Abstract

The NADPH oxidase reaction produces protons. In the case of the NADPH oxidase, NOX2, activity depends on secretion of these protons and is inhibited by blockade of the voltage-gated proton channel (Hv1). Duox1 and Duox2 activities similarly produce intracellular protons but synthesize hydrogen peroxide directly instead of superoxide. Hv1 contributes to acid secretion in some epithelia that express Duox. To test the hypothesis that Duox activity is also sensitive to Hv1 channel blockers, Duox was assayed in the presence of either Zn^{2+} or 5-chloro-2-guanidinobenzimidazole (ClGBI). Both compounds inhibited Duox activity in normal human bronchial epithelial cells but with an IC50 over 10-fold higher than that reported for Hv1 (IC50 Zn^{2+} = 0.68 mM; IC50 ClGBI = 0.07–0.14 mM). Homogenized HEK293T cells expressing either Duox1 or Duox2 showed similar IC50 values for ClGBI suggesting these compounds inhibit the enzymes through alternate mechanisms independent of Hv1 proton secretion. Inclusion of superoxide dismutase did not restore Duox hydrogen peroxide synthesis. Addition of nigericin to eliminate any possible transmembrane pH gradients in intracellular membrane-localized Duox did not alter activity in HEK293T homogenates. Extracellular Zn^{2+} blocked intracellular Ca^{2+} increases needed for Duox activity. Together the data suggest that Duox enzyme activities in epithelia are inhibited by compounds that block Hv1 but inhibition occurs through Hv1-independent mechanisms and support the idea that Hv1 is not required for Duox activity.

Keywords:
Duox
Hv1
Zinc
Bronchial epithelium

Introduction

The NADPH Oxidases are a seven-member family of ubiquitous enzymes that catalyze formation of H_2O_2, either indirectly via dismutation of superoxide or directly, in the case of Duox and Nox4 [1]. H_2O_2 participates in a variety of cellular functions including formation of antibiotic compounds, thyroid hormone synthesis and redox regulation of cellular proteins. NADPH oxidase activity releases a proton from NADPH during catalysis. Notably, in phagocytes that express a high level of Nox2, high NADPH oxidase activity increases intracellular concentration of protons and depolarizes the membrane by extrusion of superoxide. Both the intracellular acidification and the depolarization tend to inhibit Nox2 activity. However, the depolarization and intracellular acidification increase the probability of opening the voltage-gated proton channel (Hv1) that dissipates the generated H^+ gradient and repolarizes the membrane potential by the extrusion of protons [2]. Inhibition of Hv1 in phagocytes with Zn^{2+} inhibits Nox2 activity presumably by blocking the extrusion of protons, which would allow Nox2 to depolarize the membrane and acidify the cytosol [3]. Duox activity is linked to proton secretion and can contribute to intracellular acidity in bronchial epithelial cells [4–6]. Activated Duox activity in epithelial cells appears to be substantially less compared to Nox2 activity in activated phagocytes. For this reason, Fischer [6,7] suggested that Duox does not generate sufficient intracellular protons to require Hv1 activity. On the other hand Duox could generate locally acidic conditions, albeit difficult to measure, that could activate Hv1. In addition, Fischer and colleagues have shown that Hv1 contributes substantially to apical acid secretion in airway epithelia (e.g. 8).

In this study, we examined the effects of Hv1 channel blockers on Duox activity in primary human epithelia and in a cell-free assay of heterologous expressed Duox. The data showed that Duox activity is sensitive to Hv1 channel blockers but at concentrations significantly above that needed to inhibit Hv1. The data suggested that Duox activity does not rely on movement of protons by the voltage-gated channel Hv1 and is not inhibited by blockade of the major proton secretion mechanisms at work in primary bronchial epithelial cells. Instead, the data suggest that Duox activity is inhibited by compounds that block Hv1.
suggested Hv1 inhibitors reduced Duox activity through alternate mechanisms unrelated to Hv1 activity and point to a role for the H+/K+ ATPase in regulating pHi.

