# Chapter 13
# The Role of DUOX Isozymes in the Respiratory Tract Epithelium

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**Abstract**  Increasingly, reactive oxygen species such as superoxide and hydrogen peroxide are recognized to be intentionally generated intracellularly to serve important cellular functions. A key protein family responsible for the regulated generation of reactive oxygen species in multiple cell types is the NOX/DUOX enzyme family. Two family members, DUOX1 and DUOX2, appear to be highly expressed in tissues of endodermal origin including the thyroid, respiratory tract, and gastrointestinal tract. In this chapter, we will focus our review on DUOX proteins in the respiratory tract. We will discuss a brief history of the discovery of the DUOX isoforms, the estimated hydrogen peroxide-generating capacity of DUOX in respiratory tract epithelium, putative functions of the DUOX enzymes, and some regulatory factors responsible for DUOX gene expression and oxidase activity.

**Keywords**  DUOX1; DUOX2; respiratory tract; oxidase; peroxidase; host defense

## Introduction

Recent evidence suggests that reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$) are not mere byproducts of cellular respiration, but are intentionally generated to serve important cellular functions (Terada, 2006; Finkel, 2003). A key protein family responsible for the regulated generation of ROS in multiple cell types is the NOX/DUOX enzyme family (Lambeth, 2002). The prototypical member of this family is gp91*phox* (NOX2), which is responsible for the respiratory burst in neutrophils (Gabig and Babior, 1979; Dusi et al., 1995; Hohn and Lehrer, 1975). Suh et al. were the first to characterize NOX1 (initially named Mox1; for mitogenic oxidase) as a non-mitochondrial, non-phagocytic enzyme capable of regulated ROS production (Suh et al., 1999). Since this discovery,

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there has been an exponentially growing body of literature characterizing the function of the non-phagocytic NOX/DUOX family members (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) in various aspects of human biology and disease (Lambeth, 2004; Leto and Geiszt, 2006). In this chapter, we will focus our review on the expression and function of DUOX proteins in the respiratory tract.

## Discovery and Localization of DUOX Family Members

Initial identification of the DUOX2 was made by Corinne Dupuy and colleagues using biochemical techniques to isolate the known membrane-bound NADPH oxidase in thyroid tissues (Dupuy et al., 1999). In this report, they described the isolation of a 1,210 amino acid protein, p138[Tox], which contained a gp91phox-like region and two EF-hand domains. EF-hand domains contain calcium binding sites and serve as regulatory sub units for enzymatic activity. The presence of EF-hand domains suggested that p138[Tox] enzymatic activity may be regulated by calcium. Additionally, expression of p138[Tox] was upregulated by forskolin and the gene localized to Chromosome 15q15. Shortly after this discovery, Xavier De Deken described the isolation of two human thyroid NADPH oxidases (De Deken et al., 2000). These genes, thyroid oxidase 1 (ThOX1) and thyroid oxidase 2 (ThOX2), were identified by low stringency hybridization screening of thyroid cDNA libraries using a 1.3-kilobase fragment of gp91phox. Similar to Dupuy's findings, both ThOX1 and ThOX2 contained a gp91phox-like region and two EF-hand domains, and both genes were upregulated by forskolin. In addition, increased sequence information identified novel N-terminal extensions for both proteins that contained a peroxidase-like domain. The 1,210 amino acid C-terminal portion of ThOX2 was identical to p138[Tox], which strongly suggested that these two proteins were the same protein. Because both proteins express dual oxidases, a heme peroxidase domain and an NADPH oxidase domain, ThOX1 and ThOX2/p138[Tox] have been renamed DUOX1 and DUOX2, respectively.

Further characterization of DUOX enzyme expression in human tissues demonstrated *DUOX* mRNA transcripts in multiple non-thyroid human tissues including heart, kidney liver, pancreas, placenta, GI tract, prostate, testis, and the respiratory tract (Caillou et al., 2001; Edens et al., 2001; Geiszt et al., 2003; El Hassani et al., 2005). Further characterization of DUOX in the lung demonstrated that DUOX expression is likely limited to the respiratory tract epithelium (Geiszt et al., 2003). Although PCR evidence exists that several NOX family members are potentially expressed in the respiratory tract, expression levels of the other Nox family members are a thousand-fold less abundant compared to DUOX (Schwarzer et al., 2004). Based on in situ hybridization experiments, it appears that DUOX1 is predominantly located in proximal respiratory tract epithelial cells whereas DUOX2 is localized to submandibular or salivary glands during basal conditions (Schwarzer et al., 2004). However, protein confirmation of these findings has been limited due to the absence of DUOX isoform-specific antibodies.

