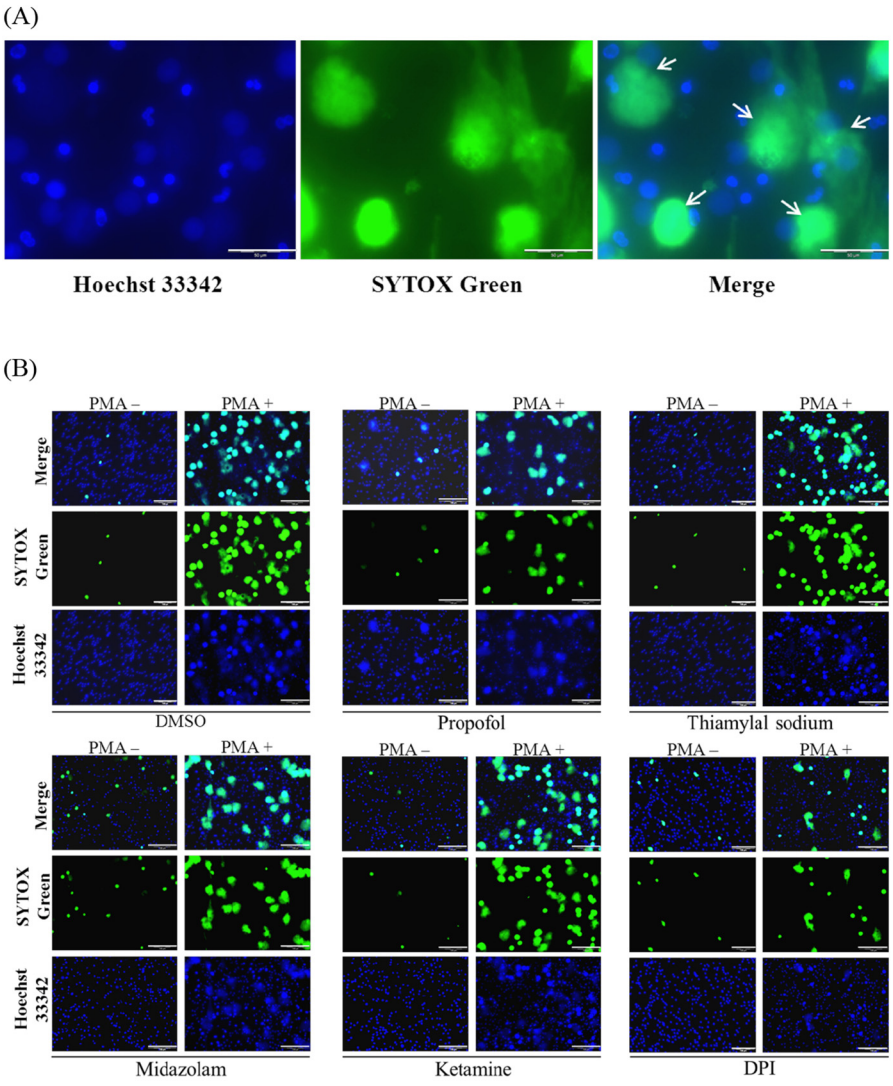


Fig. 2. Detection of NETs induced by PMA combined with or without intravenous anesthetic treatment. (A) Definition of PMA-induced NETs. NETs appear as diffused DNA stained with SYTOX Green and Hoechst 33342 by fluorescence microscopy, as shown by white arrows. The scale bar is 50 μm (B) Fluorescence microscopy images of NETs after treatment with intravenous anesthetics. Solvent (DMSO) or intravenous anesthetics alone were applied as controls. The concentrations of intravenous anesthetics applied were 6 μg/ml propofol, 30 μg/ml thiamylal sodium, 1.5 μg/ml midazolam, and 2 μg/ml ketamine. The concentration of diphenyliodonium (DPI) was 10 μM. Images were captured using a 10× objective; the scale bar is 100 μm. (C) Quantification of NETs. NET DNA fragments were quantified using a microplate reader at 485 nm excitation and 525 nm emission. The net florescence of neutrophils activated with 10 nM PMA subtracted from that of the solvent (DMSO) was used as the value for the positive control. The fluorescence of treated cells was also calculated by subtracting the baseline fluorescence of unstimulated intravenous anesthetic-treated neutrophils from that of stimulated intravenous anesthetic-treated neutrophils, which was divided by the positive control to obtain the fold change. The data are presented as the mean ± SD. * indicates a significant difference compared to the PMA positive control by one-way ANOVA followed by Dunnett's test ($p < 0.05$).

