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Research article

Nadph oxidase and epithelial sodium channels regulate neonatal mouse lung development

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Abstract: *Background:* Epithelial sodium channels (ENaC) play critically important roles in lung fluid clearance at birth. We have previously shown that Nadph oxidase (NOX)-derived reactive oxygen species signaling activates ENaC and promotes alveolar fluid clearance. In this study, we examined a new physiological role for NOX-mediated ENaC activity in mouse lung development. *Methods:* NOX isoform and ENaC subunit mRNA levels were evaluated in preterm and neonatal C57Bl6 mouse lung using real-time PCR analysis. Newborn mice were intra-nasally treated with 1 mM amiloride, 100 μM NSC 23766, or 300 μM apocynin during postnatal days 1–15 to study development. Lung development was assessed using hematoxylin and eosin (H&E) staining, coupled with radial alveolar counts (RAC) and mean linear intercept (MLI) measurements. *Results:* ENaC subunits and NOX1-4 mRNA were detected in mouse lung during late gestation, birth, and postnatally. Inhibition of Rac-1-mediated-NOX signaling indicates functional (Rac-dependent) NOX1-3 isoforms in newborn lung, determined by dihydroethidium (DHE) detection of reactive oxygen species production in postnatal (PN) day 7 mouse lung. Amiloride inhibition of ENaC activity, NSC 23766 inhibition of Rac1, and apocynin inhibition of pan NOX activity attenuated normal alveolar development in mouse lung. *Conclusion:* NOX and ENaC play important roles in mouse lung development.

Keywords: epithelial sodium channels; Nadph oxidase; oxygen therapy; preterm birth

1. Introduction

Epithelial sodium channels (ENaC) are made up of α-, β-, and γ-ENaC subunits. Together, these

subunits form a trimeric structure that transports Na^+ from the airway lumen into the cytosol. As a result of net Na^+ movement across airway epithelia via ENaC, an osmotic gradient is generated which favors net absorption of water across the lung epithelium (clearing the airways and breathing space of excess fluid). Because of its role in maintaining lung fluid volumes, normal regulation of ENaC plays a critically important role in adult and newborn lung health [1]. Glucocorticoids (GC) are classical steroid regulators of ENaC, and prenatal GC therapy has been shown to decrease morbidity and mortality in preterm infants at risk for respiratory distress syndrome [2]. A study by O'Brodovich et al indicates that at birth increased P_{O2} acts with GC through an mTOR-related pathway to increase α-ENaC protein synthesis, thereby promoting fluid clearance in the new born lung [3,4]. Despite advances and understanding of GC therapy, newborn infants are still at risk for severe lung injury for reasons that require further investigation.

A better understanding of non-steroidal molecular regulators of lung ENaC activity can lead to novel therapeutic approaches for treating pulmonary disorders related to oxidative stress in adults and newborns.

We evaluate ENaC regulation in a mouse model of preterm birth because immature lungs are at a physiological disadvantage for fluid clearance and edematous lung injury. The immature lung is lined with thick cuboidal cells and capillaries that are still distant from the epithelium [5]. Coupled with lower ENaC expression [6,7], preterm lungs have a greater burden than the term lung in absorbing fluid at birth. Studies have shown that lung water in preterm infants is 25% greater than term infants [8], suggesting that decreased ENaC expression and/or activity may be compromised in preterm infants. Studies have also shown that inability to effectively absorb fluid near the time of birth leads to respiratory distress. Herein, we evaluate the molecular mechanisms that may be responsible for hindering normal fluid clearance in preterm lung, and the developmental consequences of inappropriate fluid removal near the time of birth.

Premature infants are often supplemented with O_2 therapy in the intensive care unit. Acutely, supplemental oxygen is a beneficial therapy for preterm lungs. However, prolonged O_2 exposure can lead to oxidative stress, fibrosis, inflammation, and even increased mortality due to excessive oxidative stress [9-12].

The molecular mechanisms underlying the positive and negative effects of oxygen exposure in the lung are not clearly understood. Since O_2 is the precursor to several reactive species, we evaluated a family of Nadph oxidases responsible for producing reactive oxygen species (ROS) in cells. Specifically, we examined the expression profile of Nadph oxidase isoforms 1–4 (NOX 1–4), in a mouse model of human preterm lung.

The spatial and functional relationship between NOX isoforms and lung ENaC activity in distal alveolar cells are illustrated in Figure 1. We have previously reported that NOX-derived reactive oxygen species up-regulate ENaC [13,14], and now hypothesize that loss of NOX-mediated ENaC activity in the preterm lungs compromise lung development.

2. Materials and Methods

Chemicals and reagents. Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich.

