NOX2 (gp91phox) is a predominant O2 sensor in a human airway chemoreceptor cell line: biochemical, molecular, and electrophysiological evidence

Josef Buttigieg,* Jie Pan,* Herman Yeger, and Ernest Cutz

Division of Pathology, Department of Pediatric Laboratory Medicine, the Research, Institute, The Hospital for Sick Children and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Submitted 21 May 2012; accepted in final form 31 July 2012

Buttigieg J, Pan J, Yeger H, Cutz E. NOX2 (gp91phox) is a predominant O2 sensor in a human airway chemoreceptor cell line: biochemical, molecular, and electrophysiological evidence. Am J Physiol Lung Cell Mol Physiol 303: L598–L607, 2012. First published August 1, 2012; doi:10.1152/ajplung.00170.2012.—Pulmonary neuroepithelial bodies (NEBs), composed of clusters of amine [serotonin (5-HT)] and peptide-producing cells, are widely distributed within the airway mucosa of human and animal lungs. NEBs are thought to function as airway O2-sensors, since they are extensively innervated and release 5-HT upon hypoxia exposure. The small cell lung carcinoma cell line (H146) provides a useful model for native NEBs, since they contain (and secrete) 5-HT and share the expression of a membrane-delimited O2 sensor [classical NADPH oxidase (NOX2) coupled to an O2-sensitive K+ channel]. In addition, both native NEBs and H146 cells express different NADPH oxidase homologs (NOX1, NOX4) and its subunits together with a variety of O2-sensitive voltage-dependent K+ channel proteins (Kv) and tandem pore acid-sensing K+ channels (TASK). Here we used H146 cells to investigate the role and interactions of various NADPH oxidase components in O2-sensing using a combination of coimmunoprecipitation, Western blot analysis (quantum dot labeling), and electrophysiology (patchclamp, amperometry) methods. Coimmunoprecipitation studies demonstrated formation of molecular complexes between NOX2 and Kv3.3a and Kv4.3 ion channels but not with TASK1 ion channels, while NOX4 associated with TASK1 but not with Kv channel proteins. Downregulation of mRNA for NOX2, but not for NOX4, suppressed hypoxia-sensitive outward current and significantly reduced hypoxia-induced 5-HT release. Collectively, our studies suggest that NOX2/Ko complexes are the predominant O2 sensor in H146 cells and, by inference, in native NEBs. Present findings favor a NEB cell-specific plasma membrane model of O2-sensing and suggest that unique NOX/K+ channel combinations may serve diverse physiological functions.

PULMONARY NEUROEPITHELIAL bodies (NEBs) consist of clusters of neuroendocrine cells widely distributed within the airway mucosa of human and animal lungs (35). Although the precise function of NEBs is at present unknown, they are thought to function as hypoxia-sensitive airway sensors possibly involved in the control of breathing (2, 9). NEB cells produce amine [serotonin (5-HT)] and peptide(s), and are extensively innervated including vagal afferents derived from nodose ganglia (3). Hypoxia-induced 5-HT release from NEB cells in vivo and in vitro has been previously documented (15, 21). In addition, earlier studies have demonstrated that NEB cells express a membrane delimited O2-sensing molecular complex consisting of an O2-sensing protein coupled to an O2-sensitive K+ channel (37, 40). Initially, the heme-linked nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), similar to the one identified in neutrophils (29) was postulated as the universal O2-sensing protein in all peripheral O2-sensing cells, including the glomus cells of the carotid body and pulmonary artery smooth muscle cells (1). However, subsequent studies have postulated several alternate mechanisms operating in different O2-sensing cells. For example mitochondria, rather than a plasma membrane-delimited NADPH oxidase, are involved in O2 sensing in pulmonary artery smooth muscle cells and that of neonatal adrenal medulla, while AMP kinase has been proposed to play a key role in the carotid body (4, 12, 13, 34). In contrast, several lines of evidence support NADPH oxidase as the principal O2 sensor in NEBs, which may be specific to this cell type in the lung. This evidence includes coexpression of various components of NOX (i.e., gp91phox and p22phox) and O2-sensitive voltage-gated K+ channel subunit Kc.3.3a (8, 37). Further support for the role of NADPH oxidase in NEBs O2-sensing is derived from studies of NADPH oxidase deficient mouse (OD; gp91phox k/o) that demonstrated abrogated response to hypoxia in NEB cells of neontates both in vitro and in vivo (16, 18).

