Oxidant-antioxidant balance in alveolar macrophages from newborn rats

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Oxidant-antioxidant balance in alveolar macrophages from newborn rats. C. Delacourt, M-P. d’Ortho, I. Macquin-Mavier, S. Pezet, B. Housset, C. Lafuma, A. Harf. ©ERS Journals Ltd 1996.

ABSTRACT: An oxidant-antioxidant imbalance in neonatal alveolar macrophages (AMs) may contribute to the increased susceptibility to lung injury described in the neonatal period.

We therefore evaluated oxygen radical production by rat AMs at various post-natal ages, and measured in parallel cellular antioxidant enzyme activities. AMs were obtained by bronchoalveolar lavage from rats aged <24 h, 21 days and 5 weeks, and results were compared to those obtained with adult rat AMs.

Intracellular production of oxygen radical species, estimated fluorometrically using 2',5'-dichlorofluorescein diacetate as the substrate, was significantly reduced in neonates as compared with adults, both in the presence and in the absence of cell stimulation with phorbol myristate acetate (PMA) or opsonized zymosan. A similar pattern was observed for the extracellular release of oxygen radical species, evaluated by lucigenin-enhanced chemiluminescence (CL) or peroxidase-catalysed CL oxidation of luminol: peak CL values measured after cell stimulation with PMA or opsonized zymosan remained significantly lower for AMs from newborn rats than for AMs from adults. By contrast, high values for antioxidant enzyme activities (superoxide dismutase and glutathione peroxidase) in AMs were demonstrated in newborns as compared to adults.

We conclude that high antioxidant activity in rat AMs after birth may be at least partly responsible for the low production of oxygen metabolites observed during the same period.

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Alveolar macrophages (AMs) represent the main cellular component of the defence system that maintains the integrity of the lower respiratory tract [1]. Macrophages modulate a variety of complex host functions, including immunoregulatory, phagocytic and secretory processes. Among the numerous secretory products of AMs are the reactive oxygen metabolites [2]. After in vitro stimulation of AMs, via either phagocytosis or specific or nonspecific stimuli, oxygen is reduced by the enzyme nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase to the superoxide anion (O2–), which quickly dismutates to hydrogen peroxide (H2O2) [1]. H2O2 can then either generate hydroxyl radical (OH·) in the presence of transition metals or be converted to hypochlorous acid (HOCl) by peroxidases [3]. These intermediate compounds are highly reactive and can cause cell injury and death by altering a variety of cell structures and functions. However, cells also contain antioxidant systems, whose effect is to eliminate O2 radicals and hydroperoxides with a potential for oxidizing crucial cellular structures [4]. Superoxide dismutase (SOD) catalyses the dismutation of O2- to H2O2; the latter compound is further detoxified to H2O by reactions involving glutathione peroxidase (GPX) and catalase.

Whether neonatal AMs are capable of mounting an adequate protective response to lung injuries has been questioned, since the neonatal lung is particularly exposed to impaired development and long-term sequelae after injuries, such as hyperoxia [5–7] or infections [8, 9]. Decreased oxidant production by neonatal macrophages has been found in some studies [10–12], but not in others [13]. Similarly, studies of AM intracellular antioxidants are contradictory, showing either low SOD activity [14] or high SOD activity [15] immediately after birth. Simultaneous estimation of both oxidant production and antioxidant enzyme activities in neonatal alveolar macrophages has not been performed. We therefore undertook this study to evaluate the oxidant-antioxidant balance in neonatal alveolar macrophages and its changes with postnatal age.

Methods

Reagents

Dulbecco’s modified Eagle Medium (DMEM) was obtained from Gibco Laboratories (Paisley, UK). 2',5'-dichlorofluorescein diacetate (DCFH-DA) was supplied...
by Molecular Probes Inc. (Eugene, OR, USA). All other reagents were supplied by Sigma (Saint Quentin Fallavier, France), unless otherwise stated.

Animals

Pregnant Sprague Dawley rats (Charles River, Saint Aubin-les-Elbeuf, France) were obtained at 17 days of gestation. They were housed in individual plastic cages until spontaneous delivery (term day=22 days of gestation). Newborn rats were then kept with their mother and tested during the first 24 h of life, and on Day 21 after birth, when major postnatal changes in lung structures are accomplished [16, 17]. Adult rats of either sex weighing 300–400 g were also tested.