Animal model. Timed pregnant C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) at embryonic day 11 (E11) or bred in-house. All animal studies were performed in

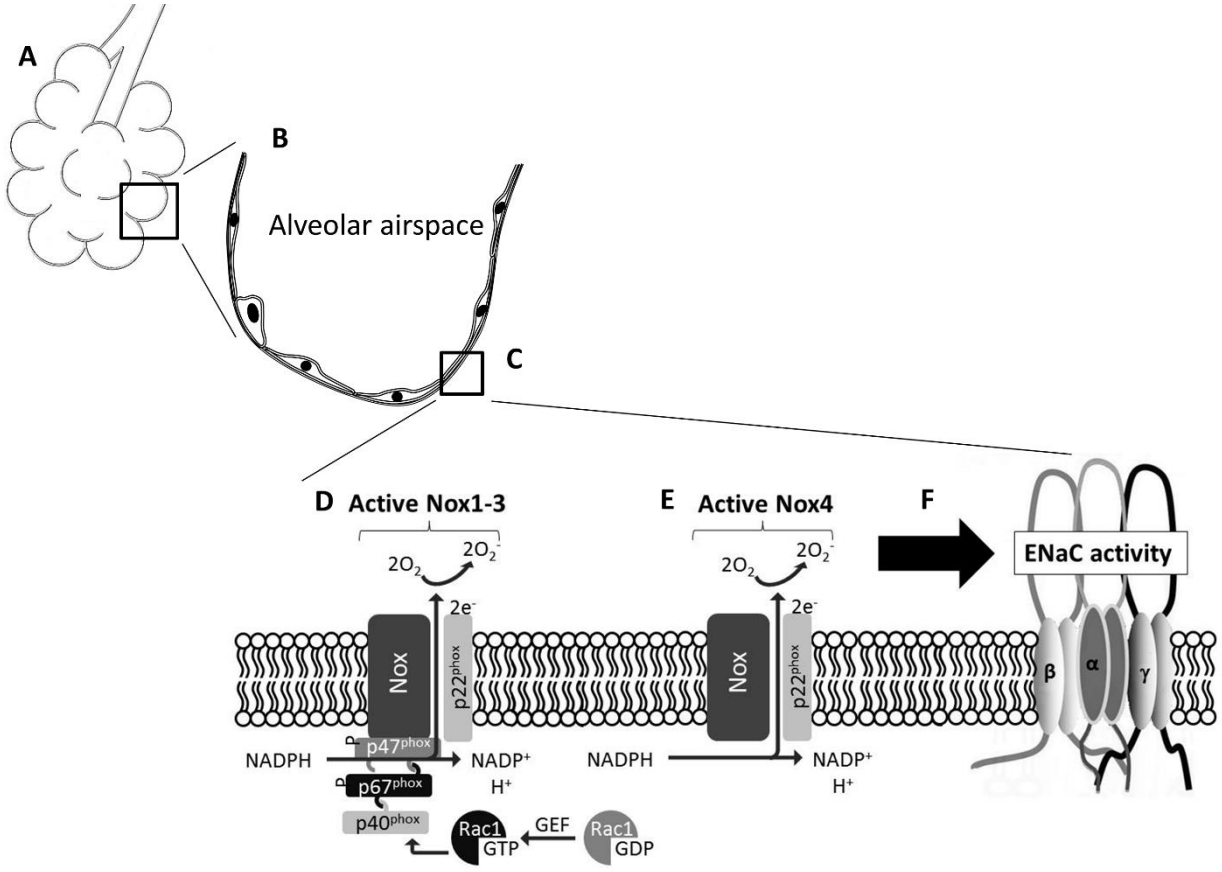

Figure 1. Diagram of membrane bound NOX isoforms and ENaC in alveolar epithelial cells. (A) Alveolar structures at a terminal bronchiole. (B) The alveolar epithelium consists of 95% alveolar type 1 (squamous) and 5% alveolar type 2 (cuboidal) cells by surface area. (C) NOX1–4 and ENaC are found on the membranes of both AT1 and AT2 cells. (D) The activation of NOX1–3 is mediated by the proper assembly of cytoplasmic subunits p40phox, p47phox, and p67phox by Rac1-GTP. Guanine nucleotide exchange factors (GEFs) are required to activate Rac1 by stimulating the release of GDP to allow binding of GTP. E) The activation of NOX4 is independent of cytoplasmic subunits and only requires the association with the stabilizing domain, p22phox. (F) Active NOX complexes produce superoxide (O_2^-), which regulate ENaC activity.

accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and were approved by the Institutional Animal Care and Use Committee.

