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 \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \).

**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 \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) was determined by chemiluminescence. Thirty minutes before stimulation with 1 \( \mu \)M 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 \( \mu \)M). The effects of arachidonic acid, zymosan and *Staphylococcus aureus* on \( \text{H}_2\text{O}_2 \) production were determined. Cell membrane fractions from type II cells and macrophages were assayed for NADPH oxidase activity.

**Results** – After exposure to 1 \( \mu \)M PMA, \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) 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 \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) generation by type II cells was completely prevented by 1 mM KCN. Pre-exposure to GF109203x, staurosporine, or DPI completely prevented the rise in \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) generation. Mean (SD) NADPH oxidase activity of 138 (38) nmol \( \text{O}_2^-/\text{min/mg} \) protein was found in membrane fraction I of the type II cells, and 102 (31) nmol \( \text{O}_2^-/\text{min/mg} \) 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 \( \text{H}_2\text{O}_2 \) generation, exposure to zymosan increased \( \text{H}_2\text{O}_2 \) 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 \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) production. Type II cells might thus play an active role in host defence.

Keywords: bacteria, alveolar macrophage, protein kinase C.

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\(^1\) 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 \( \text{H}_2\text{O}_2 \) and the levels of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) 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.\(^5\) 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 \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \), 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 \( \text{O}_2^- \), catalysed by the plasma membrane bound enzyme NADPH oxidase.\(^7\) NADPH oxidase is normally dormant, but the enzyme can be activated by the addition of both soluble and
particulate stimuli such as zymosan. 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. 

It is still unknown, however, whether type II cells generate O$_2^-$ and H$_2$O$_2$ via an NADPH oxidase-like system known to be implicated in the bactericidal activity of phagocytes, and whether exposure of the type II cells to soluble or particulate stimuli known to activate NADPH oxidase in macrophages also triggers H$_2$O$_2$ production.

Methods

**ANIMALS AND MATERIALS**

Male Wistar rats (140–170 g) were obtained from an in-house strain. Potassium cyanide, trypsin type I (EC 3.4.21.4), trizma base (Tris), bovine serum albumin (BSA), DNase I, β-nicotinamide-adenine-dinucleotide phosphate, reduced form (β-NADPH), Percoll, dimethyl-sulfoxide (DMSO), cytochrome C from bovine heart, potassium superoxide, phorbol-12-myristate-13-acetate (PMA), staurosporine (antibiotic AM-2282), 3-(N-morpholino)-propanesulfonic acid (MOPS), p-aminobenzene-sulfonamide (sulfamamide), N-(1-naphthyl)-ethylenediamine, luminol, 10,10′-dimethyl-9′-bircridinium 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 as H$_2$O$_2$ from Aldrich, and 4-μM-phorbol-12,13-dibutyrate and GFL109203x 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 Heps and 2.6 mM Na$_2$HPO$_4$, 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. and Hoet et al. Briefly, rats were deeply anaeasthetised with an intraperitoneal injection of 60 mg pentobarbital/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 Burker 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$^+$ three times and were purchased from Merck, Germany, protein assay dye solution was purchased from Bio-Rad (Brussels, Belgium), diphenyliodoniumchloride (DPI) from Aldrich, and 4-μM-phorbol-12,13-dibutyrate and GFL109203x 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 Heps and 2.6 mM Na$_2$HPO$_4$, 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.

**CHEMILUMINESCENCE ASSAY**

Luminol and lucigenin-dependent chemiluminescence was used to detect H$_2$O$_2$ and O$_2^-$/; respectively, at 37°C with a BIQ Bioview Camera System. 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 mm²) was used for comparisons between groups and for calculating the absolute amount of O₂⁻ and H₂O₂ generated per minute compared with a standard curve made with potassium superoxide (KO₂, 0–100 µM) or H₂O₂, respectively. Results were expressed as percentage of control (with vehicle) or as nmol O₂⁻ or H₂O₂ generated per min/mg protein. Protein was measured according to the method of Bradford using bovine serum albumin as standard. All determinations were done in triplicate.

ROLE OF PKC AND NADPH OXIDASE IN O₂⁻ AND H₂O₂ 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, 4xPDBU (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 O₂⁻ and H₂O₂ 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. Freshly isolated type II cells or macrophages pooled from three rats per experiment (25 x 10⁶ 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 x 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. 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 MgCl₂, 1 mM NaH₂PO₄, 1 mM CaCl₂, 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 x 10⁻³ M⁻¹.cm⁻¹.

