The purpose of this study was to determine the role of NADPH oxidase in H⁺ secretion by airway epithelia. In whole cell patch clamp recordings primary human tracheal epithelial cells (hTE) and the human serous gland cell line Calu-3 expressed a functionally similar zinc-blockable plasma membrane H⁺ conductance. However, the rate of H⁺ secretion of confluent epithelial monolayers measured in Ussing chambers was 9-fold larger in hTE compared with Calu-3. In hTE H⁺ secretion was blocked by mucosal ZnCl₂ and the NADPH oxidase blockers acetovanillone and 4-(2-aminoethyl)benzenesulfonfyl fluoride (AEBSF), whereas these same blockers had no effect in Calu-3. We determined levels of trans-sulfonyl fluoride (AEBSF), whereas these same blockers block the Na⁺/H⁺ exchanger, intracellular pH in hTE acidic compared with plasma. Initially, pH ASL (pHASL), which has been shown to affect various epithelial functions such as ion transport (2, 3), ciliary beating (4, 5), and treatment with AEBSF blocked acidification. These data suggest a role for an apically located Duox-based NADPH oxidase during intracellular H⁺ production and H⁺ secretion, but not in H⁺ conduction.

The composition of the airway surface liquid (ASL)³ is critically important for the normal function of the airway epithelium, including bacterial killing and mucociliary clearance (1). Recently, considerable attention has focused on the pH of the ASL (pH₈SL), which has been shown to affect various epithelial functions such as ion transport (2, 3), ciliary beating (4, 5), and epithelial integrity (6, 7). pH₈SL has been found to be slightly acidic compared with plasma. Initially, pH₈SL in the ferret trachea was found to be 6.85 using microelectrode measurements (8). Using a pH-sensitive fluorophore, pH₈SL was found to be 6.8 in bovine cultures and 7.1 in vivo in mice (9). In the inflammatory airways diseases asthma, cystic fibrosis, and chronic obstructive pulmonary disease an acidic pH₈SL or acidic exhaled breath has been demonstrated (10–12). These observations suggested a regulated mechanism of acid secretion by the airways that is up-regulated during inflammation. Recently we found that human airway cells express a voltage-activated, zinc-sensitive H⁺ conductance and secrete acid equivalents into the mucosal bath (13).

H⁺-selective, voltage-activated currents were first recorded from snail neurons (14) and since then have been found in many other cell types (15). The transmembrane protein complex NADPH oxidase has been suggested to mediate the H⁺ conductance in phagocytic cells (16), although this notion has been challenged recently (17). In phagocytic cells NADPH oxidase plays a crucial role in killing invading organisms by producing superoxide radicals (O₂⁻) by transferring an electron from intracellular NADPH across the membrane to extracellular O₂. During the process of NADPH oxidation in neutrophils, intracellular H⁺ is released from NADPH and thereby contributes to intracellular acid production (18). The phagocytic NADPH oxidase is a multisubunit enzyme consisting of two transmembrane subunits (the cytochrome gp91 phox and p22 phox), three cytosolic subunits (p40phox, p47phox, and p67phox). Anti-Duox antibody staining resulted in prominent apical staining in hTE but no significant staining in Calu-3. When treated with amiloride to block the Na⁺/H⁺ exchanger, intracellular pH in hTE acidified at significantly higher rates than in Calu-3, and treatment with AEBSF blocked acidification. These data suggest a role for an apically located Duox-based NADPH oxidase during intracellular H⁺ production and H⁺ secretion.

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gp91 phox is a member of the Nox gene family, where it is referred to as Nox2. In addition to the small Nox homologs Nox1 through -5, two large homologs have been described recently, called dual oxidases Duox1 and -2 (21–23). Expression of Duox1 and -2 has been found in the epithelium of the thyroid gland by immunofluorescence (22), and expression of Duox1 transcript by in situ hybridization and Duox1 antisense-sensitive H₂O₂ production by airway epithelial cultures has been demonstrated (24).