## Functional Role(s) of DUOX

The capacity for respiratory tract epithelium to generate $H_2O_2$ appears to be dependent upon the specific cell type utilized. For example, in unstimulated human primary tracheobronchial epithelial cells, the rate of $H_2O_2$ production was 0.5–2.5 nmol/10 min/$10^6$ cells and this production increases to 2–5 nmol/10 min/$10^6$ cells after stimulation with ATP (Moskwa et al., 2007). In HBE1 cells, a papilloma-transformed human tracheobronchial epithelial cell line (Yankaskas et al., 1993). $H_2O_2$ production was 0.03–0.17 nmol/10 min/$10^6$ cells and this production increases to 0.26–0.45 nmol/10 min/$10^6$ cells after stimulation with ATP (Wesley et al., 2007). In a human pulmonary mucoepidermoid carcinoma cell line, $H_2O_2$ production increased from 0.002–0.003 nmol/10 min/$10^6$ cells to 0.012–0.014 nmol/10 min/$10^6$ cells after PMA stimulation (Nakanaga et al., 2007). Although laboratory to laboratory variation may account for these differences, it appears that primary respiratory tract epithelial cells have the highest capacity for DUOX-mediated $H_2O_2$ generation. In parallel, we have observed that *DUOX1* or *DUOX2* mRNA expression and functional activity is higher in primary tracheobronchial epithelial cells compared to HBE1 cells (Xu and Haper, 2007).

It is well-accepted that DUOX2-mediated $H_2O_2$ production is critical for iodide oxidation during thyroid hormone synthesis (Igo et al., 1964; Degroot et al., 1972; Clark et al., 1975; Lissitzky, 1976; Rigutto et al., 2007; Song et al., 2007). Observations that mutations in *DUOX2* gene result in congenital hypothyroidism (Moreno et al., 2002; Vigone et al., 2005) confirm the available biochemical and expression data. However, similarly convincing evidence for the function of DUOX isoforms in the respiratory tract are lacking. Several compelling models to explain the expression and function of DUOX isoforms in the respiratory tract have been proposed. The location of DUOX isoforms in the respiratory tract epithelium and their homology to gp91phox strongly implicated the role of DUOX proteins in host defense.

Several mechanisms exist to provide innate host defense in the respiratory tract epithelium (Antunes and Cohen, 2007; Thacker, 2006; Grubor et al., 2006; Beisswenger and Bals, 2005; Ng et al., 2004; Zhang et al., 2000). The upper respiratory tract epithelium and conducting airways are structured to provide multiple layers of protection against invading organisms. This includes a tight epithelial barrier where the apical surface of the respiratory tract is in close apposition to prevent invasion. This apical surface is bathed in an airway surface liquid that is rich in antimicrobial peptides hostile to bacterial and viral products. Resting on top of this airway surface liquid is a mucous layer that provides a physical barrier against infection. Mucus that has ensnared bacteria and virus particles is cleared from the airway by ciliary action provided by the respiratory tract epithelial cells. The generation of hypothiocyanate in the airway surface liquid appears to be a critical part of the host defense for those bacteria and viral particles that are able to penetrate the initial mucociliary barrier (Goldman and Smith, 1973; Ratner and Prince, 2000).

Conner and others previously described the importance of lactoperoxidase (LPO) in maintaining sterility in the respiratory tract (Gerson et al., 2000; Conner

et al., 2002; Salathe et al., 1997; Wijkstrom-Frei et al., 2003). In this model, LPO catalyzes a reaction between $H_2O_2$ and thiocyanate ($SCN^-$) to generate the bactericidal product hypothiocyanate ($OSCN^-$) (Gerson et al., 2000; Conner et al., 2002; Salathe et al., 1997; Wijkstrom-Frei et al., 2003; Conner et al., 2007).

$$H_2O_2 + LPO \rightarrow LPO - O + H_2O$$

$$LPO - O + SCN^- \rightarrow OSCN^- + LPO$$
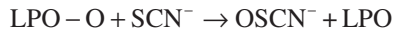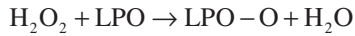
Absence of this abundant protein, nearly 1% of the total protein in airway surface liquid, results in significant colonization and infection of respiratory tract epithelium with bacteria. Although the source of $H_2O_2$ in the respiratory tract was not initially known, DUOX isoforms have since been verified to be the predominant enzymes responsible for $H_2O_2$ generation in the respiratory tract. In addition, Geiszt et al. demonstrated evidence to suggest that DUOX localization next to LPO production is consistent with this model (Geiszt et al., 2003).

