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Original Contribution

Lipid hydroperoxide formation regulates postnatal rat lung cell apoptosis and alveologenesis

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ABSTRACT

An acute increase in oxygen tension after birth imposes an oxidative stress upon the lung. We hypothesized that the resultant increase in reactive oxygen species, specifically lipid hydroperoxides, would trigger postnatal alveologenesis and physiological lung cell apoptosis in the neonatal rat. Neonatal rats were either untreated or treated daily with subcutaneous vehicle or diphenyl phenyl diamine, a scavenger of lipid hydroperoxides and inhibitor of lipid peroxidation, from day 1 to 6 of life. Alveolar formation and physiological lung cell apoptosis were assessed by morphometry, immunohistochemistry, and Western blot analyses on day 7 samples. Substitution experiments were conducted using the prototypic lipid hydroperoxide *t*-butylhydroperoxide. At a minimum effective dose of 15 μg/g body wt, treatment with diphenyl phenyl diamine resulted in a significant increase in tissue fraction and mean linear intercept and significant reductions in small peripheral blood vessels, secondary crest formation, lung and secondary crest cell DNA synthesis, and estimated alveolar number. Decreased numbers of apoptotic type II pneumocytes and mesenchymal cells, and decreased contents of proapoptotic cleaved caspase-3 and -7 and cytoplasmic cytochrome *c*, and an increase in antiapoptotic Bcl-xL were found in lungs treated with diphenyl phenyl diamine. A prevention of selected changes induced by diphenyl phenyl diamine was observed with concurrent treatment with intraperitoneal *t*-butylhydroperoxide, at a minimally effective dose of 187 μg/g body wt. We conclude that oxidative stress after birth induces lipid hydroperoxide formation, which, in turn, triggers postnatal alveologenesis and physiological lung cell apoptosis in the neonatal rat.

The human fetus has an arterial partial pressure of oxygen of ≈20 mm Hg, and that in the forming alveolar spaces will be even lower after diffusion across lung tissue into fetal lung liquid. After birth, cells at the gas–liquid interface experience a markedly increased partial pressure of oxygen to ≈140 mm Hg, and the arterial partial pressure of oxygen increases to ≈70 mm Hg in the first few hours after birth. The changes in oxygen tension around the time of birth result in an increased production of reactive oxygen species (ROS)[2] in lung tissue [1–3]. Given that ROS are now recognized to play critical roles as second messengers during various biological processes, including DNA synthesis, apoptosis, and vasculogenesis [4–6], we hypothesized that the increase in intrapulmonary ROS occurring around the time of birth is the trigger for the onset of alveolarization in rodents such as rats and mice, in which alveolar formation is an entirely postnatal event [7].

In support of our hypothesis, that increased intrapulmonary ROS formation around the time of birth triggers postnatal alveologenesis in rodents, we have previously reported [8] that treatment with a 21-aminosteroid antioxidant impaired lung DNA synthesis in air-exposed rat pups. In a subsequent series of studies, we also observed that increasing the inspiratory oxygen concentration from 21 to 60%, under conditions in which inflammatory responses were suppressed [9–12], stimulated lung cell DNA synthesis and alveolar formation in the neonatal rat.

The process of alveolarization commences on day 3 of life in rats and occurs by the in-growth of secondary crests into large precursor saccules, subdividing them into smaller alveoli [13]. An integral part of alveolar formation is a physiological apoptosis, which serves to remove excess cells to optimize gas exchange [14,15]. Increased ROS formation is a well-recognized activator of apoptosis [16].

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[2] *Abbreviations used*: Bax, Bcl-2-associated X protein; Bcl-xL, B-cell lymphoma extra large; BrdU, bromodeoxyuridine; DPPD, diphenyl phenyl diamine; FGF-2, fibroblast growth factor-2; FGF-R1, fibroblast growth factor receptor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T1α, T-1α protein (podoplanin); ROS, reactive oxygen species; PBS, phosphate-buffered saline; *t*-BuOOH, *t*-butyl hydroperoxide.

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Our initial observations using a 21-aminosteroid antioxidant intervention [8] were consistent with, but not conclusive of, lipid hydroperoxides being critical mediators of lung growth, nor had we specifically addressed the question of whether the antioxidant had impaired the process of alveolar formation. In the studies described below, we have used diphenyl phenyl diamine (DPPD), which is both an inhibitor of lipid peroxidation [17,18], perhaps through its reaction with ferric iron [19], and a scavenger of the products of lipid peroxidation [20,21], as an intervention to assess the role of lipid hydroperoxides in initiating both alveologenesis and physiological apoptosis.

Materials and methods

Cell culture

Primary cultures of day 19 rat fetal distal lung epithelial cells were prepared as previously described [22–24] and then maintained in 48-well culture plates at a PO_2 of 20 mm Hg to match the in utero fetal arterial PO_2, with phenolphthalein-free Dulbecco's modified Eagle medium and 10% (vol/vol) fetal bovine serum, until approximately two-thirds confluent. Cell monolayers were washed thoroughly and placed in serum-free medium for 24 h before the addition of *t*-butylhydroperoxide (*t*-BuOOH) for a further 24 h in serum-free culture. For assays of cytotoxicity, cells were preincubated for 2 h with 0.2 μCi/ml [8-^{14}C]adenine (Sigma–Aldrich, Oakville, ON, Canada) before being washed and the addition of *t*-BuOOH. We have previously reported that release of preincorporated [8-^{14}C]adenine correlates well with other cytotoxicity assays, such as release of cellular lactate dehydrogenase and trypan blue exclusion in these cells [25,26]. For the assessment of cell DNA synthesis, 0.5 μCi/ml [^{3}H]thymidine (MP Biomedical, Irvine, CA, USA) was added to the medium at the same time as the addition of *t*-BuOOH. At the end of the 24-h incubation, the incorporation of [^{3}H]thymidine into cell DNA [27] was assessed and expressed as a percentage of that incorporated by control cells.

