Developmental regulation of DUOX1 expression and function in human fetal lung epithelial cells

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Fischer H, Gonzales LK, Kolla V, Schwarzer C, Miot F, Illek B, Ballard PL. Developmental regulation of DUOX1 expression and function in human fetal lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 292: L1506–L1514, 2007. First published March 2, 2007; doi:10.1152/ajplung.00029.2007.—The purpose of this study was to determine the expression and cellular functions of the epithelial NADPH oxidase DUOX1 during alveolar type II cell development. When human fetal lung cells (gestational age 11–22 wk) were cultured to confluency on permeable filters, exposure of cells to a hormone mixture (dexamethasone, 8-Br-cAMP, and IBMX, together referred to as DCI) resulted in differentiation of cells into a mature type II phenotype as assessed by expression of lamellar bodies, surfactant proteins, and transepithelial electrical parameters. After 6 days in culture in presence of DCI, transepithelial resistance (2,616 ± 529 Ω·cm−2) and potential (−8.5 ± 0.6 mV) indicated epithelial polarization. At the same time, treatment with DCI significantly increased the mRNA expression of DUOX1 (~21-fold), its maturation factor DUOX1 (~12-fold), as well as DUOX protein (~12-fold), which was localized near the apical cell pole in confluent cultures. For comparison, in fetal lung specimens, DUOX was not detectable at up to 27 wk of gestational age but was strongly upregulated after 32 wk. Function of DUOX1 was assessed by measuring H2O2 and acid production. Rates of H2O2 production were increased by DCI treatment and blocked by small interfering RNA directed against DUOX1 or by diphenylene iodonium. DCI-treated cultures also showed increased intracellular acid production and acid release into the mucosal medium, and acid production was largely blocked by knockdown of DUOX1 mRNA. These data establish the regulated expression of DUOX1 during alveolar maturation, and indicate DUOX1 in alveolar H2O2 and acid secretion by differentiated type II cells.

nicotinamide adenine dinucleotide phosphate oxidase; alveolar type II cells; hydrogen peroxide; intracellular pH

THE NADPH OXIDASE IS A PROMINENT mechanism of innate defense best described in professional phagocytes where NADPH oxidase-derived superoxide (O2–•) in the phagosome is critically important for bacterial killing (42). A number of isoforms of the phagocytic NADPH oxidase have been identified in non-myeloid cell types, resulting in the recognition of the NOX gene family consisting of five small (NOX1 to NOX5) and two large members (DUOX1 and DUOX2). All of these genes encode membrane proteins that share the structural characteristics of intracellular NADPH and FAD bindings sites and two membrane-embedded hemes that support electron transfer from intracellular NADPH to extracellular oxygen (5). NADPH oxidase activity further results in the production of intracellular H+ (from NADPH; Ref. 14).

Two DUOX isoforms were initially identified and cloned from the thyroid (12, 15) where DUOX is expressed in the apical membrane and provides follicular H2O2 for the oxidation of iodide to iodine during the synthesis of thyroid hormone (11). Apart from the thyroid, DUOX has been mainly found in epithelial tissues that are continuously exposed to microbes, where DUOX is considered to function as a component of the innate defense barrier. DUOX was found to be expressed in rectal mucosa (22), colon (16), the entire intestinal tract (17), salivary glands (22), airways (20, 22, 40), and the lung (16). The DUOX1 and DUOX2 proteins are 77% identical and 83% similar; however, the specific roles for these highly similar proteins are currently unclear. Recently, two endoplasmic reticulum (ER)-associated DUOX maturation factors (DUOX1A and DUOX2A) were identified that were required for normal translocation of DUOX1 and DUOX2 protein, respectively, to the plasma membrane (24).

Although the primary product of the small NOX homologs is O2–•, DUOX has been found consistently to produce H2O2 and not O2–• (1, 11, 20, 22). In previous studies, isolated rat alveolar type II cells have been shown to release substantial amounts of H2O2 that were partially identified as NADPH oxidase mediated (27, 46). In the current study, we sought to identify the NADPH oxidase expressed by human alveolar cells to determine its developmental and hormonal regulation and to investigate its cellular function. This was investigated in a cell culture model of human primary fetal epithelial cells that were differentiated into an adult type II cell phenotype by hormone treatment. We found that hormone-differentiated alveolar type II cells showed upregulated DUOX1 in vitro and, similarly, during gestational development shortly before birth in vivo. DUOX1 expression resulted in release of H2O2 and acid into the mucosal medium of differentiated cultures. We propose that the developmental upregulation of DUOX1 before birth prepares the lung host defenses for extraterine exposure to pathogens.