Recently, Conner and Banfi independently, characterized the transport of $SCN^-$ from the basolateral surface to the apical surface of the respiratory tract where enzymatic activation of $SCN^-$ to $OSCN^-$ will occur (Moskwa et al., 2007; Conner et al., 2007). This transport appears to be the rate-limiting step in the respiratory tract and is dependent upon the presence of functional CFTR chloride channels. The rate of SCN- transport (1.5–1.8 nmol/10 min/cm$^2$) (Conner et al., 2007) parallels the rate of OSCN- formation (1.4–1.7 nmol/10 min/cm$^2$) (Moskwa et al., 2007) in respiratory tract epithelium. This is consistent with the notion that $SCN^-$ is immediately converted to $OSCN^-$ once $SCN^-$ is transported to the apical surface. However, two different model systems, rat versus human, were used in these reports, which limits our ability to directly compare the two studies. Of importance, LPO, DUOX-mediated $H_2O_2$, and apical $SCN^-$ are all required for bacterial killing of S. aureus or P. aeruginosa (Moskwa et al., 2007). The capacity of DUOX isoforms to generate $H_2O_2$ in respiratory tract epithelium (5 nmol/10 min/cm$^2$) is more than adequate to support this model.

In parallel to the putative antibacterial role for DUOX enzymes, our recent data suggest DUOX isoforms are important for innate antiviral host defense. The observation that DUOX2 expression is substantially increased by IFN-γ and rhinovirus provides circumstantial evidence for this notion (Harper et al., 2005). In addition, respiratory tract epithelial cells are known to generate $H_2O_2$ in less than 1h after RV infection (Kaul et al., 2000; Biagioli et al., 1999). RV-induced $H_2O_2$ production is inhibited by diphenyleneiodonium (DPI), which indicates the involvement of a flavoprotein, likely an NADPH oxidase, in this process. Therefore, DUOX appears to be the best candidate responsible for RV-induced $H_2O_2$ production in respiratory tract epithelium. The presence of EF-hand domains on DUOX isoforms suggests a mechanism by which RV infection can immediately induce DUOX-mediated $H_2O_2$ production (e.g. membrane-associated calcium flux (Geiszt et al., 2003; Forteza et al., 2005). The specific contribution of DUOX isoforms in respiratory tract epithelial innate host defense against viral infection remains to be determined.

Alternative functions for DUOX isozymes in the respiratory tract have recently been suggested. Hydrogen ion concentration at the apical surface of respiratory tract epithelium serves important host defense functions. Not only does lower pH increase the efficiency of known respiratory tract peroxidases such as LPO, but an acidic environment is known to decrease viability of some respiratory viruses (Greenberg, 2007; Whitton et al., 2005; Hayden, 2004). Therefore, an attractive model for DUOX is the ability of this protein to simultaneously acidify the airway surface liquid to optimize LPO enzymatic activity and provide the $H_2O_2$ needed for LPO to convert $SCN^-$ to $OSCN^-$. Recent data from Horst Fischer and colleagues support the role of DUOX in $H^+$ production and $H^+$ secretion. By using chemical inhibitors of DUOX (Schwarzer et al., 2004) and DUOX1-specific RNAi transcripts, they were able to demonstrate an approximately 50% reduction in intracellular acid production. These data suggest that under basal conditions there is DUOX-mediated turnover of NADPH to NADP + that is responsible for approximately 50% of H + production. Based on this model, it is presumed that activation of NADPH oxidase activity in the respiratory tract, and specifically activation of DUOX1, will result in a parallel increase in H + secretion. However, this presumption has not been formally tested. Similarly, it is not known if increased activation of DUOX2 will result in a similar increase in intracellular H + production or H + secretion.

DUOX1 appears to perform a specific role in signaling events related to mucin secretion and wound healing. Wesley et al. recently demonstrated that ATP-mediated P2 purinergic receptor activation was necessary for cell migration and wound closure in primary and virus-immortalized tracheobronchial epithelial cell cultures (Wesley et al., 2007). This functional activity correlated with increased DUOX1-dependent $H_2O_2$ production. Wound closure was significantly decreased in cells expressing siRNA against DUOX1, but not in cells expressing non-target siRNA transcripts. Together, these data strongly suggested that DUOX1-generated $H_2O_2$ is a necessary signal for adequate wound closure in human tracheobronchial epithelium. Although not explicitly tested, it is likely that release of intracellular ATP during injury induces purinergic receptor-mediated calcium flux, which directly triggers DUOX1 oxidase activity via EF-hand domain activation. Similarly, Shao et al. demonstrated that DUOX1 appears to be a critical protein necessary for phorbol 12-myristate 13-acetate- or human neutrophil elastase-mediated MUC5AC production in human tracheobronchial epithelial cell cultures (Shao and Nadel, 2005).