Recent studies in our laboratory identified a zinc-sensitive H⁺ conductance and H⁺ secretion across the apical membrane of well differentiated human airway epithelia (13). We were intrigued by the notion that this physiological process was provided by the NADPH oxidase complex. This was addressed by studying H⁺ conductance, H⁺ secretion, NADPH oxidase expression, and intracellular H⁺ production in two functionally different types of human airway epithelia. Ciliated cells from the airway surface epithelium function to absorb fluid and acidify the airway lumen slightly (8, 9, 13), whereas cells from the submucosal serous glands secrete a bicarbonate-rich, alkaline fluid (25, 26). In this report we compare the properties of airway surface versus submucosal gland cells by using primary
cultures of human tracheal surface epithelia (hTE) and the Calu-3 submucosal gland cell line. These cells were chosen to relate high (hTE) and low rates (Calu-3) of \( H^+ \) secretion to the expression of NADPH oxidase and its function. The experiments in this report demonstrate the localization of the dual oxidase Duox in the apical membrane of hTE surface cells but not in Calu-3 gland cells. Our data suggest that a Duox-based NADPH oxidase complex supplies extracellular \( H^+ \), which provides a \( H^+ \) gradient across the apical cell membrane. The plasma membrane \( H^+ \) conductance did not correlate with Duox expression suggesting that intracellular \( H^+ \) exited through a parallel pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Human primary tracheal epithelial cultures (hTE) were isolated as previously described (27). Cells were cultured in Dulbecco’s modified Eagle’s medium:brunhadal epithelial cell base medium (1:1) as described by Gray et al. (28). Compared with our previous report (13), this is a change in culture conditions, which resulted in epithelial cultures with significantly lower transepithelial resistances \( (R_t = 569 \pm 36 \ \Omega \cdot \text{cm}^2, n = 12) \) and higher potentials \( (V_t = -26.0 \pm 2.1 \ \text{mV}) \). Sachs et al. described the effects of both culture conditions (29). Calu-3 cells, a human airway cell line with characteristics of airway serous cells, were cultured in a Dulbecco’s modified Eagle’s medium:serum mix supplemented with 10% fetal bovine serum. For patch clamping or ‘s medium/F-12

**HEPES**

Finally free of \( \text{HCO}_3^- \) and resuspended in (in millimolar): 120 Hepes, 70 gluconic acid, 100 magnesium gluconate, 3.3 magnesium ATP, 0.07 lithium GTP (pH 5.3). Cells were whole cell patch-clamped as previously described (13, 33). Briefly, cultures were bathed serosally with

**Time constants of current activation**

Current-voltage step protocols from measured by fitting the current transients caused by a 10-mV pulse using pClamp 8 (Axon Instruments, Fremont, CA). Currents during current-voltage step protocols were sampled at 500 Hz and filtered at 200 Hz. For the plots in the figures, original currents are 200 Hz by averaging adjacent samples. In the figures original currents are

**Protein Secretion and Duox in Airways**

Proton secretion was measured using the pH-stat titration technique in an Ussing chamber as shown without leak subtraction or corrections. Measurements were done at 37 °C.

**Molecular Expression of NADPH Oxidase Subunits**—Total RNA was prepared from 2 \( \times 10^6 \) hTE and Calu-3 cells, respectively, grown on permeable filter inserts, and poly(A) mRNA was isolated using the RNeasy and Oligotex mRNA kits (Qiagen, Valencia, CA). Reverse transcribease-PCR was performed using 100 ng of mRNA, 40 units of avian myeloblastosis virus reverse transcriptase, 20 units of RNase inhibitor, 4 \( \mu \)l of 5× buffer (Roche Applied Science), 2 \( \mu \)l of 5 mM dNTPs, and 2.5 \( \mu \)l random hexamer oligonucleotides (Applied Biosystems, Foster City, CA) for 60 min at 42 °C. One microliter of first strand cDNA was used as template in polymerase chain reactions with NADPH oxidase-specific oligonucleotides (see Table I). PCR was performed in a DNA ther
camal cycler using AmpliTaq Gold® DNA polymerase (Applied Biosys
tems). Following a 10-min 94 °C incubation, reactions proceeded for 30 s at 94 °C then 60 s at 60 °C for 43 cycles followed by 1 min at 72 °C.

**Immunodetection of Duox**—For immunoblotting, hTE cultures were washed with phosphate-buffered saline and cells were transferred directly into SDS-PAGE sample buffer. Proteins were separated by 6% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 1% casein and probed with a rabbit anti-Duox antibody (kindly provided by F. Miot (22)), and horseradish peroxidase-conjugated secondary anti-rabbit antibody labeling was visualized using the ECL Western blotting analysis system (Amersham Biosciences). For immunocytochemistry, hTE cells were dissected from confluent cultures, fixed with 2% paraformaldehyde, and incubated with 0.3% (v/v) Triton X-100 followed by 1% (w/v) bovine serum albumin in phosphate-buffered saline. Cells were immunostained for Duox, and the tight junction protein ZO-1 was used as a marker of the apical plasma membrane. A monoclonal antibody (Z0-1) and secondary antibodies were Alexa Fluor 488 anti-rabbit (Molecular Probes) for Duox and Alexa Fluor 546 anti-mouse for ZO-1. Cells were embed
ded in Crystal Mount (Biomedia) and observed with a 63×/1.4 numerical aperture oil-immersion objective on a Solamare spinning disc con
cocal microscope (excitation, 457 nm; emission, 535 ± 20 nm for Duox, and excitation, 514 nm; emission, 605 ± 20 nm for ZO-1).

