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Propofol and Intralipid interact with reactive oxygen species: a chemiluminescence study

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Summary

We have studied the ability of propofol and Intralipid to inhibit reactive oxygen species generated either by stimulated human leucocytes or cell-free systems using luminol chemiluminescence. Human leucocytes were stimulated by a chemotactic peptide, FMLP 1 μmol $litre^{-1}$, or by a phorbol ester, PMA (protein kinase C activator) 0.1 μmol $litre^{-1}$. In cell-free experiments, superoxide–hydrogen peroxide, hypochlorous acid or hydroxyl radical-induced chemiluminescence responses were initiated by xanthine 0.1 mmol $litre^{-1}$ with xanthine oxidase 10 mu. ml^{-1}, NaOCl 70 μmol $litre^{-1}$ and $FeSO_4$ 3 μmol $litre^{-1}$, respectively. Propofol with Intralipid, and to a lesser degree Intralipid alone, produced a concentration-dependent reduction in chemiluminescence from stimulated leucocytes. Similar attenuations were also observed using propofol with Intralipid on xanthine with xanthine oxidase-, HOCl- and ferrous iron-induced chemiluminescence. However, Intralipid produced a reduction only at high concentrations. Intralipid produced marked decreases in ferrous iron-induced chemiluminescence. This study suggests that propofol had a direct scavenging activity against HOCl, superoxide–hydrogen peroxide and hydroxyl radical in the concentrations used. These direct scavenging effects may contribute to the effect of propofol on human leucocyte chemiluminescence. (*Br. J. Anaesth.* 1998; **80**: 649–654)

Keywords: measurement techniques chemiluminescence; anaesthetics i.v. propofol; oxygen free radicals; blood leucocytes

Free radicals are believed to contribute to tissue injury associated with a wide range of chronic and acute diseases, such as acute lung injury, severe sepsis and multi-system organ failure. There is strong evidence that superoxide and hydrogen peroxide are intimately involved in initiating free radical damage and these oxidants lead to loss of cell integrity, tissue necrosis and organ failure.[1] Free radical scavengers, or antioxidants, represent an important component of the body's defences against such free radical mediated injury. Anaesthetic agents with antioxidant or free radical scavenging activity might be beneficial in some diseases where free radicals are believed to be involved. Moreover, most anaesthetic agents inhibit some aspects of immune function and this may be clinically important when prolonged infusions are used in the intensive care unit.[2]

Propofol (2, 6-diisopropylphenol) is a widely used i.v. anaesthetic agent with a rapid onset, short duration of action and rapid elimination.[3] It is related structurally to the commonly used phenolic antioxidants, namely butylated hydroxytoluene and α-tocopherol (vitamin E).[4 5] Propofol has demonstrated antioxidant activity against lipid peroxidation in human plasma,[4] rat liver mitochondria,[6 7] microsomes[6 8] and brain synaptosomes.[6] Propofol also attenuates ischaemia–reperfusion-induced lipid peroxidation in humans.[9] However, there are conflicting reports on the effects of propofol on leucocyte function. Although propofol has been shown to inhibit phagocytosis in one study,[10] it failed to produce significant effects on phagocytosis and respiratory burst activities at similar concentrations in another study.[11]

Chemiluminescence is used widely as a sensitive assay for monitoring free radicals and reactive oxygen metabolites from enzyme, cell or organ systems.[12–14] It is well established that polymorphonuclear (PMN) leucocytes, which are stimulated by a variety of both soluble and particulate stimuli, generate a series of oxygen-derived species. The initial product of oxygen reduction is superoxide ($^{\cdot}O_2^-$), generated during a respiratory burst. After $^{\cdot}O_2^-$ generation, other oxygen metabolities may be formed, including H_2O_2, $^1O_2$, $^{\cdot}OH$ and HOCl, the latter being formed in a reaction catalysed by myeloperoxidase.[1] Generation of reactive oxygen species and metabolites emits light which can be monitored by a variety of luminometers.[15] Light emission can be amplified markedly by luminol which measures a mixture of oxygen-derived species.[13 15]