Harvesting of alveolar macrophages

Alveolar macrophages (AMs) were obtained by in situ bronchoalveolar lavage (BAL) using a technique described previously [18]. Briefly, prior to BAL, rats were anaesthetized with 5 mg·100 g⁻¹ body weight (BW) of intraperitoneally-injected sodium pentobarbital and then killed by exsanguination. The thorax was opened wide to expose the lungs and trachea. A small length of tubing was inserted into the trachea and ligated. BAL was carried out using 8–10 separate aliquots of warmed saline (37°C). Volumes ranged from 0.2 mL per aliquot for newborns (6 g) to 2.5 mL per aliquot for adults. Lavage fluids from several animals were pooled to obtain a sufficient number of cells (from two rats at 21 days to 12 newborns (6 g) to 2.5 mL per aliquot for adults). Lavage fluids were centrifuged, resuspended in HBSS to a final concentration of normal rat serum for 30 min at 37°C. Opsonized zymosan was used as a stimulant. PMA was dissolved as a 10⁻⁵ M stock solution in dimethyl sulphoxide (DMSO) and stored at -80°C before use in enzymatic assays. Cell lysates were obtained using 0.1% Triton X-100 and contained 10⁵ AMs (final volume 300 µL) were harvested in a LKB-Wallac 1251 luminometer (Wallac Co., Turku, Finland) connected to a microcomputer. Two different luminescent probes were used: 1) O₂⁻ production was evaluated using lucigenin enhanced CL [21]. Lucigenin (5 mg) was dissolved in HBSS to obtain a 10⁻³ M solution. Further dilutions were in DMEM and the final concentration was 10⁻⁵ M; 2) H₂O₂ release was evaluated using peroxidase-catalysed CL oxidation of luminol [22, 23]. Luminol (5 mg) was suspended in DMEM to a final concentration of 10⁻⁴ M. Horseradish peroxidase (HRP) was added at a concentration of 0.2 U·10⁻⁵ cells to optimize the reaction between luminol and H₂O₂.

All procedures were performed in the dark. Each vial contained 10⁵ AMs (final volume 300 µL) and was placed in a luminometer at 37°C with continuous stirring. The luminescent probe was added first, and two measurements 1 min apart were obtained in the absence of stimulation. Either PMA (10⁻⁷ M) or zymosan (40 µg·10⁻⁵ cells) was then added, and measurements were performed every minute for 60 min. Controls were performed using AMs without PMA or zymosan. CL peak after stimulation was determined as the maximal increase above control value. Results were expressed as mV·10⁻⁵ cells.

To validate the chemical specificity of chemiluminescent probes under these experimental conditions, separate experiments were performed. Firstly, we compared in vitro the relative sensitivity of lucigenin and luminol to H₂O₂, by adding incremental doses of H₂O₂ (10 nM–100 mM) to the reaction mixture. Secondly, using adult rat AMs stimulated with opsonised zymosan, we tested the inhibitory effect of SOD (500 U) and catalase (1,000 U) on lucigenin and luminol-enhanced CL, respectively. Furthermore, the dependence of luminol-induced light emission under the presence of peroxidase was also tested.
Antioxidant enzyme activities

Peroxidases are the key enzymes in the redox cycle responsible for the reduction of hydroperoxides [4]. Experimental and clinical data suggest that the glutathione redox cycle is the most important antioxidant peroxidase system in mammalian species [24]. GPX activity was measured as described by PAGLIA and VALENTINE [25]. The assay mixture contained 1 mM glutathione (reduced), 150 µM β-NADPH, 1.5 U glutathione reductase, 200 µM t-butyl hydroperoxide and sample, in a final volume of 1.65 mL 50 mM Tris buffer, pH 7.6. Reaction rates were measured at 340 nm. One unit of GPX activity was measured as the reduction of 1 nmol hydroperoxide per min.