**Measurement of Intracellular pH**

Cells on glass coverslips were incubated for 30 min with 20 \( \mu \)M BCECF-AM (2′,7′-bis-(2-carbox
eythyl)-5-(and-6-carboxyl fluorescein), acetoxyethyl ester, Molecular Probes Inc.), washed, and allowed to cleave the dye for 60 min. Cellular fluorescence was investigated in an open perfusion chamber on an inverted microscope. Excitation wavelengths were 440 and 495 nm, and emission was collected between 525 and 550 nm with a cooled charge-coupled device camera (Photometrics CoolSnap HQ, Roper Scientific) controlled by a computerized imaging system (Metafluor, Universal Imaging Corp.). Emission intensities of individual cells were background-subtracted, and ratios of 495 to 440 nm were calculated. \( pH \) was calculated from ratios using a four-point \( pH \) calibration in presence of 20 \( \mu \)M nigericin in KCl solution. Calibration points were \( pH \) 7.5, 7, 6.5, and 6.0. Resulting calibration ratios for individual cells were fitted to second order polynomials, and fitted parameters were used to calculate \( pH \) from measured ratios. Individual rates of acidification of cells during the first 15 min of treatment with amiloride were determined by regression analysis and exponential normalized to \( C_{m} \).

**RESULTS**

**Proton Conductances in Airway Cells**—Single hTE and Calu-3 cells were whole cell patch-clamped to characterize and compare their \( H^+ \) conductance under conditions selective for

**Quantification of Proton Secretion**

Proton secretion was measured using the pH-stat titration technique in an Ussing chamber as described previously (13). Briefly, cultures were bathed serosally with Heps-buffered solution and mucosally with buffer-free solution (3 ml each). Solutions were constantly gassed with oxygen and were monon
tially free of \( \text{HCO}_3^-/\text{CO}_2 \). Standard NaCl Ringer solutions contained in (millimolar): mucosal, 140 NaCl, 2 KCl, 15 glucose, 2 CaCl\(_2\), 1 MgCl\(_2\); serosal, 140 NaCl, 2 KCl, 5 glucose, 10 Heps, 2 CaCl\(_2\), 1 MgCl\(_2\) (pH 7.3) with HCl/NaOH. During the experiment the \( pH \) of the mucosal solution was continuously measured and titrated to a target value of \( pH \) 7.3 (range, 7.25–7.35) with 10 \( \mu \)l NaOH to determine \( H^+ \) secretion \( \left(I_{H^+}\right) \) by the cultures. Rates are expressed in pmol cm\(^{-2}\) cm\(^{-2}\) and positive rates refer to acidification of the mucosal medium. Measurements were done at 37 °C.

**Proton Secretion and Duox in Airways**

Proton Secretion and Duox in Airways (18). Some cultures were treated for 1 h with 100 \( \mu \)M nigericin, which provides a \( H^+ \) gradient across the apical cell membrane. The plasma membrane \( H^+ \) conductance did not correlate with Duox expression suggesting that intracellular \( H^+ \) exited through a parallel pathway.
H\(^+\) currents with a pipette to bath pH gradient of 5.3 to 7.3. Calu-3 are large round cells with an average C\(_m\) of 29.9 ± 5.9 pF (n = 7). hTE were visibly smaller with a C\(_m\) of 10.4 ± 1.4 pF (n = 6, significantly lower than Calu-3, p = 0.012, t test). hTE had visible cilia on one pole of the elongated cells. Reported conductances were normalized to C\(_m\) to account for the difference in cell size.

Measured currents were identified as H\(^+\) currents by the established characteristics: (i) H\(^+\) -selective reversal potentials, (ii) zinc sensitivity, and (iii) activation by depolarization with time constants in the order of seconds (34). Both airway cell types expressed whole cell H\(^+\) currents, H\(^+\) -selective reversal potentials, and activation by depolarizing pulses. Continuous H\(^+\) current recordings and block by ZnSO\(_4\) are shown in Fig. 1A for hTE and Fig. 1B for Calu-3. During the recordings the membrane potential was clamped to −50 mV and pulsed to 20 mV (as shown in the voltage traces, lower panels of Fig. 1, A and B) to continuously monitor the depolarization activation of H\(^+\) currents. Note the typically slow current activation during the pulses in both cell types. Addition of 100 μM ZnSO\(_4\) to the bath fully blocked the depolarization-activated currents. The current responses to the voltage step protocols from Fig. 1 (A and B) are shown in detail in Fig. 1 (C and D) for both cell types before (top panels) and after (bottom panels) addition of 100 μM ZnSO\(_4\). Outward H\(^+\) currents activated slowly during depolarizing potentials and were blocked after addition of ZnSO\(_4\). Steady-state current-voltage relations are shown in Fig. 2 (E and F). Current-voltage relations recorded under control conditions (triangles) showed outward rectification and negative reversal potentials indicative for H\(^+\) currents. Control steady-state currents reversed at −21.6 ± 4.7 mV (hTE) and −25.9 ± 6.1 mV (Calu-3). Addition of Zn\(^{2+}\) shifted the reversal potential to positive values in both cell types (Fig. 1, E and F, circles, by on average 37.8 ± 3.5 mV, n = 11; not different between cell types). On average, hTE and Calu-3 expressed similar specific H\(^+\) conductances of 19.6 ± 3.1 pS/pF and 11.6 ± 2.8 pS/pF, respectively (p = 0.085, Fig. 1G).

