Involvement of an NAD(P)H oxidase-like enzyme in superoxide anion and hydrogen peroxide generation by rat type II cells

R J van Klaveren, C Roelant, M Boogaerts, M Demedts, B Nemery

Abstract

***Background* – Although alveolar macrophages are considered to be the primary cellular mediators of host defence in the lung, there is increasing evidence that type II cells may also play an active role in host defence. A study was undertaken to investigate whether type II cells generate $O_2^{-\cdot}$ and H_2O_2 via an NADPH oxidase-like system and whether exposure of the type II cells to soluble or particulate stimuli known to activate NADPH oxidase in macrophages also leads to increased production of H_2O_2.**

***Methods* – Rat type II cells and alveolar macrophages were exposed to 10, 100, or 1000 nM phorbol-12-myristate-13-acetate (PMA) and the production of $O_2^{-\cdot}$ and H_2O_2 was determined by chemiluminescence. Thirty minutes before stimulation with 1 μM PMA type II cells were also exposed to the same concentrations of a protein kinase C (PKC) antagonist GF109203x, the non-selective protein kinase inhibitor staurosporine (1, 10, or 100 nM), or the NADPH oxidase inhibitor diphenyliodonium chloride (DPI) (1, 10, 100, or 1000 μM). The effects of arachidonic acid, zymosan and *Staphylococcus aureus* on H_2O_2 production were determined. Cell membrane fractions from type II cells and macrophages were assayed for NADPH oxidase activity.**

***Results* – After exposure to 1 μM PMA, $O_2^{-\cdot}$ and H_2O_2 generation increased 6.3-fold and 9.0-fold, respectively, in type II cells and 2.4-fold and 5.2-fold, respectively, in macrophages. In contrast to the macrophages, the increase in $O_2^{-\cdot}$ and H_2O_2 generation by type II cells was completely prevented by 1 mM KCN. Pre-exposure to GF109203x, staurosporine, or DPI completely prevented the rise in $O_2^{-\cdot}$ and H_2O_2 generation. Mean (SD) NADPH oxidase activity of 138 (38) nmol $O_2^{-\cdot}$/min/mg protein was found in membrane fraction I of the type II cells, and 102 (31) nmol $O_2^{-\cdot}$/min/mg protein in fraction II. Macrophages showed higher NADPH oxidase activity in membrane fraction II. In type II cells exposure to arachidonic acid led to a significant 5.3-fold increase in H_2O_2 generation, exposure to zymosan increased H_2O_2 generation 46-fold, and exposure to *S aureus* 25-fold with a maximum 30–50 minutes after addition of the bacteria.**

***Conclusions* – Type II cells generate $O_2^{-\cdot}$ and H_2O_2 via a PKC-mediated activation of an NAD(P)H oxidase-like membrane bound enzyme. Arachidonic acid, zymosan, and bacteria also give rise to increased H_2O_2 production. Type II cells might thus play an active role in host defence.**

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Type II pneumocytes represent about 15% of the total lung cell population and make up two thirds of alveolar epithelial cells by number.[1] They play a critical role in preserving the functional integrity of the alveolar surface of the lung and in the production of surfactant. They are also the progenitor cells of the type I pneumocytes[2] and, in conjunction with the type I pneumocytes, they serve as a permeability barrier. Type II cells have been studied in models in which they were subjected to toxic compounds or reactive oxygen species from various origins (cigarette smoke, alveolar macrophages). The possible role of the type II cells, however, as producers of free radicals in tissue injury and host defence has been much less investigated. Previous investigators have demonstrated that baseline production of H_2O_2, and the levels of H_2O_2 and $O_2^{-\cdot}$ produced by type II cells after stimulation with phorbol-12-myristate-13-acetate (PMA), are of the same magnitude as those observed with PMA-stimulated alveolar macrophages.[3–5] Furthermore, it has been found that type II cells spontaneously release levels of nitric oxide (NO) that are similar to those produced by macrophages.[6] These findings are surprising because alveolar macrophages were generally considered to be the only primary cellular mediators of host defence in the lung. The precise role of the type II cell in host defence and oxidant-mediated lung toxicity, and the mechanism by which type II cells produce H_2O_2 and $O_2^{-\cdot}$, is not fully understood. The rationale for the present study was to clarify the latter question.