Other potential candidates for an O2-sensor in NEB cells include the recently identified homologues of NADPH oxidase (also referred to as low-output oxidases) found in a variety of nonphagocytic cells (19). The founder protein, gp91phox [NADPH oxidase 2 (NOX2)] is predominantly expressed in phagocytic cells where it plays a critical role in host defense (31). Whereas NOX1 is expressed mostly in colonic epithelium, NOX3 is expressed mainly in the fetal kidney and inner ear (7, 20). In contrast NOX4, originally described as a renal oxidase, is widely expressed in different tissues, including lung, placenta, pancreas, bone, and blood vessels where it may be involved in various cellular processes, such as cell proliferation, apoptosis, and receptor signaling (19).

In NEB cells there are several potential K+ channels that can mediate the hypoxic response. The tandem pore acid-sensing K+ channels (TASK), are outwardly rectifying channels sensitive to changes in extracellular pH, are inhibited by extracellular acidification and are known to be involved in O2-sensing in several systems (6, 22). In addition, there are the Kv channels that are sensitive to TEA and changes in O2-availability (14, 23). However, the precise mechanism is unknown as to how these channels contribute to hypoxia sensing.

The immortalized cell line, small cell lung carcinoma cell line H146, representative of human airway chemoreceptor...
cells, offers several advantages over native NEBs. This includes an unlimited source of cells that are amenable to cellular and molecular studies difficult to perform, or not feasible, on native NEBs. H146 cells share many features with native NEB cells (17) including O$_2$-sensitive K$^+$ channels, NADPH oxidase, and serotonin content (9, 21, 25, 37). In our recent studies using multigene profiling arrays on NEB cells isolated by laser capture microdissection from human neonatal lungs, and in extracts of H146 cells we have identified expression of several novel oxidases and related protein subunits together with a variety of O$_2$-sensitive K$^+$ channels (10). In the present study, we investigated the function of NOX protein isoforms in mediating the hypoxic response in H146 cells using a combination of biochemical, molecular, and electrophysiological approaches. Our findings confirm the critical role of NOX2 and possibly NOX4 in O$_2$-sensing via formation of specific NOX/K$^+$ (O$_2$) channel complexes.

**MATERIALS AND METHODS**

**Cell lines.** The classical small cell lung carcinoma cell line H146 was obtained from ATCC (Manassas, VA). A reference cell line, the promyelocytic cell line (HL60), representative of neutrophils was also obtained. Both H146 and HL60 cells were cultured as previously described (10, 25) using RPMI 1640 culture medium (containing l-glutamine) supplemented with 10% FCS, 2% sodium pyruvate, and 10 glucose, 10 HEPES, at pH 7.4, and centrifuged at 800 g for 10 min at 4°C. The pellets were extracted in PBS containing 0.001% Tween 20 and 1X protease inhibitor cocktail. For coimmunoprecipitation studies, 300 µg protein from control and shRNA-treated cells were coimmunoprecipitated with either NOX2, NOX4, or K$^+$ channels, and TASK1 antibodies [for type and source see Cutz et al., (10)] using the Seize@X Protein G Immunoprecipitation kit (Pierce) and analyzed by Western blot analysis. Specific proteins were then detected with a triple-Quantum Dot (Q-dot) labeling method. Essentially, immunoprecipitated samples trapped on beads were eluted, 5 µl of whole cell lysate [abundant in actin] was added to provide the loading control, and the mixture was separated in 7% SDS-PAGE gels (30 µl/lane) and electrophoresed. Proteins were then transferred onto a polyvinylidene difluoride membrane. After incubating with Fab fragments to light and heavy chains to block nonspecific detections of antibodies, polyvinylidene difluoride membranes were incubated for NOX/K$^+$ channel associations through immunoblotting with goat anti-NOX2, rabbit anti-K, 3.3 or K,4.3; or rabbit anti-NOX4, goat anti-TASK1, and mouse anti-$\beta$-actin for a loading control with overnight incubation. Bound NOX and K$^+$ channel antibodies were then detected by incubation in Qdot Secondary Antibody Conjugates Qdot705 goat anti-rabbit IgG (red)/Qdot655 horse anti-goat IgG (green) and donkey anti-mouse-AMCA (blue) conjugates diluted 1:1,000 in blocking buffer (10). To assess the strength of the NOX/K$^+$ channel association, immunoprecipitated complexes on beads were incubated in a low concentration of 0.005% Triton X-100 prior to gel analysis and immunoblotting. ImageStation 2000MM system with Filtered Epi Illumination (KODAK 415/100 bp) was used to capture images by charge-coupled device camera.