We also evaluated SOD which reacts specifically with O2-. SOD activity was determined by the SOD-525 kit (Bioxytech SA, Bonneuil sur Marne, France) whose characteristics have been described previously [26]. Briefly, the assay is based on the SOD-mediated increase in the rate of auto-oxidation of 5,6,6α,11β-tetra-hydro-3,9,10-trihydroxybenzo(c)fluorene (BXT-01050) in aqueous alkaline solution. This auto-oxidation yields a chromophore with a maximal absorbance wavelength of 525 nm, which is perfectly stable during the time of measurement. The optimized assay of SOD activity is performed at pH 8.8, 37°C, in 50 mM air-saturated 2-amino-2-methyl-1,3-propanediol buffer containing 3 mM boric acid and 0.1 mM diethylenetriamine penta-acetic acid. With such conditions, optimal assay sensitivity is achieved without affecting activities of known SODs such as Cu/Zn-, Mn-, or Fe-SOD. As the cyanide inhibition of Cu/Zn-SOD will lead to erratic results, it is recommended to perform the assay without added cyanide. The precision of this method was determined by performing 30 series of measurements under the same experimental conditions within a single day. The reproducibility was measured by performing the same experiment 3 days later, which again gave standard errors lower than 5% in the two experimental series. Results are expressed as U·10-6 AMs.

Data analysis and statistics

At least three pools of AMs were tested in each experiment. Results are expressed as mean ± SEM. Analysis of variance and Fisher’s protected least-squares difference test was used for statistical analysis of differences in oxidant or antioxidant production according to the age. A p-value of <0.05 was considered significant.

Results

Intracellular production of oxygen radical species

The fluorescent product was identified as DCF based on the fact that emission spectra of both reagent DCF and AMs preincubated with DCFH-DA were identical with 488 nm excitation, with a maximal emission wavelength at 520 nm.

Oxygen radical production patterns by AMs are shown in figure 1. AMs from neonatal rats (<24 h of life) produced significantly less oxygen radical species than adult AMs, both in the absence of stimulation ( ) or in the presence of 10-5 M PMA ( ) or opsonized zymosan ( ). Values are presented as mean±SEM. Tests were performed at three different ages: <24 h of life (n=3); 21 days of life (n=3); and adults (n=4). DCFH-DA: 2’5’-dichlorofluorescein diacetate; PMA: phorbol myristate acetate. *: p<0.05, as compared with adult values.

Chemiluminescence assays

Validation experiments. The sensitivity for H2O2 of peroxidase-catalysed CL oxidation of luminol was determined. Under our reaction conditions (10-4 M luminol, 0.2 U HRP, 300 µL), the lowest concentration of H2O2 that could be detected was 10 µM. A linear increase in light emission was observed when H2O2 concentrations were increased from 10 to 100 µM (fig. 2a). Catalase (1,000 U) completely inhibited the HRP-luminol-enhanced CL induced by 100 µM H2O2. When no HRP was added to the reaction mixture, 100 µM H2O2 did not induce any increase in chemiluminescence above the background level. From this dose-response curve, it could be estimated that higher CL peaks obtained in the present experimental conditions with adult rat stimulated macrophages always corresponded to H2O2 concentrations lower than 40 µM. We therefore verified that this H2O2 concentration range (10–100 µM) did not produce light when combined with lucigenin in our reaction conditions (10-5 M lucigenin, 300 µL). It was found that lucigenin produced light only when combined with 10 mM H2O2.
Thus, under the present experimental conditions, lucigenin-enhanced CL was not related to H$_2$O$_2$ production.

Finally, in experiments with adult rat AMs stimulated with opsonized zymosan, it was demonstrated that lucigenin-enhanced CL was totally inhibited by SOD (500 U) and that HRP-luminol-enhanced CL was inhibited by catalase (1,000 U) or by the absence of added peroxidase (fig. 2b and c).

