Published in final edited form as:

Int Forum Allergy Rhinol. 2013 May ; 3(5): 376–383. doi:10.1002/alr.21133.

Expression of dual oxidases and secreted cytokines in chronic rhinosinusitis

Do-Yeon Cho, MD^{1,2}, Jayakar V. Nayak, MD, PhD¹, Dawn T. Bravo, PhD¹, Wei Le, MD¹, Alan Nguyen, BA¹, Justin A. Edward, MS¹, Peter H. Hwang, MD¹, Beate Illek, PhD², and Horst Fischer, PhD²

¹Division of Rhinology, Department of Otolaryngology–Head and Neck Surgery, Stanford University School of Medicine, Stanford, CA

²Children's Hospital Oakland Research Institute, Oakland, CA

Abstract

Background—The airway epithelium generates reactive oxygen species (ROS) as a first line of defense. Dual oxidases (DUOX1 and DUOX2) are the H₂O₂-producing isoforms of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family in the airway epithelium. The purpose of this study was to explore the molecular expression, function, and regulation of DUOXs in chronic rhinosinusitis (CRS).

Methods—Human nasal tissue samples and nasal secretions were collected from 3 groups of patients undergoing sinus surgery (normal, n = 7; CRS with polyposis [CRSwP], n = 6; CRS without polyposis [CRSsP], n = 6). Nasal secretions were studied for cytokine and H₂O₂ content. Tissue samples were used to determine DUOX mRNA and protein expression.

Results—DUOX1 mRNA level (80.7 ± 60.5) was significantly increased in CRSwP compared to normal (2.7 ± 1.2) and CRSsP (2.3 ± 0.5 , $p = 0.042$). DUOX2 mRNA levels were increased in both CRSwP (18.6 ± 9.9) and CRSsP (4.0 ± 1.3) compared to normal (1.1 ± 0.3 ; $p = 0.008$). DUOX protein was found in the apical portion of the nasal epithelium and protein expression was increased in CRSwP and CRSsP. H₂O₂ production was significantly higher in CRSwP (160.9 ± 59.4 nM) and CRSsP (81.7 ± 5.6 nM) compared to normal (53.5 ± 11.5 nM, $p = 0.032$). H₂O₂ content of nasal secretions correlated tightly with DUOX expression ($p < 0.001$). Cytokines (eotaxin, monokine-induced by interferon γ [MIG], tumor necrosis factor [TNF]- α , interleukin [IL]-8) showed significantly higher levels in nasal secretions from CRSwP compared to normal ($p < 0.05$). Levels of eotaxin, MIG, and TNF- α correlated closely with DUOX expression.

Conclusion—DUOX1 and DUOX2 were identified as factors upregulated in CRS. Close correlations between DUOX expression and H₂O₂ release, and correlation between key inflammatory cytokines and DUOX expression, indicate DUOX in the inflammatory response in CRS.

Correspondence to: Horst Fischer, PhD, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609; hfischer@chori.org.

Potential conflict of interest: None provided.

Presented orally at the Annual ARS Meeting on September 8, 2012, Washington, DC.

Keywords

DUOX; NADPH oxidase; chronic rhinosinusitis; Luminex assay; qRT-PCR; cytokines

The airway epithelium has a mucosal surface that is covered with a thin layer of fluid called the airway surface liquid (ASL) that is critically important for its normal function.^{1,2} Mucosal surfaces that communicate with the outside are protected by the presence of a number of secreted antibacterial factors, such as, lactoferrin, lysozyme, defensins, and lactoperoxidase (LPO).³ LPO is unique because it protects epithelial surfaces from bacteria, viruses, and fungi by producing reactive oxygen species (ROS) as a first line of innate defense.^{4–6} LPO requires H₂O₂ to oxidize thiocyanate (SCN[−]) and thereby generate the antimicrobial compound hypothiocyanite (OSCN[−]).³ The primary source for H₂O₂ production in normal human airways is provided by the dual oxidases DUOX1 and DUOX2, which are homologues of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase found in phagocytes.⁷ NADPH oxidases (NOX) comprise a group of membrane flavoproteins that use NADPH as an electron donor to generate extracellular superoxide and H₂O₂. It has been shown that DUOX1 and DUOX2 are the major NADPH oxidases in pulmonary epithelial cells. The DUOXs provide H₂O₂ to support the LPO system of bacterial defense on mucosal membranes.^{8–11} Thus, the regulation of DUOX expression and function is an important regulator of epithelial defense. DUOX1 and DUOX2 are strongly regulated in the pulmonary epithelium.^{11,12} In primary airway epithelial cultures, the expression of DUOX1 is specifically upregulated by interleukin 4 (IL-4) and IL-13 and DUOX2 expression is highly induced by interferon γ (IFN- γ).¹¹ Joo et al.¹³ recently found that DUOX2 expression is upregulated in the nasal mucosa during acute sinusitis. There have been no studies to investigate DUOX1 and DUOX2 in human sinonasal epithelium to delineate their expression and function in chronic rhinosinusitis (CRS) patients with or without polyposis. Therefore, the purpose of this study was to identify the molecular expression, function, and regulation of DUOX1 and DUOX2 in the nasal epithelium in healthy and CRS patients with and without polyposis, and to explore a relation of DUOX expression to inflammatory mediators found in CRS.