Phagocytic cells, including neutrophils, eosinophils, monocytes and macrophages, are part of the host defence system against infections. On binding to the target organism there is an increase in oxygen consumption after phagocytosis accompanied by the release of $O_2^{-\cdot}$, catalysed by the plasma membrane bound enzyme NADPH oxidase.[7 8] NADPH oxidase is normally dormant, but the enzyme can be activated by the addition of both soluble and

particulate stimuli such as zymosan.[7] The most commonly used soluble stimulus is PMA which activates NADPH oxidase through the activation of protein kinase C (PKC). Other non-phagocytic cells, such as fibroblasts, mesangial, carotid body, thyroid and endothelial cells, have also been found to contain an NADPH oxidase-like enzyme.[9–11] It is still unknown, however, whether type II cells generate $O_2^{-\cdot}$ and H_2O_2 through an NADPH oxidase-like enzyme complex.

The aim of our study was to investigate (1) whether type II pneumocytes do generate $O_2^{-\cdot}$ and H_2O_2 via an NADPH oxidase-like system known to be implicated in the bactericidal activity of phagocytes, and (2) whether exposure of the type II cells to soluble or particulate stimuli known to activate NADPH oxidase in macrophages also triggers H_2O_2 production.

Methods

ANIMALS AND MATERIALS

Male Wistar rats (140–170 g) were obtained from an in-house strain. Potassium cyanide, trypsin type I (EC 3.4.214), trizma base (Tris), bovine serum albumin (BSA), DNase I, β-nicotinamide-adenine-dinucleotide phosphate, reduced form (β-NADPH), Percoll, dimethylsulfoxide (DMSO), cytochrome C from bovine heart, potassium superoxide, phorbol-12-myristate-13-acetate (PMA), staurosporine (antibiotic AM-2282), 3-(N-morpholino)-propanesulphonic acid (MOPS), p-aminobenzenesulfonamide (sulfanilamide), N-(1-naphthyl)-ethylenediamine, luminol, 10,10′-dimethyl-9,9′-biacridinium dinitrate (lucigenin), zymosan, arachidonic acid, and glutaraldehyde 25% were all purchased from Sigma Germany (Filter Service NV/SA, Eupen, Belgium). Waymouth's 752/1 medium, fungizone (amphotericin 250 μg/ml), L-glutamine (200 mM), penicillin-streptomycin solution (10 000 U-10 000 μg/ml, respectively), HEPES, and fetal calf serum (FCS) were purchased from Gibco (Merelbeke, Belgium), hydrogen peroxide was purchased from Merck, Germany, protein assay dye solution was purchased from Bio-Rad (Brussels, Belgium), diphenyliodoniumchloride (DPI) from Aldrich, and 4-α-phorbol-12,13-dibutyrate and GF109203x were purchased from Biomol (Sanvertech, Boechout, Belgium). All other chemicals were purchased from U.C.B. Belgium (Vel NV/SA, Leuven, Belgium). PBS^- is phosphate buffered saline (130 mM NaCl, 5.2 mM KCl, 10.6 mM Hepes and 2.6 mM Na_2HPO_4, pH 7.4) and PBS^+ is the same buffer with the addition of $CaCl_2$ (1.9 mM) and $MgCl_2$ (1.3 mM). Sterile plastic 96-well and 24-well cell culture plates were purchased from Corning, and the sterile 96-well culture plates for chemiluminescence were obtained from Packard.