Intranasal Treatment Protocol. Beginning on PN day 1 up to PN day 15, newborn C57BL/6 mice were intra-nasally treated with ENaC or NOX inhibiting compounds. Specifically, 1 mM amiloride, a direct blocker of ENaC activity; 100 μM NSC 23766, a specific inhibitor of Tiam1-mediated Rac1 activation; or 300 μM apocynin, an inhibitor of p47phox translocation and activation of Nox1–3, were intra-nasally delivered in 5 μL volume on PN days 1, 3, 5, and then increased to 20 μL volume on days 7, 9, 11, 13, and 15. Vehicle treatment consisted of corresponding volumes of phosphate buffered saline (PBS). Nasal administration of 5 μL of PBS was given to all treatment and control groups to ensure inhalation delivery. Cryoslices were prepared on PN day 17.

Cryosectioning and H&E staining. At PN day 17, lungs were perfused with PBS via the pulmonary artery until blanch and then uniformly inflated with Tissue-Tek OCT Compound (Sakura Finetek USA) via a tracheal cannula. The lung and heart tissue was removed en bloc and embedded in OCT in cryomolds. Molds were dipped in liquid nitrogen to flash freeze the tissue. Rapid freezing reduces the formation of ice crystals and minimizes morphological damage. Samples were stored at −80 °C until ready for sectioning. Tissues were cryosectioned using a Leica CM1520 cryostat (Leica Biosystems); 10 μm sections were fixed with acetone and stained with hematoxylin and eosin (H&E) for analysis of lung histology and morphology.

Radial alveolar counts (RAC) and mean linear intercepts (MLI). Lung alveolar morphology of H&E stained sections was visualized using an Olympus IX71 microscope and compatible camera attachment. RAC were calculated as described by Emery and Mithal [15]. MLI were measured as described by Dunnill [16], and using the equation $MLI = \frac{N \cdot L}{m}$, where N is the number of traverses, L is the length of the traverse, and m is the total number of intercepts by the traverses. Images from a minimum of ten 100X lung fields were taken from each pup, consisting of a mixture of images from the apex, middle, and base of each lung. RAC and MLI measurements were blinded.

AT2 cell isolation and lung tissue slices. Newborn mice (postnatal (PN) days 1–7) were anesthetized with an intraperitoneal injection of xylazine/ketamine and euthanized by exsanguination via the renal artery. Under a Zeiss Stemi 2000-C Stereo microscope, mouse pup lungs were lavaged three times with 30 μL PBS via a tracheal cannula to remove alveolar macrophages. Lungs were subsequently perfused via the pulmonary artery with PBS until blanch and then instilled with 0.3 mL dispase (1 U/mL) via the tracheal cannula until fully inflated. The trachea was then tied off and the lungs were removed en bloc and incubated at room temperature in 3 mL of dispase (1 U/mL) for 45 minutes. Afterwards, lung lobes were teased away from heart and bronchial tissue and minced in a 1:1 solution of neonatal calf serum (NCS) and 0.01% DNase 1 in mouse cell media (DMEM/F12 media with 10 mM HEPES, 0.04 mM L-glutamine, 1x MEM non-essential amino acids, and 1 mg/mL Primocin™). Cell suspension was sequentially filtered through a 100 and 40 μm filter for selection.

ROS measurements. PN day 7 mouse lung slices were treated with vehicle (PBS), 100 μM NSC 23766, or 300 μM apocynin for 20 min followed by incubation with dihydroethidium (DHE) and 4',6-diamidino-2-phenylindole (DAPI) to detect ROS and nuclei, respectively.

Primer design and mRNA analysis. Primer pairs (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA) and were designed to produce short amplicons (70–150 bp). Amplification efficiency of the primer pairs were tested using a calibration curve where cycle threshold values (Ct) were graphed against template concentration expressed as log relative dilution (data not shown). In this way, we determined that the amplification efficiency of primer pairs presented in Table 1 are near 100%.

Transcript levels of genes of interest were measured by quantitative real-time PCR. RNA samples were prepared from mouse pups at PN days 1–7. Total RNA was extracted from whole lungs using TRIzol (Invitrogen) and column-purified using the RNeasy isolation kit (Qiagen) per manufacturer protocol. RNA was treated was DNase 1 and reverse transcribed using Superscript II RNaseH-reverse transcriptase (Invitrogen). Cycle threshold levels of mRNA expression were normalized to mouse GAPDH levels.

Statistical analysis. Data are reported as means ± SE. Multiple comparisons were performed using one-way ANOVA followed by Scheffe's post-hoc test. All statistical analysis was performed using Statistical Analysis System (SAS 9.3) software. Results were considered significant if $P < 0.05$.

Table 1. Forward and reverse primer pairs used for transcript studies.