As a biophysical identifier of the H\(^+\) conductance we used the time constants of current activation when stepping the membrane potential from −50 mV to 20 mV (τ\(_{20}\)) and from −50 mV to 40 mV (τ\(_{40}\)). Fig. 2 shows current activations evoked by voltage pulses in hTE (Fig. 2A) and Calu-3 (Fig. 2B). In both cell types the depolarization-activated currents were fitted well with a single exponential (lines in Fig. 2, A and B) yielding τ\(_{20}\) and τ\(_{40}\) (Fig. 2C). Fitted time constants at either potential were not different between cell types (p > 0.4, t tests) suggesting that hTE and Calu-3 cells expressed a biophysically similar type of H\(^+\) conductance. On average τ\(_{20}\) = 3.97 ± 0.52 s and τ\(_{40}\) = 2.77 ± 0.39 s. τ\(_{20}\) was significantly longer than τ\(_{40}\) (p = 0.01), consistent with a voltage-activated opening transition. For comparison, the native H\(^+\) conductance of rat alveolar cells (τ\(_{40}\) = 2.5 s (35)) or eosinophils (τ\(_{40}\) = 2.4 s (36)) showed values similar to our data under comparable recording conditions. These data showed that hTE and Calu-3 cells expressed functionally similar H\(^+\) conductances, as judged by the quantitatively similar average conductances, rates of voltage-activated currents, and zinc sensitivity.

Acid Secretion by hTE and Calu-3 Cultures—Acid secretion of intact epithelia was measured using the pH-stat titration technique in Ussing chambers. Block of H\(^+\) channels (by ZnCl\(_2\)) and block of NADPH oxidase (by acetovanillone or AEBSF) was used to 1) determine the role of H\(^+\) channels in epithelial acid secretion, 2) verify an apical location of H\(^+\) channels in polarized epithelial monolayers, and 3) determine the role of NADPH oxidase during acid secretion. Both cell types showed basal acid secretion into the mucosal compartment. In hTE, J\(_{H} = 939 ± 49\) nmol h\(^{-1}\)cm\(^{-2}\), n = 12. Calu-3 showed a 9-fold smaller J\(_{H}\) of 106 ± 9 nmol h\(^{-1}\)cm\(^{-2}\), n = 18 (p < 0.0001, Fig. 3). When cultures were pre-treated for 1 h with ZnCl\(_2\) (100 μM mucosally), acid secretion by hTE was significantly reduced (to 480 ± 29 nmol h\(^{-1}\)cm\(^{-2}\), n = 6, p < 0.0001, unpaired t test). In contrast, no significant effect of ZnCl\(_2\) was found on acid secretion by Calu-3 (block to 84 ± 4 nmol h\(^{-1}\)cm\(^{-2}\), n = 5, p = 0.2, Fig. 3).

In addition we tested the NADPH oxidase blockers AEBSF and acetovanillone (500 μM each) on H\(^+\) secretion by hTE and Calu-3. After incubation of cultures for 14–16 h with blockers, H\(^+\) secretion was inhibited significantly in hTE (p < 0.0001,
Expression of NADPH Oxidase Genes in Airway Cells—We investigated the expression of described NADPH oxidase subunits, including the transmembrane subunits Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, Duox2, and p22^phox^, and the intracellular subunits p40^phox^, p47^phox^, and p67^phox^. Actin and generaldehyde-3-phosphate dehydrogenase were included as control genes. The sequence-specific primers used for these genes are shown in Table I. Fig. 4A shows the expression pattern of mRNA of NADPH oxidase subunits in Calu-3 (top panel) and hTE (bottom panel) using high amplification (43 cycles). Transcripts for Nox1, Nox2, and Nox5 were detected in both cell types. Nox4 was expressed at low levels in hTE but not detected in Calu-3 cells. The large Nox genes, Duox1 and Duox2, were major isoforms in hTE; in contrast, Duox1 was not detected and Duox2 was expressed at comparably low levels in Calu-3 cells. Nox3 was not detected in either cell type (not shown). The small transmembrane subunit of NADPH oxidase, p22^phox^, was expressed highly in both cell types. Quantification of the relative expression of the transmembrane subunit in hTE using real-time PCR showed that in hTE transcripts for Duox1, Duox2 and p22^phox^ were expressed at 1000-fold higher levels than the small transmembrane Nox isoforms (Fig. 4B). The cytosolic subunits p40^phox^, p47^phox^, and p67^phox^ (Fig. 4A, right panels) were expressed in hTE, whereas Calu-3 expressed p67^phox^ but lacked transcripts for the two smaller subunits p40^phox^ and p47^phox^. Thus the expression of NADPH oxidase subunits showed significant differences between hTE and Calu-3 cells. The lack of Duox1 and the comparably low expression of Duox2 in Calu-3, both of which were highly expressed in hTE, suggested a role for Duox during epithelial acid secretion.