MATERIALS AND METHODS

Cell culture. Human fetal lung tissues of 11–22 wk gestation abortuses were obtained from Advanced Bioscience Resources (Alameda, CA) and/or the Birth Defects Laboratory of the University of California, Oakland, CA 94609-1673 (e-mail: hfischer@chori.org).

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of Washington (Seattle, WA). Lung epithelial cells of ∼88% purity (47) were isolated and cultured as previously described (23) under approved institutional review board protocols. Briefly, the tissue was digested with trypsin, collagenase, and DNase, fibroblasts were removed by differential adherence, and nonadherent cells were plated on transparent permeable filters (12-nm Costar Snapwell, 0.4 μm pore size; Corning) at a density of ∼0.5–0.75 × 10^6 cells per filter in Waymouth’s medium supplemented with 10% fetal calf serum. After overnight culture (day 1), attached cells were cultured an additional 3–9 days in serum-free Waymouth’s medium alone (control) or with addition of DCI (10 nM dexamethasone, 0.1 mM 8-Br-cAMP, 0.1 mM IBMX) to induce type II cell differentiation (23). Transepithelial resistance (Rt) and voltage (Vt) were measured daily under sterile conditions using a two-electrode volt-ohm-millimeter (EVOM, World Precision Instruments) as previously described (26); Vt is reported as referenced to the serosal side. Determination of the developmental expression of DUOX was done on banked tissue samples of pre- and postnatal lungs that were obtained through Advanced Bioscience Resources, the Birth Defects Laboratory of the University of Washington, and the Children’s Hospital of Philadelphia Department of Pathology.

Staining for differentiation markers. Alveolar cultures grown on permeable support in absence or presence of DCI were used. Differentiation of cells was assessed using the lipid stain Nile red (0.1 mg/l) and antibodies against surfactant proteins B (SP-B) and C (SP-C). For immunocytochemistry, cells were fixed with cold methanol and immunostained with antibodies for SP-B (1:100 dilution rabbit anti-bovine SP-B, Chemicon) and SP-C (Byk Gulden rabbit anti-SP-C antibody) as previously described (37).

Molecular expression of DUOX mRNA. Total RNA was prepared from 2 × 10^6 cells grown on permeable filter supports, and poly(A) mRNA was isolated using the RNasy kit and Oligotex mRNA kit (Qiagen, Valencia, CA). RT-PCR was performed using 100 ng of mRNA, 40 units of reverse transcriptase, 20 units of RNase inhibitor, 5 μg of random hexamer oligonucleotides (Applied Biosystems) for 60 min at 42°C. Real-time PCR was performed on an ABI Prism cycler using SYBR green or TaqMan reporters designed to detect human DUOX1, DUOX2, and DUOXA1. Oligonucleotide primers for DUOX1, DUOX2, and GAPDH were as previously described (40); primers for DUOXA1 were forward 5'-tggggtgatcaacctac3' and reverse 5'-cctgtaatgcttctacaggc3'. mRNA detection was performed compared with the GAPDH gene using the difference of PCR cycles to reach a threshold amplification and by relating treatment groups to controls. Standard curves were generated for both target and endogenous control genes using serial dilutions of cDNA using ABI reagents, 0.4 μM forward and reverse primers, and 50 ng of cDNA. To control for specific PCR products, a dissociation curve was generated after the end of the last cycle. Because ratios of mRNA levels of treatment-to-control varied considerably between experiments, averages were calculated from log-transformed ratios.

Immunodetection of DUOX. Total cell extract protein (20 μg) was separated by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membrane and then blocked for 2 h with 5% powdered milk. 40 mM Tris, pH 7.5, 0.1% Tween 20. A polyclonal antibody raised against DUOX1 (11) was used for immunoblots. Although this antibody has a slightly higher affinity for DUOX1 than for DUOX2, it cannot be used to distinguish between the two isoforms, and thus detected protein is referred to as DUOX to indicate this fact. DUOX antibody (1:2,000) was added for overnight incubation at 4°C. After washing, the mouse monoclonal anti-GAPDH antibody (1:20,000) was added for 1 h, the blot was washed, and the secondary antibodies (1:10,000, goat anti-rabbit IgG-infrared dye 800; 1:10,000, goat anti-mouse IgG-Alexa Fluor 680) were added for 1 h. Stained proteins were examined with the Odyssey Infrared Imaging System. For immunocytochemistry, cells grown on permeable support were fixed with 2% paraformaldehyde and incubated with 0.3% (vol/vol) Triton X-100 followed by 1% (wt/vol) BSA in PBS. Cells were immunostained for DUOX and the tight junction protein ZO-1 using a monoclonal anti-ZO-1 antibody (BD Biosciences). Secondary antibodies were Alexa Fluor 488 anti-rabbit (Molecular Probes) for DUOX and Alexa Fluor 546 anti-mouse for ZO-1. Cells were embedded in Crystal/Mount (Biomedia) and observed with a ×63 (1.4 numerical aperture) oil-immersion objective on a spinning disc confocal microscope (Solamere Technology Group).