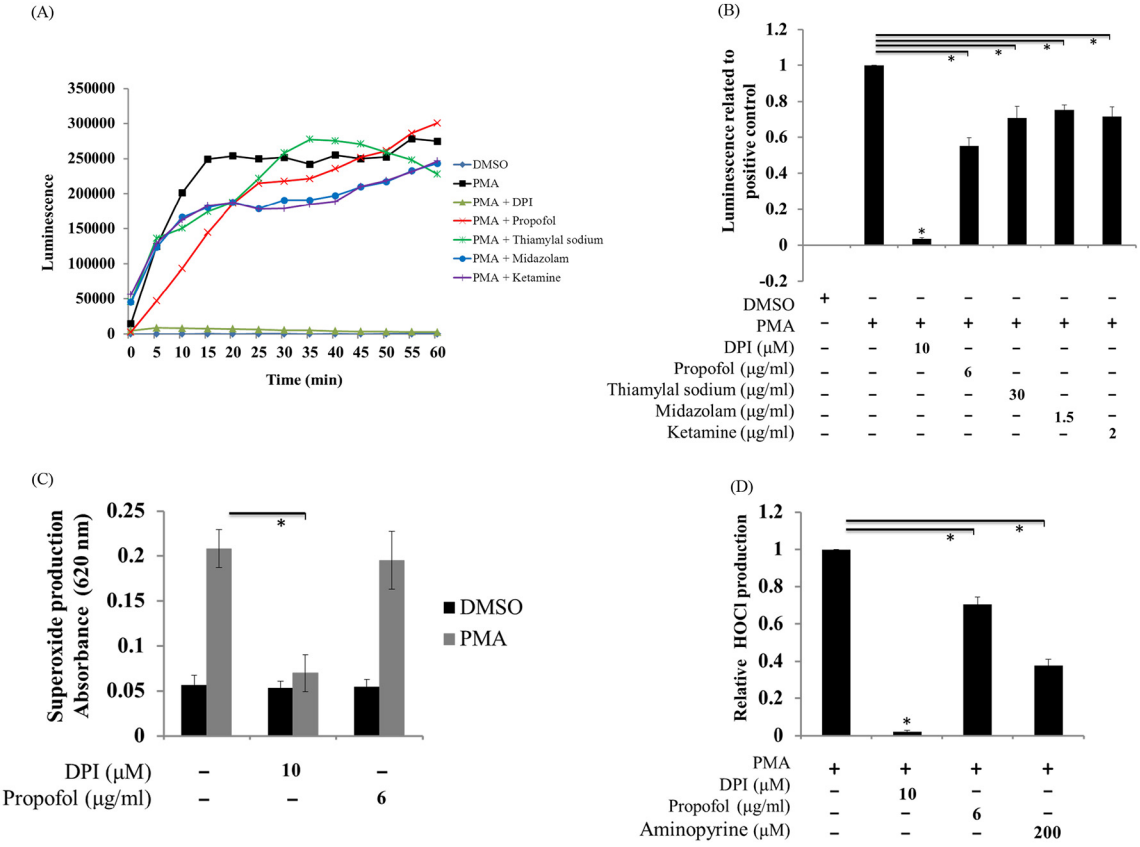


Fig. 3. Influence of intravenous anesthetics on ROS production by neutrophils. Total ROS production induced by PMA coincubated without or with intravenous anesthetics was measured using a luminol chemiluminescence assay. (A) The time kinetics of the total ROS level (chemiluminescence) of one representative result among 7 individual donors. DMSO was used as the solvent control without PMA stimulation; diphenyliodonium (DPI, 10 μM) was used as a negative control to block ROS production. (B) The peak value of total ROS production by neutrophils. The total ROS level induced by PMA at 15 min was used as the reference control. The relative ratio was assessed by subtracting the luminescence value of the anesthetic control from that obtained from coincubation of PMA and the anesthetic and then dividing by the luminescence of PMA alone. The data are presented as the mean ± SD of three independent experiments. (C) Propofol was unable to reduce intracellular superoxide production. The effect of propofol and on PMA-induced superoxide production was detected using an NBT assay. Absorbance at 620 was used to measure superoxide production. The data are expressed as the mean ± SD of four independent experiments. (D) Propofol reduced HOCl generation in stimulated neutrophils. Human neutrophils were either left untreated or treated with PMA in the absence or presence of aminopyrine (200 μM), DPI (10 μM), and propofol (6 μg/ml). The amount of taurine-chloramine produced from the reaction of taurine and HOCl was quantified by TMB. The absorbance (655 nm) of the supernatant from the DMSO control subtracted from that of the PMA-stimulated neutrophils was used as the reference. The relative ratio was calculated by determining the net absorbance