Patients and methods

Subjects and sample collection

This study was approved by the institutional review board at Stanford University. We considered normal nasal mucosa as tissue from the lateral nasal wall (turbinate or uncinate process) tissue that was obtained intraoperatively from patients undergoing endoscopic transnasal surgery for benign pituitary lesions, benign sinonasal tumors, or lacrimal obstruction. Alternatively, diseased nasal mucosa (turbinate or uncinate process) was harvested from patients undergoing endoscopic sinus surgery (ESS) for medically refractory CRS patients with or without polyposis. Of note, all patients fulfilled diagnostic criteria for CRS developed by the Task Force for Defining Adult Chronic Rhinosinusitis and endorsed by the American Academy of Otolaryngology, and had failed medical management.¹⁴

Tissue samples for real-time quantitative polymerase chain reaction (RT-qPCR) were taken to the laboratory and immediately placed into RNAlater (Invitrogen, Carlsbad, CA) solution and stored at -80°C until needed for analysis. Other tissue samples were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4) and stored overnight at 4°C in hematoxylin and eosin (H&E) for immunofluorescence staining.

Collection of nasal secretions

Nasal secretions were collected based on the method described by Watelet et al.¹⁵ for immunological analysis. Patients were not given decongestant sprays in the preoperative area, and just after intubation, but prior to starting the operation, polyurethane sponges (20×10 mm size, Mepilex® Absorbent Foam Dressing Sponge; Mölnlycke Health Care, Göteborg, Sweden) were placed into both nasal cavities along the floor, between the septum and inferior turbinate. After 10 minutes, the sponges were removed and placed into Falcon tubes (Blue Max Jr. 15-mL polypropylene conical tube; Becton Dickinson, Franklin Lakes, NJ). To retrieve secretions out of sponges, 1 mL of 0.9% NaCl solution was added to each Falcon tube and stored at 4°C for about 2 hours, after which each sponge was placed into the barrel of a 5-mL syringe and secretions were released through mechanical piston action. Additionally, the syringe containing the sinus pack was replaced into a Falcon tube and centrifuged at $1500g$ for 10 minutes at 4°C to recover all fluid. Aliquots of 300 μL each were prepared and stored at -80°C for further analysis.

Luminex multiplex analysis

Human 51-plex kits were purchased from Affymetrix (Santa Clara, CA) and used according to the manufacturer's recommendations with modifications as described below. Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 hours followed by overnight incubation at 4°C . Plates were vacuum-filtered and washed twice with buffered Wash solution (Invitrogen, Carlsbad, CA), followed by incubation with biotinylated detection antibody for 2 hours at room temperature. Samples were then filtered and washed twice as above and resuspended in streptavidin- phycoerythrin (PE). After incubation for 40 minutes at room temperature, two additional vacuum washes were performed, and the samples resuspended in Reading Buffer (Invitrogen, Carlsbad, CA). Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower limit of 100 beads per sample per cytokine. Eight inflammatory cytokines were analyzed: eotaxin, IFN- γ , IL-2, IL-4, IL-8, IL-13, tumor necrosis factor (TNF)- α , and monokine-induced by IFN- γ (MIG). Data were analyzed using MasterPlex software (Hitachi Software Engineering America Ltd., MiraiBio Group, South San Francisco, CA), and are reported as fluorescence intensities.

RT-qPCR

Total RNA was extracted from sinonasal mucosal specimens using RNA later and RNA was quantified spectroscopically. One microgram (1 μg) of total RNA from each sample was added to 20 μL of reaction mixture. Reverse transcription was performed with $5 \times$ iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA) at 42°C for 30 minutes. Amplification of cDNA was performed by SsoAdvanced SYBR Green Supermix (Bio-Rad,

Hercules, CA) in a Bio-Rad CFX 384 Touch Real-Time PCR detection system. Because DUOX1 and DUOX2 are expressed in the epithelial cells, epithelial cell adhesion molecule (EPCAM) was used as an internal RNA control for this study. Sequence of primers for DUOX1, DUOX2, and EPCAM (Integrated DNA Technology, Coralville, IA) are provided in Table 1. A threshold cycle (C_t) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the delta delta C_t values, and the relative quantification was expressed as $2^{-\Delta C_t}$.