Measurement of H2O2 release. Production of H2O2 was measured using Amplex Red in presence of horseradish peroxidase (Molecular Probes). Fetal lung cells were seeded in 96-well plates and incubated with or without DCI for 4 days. Then, cells were incubated in 50 μl of glucose-supplemented PBS solution containing 50 μM Amplex Red reagent combined with 0.1 U/ml of horseradish peroxidase. Amplex Red fluorescence was measured at 37°C in a microplate reader (Wallac Victor2 multilabel counter) and excited at 530 ± 20 nm, and emission was measured at 590 ± 10 nm. Each experimental condition was performed at least in quadruplicate. A calibration curve was performed for every time point to correct for time dependence. Epithelial area in each well was 0.316 cm², and values are reported as pmol cm⁻².

Measurement of intracellular pH. Cells grown on transparent permeable filters were incubated for 30 min with 20 μM BCECF-AM (Molecular Probes), washed, and allowed to cleave the dye for 60 min. Cellular fluorescence was measured in an open perfusion chamber on an inverted microscope exactly as previously described (40). Briefly, excitation wavelengths were 440 and 495 nm and emission was collected between 525 and 550 nm. Intracellular pH (pHi) was calculated from the calibration of emission intensity ratios of 495-to-440 nm excitation using a four-point pH calibration in presence of 10 mM nigericin in KCl solution. Dye loading and experiments were done at 26°C. NaCl solution composition was (in mM) 145 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 12.5 HEPES, pH 7.4. pH calibration was performed in (in mM) 5 NaCl, 145 KCl, 1 CaCl₂, 1 MgCl₂, and HEPES and/or MES (12.5 mM total buffer).

Measurement of acid secretion. Acid secretion was measured using the pH stat titration technique in an Ussing chamber as described previously (19). Briefly, cultures were bathed serosally with HEPESTM buffered solution and mucosally with buffer-free solution (3 ml each). Solutions were constantly gassed with oxygen and were nominally free of HCO₃⁻/CO₂. Standard NaCl Ringer solutions contained (in mM): mucosal, 140 NaCl, 2 KCl, 15 glucose, 2 CaCl₂, 1 MgCl₂; serosal, 140 NaCl, 2 KCl, 5 glucose, 10 HEPES, 1 MgCl₂, 140 NaCl, pH 7.3 with HCl/NaOH. During the experiment, the pH of the mucosal solution was continuously measured and titrated (with 10 mM NaOH) to a target value of pH 7.3. Epithelial area of measurement was 1 cm². Experiments were done at 37°C.

Transfection with small interfering RNA. Three DUOX1-specific oligonucleotide small interfering RNAs (siRNAs) obtained from Ambion (Austin, TX) were designed to bind positions 2598 to 2622, 2725 to 2749, and 2884 to 2912. siRNAs are referred to here by their first nucleotide binding position, i.e., 2598, 2725, and 2884. A scrambled sequence of 2598 was used as control. Human fetal lung epithelial cells were isolated, and 3 × 10⁶ cells were either mock-transfected (i.e., no siRNA) or transfected with siRNAs (150 nM each) by electroporation using a nucleofector device (Amazxa Biosystems). Subsequently, cells were plated and allowed to adhere for 8–12 h, treated with DCI, and further cultured for 3–4 days.