The direct effects of propofol on oxygen-derived free radicals are unknown. As propofol is dissolved in 10% Intralipid for i.v. use and parenteral lipid emulsions have been reported to influence the cellular immune response,[16] the effects of Intralipid also need to be investigated. Therefore, in this study we investigated the effects of propofol and Intralipid on

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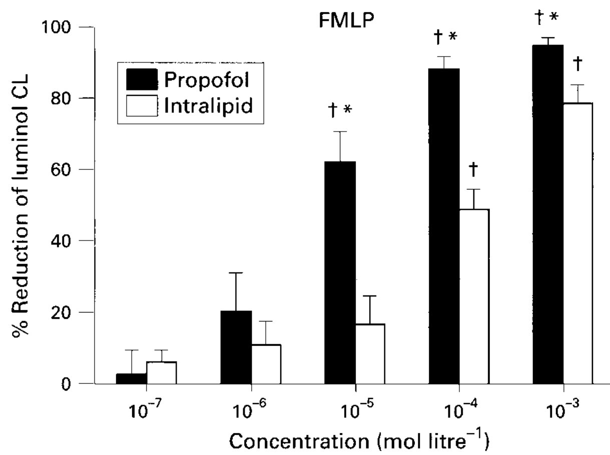

Figure 1 Concentration-dependent effects of propofol ($n=4$–7) and Intralipid ($n=5$–7) on FMLP 1 μmol litre^{-1}-induced luminol chemiluminescence (CL). Data are mean (SEM). *$P<0.05$ compared with corresponding Intralipid response; †$P<0.05$, significantly less than its control value.

chemiluminescence from stimulated leucocytes and examined direct scavenging activity in cell-free systems.

Materials and methods

ISOLATION AND SEPARATION OF LEUCOCYTES

Human venous blood was obtained from healthy volunteers and leucocytes were isolated according to methods described previously with slight modification.[14] Blood (9 ml) was obtained into tubes containing 3.8% sodium citrate 1 ml. Dextran was added and allowed to sediment at room temperature for 60 min. The leucocyte-rich supernatant was removed and centrifuged at 900 rpm for 20 min. Erythrocyte lysis was performed by washing cells with 0.2% NaCl for 30 s and mixing immediately with a double volume of 1.6% NaCl. Leucocytes were then centrifuged at 900 rpm for 15 min and the pellet resuspended in Hanks' buffered salt solution (HBSS) containing Ca^{2+} 1 mmol litre^{-1} (pH 7.4). Leucocytes were washed with HBSS three times. After leucocyte counting using a light microscope, cell yield was adjusted to 10^7 cells ml^{-1} (stock cell suspension) by adding HBSS. Cell viability was assessed by a Trypan blue exclusion test and more than 98% of the cells were found to be viable leucocytes ($n=6$). The stock cell suspension was stored at room temperature until use.

PMA- AND FMLP-INDUCED CHEMILUMINESCENCE

Stock leucocyte cell suspension (0.1 ml) was diluted with HBSS in a cuvette (total volume 1 ml) and luminol 20 μl (50 μmol litre^{-1}; final cuvette concentration) was added, producing a final cell yield of 10^6 cells ml^{-1}. Stimulant (phorbol 12-myristate 13-acetate (PMA) or N-formyl-methionyl-leucyl-phenylalanine (FMLP)) was then added to yield final cuvette concentrations of 10^{-7} mol litre^{-1} and 10^{-6} mol litre^{-1}, respectively. Luminol chemiluminescence was measured at 37°C using a chemiluminometer (Bio-Orbit 1250 Luminometer, Turku, Finland). The chemiluminescence produced was measured continuously and recorded on a computer using the Luminometer 1250 program (version 1.12, Bio-Orbit).

XANTHINE–XANTHINE OXIDASE-INDUCED CHEMILUMINESCENCE

Superoxide and hydrogen peroxide were generated enzymatically by the xanthine–xanthine oxidase reaction system.[13,14] In order to characterize xanthine–xanthine oxidase-induced chemiluminescence, 0.9 ml of phosphate-buffered saline (PBS: KH_2PO_4 10 mmol litre^{-1} and NaCl 150 mmol litre^{-1}, pH 7.4) were mixed with luminol 0.1 ml (250 μmol litre^{-1}; final cuvette concentration) in a cuvette. After further addition of 10 μl of xanthine 0.1 mmol litre^{-1}, 25 μl of xanthine oxidase 10 mu. ml^{-1} was injected into the cuvette, and the chemiluminescence produced was measured continuously for 5 min.

