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Production of Hydrogen Peroxide by Alveolar Macrophages. Effect of Barbiturates

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Summary

Production of hydrogen peroxide by rat lung alveolar macrophages represents one of the key events in the inflammatory process. For the interpretation of the *in vitro* measurements it is important to control all possible interfering influences. The present work documents that the type of anaesthesia might critically influence the observed results. H_2O_2 production was measured in isolated rat alveolar macrophages by luminol chemiluminescence catalyzed by horseradish peroxidase. Three different mechanisms of H_2O_2 production were observed after stimulation of cells with a chemotactic peptide (FMLP), phorbol ester (PMA), and during cell adherence. All these activities were influenced independently by the treatment with barbiturates, which both stimulated or inhibited the H_2O_2 production, depending on the barbiturate concentration. As the effective barbiturate concentrations were found to be within the range used for the anaesthesia of experimental animals, the presented results imply that barbiturates are not suitable for experiments in which the production of reactive oxygen species by phagocytes is measured, and that other anaesthetics should be tested.

Key words

Alveolar macrophages – Anaesthesia – Hydrogen peroxide – Chemiluminescence

Introduction

Macrophages, similarly as other phagocytic cells, are known to respond by production of reactive oxygen species (ROS) to phagocytic stimuli (Babior 1978). ROS can be detected with the use of luminol-dependent chemiluminescence (LDCL). This technique is prone to interference with several factors, but under carefully controlled conditions it may serve as a very sensitive tool for ROS measurements (Vilím and Wilhelm 1989). Stimulated alveolar macrophages (AMs) produce both superoxide and hydrogen peroxide. In a previous report (Wilhelm *et al.* 1987), we showed that the production of superoxide is transient. Therefore, in the present study we have measured the production of H_2O_2 on the basis of its reaction with horseradish peroxidase (HRP) and luminol. The kinetics of this reaction are known in great detail (Cormier and Prichard 1968) and for a cell-free system it was described by Wilhelm and Vilím (1986).

Animal experiments where the ROS production by phagocytes is studied are usually performed on anaesthetized animals. However, it is often forgotten that anaesthetics are not chemically inert and that they may take part in the measured effects. In a preliminary unpublished study, we observed a tenfold scatter in LDCL response within groups of animals anaesthetized with pentobarbital or thiopental. We decided therefore to measure the effects of barbiturates on LDCL related to H_2O_2 production by isolated rat alveolar macrophages *in vitro*. The production of H_2O_2 was induced by three independent stimuli: cell adherence, then the receptor-mediated response was triggered by N-formyl-methionyl-leucyl-phenylalanine (FMLP), and finally the protein kinase C dependent response was initiated by phorbol 12-myristate,13-acetate (PMA).

Materials and Methods

Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) was purchased from Sigma. A stock solution of 1 mM luminol was prepared by dissolving it in 0.05 M NaOH, pH was adjusted to 7.4 with HCl.

Horseradish peroxidase (HRP) from Boehringer was diluted to 250 U/ml with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS), pH 7.4. FMLP from Sigma was made to 0.1 mM stock solution by dissolving it in 0.5 mM NaOH. 160 μM PMA stock solution was prepared by dissolution in dimethylsulfoxide (Sigma).

Male Wistar rats (250–300 g, Velaz, Czech Republic) were killed by cervical dislocation. AMs were isolated according to the method of Lavnikova *et al.* (1993). Briefly, the lungs were perfused *via* pulmonary artery with 50 ml of HBSS containing EDTA (0.6 mM). During the perfusion, lungs were ventilated with a normoxic gas mixture (21 % O_2 + 5 % CO_2 + 74 % N_2) at the rate of 40 ml/min with positive inspiratory pressure of 12 cm H_2O and end expiratory pressure of 2.5 cm H_2O. Then the lung and trachea were excised, and lavaged 8 times by slowly instilling and withdrawing 8 ml of warm HBSS. During the procedure the lung was gently massaged. Cells were collected by centrifugation (400x *g*, 10 min, 20 °C), washed in HBSS and sedimented under the same conditions. The cell number was counted under the microscope, and cells were kept at room temperature.