Statistics. Data are presented as original values or as means ± SD; n refers to the number of cultures investigated. Comparison between two treatment groups was done using t-tests. Effect of multiple treatments was tested using ANOVAs followed by Holm-Sidak corrected t-tests. Relation between parameters was tested by linear regression analysis. Statistical testing was done using StatView (version 4.57, Abacus Concepts) or SigmaStat (version 3.5, Systat Software). Resulting P values are given, and P < 0.05 was considered significant.
RESULTS

Treatment of undifferentiated fetal lung epithelial cultures with DCI results in a mature epithelial phenotype. Previously, we (10, 23, 47) have shown that treatment of fetal epithelial cultures on plastic with DCI (10 nM dexamethasone, 0.1 mM 8-Br-cAMP, 0.1 mM IBMX) resulted in an upregulation of genes involved in surfactant production and secretion, epithelial transport, and junctional formation. Here, we investigated the phenotypical effects of DCI treatment on the formation of differentiated epithelia when cultured on permeable supports. Differentiation was assessed by lamellar body formation, surfactant expression, and the formation of high-resistance epithelia, which are typical markers of mature alveolar type II cell cultures. Cells on permeable supports were cultured in the absence or presence of DCI for 4–6 days. Figure 1, A–F, illustrates the typical staining patterns observed in control (Fig. 1, A, C, and E) and DCI-treated cell cultures (Fig. 1, B, D, and F). Staining with the lipid dye Nile red showed diffuse staining in control cultures (Fig. 1A), whereas DCI treatment resulted in enhanced staining of large intracellular inclusions typical for lamellar body staining (Fig. 1B). Similarly, immunostaining for SP-B and SP-C resulted in well-defined SP-B and SP-C staining of inclusions in the cell periphery typical for lamellar body staining (Fig. 1, D and F).

In addition, epithelial formation and polarization was assessed by measuring $R_t$ and $V_t$. Figure 1, G and H, shows the time course of $R_t$ and $V_t$, respectively, in control vs. DCI-treated cultures over a period of 6 days in culture. DCI-treated cultures consistently increased $R_t$ and $V_t$ during the growth period. At day 6, DCI-treated cultures reached $R_t = 2.616 \pm 0.005 \mu \Omega \cdot \text{cm}^2$ and $V_t = -8.5 \pm 0.6 \mu \text{V}$ ($n = 6$), indicative for tight, confluent, and polarized epithelia exhibiting active vectorial ion transport. In contrast, cells cultured in the absence of DCI initially formed resistive monolayers, but both $R_t$ and $V_t$ declined after 2–3 days in culture; visual examination of these cultures confirmed that cells covered the entire surface of the cell culture insert, so that this failure to form a resistive cell layer could not be attributed to impaired spreading and confluence. These data show that the treatment of undifferentiated human fetal alveolar cultures with DCI resulted in a phenotype typical for adult alveolar type II epithelia.

DCI treatment upregulates DUOX1 mRNA and protein expression. Our (47) previous gene array study indicated that DUOX1 transcript was expressed and upregulated in fetal alveolar cells when treated with DCI, whereas DUOX2 was not detected. This was further investigated using real-time PCR (Fig. 2) on cultures grown to confluence on permeable supports with and without DCI for 4 days. DCI treatment resulted in a substantial upregulation of DUOX1 mRNA expression compared with untreated controls (Fig. 2A, closed circles; $P = 0.005$). For comparison, the expression of the highly similar isofrom DUOX2 was low and was not affected by DCI treatment (Fig. 2A, open circles). We also tested whether incubation of cultures with dexamethasone alone or with 8-Br-cAMP plus IBMX exerted similar effects and found no significant induction of DUOX1 mRNA (not shown).

In a second set of experiments (6 cultures per group), cultures were treated with DCI (compared with untreated controls) and were investigated for mRNA expression of both DUOX1 and its maturation factor, DUOXA1 (24). DUOXA1 is an ER-resident transmembrane protein necessary for the ER-to-Golgi transition of DUOX1 protein and thus for proper maturation and expression at the plasma membrane (24). Treatment of cultures with DCI resulted in a closely correlated induction of DUOX1 and DUOXA1 mRNA expression (Fig. 2, B and C).

Protein expression was investigated using an antibody raised against DUOX1 (12). Figure 3 shows immunoblotting and immunocytochemistry from control and DCI-treated cultures. All...
though the antibody does not distinguish well between DUOX1 and DUOX2 protein (and is thus referred to here as DUOX antibody), the detected protein found in these blots is most likely DUOX1 since the mRNA expression of DUOX2 was unchanged in DCI-treated cultures (Fig. 2A), but the detected DUOX protein induction correlated well with DUOX1 mRNA (cf., Fig. 3D). Previously, DUOX1 has been shown to generate a fully glycosylated product at \( \sim 185 \text{ kDa} \) in Western blots (12). Figure 3A shows immunoblots from cultures isolated from four different donor lung cell preparations (labeled lungs 1–4) cultured in the absence or presence of DCI for 5 days. Protein recognized by the DUOX antibody is shown in green, and for comparison, GAPDH protein is shown in red in Fig. 3A. Bands at 185 kDa were significantly increased in DCI-treated cultures in each lung preparation. Quantification of the 185-kDa bands in this blot indicated a 5- to 59-fold increase in