2. Materials and methods

2.1. Cell culture

Primary normal human bronchial epithelial (NHBE) cells were isolated from organ donor lungs rejected for transplant and obtained by the Life Alliance Organ Recovery Agency of the University of Miami according to IRB approved protocols. Isolation, air-liquid interface (ALI) culture and redifferentiation of NHBE were previously described [9,10]. All experiments were performed with date, passage and lung matched control cultures. shRNA-mediated knockdown in NHBE culture used MISSION® shRNA in pLKO.1 lentiviruses as described [11]. HEK293T (ATCC, CRL-3216) were cultured as recommended by ATCC. All cultures were discarded after 10 passages. HEK293T cell lines, expressing either Duox1 or Duox2 activity, were constructed using pCDH lentiviral vectors (System Biosciences, Palo Alto CA). See Supplemental methods for details.

2.2. NHBE H2O2 synthesis activity

NHBE cell H2O2 synthesis was measured using modifications of an Amplex Red assay described previously [12]. For Zn2+ inhibition experiments, apical phosphate buffers were replaced by either HEPES Ringers, pH 7.4 or Tris Ringers pH 7.4 [13] to prevent Zn2+ precipitation. Zn2+ containing buffer was added to apical surface for 30 min prior to assays. For CIGBI inhibition, either PBS or HEPES Ringers were used (see Supplemental methods for composition) and CIGBI was added to the basolateral solution 30 min before assay. Buffer containing 25–50 μM Amplex Red (AR) (10-Acetyl-3,7-dihydroxyphenoxazine, Cayman Chemical, Ann Arbor, MI) and 0.05–0.1 U/ml HRP (SigmaAldrich, P2088) was added to the apical surface of NHBE cultures and recorded continuously in a Biotek SynergyH1 plate reader (530 nm ex/590 nm em). After a 5–10 min baseline, cultures were stimulated by addition of 100 μM ATP. When assays used Fura-2 loaded cells, excitation was at 545 nm. Fluorescence was recorded at 30–40 s intervals and H2O2 synthesis rates were calculated from the slope of RFU change over time using a 1–2 min sliding window with a 30–40 s step. Initial rates were normalized to cultures treated with vehicle alone and IC50 values were calculated by nonlinear regression using PRISM 5.0b (GraphPad Software). See Supplemental methods for details on

**Abbreviations**

AR  10-Acetyl-3,7-dihydroxyphenoxazine, Amplex Red  
ALI  air-liquid interface  
CIGBI  5-chloro-2-guanidinobenzimidazole  
DPBS  Dulbecco’s Phosphate buffered saline  
NHBE  normal human bronchial epithelia  
pHi  intracellular pH

Fig. 1. High concentrations of Hv1 channel blockers inhibit Duox H2O2 synthesis. H2O2 synthesis by fully differentiated NHBE cells was assayed in the absence and presence of either Zn2+ (panel a, Tris-Ringers solution) or CIGBI (panels b–d, PBS). Rates of AR oxidation were normalized to assays in the absence of inhibitors but with vehicle. ATP (100 μM) was used to stimulate Ca2+ dependent Duox activity. All values are means ± s.e.m. Panel a, Zn2+ inhibited ATP stimulated Duox H2O2 synthesis (IC50 = 0.68 mM, 3–5 lung donors, triplicate cultures each donor). Panels b and c, CIGBI inhibited both baseline Duox activity (IC50 = 0.070 mM, n = 3–6 lung donors, triplicate cultures each donor) and stimulated activity (IC50 = 0.12 mM, n = 3–6 lung donors, triplicate cultures each donor). Panel d, Inhibition of stimulated Duox activity by CIGBI (0.3 mM) was not rescued by addition of superoxide dismutase (20 Units/ml), n = 6 cultures, 3 lung donors.
HEK293T homogenization and H$_2$O$_2$ synthesis assay.