Primer Pairs	
NOX subunits	
p22phox-F	5'- AAC GAG CAG GCG CTG GCG TCC G -3'
p22phox-R	5'- GCT TGG GCT CGA TGG GCG TCC ACT -3'
p47phox-F	5'- CCA CAC CTG CTG GAC TTC TT -3'
p47phox-R	5'- ATC TTT GGG CAC CAG GTA TG -3'
NOX isoforms	
Nox1-F	5'- CAG TTA TTC ATA TCA TTG CAC ACC TAT -3'
Nox1-R	5'- CAG AAG CGA GAG ATC CAT CCA -3'
Nox2-F	5'- CAG GAA CCT CAC TTT CCA TAA GAT -3'
Nox2-R	5'- AAC GTT GAA GAG ATG TGC AAT TGT -3'
Nox3-F	5'- GCT GGC TGC ACT TTC CAA AC -3'
Nox3-R	5'- AAG GTG CGG ACT GGA TTG AG -3'
Nox4-F	5'- CCC AAG TTC CAA GCT CAT TTC C -3'
Nox4-R	5'- TGG TGA CAG GTT TGT TGC TCC T -3'
ENaC subunits	
α-ENaC-F	5'- TGC TCC TGT CAC TTC AGC AC -3'
α-ENaC-R	5'- CCC CTT GCT TAG CCT GTT C -3'
β-ENaC-F	5'- CCC CTG ATC GCA TAA TCC TA -3'
β-ENaC-R	5'- GCC CCA GTT GAA GAT GTA GC -3'
γ-ENaC-F	5'- ACC CTT TCA TCG AAG ACG TG -3'
γ-ENaC-R	5'- CCT CTG TGC ACT GGC TGT AA -3'
Reference gene	
GAPDH-F	5'- CAA GGT CAT CCA TGA CAA CTT TG -3'
GAPDH-R	5'- GGC CAT CCA CAG TCT TCT GG -3'

3. Results

3.1. Perinatal ENaC and NOX expression

Newborn mice clear fluid within hours of birth [17]. At birth, the newborn mouse lung is in the saccular stage of lung development and are still several days away from complete alveolar development. Infants born prematurely (between weeks 24–36 of gestation) are also undergoing lung development in the saccular stage. Unlike human lungs, however, the mouse lung completes alveolar development between post-natal (PN) days 1–20. Although newborn mice may not secrete surfactant nor develop respiratory distress as seen in human infants, newborn mice are still an ideal animal model for examining the acute signaling mechanisms responsible for triggering ENaC activity in a mammalian system.

We measured the transcript level of ENaC subunits and NOX enzymes at embryonic day 17, on the day of birth, and on post-natal day 7. The results are shown in Figure 2A. Antenatal evaluation of ENaC transcript expression reveals that α-, β-, and γ-ENaC subunits significantly increase between embryonic day 17 (E17) and birth, with α-ENaC exhibiting the sharpest rise in expression level

between birth and PN day 1. Between PN days 2–7, all ENaC subunit expression levels stabilize and are expressed at near equal (normalized) value in mouse lung. Of the four NOX isoforms evaluated in mouse lung (Figure 2B), NOX4 transcript expression levels were significantly higher than NOX 1–3 throughout the perinatal period observed. NOX1 and NOX3 transcript levels remain unchanged between PN days 1–7; NOX2 transcript levels elevated in a linear manner, but failed to reach statistical significance.