![Figure 2](http://ajplung.physiology.org/)

Fig. 2. Expression of DUOX1 and DUOX1 mRNA is increased by DCI treatment. A: expression of DUOX1 and DUOX2 by real-time PCR. Treatment with DCI increased expression of DUOX1 \( (P = 0.005) \) but not DUOX2 \( (P = 0.051; n = 3 \) per symbol). B and C: expression of both DUOX1 and its maturation factor, DUOX1, was upregulated by DCI and correlated tightly (regression line in C, slope = 0.66 ± 0.23, \( r^2 = 0.68, ^*P = 0.045 \)); note the log scaling of axes. Dashes in B represent the logarithmic means of data (circles). \( \Delta C_T \), threshold amplification.

![Figure 3](http://ajplung.physiology.org/)

Fig. 3. DCI treatment induces DUOX protein expression in human fetal lung epithelial cells. A: immunoblots of cell cultures prepared individually from 4 different lungs (17–20 wk gestation) were cultured in the absence or presence of DCI (as indicated); green, DUOX antibody staining; red, GAPDH protein staining. Densitometric analysis resulted in treatment effects of DCI (compared with control) on DUOX expression of lung 1 (58), lung 2 (178), lung 3 (598), and lung 4 (58). B: immunostaining of cultures for ZO-1 (red) and DUOX (green). Cells were grown on permeable transparent supports in presence or absence of DCI. Confocal images are focused on the apical pole (i.e., the ZO-1 signal) of the cell layers. Original magnification was \( \times 600 \). Images are merged pseudo-color images with the ZO-1 signal occupying the red channel and the DUOX signal the green channel of a red-green-blue-coded image. C, bottom: induction of DUOX protein and DUOX1 mRNA by DCI treatment over time; top: protein blots at times (in hours) as indicated, with “C” denoting time controls at 8 and 72 h. D: correlation of DCI induction of DUOX protein and mRNA expression (slope = 0.33 ± 0.05, \( r^2 = 0.89, P = 0.001 \)).
DUOX expression after DCI treatment. The DUOX antibody also recognized a lower-weight band at ~120 kDa, for which expression was similarly regulated in DCI-treated cells. A corresponding 100- to 120-kDa band was previously found in thyroid gland (F. Miot, unpublished results) and might be a degradation product of DUOX1. This band was not consistently present (e.g., see Fig. 3C, top) and was not further investigated.

Expression and localization of DUOX in alveolar cultures were investigated using confocal microscopy on confluent cultures grown on permeable supports. Cultures were stained for DUOX and for ZO-1 as a marker for the junctional rings and the apical cell pole. In the images shown in Fig. 3B, the confocal plane was focused on the ZO-1 signal (red). Cultures treated with DCI for 4 days showed prominent DUOX staining (green). In untreated control monolayers, the DUOX signal was weak, diffuse, and nonspecific.

The expression of DUOX1 mRNA and protein correlated closely over time of DCI treatment (Fig. 3C, bottom). Isolated undifferentiated human fetal lung epithelial cells were cultured for 3 days in control media, then DCI was added, and cells were harvested at various times of hormone exposure (4–72 h). DUOX1 mRNA increased by ~4–8 h of treatment and continuously increased at later times. The increase in DUOX protein content was delayed relative to DUOX1 mRNA but then also increased after 24–72 h. Figure 3C, top, shows staining intensities of the corresponding immunoblots with untreated controls (labeled “C”) shown for 8- and 72-h time points. Figure 3D shows the data from Fig. 3C, bottom, replotted to display the correlation between DUOX protein and mRNA induction during DCI treatment (slope = 0.33 ± 0.05, r² = 0.89, P = 0.001).

DUOX expression in fetal lung tissue is developmentally regulated and increases before birth. For comparison and as a control for the upregulation of DUOX1 in vitro cell cultures, the developmental expression of DUOX was investigated in human peripheral lung tissue specimens of 15–32 wk of gestational age and in adult tissues using immunoblotting (Fig. 4). No DUOX-specific bands were detected in 15- to 27-wk gestational age lungs. Substantial signals were found in a 32-wk gestational age specimen and in two adult specimens (Fig. 4). No other specimen of gestational age older than 32 wk became positive for DUOX and for ZO-1 as a marker for the junctional rings. The 2725 siRNA was particularly effective and was further utilized for functional studies. For comparison, and to test for any effects of the scrambled siRNA, cells were also treated using the identical electroporation and culture procedure but without siRNA (mock), which resulted in DUOX1 expression similar to the treatment with scrambled siRNA.