of PMA-stimulated neutrophils incubated with the anesthetics and dividing that value by the reference. The data are expressed as the mean ± SD of three experiments. In B to D, * indicates a significant difference compared to the PMA positive control by one-way ANOVA followed by Dunnett's test ($p < 0.05$).

2.10. Statistical analysis

Results are provided as the mean of at least three independent experiments ± SD (standard deviation). The statistical analysis was performed using one-way ANOVA with SPSS (IBM SPSS Statistical Version 21) followed by Dunnett's test. A statistically significant difference was considered at $p < 0.05$.

3. Results

3.1. Inhibitory effect of intravenous anesthetics on NET formation

To avoid cellular cytotoxicity due to intravenous anesthetic treatment, neutrophil viability under clinically relevant concentrations of four intravenous anesthetics was monitored using a CCK-8 assay and found to be almost 100% up to 2 h (Fig. 1). We then applied the same concentrations of intravenous anesthetics to assess the influence of these anesthetics on NET formation. According to fluorescence microscopy results (Fig. 2A), NET generation was clearly induced by treating neutrophils with PMA. In Fig. 2A, NETs are shown as diffused DNA stained with SYTOX Green and Hoechst 33342 that are larger than the cells with a blue-stained nucleus.

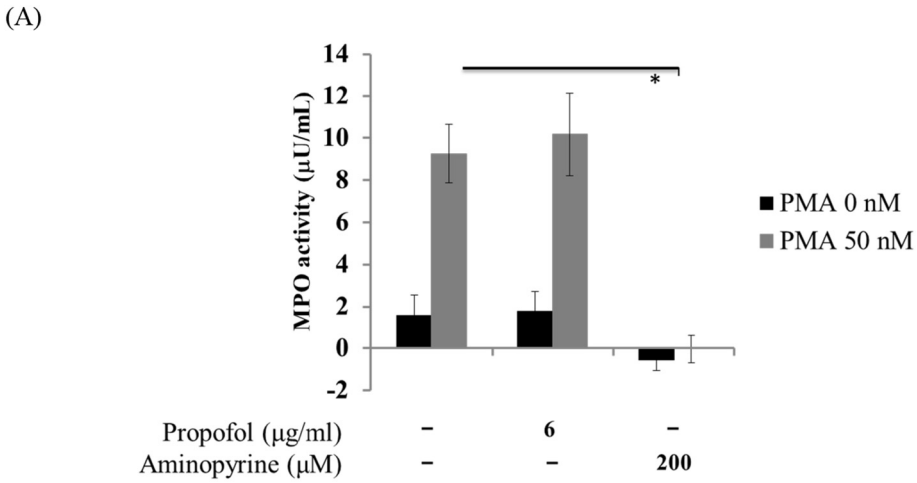
Three intravenous anesthetics (propofol, midazolam and ketamine) could decrease the number of neutrophil-released NETs, with propofol having the greatest effect (Fig. 2B). The results were confirmed by NET quantification (Fig. 2C). Overall, inhibition of NET release by thiamylal sodium was highly variable for neutrophils from different individuals, with a large standard deviation.