EFFECT OF NADPH OXIDASE STIMULATING AGENTS ON H₂O₂ 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 Tryptcase soya broth, washed and suspended in 150 µl PBS, and finally added to the confluent monolayers. H₂O₂ 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 (10⁻⁴ 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 H₂O₂ AND O₂⁻ GENERATION BY TYPE II CELLS

Mean (SD) baseline O₂⁻ production by type II pneumocytes was 0.03 (0.02) nmol/min/mg protein, and baseline H₂O₂ 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 $O_2^-$ 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).

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 $O_2^-$ 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 $O_2^-$/min/mg protein) in cell membrane fractions of rat alveolar macrophages and type II cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Macrophages</th>
<th>Type II cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>190 (12)</td>
<td>138 (38)*</td>
</tr>
<tr>
<td>II</td>
<td>414 (21)</td>
<td>102 (31)**</td>
</tr>
</tbody>
</table>

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

H$_2$O$_2$ production for all doses studied (fig 3A). When the type II cells were pre-exposed to 1 μM DPI, $O_2^-$ 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$_2$O$_2$ 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 $O_2^-$/min/mg protein was found in membrane fraction I of the type II pneumocytes, and 102 (31) nmol $O_2^-$/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$_2$O$_2$ generation only at the highest concentration (1000 nM). However, exposure to 0.125, 0.25, or 0.50 mg/ml zymosan increased H$_2$O$_2$ generation 4.2-fold, 4.9-fold, and 4.6-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$_2$O$_2$ 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 $O_2^-$ and H$_2$O$_2$ production than type II cells. However, after stimulation with PMA the increases in $O_2^-$ and H$_2$O$_2$ 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 $O_2^-$ and H$_2$O$_2$. 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$_2$O$_2$ generation, but exposure to
zymosan particles and S aureus bacteria led to a significant increase in H$_2$O$_2$ 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$$^\cdot$ and H$_2$O$_2$, respectively. To measure O$_2$$^\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$_2$O$_2$ 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$_2$O$_2$ 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.

**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:

\[
\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{O}_2^-
\]

NADPH oxidase, which is a membrane bound flavoprotein that catalyses the generation of O$_2$$^\cdot$, is dormant in resting neutrophils but is activated when the cells are exposed to bacterial targets.$^5$ 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$. 

**Figure 3**  Effect of stimulation of rat type II cells with 1 μM PMA (15 min) on generation of O$_2$$^\cdot$ and H$_2$O$_2$ 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).

**Figure 4** Increase in H$_2$O$_2$ 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 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).
and \(\text{H}_2\text{O}_2\) in rat alveolar type II pneumocytes. However, \(\text{O}_2^-\) 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 \(\text{O}_2^-\), generation compared with baseline levels. Moreover, \(\text{O}_2^-\) generation could be inhibited by the NADPH oxidase inhibitor DPI. These arguments almost completely exclude the possibility that \(\text{O}_2^-\) 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 a membrane bound 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 \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) production in the presence of the NADPH oxidase inhibitor DPI (>10 \(\mu\text{M}\)),\(^{24}\) and we found that the PKC inhibitor, GF109203x,\(^{25}\) also prevented the stimulatory effects of PMA. Stauropsinine 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 stauropsinine 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 \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) 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 \(\mu\text{M}\) of DPI increased \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) production while at 10 or 100 \(\mu\text{M}\) \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) production was decreased. As we have shown that 1 \(\mu\text{M}\) DPI efficiently inhibits NO production in type II cells (unpublished data), the increases in \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) generation at 1 \(\mu\text{M}\) could be due to a relative overproduction of \(\text{O}_2^-\) in the absence of the \(\text{O}_2^-\) scavenger NO. At higher concentrations of DPI, NADPH oxidase inhibition is probably more effective so that a decrease in \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) generation is found.

Stimulation of NADPH oxidase with the particulate stimulus zymosan led to marked increases in \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) generation. Because of this resemblance with neutrophils and macrophages, we exposed the type II cells to bacteria — a real physiological stimulus — and found that \(\text{H}_2\text{O}_2\) generation also increased severalfold. This increase in \(\text{H}_2\text{O}_2\) 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.\(^{8}\) 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 \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) 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.

The research reported herein was supported in part by grants from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek and by the European Union (BMH1-CT92-1229). B. Nemery is holder of the Dr P Tuytens leertaal in toxicologie.
NAD(P)H oxidase-like activity in type II cells