For the study of the effects of anaesthetics produced *in vivo*, three groups of ten animals each were used. Animals killed by cervical dislocation served as controls, the second group was anaesthetized by breathing halothane and the third group received an intraperitoneal injection of thiopental (10 mg/100 g b.w.). The isolation of AMs was done according to the same protocol in all three groups.

In the experiments studying the effects of barbiturates, the cell suspension was incubated with increasing concentrations of thiopental (sodium 5-ethyl-5-(1-methylbutyl)-2-thiobarbiturate, Spofa, Czech Republic) for 5 min before initiation of LDCL.

LDCL was measured on a Luminometer 1250 (LKB-Wallac Oy, Finland), equipped with a thermostated cell holder. The instrument was coupled through a custom-made interface to a computer, where the data was collected at 1 s periods. The peak intensity of LDCL was expressed in mV of the photomultiplier response. The measurements were carried out at 37 °C in a plastic measuring cuvette. The reaction mixture of total volume of 1 ml consisted of 100 μl luminol (final concentration 0.1 mM), 10 μl HRP (2.5 U/ml), 50 μl FMLP (5 μM), 10 μl PMA (1.6 μM) and a cell suspension containing one million cells.

The measurements of the effects of cell adherence on LDCL was started by pipetting the HRP solution into a mixture of cells with buffer and luminol. When LDCL was triggered by PMA, it was added to the cuvette together with HRP. In some experiments the effects of FMLP were investigated subsequently to previous stimulation of the cells by adherence or by PMA. In these cases, the data collection was interrupted at exactly 500 s from the beginning of the measurement, FMLP was added and the data recording was restarted within 5 s.

When testing the effects of thiopental on the light production by the reaction between hydrogen peroxide and luminol catalyzed by HRP, hydrogen peroxide was pipetted through a septum into a cuvette containing the mixture of thiopental and HRP placed in the measuring position because the maximum light production was achieved within several seconds.

Analysis of variance was used for the statistical evaluation of the effects of anaesthetics. *In vitro* effects of thiopental were evaluated the unpaired t-test. Five animals were used, all measurements were run in triplicates.

Results

During adherence of isolated AMs to plastic cuvettes the LDCL response started to develop after 100 s. Around 450 s from the beginning of the reaction, LDCL reached a steady state and from that time on the luminescence intensity was constant for at least the next 10 min. When FMLP was added to the reaction mixture at 500 s, a new rise of LDCL was observed. Agitation of the cell suspension at one-minute intervals greatly diminished the LDCL response which implied the role of cell adherence in the stimulation of ROS production (Fig. 1).

The measured LDCL was fully dependent on H_2O_2 since practically no luminescence was observed in the absence of HRP. As is illustrated in Fig. 2, the addition of HRP at 500 s triggered a burst of luminescence which was caused by the reaction with H_2O_2 accumulated during cell adhesion.

Stimulation of AMs in the presence of HRP with PMA which activates protein kinase C, resulted in LDCL production which was about one order of magnitude higher than the luminescence induced by adherence (Fig. 3). Subsequent addition of FMLP triggers a new rise of LDCL, indicating an independent mechanism of activation.

The above characteristics were used for the evaluation of the effects of anaesthesia on the activities of AMs. We measured the maximum obtainable response, i.e. the combined effects of adherence, PMA and FMLP, of the AMs isolated from animals killed by cervical dislocation without anaesthesia (controls) and from those anaesthetized by thiopental or halothane. The values expressed as mV of the peak response were 88.5±6.9 for the controls, 91.9±33.8 after thiopental anaesthesia, and 108.6±14.1 after anaesthesia with halothane (mean±S.D.). The differences between these measurements were not statistically significant as assessed by analysis of variance. A marked feature was the great dispersion of the data after thiopental anaesthesia, and we consequently studied its effects more closely.