ISOLATION OF RAT TYPE II PNEUMOCYTES

The isolation procedure was based on the methods of Richards *et al*[12] and Hoet *et al.*[13] Briefly, rats were deeply anaesthetised with an intraperitoneal injection of 60 mg pentobarbitone/kg body weight (Nembutal, 60 mg/ml). After perfusion of the lungs with 0.9% NaCl the lungs and trachea were removed from the thoracic cavity and 6–8 ml 0.9% NaCl were instilled (using a syringe) and poured out (by simple inversion of the lungs) five times via a tracheal cannula. This lavage fluid was centrifuged at 250 *g* for 10 minutes (4°C) and the cells (>95% macrophages) were cultured in the same medium as the type II cells (see below) on plastic culture plates for 48 hours. After the last lavage the lungs were filled with a trypsin solution (500 mg/200 ml PBS^+) for 30 minutes at 37°C. After this digestion period the trachea and main bronchi were removed from the lungs, the lungs were chopped, 5 ml FCS and 15 ml DNase solution (250 μg/ml PBS^-) were added and the tube was shaken by hand for five minutes. The digest was filtered through a cotton gauze and two nylon filters and centrifuged at 250 *g* for 20 minutes (4°C) on a discontinuous Percoll gradient (density 1.089 g/ml and 1.040 g/ml). The creamy layer above the heavy gradient was collected, rinsed in PBS^-, suspended in Waymouth's medium supplemented with DNase 50 μg/ml, 2% FCS, 2% fungizone and 2% penicillin-streptomycin solution, and incubated for one hour in a Petri dish placed in a CO_2 incubator (10% CO_2, 37°C) in order to let the macrophages attach. The unattached cells were then spun down (250 *g*, 10 minutes, 4°C) and resuspended in the final Waymouth's medium containing 10% (v/v) FCS, 1% fungizone, 1% penicillin-streptomycin solution, and 1% L-glutamine. The cells were counted in a Bürker chamber and plated on sterile plastic 96-well (100 000 type II cells/200 μl/well) or 24-well plates (600 000 type II cells/1200 μl/well).

Immediately after isolation (density gradient centrifugation and differential attachment) the purity of the type II cells was 85 (7)%. Contamination was mainly caused by alveolar macrophages. The type II cells were used 48 hours following plating after they had been rinsed with PBS^+ to remove non-adherent cells and macrophages. As in previous experiments, we demonstrated that, after rinsing the plates, monolayers of 90–95% pure type II cells were obtained as judged by alkaline phosphatase staining and electron microscopy.[13] In the experiment in which freshly isolated type II cells were not plated (cell membrane preparation) purity was further increased by performing the differential attachment twice. In addition, for some experiments type II cells were also plated on the bottom of 5 ml glass scintillation vials (Packard) (10^6 type II cells/1500 μl/vial); after 24 hours the cells were washed to remove non-adherent cells and confluency was found after 48 hours as assessed by light microscopy.

CHEMILUMINESCENCE ASSAY

Luminol and lucigenin-dependent chemiluminescence was used to detect H_2O_2 and $O_2^{-\cdot}$, respectively, at 37°C with a BIQ Bioview Camera System.[14] The culture medium of the type II cell monolayers was removed and 15

minutes after the addition of 100 µl PBS^+, with or without the compound to be investigated (e.g. PMA), 50 µl of 100 µM lucigenin or 100 µM luminol were added. The luminol-dependent chemiluminescence reaction was amplified by the addition of 5 µM haematin. The peak of the chemiluminescence produced (counts/min/10 mm^2) was used for comparisons between groups and for calculating the absolute amount of $O_2^{-\cdot}$ and H_2O_2 generated per minute compared with a standard curve made with potassium superoxide (KO_2, 0–100 µM) or H_2O_2, respectively. Results were expressed as percentage of control (with vehicle) or as nmol $O_2^{-\cdot}$ or H_2O_2 generated per min/mg protein. Protein was measured according to the method of Bradford using bovine serum albumin as standard.[15] All determinations were done in triplicate.

ROLE OF PKC AND NADPH OXIDASE IN $O_2^{-\cdot}$ AND H_2O_2 GENERATION BY TYPE II CELLS

Twenty four hours after their isolation, type II cells were exposed to 10, 100, or 1000 nM PMA in PBS^+ for 15 minutes at 37°C. Thirty minutes before stimulation with 1 µM PMA the type II cells were also exposed to (1) an inactive PKC agonist, 4αPDBU (10, 100, or 1000 nM), (2) a selective PKC antagonist, GF109203x (10, 100, or 1000 nM), (3) a non-selective protein kinase inhibitor, staurosporine (1, 10, or 100 nM), or (4) an NADPH oxidase inhibitor, DPI (1, 10, 100, or 1000 µM). The effect of 1 µM PMA on baseline $O_2^{-\cdot}$ and H_2O_2 generation by alveolar macrophages and type II cells was also evaluated with and without 1 mM KCN.