HOCL-INDUCED CHEMILUMINESCENCE

HOCl was prepared as described previously by Vissers and colleagues.[17] NaOCl was diluted with PBS and the pH of the solution readjusted to 7.4 immediately before addition to the chemiluminescence cuvette. At this pH, the solution contains approximately 1 : 1 HOCl and OCl^{-1} and is subsequently referred to as HOCl. HOCl 70 μmol litre^{-1} was injected into the HBSS and luminol 250 μmol litre^{-1} mixture to induce chemiluminescence.

FERROUS IRON-INDUCED CHEMILUMINESCENCE

Hydroxyl radicals were generated by addition of ferrous iron to the buffer solution, as described previously by Green, Bennett and Nelson.[5] Ferrous iron reduces molecular oxygen to superoxide radical, which in turn dismutates to hydrogen peroxide. Further reduction of H_2O_2 by Fe^{2+} produces the highly reactive hydroxyl radical. Freshly prepared $FeSO_4$ 3 μmol litre^{-1} was injected into the PBS and luminol 250 μmol litre^{-1} mixture, and chemiluminescence was recorded continuously for 5 min.

EXPERIMENTAL PROCEDURE

The effects of various concentrations of propofol, Intralipid or enzyme inhibitors were examined by addition before the stimulant. In PMA-stimulated leucocytes, the effects of propofol and Intralipid were also studied when the chemiluminescence signal reached a peak. Duplicate assays were performed in all experiments. Results were calculated as peak chemiluminescence or as a percentage of peak chemiluminescence, and expressed as mean (SEM). n refers to the number of individual volunteers (for leucocyte experiments) and number of experiments (for cell-free assays). Comparisons of the differences between mean values were performed using an unpaired Student's t test. Analysis of variance (ANOVA) was used to assess the observed differences in chemiluminescence between concentrations. If significant differences were detected by ANOVA, individual means were compared with control using a Dunnett test. Differences were considered to be statistically significant when $P<0.05$.

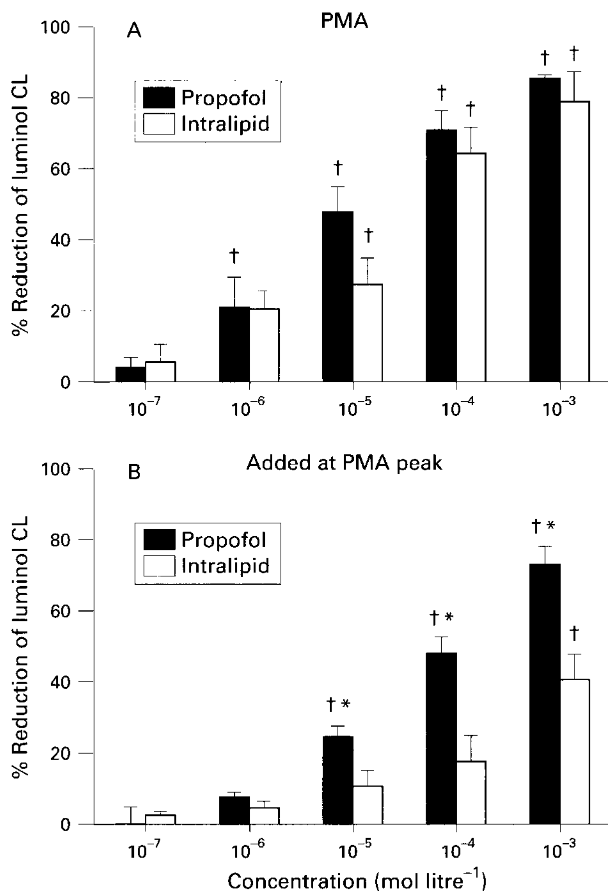

Figure 2 Concentration-dependent effects of propofol ($n=6$–7) and Intralipid ($n=7$) on PMA 0.1 μmol litre^{-1}-induced luminol-enhanced chemiluminescence (CL) when added before PMA (A) or at the peak of the PMA response ($n=4$–6 for propofol and $n=5$–6 for Intralipid) (B). Data are mean (SEM). *$P<0.05$ compared with corresponding Intralipid values; †$P<0.05$, significantly less than its control value.