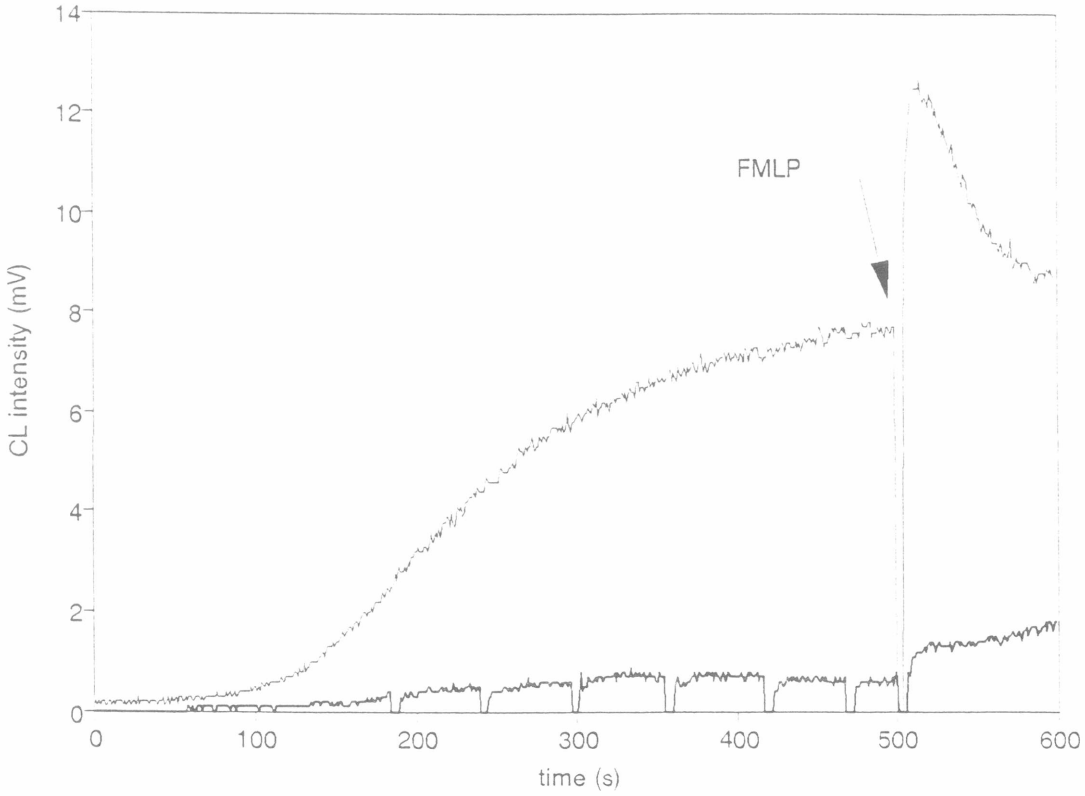

Fig. 1
LDCL during cell adherence and after stimulation with FMLP. HRP was present in the reaction mixture from the beginning. The AMs were left to adhere for 500 s, then FMLP was added (upper curve). If the cell suspension was agitated at 60 s intervals (lower curve), the LDCL response was greatly damped.