3.2. Inhibitory effect of intravenous anesthetics on total ROS production

Among the four intravenous anesthetics tested, propofol was the most effective at inhibiting ROS production within 20 min (Fig. 3A). The maximal level of ROS induced by PMA occurred at 15 min, and at this time point, neutrophils treated with propofol exhibited a 34% reduction in ROS compared with that of PMA-treated control neutrophils (Fig. 3B). When the PMA concentration was as low as 10 nM, the peak in ROS production appeared at 40 min, and a similar inhibition pattern for intravenous anesthetics was found (data not shown).

3.3. Propofol inhibited HOCl-mediated NET formation

Previous studies have revealed that NADPH oxidase and MPO are required for NET release [9,20], and MPO-derived HOCl is among the



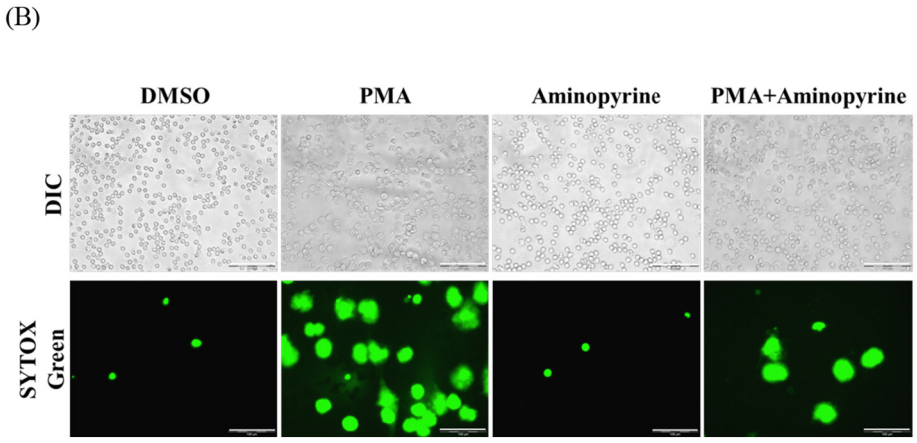


Fig. 4. (A) Propofol was unable to inhibit MPO activity. The supernatants of PMA-stimulated neutrophils treated with propofol or aminopyrine were collected to assess MPO activity. Values are expressed as the mean ± SD of three independent experiments. * indicates a significant difference compared to the PMA positive control by one-way ANOVA followed by Dunnett's test ($p < 0.05$). (B) The inhibitory effect of aminopyrine on PMA-induced NET formation was observed by staining with SYTOX Green. Solvent (DMSO) and aminopyrine (200 μM) alone were used as controls. The images were captured using a 10× objective; the scale bar is 100 μm.

ROS directly mediating NET formation [21]. To specify the individual ROS product inhibited by propofol, superoxide ($O_2 \cdot^-$) was detected using an NBT assay. The generation of superoxide radicals was significantly enhanced in neutrophils incubated with PMA compared with untreated neutrophils, and this enhancement was prevented by culturing the PMA-stimulated neutrophils with an NADPH oxidase inhibitor (DPI). However, propofol was unable to reduce PMA-induced superoxide generation (Fig. 3C), even though it did significantly reduce the level of HOCl induced by PMA (Fig. 3D). Thus, HOCl reduction was not due to propofol-mediated inhibition of MPO activity (Fig. 4A). The MPO inhibitor aminopyrine markedly decreased MPO activity (Fig. 4A), HOCl production and the formation of PMA-induced NETs (Figs. 3D and 4B). These data suggest that propofol may inhibit PMA-induced NETs by decreasing HOCl production.

