Flow cytometry evaluation of the in vitro influence of four i.v.
aeasthetics on respiratory burst of neutrophils

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Summary
Exposure of neutrophils to anaesthetic agents may alter their functional characteristics and in patients undergoing long-term sedation this may be clinically relevant. We have investigated the in vitro influence of propofol, thiopentone, methohexitone and midazolam on phorbol 12-myristate 13-acetate (PMA)-induced respiratory burst of neutrophils by the intracellular oxidative transformation of dihydorhodamine-123 to the fluorescent dye rhodamine-123 via flow cytometry. We tested in vitro concentrations similar to sedating, anaesthetic, 10-fold sedating and 10-fold anaesthetic plasma concentrations. All drugs showed similar inhibition of respiratory burst at sedating concentrations (1–6%). At anaesthetic concentrations, propofol produced significantly higher mean inhibition (7.3%) compared with thiopentone (4.5%) and methohexitone (0.9%). At 10-fold anaesthetic concentrations inhibition of respiratory burst by propofol was almost complete (90.8%) and significantly higher than that by thiopentone (29.2%) and methohexitone (1.8%). Methohexitone and midazolam had only minimal effects at all concentrations. The effect of the solvent of propofol (10% Intralipid) was similar to that of propofol. Thus suppression of respiratory burst of neutrophils by propofol may be caused by this lipid carrier. (Br. J. Anaesth. 1996;77:387–392)

Key words

Blood neutrophils are one of the major cell populations acting against microbial infections. Alteration of neutrophil function by i.v. anaesthetics is currently under intensive laboratory investigation. O'Donnell and colleagues found significant suppression of neutrophil polarization by propofol and thiopentone, which is a change in morphology as a response to a chemotactic stimulus. Another group described a decrease in chemotaxis produced by propofol and its lipid carrier Intralipid after zymosan stimulation.

Another important aspect for bacterial killing is neutrophil respiratory burst. The respiratory burst enzyme in the plasma membrane of neutrophils catalyses the oxidation of NADPH which leads to the production of superoxide anion. This production of oxygen radicals is responsible for killing phagocy-tosed micro-organisms. The importance of respiratory burst for defence of bacterial infections is evident in patients with chronic granulomatous disease, which is a congenital disturbance of respiratory burst enzyme activity. These young patients suffer from recurrent severe bacterial infections because their phagocytes are unable to produce superoxide anion, or at best produce only very low levels. Thus it is possible that impairment of neutrophil respiratory burst by i.v. anaesthetics may be hazardous, especially for intensive care patients at risk.

Rothe, Oser and Valet have shown that oxygen radical production during respiratory burst can be quantified in real-time with multiparameter flow cytometry (MFC) using the dye dihydorhodamine-123 (DHR) which reacts with free radical-derived oxidants to become the brightly green fluorescent rhodamine-123 (rho-123)\(^7\). MFC simultaneously assesses five variables per measured event: relative cell (particle) size, density (granularity) and three fluorescences, representing surface, intracellular or cell cycle characteristics. Compared with chemiluminescence, which estimates only non-specific oxygen radical generation, the methodology of this study has the advantage of assessing specific intracellular oxygen radical production by plasma membrane respiratory burst oxidase of a certain cell population such as neutrophils.

Thus we have evaluated if propofol, thiopentone, methohexitone or midazolam can modulate phorbol 12-myristate 13-acetate (PMA)-induced respiratory burst of human neutrophils. The concentrations of the drugs tested in vitro were adjusted to be similar to plasma concentrations reported for anaesthesia or sedation and also 10-fold higher concentrations. As propofol is dissolved in 10% Intralipid for i.v. use and it has been reported that parenteral lipid emulsions can influence the cellular immune response, we also investigated the effect of 10% Intralipid on respiratory burst.

\(^{10–13}\)
Subjects and methods

The study was approved by the Institution Ethics Committee. Informed consent was obtained from the blood donors.

ANAESTHETIC AGENTS

Different concentrations of propofol (Disoprivan, ICJ Pharma, Plankstadt, Germany), its lipid carrier, 10% Intralipid (Pharmacia, Erlangen, Germany), thiopentone (Trapanal, Byk Gulden, Konstanz, Germany), methohexitone (Brevimytal Natrium, Eli Lilly, Bad Homburg, Germany) and midazolam (Dormicum, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) on neutrophil respiratory burst were studied. As midazolam is suitable for anaesthesia only in combination with other agents, there are no anaesthetic plasma concentrations defined. Thus midazolam was analysed only at reported sedating doses. Thiopentone and methohexitone were purchased as dry material and dissolved in isotonic saline solution (0.9% NaCl). The desired concentrations of all tested drugs were standardized with phosphate-buffered saline (PBS, Dulbeco’s without Ca\(^{2+}\) and MgCl\(_2\), Gibco BRL, Eggenstein, Germany).