## Regulation of DUOX Expression and Enzymatic Activity

As mentioned, the initial identification of DUOX isoforms demonstrated that *DUOX* mRNA is upregulated by forskolin in thyroid tissues (Dupuy et al., 1999; De Deken et al., 2000). Thyroid stimulating hormone also increased *DUOX1* and *DUOX2* mRNA expression in thyroid tissues after 24 h of treatment (Pachucki et al., 2004). However, DUOX promoter activity does not appear to depend upon thyroid-specific regulators consistent with the expression of DUOX in multiple tis-

sue types (Christophe-Hobertus and Christophe, 2007). Data for the regulation of DUOX expression in the respiratory tract is similarly limited.

We recently demonstrated differential expression of *DUOX1* and *DUOX2* after treatment of human tracheobronchial epithelial cells with inflammatory cytokines. *DUOX1* increased in response to the Th2 cytokines interleukin-4 (IL-4) and inter-leukin-13 (IL-13). DUOX2 mRNA expression increased significantly in response to the Th1 cytokine interferon-γ (IFN-γ), and increased modestly to interleukin-1a or interleukin-1b. Of potential importance, the nature of gene expression was signifi-cantly different between the two genes in response to their respective cytokines. DUOX1, for example, increased maximally three- to sixfold in response to IL-4 at varying doses of the cytokine. In comparison, DUOX2 increased linearly with increasing doses of IFN-γ more than 15- to 20-fold. Although the fold-induction was substantially higher for DUOX2 compared to DUOX1, DUOX2 expression levels began to wane after 48 h. However, DUOX1 levels continued to increase 72 h after cytokine treatment. These qualitative differences in regulation and the differential regulation of DUOX1 and DUOX2 by cytokines suggest both enzymes perform distinct functions in the respiratory tract epithelium. Localization of DUOX1 and DUOX2 protein in the respiratory tract epithelium, and identifying proteins that co-localized with the DUOX isoforms will be instrumental in determining the functions of each protein.

Current evidence suggests that constitutive DUOX-mediated oxidase activity is directly proportional to the total DUOX protein available in respiratory tract epithelial cells. Based on mRNA data, DUOX1 is the predominant isoform expressed under basal conditions in airway cell cultures. Therefore, it is likely that DUOX1 provides the majority of DUOX-mediated $H_2O_2$ under basal condi-tions. Cytokine treatments that increase *DUOX* mRNA levels, and presumably DUOX protein levels, induce a parallel increase in $H_2O_2$ production (Harper et al., 2005). This suggests that one method to regulate DUOX oxidase activity is to regulate the absolute level of protein in respiratory tract epithelium. Based on our previous data, it appears that maximal inducible expression of *DUOX1* or *DUOX2* result in similar levels of $H_2O_2$ generation in respiratory tract epithelium (Harper et al., 2005). Although DUOX2 is more inducible by cytokine stimulation com-pared to DUOX1, it is possible that the protein levels of DUOX2 or DUOX1 are similar when maximally induced by their respective cytokines. Antibodies that differentiate between DUOX1 and DUOX2 will provide insights into the absolute protein levels for each isoform.

DUOX-mediated $H_2O_2$ generation can also be rapidly and robustly induced above constitutive levels in respiratory tract epithelium (Geiszt et al., 2003; Wesley et al., 2007; Forteza et al., 2005). Prior to the identification of DUOX2 in thyroid tissue, it was established that thyroid cells required calcium for $H_2O_2$ generation (Ameziane-El-Hassani et al., 2005; Leseney et al., 1999; Nakamura et al., 1987). Similar to Nox5, DUOX family members contain two EF-hand domains suggesting that DUOX oxidase activity will increase in response to calcium (De Deken et al., 2000; Lambeth et al., 2007; Dupuy et al., 1988). Further evidence in thyroid and respiratory tract epithelium has confirmed that $Ca^{++}$ influx is likely the predominant

factor regulating inducible DUOX-mediated NADPH oxidase activity. Treatment of respiratory tract epithelial cells with agonists for calcium mobilization, including ionomycin, thapsigargin, or ATP, has resulted in the rapid increase in $H_2O_2$ flux. This activity was blocked by nonspecific inhibitors of DUOX enzymes or siRNA against DUOX1.