Immunofluorescent staining

Immunofluorescent staining were performed using sinonasal tissue embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA) from 3 patient groups. Samples were cut into 9- μ m sections by a cryomicrotome (CM1950; Leica Microsystems Buffalo Grove, IL). Monoclonal rabbit anti-human DUOX2 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. No specific anti-human DUOX1 monoclonal antibody was available. However, owing to the high similarity of DUOX1 and DUOX2, antibodies were described to detect both isoforms.¹⁶ The sections were washed and blocked in 10% goat serum for 1 hour at room temperature and then incubated with the primary antibody overnight at 4°C. Sections were then washed and incubated for 1 hour at 37°C with a cyanin 5 (CY5)-labeled donkey anti-rabbit immunoglobulin G (IgG) antibody (1:100; KPL, Inc., Gaithersburg, MD) for DUOX2. 4',6-Diamidino-2-phenylindole (DAPI) and commercial EPCAM antibody were used as a counterstain to identify all cellular nuclei in the tissue specimen. Immunofluorescence microscopy was performed on a confocal microscope (LSM 710; Carl Zeiss Microimaging, Thornwood, NY).

Ex vivo measurement of nasal airway H₂O₂

H₂O₂ production was measured using the Amplex Red hydrogen peroxide assay kit (Invitrogen, Eugene, OR). Snapfrozen collected nasal secretions (described in Collection of nasal secretions) were thawed before the assay. Each sample was collected exactly same among 3 groups. Nasal secretions (standard curve samples, control, and experimental samples) were mixed with buffer solution of 100 μ M Amplex® Red reagent and 0.2 U/mL horseradish peroxidase (HRP) to a final volume of 100 μ L. Amplex Red reacts in a 1:1 stoichiometry with H₂O₂ to produce red fluorescent resorufin, which was detected using a fluorescence plate reader after 30 minutes and calibrated against a standard curve generated from serial dilutions of H₂O₂.

Statistical analysis

Statistical analysis was accomplished using SPSS software (version 17.0; SPSS Inc., Chicago, IL), and $p < 0.05$ was considered significant. Results are given as mean $\pm$ SE. Between-group differences were assessed for significance by 1-way analysis of variance (ANOVA), Kruskal-Wallis 1-way ANOVA, followed by Dunn's test, Mann-Whitney, or the Pearson's chi-square test, as appropriate. Linear regression analyses were performed using Sigmaplot 11 (Systat Software, Inc., San Jose, CA). In part, regression analyses were performed on log-transformed data to achieve normal data distribution.

Results

Patient characteristics

Mean ages were 48.6 ± 3.4 years in the control group ($n = 7$), 50.1 ± 8.6 years in CRS patients without polyposis (CRSsP; $n = 6$), and 51.4 ± 4.8 in CRS patients with polyposis (CRSwP; $n = 6$, 1-way ANOVA, $p = 0.923$, Table 2). In the normal group, there were 3 male and 4 female subjects; in CRSsP, females outnumbered males by 4 to 2; and in CRSwP there were 3 male and 3 female subjects (chi-square, $p = 0.842$). One patient in the CRSwP group had Samter's triad. There was no difference in the patient characteristics in each group.

DUOX1 and DUOX2 mRNA expression in CRS with and without polyposis

We explored the mRNA expression of DUOX1 and DUOX2 in nasal mucosal tissues from CRSwP and CRSsP patients in comparison to noninflamed control nasal tissue. In nasal tissues from CRSwP patients both DUOX1 and DUOX2 were highly upregulated compared to controls (30-fold and 17-fold, respectively; Fig. 1A and B). Tissues from CRSsP patients showed unchanged DUOX1 expression but a 3.6-fold increase in expression of DUOX2 compared to controls (Fig. 1B). DUOX2 showed distinctly different mRNA expression levels in all 3 groups, suggesting a differential regulation of DUOX2 expression by CRSwP and CRSsP.

In previous cell culture studies, DUOX1 and DUOX2 were shown to release H_2O_2 into the mucosal fluid layer.⁷ Here, we measured H_2O_2 in nasal secretions collected before sinus surgery and correlated these to epithelial DUOX1 and DUOX2 expression. Figure 1C shows the H_2O_2 concentrations found in nasal secretions grouped by disease state. H_2O_2 was detected in all groups and H_2O_2 concentrations were significantly different between all groups. In nasal secretions from normal subjects we found an H_2O_2 concentration of 53.5 ± 11.5 nM. In nasal secretions from CRSsP patients the H_2O_2 concentration was significantly increased (81.7 ± 5.6 nM) and in CRSwP patients the H_2O_2 concentration was increased further (161 ± 59 nM) more than 3 times over control levels. These findings suggested a correlation between H_2O_2 concentrations found in nasal secretions and the molecular expression of DUOX in nasal tissue. Figure 1D shows a close correlation between DUOX mRNA expression and H_2O_2 concentrations in corresponding nasal secretions. For quantifications see the legend for Figure 1.