3.4. Propofol decreased the level of p-ERK involved in NET formation

Previous studies have demonstrated the involvement of p-ERK in PMA-induced NET formation [10,11]. In this study, involvement of p-ERK in NET formation was confirmed by using PD98059 to reduce the level of p-ERK (Fig. 5A and B). The NADPH oxidase inhibitor DPI inhibited expression of p-ERK (Fig. 5B), but PMA-stimulated intracellular superoxide levels were not reduced by PD98059 (absorbance 620 nm: 0.21 ± 0.01), which was close to that of the PMA-stimulated neutrophils shown in Fig. 3C. These data suggest that NADPH oxidase is upstream of p-ERK.

According to western blotting results (Fig. 5B), p-ERK involvement in PMA-induced NET formation was strongly inhibited by propofol. Propofol also inhibited the PMA-induced HOCl production associated with NET formation (Fig. 3D). Thus, we further investigated whether existence of crosstalk between the ERK and HOCl pathways during NET formation. PD98059 did not significantly decrease PMA-induced HOCl generation (almost 6%), and the MPO inhibitor did not decrease ERK phosphorylation (Fig. 5B). These results indicate that propofol inhibited PMA-induced NET generation by decreasing two independent pathways, p-ERK and HOCl.

4. Discussion

NETs are net-like chromatin fibers that have been identified as an alternative mechanism for trapping and killing microorganisms [3,5]. PMA can stimulate NET formation through the ROS-dependent pathway [7,8], and NADPH oxidase activity and MPO-derived ROS are involved in NET release [9]. However, there are inconsistent results regarding the signaling pathway involved in PMA-induced NET formation [10,11], and whether intravenous anesthetics (propofol, thiamylal sodium, midazolam, and ketamine) are able to inhibit NET formation has not yet been investigated. In the present study, we found propofol to be the most effective among four intravenous anesthetics at inhibiting PMA-induced NET formation and total ROS production. The inhibition of NET formation caused by propofol was mediated by two independent mechanisms: decreases in p-ERK and HOCl levels.

Previous studies have demonstrated the anti-inflammatory effects of intravenous anesthetics, including the inhibition of phagocytosis, chemotaxis, cytokine secretion and ROS generation in phagocytes [12–15]. The current results provide evidence that the inhibitory effects of four intravenous anesthetics on PMA-induced NET formation and the decrease in NET release mediated by thiamylal sodium depend on an