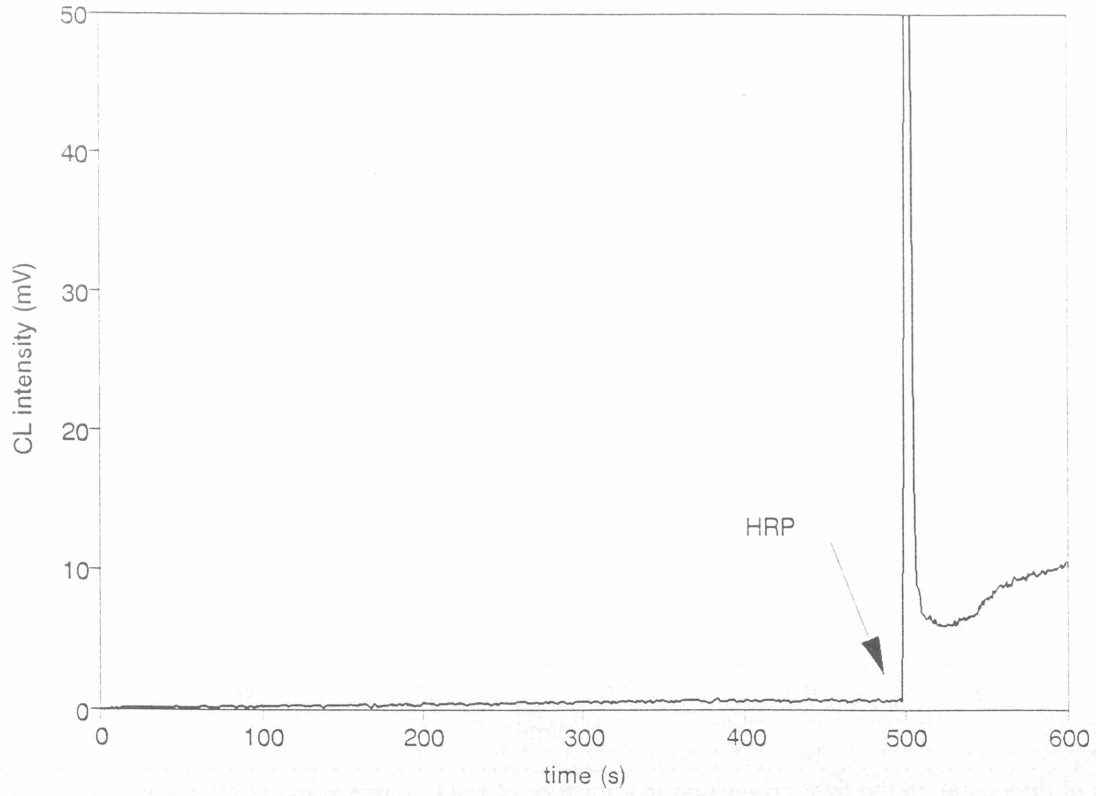

Fig. 2
The effect of HRP on LDCL. When HRP was absent from the reaction mixture, no luminescence production was observed. After its addition at 500 s a LDCL burst resulted.