**Electrophysiology.** Voltage clamp data were obtained using the nystatin perforated patch technique as previously described (4). The pipette solution contained (in mM): 110 K gluconate, 25 KCl, 5 NaCl, 2 CaCl$_2$, 10 HEPES, at pH 7.2, and nystatin (300–450 µg/ml). Experiments were conducted at 37°C in HEPES-buffered extracellular medium that contained (in mM): 135 NaCl, 5 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 1.8 K$_2$HPO$_4$, 10 HEPES, at pH 7.4, and with or without 0.5 µM TTX. Hypoxic solutions (PO$_2$ = 5–15 mmHg) were generated by bubbling N$_2$ gas and were applied to the cells by gravity flow. In voltage-clamp experiments, cells were held at −60 mV and step-depolarized to the indicated test potential (between −100 and +80 mV in 10 mV increments) for 100 ms at a frequency of 0.1 Hz. An Axopatch 200B amplifier was used to record whole cell currents (voltage-clamp) or membrane potentials (current-clamp). Voltage- and current-clamp protocols, data acquisition, and analysis were performed using pClamp 9.0 software and DigiData 1200B interface (Axon Instruments). All electrophysiological data are expressed as means ± SE and compared using the paired or independent Student’s t-tests (Micoral Origin version 7.0). Drugs were prepared fresh on the day of experiments.

**Carbon fiber amperometry.** Real-time secretion of 5-HT released from individual H146 cells (control vs. cells transfected with siRNA for NOX2 or NOX4) was monitored using carbon fiber amperometry after the culture dish was placed on the stage of a Nikon inverted microscope (model Optiphot-2UD; Nikon, Tokyo, Japan) equipped with a magnification, ×40 lens. The culture was perfused via gravity with an extracellular solution containing (in mM): 135 NaCl, 5 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 10 glucose, 10 HEPES, at pH 7.4 and 37°C. In some experiments, high K$^+$ (30 mM) solutions were used after equilibrating for NaCl to depolarize cells and test for secretion. Hypoxic solution (PO$_2$ = 15–20 mmHg) was obtained by continuously bubbling with N$_2$. Secretion was monitored from single cells that were usually part of a cell cluster with a ProCFE low noise carbon fiber electrodes (5-µm diameter tip; Dagan) connected to a CV...
23BU headstage and an Axopatch 200B amplifier set at 800 mV, a potential more positive than the oxidation potential for 5-HT. The electrode was backfilled with 3M KCl. Data acquisition and analysis were performed using Clampfit 9 (Axon Instruments); currents were filtered at 100 Hz, digitized at 250 Hz, and stored on a personal computer. Individual secretory events were quantified by measuring the charge, calculated by integrating the area under each amperometric spike. Events smaller than 3 pA were excluded from the analysis, and spike frequency was calculated as the number of spike events per minute. Samples were compared using Student’s t-test, and level of significance was set at \( P < 0.05 \). Unless otherwise indicated, the data are expressed as means ± SE.

**RESULTS**

Downregulation of NOX mRNAs by siRNAs. Our previous work, and that of others, suggests that pulmonary neuroendocrine cells and their immortalized derivatives (e.g., H146 cells) express a wide range of \( K_v \) channels and NOX isoforms (10, 23). While the mechanism by which membrane depolarization occurs is unknown, there is evidence that suggests that the interaction between NOX isoforms and certain \( K_v \) channels plays a key role (27). To test the potential involvement of NOX2 and NOX4 in \( O_2 \)-sensing, we first generated H146 cell lines deficient in either NOX2 or NOX4 isoforms. To verify the efficiency of siRNA downregulation of NOX proteins, H146 cells deficient in either NOX2 or NOX4 (termed NOX2-D or NOX4-D) cells were analyzed using immunohistochemistry, Western blot analysis, and RT-PCR. In control H146 cells, immunoreactivity for gp91phox (NOX2) and p22phox were colocalized to the plasma membrane or submembrane regions (Fig. 1A). In H146 cells transfected with NOX2 siRNA there was total loss of NOX2 (gp91phox) immunoreactivity, while p22phox immunoreactivity was preserved (Fig. 1B). In contrast, H146 cells transfected with NOX4 siRNA showed preservation of both NOX2 and p22phox immunoreactivities (Fig. 1C). The loss of respective NOX mRNAs expression was confirmed by RT-PCR. In H146 cells exposed to NOX2

![Fig. 1. Downregulation of NOX2 and NOX4 by siRNA.](image-url)
siRNA, there was a significant downregulation of NOX2 mRNA without an effect on NOX4 mRNA, while in cells exposed to NOX4 siRNA only NOX4 mRNA expression was reduced, leaving NOX2 mRNA intact (Fig. 1D). These findings were further corroborated by Western blot analysis demonstrating that pretreatment with NOX2 siRNA resulted in significant reduction in NOX2 protein expression, whereas incubation with NOX4 siRNA caused almost complete loss of NOX4 protein expression (Fig. 1D). In control cells exposed to scrambled transcripts (SC), the expression of both NOX mRNAs (Fig. 1D) and proteins were preserved (Fig. 1E).