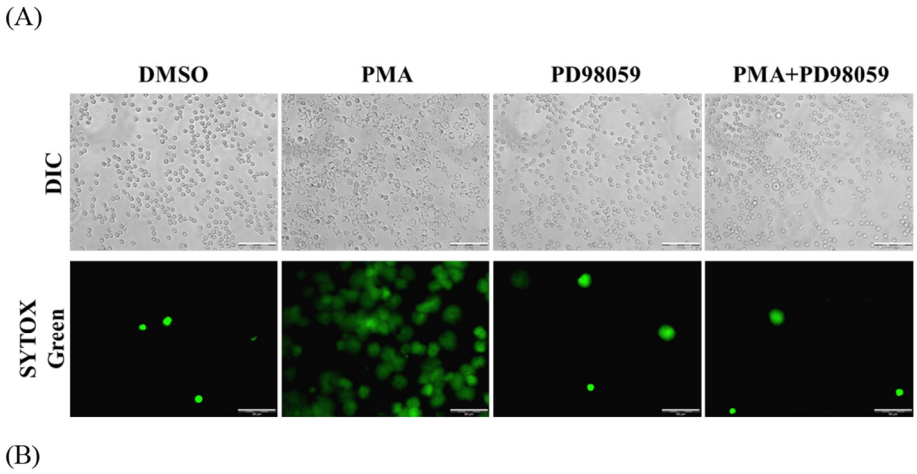


Fig. 5. Involvement of p-ERK in propofol-mediated inhibition of NET formation. (A) An ERK inhibitor (PD98059) reduced NET formation. PMA-stimulated NET formation in the presence of the ERK inhibitor was assessed by staining with SYTOX Green and visualized by fluorescence microscopy using a 10× objective. (B) The level of ERK phosphorylation in PMA-stimulated neutrophils was reduced by propofol treatment. Western blotting was employed to analyze ERK in PMA-treated neutrophils incubated with propofol (6 μg/ml), PD98059 (20 μM), DPI (10 μM) or aminopyrine (200 μM). Numbers on top of the bands indicate the changes in p-ERK levels compared with that of PMA positive control as determined by densitometric scanning of the immunoreactive bands with correction for the actin loading control. Similar results were observed in replicate experiments.

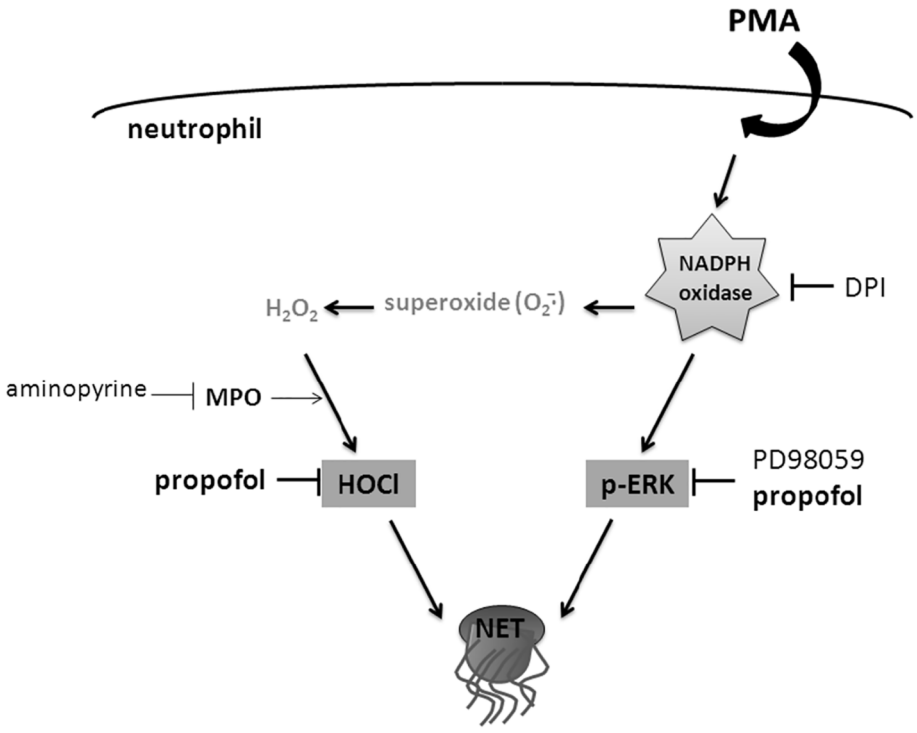


Fig. 6. The main effects of propofol on NET formation. Inhibition of p-ERK and HOCl is responsible for propofol-mediated reductions in PMA-induced NET formation. The inhibitors used in this study are helpful for demonstrating propofol-involved pathways; PD98059, DPI and aminopyrine were used as inhibitors of ERK, NADPH oxidase and MPO, respectively. Myeloperoxidase is abbreviated as MPO.