Similar observations were made using immunofluorescence staining of nasal tissues by an antibody directed against DUOX2 (Fig. 2, green). Although this antibody was raised against an epitope of DUOX2, cross-reactivity with DUOX1 is likely owing to the high similarity of the 2 proteins.¹⁶ Epithelial cells were identified using EPCAM antibody staining (Fig. 2, red) and nuclei were stained with DAPI (Fig. 2, blue). DUOX2 was expressed in the apical (luminal) surface of epithelial cells in EPCAM-positive nasal epithelial cells. DUOX2 appeared to be not present in mucin producing cells (Fig. 2B). Markedly higher DUOX2 immunoreactivity was noted in nasal tissue from CRSwP patients compared to normal control tissue and tissue from CRSsP patients (Fig. 2C and D).

Upregulation of selected inflammatory cytokines in human nasal secretions

Inflammatory mediators regulate the expression of DUOX1 and DUOX2 in the epithelium of the airways.¹¹ Currently there is no information available about the role of inflammatory mediators found in CRS on the expression of DUOX1 and DUOX2 in the nasal epithelium. In this part of the study we used an exploratory approach by measuring eight common cytokines (eotaxin, IFN- γ , IL-2, IL-4, IL-8, IL-13, TNF- α , MIG) in nasal secretions collected from CRS patients before surgery. Levels of secreted cytokines were then correlated with the expression of DUOX1 and DUOX2 mRNA measured in surgical tissue specimen. This approach allowed us to identify secreted cytokines selectively upregulated in CRSsP and CRSwP and to identify any correlation of cytokine levels with DUOX1 and DUOX2 expressing.

Figure 3 shows for each cytokine the relative average concentrations found in nasal secretions collected from normal subjects and CRS patients (left panel bar charts) and the relation of each cytokine to the tissue expression of DUOX1 and DUOX2 mRNA (right panels). In CRSwP, 4 of the 8 cytokines measured in nasal secretions were increased more than 2.5-fold. CRSwP (but not CRSsP) was characterized by a selective increase of eotaxin (14-fold), MIG (7.5-fold), TNF- α (3.2-fold), and IL-8 (2.6-fold) above control levels (Fig. 3A–D, left panels). In addition levels of eotaxin, MIG, and TNF- α correlated significantly with mRNA levels of both DUOX1 and DUOX2 in nasal tissues (Fig. 3A–C, right panels).

Nasal secretions collected from CRSsP patients showed cytokine levels that were little changed compared to control. IL-2 was the only cytokine that was selectively increased (2.2-fold) in CRSsP but not in CRSwP (Fig. 3G). IL-4 was the only cytokine that was increased in both CRSsP and CRSwP, although at low levels (1.9-fold over control; Fig. 3F). Despite the small effects on IL-4 levels, there was a significant relation between IL-4 levels and DUOX2 (but not DUOX1) expression. Similarly, IFN- γ showed a significant relation to DUOX2 expression despite low IFN- γ levels in secretions. This suggests that DUOX2 is more sensitive to regulation by inflammatory mediators than DUOX1, as noted previously.¹¹ These results suggest that changes in the proinflammatory cytokine milieu may play a role in the regulation of DUOX1 and DUOX2 expression in sinonasal disease states.

Discussion

In this study we investigated the expression and function of DUOX1 and DUOX2 in the nasal airway epithelium. Immunohistochemistry studies showed an apical localization of DUOX2 at the luminal surface of nasal epithelial cells. DUOX1 and DUOX2 expression was strongly upregulated in CRSwP. Notably, H₂O₂ content of nasal secretions correlated tightly with levels of DUOX expression in the 3 groups investigated. To our knowledge, this is the first study demonstrating DUOX1 and DUOX2 expression and function in freshly isolated human nasal epithelial tissue.