**Lucigenin-enhanced CL: O$_2^-$ generation.** CL peak values obtained after stimulation increased gradually with age (PMA: $p<0.005$; zymosan: $p=0.0001$) (fig. 3). Mean CL peak was significantly lower for neonatal AMs than for adult AMs, both after $10^{-7}$ M PMA stimulation (1.4±0.1 versus 8.2±2.2 mV·10$^{-5}$ AMs, respectively), and after zymosan stimulation (4.8±2.0 versus 29.5±7.6 mV·10$^{-5}$ AMs, respectively). Since CL peak values for 21 day old rat AMs remained significantly lower than adult values, AMs from 5 week old rats were also tested. At this age, CL peaks after PMA or zymosan stimulation were 5.4±0.4 and 12.0±4.6 mV·10$^{-5}$ AMs, respectively. Although these values remained lower than adult values, the difference did not reach statistical significance.
SOD activity was 1.33±0.22 U·10⁻⁶ AMs in neonates and fell to 0.99±0.06 U·10⁻⁶ AMs at 21 days of age and to 0.12±0.05 U·10⁻⁶ AMs in adults. Similarly, GPX activities were 98.2±21.4, 40.6±4.0 and 28.1±5.8 U·10⁻⁶ AMs in cells from neonates, 21 day old rats, and adults, respectively.

By plotting PMA-induced CL responses both for O₂⁻ and H₂O₂ with the levels of SOD and GPX activities, respectively, we demonstrated an inverse relationship between CL response and antioxidant activity (fig. 6).

**Antioxidant enzyme activities in alveolar macrophages**

Gradual decreases in activity as postnatal age increased were observed for SOD (p=0.0064) and GPX (p=0.01) (fig. 5). SOD activity was 1.33±0.22 U·10⁻⁶ AMs in neonates and fell to 0.99±0.06 U·10⁻⁶ AMs at 21 days of age and to 0.12±0.05 U·10⁻⁶ AMs in adults. Similarly, GPX activities were 98.2±21.4, 40.6±4.0 and 28.1±5.8 U·10⁻⁶ AMs in cells from neonates, 21 day old rats, and adults, respectively.

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Discussion

A decreased ability of AMs to generate $O_2^-$ was reported during the neonatal period, and an inadequate production of oxygen radicals may contribute to the increased risk of pulmonary infection in neonates [11, 12]. However, the mechanisms underlying this decreased $O_2^-$ production remain to be established. Because spontaneous increases in antioxidant activity have been demonstrated in neonatal lung homogenates [27], we hypothesized that the low oxidant-generating capacity of neonatal AMs may be related to high levels of cellular antioxidant activity. Our estimations of oxidant production levels and antioxidant enzyme activities in rat AMs at various postnatal ages showed inversion of the oxidant-antioxidant balance in these cells with postnatal maturation. Increases in production of oxygen radical species and decreases in SOD and GPX activities occurred as postnatal age increased.

Among endogenous free radicals, some are generated and act intracellularly, whereas others are produced within the cell and are subsequently released into the surrounding area [28]. In our study, oxygen metabolite production in AMs was monitored both by chemiluminescence (release of free radicals) and by intracellular oxidation of DCFH (intracellular free radicals). We found a defective response to exposure to PMA or to opsonized zymosan particles in neonatal AMs with the chemiluminescence method, in keeping with results of earlier studies [10–12]. In addition, this study demonstrated that intracellular production of oxygen metabolites was also decreased during the neonatal period. This simultaneous depression both of extracellular and intracellular $O_2^-$ radical production in neonatal AMs argues for a common developmental control mechanism. Furthermore, the deficiency in $O_2^-$ radical production persisted in AMs from 21 day old rats and was perhaps also present in cells from 5 week old rats, since these were characterized by decreased light emission.

This defect in the $O_2^-$ radical production response has generally been ascribed to AM immaturity during early postnatal development [11, 12, 29], but our previous report of high gelatinase activity from neonatal rat AMs [18] suggests that cell immaturity, if present, may not affect all AM functions. Alternatively, the low capacity of neonatal AMs for producing oxygen metabolites may be due to the presence of inhibitory factor(s) in the surface lining material of airways, such as pulmonary surfactant [10]. However, this finding may be species-dependent, since pretreatment of rat AM with surfactant has been reported to increase their luminol-dependent CL response [30]. Thus, surfactant may not account for the defective AM response observed in young rats. From the present data, we propose that upregulation of intracellular antioxidant activity is, at least in part, responsible for the observed defective response in $O_2^-$ radical production.