Figure 5B shows H2O2 production into the extracellular medium. All cultures, treated or not, continuously released H2O2 into the medium. This baseline signal was not observed in the absence of cells (data not shown). Treatment with DCI significantly increased H2O2 release resulting in a rate of 196 ± 21 pmol·h⁻¹·cm⁻². DCI-stimulated H2O2 release was blocked in cells treated with DUOX1 siRNA or by the NADPH oxidase blocker diphenylene iodonium (DPI, 5 μM; Fig. 5B). Thus DCI-treated cells express a functional DUOX1-based NADPH oxidase activity that generates H2O2 in the extracellular medium. Baseline H2O2 generation in untreated cells may represent other H2O2 sources (such as mitochondrial activity) because it was insensitive to DUOX1 siRNA. DPI, on the other hand, blocks several flavoenzymes (34) including the mitochondrial NADH dehydrogenase complex I of the mitochondrial respiratory chain (7), which may represent one reason for the additional blocking effect of DPI compared with treatment with DUOX1 siRNA.

DCI-stimulated expression of DUOX1 results in increased intracellular acid production. DUOX1 function is expected to acidify the pH owing to its NADPH oxidase activity (14, 40). To release intracellularly produced acid, adult rat alveolar type II cells were shown to express basolateral Na⁺/H⁺ exchanger (28) and apical H⁺ channel activities (13). Both are activated by intracellular acidification (8, 36). pH₁ was recorded from BCECF-loaded cells grown to confluency on transparent, permeable supports. Cells within a confluent monolayer were monitored individually, and readings from 15 to 20 cells were averaged for each experiment. Figure 6A shows pH₁ recordings from control and DCI-treated cells, and Fig. 6B shows average steady-state pH₁ values before and after treatment with blockers. Initial pH₁ was similar in DCI-treated and untreated cells (on average, pH₁ = 7.31 ± 0.28). Addition of 1 mM amiloride (to block the Na⁺/H⁺ exchanger) and the subsequent addition of 100 μM ZnCl₂ (to block H⁺ channels) significantly acidified DCI-treated cells but showed no effects in control cells (Fig. 6, A and B). The effect of DUOX1 knockdown by 2725 siRNA on intracellular H⁺ generation was measured in DCI-treated cells...
Electroporation with DUOX1 siRNA had no measurable effect on baseline pH (mock, 7.44 ± 0.10; siRNA, 7.27 ± 0.19; P = 0.19). However, addition of amiloride plus ZnCl₂ resulted in a significantly larger acidification in cells treated with scrambled siRNA compared with DUOX1 siRNA-treated cells. These data indicate that DUOX1 activity in DCI-treated cells acidified pH. Intracellular acid was continuously released by the active Na⁺/H⁺ exchanger and H⁺ channels in DCI-treated alveolar cultures.

**DISCUSSION**

**Expression of DUOX1 in alveolar cells.** In this study, we investigated the expression and function of the NADPH oxidase isoform DUOX1 in the alveolar epithelium. Using a hormonally induced primary epithelial cell culture model of human alveolar epithelium, we found increased DUOX1 expression, H₂O₂ release, and acid production in vitro during differentiation of precursor cells to epithelial alveolar type II cells, and in vivo the expression of DUOX was upregulated at gestational age of 32 wk.

DUOX1 and DUOX2 are highly similar proteins (83% sequence similarities; Ref. 12). We focused our study on DUOX1 based on our (47) previous observation in a microarray study that DUOX1 mRNA expression was increased during alveolar type II cell differentiation. This observation was further confirmed in the current study using quantitative PCR (Fig. 2A). In contrast, the expression of DUOX2 mRNA appeared lower (Fig. 2A), was previously not detected by
microarray (47), and was not increased during in vitro differentiation (Fig. 2A). Consistent with these data, in a recent quantitative PCR study on rat lung maturation, DUOX2 mRNA was found to be slightly downregulated between gestational day 18 and adult rat lung (49). In addition, we found that mRNA levels of the maturation factor DUOX1A closely followed DUOX1 mRNA (Fig. 2C), consistent with an increased production of membrane-localized, functional DUOX1 protein in differentiated cells in vitro. Similarly, in a previous tissue expression profiling study, DUOX1A was highly expressed in lung tissue, whereas the corresponding maturation factor DUOX2A was not detected (24). These reports support our observation that DUOX1 is the dominant DUOX isoform in differentiated adult lung epithelia.