There is some evidence that additional regulatory proteins are required for DUOX-mediated enzymatic activity. Schwarzer et al. demonstrated that known NOX2 regulatory proteins, p40phox, p47phox, and p67phox, are co-expressed with the DUOX isozymes in respiratory tract epithelium (Schwarzer et al., 2004). Shao et al. demonstrated that p47phox and p67phox proteins were identified in the membrane fraction of respiratory tract epithelial cells after PMA treatment. This localization corresponded to PMA-induced, DUOX1-mediated $H_2O_2$ production (Shao and Nadel, 2005). However, family members that are more closely related to NOX2 do not use these phox regulatory units for enzymatic activity (Banfi et al., 2003). There is direct evidence in thyroid tissues that cytosolic phox regulators, including Rac, do not provide regulatory functions for DUOX. And, patients with autosomal recessive chronic granulomatous disease, in which there are defects in cytosolic phox regulators, do not exhibit hypothyroidism. Together, these latter data suggest that the DUOX isozymes do not rely on these cytosolic phox regulatory units. However, differences in the predominant isoform found in thyroid tissue (DUOX2) versus respiratory tract epithelium (DUOX1) may be responsible for these contradictory data. Further studies with knock down of various phox regulatory units in respiratory tract epithelium will be able to definitively settle this issue.

The model that DUOX isoforms are simply $H_2O_2$ generators in the respiratory tract epithelium is consistent with current data, but presents potential teleological inconsistencies. For example, if the predominant function of the DUOX proteins is to generate $H_2O_2$, why are *two* bulky DUOX proteins maintained in the airway rather than a single, and smaller, isoform of the NOX/DUOX family? This question can be partially addressed by the observation that DUOX1 and DUOX2 are differentially regulated in respiratory tract epithelium. Although $H_2O_2$ is produced by both DUOX proteins, it is possible that the functional signalasomes that assemble around DUOX1 are substantially different from the proteins that cluster around DUOX2. The presence of two different oxidases in the same cell type, allow efficient utilization of $H_2O_2$ as a single signal that produces two very distinct effects.

Similarly, it is unclear why two peroxidase-containing DUOX proteins are present in airway epithelium if several other heme peroxidases (e.g. LPO, MPO, and others) are abundantly present in airway surface liquid (Gerson et al., 2000; Salathe et al., 1995). Several investigators have suggested that the peroxidase domains within DUOX1 or DUOX2 are not functional (Lambeth et al., 2007; Dupuy et al., 1988; Banfi et al., 2003; Salathe et al., 1995; Geiszt and Leto, 2004). It is possible that the peroxidase domains represent the vestigial remains of an ancestoral protein that has not been selected against. Or, the peroxidase domains may provide regulatory functions for the DUOX isozymes independent of heme peroxidase activity.

However, biochemical analysis of purified DUOX protein suggests that DUOX has functional heme peroxidase activity (Edens et al., 2001; Ha et al., 2005). DUOX peroxidase domains isolated from humans, C. elegans, and Drosophila demonstrated peroxidase activity. In addition, we have recently shown that *DUOX2* is required for functional peroxidase activity in respiratory tract epithelium (Harper et al., 2006). Therefore, based on physical proximity, it is likely that DUOX2-generated $H_2O_2$ is utilized by its own peroxidase rather than diffusing to LPO some distance away. Together, these data provide strong evidence that DUOX is not solely an $H_2O_2$ generator to assist the antibacterial functions of LPO but is performing distinct functions apart from other respiratory tract peroxidases. The demonstration of heme incorporation into the DUOX peroxidase domain has not been confirmed to date, and therefore, the functional capacity of the DUOX peroxidase domain remains controversial. Therefore not only are there questions related to the levels of oxidants generated via the DUOX pathway – i.e. oxidative balance but in addition there are questions related to the balance of oxidant generation between the DUOX isoforms and what these may signal.

## Summary

There is growing interest in understanding the function and importance of DUOX proteins in the respiratory tract. Based on the current body of literature, it is clear that DUOX is important for antibacterial host defense functions in the respiratory tract as part of the DUOX/LPO/SCN⁻ system. Evidence that DUOX isozymes are important for mucin secretion, proton secretion, and wound healing strongly supports the notion that these family members are critical for multiple functions in the respiratory tract. Regulation of DUOX enzymatic activity by calcium flux and regulation of protein expression by immune-modulating cytokines provide insights into additional potential functions for these family members. Protein isolation techniques that allow specific identification of each isoform are still required to fully characterize the role of DUOX1 and DUOX2 in the respiratory tract epithelium.

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