Mini review: immunologic functions of dual oxidases in mucosal systems of vertebrates

H. T. Yang*a, Y. H. Huanga and G. W. Yanga

aShandong Provincial Key Laboratory of Animal Resistance Biology, College of Life Sciences, Shandong Normal University, 250014, Jinan, Shandong, China
*e-mail: yanghuiting@sdnu.edu.cn

Received: September 15, 2018 – Accepted: May 8, 2019 – Distributed: November 30, 2020
(With 1 figure)

Abstract
Mucosal epithelial cells act as the first immunologic barrier of organisms, and contact directly with pathogens. Therefore, hosts must have differential strategies to combat pathogens efficiently. Reactive oxygen species (ROS), as a kind of oxidizing agents, participates in the early stage of killing pathogens quickly. Recent reports have revealed that dual oxidase (DUOX) plays a key role in mucosal immunity. And the DUOX is a transmembrane protein which produces ROS as their primary enzymatic products. This process is an important pattern for eliminating pathogens. In this review, we highlight the DUOX immunologic functions in the respiratory and digestive tract of vertebrates.

Keywords: NADPH oxidases, dual oxidase, innate immunity, mucosal immunity.

1. Dual Oxidase is a Member of NADPH Oxidases
DUOXs belong to the family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and are the primary sources of H₂O₂ production in a wide range of organisms. NADPH oxidases (NOXs) represent a family of enzymes, which directly regulate cellular production of reactive oxygen species (ROS) and play various functional roles in physiology (Laurindo et al., 2014). ROS includes H₂O₂, hydroxyl radical (-OH), superoxide anion (O₂⁻) and singlet oxygen (¹O₂) species (Cao et al., 2017; Li et al., 2012; Pang et al., 2011). There are 7 members, including 5 NOX enzymes (NOX1-5) and 2 DUOX enzymes (DUOX1 and DUOX2), in the human genome. DUOXs were originally identified as NADPH oxidases producing H₂O₂ necessary for thyroid hormone biosynthesis for the iodination process in the thyroid gland (De Deken et al., 2000; Dupuy et al., 1999). NOXs have a conserved catalytic core, which is responsible for transmembrane electron transfer. The intracellular electron donors are transferred to the extracellular compartment, and subsequently, generate superoxide or H₂O₂ (De Deken et al., 2014). Under normal conditions, most NOX isoforms have very low or no constitutive activity but their expression can be increased after pathogens’ invasion. Under these conditions, the activated NOXs can generate high ROS levels leading to increased oxidative stress (Sarr et al., 2018). Oxidative stress commonly is used as a defense mechanism for killing foreign microorganisms (Xiong et al., 2018). The role of ROS as microbicidal effectors is obvious during the oxidative burst of phagocytes (Babior, 2004).

In the absence of the NOX2 gene, humans are prone to severe microbial infection. Therefore, ROS generated by NOXs is important in immunity.

The molecular structure of the NOX family members share high homology between them. NOX2 contains two heme groups in the N-terminal region, which is composed of six transmembrane α-helical domains, and one NAD
binding domain and one flavin adenine dinucleotide binding domains in the C-terminal region. The basic structure of NOX1, NOX3 and NOX4 is similar to that of NOX2. NOX5 has a short N-terminal extension of Ca\(^{2+}\)-responsive EF hand motifs (Ameziane-El-Hassani et al., 2005; Bae et al., 2010; Grandvaux et al., 2015) (Figure 1).

In phagocytes, the catalytic protein NOX2 serves as a core protein in the oxidase complex (Segal, 2005). Excessive ROS production is also very detrimental to the host, therefore there are many signaling pathways which can inhibit the NOXs’ activity (Lambeth et al., 2007; Leto et al., 2009). To gain full activation, it is necessary that five molecules must combine with NOX2, including one membrane-integrated protein (p22phox), three cytosolic proteins (p47phox, p40phox and p67phox), and one small G-protein Rac. After pathogen challenge, NOX2 tightly associates with p22phox to form cytochrome b558, which leads to the recruitment of cytosolic proteins and Rac (Bae et al., 2010). NOX2 produces superoxide from molecular oxygen (Babior et al., 1973; Leto and Geiszt, 2006). Superoxide then dismutates into \( \text{H}_2\text{O}_2 \) enzymatically or non-enzymatically. \( \text{H}_2\text{O}_2 \) can then be converted to the highly microbicidal \( \text{HOCl} \) by an enzyme known as myeloperoxidase (Klebanoff, 2005).