The mode of regulation of DUOX1 gene expression is not known. DUOX1 is located on chromosome 15 in a complex locus containing both DUOX1 and its maturation factor, DUOX1A (12, 24, 35), which are arranged head to head and might share a bidirectional promoter (24). Recent genome-wide analyses indicate that bidirectionally promoted gene pairs represent ~9% of all transcriptional units and have a higher probability of coordinately expressed than random pairs of genes (18, 43, 44). In support of this notion, we found a close correlation between the level of induction for DUOX1 and DUOX1A expression in our study when cells were stimulated with DCI (Fig. 2C).

In our study, 8-Br-cAMP alone had no effect on the expression of DUOX1 in lung cells; however, DUOX1 and DUOX2 mRNA levels in Northern blots of dog thyroid (12) or DUOX2 in porcine thyroid follicles (29) were found to be upregulated when cultured in forskolin for 2–3 days, whereas the effects in human thyroid were small (12). In a later investigation of the promoter regions of the DUOX isoforms, a cAMP regulation of the human promoters was not found (35). Combined treatment with cAMP, IBMX, and dexamethasone strongly upregulated DUOX1 (but not DUOX2) expression in our study (Fig. 2A). The details of this combined regulation are currently not understood. Our data also indicate that DUOX1 and DUOX2 are regulated independently. Our finding of developmental regulation of DUOX protein in fetal lung in vivo (Fig. 4) is consistent with the inducibility of DUOX1 mRNA and protein by glucocorticoid plus cAMP in cultured cells. There is considerable evidence that these hormones modulate in vivo differentiation of type II cells, including prominent regulation of surfactant components and other proteins involved in specialized functions of these cells (4).

For comparison, in primary airway epithelial cultures, DUOX1 expression was shown to be regulated by inflammatory cytokines (28). Specifically, DUOX1 mRNA expression measured by PCR was increased ~5-fold by treatment of cultures with IL-4 or ~3-fold by treatment with IL-13. These cytokines are expected to initiate very different signaling cascades compared with the effects of glucocorticoids and cAMP used in our study. The relation between these pathways and their expression in different cell types is currently not known.

H2O2 production by the alveolar epithelium. Our data indicate that DCI-differentiated alveolar epithelia secrete substantial amounts of H2O2 into the extracellular medium. The involvement of DUOX1 in H2O2 production was identified by its sensitivity to DUOX1 siRNA (Fig. 5A), and the NADPH oxidase activity of DUOX1 was verified by its sensitivity to 5 μM DPI (34). There were previous indications that the alveolar epithelial cells generate H2O2. Kinnula et al. (27) found that freshly isolated rat alveolar type II cells released H2O2 into the medium that was tentatively assigned to a plasma membrane source. Van Klaveren et al. (46) investigated rat alveolar type II cells using luminol and lucigenin chemiluminescence assays to detect both superoxide and H2O2, which were assigned to NADPH oxidase activity based on the effects of DPI, albeit in that study, very high concentrations (100–1,000 μM) were necessary to block oxidant production (46). Wallaert et al. (48) isolated type II cells from guinea pigs and also identified both superoxide (by ferricytochrome c reduction) and H2O2 (by phenol red oxidation) release. On the other hand, alveolar epithelial cells and the alveolar lining fluid contain considerable amounts of glutathione (6) and antioxidant enzymes, most notably glutathione peroxidase (GPx) and catalase (2, 3, 9), which degrade H2O2 and maintain a relatively reduced alveolar environment. GPx1 and catalase mRNA expression were found to be upregulated in adult compared with fetal human lung tissue (2), and in a recent microarray study, we (47) found that Gpx3 mRNA expression was increased 3.7-fold by DCI treat-
ment of human fetal alveolar cultures. The upregulation of both oxidant production (DUOX1) and antioxidant mechanisms during type II cell differentiation suggests that these two systems interact to maintain a controlled redox equilibrium.