SAMPLE PREPARATION

Heparinized (10 u. ml\(^{-1}\), Liqueemin N, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) venous blood samples (3 ml) were obtained from healthy blood donors (\(n = 20\)). Blood was layered on equal Ficoll quantity (Ficoll-Paque: density 1.077 g dl\(^{-1}\), Biocrom, Berlin, Germany). PBS was portioned (1 ml) and warmed to 37 °C. The nucleated blood cells of the supernatant were harvested after 1 g sedimentation at 22 °C for 45 min and resuspended in PBS. Then 30 μl of the leucocyte sample (containing 5 × 10\(^6\) cells ml\(^{-1}\)) and DHR 15 μl (1 × 10\(^4\) mol, MoBiTec, Goettingen, Germany) were added to the PBS and incubated at 37 °C for 5 min. Subsequently, the cell suspensions were incubated with the drug and concentrations tested at 37 °C for 10 min. Neutrophils from the samples were stimulated with PMA 10 μl (1 × 10\(^{-4}\) mol, Sigma, Deisenhofen, Germany) at 37 °C for 20 min. Stimulation was then terminated by transferring the samples onto ice. Viability discrimination was performed by addition of propidium iodide (PI) 10 μl (3 × 10\(^{-3}\) mol, Serva, Heidelberg, Germany) just before measurement. Internal positive (full PMA stimulation) and negative (no PMA) controls for each sample were carried out without addition of the tested substances. Full stimulation of neutrophil respiratory burst for the positive controls was titrated and considered 1 × 10\(^{-6}\) mol PMA (data not shown).

MULTIPARAMETER FLOW CYTOMETRY

Samples were analysed on a fluorescence-activated cell scanner (FACScan, Becton Dickinson Immunocytometry Systems- BDIS, Heidelberg, Germany). A minimum of 15,000 events per sample were acquired. The flow cytometer was equipped with an argon ion laser, adjusted to an excitation wavelength of 488 nm and 0.5 W. Rho-123 fluorescence emission was filtered and acquired from the corresponding photomultiplier (FL1) in the band spectrum 515–545 nm. The photomultiplier for FL2 and FL3 was used for acquisition of PI emission over the range 563–607 nm. Forward light scatter (FSC), sideward light scatter (SSC) and fluorescence signals were adjusted with the negative and positive control samples. SSC data were collected in linear mode whereas FSC and the fluorescences were collected in logarithmic amplification mode without compensation. A relatively high threshold level of FSC (channels > 10\(^3\)) was set to exclude erythrocytes and cell debris. Data files were stored in list mode using FACSscan software and analysed with the PC-Lysis software (BDIS). Two analytical gates were used for precise discrimination of the desired neutrophils (fig. 1A, C and D). Briefly, neutrophils were included by setting an arbitrary leucocyte right-angled gate in FSC vs SSC (fig. 1A). Further restriction of neutrophils was performed after zooming leucocytes (R1) in the same dot plot (fig. 1B), as shown elsewhere\(^{14}\). Additionally, dead cells were gated and excluded (R2) because of the high fluorescence in FL3 as a result of intracellular PI content (fig. 1C). Thus inhibition of neutrophil respiratory burst was estimated on approximately 5000 remaining events using the negative and positive controls, and an appropriate threshold in SSC vs FL1 (fig. 1D).

CALCULATION AND STATISTICS

The percentage of respiratory burst inhibition by the drugs was calculated using the formula:

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\text{% inhibition} = \left( \frac{\text{positive control} - \text{tested drug}}{\text{positive control}} \right) \times 100
\]

where positive control = number of rho-123 positive events by full stimulation with PMA; negative control = number of rho-123 positive events without stimulation with PMA and without drug; tested drug = number of rho-123 positive events by stimulation with PMA in the actual drug incubated sample; viable neutrophils = PI negative neutrophils.

As numeric data showed a Gaussian distribution, descriptive statistics, mean (SD), paired two-tailed Student’s \(t\) test for inter-group significance (\(P<0.05\), 95% confidence intervals) and Forman’s regression were used for statistical analysis of the flow cytometry data (\(n = 20\) for each drug and concentration). Otherwise, the non-parametric Mann-Whitney \(U\) test was used (SPSS/PC V6.01 software package, SPSS, Munich, Germany).

Results

Overall inhibition of neutrophil respiratory burst by propofol, thiopentone, methohexitone and midazolam is illustrated in figures 2 and 3. The percentages of PI positive neutrophils were low for all drugs and concentrations tested (<5%).