PREPARATION OF TYPE II CELL OR MACROPHAGE MEMBRANES

To obtain the membrane fractions of type II cells and macrophages a combination of different procedures was used.[16–18] Freshly isolated type II cells or macrophages pooled from three rats per experiment ($\pm 25 \times 10^6$ cells) were cooled on ice, centrifuged (800 *g*, 10 minutes, 4°C), suspended in 1 ml 0.34 M sucrose, and sonicated on ice (MSE sonicator equipped with an exponential probe, 20 kHz, amplitude 12 µm, 3×10 s). EDTA 0.5 mM was added and the suspension was spun down (800 *g*, five minutes) to remove nuclei and unbroken cells. The supernatant was centrifuged at 20 000 *g* for 20 minutes at 4°C to obtain a pellet of plasma membranes and granules. This was called fraction I. The supernatant was centrifuged again at 48 000 *g* for one hour and this pellet was called fraction II. Both fractions were assayed for NADPH oxidase activity.

NADPH OXIDASE ACTIVITY

NADPH oxidase activity was assayed according to the method of Jones *et al.*[19] Fifty µl of the cell membrane fractions I or II were added to 850 µl buffer (50 mM HEPES, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 2 mM glucose, pH 7.4) and 50 µl 2 mM cytochrome C in 1 M MOPS. The reaction was performed at room temperature and started by the addition of 50 µl 2 mM NADPH. The change in absorbance was measured spectrophotometrically at 550 nm using a molar extinction coefficient of $21 \times 10^3\ M^{-1}.cm^{-1}$.

EFFECT OF NADPH OXIDASE STIMULATING AGENTS ON H_2O_2 PRODUCTION BY TYPE II CELLS

Type II cells were also exposed for 15 minutes at 37°C to arachidonic acid (10, 100, or 1000 nM), unopsonised zymosan (0.125, 0.25, or 0.5 mg/ml in PBS^+), and *Staphylococcus aureus* bacteria. The *S aureus* bacteria (ATCC 25923) had been cultured overnight at 37°C in Trypcase soya broth, washed and suspended in 150 µl PBS^+, and finally added to the confluent monolayers. H_2O_2 production was measured 20, 50, 85, and 110 minutes later as described above. In addition, chemiluminescence was measured in monolayers of type II cells cultured on the bottom of 5 ml glass scintillation vials for 48 hours using a Beckman LS 7500 ambient temperature liquid scintillation counter. Measurements were made in the dark with exposure limited to red light. Data were collected using the single photon monitor feature of the instrument in which the coincidence circuit is not used. The counts represent an average of the total number of events seen by each photomultiplier tube during the counting period. The cells were washed three times with PBS^+, luminol was added (10^{-6} M in 2 ml PBS^+), and background registrations were made after a stabilisation period of 10 minutes. Next, 100 µl of the bacterial suspension were added and every 20 minutes the increase in counts/min (% control values) was determined for five minutes and compared with cell cultures not containing bacteria.

DATA ANALYSIS

In all instances cells were isolated from three rats and each cell culture was considered as a single unit. The cells from each rat were divided into control and test cells. Triplicate measurements were made for each condition and then averaged. Thus, three independent values (each the average of three measurements) were available. This allowed the computation of a mean and statistical deviation, as well as unpaired and paired (control versus treated) *t* tests. In total, 24 rats were used for the different studies. Statistical analysis was performed using the SAS/STAT package (6th version). The level of significance was set at $p<0.05$.

Results

ROLE OF PKC AND NADPH OXIDASE IN PMA-INDUCED H_2O_2 AND $O_2^{-\cdot}$ GENERATION BY TYPE II CELLS

Mean (SD) baseline $O_2^{-\cdot}$ production by type II pneumocytes was 0.03 (0.02) nmol/min/mg protein, and baseline H_2O_2 production was 0.02 (0.01) nmol/min/mg protein. Stimulation of the type II cells with PMA resulted in a dose