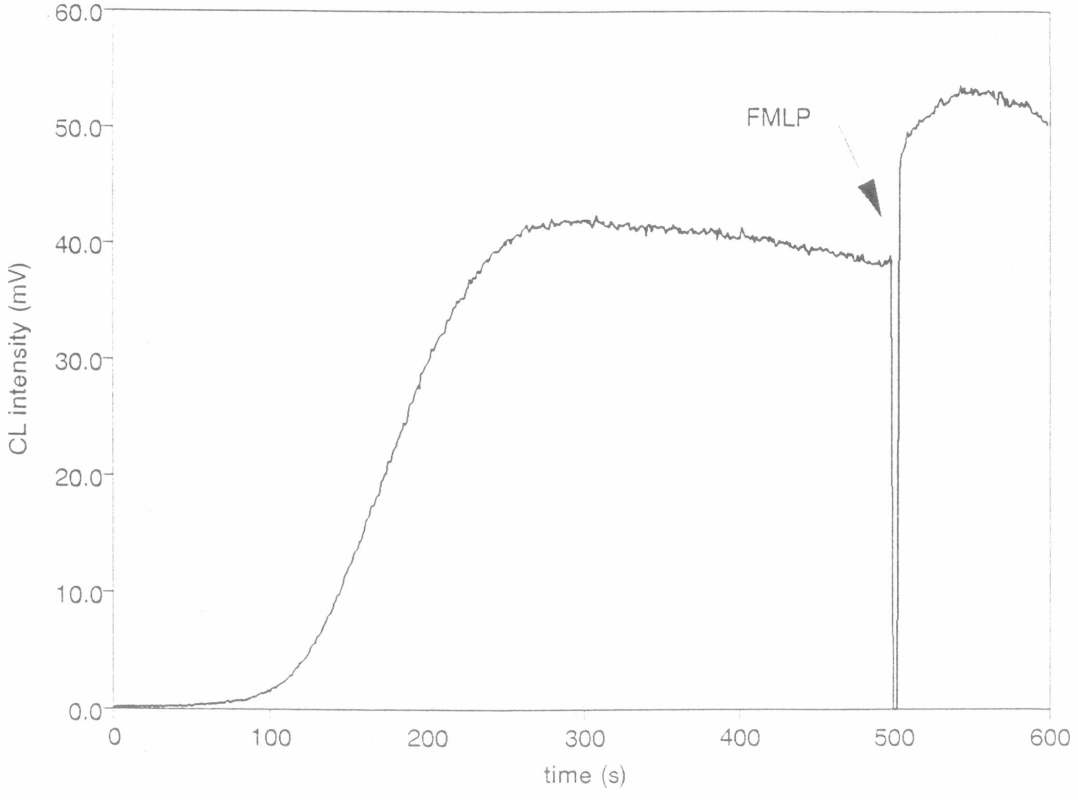

Fig. 3
The effects of PMA and FMLP on LDCL. PMA was present from the beginning of the measurement, FMLP was added at 500 s from the start.

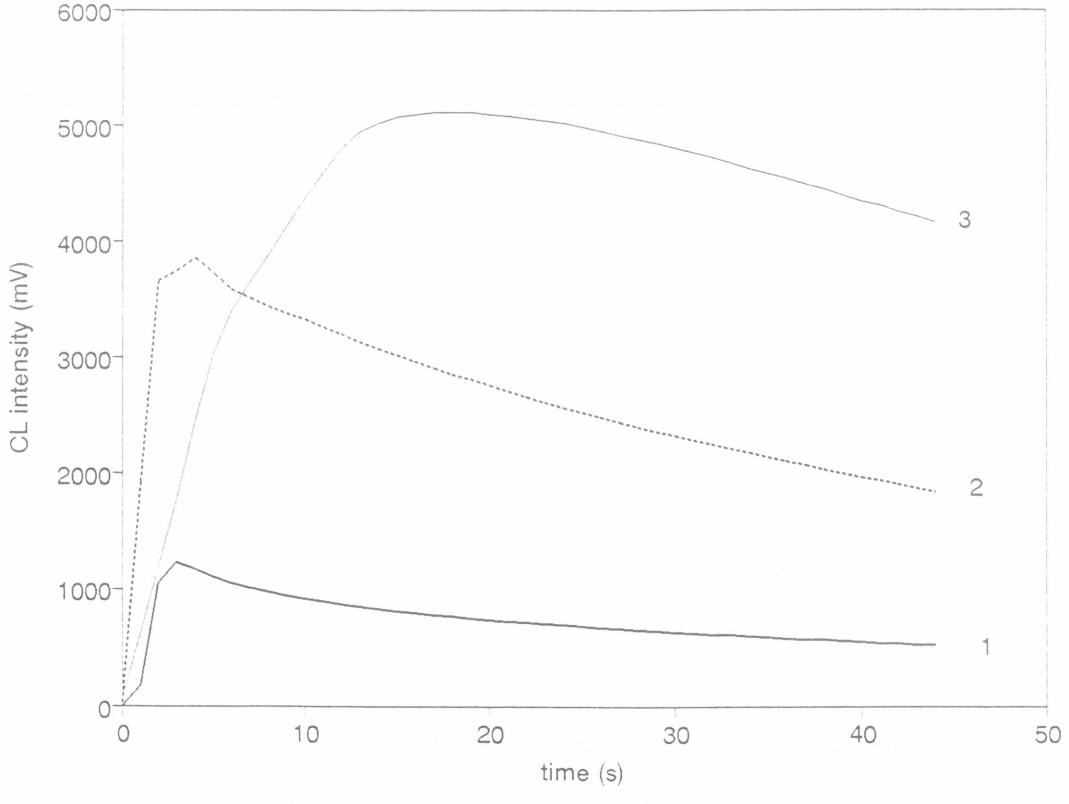

Fig. 4
The effect of thiopental on the light production in a mixture of H_2O_2, luminol and HRP. Curve 1 – no thiopental; curve 2 – 50 μg/ml, curve 3 – 100 μg/ml of thiopental.