Localization of Duox—We investigated the localization of Duox in hTE using immunofluorescence and confocal microscopy. A polyclonal antibody raised against Duox1 (22) was used to immunostain airway cells. Note that this antibody does not distinguish well between the Duox1 and -2 isoforms and is thus referred to as Duox antibody. Fig. 5A shows an immunoblot for Duox of cell lysates from hTE and Calu-3. A confocal image slice of the apical region of Calu-3 shows for comparison immunostaining for the tight junction protein zonula occludens 1 (ZO1) as a marker for the luminal side of the epithelium. The staining pattern indicates luminal localization of Duox. Fig. 5D shows another group of hTE cells in a confocal side view with cilia clearly visible on one pole of the cells. Prominent anti-Duox immunostaining of the ciliated pole indicates apical localization of Duox. For comparison and as a negative control, Calu-3, which expressed low levels of Duox2 mRNA but no detectable transcripts for Duox1 (Fig. 4A), were immunostained with anti-Duox (Fig. 5E) and anti-ZO1 (Fig. 5F). A confocal image slice of the apical region of Calu-3 is

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F. Mirot, personal communication.
Proton Secretion and Duox in Airways

Table 1

| Gene   | GenBank™ accession no. | Forward primer            | Reverse primer            |
|--------|-----------------------|---------------------------|--------------------------|
| Nox1   | AF166327              | tatgaagtgcctgctgttgt      | gaggttgttgtgcacactgt     |
| Nox2   | NM_006897             | tggtagcctggagttgtga       | teagttgtgggtactgtgta     |
| Nox3   | NM_015718             | teacaacttgctgctagtga      | aggctgctgctgactaga       |
| Nox4   | NM_018831             | gctgctgtgcctgtagtga       | acaactagtggcactaga       |
| Nox5a  | AF317889              | actgactgccacctgtga        | aceactgacactgacaetct     |
| Duox1  | AF213465              | egaactgagctagcgaaga      | ctgtagggacagctagctct     |
| Duox2  | AF267981              | aacactgcagctgactaga       | caagacaagctgagctgta      |
| p40b   | NM_001010             | actgactgccacctgtga        | cagacaagctgagctgta      |
| p47b   | NM_000285             | actgactgccacctgtga        | cagacaagctgagctgta      |
| p67b   | M32011                | actgactgccacctgtga        | cagacaagctgagctgta      |
| GAPDHb | NM_002046             | actgactgccacctgtga        | cagacaagctgagctgta      |
| Actin  | BC016945              | actgactgccacctgtga        | cagacaagctgagctgta      |

a The primer for AF317889 does not distinguish between different Nox5 isoforms.
b GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 4.** Expression of NADPH oxidase subunits in airway cells. A, PCR gels of transmembrane subunits (left panels) and intracellular subunits (right panels) of Calu-3 (top) and hTE (bottom). Arrows show the 100-bp band in the marker lanes. Glyceraldehyde-3-phosphate dehydrogenase and actin are shown as controls. Primers for the reactions are given in Table I. Reaction was run for 43 cycles to visualize also low expressing small Nox isoforms. B, quantification of expression of transmembrane subunits in hTE using real-time PCR. Note log scaling of the y-axis. Each bar represents determination in triplicate. Signal was normalized to glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 5.** Immunostaining of airway cultures. A, Western blot for Duox on cell lysates from Calu-3 and hTE cells. B and C, confocal images showing a group of hTE cells stained with a polyclonal anti-Duox1 antibody (B) and anti-ZO1 as a marker of the luminal side (C). D, confocal side view of a group of hTE cells stained with an anti-Duox1 antibody. Note clear staining of the apical pole and the cilia. E and F, Calu-3 cell monolayer, confocal top view focused on apical membrane layer. Anti-Duox1 antibody did not result in specific staining (E), for comparison, anti-ZO1 staining of the layer shown in F.

shown (identified by ZO1 expression). No specific staining of Duox was detected indicating that Calu-3 do not express Duox at their luminal membranes.