DUOX1 and DUOX2 are H₂O₂ generators in nasal epithelium

The two large NADPH oxidase (NOX) homologues DUOX1 and DUOX2 were initially identified and cloned from the epithelium of the thyroid gland.¹⁷ Further investigations found that the DUOXs are widely expressed in epithelial cells, including the respiratory tract.⁷ Human DUOX1 and DUOX2 are highly similar transmembrane proteins (77% identical and 83% similar). Both DUOX isoforms release extracellular H₂O₂ on mucosal surfaces.⁷ It has been reported that H₂O₂ production increases with increasing DUOX expression at the plasma membrane.¹⁸ A model of bacterial killing at the airway epithelial cells is now considered that is similar to the killing mechanism of the NADPH oxidase in phagocytes.¹⁹

In phagocytes, the NOX2-based NADPH oxidase generates superoxide inside the phagosome, that dismutates to H₂O₂ by phagosomal superoxide dismutase (SOD) and is further converted to bactericidal HOCl by myeloperoxidase (MPO).²⁰ A similar oxidative process was proposed for the airway epithelium as model for an antibacterial mechanism based on the DUOX-LPO system.⁹ In normal respiratory airways, H₂O₂ is continuously released into the airway surface liquid (ASL) where LPO generates antibacterial OSCN⁻ from H₂O₂ and SCN⁻. A key functional difference between the airway DUOX-LPO system and the phagocytic NOX2-MPO system is that the system in phagocytes is active only during the respiratory burst whereas DUOX generates H₂O₂ continuously and, thus, maintains a continuous defense shield.

Normal subjects continuously exhale H₂O₂, which is thought to originate from the airway mucosa.⁷ In most studies, airway disease groups exhaled 4 to 5 times higher levels of H₂O₂. It was found that the level of inflammation, the white blood cell count, and disease exacerbation correlated positively with exhaled breath H₂O₂, which is expected to be produced both by the epithelium and by invading leukocytes during airway inflammation.²¹ Our measurements of H₂O₂ in nasal secretions cannot distinguish between the H₂O₂ generated by the epithelium or by leukocytes present in the mucosa and, therefore, it is difficult to quantify the fraction of DUOX-mediated H₂O₂ production. Nevertheless, the tight correlation between DUOX expression and H₂O₂ concentrations in the nasal secretion indicates that DUOX function generates mucosal H₂O₂. However, there are observations that DUOX-generated H₂O₂ attracts leukocytes.²² And, as a result, a close correlation between H₂O₂ and DUOX expression could also involve concomitant H₂O₂ released from leukocytes.

Relation of inflammatory cytokines to DUOX1 and DUOX2 expression

Little previous information is available about the regulation of DUOX expression and its relation to CRS. We used an exploratory approach by measuring levels of 8 common cytokines in nasal secretion. The most striking effects were noted for eotaxin, MIG, and TNF- α in CRSwP patient samples. Eotaxin and MIG are specific chemoattractants and activators of inflammatory leukocytes, and TNF- α mediates the acute phase response of inflammation. In our study, levels of eotaxin, MIG, and TNF- α were greatly increased in CRSwP and their levels in nasal secretions correlated tightly with both DUOX1 and DUOX2 expression (Fig. 3A–C). These observations indicate epithelial DUOX expression

as part of the inflammatory response in the nasal epithelium and in leukocyte attraction. The mechanism of interaction between DUOX expression and eotaxin, MIG, or TNF- α in secretions is unclear; however, it conforms to previous observations showing leukocyte recruitment by DUOX-generated H₂O₂.²²

For comparison, in CRSsP patients, DUOX1 expression was not elevated and DUOX2 expression and H₂O₂ release were slightly increased (Fig. 1). Also, the cytokine profile showed comparable small changes suggesting a distinct inflammatory environment in CRSsP compared to CRSwP. A similar conclusion was drawn previously in a study of tissue cytokines in CRS where CRSsP was found to show a Th1 polarization and CRSwP was characterized as Th2 polarized.²³ Our data support a differential expression of immune factors in CRSsP vs CRSwP, including the epithelial expression of DUOX.

Previously, Harper et al.¹¹ showed in an in vitro study on airway epithelial primary cultures that the level of expression of DUOX1 and DUOX2 is selectively regulated by a small number of cytokines. DUOX1 mRNA was specifically increased by the T-helper 2 (Th2) cytokines IL-4 and IL-13, and DUOX2 mRNA was upregulated by the Th1 cytokine IFN- γ when added to the culture medium. In our study, IFN- γ was little affected by disease state (Fig. 3E); however, we found a correlation between IFN- γ and DUOX2 mRNA expression supporting the previous cell culture findings. Changes in IL-4 and IL-13 may have been too small in our study as no effects were seen on DUOX1 expression.