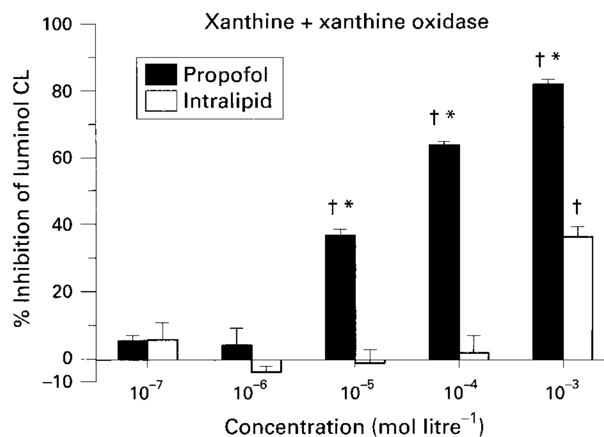

Figure 3 Concentration-dependent effects of propofol ($n=6$–9) and Intralipid ($n=5$–10) on xanthine 0.1 mmol litre^{-1} with xanthine oxidase 10 mu. ml^{-1}-induced luminol chemiluminescence (CL). Data are mean (SEM). *$P<0.05$ compared with corresponding Intralipid response; †$P<0.05$, significantly less than its control value.

MATERIALS

Commercially available preparations of propofol (Diprivan, Zeneca Ltd, Macclesfield, Cheshire, UK) and Intralipid (Lipovenös 10%, Fresenius AG, Germany) were used and diluted with saline. Intralipid 10% is the vehicle for commercially available propofol, and contains 10% soybean oil, 2.5% glycerol and 1.2% purified egg phosphatide. PMA and FMLP were dissolved in ethyl alcohol and fresh dilutions were made by PBS before use. Luminol was prepared daily in NaOH 2 mol litre^{-1} (2.5%) and diluted with PBS. Xanthine (sodium salt) and xanthine oxidase (grade I, from buttermilk) were dissolved in distilled water. $FeSO_4$ was prepared in distilled water before use. Superoxide dismutase (from bovine erythrocytes), catalase (from bovine liver), D-mannitol and NaOCl were dissolved in PBS before use. All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Results

Concentrations of 10 mu. ml^{-1}, 70 μmol litre^{-1} and 3 μmol litre^{-1} were selected for xanthine oxidase, HOCl and $FeSO_4$, respectively, and generated chemiluminescence peaks (1898 (117) mV ($n=11$) for xanthine–xanthine oxidase, 1855 (45) mV ($n=22$) for HOCl and 2064 (75) mV ($n=34$) for $FeSO_4$) were found to be comparable with that produced by PMA 0.1 μmol litre^{-1} in leucocytes (2053 (208) mV ($n=15$)). Both receptor-mediated (FMLP) and non-receptor-mediated (PMA, a protein kinase C activator) stimulants were used for activation of leucocytes.