**Intracellular Acid Production by NADPH Oxidase—**Although hTE and Calu-3 both expressed a similar Zn$^{2+}$-sensitive plasma membrane H$^+$ conductance, only hTE secreted acid in a Zn$^{2+}$-blockable manner. These results suggested that the electrochemical gradient for H$^+$ exit across an apical H$^+$ conductance was larger in hTE than in Calu-3. In the following experiments we tested the notion that the activity of NADPH oxidase generates an intracellular H$^+$ gradient that drives H$^+$ through a plasma membrane H$^+$ conductance. Under control conditions cytosolic pH in hTE, Calu-3, or in hTE treated with AEBSF was not significantly different (Fig. 6C, black bars). Treatment of cells with amiloride (1 mM) acidified all cells (Fig. 6, A and C), but hTE acidified at a significantly higher rate than Calu-3 (Fig. 6B), and after 90 min of amiloride treatment pH$_i$ was significantly more acidic in hTE than in Calu-3 (Fig. 6C, gray bars). The amiloride-induced acidification was significantly reduced by treating hTE with AEBSF (Fig. 6A), which reduced the rate of acidification (Fig. 6B) and increased pH$_i$ after 90 min of amiloride treatment over untreated hTE (Fig. 6C, gray bars), suggesting that the activity of NADPH oxidase significantly contributed to the intracellular acid production of hTE. These measurements are consistent with an NADPH oxidase-mediated acidification of pH$_i$ in hTE. This process likely provides the driving force for H$^+$ exit through apical membrane H$^+$ channels in intact epithelia. For comparison, Calu-3 showed small rates of intracellular acid production and thus may not generate an appreciable H$^+$ gradient across an apical membrane H$^+$ conductance, consistent with the low rates of H$^+$ secretion (Fig. 3) by these cells.

**DISCUSSION**

The data presented in this report suggest a close correlation between intracellular acid production, acid secretion, NADPH oxidase function, and Duox expression in airway epithelial...
cells: hTE showed high expression of Duox1 and -2, high acid secretion, and high rates of intracellular acid production, which were reduced by blocking NADPH oxidase function, whereas Calu-3 showed no expression of Duox1 and low expression of Duox2, little acid secretion and intracellular acid production, and no effects of NADPH oxidase blockers on these measurements. At the same time both cell types expressed a functionally similar plasma membrane H^+ conductance. These observations suggest a model for acid secretion by airway surface epithelia in which a Duox-based NADPH oxidase generates intracellular H^+ adjacent to the apical membrane, and this H^+ both activates an apical membrane H^+ conductance and provides the driving force for H^+ secretion across this H^+ conductance. This model and its implications are discussed below.

**Acid Secretion Is Driven by a H^+ Gradient Generated by the Activity of NADPH Oxidase**—Activation by intracellular acidity and membrane depolarization are typical characteristics of H^+ conductances in various cell types (15). In our study, whole cell patch clamp measurements were performed with an inside-to-outside H^+ gradient that was used both to activate the H^+ conductance and to provide a driving force for H^+ movement, and the membrane voltage was controlled. Under these conditions both airway cell types showed similar specific H^+ conductances with common biophysical characteristics and zinc sensitivity, suggesting a similar type of H^+ channel in the plasma membrane of hTE and Calu-3 (Fig. 1). However, in intact epithelial preparations, hTE showed zinc-sensitive H^+ secretion, whereas Calu-3 did not (Fig. 3). These observations suggest that in Calu-3 epithelia either the H^+ conductance was inactive or there was no proton-motive force driving force across the apical membrane to support epithelial H^+ secretion.

In intact cells several factors affect the electrochemical driving forces for H^+ exit, such as the apical H^+ gradient and the apical membrane potential (V_m), the latter of which in turn is determined by the transepithelial resistance (R_t), the relative resistances of the tight junctions and the cells, and the relative conductance of the apical membrane to Na^+ and Cl^- . Compared with Calu-3 cells, hTE have larger R_t (569 versus 257 Ω·cm^2), more negative V_m (-26 versus -5.6 mV), and a larger apical Na^+ conductance (30, 38). Thus, compared with Calu-3, the hTE cultures can be expected to possess relatively depolarized V_m values. Consistent with the observed transepithelial differences in our report, the reported V_m for hTE was V_m = -26 mV (38) or V_m = -19 mV (39), and in Calu-3 cells V_m = -52 mV (40). Thus, one explanation for the lack of zinc-sensitive H^+ secretion in Calu-3 is the hyperpolarized V_m, which is expected both to inactivate the H^+ conductance (15) and to reduce the electrochemical driving force for H^+ exit. The presence of conductive H^+ secretion by hTE in the face of a negative V_m indicates an intra- to extracellular H^+ gradient across the apical membrane (13). In alveolar type II cells it was shown that intracellular acidification and a H^+ gradient were necessary both to activate the H^+ conductance and to drive H^+ currents (41). In neutrophils, activation of NADPH oxidase caused the cytosol to acidify from pH 7 to 6.2 when the Na^+-H^+ exchanger was blocked by Na^+ free solutions (18). In airway epithelia the Na^+/H^+ exchanger was shown to be a major regulator of intracellular pH, however, it is present only in the basolateral membrane (42). Therefore, any H^+ produced apically by NADPH oxidase may not be readily transported by the Na^+/H^+ exchanger but likely, instead, to cause a local acidification of the intracellular face of the apical membrane.

