Duox2 exhibits potent heme peroxidase activity in human respiratory tract epithelium

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Abstract The dual oxidase isozymes Duox1 and Duox2 exhibit functional NADPH:O2 oxidoreductase activity in thyroid and respiratory tract cells and are thought to be essential for H2O2 generation in these tissues. However, it is not universally accepted that the heme peroxidase domains of the Duox isozymes are functional. To address this question, we modulated Duox2 expression in human tracheobronchial epithelial (TBE) cell culture systems and quantified peroxidase activity. We discovered that interferon-gamma (IFN-γ) induced robust peroxidase activity in TBE cells that paralleled Duox2 expression. IFN-γ-induced peroxidase activity was abolished in the presence of sodium azide, which implicated the activation of a heme peroxidase. IFN-γ-induced peroxidase activity was abolished in TBE cell lines expressing anti-Duox2 short hairpin RNA transcripts. Together, these data unequivocally demonstrated that Duox2 contains a functional heme peroxidase in intact respiratory tract epithelium.

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1. Introduction

The observation that gp91phox (Nox2) was a necessary component of neutrophil-mediated host defense [5,6] provided evidence that specific proteins function to purposefully generate reactive oxygen species. This discovery provided the foundation for the detection of multiple Nox2 homologues in several non-phagocytic tissues [7]. It is now well accepted that regulated generation of reactive oxygen species is essential for several basic cellular processes including host defense, growth, apoptosis, and cell signaling.

In addition to the NADPH oxidase domain present in all Nox family members, two Nox proteins, Duox1 and Duox2, each contain an additional heme peroxidase domain. Current models to explain the function of these dual oxidase (Duox) isozymes primarily focus on the ability of these proteins to generate H2O2, and largely ignore the contribution of the peroxidase domain [1–4,8,9]. Because critical histidine residues, necessary for heme binding, are missing from the primary structure of the Duox peroxidase domains, it is reasonable to suggest that these motifs are functionally inactive [8]. Evidence that the human Duox1 peroxidase domain has functional activity when expressed in Escherichia coli [10] or in Drosophila [11] suggests otherwise. To address this, we assayed human tracheobronchial epithelial (TBE) cells, which normally express Duox isozymes and are the likely source of H2O2 production in these cells [2,4,12,13], for the presence of Duox-dependent heme peroxidase activity.

2. Methods

2.1. Cell culture from human tissues

HBE1 cells, a papilloma virus-immortalized human TBE cell line kindly provided by Dr. J. Yankaskas from the University of North Carolina [14], were plated on Transwell® (Corning Costar, Corning, NY) chambers (24 mm) or standard six-well cell culture plates (BD Falcon, San Jose, CA) at 1–2 × 10^5 cells/cm², in a Ham’s F12/Dulbecco’s modified Eagle’s medium (DMEM) (1:1) supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), epidermal growth factor (10 ng/ml), dexamethasone (0.1 µM), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 µg/ml), bovine serum albumin (0.5 mg/ml), and all-trans-retinoic acid (30 nM) as described previously [15]. After one week, cells were transferred to air–liquid interface (ALI) culture conditions for one additional week followed by cytokine treatment for 48 h (IL-4 (10 ng/mL), IL-13 (10 ng/mL), or IFN-γ (100 U/mL)). For tissue culture conditions, cells were treated with cytokines after the cells reached 100% confluence.