The cytokine profile that we found in this study in nasal secretions is in part comparable to findings of cytokines determined in tissue samples in previous studies. CRSwP is typically characterized by high levels of eotaxin and IL-8 as was also found here.²³ In addition we found increased levels of TNF- α and IL-4, which have been shown to elevate the release of eotaxin from fibroblasts in nasal polyps;²⁴ however, in other studies TNF- α and IL-4 in nasal tissue were only marginally increased.^{23,25} CRSsP is typically characterized by elevated IFN- γ .²³ In our measurement, IFN- γ showed small, insignificant changes between groups. Although CRSwP and CRSsP are characterized by typical inflammatory patterns of mediators, these are also affected by the microbial environment present on the nasal surface and thus variability between patients can be expected. This was not controlled in our study, which is expected.²⁶

Role of DUOX in airway defense

The airway epithelium is the first line of defense against inhaled pathogens, and the epithelium secretes a number of antimicrobial factors, which act in combination with mucociliary transport in the ASL. Several antimicrobial factors have been isolated from airway secretions indicating the role of the airways in bacterial defense.² Recognizing the general involvement of NADPH oxidases in bacterial killing by production of ROS in various cell types, the expression of the NADPH oxidases DUOX1 and DUOX2 in the human nasal airway epithelium represents a likely innate defense function as previously indicated by supporting the function of the airway LPO system with regulated release of H₂O₂. In addition, our study provides indications for a close relation between the expression and function of DUOX and the leukocyte-based inflammatory signaling response. As our

study was largely exploratory and non-interventional, the details of this interaction are currently unclear.

Conclusion

We investigated the DUOX1 and DUOX2 mRNA expression in freshly excised sinonasal mucosa from CRSwP and CRSsP patients as well as control tissues. Significant up-regulation of DUOX1 was noted in CRSwP and DUOX2 expression was significantly upregulated in CRSwP and CRSsP. H₂O₂ levels in nasal secretion were 3 times higher in CRSwP compared to control subjects, and the upregulated levels of DUOX1 and DUOX2 correlated tightly with the H₂O₂ content of nasal secretions. Immunohistochemistry demonstrated an apical localization of DUOX2 in the luminal surface of nasal epithelial cells. Secreted levels of eotaxin, MIG, and TNF- α were significantly higher in CRSwP and correlated with DUOX1 and DUOX2 expression. The levels of secreted inflammatory cytokines in CRSsP were generally low. The expression of DUOX1 and DUOX2 in the human nasal airway epithelium represents a novel mechanism that appears to be a factor in innate defense signaling and the inflammatory response of the nasal mucosa.

Acknowledgments

This work was supported mainly by generous funding from the Department of Otolaryngology–Head and Neck Surgery at Stanford University (to R.K. Jackler). We acknowledge the Stanford Human Immune Monitoring Center (HIMC) for LUMIX assay data contribution.

Funding sources for the study: NIH NHLBI 5R01HL086323 (to HF) and Cystic Fibrosis Research Inc. (to HF).

References

1. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest*. 2002; 109:571–577. [PubMed: 11877463]
2. Schwarzer C, Machen TE, Illek B, Fischer H. NADPH oxidase-dependent acid production in airway epithelial cells. *J Biol Chem*. 2004; 279:36454–36461. [PubMed: 15210697]
3. Gattas MV, Forteza R, Fragoso MA, et al. Oxidative epithelial host defense is regulated by infectious and inflammatory stimuli. *Free Radic Biol Med*. 2009; 47:1450–1458. [PubMed: 19703552]
4. Lenander-Lumikari M. Inhibition of *Candida albicans* by the peroxidase/SCN-H₂O₂ system. *Oral Microbiol Immunol*. 1992; 7:315–320. [PubMed: 1494457]
5. Pourtois M, Binet C, Van TN, Courtois P, Vandenabeele A, Thiry L. Inhibition of HIV infectivity by lactoperoxidase-produced hypothiocyanite. *J Biol Buccale*. 1990; 18:251–253. [PubMed: 2128884]
6. Reiter B, Perraudin JP. The antibacterial activity of lactoferrin and neonatal *E. coli* infections. A selective and critical review. *Adv Exp Med Biol*. 1998; 443:175–188. [PubMed: 9781357]
7. Fischer H. Mechanisms and function of DUOX in epithelia of the lung. *Antioxid Redox Signal*. 2009; 11:2453–2465. [PubMed: 19358684]
8. El Hassani RA, Benfares N, Caillou B, et al. Dual oxidase2 is expressed all along the digestive tract. *Am J Physiol Gastrointest Liver Physiol*. 2005; 288:G933–G942. [PubMed: 15591162]
9. Forteza R, Salathe M, Miot F, Forteza R, Conner GE. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol*. 2005; 32:462–469. [PubMed: 15677770]