SEDATING AND 10-FOLD SEDATING DOSES (FIG. 2)

All drugs showed identical low mean inhibition of respiratory burst at the corresponding sedating concentrations (1% and 6%). At 10-fold sedating doses, propofol 30 μg ml\(^{-1}\) produced significantly (\(P<0.001\)) more inhibition (68.6 (11.2)%)) compared with the three other drugs. Thiopentone 200 μg ml\(^{-1}\) inhibited
Influence of i.v. anaesthetics on respiratory burst

respiratory burst more (14.6 (10.7)%) than methohexitone 50 μg ml\(^{-1}\) (1.3 (1.4)%) and midazolam 6 μg ml\(^{-1}\) (3.4 (3.9)%) at 10-fold sedating concentrations \((P<0.001)\). The difference between methohexitone and midazolam was also significant \((P<0.05)\).

ANAESTHETIC AND 10-FOLD ANAESTHETIC DOES (FIG. 3)

At concentrations normally required for anaesthesia, the following significant differences were observed: propofol 6 μg ml\(^{-1}\) and thiopentone 40 μg ml\(^{-1}\) produced greater suppression (7.3 (6.0)%) and 4.5 (5.3)%, respectively \((P<0.001)\) than methohexitone 10 μg ml\(^{-1}\) (0.9 (1.6)%). At the highest (10-fold anaesthetic) concentrations, inhibition by propofol 60 μg ml\(^{-1}\) was almost complete (90.8 (3.9)%) and greater \((P<0.001)\) than that by thiopentone 400 μg ml\(^{-1}\) (29.2 (15.1)%) and methohexitone 100 μg ml\(^{-1}\) (1.8 (1.7)%), which had only minimal effects.

Significant correlations \((P<0.001)\) for the four concentrations with inhibition of respiratory burst were observed with propofol \((r=0.93)\) and thiopentone \((r=0.72)\). Methohexitone did not inhibit respi-
In this study, five variables of 15,000 cells for each selected cell population is investigated and several variables of each cell are evaluated at the same time. Only the intracellular oxygen radical content of one molecule is measured. Moreover, with low coefficients of variation. The objectivity of the procedure was maintained by repeated device adjustment and the use of external and internal positive and negative controls. Cell toxicity as a possible explanation for the effects of the drugs studied was excluded by staining with fluorochrome propidium iodide (PI). PI can only attach to cellular DNA after destruction or disintegration of cell membranes, usually subsequent to cell death. Only PI-negative cells (i.e. viable cells) were included in the estimation.

THIOPENTONE

The thiobarbiturate thiopentone produced dose-related suppression of respiratory burst. The highest mean suppression of up to 30% was reached with 10-fold the concentrations found in plasma during anaesthesia. The oxybarbiturate methohexitone had only minimal effects at all concentrations studied, without any dose–effect correlation. A former study showed that thiopentone suppressed N-formylmethionyl-leucyl-phenylalanine (FMLP)-induced neutrophil chemiluminescence and chemotaxis, even at plasma concentrations in the therapeutic range. Suppression of neutrophil migration and chemiluminescence by thiobarbiturates was 10–100 stronger than that by oxybarbiturates and was attributed to the sulphur atom in the thiobarbiturate molecule. Additionally, lymphocyte functions such as mitogen-induced blast transformation and cell proliferation appeared to be markedly more impaired in the presence of thiopentone than other barbiturates. Eberhardt and colleagues reported an increase in pulmonary infections of up to 40% in patients with brain oedema undergoing mechanical ventilation and receiving thiopentone. Higher doses of thiopentone were associated with development of infections. The reported observations and our own observations of increased intracellular oxygen radical production by thiopentone may favour the use of oxybarbiturates in

**Discussion**

**METHODOLOGY**

We have studied the in vitro effects of thiopentone, methohexitone, midazolam and propofol on respiratory burst of neutrophils from healthy blood donors after PMA stimulation via multiparameter flow cytometry (MFC). The discrepancy between the published data on the influence of different drugs on in vitro neutrophil function is probably because of the variety of techniques used and the sensitivity and fragility of neutrophils. The applied MFC technique has different advantages compared with other methodologies measuring non-specific oxygen radical production, such as chemiluminescence. With the present MFC technique it is possible to measure the direct effects on the responsible enzyme complex, the intracellular respiratory burst oxidase. Moreover, only the intracellular oxygen radical content of one selected cell population is investigated and several variables of each cell are evaluated at the same time. In this study, five variables of 15,000 cells for each sample were assessed and four variables of approximately 5000 viable neutrophils were analysed. Thus the high number of analysed events resulted in data

![Figure 3](image3.png) **Figure 3** Percentage inhibition of neutrophil respiratory burst produced by propofol (open bars) 6 and 60 µg ml⁻¹, thiopentone (shaded bars) 40 and 400 µg ml⁻¹ and methohexitone (solid bars) 10 and 100 µg ml⁻¹ at concentrations likely to be required for anaesthesia and 10-times these concentrations.