individual's neutrophil diversity. Moreover, compared with the other anesthetics tested, propofol was best at inhibiting NET formation (Fig. 2B and C). Propofol is widely used in surgery and sedation in ICUs. Visvabharathy et al. reported that even brief exposure to propofol is sufficient to significantly increase the susceptibility of mice to *Listeria monocytogenes* and bloodstream MRSA infections, although ketamine or pentobarbital exposure did not enhance susceptibility to systemic infection by *L. monocytogenes* in mice [22,23]. In addition, propofol exposure increases neutrophilic infiltrates in the kidneys as well as bacterial dissemination throughout kidney tissue [23]. These results indicate that it is worth further investigating in an *in vivo* model whether propofol also interferes with the NET formation related to bacterial killing.

Neutrophils produce high amounts of ROS, which act as antimicrobial agents, signaling molecules or initiators during PMA-induced NET formation. Of the intravenous anesthetics used in this study, propofol was the best at decreasing PMA-induced ROS levels (Fig. 3A and B). Our previous study also demonstrated that propofol is the most effective anesthetic at inhibiting total ROS production in *Staphylococcus aureus*-infected RAW264.7 cells [24]. In addition to propofol, midazolam and ketamine have been demonstrated to suppress ROS production in phagocytes [12,14,25], and Yang et al. [12] reported that propofol significantly reduced superoxide generation in fMLF-activated human neutrophils. Conversely, our results indicated that propofol reduced the total amount of ROS (Fig. 3A and B) but not the superoxide (Fig. 3C) produced in response to PMA. This discrepancy may be due to the different stimulators used because our results are supported by those of Davidson et al. [26]. Inhibitors of NADPH oxidase and MPO reduce PMA-induced NET release, indicating that superoxide and MPO-derived HOCl are involved in NET release [9]. In our study, propofol specifically decreased the level of HOCl (Fig. 3D), which was related to the reduction in total ROS production stimulated by PMA (Fig. 3A and B). This propofol-driven reduction in the HOCl level may further mediate the decrease in NET formation observed with the MPO inhibitor aminopyrine (Fig. 4B). However, the decreased HOCl level induced by propofol treatment was not directly mediated by inhibiting the activity of MPO (Fig. 4A) and may instead be due to the HOCl-scavenging activity of propofol [27].

Two main studies have reported related pathways involved in PMA-induced NET formation [10,11]. Hakkim et al. [10] showed that RAF, MEK and ERK inhibitors can each block ROS production and that phosphorylation of ERK was not prevented by DPI treatment, indicating that the RAF-MEK-ERK pathway is upstream of NADPH oxidase. In contrast, Keshari et al. [11] found that the ERK inhibitor U0126 failed to reduce PMA-induced superoxide generation and DPI that inhibited ERK phosphorylation, suggesting that ERK is downstream of NADPH oxidase. This discrepancy may be because of the neutrophil diversity among individuals or the detection method and concentrations of the different inhibitors used [11]. In addition, Hakkim et al. [10] did not detect superoxide and did not clearly describe the method used to measure total ROS. In the present study, we found that the level of PMA-induced superoxide was not influenced by the ERK inhibitor PD98059 (Fig. 3C), which is consistent with the study by Keshari et al. [11]. Furthermore, we demonstrated that propofol inhibited PMA-induced NET formation by decreasing the level of p-ERK (Figs. 2B and 5B) but not that of superoxide (Fig. 3C). These data indicate that propofol has the potential to inhibit NET formation *in vivo*. Further investigation of NET formation in sepsis patients with propofol sedation would be interesting.

5. Conclusions

By comparing the effects of four intravenous anesthetics, propofol was found to be the most effective at inhibiting PMA-induced NET formation. This inhibition was mediated by two pathways: reduced ERK phosphorylation and HOCl levels (Fig. 6). The two pathways are independent because ERK and MPO inhibitors did not reduce the levels of the product of the other pathway. In the present study, we define a new immunomodulatory function of intravenous anesthetics, particularly propofol, against NET formation.

Acknowledgments

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