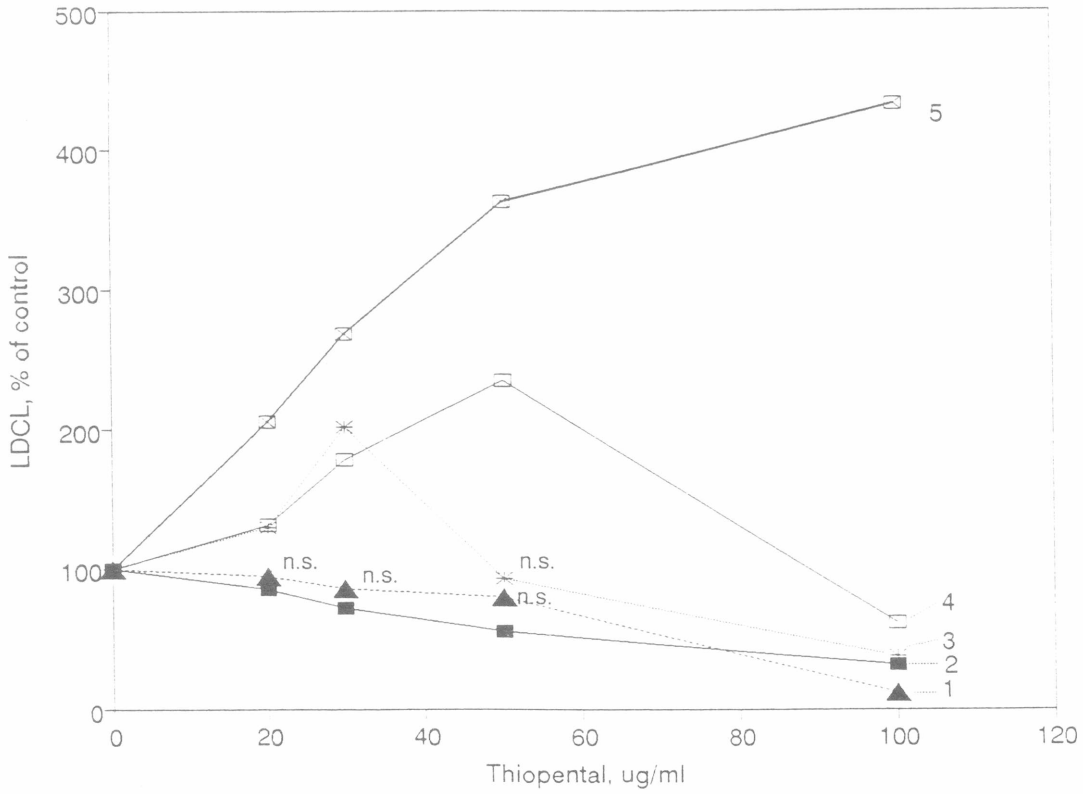

Fig. 5
Thiopental effects expressed as percentage. The control (zero thiopental concentration) is taken as 100 %. LDCL was initiated by adherence (curve 4), PMA (curve 3), FMLP applied after the action of PMA (curve 2), and FMLP applied after adherence (curve 1). Curve 5 illustrates the effects in a cell-free system containing 10 mM H_2O_2.

Thiopental, in the concentration range observed in the anaesthetized animals, influenced the chemistry of light production by a reaction between luminol and hydrogen peroxide catalyzed by HRP. As is shown in Fig. 4, it increases the maximum light output and also changed the kinetics of the reaction. Therefore, to characterize its effects we integrated the light production over the first 20 s of the reaction which provided a reproducible parameter. Its value for the controls (without thiopental) was 18 674±608 mV, at 100 μg of thiopental/ml it was 80 897±2 272 mV. The effects of the whole range of measured concentrations is illustrated in Fig. 5 (curve 5) as percentage of the control value.