In analogy to acid production by NADPH oxidase in neutrophils, addition of amiloride (to block the Na^+/H^+ exchanger) caused an AEBSF-blockable acidification of hTE cells (Fig. 6). Basal pH_i was not affected by treatment with AEBSF (Fig. 6), indicating a tight control of pH_i by the Na^+/H^+ exchanger under control conditions. Thus, treatment with amiloride uncovered intracellular acid production by NADPH oxidase activity in hTE cells.

The effect of AEBSF on both the rates of intracellular acidification (Fig. 6C) and epithelial acid secretion by hTE (Fig. 3) is consistent with the notion that NADPH oxidase-generated intracellular H^+ both activates the plasma membrane H^+ conductance and provides a driving force for H^+ exit across the apical membrane. When assuming that the pH_i measured in the presence of amiloride corresponds to the local pH at the cytosolic face of the apical membrane in untreated epithelia, then, with the measured pH gradient (6.89 to 7.4) and an average V_m of -22.5 mV (43, 44), an outwardly directed proton-motive force of 11.5 mV is predicted using the Nernst equation for conductive H^+ secretion across the apical membrane. The critical role of intracellular acidification for H^+ secretion is further supported by the data found in Calu-3 cells, which show little intracellular acid production and no evidence for an active, zinc-sensitive H^+ secretion into the mucosal solution despite a significant plasma membrane H^+ conductance.

**Expression of NADPH Oxidase in Airway Epithelia**—Duox1 and -2 were initially identified in thyroid epithelia where they were found to be expressed in the apical membrane (22, 45).
In this model a Duox-based NADPH oxidase releases intracellular H⁺ from NADPH and acidifies pH. Intracellular H⁺ exits across a Zn²⁺-sensitive H⁺-ATPase and an ouabain-sensitive K⁺/H⁺-ATPase. Percentages give relative contribution to total H⁺ secretion as determined from blocker experiments. Residual, non-blockable H⁺ secretion may be through a separate pathway, or may be due to incomplete inhibition of transporters by used blockers. Quantitative contribution of individual H⁺ secretory mechanisms may be different under experimental circumstances. Note that the source of H⁺ for the H⁺-channel is NADPH (which results in an acidification of pH, as observed in this study), whereas the ATPases release H⁺ from water resulting in an alkalinization of pH, in this model. For simplicity all transporters are shown located in one cell. Electron transport of NADPH oxidase is not shown in this model.

Fig. 7. Model of acid release in the apical membrane of airway surface epithelial cells. In this model a Duox-based NADPH oxidase releases intracellular H⁺ from NADPH and acidifies pH. Intracellular H⁺ exits across a Zn²⁺-sensitive H⁺-ATPase and an ouabain-sensitive K⁺/H⁺-ATPase. Percentages give relative contribution to total H⁺ secretion as determined from blocker experiments. Residual, non-blockable H⁺ secretion may be through a separate pathway, or may be due to incomplete inhibition of transporters by used blockers. Quantitative contribution of individual H⁺ secretory mechanisms may be different under experimental circumstances. Note that the source of H⁺ for the H⁺-channel is NADPH (which results in an acidification of pH, as observed in this study), whereas the ATPases release H⁺ from water resulting in an alkalinization of pH, in this model. For simplicity all transporters are shown located in one cell. Electron transport of NADPH oxidase is not shown in this model.

Investigation of a panel of tissues showed a high expression of Duox1 and -2 transcripts in thyroid and of Duox1 in whole lung (23). In this report we show that in human tracheal surface epithelia both Duox1 and -2 are expressed at levels ~1000-fold higher than other Nox isoforms (Fig. 4) and thus represent the major Nox isoforms in airways. In contrast, in Calu-3 cells Nox isoforms were generally expressed at low levels, and no transcript for Duox1 was found. The high expression of Duox1 in hTE, its apical localization, and the high rates of acid secretion, compared with the lack of expression of Duox1 in Calu-3, which also showed no zinc-sensitive acid secretion, suggest Duox1 as the isoform responsible for acid production and secretion in airways. The effects of block of NADPH oxidase by AEBSF on both H⁺ production and H⁺ secretion underlie the correlation between these two functional parameters and Duox-expression.

Regulation and function of the NADPH oxidase of phagocytes greatly depends on the proper assembly of Nox2 with its subunits (46). Currently there is no known subunit that interacts with Duox. Interestingly, hTE express at high levels p22phox, p40phox, p47phox, and p67phox (Fig. 4). In contrast Calu-3 lacked the two subunits p40phox and p47phox. Although we currently have no evidence for a functional interaction between Duox and additional subunits of NADPH oxidase, our expression data support the notion of a fully functional NADPH oxidase complex in hTE that allows for acid production, whereas Calu-3 may not assemble a functional complex and do not generate H⁺ by this mechanism.