10. Geiszt M, Witta J, Baffi J, Lekstrom K, Leto TL. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J*. 2003; 17:1502–1504. [PubMed: 12824283]
11. Harper RW, Xu C, Eiserich JP, et al. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett*. 2005; 579:4911–4917. [PubMed: 16111680]
12. Fischer H, Gonzales LK, Kolla V, et al. Developmental regulation of DUOX1 expression and function in human fetal lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2007; 292:L1506–L1514. [PubMed: 17337509]
13. Joo JH, Ryu JH, Kim CH, et al. Dual oxidase 2 is essential for the toll-like receptor 5-mediated inflammatory response in airway mucosa. *Antioxid Redox Signal*. 2012; 16:57–70. [PubMed: 21714724]
14. Benninger MS, Ferguson BJ, Hadley JA, et al. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. *Otolaryngol Head Neck Surg*. 2003; 129(3 Suppl):S1–S32. [PubMed: 12958561]
15. Watelet JB, Gevaert P, Holtappels G, Van Cauwenberge P, Bachert C. Collection of nasal secretions for immunological analysis. *Eur Arch Otorhinolaryngol*. 2004; 261:242–246. [PubMed: 14551791]
16. De Dekken X, Wang D, Many MC, et al. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem*. 2000; 275:23227–23233. [PubMed: 10806195]
17. Dupuy C, Ohayon R, Valent A, Noel-Hudson MS, Deme D, Virion A. Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cDNAs. *J Biol Chem*. 1999; 274:37265–37269. [PubMed: 10601291]
18. Rigutto S, Hoste C, Grasberger H, et al. Activation of dual oxidases Duox1 and Duox2: differential regulation mediated by camp-dependent protein kinase and protein kinase C-dependent phosphorylation. *J Biol Chem*. 2009; 284:6725–6734. [PubMed: 19144650]
19. Leto TL, Geiszt M. Role of Nox family NADPH oxidases in host defense. *Antioxid Redox Signal*. 2006; 8:1549–1561. [PubMed: 16987010]
20. DeCoursey TE. Voltage-gated proton channels and other proton transfer pathways. *Physiol Rev*. 2003; 83:475–579. [PubMed: 12663866]
21. Loukides S, Bouros D, Papatheodorou G, Panagou P, Siafakas NM. The relationships among hydrogen peroxide in expired breath condensate, airway inflammation, and asthma severity. *Chest*. 2002; 121:338–346. [PubMed: 11834641]
22. Niethammer P, Grabher C, Look AT, Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature*. 2009; 459:996–999. [PubMed: 19494811]
23. Van Zele T, Claeys S, Gevaert P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy*. 2006; 61:1280–1289. [PubMed: 17002703]
24. Yoshifuku K, Matsune S, Ohori J, Sagara Y, Fukuiwa T, Kurono Y. IL-4 and TNF-alpha increased the secretion of eotaxin from cultured fibroblasts of nasal polyps with eosinophil infiltration. *Rhinology*. 2007; 45:235–241. [PubMed: 17956026]
25. Liu T, Wang BQ, Yang PC. A possible link between sinusitis and lower airway hypersensitivity: the role of Staphylococcal enterotoxin B. *Clin Mol Allergy*. 2006; 4:7. [PubMed: 16677400]
26. Bachert C, Zhang N, Patou J, Van ZT, Gevaert P. Role of staphylococcal superantigens in upper airway disease. *Curr Opin Allergy Clin Immunol*. 2008; 8:34–38. [PubMed: 18188015]

Figure 1.

mRNA expression levels of DUOX1 and DUOX2 and H₂O₂ production in human nasal mucosa from normal, CRSsP, and CRSwP. (A) DUOX1 mRNA expression. Tissue from CRSwP demonstrated significant increase in DUOX1 expression (80.71 ± 60.53) compared to nasal mucosa from normal control (2.69 ± 1.2) and CRSsP (2.30 ± 0.75 ; $p = 0.048$, Kruskal-Wallis 1-way ANOVA). (B) DUOX2 mRNA expression. Tissue from CRSwP (18.58 ± 9.8) showed the highest expression of DUOX2, followed by CRSsP (4.04 ± 1.32) and normal tissue (1.11 ± 0.26 ; $p = 0.038$, Kruskal-Wallis 1-way ANOVA), * $p < 0.05$. (C)

Concentration of H_2O_2 in human nasal secretion. The concentration of H_2O_2 was highest in nasal secretion from CRS patient with polyposis (160.9 ± 59.4 nM) among three groups, followed by CRS without polyposis (81.7 ± 5.6 nM) and normal controls (53.5 ± 11.5 nM; $p = 0.032$, Kruskal-Wallis 1-way ANOVA followed by Dunn's comparisons). (D) H_2O_2 in nasal secretion correlated tightly with DUOX expression levels. DUOX1 (gray circles), $p < 0.001$, slope: $0.27 \pm 0.045 \log \text{H}_2\text{O}_2 / \log \text{DUOX1 mRNA}$. DUOX2 (black squares), $p < 0.001$, slope: $0.36 \pm 0.084 \log \text{H}_2\text{O}_2 / \log \text{DUOX2 mRNA}$; by linear regression of log-transformed data. Numbers in parentheses give number of analyzed patient samples for each bar. ANOVA = analysis of variance; CRS = chronic rhinosinusitis; CRSsP = CRS without polyposis; CRSwP = CRS with polyposis; DUOX = dual oxidase; mRNA = messenger RNA.