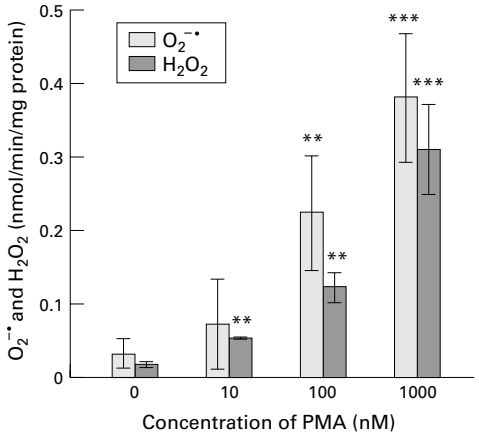

*Figure 1 Effect of different concentrations of PMA (15 min) on generation of $O_2^{-\cdot}$ and H_2O_2 by rat type II cells in culture 24 hours after their isolation. ** p<0.01, *** p<0.001 compared with baseline production without stimulation with PMA (paired t test, n=3 rats). Values are mean (SD).*

dependent increase in $O_2^{-\cdot}$ and H_2O_2 generation (fig 1). Although macrophages produced more $O_2^{-\cdot}$ and H_2O_2 in the baseline situation (0.36 (0.29) nmol/min/mg protein (p<0.05) and 0.08 (0.03) nmol/min/mg protein (p<0.05), respectively), the increases in $O_2^{-\cdot}$ and H_2O_2 generation after exposure to 1 μM PMA were higher in type II cells (6.3-fold and 9.0-fold, respectively) than in macrophages (2.4-fold and 5.2-fold, respectively; fig 2). In contrast to the macrophages, the increase in $O_2^{-\cdot}$ and H_2O_2 generation by type II cells was completely prevented by 1 mM KCN (p<0.01; fig 2).

By itself, 4αPDBU increased baseline $O_2^{-\cdot}$ generation 1.5-fold and H_2O_2 generation 2.4-fold. Baseline $O_2^{-\cdot}$ and H_2O_2 generation were also increased by GF109203x (1.8-fold and 1.4-fold, respectively), staurosporine (1.9-fold and 1.9-fold, respectively), and DPI (1.8-fold and 1.8-fold, respectively). These effects were independent of the doses of GF109203x, staurosporine, or DPI used. However, pre-exposure to GF109203x before stimulation of the type II cells with 1 μM PMA completely prevented the stimulatory effect of PMA on $O_2^{-\cdot}$ and

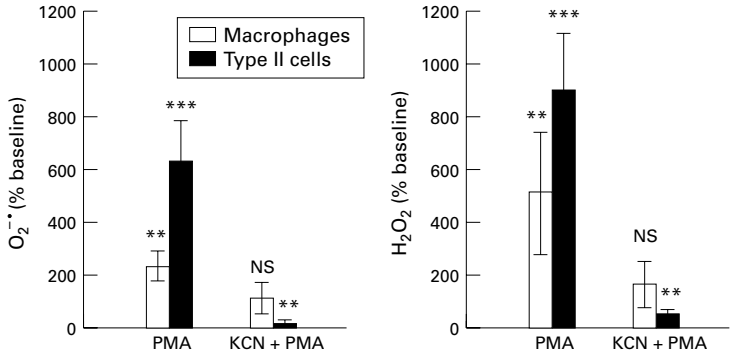

*Figure 2 Effect of stimulation of rat alveolar macrophages and type II cells with 1 μM PMA (15 min), with or without 1 mM KCN, on production of $O_2^{-\cdot}$ and H_2O_2. ** p<0.01, *** p<0.001 compared with baseline (values given in the text). NS=not significant, ** p<0.01 compared with stimulation without KCN (paired t test, n=3 rats). Values are mean (SD).*

Table 1 Mean (SD) NAD(P)H oxidase activity (nmol $O_2^{-\cdot}$/min/mg protein) in cell membrane fractions of rat alveolar macrophages and type II cells

	Macrophages	*Type II cells*
Fraction I	190 (12)	138 (38)*
Fraction II	414 (21)	102 (31)**

* p<0.05, ** p<0.01 (unpaired *t* test), three experiments, three rats per experiment.

H_2O_2 production for all doses studied (fig 3A). When the type II cells were pre-exposed to 1 μM DPI, $O_2^{-\cdot}$ production increased (p<0.01) but at higher concentrations (100 or 1000 μM) the effect of PMA was completely abolished compared with the baseline value. The same pattern was found with H_2O_2 generation, although the initial increase at 1 μM DPI was less prominent, again in comparison with the baseline value (p<0.05; fig 3B). Staurosporine led to almost complete inhibition at 1 nM, whereas complete inhibition was achieved at 10 and 100 nM (fig 3C).