**Figure 1.** NOXs and DUOXs structure of the NADPH oxidase family. (A) The structure of NOX1–4; (B) The structure of NOX5; (C) The structure of DUOXs from humans; (D) The structure of DUOX from *Caenorhabditis elegans*. PHD = peroxidase homology domain; EF hands = calcium-binding region; FAD = FAD-binding domain; NADPH = NADPH-binding domain.
2. DUOX’s Characterization

DUOXs share conserved motifs, including one peroxidase homology domain in the N-terminal region, which is a crucial extracellular matrix with tyrosine-crosslinking activity, two Ca\(^{2+}\) binding domains (EF hand), five or six transmembrane domains, one FAD binding domain and one NAD binding domain (Figure 1) (Bae et al., 2010; Rigutto et al., 2009; Yang et al., 2016). Once thought to be an exclusive feature of phagocytes, ROS production by DUOXs has now also been described in several different tissues and cells in many animals (Bedard and Krause, 2007).

DUOX-mediated generation of ROS needs to be regulated by enzymatic activity and gene expression. Previous reports indicate that uracil acts as a ligand that induces the activity of the Hedgehog (Hh) signaling pathway. In this pathway, a kind of calcium-dependent cell adhesion molecule, Cadherin 99C, expression levels increase can induce Ca\(^{2+}\) mobilization together with phospholipase C\(\beta\) and protein kinase C. The high Ca\(^{2+}\) concentration in the cytoplasm initiates DUOX enzymatic activity (Lee et al., 2015a). Additionally, the induction of lipid raft formation, lipolysis, and CaMKII can promote DUOX enzymatic activity (Lee et al., 2018, 2015b). DUOX-mediated ROS production requires the formation of a DUOX complex rather than an individual DUOX molecule. DUOX activator 2 (DUOXA2) can associate with DUOX2 and form a stable complex. The complex is crucial for H\(_2\)O\(_2\) generation by DUOX2. The disulfide bridge formed between DUOX2 and DUOXA2 has been reported to be very important for the function of the complex (Casas et al., 2015).

DUOX gene expression is highly upregulated in response to pathogen infections (Geiszt et al., 2003; Ha et al., 2005). Some studies have shown that the NF-\(\kappa\)B pathway triggered by bacteria can elicit and promote DUOX expression (Ha et al., 2009). Pseudomonas aeruginosa flagellin can induce DUOX expression and ROS production in human bronchial epithelial cells (Gattas et al., 2009). DUOX1 mRNA levels are moderately upregulated by Th2 cytokines such as IL-4 and IL-13. DUOX1 expression is promoted by IL-4 in the thyroid of transgenic mice (Eskalli et al., 2016). However, DUOX2 mRNA levels are enhanced by cytokine interferon-\(\gamma\) in primary respiratory tract epithelial cells (Harper et al., 2005). DUOX2 expression has also been reported to be elevated by polycytidylic acid (poly (I:C)) and rhinovirus infection in primary respiratory epithelial cells. These findings indicate that the two types of DUOXs have distinct functions under different physiological processes. Mucosal immune dysbiosis could increase the expression of DUOX2 in the mice intestine (Grasberger et al., 2015). Mitogen activated protein p38c is expressed in the midgut and can induce the expression of DUOX (Chakrabarti et al., 2014). However, the accurate intracellular signaling mechanism by which DUOX expression is regulated has not yet been elucidated.

3. DUOX’s Immunologic Functions

DUOX1 and DUOX2 were first cloned from porcine and human thyroid glands. In DUOX2 mutated mice, congenital hypothyroidism was observed. This demonstrated that DUOX2 has thyroid hormone biosynthesis function (Louzada et al., 2018; Moreno et al., 2002; Weber et al., 2013). However, DUOX expression is not confined to the thyroid gland; the enzyme is present in various epithelial cell types on the mucosal surfaces of the respiratory and gastrointestinal tract (Geiszt et al., 2003; Hampton et al., 1998; Rada and Leto, 2008). This suggests that DUOXs have important roles in innate immunity pathways. DUOXs produce H\(_2\)O\(_2\), which is an effective antimicrobial compound against a broad range of bacteria (Gerson et al., 2000; Oram and Reiter, 1966).