H⁺ Channels in Airways—Several small Nox isoforms have previously been suggested to function as H⁺ channels, including Nox2 (16), Nox5 (47), and a truncated form of Nox1 (48). However, DeCoursey and colleagues (34) challenged this conclusion owing to (i) the expression of the Nox constructs in cell systems that already expressed native H⁺ currents, (ii) a lack of H⁺ selectivity of the resulting currents, and (iii) the much shorter time constants of current activation recorded after Nox expression in these studies when compared with time constants of native H⁺ currents. Several additional studies then presented evidence that expression of Nox2 did not correlate with H⁺ channel function, but a parallel non-Nox H⁺ channel structure was suggested to be the H⁺ conductive site (summarized in Ref. 34). Thus the identity of the H⁺ channel and the H⁺ conductance of the NADPH oxidase is controversial. Because the putative H⁺ permeation pathway across Nox2 involves strictly conserved histidine residues (16, 49), the argument made for Nox2 can be extended to the other Nox family members, including Duox.

We found in airway cells a H⁺ conductance with characteristics very similar to the native H⁺ conductances found in white blood cells or alveolar type II cells: H⁺ currents activated slowly with time constants in the order of seconds, steady-state H⁺ currents were outwardly rectifying and showed H⁺ selectivity, and ZnSO₄ readily blocked the depolarization-activated currents (Figs. 1 and 2). Both hTE and Calu-3 expressed qualitatively and quantitatively similar H⁺ conductances suggesting the same type of H⁺ channel was active in both cell types. However, the high expression of Duox1 and -2 in hTE, and the total lack of Duox1 and low expression of Duox2 in Calu-3, suggest that the Duox isoforms do not function as H⁺ channels in airways, but a parallel non-Duox entity serves as a H⁺ channel. In analogy this argument may be extended to the other Nox family members based on the high homology between Duox and Nox in the histidine-containing transmembrane domains. However, our data cannot exclude the possibility that the lowly expressed isoforms Nox1, -2, and -5 may function as H⁺ channels, because they were found in both cell types. Thus a general conclusion about the channel characteristics of the Nox family from our data is precluded by the expression of multiple Nox isoforms in our cell system. Our data indicate that in airways a Duox-based NADPH oxidase generates intracellular H⁺ and a proton-motive force and H⁺ is conducted across the membrane through a parallel non-Duox-mediated pathway.

Two previous reports showed acid secretion of airway epithelia governed by different mechanisms. Coakley et al. (12) reported a ouabain-sensitive non-gastric K⁺/H⁺-ATPase in human primary cultures, and Inglis et al. (37) found a bafilomycin-sensitive vacuolar-type H⁺-ATPase in distal pig bronchi. Using the effects of ouabain and bafilomycin as indicators for these mechanisms, in this report we found their quantitative contributions to be 15 and 11%, respectively, of total acid secretion by hTE airway epithelia in pH-stat experiments. Although the quantitative contribution of these mechanisms is likely dependent on a number of factors, such as tissue origin, species, and experimental pH values, these observations suggest that the airway epithelium expresses additional mechanisms to acidify the airway surface liquid. Fig. 7 shows a model that displays the relation of acid transporting mechanisms and NADPH oxidase in the apical membrane of airway cells as derived from our study. See Fig. 7 legend for details.
Function of Duox and NADPH Oxidase in the Airway Epithelium—In the thyroid gland the function of Duox has been identified as a source of oxidant for the iodination and cross-linking of tyrosine to generate thyroid hormone (21). In a similar reaction Duox of C. elegans was shown to be involved in the oxidative cross-linking of tyrosine residues for the stabilization of the cuticle of the worm (23). In contrast, for the airway epithelium, it appears prudent to assume a functional role of Duox during bacterial killing, which is the major function of the airways. The airway epithelium is the first line of defense against inhaled bacterial pathogens, and the epithelium secretes a number of antimicrobial factors, which act in combination with the mechanical movement of the ciliary escalator to keep the distal lungs sterile. Several antimicrobial factors have been isolated from airway secretions indicating the role of the airways in bacterial defense. Production of O$_2$ by airway cultures by NADPH oxidase has been shown (24). Acidification of the airway surface liquid during acid secretion by the epithelium is expected to support conversion of O$_2$ into H$_2$O$_2$ and HOCl, which show bactericidal activity (1). Thus we suggest that the expression of NADPH oxidase in the airway epithelium and acid secretion reaction products. Therefore we suggest that the expression of O$_2$ production of O$_2$ and the presence of significant lactoperoxidase activity in airway gland secretions suggest a rapid turnover of O$_2$ in support of bactericidal reaction products. Thus we suggest that the expression of NADPH oxidase in the airway epithelium and acid secretion represents an innate defense function of the airway epithelium.

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