![Figure 4](image4.png) **Figure 4** Regression analysis of dose–response relationship of respiratory burst to the same extent and with significant correlation between the dose inhibition relations of propofol (r=0.93) and 10% Intralipid (r=0.90). There were no significant differences between the effects of both drugs (fig. 4).

Only in samples incubated with the highest concentration (10-fold anaesthetic) of propofol 3, 6, 30 and 60 µg ml⁻¹, inhibited respiratory burst to the same extent and with significant correlation between the dose inhibition relations of propofol (r=0.93) and 10% Intralipid (r=0.90). There were no significant differences between the effects of both drugs (fig. 4).

In vitro concentrations of propofol (solid line) likely to be required in plasma during sedation, anaesthesia and 10 times these concentrations (3, 6, 30 and 60 µg ml⁻¹) and the respective volumes of 10% Intralipid (broken line) (0.3, 0.6, 3 and 6 µl ml⁻¹)
Influence of i.v. anaesthetics on respiratory burst induced barbiturate coma during brain oedema than thiobarbiturates.

**MIDAZOLAM**
The influence of midazolam on respiratory burst after PMA stimulation was negligibly low. Finnerty and colleagues described stimulus-dependent inhibition of superoxide anion production by benzodiazepines measured by chemiluminescence. Benzodiazepines specifically repressed FMLP- but not PMA-stimulated neutrophil superoxide anion production\(^1\). Stimulation by FMLP is receptor-mediated by a formyl peptide receptor on the surface of the neutrophil granulocytes, whereas PMA activates respiratory burst oxidase through its ability to stimulate protein kinase C\(^6\). Suppression of neutrophil respiratory burst oxidase by benzodiazepines was found to be mediated by a specific peripheral receptor\(^8\). Thus stimulation with FMLP may not correlate with PMA stimulation. However, the clinical relevance of this phenomenon remains unclear.

**PROPOFOL**
Propofol produced the greatest inhibition of respiratory burst with tested concentrations. At 10-fold anaesthetic concentrations, suppression was almost complete (90.8%). It is not clear if the effects of propofol on leucocyte function are induced by its lipid carrier, 10% Intralipid. In a previous study, propofol was found to suppress whereas 10% Intralipid augmented neutrophil polarization\(^2\), which is in contrast with another study, where propofol and 10% Intralipid suppressed neutrophil chemotaxis\(^3\).

**INTRALIPID**
We found that propofol and equal volumes of 10% Intralipid inhibited respiratory burst to the same extent and with a significant dose–response correlation. Thus the effects of propofol on respiratory burst may be attributed to its lipid carrier. Moreover, it is well known that parenteral lipid emulsions suppress some aspects of leucocyte function. Numerous authors have described impairment of antimicrobial defence\(^6\ 10\ 21\), chemotaxis\(^22\ 22\) and T cell-mediated immunity\(^11\ 12\ 23\). Additionally, accumulation of parti-

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*Figure 5* Decrease in SSC signals of neutrophils incubated with 10-fold anaesthetic doses of propofol (60 µg ml\(^{-1}\)) compared with the negative control. A (negative control) and C (propofol 60 µg ml\(^{-1}\)) are depicted as acquired on the flow cytometer. Changing FSC from logarithmic to linear scale and gating the neutrophils (R1) leads to figures B (negative control) and D (propofol 60 µg ml\(^{-1}\)). The SSC signals were decreased from B to C. As estimated in a SSC histogram over the gated neutrophils (R1), peak, mean (SD) and median channel numbers were as follows: 456, 452 (75) and 450 for propofol, and 553, 550 (143) and 550 for the negative control, respectively.
icles was described in the cytoplasm of neutrophils, which correlated with reduced cell function after parenteral i.v. lipid infusions. This observation correlates with the fact that polyunsaturated fatty acids can be incorporated rapidly into cell membranes and increase membrane rigidity.

Intralipid 10% is composed of high concentrations of long chain triglycerides (LCT) containing unsaturated fatty acids. The increase in membrane rigidity in the presence of LCT may result in the observed reduction in chemotactic properties. These changes in membrane structure may also provide an explanation for the decrease in SSC signals of neutrophils found in this study, because SSC characterizes cell granularity, density or membrane folding. This decline was observed only with the highest concentrations of propofol (10-fold anesthetic dose, 60 μg/ml) and the corresponding volume of 10% Intralipid (6 μl/ml) alone. Cell death and a coating phenomenon were excluded, because both fluorescence (FL 1 and 3) were not influenced. Many studies and the results of our study suggest that parenteral lipid emulsions can inhibit some leucocyte functions.

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References