3.1. DUOXs’ immunologic functions in the respiratory tract

Both DUOX1 and DUOX2 are expressed in the respiratory epithelium. Earlier on, DUOX1 was considered as the main isoform responsible for extracellular H\(_2\)O\(_2\) production in response to bacterial stimulation (Boots et al., 2009; Forteza et al., 2005; Koff et al., 2008). In contrast, the function of DUOX2 was mainly considered to be involved in differentiated airway epithelial cells (Gattas et al., 2009; Linderholm et al., 2010). However, in recent years, reports have suggested that DUOX2 produces extracellular H\(_2\)O\(_2\) which also has antimicrobial function in mammalian airway epithelial cells (Moskwa et al., 2007). The bacterial component flagellin induces the airway mucosa to promote the DUOX2-mediated ROS release and trigger innate immune responses via TLR5 activation (Joo et al., 2012).

Pseudomonas aeruginosa obtained from long-term cultures inhibits DUOX1-dependent hydrogen peroxide release, and purified pyocyanin from P. aeruginosa can inhibit the dual oxidase-based antimicrobial system of airway epithelial cells (Rada et al., 2008).

Virus infection of the respiratory tract usually results in severe respiratory diseases (He et al., 2016; Hou et al., 2017, 2018; Liang et al., 2010; Zheng et al., 2018). DUOX2 and its maturation factor DUOXA2 in the respiratory mucosa could be upregulated by viral infection and some cytokines (Interleukin I (\(\alpha\) and \(\beta\)) and II (\(\gamma\)) IFNs and Th1) (Fink et al., 2013; Strengert et al., 2014). Influenza A virus (IAV) infection can lead to coordinated up-regulation of DUOX2, which generates H\(_2\)O\(_2\). After H\(_2\)O\(_2\) decomposition, the IAV replication levels are augmented. Furthermore, when the expression of DUOX2 was blocked, 2009 pandemic H1N1 influenza virus was more likely to load on intranasal infection (Strengert et al., 2014). Respiratory syncytial virus (RSV) is the most frequent cause of bronchiolitis in infants and children worldwide. In a perinatal lamb model, after RSV infection, DUOX expression levels were increased under bronchiolitis (Derscheid and Ackermann, 2012). Retinoic acid inducible gene 1 and melanoma differentiation-associated protein 5 also can induce DUOX2-mediated antiviral responses (Kim et al., 2015). In chronic rhinosinusitis (CRS), DUOX mRNA levels were significantly elevated. The expression levels of eotaxin and TNF-\(\alpha\) closely correlated with the expression of DUOX. These results indicate that DUOX is implicated in the inflammatory response in CRS (Cho et al., 2013).
In some cases, viral infection can also induce the expression of DUOX1 (Grandvaux et al., 2015). However, DUOX1 is a commonly critical protein in the pathogenesis of allergic airways according to its association with type 2 immune responses and the its ability to interact with type 2 cytokines (Comhair and Erzurum, 2010; Sugiuira and Ichinose, 2008; Suzuki et al., 2016; Van der Vliet et al., 2018). IL-4 and IL-13, can recruit neutrophils in airways to promote allergic asthma (Chang et al., 2013). It has been reported that DUOX1 mRNA and protein levels increased and promoted IL-33 secretion in cultured nasal or bronchial epithelial cells from humans or model animals with allergic asthma (Habibovic et al., 2016; Hristova et al., 2016; Wan et al., 2016).

### 3.2. DUOX immune function in the digestive tract

Vertebrate guts harbor large amounts of microorganisms. In the human gut, there are approximately 10^{14} microbial cells from over 500 species which reside in the barrier epithelia (Goodacre, 2007; Savage, 1977; Tannock, 2005). It has been confirmed that DUOX2 is expressed all along the digestive tract, where it provides a novel source of H₂O₂, particularly in the cecum, ileum and colon (El Hassani et al., 2005; Sommer and Backhed, 2015).