NADPH oxidase-like activity

An NADPH oxidase-like activity of 138 (38) nmol $O_2^{-\cdot}$/min/mg protein was found in membrane fraction I of the type II pneumocytes, and 102 (31) nmol $O_2^{-\cdot}$/min/mg protein in membrane fraction II. Macrophages showed higher NADPH oxidase activity (p<0.05), especially in membrane fraction II (table 1).

NADPH oxidase activating compounds

Exposure of the type II cells to arachidonic acid led to a significant 5.3-fold increase in H_2O_2 generation only at the highest concentration (1000 nM). However, exposure to 0.125, 0.25, or 0.50 mg/ml zymosan increased H_2O_2 generation 42-fold, 49-fold, and 46-fold, respectively.

Exposure of the type II cell monolayers to *S aureus* led, in three separate experiments, to 26-fold, 12-fold, and 21-fold increases in H_2O_2 generation with a maximum 50 minutes after the addition of the bacteria, as observed using the BIQ Bioview camera. In the β counter experiment a 30 (14)-fold increase was found 40 minutes after the start of the exposure (fig 4).

Discussion

Macrophages showed a higher baseline $O_2^{-\cdot}$ and H_2O_2 production than type II cells. However, after stimulation with PMA the increases in $O_2^{-\cdot}$ and H_2O_2 generation were higher in the type II cells and, in contrast to the macrophages, they were completely inhibited by KCN. Pre-exposure to the PKC inhibitors GF109203x and staurosporine, or the NADPH oxidase inhibitor DPI, completely prevented the PMA-induced rise in $O_2^{-\cdot}$ and H_2O_2. In the cell membrane fractions of freshly isolated rat type II cells NADPH oxidase-like activity was demonstrated. Exposure to the NADPH oxidase activator arachidonic acid had little effect on H_2O_2 generation, but exposure to

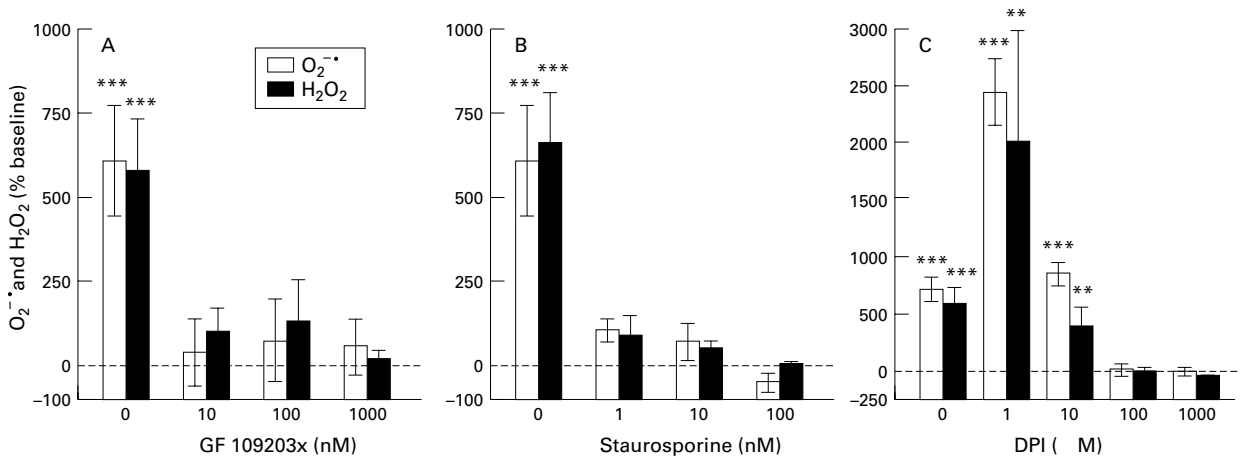

*Figure 3 Effect of stimulation of rat type II cells with 1 μM PMA (15 min) on generation of $O_2^{-\cdot}$ and H_2O_2 after pre-exposure (30 min) and in the presence of different concentrations of (A) the PKC antagonist GF109203x, (B) the protein kinase inhibitor staurosporine, and (C) the NADPH oxidase inhibitor DPI. ** p<0.01, *** p<0.001 compared with the baseline values without PMA (paired t test, n=3 rats). Values are mean (SD).*

zymosan particles and *S aureus* bacteria led to a significant increase in H_2O_2 generation, with a maximum obtained after 30–50 minutes.