EFFECTS OF PROPOFOL WITH INTRALIPID AND INTRALIPID ALONE ON LUMINOL CHEMILUMINESCENCE IN LEUCOCYTES

In luminol chemiluminescence, FMLP 1 μmol litre^{-1} induced response (1620 (162) mV, $n=6$) was depressed by either propofol with Intralipid or an equivalent volume of Intralipid in a concentration-dependent manner. The Intralipid concentrations used in this study were 1.78×10^{-4} to 17.8 μl ml^{-1}, which were carrying corresponding concentrations of propofol of 0.01 μmol litre^{-1} to 1 mmol litre^{-1}, respectively. There was a marked reduction in FMLP-induced luminol chemiluminescence by the combination of propofol and Intralipid, with an IC_{50} value of 4.6 μmol litre^{-1} (fig. 1). To exclude the possibility that the inhibitory effect of propofol and Intralipid on luminol chemiluminescence was specific to FMLP, the effects of these drugs were also tested on PMA-activated leucocytes. Propofol with Intralipid produced concentration-dependent depression of luminol chemiluminescence in PMA-activated leucocytes when added before PMA, with an IC_{50} value of 7 μmol litre^{-1} (fig. 2). Propofol with Intralipid started to decrease PMA-induced luminol chemiluminescence at 1 μmol litre^{-1} (21 (8)%, $n=6$). At the concentrations tested, maximum reduction with the propofol and Intralipid combination was observed at 1 mmol litre^{-1} (86 (1)%, $n=7$). Intralipid caused significant attenuation similar to that produced by propofol with Intralipid (79 (9)%, $n=7$ at 17.8 μl ml^{-1}) (fig. 2A). Addition of propofol with Intralipid and Intralipid alone at the peak of chemiluminescence also produced concentration-dependent reduction in PMA-activated leucocytes. This inhibition was similar to that seen by addition of propofol with Intralipid before the stimulant (73 (5)%, $n=4$, at 1 mmol litre^{-1}). In contrast, Intralipid produced only a 40 (8)% ($n=6$) decrease at 17.8 μl ml^{-1} on PMA-induced luminol chemiluminescence (fig. 2B).

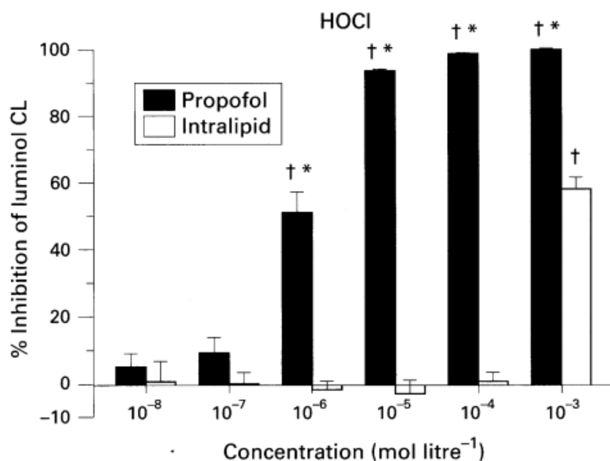

Figure 4 Concentration-dependent effects of propofol ($n=6$–9) and Intralipid ($n=5$–9) on HOCl 70 μmol litre^{-1}-induced luminol-enhanced chemiluminescence (CL). Data are mean (SEM). *$P<0.05$ compared with corresponding Intralipid values; †$P<0.05$, significantly less than its control value.

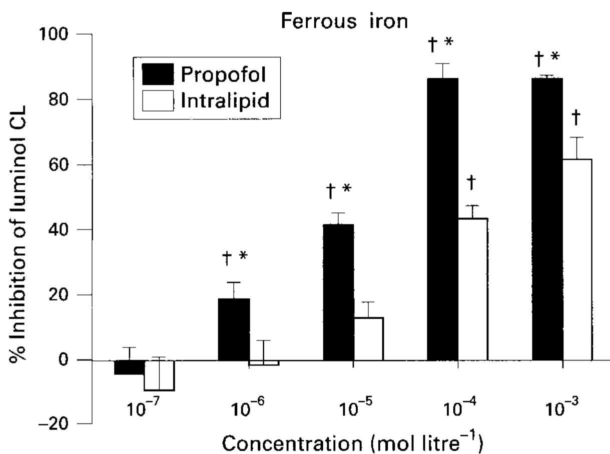

Figure 5 Concentration-dependent effects of propofol ($n=6$–14) and Intralipid ($n=6$–8) on $FeSO_4$ 3 μmol litre^{-1}-induced luminol chemiluminescence (CL). Data are mean (SEM). *$P<0.05$ compared with corresponding Intralipid values; †$P<0.05$, significantly less than its control value.

EFFECTS OF PROPOFOL WITH INTRALIPID AND INTRALIPID ALONE ON LUMINOL CHEMILUMINESCENCE IN THE CELL-FREE SYSTEMS

In luminol chemiluminescence, propofol with Intralipid inhibited xanthine–xanthine oxidase-induced responses in a concentration-dependent manner, with an IC_{50} value of 18.5 μmol litre^{-1} (fig. 3). At the concentrations tested, maximum inhibition was recorded at 1 mmol litre^{-1} (82 (2)%, $n=9$). However, Intralipid was only effective at the highest concentration (17.8 μl ml^{-1}) and produced 36 (3)% ($n=8$) inhibition (fig. 3).