Segmented filamentous bacteria up-regulate the expression of DUOX2 in the mice intestinal tract. Mucosal cytokine IL-22 increases the expression of DUOX2. However, IL-17 or IL-22 are not required for DUOX2 transcription (Grasberger et al., 2015). In the inflamed human colon, DUOX protein expression is highly elevated in the apical, lateral and perinuclear membrane along the whole length of the gland. DUOX2 induces exfoliation of the crypt epithelium, which is a major contributor to inflammation. Therefore, DUOX2 may be a potential therapeutic target for treating ileocolitis inflammatory bowel disease (Chu et al., 2017).

*Helicobacter pylori* infection causes the most common human chronic infection. In wild-type mice, infection with *Helicobacter felis* induces the expression of DUOX2 and DUOXA2 in the stomach (Grasberger et al., 2013). In a colon study, it was demonstrated that a sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. This goblet cell nonspecifically endocytoses and reacts to the TLR2/1, TLR4, and TLR5 ligands, and then induces the DUOX-dependent ROS synthesis against persisting pathogens (Birchenough et al., 2016). In intestinal immunity of aquatic animals, the function of DUOX has been studied. Based on the advantages of the zebrafish genetic background, it has been used as good animal model to study intestinal immunity. Zebrafish DUOX is highly expressed in intestinal epithelial cells. After knockdown of DUOX, zebrafish larvae had impaired capacity to control enteric *Salmonella* infection (Flores et al., 2010). It has been reported that DUOX is activated following ligand-dependent stimulation of TLRs via interacting microbial components. However, the mechanism by which TLR stimulation leads to DUOX activation is less clear. Previous experiments have shown that DUOX is physically associated with at least some members of the TLR family, such as the TLR2 and the TLR5 (Joo et al., 2012). In the common carp (*Cyprinus carpio* L.), many mucosal immunity associated molecules, such as the Rig-I-like receptors, TLRs, plgR-like molecule, interferon regulatory factors, interleukin-1 receptor-associated kinase 1, and antimicrobial peptides, have shown immunity functions (Chen et al., 2018; Li et al., 2013, 2017; Rombout et al., 2014; Shan et al., 2015; Yang et al., 2014, 2017; Zhang et al., 2015; Zhu et al., 2016a, b). Various TLRs have been identified in *Cyprinus carpio* L., including the TLR4, 18, 21, 22. In addition, these TLRs can participate in various antimicrobial and antiviral responses (Li et al., 2018; Shan et al., 2018; Westfall et al., 2017). However, there are no reports that clearly show the relationship between TLRs and DUOX activation in fish.

Enterovirus infection usually results in host’s diarrhea, and weak or severe fever. The microbiota diversity was increased in the intestine after humanized pigs were infected with human rotavirus (Kumar et al., 2018). Transmissible gastroenteritis virus (TGEV) causes pig diarrhea. After TGEV infection, the mRNA levels of cytokines, including IL-1β, IL-6, IL-8 TNF-α and IL-10, were significantly enhanced. Additionally, the number of *Lactobacillus* decreased, while the number of *Enterobacteriaceae* increased (Xia et al., 2018; Zhang et al., 2018). Newcastle disease virus can induce oxidative stress and tissue damage in the intestine of chickens. Vitamin E supplementation alleviates this condition (Rehman et al., 2018). However, few studies have reported the relationship of DUOXs with viral infection in the intestine.

### 4. Conclusion

In this review, we summarized the current knowledge with respect to the importance of DUOX enzymes in mucosal epithelia, especially in the respiratory tract and the digestive tract. In the respiratory tract, DUOX1 mainly participates in antimicrobial responses, and also in allergic airways. However, DUOX2 plays an important role against viral infection in the respiratory mucosa. The mucosal immunity in the intestine is necessary for the host’s health, considering the complexity of the local environment. DUOXs can indeed participate in antibacterial processes. However, the signaling pathways regulating the DUOXs have not been revealed. It is merely reported that enterovirus infection induces oxidase stress. Whether viral infection induces DUOXs expression and activation has not been uncovered. Additionally, it is necessary to study the expression and activation of the molecular mechanism of function of DUOXs in mucosal immunity.

### Acknowledgements

This work was supported by grant from Shandong University Scientific Research Project (No. J18KB081).
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