TECHNICAL ASPECTS

We have used the lucigenin and luminol-dependent chemiluminescence technique to measure $O_2^{-\cdot}$ and H_2O_2, respectively. To measure $O_2^{-\cdot}$ the traditional cytochrome c assay is specific and convenient, but it has a low sensitivity. To improve this sensitivity we used the lucigenin-dependent chemiluminescence technique which has been carefully standardised for the purpose of routine measurements and has wide application in clinical practice.[20] To measure H_2O_2 the luminol chemiluminescence technique is also convenient, quick, and reproducible, but it has the disadvantage that various other reactive oxygen species such as $O_2^{-\cdot}$ and $OH^{\cdot}$ also react with luminol[21] which prevents a proper discrimination between $O_2^{-\cdot}$ and H_2O_2 generation, but this is inherent to the technique.[14] To strengthen the BIQ Bioview camera results we also used the even more sensitive liquid scintillation counter technique to detect free radical generation by the type II cells during exposure to *S aureus*.

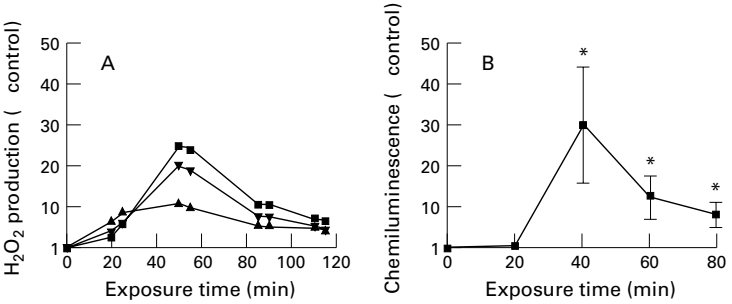

*Figure 4 Increase in H_2O_2 generation by type II cells after their exposure to S aureus by using (A) a BIQ Bioview camera or (B) a Beckman LS 7500 liquid scintillation β counter. In (A) the data from three experiments on type II cell monolayers are presented and in (B) the mean values are given. * p<0.05 compared with the baseline values before exposure to S aureus (paired t test, n=3 rats). Values are mean (SD).*

An important issue in our study is that of possible contamination of our type II cells with macrophages. We have carefully examined by alkaline phosphatase staining and light microscopic examination (magnification 40×) the number of contaminating macrophages in at least three fields of the epithelial type II cell monolayers 48 hours after the isolation. No more than 5% contamination was found after extensive washing with PBS^+. Such a proportion is unlikely to explain our results. Moreover, the observation that the generation of $O_2^{-\cdot}$ and H_2O_2 by the type II cell monolayers was cyanide sensitive supports our findings that contamination with macrophages did not significantly contribute to the generation of $O_2^{-\cdot}$ and H_2O_2 because $O_2^{-\cdot}$ and H_2O_2 generation by stimulated neutrophils and macrophages is insensitive to cyanide.[7 22]

INTERPRETATION

The principal function of neutrophils and other phagocytic cells such as macrophages is the destruction of invading bacteria. Bacterial killing is largely accomplished by means of an oxygen-dependent microbicidal system which produces $O_2^{-\cdot}$ through the action of NADPH oxidase:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{-\cdot}$$

NADPH oxidase, which is a membrane bound flavoprotein that catalyses the generation of $O_2^{-\cdot}$, is dormant in resting neutrophils but is activated when the cells are exposed to bacterial targets.[7] Although the neutrophil has been the prototype source of reactive oxygen species, many other cells – including macrophages and vascular endothelial cells – have also been identified as potential producers of reactive oxygen species. Superoxide production by endothelial cells has been shown in response to cytokines, bradykinin, phorbol ester, and hypoxia followed by reoxygenation.[9]