2.2. Development of short hairpin RNA transcripts (shRNA) expression clones

We established several HBE1 cell lines which stably expressed anti-Duox2 shRNA transcripts after lentivirus infection [16]. Briefly, anti-Duox2 small interfering RNA transcripts (Ambion Inc., Austin, TX) were transiently transfected into HBE1 cells to identify two sequences that demonstrated significant knock down of Duox2 mRNA (data not shown). Oligonucleotides were then designed for insertion into the pSi-Lentilla plasmid, which resulted in the expression of shRNA transcripts [16]. To insert these sequences, we digested the pSiColR plasmid with HpaI and XhoI and ligated to the following annealed oligos: Duox2-S1 F, TGGTGAAGAGTGCAATAGATTCCAGAGAA-GATTTGATCCTTTTCACCT-T-TTTTC; Duox2-S1 R, ACCACT-TTCCATGTATCATCAAGGTTCTTCTAGACCAT-GGAATTGTGGAATATGAGGCT or Duox2-S5 F, TGGTCTGAAACAGGGTTTTTTTC-CCAAGAGAAACCCCTTGTCAGGACCTTTT-
ttc; duox2-s5 r, accagcgact-gttctccccaaaaaggttc-tcttttgggaaacaagtctggaагагдацт. the resulting constructs were packaged into a lentivirus delivery system as previously described [17]. hbe1 cells were seeded in 100 mm tissue culture plates (bd falcon) at a density of 4 x 10^6 cells/ml. after 24 h, 5 μl of concentrated lentivirus particles was added to cell culture media, and cells were expanded for three passages prior to cell sorting. infected cells were purified by gfp-triggered cell sorting and gfp-enriched cells were separated into more than 30 different isolates. each isolate was treated with ifn-γ and, three clones that had low to absent levels of functional peroxidase activity (clones 5-2-k, 5-2-d, and 5-4-o) were selected for subsequent experiments. similarly, hbe1 cells expressing shiro-r without the shrna insert (clone psr-1) were developed to serve as negative control.

2.3. cytokine treatment
recombinant human ifn-γ, il-4, and il-13 cytokines were purchased from r&d systems inc. (minneapolis, mn). cytokines (0-100 ng/ml) were dissolved in phosphate-buffered saline (pbs) with 1% bovine serum albumin (bba) and added directly to the media of tbe tissue cultures or both the apical and basal sides of ali tbe cultures. for untreated conditions, the same amount of pbs-1% baa, without cytokine, was added to the media.

2.4. real-time rt-pcr expression analysis
real-time rt-pcr was carried out as previously described [18]. briefly, 5 μg of total rna was reverse transcribed with momlv-reverse transcriptase (promega inc., madison, wi) using oligo-dt primers. diluted sample cednas were used in real-time pcr reactions with either an abi 5700 or abi prism™ 7900ht sequence detection system (applied biosystems inc., foster city, ca). reactions were carried out in 96-well optical reaction plates in a 50 μl final volume containing 25 μl of the sybr® green (applied biosystems) pcr master mix, 1 μl of each gene-specific primer, 2 μl of diluted sample cedna, and 21 μl of water. relative expression values were normalized using glyceraldehyde-3-phosphate dehydrogenase (gapdh). pcr primer pairs were designed based on published sequences for each gene as follows: duox1 f 5'-ttcagcagctgtcgtgcaaa-3', duox1 r 5'-agaagcacagcaagttc-3'; duox2 f 5'-agcacagctgtcgtgcaaaagttc-3', duox2 r 5'-tgatgagagccgaacctcga-cgc-3'; lpo f 5'-cagaatgtgatcaccacaata-3', lpo r 5'-ctgggtcaagggctggcag-3'; mpo f 5'-agaggcttagacccctcagtg-3', mpo r 5'-tgcaatgcaatggcctggca-3'; dpo f 5'-acagcacagtagcatcaccacaata-3', dpo r 5'-tcctacatcattgtaaccctg-3'; epo f 5'-actgtagaatgagctggccttgca-3', epo r 5'-gaatgtgatcaccacaata-3'; gapdh f 5'-ggagtcaagagcaaccacagt-3', gapdh r 5'-gaagaggcttagacccctcagtg-3'. real-time pcr was conducted in triplicate for each sample and the mean value was calculated. final figures represent the results from at least three independent experiments.

2.5. peroxidase measurements
peroxidase activity was assessed using 3,3',5,5'-tetramethylbenzidine (tmb; sigma–aldrich, st. louis, mo) as a peroxidase substrate in the presence of excess h2o2. briefly, media were removed from untreated and cytokine-treated cells and cells were washed twice in pbs. one hundred microliters of suspension buffer (100 mM NaH2PO4, pH 7.0) was added to each well of a 96-well plate followed by cell scraping using a rubber spatula. suspended intact cells (10-20 μl) were placed into a 96-well plate followed by the addition of tmb solution (300 mM NaAcetate, pH 5.4, 1.2 mM TMB, 0.3 mM H2O2). of note, detergents in the suspension buffer significantly attenuated peroxidase activity, and therefore we avoided the use of lysis buffers. to inhibit heme peroxidase activity, 100 μM NaN3 was added to duplicate samples. because the tmb assay is temperature-sensitive, all assays were performed at room temperature. absorbance at 630 nm was measured over 15-40 min. for each treatment condition, duplicate to triplicate samples were assayed, and each experiment was repeated a minimum of three times.