Figure 2.

Localization of DUOX2 in human nasal mucosa using immunofluorescent microscopy. (A) Normal ($\times 200$, scale bar = 50 μm). (B) CRSsP ($\times 200$, scale bar = 50 μm). (C) CRSwP ($\times 200$, scale bar = 50 μm). DUOX2 was expressed in the apical (luminal) surface of epithelial cell as a linear dot pattern (bright green) in the human nasal epithelial cells. Markedly higher DUOX2 immunoactivity was noted in nasal tissue from CRSwP in immunofluorescent staining. There is a trend toward the increased expression of DUOX2 in diseased mucosa (CRSsP and CRSwP) compared to normal control. (blue: DAPI; green: DUOX2; red: EPCAM). CRS = chronic rhinosinusitis; CRSsP = CRS without polyposis; CRSwP = CRS with polyposis; DAPI = 4',6-diamidino-2-phenylindole; DUOX = dual oxidase; EPCAM = epithelial cell adhesion molecule.

Figure 3.

Cytokines in nasal secretion by disease state and relation to DUOX1 and DUOX2 expression. For each cytokine, the left panel shows average levels in nasal secretions per group (normal, CRSsP, CRSwP). Numbers in parentheses give the number of patients in each group; * $p < 0.05$ by ANOVA. The right panel shows the relation of cytokine to DUOX1 (black) and DUOX2 (red) expression for data of all groups. Corresponding regression lines are shown if significant; levels of significance are stated in the panel. (A) Eotaxin, significantly increased in CRSwP ($p = 0.015$) and significantly related to DUOX1 ($p < 0.001$) and DUOX2 ($p < 0.001$). (B) MIG, significantly increased in CRSwP ($p =$

0.018) and significantly related to DUOX1 ($p < 0.001$) and DUOX2 ($p < 0.001$). (C) TNF- α , significantly increased in CRSwP ($p = 0.04$) and significantly related to DUOX1 ($p < 0.023$) and DUOX2 ($p < 0.033$). (D) IL-8, significantly increased by CRSwP ($p = 0.011$) but unrelated to DUOX expression. (E) IFN- γ , unaffected by disease group; significantly related to DUOX2 ($p < 0.018$) but not DUOX1. (F) IL-4, significantly increased by CRSwP and CRSsP ($p = 0.049$) and significantly related to DUOX2 ($p < 0.01$) but not DUOX1. (G) IL-2, significantly increased by CRSsP ($p = 0.022$) but unrelated to DUOX expression. (H) IL-13, unaffected by disease condition and unrelated to DUOX expression. ANOVA = analysis of variance; CRSsP = chronic rhinosinusitis without polyposis; CRSwP = chronic rhinosinusitis with polyposis; DUOX = dual oxidase; IL = interleukin; MIG = *monokine-induced by interferon γ* ; ns = not significant; TNF = tumor necrosis factor.

TABLE 1

Primer pairs used in RT-qPCR

Gene	Primer	Sequence
DUOX1	Forward	5'-CGACATTGAGACTGAGTTGA-3'
	Reverse	5'-CTGGAATGACGTTACCTTCT-3'
DUOX2	Forward	5'-AACCTAAGCAGCTCACAAC-3'
	Reverse	5'-CAGAGAGCAATGATGGTGAT-3'
EPCAM	Forward	5'-AATGTGTGTGCGTGGGA-3'
	Reverse	5'-TTCAAGATTGGTAAAGCCAGT-3'

DUOX = dual oxidase; EPCAM = epithelial cell adhesion molecule; RT-qPCR = real-time quantitative polymerase chain reaction.

TABLE 2

Patient demographic characteristics

	Normal (n = 7)	Chronic rhinosinusitis (n = 12)		<i>p</i>
		Without polyp (n = 6)	With polyp (n = 6)	
M:F	3:4	2:4	3:3	<i>p</i> = 0.842
Age	48.6 ± 3.4	50.1 ± 8.6	51.4 ± 4.8	<i>p</i> = 0.923

F = female; M = male.