In this study we provide evidence that an NADPH oxidase-like membrane bound enzyme is involved in the generation of $O_2^{-\cdot}$

and H_2O_2 in rat alveolar type II pneumocytes. However, $O_2^{-\cdot}$ can also be generated by several other oxidases such as cytochrome oxidase and xanthine oxidase. Cytochrome oxidase is present in the mitochondria but xanthine oxidase is not a membrane bound enzyme but is cytosolic in origin.[23] In the NADPH oxidase assay we added NADPH, the substrate for NAD(P)H oxidase, but not for xanthine oxidase, and measured the change in $O_2^{-\cdot}$ generation compared with baseline levels. Moreover, $O_2^{-\cdot}$ generation could be inhibited by the NADPH oxidase inhibitor DPI. These arguments almost completely exclude the possibility that $O_2^{-\cdot}$ was generated by xanthine oxidase. The NADPH oxidase assay does not allow us, however, to differentiate between NADPH oxidase and NADH oxidase activity so we must restrict our conclusion to the presence of an "NAD(P)H oxidase-like" enzyme. Cell membrane fraction I was the largest pellet which is likely to contain larger membrane particles and cell granules, whereas the much smaller fraction II is only enriched in small cell membrane particles.[16–18] As fraction II is probably the most representative fraction for the analysis of plasma membrane enzymes, we might conclude that macrophages demonstrate NAD(P)H oxidase activity about four times higher than type II cells.

We have not only shown NAD(P)H oxidase-like enzyme activity in cell membrane fractions of the type II cells, but we have also provided other indirect evidence for the existence of NADPH oxidase activity. Stimulation of the type II cells with the phorbol ester PMA did not raise $O_2^{-\cdot}$ and H_2O_2 production in the presence of the NADPH oxidase inhibitor DPI (>10 μM),[24] and we found that the PKC inhibitor, GF109203x,[25] also prevented the stimulatory effects of PMA. Staurosporine is a non-selective inhibitor of protein kinases A, C and G inhibitor and it also has NADPH oxidase inhibitory effects.[26] As such, the staurosporine data provide a good back-up to the data from the more selective inhibitors GF109203x and DPI. We therefore conclude, by analogy with other cells, that the PMA-induced increase in $O_2^{-\cdot}$ and H_2O_2 generation probably occurs via a PKC-mediated activation of a membrane bound NADPH oxidase-like enzyme. DPI and its analogues are not only known as inhibitors of NADPH flavoproteins but they were also found to be fairly potent inhibitors of NO synthase, both iNOS from macrophages and cNOS from endothelial cells.[27] Remarkably, 1 μM of DPI increased $O_2^{-\cdot}$ and H_2O_2 production while at 10 or 100 μM $O_2^{-\cdot}$ and H_2O_2 production was decreased. As we have shown that 1 μM DPI efficiently inhibits NO production in type II cells (unpublished data), the increases in $O_2^{-\cdot}$ and H_2O_2 generation at 1 μM could be due to a relative overproduction of $O_2^{-\cdot}$ in the absence of the $O_2^{-\cdot}$ scavenger NO. At higher concentrations of DPI, NADPH oxidase inhibition is probably more effective so that a decrease in $O_2^{-\cdot}$ and H_2O_2 generation is found.

Stimulation of NADPH oxidase with the particulate stimulus zymosan led to marked increases in $O_2^{-\cdot}$ and H_2O_2 generation. Because of this resemblance with neutrophils and macrophages, we exposed the type II cells to bacteria – a real physiological stimulus – and found that H_2O_2 generation also increased several fold. This increase in H_2O_2 production was even more pronounced than after stimulation with PMA. The lag phase may be caused by the sedimentation time of the bacteria and the time needed to activate the type II cells. These data suggest that type II cells may also play a role in host defence, as has been suggested in other studies.[6] These results were also surprising because alveolar macrophages have traditionally been considered to be the primary cellular mediator of host defence in the lung.

We conclude that rat type II cells generate $O_2^{-\cdot}$ and H_2O_2 via a PKC-mediated activation of a membrane bound NAD(P)H oxidase-like enzyme. In addition to a soluble stimulus such as PMA, particulate stimuli such as zymosan or unopsonised bacteria may also increase H_2O_2 production. Type II cells seem to share several characteristics with alveolar macrophages, thus suggesting that alveolar type II cells may also play a critical role in host defence of the lung.

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