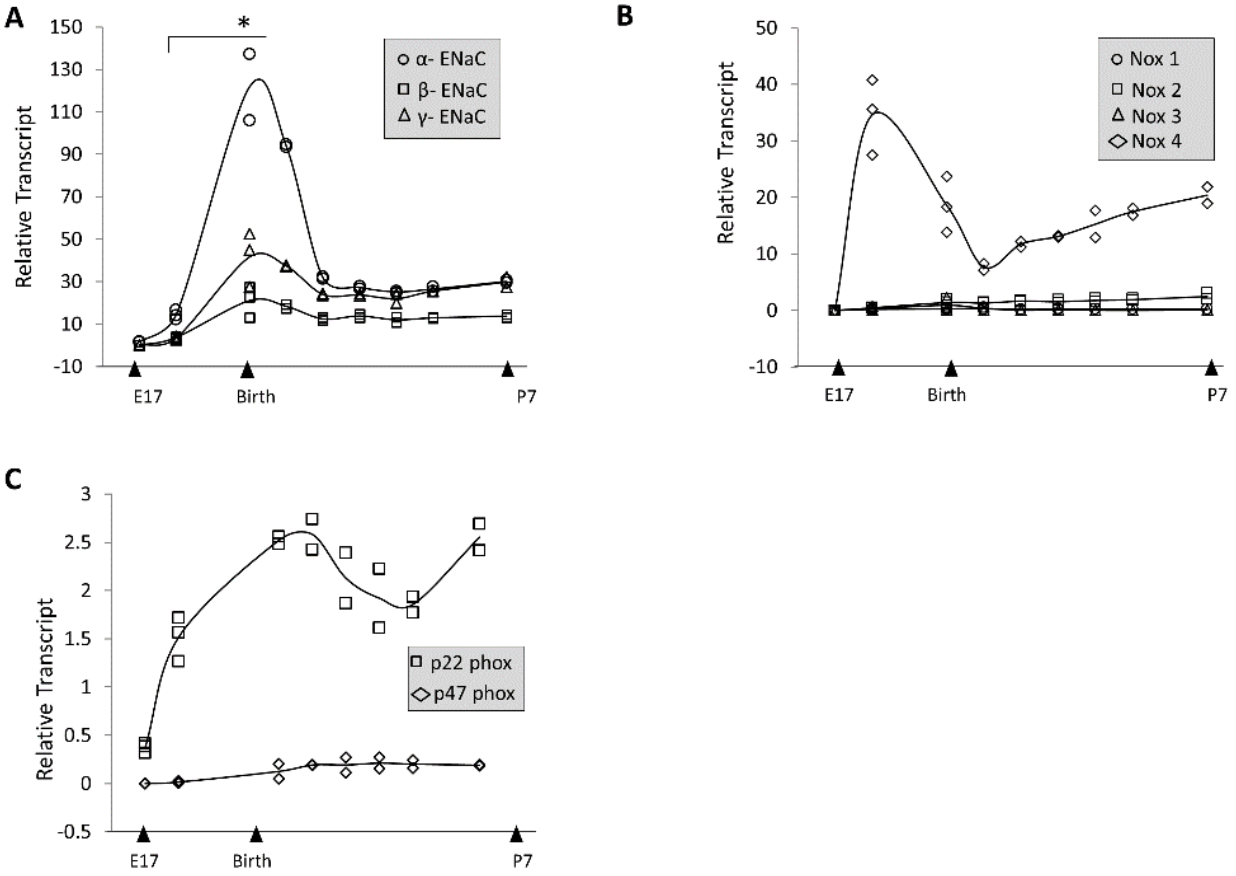

Figure 2. NOX and ENaC expression during gestational postnatal lung development periods. (A) Correlation between normalized α-ENaC, β-ENaC, γ-ENaC mRNA expression at embryonic day 17 (E17), birth, and post-natal (PN) days 1–7. (B) Correlation of NOX1-4 gp91phox subunit expression on E17, birth, PN days 1–7. (C) Comparison of p22phox and p47phox mRNA at E17, birth, and PN days 1–7. All data represents a total of 12 pups from two separate deliveries in each age group). *$P < 0.05$.

As shown in Figure 1, the catalytic domain of NOX contains an essential p22phox auxiliary domain which serves as an essential binding site for the catalytic domain that associates with gp91phox in a 1:1 complex and contributes to the maturation and stabilization of this protein. There are also corresponding regulatory domains that are turned on after activation. The p22phox auxiliary domain (for all NOX isoforms) was detected at significantly higher level than the p47 phox regulatory domain (needed for NOX1-3). After normalization, we found p22phox and p47 phox transcript levels to be very low, relative to catalytic domain expression levels (Figure 2C).

3.2. Functional NOX expression in postnatal mouse lung

Next, we treated PN day 7 lung tissue with NSC23766, a selective inhibitor of Rac1-GEF interaction, in order to study Rac1 dependent NOX1-3 generation of reactive oxygen species in newborn lung. Apocynin is an additional pan NOX inhibitor used to evaluate all NOX-derived ROS production in PN day 7 lung tissue. Figure 3 shows that NSC23766 and apocynin treatments significantly decreased detection of reactive oxygen species production in newborn mouse alveolar cells. Since we were able to detect changes in ROS levels in PN day 7 lung tissue, the data is suggestive of Rac1-dependent NOX and NOX4) enzyme functionality as early as PN day 7 in mice (albeit NOX4 transcript levels were detected at the highest level in the newborn mouse lung).