HOCl 70 μmol litre^{-1}-induced luminol chemiluminescence was also inhibited by the combination of propofol and Intralipid in a concentration-dependent manner. Inhibition was observed at 1 μmol litre^{-1} (51 (6)%, $n=7$) and there was almost complete inhibition at propofol 0.1 mmol litre^{-1} (99 (0.1)%, $n=9$) with an IC_{50} value of 1 μmol litre^{-1}. While Intralipid had no effect at 1.78 μl ml^{-1} (1 (3)%, $n=9$), it produced 58 (4)% ($n=8$) inhibition at 17.8 μl ml^{-1} (fig. 4).

Propofol with Intralipid and Intralipid alone produced concentration-dependent inhibition of $FeSO_4$ 3 μmol litre^{-1}-induced chemiluminescence response. There was 86 (0.8)% ($n=14$) and 61 (7)% ($n=7$) suppression by propofol 1 mmol litre^{-1} and Intralipid 17.8 μl ml^{-1}, respectively. Inhibition with propofol–Intralipid ($IC_{50}=8.2$ μmol litre^{-1}) was more marked compared with Intralipid alone (fig. 5). In order to determine the reactive oxygen species responsible for ferrous iron-induced chemiluminescence, superoxide dismutase 50 iu ml^{-1}, catalase 500 iu ml^{-1} and the hydroxyl radical scavenger, mannitol 100 mmol litre^{-1}, were used. Ferrous iron-induced chemiluminescence was inhibited significantly by superoxide dismutase and catalase by 88 (1) % ($n=7$) and 66 (3) % ($n=5$), respectively. Ferrous iron-induced chemiluminescence was also inhibited markedly by mannitol (83 (2) %, $n=5$) showing that $^{\bullet}OH$ was generated and involved in the chemiluminescence response (data not shown).

Discussion

Binding of FMLP to its specific membrane receptors on phagocytes stimulates NADPH oxidase, the enzyme responsible for production of superoxide radical, mainly by activation via phospholipase C and increase in Ca^{2+} influx. However, PMA binds directly to protein kinase C and activates superoxide formation in leucocytes at low intracellular Ca^{2+} concentrations.[18] In this study, propofol and Intralipid reduced chemiluminescence generated by FMLP- and PMA-activated leucocytes, suggesting that reduction occurs in a non-receptor mediated way. This attenuation does not seem to require preincubation as propofol and Intralipid were introduced just before the stimulant. Additionally, in PMA-stimulated leucocytes, propofol and Intralipid also reduced chemiluminescence when added at the peak, implying direct interaction with reactive oxygen species. Previous studies showed that free radical release from activated leucocytes was impaired after *in vitro* propofol and Intralipid exposure.[10 19–21] At anaesthetic concentrations, propofol produced significant inhibition of PMA-induced respiratory burst in human neutrophils measured by flow cytometry.[10] The effect of the vehicle of propofol has been shown to be similar to that of propofol, implying that suppression of respiratory burst of neutrophils by propofol may be caused partly by this lipid and is unrelated to cell death.[10] Clinically relevant concentrations of propofol suppressed phagocytosis, neutrophil polarization and killing of bacteria in human PMN leucocytes, while Intralipid impaired phagocytosis.[20 22] However, in another study, propofol produced no significant depression of phagocytosis and hydrogen peroxide production of human PMN leucocytes incubated for 1 h with i.v. anaesthetics at clinically relevant concentrations measured by flow cytometry.[11]

There is disagreement on the effect of Intralipid on leucocyte function. Both inhibitory[10] and stimulatory[22 23] responses have been reported. Previous studies demonstrated that neutrophils preincubated with Intralipid exhibited diminished chemotactic responses and inhibited neutrophil bactericidal activity, superoxide generation, phagocytosis[16 24] and T-cell mediated immunity *in vitro*.[23] In a previous study, it has been shown that 10% Intralipid

augmented neutrophil polarization,[22] which is in contrast with another study, where Intralipid suppressed neutrophil chemotaxis.[25] Additionally, polyunsaturated fatty acids can be incorporated rapidly into cell membranes[26] and increase membrane rigidity.[27] These changes in membrane structure may provide an explanation for the decrease in chemiluminescence response. There is concern about the potential for increased serum lipid concentrations, particularly in patients receiving prolonged infusions.