3. results and discussion
although it is well established that the duox isozymes generate H2O2 via the NADPH oxidase domain [2,4,19,20], functional activity of the heme peroxidase domain in tissues that normally express duox isozymes has not been demonstrated. our previous data in respiratory tract epithelial cells demonstrated that treatment with IL-4 or IL-13 enhanced duox1 expression and treatment with IFN-γ significantly increased duox2 expression. all three treatment conditions induced the generation of apical H2O2 [18]. therefore, we postulated that treatment of these cells with the aforementioned cytokines would induce peroxidase activity which paralleled NADPH oxidase activity.

consistent with this notion, treatment of hbe1 cells with IFN-γ resulted in a significantly enhanced rate of TMB oxidation over 15 min compared to untreated hbe1 cells (fig. 1). in addition, there was a clear dose-dependent increase in IFN-γ-inducible TMB oxidation which paralleled the dose-dependent increase in duox2 mRNA expression (figs. 2 and 4a). compared to untreated hbe1 cells, IFN-γ induced an approximate
fivefold increase in TMB oxidation at the lowest dose tested, which increased to close to 40-fold at the highest dose tested (Fig. 2).

These data suggested that IFN-γ is sufficient to induce active peroxidase activity in the extracellular compartment of respiratory tract epithelium. Based on our previous data, the proposed topology of Duox2 [7], and the localization of Duox protein on the apical surface of human TBE cells [4,13], we suspected this activity was due to Duox2 expression. Importantly, IL-4 and IL-13 did not induce measurable peroxidase activity in our cell culture system (Fig. 1), despite four to sixfold elevations in Duox1 mRNA expression (data not shown).

Sodium azide treatment completely inhibited IFN-γ-induced peroxidase activity (Fig. 3), which confirmed that IFN-γ induced a heme peroxidase. Although we inferred this peroxidase activity was due to Duox2, it is possible that Duox1, or an alternative heme peroxidase normally not identified in respiratory tract epithelium, was augmented by IFN-γ. To address this possibility, we performed real-time PCR on mRNA harvested from IFN-γ-treated HBE1 cells. As shown in Fig. 4A, Duox1 mRNA levels did not change after IFN-γ treatment. Primers designed to selectively identify four other heme peroxidase proteins including lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO), and thyroperoxidase (TPO) (see Section 2) failed to produce measurable amplicons (Fig. 4A and data not shown). Similarly, western blots using antibodies against MPO or EPO failed to produce any observable signal (data not shown).

To definitively establish that Duox2 is responsible for IFN-γ-induced heme peroxidase activity in intact respiratory tract epithelial cells, we developed several stable cell lines that suppressed Duox2 expression through RNA interference mechanisms. Using this technique, we isolated three GFP-positive clones that did not exhibit IFN-γ-inducible Duox2 mRNA expression (see Section 2). Consistent with our premise, IFN-γ-induced heme peroxidase activity was observed in HBE1 cells expressing the lentivirus construct alone (pSR-1), but was not observed in HBE1 cells that stably expressed anti-Duox2 shRNA transcripts (e.g. clone 5-2-k) (Fig. 4B). Gain or loss of IFN-γ-mediated heme peroxidase activity paralleled the gain or loss of IFN-γ-mediated Duox2 mRNA expression. These data confirmed that IFN-γ-inducible heme peroxidase activity in respiratory tract epithelial cells is mediated by Duox2.

To determine if this level of heme peroxidase activity is consistent with the induction of a single protein, we estimated the number of Duox2 molecules required to produce the experimentally observed absorption values in HBE1 cells. We found that 5–20 ng of purified human MPO protein displayed similar end point absorption values at 15 min compared to cellular suspensions of IFN-γ-treated HBE1 cells (data not shown). We utilized approximately $1 \times 10^3–3 \times 10^4$ HBE1 cells per peroxidase assay. Based on these values, there were approximately $1 \times 10^3–1.5 \times 10^5$ “MPO equivalents” per cell [mols MPO × Avogadro’s number/number of cells]. If we assume a 1:1 correlation between MPO peroxidase activity and Duox2 peroxidase activity, IFN-γ induced the expression of $1 \times 10^5$
to $1.5 \times 10^6$ Duox2 molecules per cell. These numbers are consistent with values for other membrane proteins and receptors.