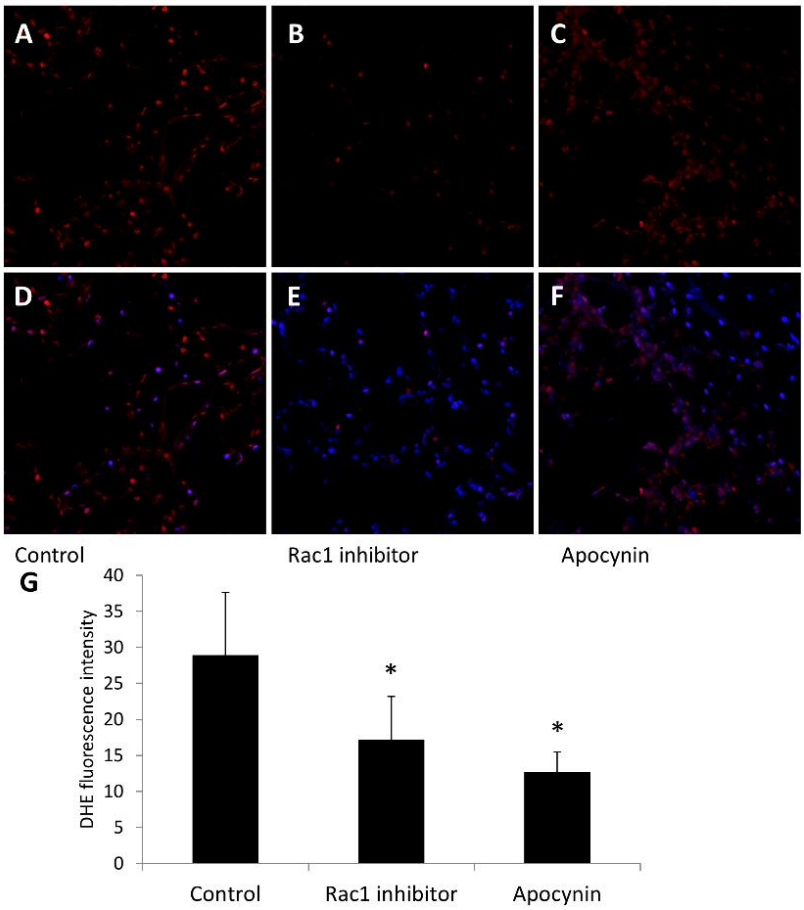

Figure 3. Inhibition of ROS production in neonatal lung epithelia by NSC 23766 and Apocynin inhibition. (A–F) Representative micrographs from PN day 7 mouse lung slice exposed (from left to right) to vehicle (n = 134 regions of interest (ROI) as shown from 2 pups from 2 separate deliveries), NSC 23766 (n = 48 ROIs from 2 pups from 2 separate deliveries), an inhibitor of Rac1 activity, or apocynin (n = 200 ROIs from 2 pups from 2 separate deliveries). Top panel shows fluorescent red detection of ROS using the superoxide indicator dihydroethidium (DHE), and bottom panel shows ROS and nuclear (DAPI; blue) labeling. (G) Quantification of DHE staining intensities using NIH ImageJ. Original 100X magnification.

3.3. NSC 23766, apocynin, and amiloride treatment inhibit postnatal lung development

It has been shown that transgenic mice lacking α- and γ- ENaC subunits fail to thrive beyond the first 16 hours of birth [18,19]. Alveolar flooding is believed to be the primary cause of death in α- and γ-ENaC knock-out mice. In order to study the physiological consequence of abnormal ENaC regulation in viable lung, we periodically disrupted ENaC function using amiloride and NOX enzyme inhibitors between PN days 1–15 in the mouse lung (see treatment protocol Figure 4). Then, we evaluated lung development in H&E stained sections of cryopreserved lungs.

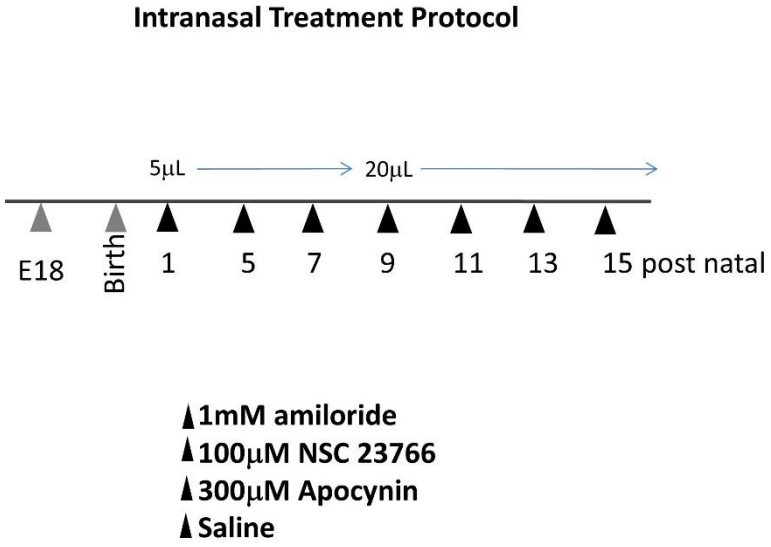

Figure 4. Intranasal Treatment Protocol. Schematic of intranasal insufflation of amiloride and NOX inhibitor compounds on postnatal (PN) days 1–15. On PN day 17 cryoslices were prepared for H&E staining.