DUOX1 mediated acid production and secretion by alveolar cells. The alveolar lining fluid in rabbit lungs has been described as slightly acidic (pH 6.9), and epithelial H⁺ secretion has been suggested (33). To remove intracellular H⁺, rat alveolar type II cells have been shown to express basolateral Na⁺/H⁺ exchanger activity (28) and apical H⁺ channels as the major H⁺ transport proteins (13). We similarly found that DCI-differentiated human cultures acidified their cytosol when treated with amiloride and ZnCl₂, suggesting continuous intracellular acid generation as well as Na⁺/H⁺ exchanger and H⁺ channel activities in these cells. Previously, H⁺ channels in rat alveolar type II cells were shown to be active only under conditions of intracellular acidification (32) and we focused on DUOX1 expression as one acid producer. Knockdown of DUOX1 mRNA levels by siRNA resulted in a considerable reduction of intracellular acid generation, indicating a role for DUOX1 in intracellular acidification. The simplest explanation of these data is that DUOX1 expression in differentiated alveolar cells generates intracellular H⁺ during NADPH oxidation, similar to the activity of NADPH oxidases in phagocytes (14) or airway epithelia (40). Whether there are other sources of intracellular acid that are upregulated in parallel to or by DUOX1 is currently not known.

Although secretion of H⁺ by the alveolar epithelium has been suggested to contribute to the acidic mucosal pH, H⁺ secretion into the mucosal bath has not been formally measured. We found that DCI-treated cells continuously acidified the mucosal bath, which appeared largely H⁺ channel-mediated. This is noteworthy because, previously, H⁺ channels have been found to be inactive under basal conditions (32) and only activated after an acidic challenge. Note, however, that the transepithelial experiments (Fig. 6) were performed at a relatively alkaline mucosal pH of 7.3, which is expected to increase the inside-to-outside gradient for H⁺ and amplify the measured H⁺ secretion. Taken together, these data are consistent with the notion that the alveolar mucosa acidifies by DUOX1-associated intracellular H⁺ production, which generates a H⁺ gradient to drive H⁺ secretion.

Possible role of DUOX1 in pulmonary innate host defense. DUOX1 is a member of the NOX gene family of NADPH oxidases. The first and most intensely investigated member of the NOX family is gp91phox (now called NOX2) of phagocytes, which is unequivocally involved in innate defense (42). DUOX1 and DUOX2 appear to be the epithelial NADPH oxidase isoforms, and there are a number of observations in Airways that support a role in innate mucosal defense (20, 22, 31). Further supporting evidence comes from a study on isolated adult rat alveolar type II cells that showed that H₂O₂ release was substantially increased when type II cells were exposed to Staphylococcus aureus or the yeast cell wall particle zymosan (46).

Although these observations support the notion of a role of DUOX1 in alveolar defense, in all other innate defense tissues, H₂O₂ (which by itself shows only weak bactericidal activity; Ref. 38) is further reduced by a class of heme-containing peroxidases to form bactericidal oxygen species. For example, myeloperoxidase is the major granule protein in neutrophils, which generates highly reactive HOCl (45). Similar reactions are catalyzed by the eosinophil peroxidase to form HOBr or HOSCN (21) or by lactoperoxidase on mucosal surfaces to form HOSCN (22, 51). In contrast to the peroxidases found in other tissues, the presence of a complementary peroxidase in alveolar cells is unclear. The only well-described peroxidase present in the alveolar lining fluid is GPx (3), which is a selenium-containing peroxidase that is structurally unrelated to the above mentioned peroxidases. In presence of the high concentrations of glutathione in the alveolar lining fluid (6), GPx reduces H₂O₂ to water and thus reduces the availability of H₂O₂ as a defense molecule. Therefore, it is currently unclear whether H₂O₂ in the alveolus is further reduced to reactive, antibacterial oxygen species.

On the other hand, there is increasing evidence suggesting that DUOX1 has additional functions in cell signaling. Low concentrations of H₂O₂ can function as an intracellular signal and activate signaling cascades involved in growth and differentiation of many cell types (39). The transcription factors NF-κB, p53, and AP-1 are redox-sensitive and provide a link to gene expression (30). DUOX1-derived H₂O₂ has been shown to act as an extracellular signal for TNF-α-converting enzyme resulting in gene expression and mucus secretion by the airway epithelium (41). That study defined a role for extracellular H₂O₂ as a diffusible mediator in the airway surface liquid. In a recent study of airway epithelial wound repair, it was shown that regulated H₂O₂ release by DUOX1 enhanced wound closure and cell migration and was associated with activation of extracellular signal-regulated kinase-1 and -2 and matrix metalloproteinase-9 (50). Thus DUOX1 may have multiple functions in epithelia.

In summary, our study has identified DUOX1 as the NADPH oxidase expressed in mature alveolar epithelial cells. Expression of DUOX1 in alveolar cells is associated with H₂O₂ and H⁺ secretion. The upregulated expression of DUOX1 before birth supports the notion that DUOX1 contributes to the function of the mature, differentiated alveolar epithelium.

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REFERENCES

DUOX IN ALVEOLAR EPITHELIUM