Propofol has been demonstrated to delay significantly the onset of lipid peroxidation compared with Intralipid.[4] The inhibitory effects of propofol on malondialdehyde production in rat liver mitochondria, microsomes and brain synaptosomes indicate that its antiperoxidative action is comparable with that of butylated hydroxytoluene.[6] Propofol also inhibited lipid peroxidation initiated by hydrogen peroxide[28] or hydroxyl, ferryl and oxo-ferryl radicals.[29] It is assumed that propofol behaves as a chain breaking antioxidant *in vitro*, and scavenges lipid peroxyl radicals by a process of hydrogen abstraction to form the relatively stable propofol phenoxyl radical.[4,7] In addition to these effects, we have shown recently that propofol also scavenges peroxynitrite, a potent toxic metabolite generated from the interaction of nitric oxide and superoxide.[30] Intralipid has been shown to have no effect on lipid peroxidation[4] but suppresses the effectiveness of propofol in scavenging free radicals.[5]

Our results showed that propofol significantly inhibited chemiluminescence generated by superoxide radical–hydrogen peroxide, hydroxyl radical and HOCl. However, the most potent inhibitory effect of propofol was for HOCl. To our knowledge, this is the first experimental evidence that propofol is a potent scavenger of HOCl. Intralipid, however, caused marked inhibition of hydroxyl radical generation and this effect is likely to be responsible for the inhibition seen in PMA- and FMLP-activated leucocytes. Our results suggest that propofol exerts scavenging activity on superoxide-hydrogen peroxide, hydroxyl radical and HOCl in addition to the previously shown organic, organoperoxyl radical species[5] and peroxynitrite.[30] Our results disagree with the studies demonstrating that propofol does not react with oxygen centred radicals (superoxide, hydrogen peroxide and hydroxyl radical).[4,5] This discrepancy is probably because of the different laboratory techniques used (chemiluminescence *vs* electron spin resonance (ESR) spectroscopy or illumination of riboflavin and measurement of degradation products of propofol). Failure to demonstrate a hydroxyl radical scavenging effect of propofol is probably because of the reaction of hydroxyl radical with the large concentrations of acetone as a solvent in ESR studies.[4]

The clinical consequences of inhibition of membrane lipid peroxidation and direct free radical scavenging activity of propofol have not been well documented in patients. At anaesthetic concentrations, propofol produced 50% inhibition of neutrophil polarization and complete inhibition was attained with higher concentrations.[22] In another *in vitro* study, Jensen, Dahlgren and Eintrei[25] found a decrease in chemotaxis of human neutrophils produced by propofol after zymosan stimulation. Clinically relevant concentrations of propofol also depressed migration of human PMN leucocytes.[25] Therefore, inhibition of the immune response may have serious effects if propofol and Intralipid are administered continuously to patients with known infection or to immunocompromised patients, and may increase the risk of postoperative infections. In contrast, in many clinical situations, endogenous antioxidant defence systems may be diminished, making cell membranes even more vulnerable to oxidative stress and causing an increase in lipid peroxidation. Thus during anaesthesia for cardiac, vascular and transplantation surgery, or during sedation of the critically ill, in patients undergoing artificial ventilation in the intensive care unit with sepsis or severe burns, propofol offers theoretical advantages by scavenging reactive oxygen species and metabolities, and inhibiting lipid peroxidation.

In summary, we found that propofol with Intralipid suppressed respiratory burst in a concentration-dependent manner in leucocyte experiments. As Intralipid per se produced a less marked decrease, some of the effects of propofol on respiratory burst may be attributed to its lipid vehicle. Although propofol was proposed as a scavenger of organic radicals rather than superoxide and hydroxyl radicals, our results showed that propofol had direct scavenging activity against superoxide radical–hydrogen peroxide, hydroxyl and HOCl. Therefore, the direct scavenging activity of propofol may contribute to the reduction in luminol chemiluminescence in PMA-activated leucocytes. Our results provide experimental support for investigating the potential benefit of using propofol as an anaesthetic agent in patients presenting pathologies associated with free radical reactions.

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