Recent evidence demonstrated that proper maturation and expression of Duox proteins does not occur in cell types that normally do not express these isoforms [21], and requires the expression of cell-type-specific adaptor proteins (DUOXA1 and DUOXA2) [22]. Our data imply that proper maturation and expression of native Duox proteins is also dependent upon the differentiation status of the cells that normally express Duox proteins. Although IFN-γ consistently induced heme peroxidase activity in all TBE cell culture conditions, ALI cell culture conditions resulted in significantly higher levels of induced heme peroxidase activity compared to tissue culture conditions (compare Figs. 2 and 3). The factors responsible for this regulation are unknown, but may depend upon the increased expression of these newly identified Duox maturation factors.

Peroxidase activity in respiratory tract epithelial cells has been observed for several decades [23]. However, the enzyme(s) responsible for this activity and the functional importance of this activity remains to be fully elucidated. Substantial evidence demonstrates that LPO is the predominant peroxidase in respiratory tract epithelium [24]. Based on these observations, it has been reasonably suggested that Duox enzymes provide $\mathrm{H}_2\mathrm{O}_2$ as a substrate for LPO-mediated generation of antibacterial hypohalous acids [2,24]. Our current evidence demonstrated that Duox2 itself provides functional heme peroxidase activity within the human respiratory tract epithelium. We propose that multiple functional peroxidases are localized in distinct subcellular compartments in the respiratory tract epithelium, and this localization is fundamental to the functional activity of each peroxidase. Verification of this notion will require the development of Duox1- and Duox2-specific antibodies or functional expression of labeled Duox1 and Duox2 isoforms.

The absence of measurable IL-4/IL-13-mediated peroxidase activity suggests Duox1 has distinct functional activities, or distinct cellular localization compared to Duox2. Two recent reports demonstrated that human Duox1 has functional peroxidase activity when overexpressed in E. coli [10] or Drosophila [11]. Based on these data and the homology between the Duox1 and Duox2 peroxidase domains, it is likely that Duox1 is a third functional peroxidase in respiratory tract epithelial tissues. We were able to observe peroxidase activity only in intact cells. Therefore, if Duox1 is located within intracytoplasmic vesicles, we would be unable to assess Duox1-mediated peroxidase activity with the methods we used. However, Duox1-mediated apical $\mathrm{H}_2\mathrm{O}_2$ production could still be measured due to the ability of $\mathrm{H}_2\mathrm{O}_2$ to freely diffuse through lipid membranes. It is intriguing to consider the possibility that Duox1 heme peroxidase activity is responsible for a portion of peroxidase-positive non-secretory vesicles previously identified in respiratory tract epithelial cells [23]; secretory vesicles with peroxidase activity likely contain LPO.

It is unclear why two heme peroxidase-containing Duox proteins are present in tissues, such as the respiratory tract or thyroid, that contain other heme peroxidases in abundance (e.g. lactoperoxidase or thyroid peroxidase). Our data, in combination with previous work, strongly supports the notion that oxidant-producing cells have developed a strategy in which oxidant generation and utilization are tightly regulated and compartmentalized. The presence of multiple functional peroxidases in the respiratory tract supports this general model. For example, abundant expression of LPO secreted into respiratory tract lining fluid provides an antimicrobial environment to prevent bacterial infection. Duox2 provides extracellular membrane-associated peroxidase activity, which may provide protection against membrane-bound pathogens such as virus. Duox1 may provide peroxidase activity in specialized intracytoplasmic vesicles whose functions still need to be determined. The specific subcellular locations of the two Duox isoforms, and the substrates utilized by their peroxidase domains will significantly contribute to our understanding of their function in the tissues in which they are expressed.

References


1 Portions of this idea were originally suggested by Tom Leto and Dave Lambeth; personal communication.