2.3. Intracellular Ca$^{2+}$ and pH measurement

Differentiated NHBE cultures were loaded with Fura-2AM (10 μM) or BCECF-AM (5 μM) (both from Molecular Probes), washed and fluorescence followed in a microplate reader until fluorescence ratios stabilized. Intracellular Ca$^{2+}$ ([$Ca^{2+}$]$_i$) was estimated using an intracellular K$_d$ = 0.25 μM. Intracellular pH (pHi) was assessed using high K$^+$ buffers containing 20 μM nigericin. See Supplemental methods for details.

2.4. PCR, western blotting and data analysis

Quantitative PCR used TaqMan gene expression kits. Western blotting of Duox in HEK293T homogenates used rabbit anti-Duox1 (SigmaAldrich, SAB2701393), see Supplemental methods. Statistical analysis was performed using JMP Pro (SAS Institute).

3. Results and discussion

3.1. Hv1 channel blockers inhibit Duox H$_2$O$_2$ synthesis

Hv1 channel inhibitor effects on Duox activity were studied in differentiated NHBE cells cultured at the ALI. These cells express Duox1 and Duox2 at levels much higher than other members of the NOX family [11] and express functional Hv1 [4,8]. Previously, NHBE H$_2$O$_2$ synthesis was shown to be due to Duox1 and Duox2 activity [12,14,15] and that Duox contributes to intracellular acid production in NHBE [4]. Extracellular Zn$^{2+}$ inhibits Hv1 and decreases H$^+$ secretion in a variety of cells, including NHBE [4,8]. Thus, cultures were assayed for H$_2$O$_2$ synthesis in various apical [Zn$^{2+}$] with and without ATP stimulation that increases [Ca$^{2+}$]$_i$ and stimulates Duox activity [12,16,17]. Assays were conducted at room temperature since it was previously shown that Zn$^{2+}$ stimulated thyroid NADPH oxidases (Duox1 and Duox2) at elevated temperature in cell free assays [18]. In NHBE cultures, Zn$^{2+}$ inhibited ATP-stimulated H$_2$O$_2$ synthesis with an apparent IC$_{50}$ = 0.68 mM (Fig. 1a) but failed to affect baseline activity. This IC$_{50}$ is substantially higher than that expected for Hv1 inhibition [19]. The solubility of free Zn$^{2+}$ at 25°C and pH 7.4 in phosphate free buffers was calculated to be 0.48 mM [20] and limits the accurate determination of the IC$_{50}$.

Since Hv1 gating is strongly temperature dependent [21,22] it was possible that the lower temperature used for Zn$^{2+}$ inhibition might prevent observation of an effect on H$_2$O$_2$ synthesis. For this reason, assays were also conducted in 5-chloro-2-guanidinobenzimidazole (ClGBI, SigmaAldrich, CAS# 70590-32-8), a cell permeable Hv1 inhibitor [23]. These assays were conducted at 37°C. ClGBI inhibited both

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**Fig. 2.** Both Duox2 and Duox1 are inhibited by ClGBI and Zn$^{2+}$ inhibits intracellular Ca$^{2+}$ increases concurrently with inhibition of Ca$^{2+}$-stimulated Duox activity. Panel a, Duox1 expression was reduced by shRNA expressing lentivirus (see Supplemental methods). Transcripts relative to β$_2$-microglobulin were reduced > 95%, n = 3 cultures, 1 donor. Panel b and c, H$_2$O$_2$ synthesis by differentiated NHBE cells with reduced Duox1 activity were assayed in PBS in the absence and presence of ClGBI. Rates of AR oxidation were normalized to assays in vehicle only. ClGBI inhibited both baseline Duox2 (IC$_{50}$ = 0.12 mM) and stimulated activity (IC$_{50}$ = 0.13 mM), n = 3 cultures, one lung donor. Panel d, Following ATP stimulation, [Ca$^{2+}$]$_i$ transiently increased in control cultures but the increase was blunted in the presence of Zn$^{2+}$ (300 μM in HEPES Ringers) (n = 2 lung donors, triplicate cultures of each donor). Panel e, Following ATP stimulation, H$_2$O$_2$ synthesis rate (Resorufin RFU/sec) increased in control cultures but was inhibited by Zn$^{2+}$ (300 μM in HEPES Ringers). Panels d and e, n = 6 cultures from 2 lung donors. RFU/sec was calculated with a 60 s sliding window and 30 s steps. Panel f, example curves of a control culture (red circles) and a Zn$^{2+}$ treated cultures used in panels d and e are shown along with tangents to the region used for slope calculations over the first 2 min following ATP stimulation.
baseline and ATP-stimulated H2O2 synthesis with an apparent IC50 = 0.07 mM and 0.12 mM respectively (Fig. 1b and c) that is about 10 fold higher than expected for Hv1 inhibition [23]. To rule out the possibility that inhibition of Hv1 resulted in synthesis of superoxide rather than H2O2, the normal product of Duox activity, superoxide dismutase was added to the assays and did not increase the measured H2O2 (Fig. 1d). Thus, the IC50 of both inhibitors for Duox activity was greater than expected based on Hv1 sensitivity [19,22,23].