We show that mice intermittently exposed to amiloride and NOX enzyme inhibitors (on PN days 1, 3, 5, 7, 9, 11, 13, and 15) have reduced lung septation and increased alveolar size (Figure 5A–E) The phenotype observed is indicative of impaired lung development. All outcomes were compared to untreated and vehicle treated mice. Quantitative analysis shows that mice exposed to NSC 23766, apocynin, or amiloride have significantly reduced radial alveolar counts (RAC) and increased mean linear intercepts (MLI) (Figure 5F–G). This data shows that both NOX signaling and ENaC activity play key roles in postnatal alveolar development in newborn mouse lung.

4. Discussion

Cellular and molecular implications of findings

To date, the mechanisms that lead to ENaC activation in the neonatal lung remain unclear, albeit oxygen signaling as the fetal lungs transition from low to high oxygen tension has been implicated in regulating lung ENaC [3,4,20,21]. Previous studies in adult lung showed that Rac-mediated NOX enzyme activity plays an important role in regulating lung and kidney ENaC activity [13]. In the

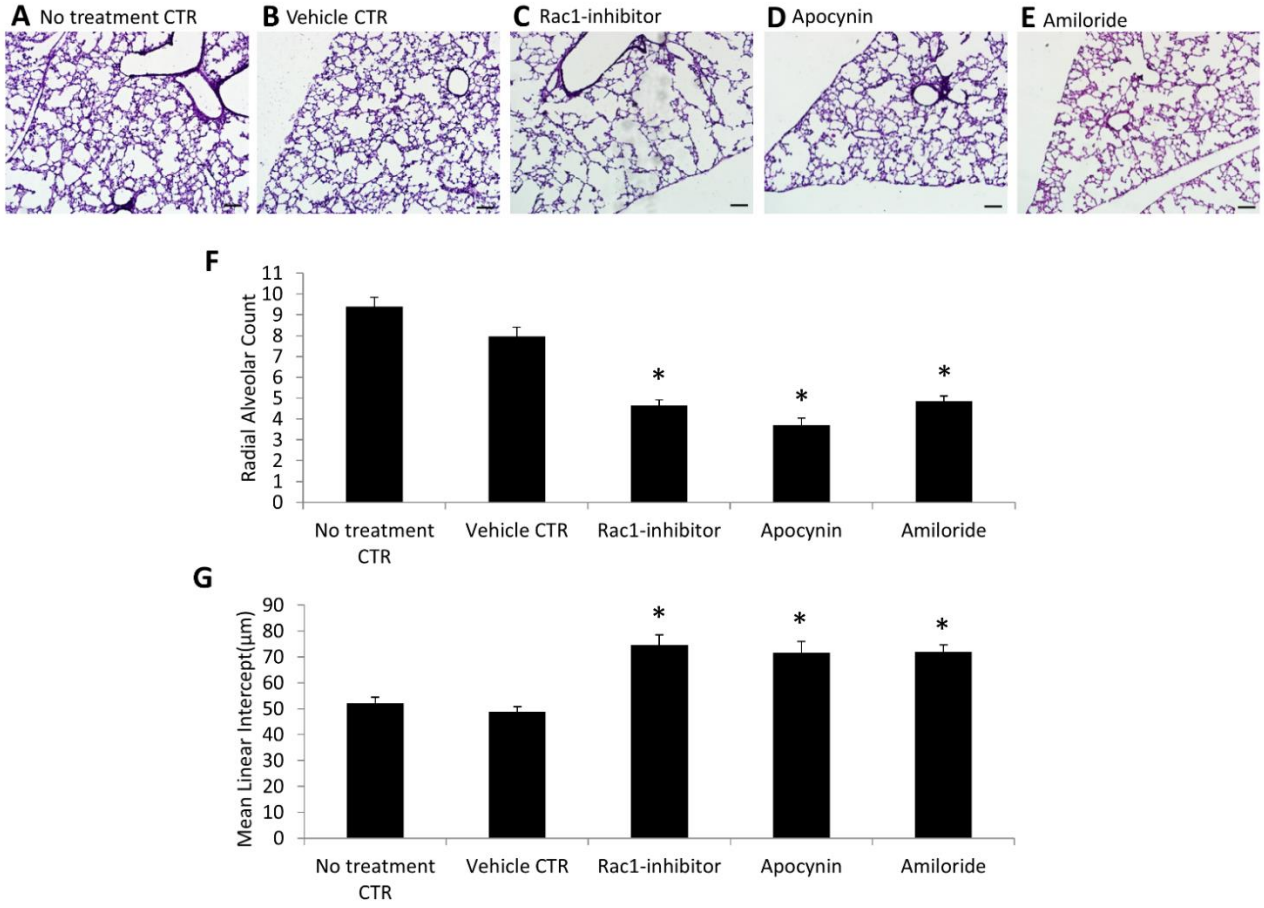

Figure 5. Inhibition of ENaC and NOX activity attenuates alveolar development. Representative H&E staining from P15 mouse pups undergoing no treatment (A), or exposed to (B) vehicle (C) NSC 23766 (D) Apocynin, or (E) amiloride. Calibration bar = 100 μm, Original 100X magnification. (F) Summary of radial alveolar counts of A–E. (G) Summary of mean linear intercepts of A–D. Number of observations: No treatment control group: n = 35 independent observations from 2 mice born from the same litter. Vehicle control group: n = 29 independent observations from 2 mice born from the same litter. Rac1-inhibitor treatment group: n = 36 independent observations from 3 mice born from the same litter. Apocynin treatment group: n = 10 independent observations from 1 mouse. Amiloride treatment group: n = 40 independent observations from 3 mice born from the same litter. *P < 0.05 compared to vehicle.

present study, we show that NOX isoforms are functionally expressed within the first week of life which suggests that NOX-derived ROS also play key roles in lung development. Each of the NOX isoform may be acting in concert to sense, and then signal, for appropriate release of ROS. As one possible mechanistic scenario, it may be possible that the Rac-mediated NOXes release low, yet sufficient levels of ROS to stimulate further H_2O_2 release from NOX4, thereby amplifying oxygen signal transduction in the newborn lung. In support of this, Pendyala et al. have shown that NOX4 is indeed redox sensitive and activated by ROS [22,23]. We found NOX4 expression levels to peak at gestational age E17. This observation indicates that NOX4 sensing of O_2 may be primed prior to birth for