In vivo interventions

Animal studies were performed according to criteria established by the Canadian Council on Animal Care and were approved by the Hospital for Sick Children Research Institute Animal Care Review Committee. Ten to twelve newborn rat pups from each of four different litters either were uninjected or received daily subcutaneous injections of DPPD in corn oil (15 μg/g) or corn oil alone (5 μl/g), from day 1 to 6 of life, into the nape of the neck via a 30-gauge needle. For *t*-BuOOH substitution experiments, pups were either uninjected or injected with DPPD in corn oil or corn oil alone, as above, but additionally received daily intraperitoneal injections, via a 30-gauge needle into the right iliac fossa, of *t*-BuOOH or saline vehicle (5 μl/g). Pups were killed on day 7 of life by inhalation of ethyl ether.

Total 8-isoprostane measurements

Total (free and esterified) 8-isoprostane (8-iso-prostaglandin $F_{2\alpha}$) content was measured as previously described [12]. An enzyme-linked immunosorbent assay kit for 8-isoprostane was from Cayman Chemical Co. (Ann Arbor, MI, USA).

In vivo DNA synthesis

For assessment of cells undergoing DNA synthesis, pups received intraperitoneal injections into the right iliac fossa of bromodeoxyuridine (BrdU) at 40 μg/g 2 h before removal of organs. A BrdU *In-Situ* Detection Kit (BD Biosciences Pharmingen, San Diego, CA, USA) was used for immunohistochemical staining of BrdU, as previously described [28].

Western blot analyses and immunoprecipitation

Excised lungs were homogenized and lysed in RIPA buffer containing phosphatase and protease inhibitors (Roche Applied Science, Laval, QC, Canada). Supernatants of lung tissue lysates, representing the cytoplasmic fractions, were stored at −80 °C. For analysis of cytoplasmic cytochrome *c*, samples were centrifuged at a relative centrifugal force of 20,817 at 4 °C for 30 min. Protein content was measured as described by Bradford [29]. Membranes were incubated overnight at 4 °C with 1:1000 dilutions of rabbit polyclonal antibodies to cleaved caspase-3, caspase-7, or Bax (Cell Signaling Technology, Boston, MA, USA) or 1:100 dilutions of mouse monoclonal antibodies to rat cytochrome *c* and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As an internal control, α-catenin was detected using a 1:4000 dilution of rabbit anti-α-catenin (Sigma). After being washed, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-mouse antibody (Calbiochem–Novabiochem, San Diego, CA, USA) at 1:10,000 dilution or goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) at 1:10,000 for 90 min. For immunoprecipitation of fibroblast growth factor receptor-1 (FGF-R1), rabbit anti-human FGF-R1 antibody (Santa Cruz Biotechnology) was added to lysates (2 μg/500 μg protein) and incubated at 4 °C overnight. Packed protein A/G agarose (40 μl beads/500 μg protein; Santa Cruz Biotechnology) was then added and incubated at 4 °C for 4 h with agitation. Agarose beads were boiled for 5 min in SDS sample buffer, before being loaded onto polyacrylamide gels. Membranes were incubated overnight at 4 °C with a 1:500 dilution of mouse monoclonal antibody to phosphotyrosine (Santa Cruz Biotechnology). Integrated band densities were calculated after subtraction of background values, as previously described [30]. Supernatants from immunoprecipitated samples were saved for immunoblotting analysis using a 1:500 dilution of rabbit polyclonal antibody to GAPDH (Santa Cruz Biotechnology) as an internal control.

Histochemistry and immunohistochemistry

While undergoing manual inflations, lungs were flushed with phosphate-buffered saline (PBS) containing 1 U/ml heparin, to clear the pulmonary circulation of blood. Lungs were then perfusion-fixed over 12 h using 4% (wt/vol) paraformaldehyde in PBS or, for immunodetection of BrdU, 10% (wt/vol) neutral-buffered formalin, while inflated with air under a constant airway pressure of 20 cm water. Elastin was identified using Hart's elastin stain. Tissue sections were stained using Weigert's resorcin–fuchsin and counterstained with tartrazine, as previously described [12]. An avidin–biotin–peroxidase complex method [31] was used for immunostaining. Slides were incubated with the primary antibody overnight at 4 °C. After the slides were incubated with biotin-conjugated secondary antibody for 1 h, the labeled Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA) was used with 3,3-diaminobenzidine (SK 4100, Peroxidase Substrate kit, DAB; Vector Laboratories) as a substrate. Hematoxylin was used as the nuclear counterstain. Slides were mounted in Permount mounting medium. A rabbit polyclonal antiserum against human cleaved caspase-3 (Cell Signaling Technology) was used at a 1:200 dilution. A goat polyclonal antibody to murine cleaved caspase-7 (Santa Cruz Biotechnology) was used at a dilution of 1:200 (vol/vol). We have previously reported [24] the specificity of these antibodies. Sections that were