Since Duox1 is responsible for the majority of ATP-stimulated H2O2 synthesis in NHBE [11], it was possible that Duox2 sensitivity to Zn2+ or CIGBI could be obscured by the larger Duox1 activity. NHBE cells, that had shRNA-reduced Duox1 expression (Fig. 2a), were assayed and the baseline and stimulated activity IC50 for CIGBI in these Duox1 knock down cultures was indistinguishable from the control cultures (IC50 = 0.12 and 0.13 mM respectively, Fig. 2b and c).

3.2. Zn2+ reduces intracellular Ca2+ transients concurrently with Duox inhibition

Zn2+ and CIGBI inhibition of H2O2 synthesis differed with respect to effects on baseline activity with Zn2+ only inhibiting ATP-stimulated activity. Duox1 and Duox2 both bind Ca2+ via an EF hand to stimulate activity. Since Zn2+ is known to block [Ca2+]i transients in epithelial cells [24,25], it was possible Zn2+’s effect on Duox H2O2 synthesis was due to reduction of the ATP-stimulated purinergic Ca2+-signaling. To assess the effect of Zn2+ on [Ca2+]i, H2O2 synthesis and changes in [Ca2+]i were measured simultaneously. Addition of Zn2+ (300 μM) reduced the transient [Ca2+]i increase following ATP stimulation with concomitant reduction of H2O2 synthesis (Fig. 2d and e). It appeared that Zn2+ inhibition of H2O2 synthesis might be due in part to suppression of Ca2+-induced Duox enzyme activity. The data are confounded by any intracellular Zn2+ that would also bind to Fura-2 [26,27], however such binding will shift Fura-2 fluorescence to give an apparent increase in [Ca2+]i rather than a decrease. Consequently, the Zn2+ effect on [Ca2+]i, seen in Fig. 2d is underestimated and a greater reduction of [Ca2+]i, supports that Zn2+ reduced the Ca2+ transient simultaneously with Duox inhibition.

3.3. Duox in HEK293T cell homogenates is inhibited by CIGBI

Several attempts were made to lower Hv1 expression in NHBE cells via HVGCN1-directed shRNA without substantial reduction. As an alternative approach, Duox1 and Duox2 were expressed along with their partners DuoxA1α and DuoxA2 in HEK293T cells (Supplemental Figure) that express barely detectable levels of HVGCN1 mRNA (data not shown) and protein (in Supplementary Fig. 2 of [28]). To further support that CIGBI inhibition of Duox is unrelated to blocking Hv1 channels, H2O2 synthesis by Duox1 and Duox 2 was assayed in homogenates of these HEK293T cells in the presence and absence of CIGBI. The assays showed that CIGBI inhibited Duox1 and Duox2 in homogenates of HEK293 cells (Fig. 3a and b) with an IC50 similar to that seen in intact NHBE cells (IC50 = 0.14 and 0.11 respectively). Since Duox 1 and 2 are also found in intracellular compartments (e.g. [29]) the data also suggest that intracellular Duox1 and Duox2 were not protected at lower inhibitor concentrations due to cellular location.