immediate sensing and then signaling to lung ENaC for fluid clearance near birth. We, and others [24-27], report significant rise in ENaC subunits immediately preceding birth to facilitate lung fluid clearance. The novelty of our report is that it offers mechanistic insight implicating oxygen sensors, the NOX family of enzymes, as important regulators of lung ENaC activity near birth. Whether there is cross-talk amongst NOX-derived ROS and other key proteolytic regulators of ENaC [28,29] requires further study. Below, we discuss possible clinical limitations of antioxidant therapy that would infringe upon NOX-mediated ENaC activity.

5. Conclusion

In adults and newborns, ENaC activity is critically important in facilitating lung fluid clearance [18,30-33]. In preterm lungs, however, any delay in fluid clearance may lead to severe pulmonary distress because the alveolar airspace responsible for effective gas exchange is not yet fully developed. Therefore, preterm infants have higher incidences of respiratory distress syndrome, transient tachypnea, and respiratory failure than term infants, and are frequently admitted into neonatal intensive care units. The molecular regulators of newborn lung pathogenesis remain unclear and requires more investigation.

Several investigative groups have shown that hyperoxia disrupts normal lung development [34-36]. The oxidative stress caused by prolonged exposure to high levels of oxygen clearly leads to lung injury in these newborn lung studies. However, our studies indicate that lower levels of tissue NOX production of ROS production may play an important role in regulation ENaC in the developing lung and possibly alongside classical signaling mechanisms induced by steroid hormones [37]. We showed that inhibiting enzymatic NOX and normal ENaC activity resulted in decreased septation and attenuation of alveolarization in newborn mouse lung. The phenotype observed in NOX and ENaC inhibited mouse lung closely resembles the phenotype commonly associated with pre-term infants born with serious respiratory distress who go on to develop bronchopulmonary dysplasia (BPD) [38-40]. Since BPD is a chronic newborn lung disorder associated with arrested structural development of the lung, and inhibition of normal lung septation and alveolarization, our findings indicate that activating tissue NOX and lung ENaC activity may have beneficial outcomes for preterm infants susceptible to developing BPD. However, more research is needed in this area of investigation to define the fine line between the beneficial effects of O_2, and oxidative stress. For example, there is need to fine tune O_2 signaling in the developing lung, since intervention therapies targeted at enhancing lung antioxidant capacity did not significantly improve health outcomes in the lungs of preterm infants [41]. It may be plausible that excessive antioxidant therapy may hinder beneficial effects of O_2 signaling, such as the permissive effects of NOX-derived ROS on ENaC activity in the developing lung.

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Conflict of interest

The authors have no conflict of interest to declare.

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