Changes in AM antioxidant enzyme activities with postnatal age have been the focus of few investigations, and these have yielded conflicting results. STEVENS and AUTOR [15] reported high basal levels of both mitochondrial SOD and catalase in neonatal rat AMs. On the contrary, NERURKAL et al. [14] found low levels of SOD activity in rabbit AMs immediately after birth. To our knowledge, no data on GPX activity are available. Our results argue for a tight connection between the antioxidant enzyme activity and the presence of critical concentrations of oxygen metabolites in AMs (fig. 5).

These results are, at first sight, at variance with the report by SPEER et al. [13], who found similar oxygen metabolite production in neonatal and adult monocyte-derived macrophages in a study involving testing of macrophages after a long incubation period (7 days). However, as demonstrated by NERURKAL et al. [14], such a delay is associated with an overall loss in antioxidant enzyme activities in cells. We suggest that the key role of antioxidants is, paradoxically, further supported by the results of SPEER et al. [13].

Developmentally, a number of studies performed in several mammalian species have demonstrated that a rise in antioxidant enzyme activity was an important event in the preparation of the foetal lung for birth [27, 31–33]. At birth, lung cells are exposed to much higher levels of oxygen tension ($P_O_2$) than those experienced in the comparatively hypoxic in utero environment. In particular, in lung homogenates of late gestation foetal rats, a rapid increase in activity was demonstrated for the three pulmonary antioxidant enzymes, SOD (up to 150%), catalase (up to 350%) and GPX (up to 250%) [27]. The present study shows that a similar increase occurs in the components of the primary antioxidant defence system in AMs. Both SOD and GPX were elevated in AMs immediately after birth, and decreased to adult levels during the first weeks of life. In the absence of such an increase, exposure of alveolar cells to threefold to fourfold increase in alveolar $O_2$ tensions would be expected to result in a marked increase in production of intracellular $O_2^-$ radical [34, 35]. On the contrary, we found that the spontaneous intracellular production of oxygen radical species was considerably lower in neonatal AMs than in adult AMs. Furthermore, this lower $O_2^-$ radical production rate in neonatal AMs persisted after AM stimulation, demonstrating a strikingly different pattern for intracellular oxidant-antioxidant balance between neonates and adults. The effect of this antioxidant shield may be to protect newborns from cytotoxic injury on initiation of air breathing. However, there is no direct evidence indicating whether or not the intracellular oxidant-antioxidant imbalance in AMs contributes to pathological changes in neonatal lungs. Intracellular antioxidants produced by phagocytic cells may help to scavenge oxygen radical species released outside the cells, thus decreasing free radical-mediated tissue injury [36]. On the other hand, intracellular antioxidants may regulate the amount of oxygen metabolites released outside the cells in some situations, such as during respiratory bursts. Indeed, increasing the intracellular antioxidant defences of phagocytes with N-acetylcysteine has been shown to reduce the chemiluminescence response of phagocytes to opsonized zymosan [37]. The present data, demonstrating a similar pattern of AM response both for intracellular and extracellular oxygen metabolites support this hypothesis. In case of prematurity, appropriate antioxidant protection should not have been developed, thus rendering the premature lung prone to $O_2^-$ radical-induced toxicity [38].
The deficiency in $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ production demonstrated in AMs immediately after birth in this study persisted during the first weeks of life, suggesting that AMs acquire their full oxidant-releasing capacity only when major pulmonary structural changes are completed, i.e. when lung tissue is more resistant to oxidant injury. Within 3 weeks, the saccular lung present at birth undergoes dramatic changes, including remodelling of lung structure, thinning of the interstitial region, and production of new alveolar walls with supporting extracellular matrix. During this period, the production of oxygen species by rat AMs remains significantly lower than during adulthood. After the third week, when production of AM oxygen species nears adult values, the rat lung exhibits an essentially mature structure, although it continues to undergo maturational changes, including an increase in capillary volume [39]. Although this developmental pattern for AM oxidant production may be an important factor in normal lung development, it may also contribute to the physiological impairment of lung antibacterial defences characteristic of the neonatal period.

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