3.4. H+/K+ ATPase plays a role in controlling NHBE pH but not Duox activity

In NHBE, Hv1 gating primarily depends on the transmembrane pH gradient, because the membrane voltage is fairly constant about −20 mV in the apical membrane [2,7,30,31]. Measurement of pH in NHBE in mucosal pH buffers used in the Duox assays (Supplemental Table) suggests that the pH gradient in our assays is insufficient to open Hv1 [31] and, indeed, addition of Hv1 inhibitors failed to lower pH. These data suggested that effects of Hv1 inhibition on Duox activity might not be detectable because it is inactive under our assay conditions. However, it is possible that intracellular vesicles were providing an opportunity to form transmembrane pH gradients since intracellular organelles can form vesicles that are sealed and any Duox localized in these vesicles could be subject to tonic transmembrane gradients. To this end, HEK293T homogenates were also assayed in the presence of nigericin to disrupt any possible H+ gradients. Nigericin requires cholesterol to intercalate into the membrane and although, most cholesterol is found in the plasma membrane, cholesterol is synthesized in the endoplasmic reticulum and traffic to and from the plasma membrane. Consequently, cholesterol is also found in intracellular membranes although at lower levels than the plasma membrane. Assay of cell homogenates in the presence of nigericin (20 μM) did not alter the CIGBI effects on H2O2 synthesis (Fig. 3c), arguing that inhibition of Duox in homogenates is not related to intracellular action of Hv1.

NHBE cells express multiple proteins to control pHi (viz. 7) including apical, Ouabain-sensitive H+ /K+ exchanger (ATP12A), shown to be highly expressed [32] in bronchial epithelia and to regulate airway surface liquid pH [32–35]. NHBE cells express approximately 10 fold higher levels of ATP12A compared to HVGCN1 (Fig. 4a). Ouabain (1 mM) addition to mucosal buffers did not block Duox H2O2 synthesis (not shown) but measurement of ASL pH of NHBE cultures at the ALI, in buffer conditions used for CIGBI studies, showed a large apical Ouabain-sensitive H+ secretion that rapidly reduced the pH (0.01 pH unit/ min) of the apical assay buffer (Fig. 4b). Neither addition of Zn2+ nor CIGBI further reduced the apical acidification suggesting that ATP12A
was controlling extracellular pH in the H$_2$O$_2$ synthesis assays. Measurement of pHi in the presence of Ouabain and/or Zn$^{2+}$ showed that Ouabain, but not Zn$^{2+}$, decreased pHi (Fig. 4c). Since Ouabain is known to have off-target effects (e.g. CFTR stimulation [36,37]), ATP12A expression was reduced by shRNA (Fig. 4d) and pHi assessed. Knockdown of ATP12A expression in NHBE cells reduced pHi to a similar extent (Fig. 4e).

4. Conclusions

These studies support the idea that Hv1 conductance is not needed to maintain Duox activity in differentiated NHBE cells. The studies also suggest that inhibition of the non-gastric H$^+$/K$^+$ ATPase leads to modest changes in pHi, but this change in pHi does not significantly alter Duox activity. In addition, the studies suggest that effects of extracellular Zn$^{2+}$ (300 μM) may be due to Zn$^{2+}$-mediated changes in [Ca$^{2+}$], and that CGBI affects other proteins in addition to Hv1. Thus, these inhibitors’ target effects can potentially complicate interpretation of data from inhibitor studies of Hv1 and NADPH oxidase.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101346.

References

[1] J.D. Lambeth, A.S. Neish, Nos enzymes and new thinking on reactive oxygen: a double-edged sword revisited, Annu. Rev. Pathol. 9 (2014) 119–145 PubMed PMID: 24050626.
[2] T.E. DeCoursey, Voltage-gated proton channels find their dream job managing the respiratory burst in phagocytes, Physiology 25 (1) (2010) 27–40 PubMed PMID: 20134026.
[3] T.E. DeCoursey, D. Morgan, V.V. Cherny, The voltage dependence of NADPH