In Fig. 5 the effect of thiopental on the LDCL response of AMs isolated from unanaesthetized animals is expressed as a percentage value related to the control (zero thiopental concentration) representing 100%. All values were statistically significant ($p<0.05$) except those labelled as n.s. (non-significant). Especially insensitive to the effect of thiopental was the action of FMLP applied after adherence, the only significant decrease was observed at the highest concentration of thiopental tested (curve 1). On the contrary, LDCL produced after adherence alone (curve 4) was stimulated by thiopental up to 50 μg/ml, at 100 μg/ml of thiopental inhibition was also observed. PMA-stimulated LDCL (curve 3) was enhanced by thiopental up to 30 μg/ml, at 50 μg/ml the value returned to the control level and at 100 μg/ml the inhibition was studied. LDCL induced by FMLP applied after the action of PMA was inhibited by all thiopental concentrations tested (curve 2).

Discussion

In the present study, H_2O_2 generation by AMs was documented using measurement of LDCL in the presence of HRP. Three independent mechanisms eliciting H_2O_2 production were observed: cell adherence, receptor-mediated cell activation by FMLP, and direct activation of protein kinase C by PMA. The greatest response was observed after the combined action of PMA and FMLP.

Hydrogen peroxide produced during cell adherence accumulates in the medium and might cause biological effects under *in vivo* conditions. Though this

possibility is probably limited in the natural environment of AMs, as we have shown earlier that the chemiluminescence response of AMs to quartz dust particles was inhibited by the supernatant of lavage fluid (Vilím *et al.* 1987). It has also recently been documented that surfactant apoprotein exhibits antioxidant effects (Katsura *et al.* 1993). However, it can be speculated that under pathological conditions resulting in changes of surfactant composition this inhibitory effect of surfactant may be released.

Contrary to the observations made on mouse peritoneal macrophages (Lefkowitz *et al.* 1993), HRP did not directly activate AMs to generate ROS. If present, this reaction was of small importance.

Though anaesthetics are widely used for the sedation of experimental animals, their effects on phagocytic cells were not studied in great detail. In neutrophils, the barbiturate methohexitone inhibited cell adherence (Krumholz *et al.* 1988) and chemotaxis (Moudgil *et al.* 1977). It was also reported that methohexitone at clinically relevant concentrations had no effect on H_2O_2 production by human neutrophils (Krumholz *et al.* 1993). The detection of H_2O_2 in this study, however, has been carried out by a spectrophotometric method which might not be sufficiently sensitive for the detection of minute amounts of H_2O_2 produced. Nevertheless, the effect of individual anaesthetics might be quite specific, as we have observed increased production of hydrogen peroxide during adherence in the presence of thiopental.

We have found both activation and inhibition of H_2O_2 production by AMs *in vitro* that was related to the barbiturate concentration in the reaction mixture. In addition, various mechanisms of H_2O_2 production reacted differently to a given concentration of thiopental. This indicates that thiopental always plays a specific role in a particular pathway and does not act through a general mechanism such as inhibition of ATP production. The pathway triggered by FMLP after the action of PMA was inhibited even at low thiopental concentrations which activated PMA- and adherence-initiated H_2O_2 production.

In view of the fact that thiopental produced only activation of light production in a cell-free system, we can look upon all inhibitions of LDCL as cell-specific.

The lowest barbiturate concentrations that were effective *in vitro* corresponded roughly to one tenth of the concentration used for animal sedation, assuming uniform distribution of the drug in the whole animal. We therefore believe that the great scatter of LDCL values that we have found within a group of animals sedated by a unit dose of thiopental related to the body mass was primarily caused by interference of thiopental with various pathways of H_2O_2 production. In addition, the duration of cell exposure to thiopental may be another factor which should be considered.

Based on the present results, all the measurements of ROS production by phagocytes should be interpreted with regard to the effect of the anaesthetic used. Halothane anaesthesia seems to be more suitable for that purpose than that of barbiturates.

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Reprint Requests

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