**NOX/K^+ channel protein-protein associations.** To study the protein-protein association between NOX and O2-sensitive K^+ channels we used isolated cell membranes of H146 cells and a coimmunoprecipitation method with detection by multicolor Q-dot immunoblotting technique. In experiments with antibodies against K_3.3/NOX2 or K_4.3/NOX2 distinct complexes of the two proteins were observed (Fig. 2A, upper and middle). When cells were first pretreated with siRNA for NOX2, the protein band corresponding to NOX2 was absent; however, the cell membrane fraction still showed expression of the K_3.3 and K_4.3 ion channel proteins respectively (Fig. 2A, lane 3). Interestingly these protein-protein complexes appear to be weakly associated, since exposure to 0.005% Triton X-100, a nonionic surfactant, caused these complexes to dissociate (Fig. 2A, lanes 4 and 5 compared with lanes 1 and 2). In comparison, in immunoblots testing for the association between NOX2 and TASK1 channel, no complexes had formed (Fig. 2A, bottom). In contrast, while NOX4 did not associate with either K_3.3. or K_4.3 (Fig. 2B, upper and middle), it readily formed a complex with TASK1 (Fig. 2B, lanes 1 and 2, bottom). In a similar pattern to NOX2 downregulation, the down-regulation of NOX4 by siRNA did not affect TASK1 channel protein expression (Fig. 2B, lane 3, bottom). As before, exposure to 0.005% Triton X-100 led to the dissociation of the NOX4/TASK1 protein complex (Fig. 2B, lanes 4 and 5, bottom), revealing that NOX/K^+ channel interactions were relatively weak and...
easily dissociated by a low concentration of the nonionic detergent Triton X-100.

**H146 cells exposed to acute hypoxia show decrease in reactive oxygen species generation.** In agreement with previous reports (24, 28, 32), acute hypoxia (PO$_2$ ~5 mmHg) inhibited outward current in H146 cells (Fig. 3A). At a step to +30 mV, hypoxia elicited a 30.1 ± 3.6% (P < 0.05; n = 22) decrease in current density in H146 cells. During current-clamp experiments, hypoxia elicited an 8.2 ± 4.8 mV (n = 22) increase in membrane potential (Fig. 3C). No spontaneous depolarization events were found at either resting membrane potential or when stimulated with hypoxia. It was previously suggested that a decrease in reactive oxygen species (ROS) may mediate the hypoxic response in H146 cells (24). This appears to be the case since a concomitant exposure of H146 cells to hypoxia and H$_2$O$_2$ (50 μM) abolished hypoxic inhibition of outward current (Fig. 3A; n = 20). Exposure to higher concentrations of H$_2$O$_2$ resulted in either an increase in outward current beyond that seen in control or caused the loss of pipette/membrane seal (data not shown). Addition of the antioxidants N-acetyl-cysteine (NAC; 50 μM), mimicked hypoxia exposure by inhibiting outward K$^+$ current. For example in Fig. 3B, bath application of 50 μM NAC significantly reduced outward K$^+$ current at more positive potentials. The current density, at +30 mV, was reduced from a control value of 40.2 ± 5.2 pA/pF to 29.4 ± 3.8 pA/pF in the presence of 50 μM NAC (n = 21; Fig. 3B). When hypoxia and NAC were applied together (NAC+hyp; Fig. 3B), the outward current density for these cells was 28.9 ± 3.1 pA/pF, a value not significantly different from NAC alone (P > 0.05). Finally, we stimulated H146 cells with TEA (10 mM), a known blocker of several O$_2$-sensitive K$^+$ channels, as an indirect indication of ion channel function (Fig. 3D). It appears that K$^+$ channels are open at resting potential since TEA elicited a −6.4 ± 3 mV increase in membrane potential (n = 21; Fig. 3D).

---

**Fig. 3. The effects of reactive oxygen species (ROS) manipulation on outward currents in H146 cells.** Current-voltage (I-V) plot for a cell exposed to normoxic control (C), hypoxia (Hyp), or modulation of ROS. Current recordings for a voltage step to +30 mV from a holding potential of −60 mV during hypoxia are shown in the *inset*. While hypoxia caused a decrease in outward current compared with normoxic control (A), application of H$_2$O$_2$ reversed the effects of hypoxia. B: administration of N-acetyl cysteine (NAC) caused a decrease in outward current, similar to hypoxia. Coadministration of hypoxia and NAC did not result in an additive response. C: during current-clamp studies hypoxia caused a reversible increase in membrane potential in H146 cells. D: bath application of tetraethylammonium (TEA) also caused an increase membrane potential in H146 cells. E: use of catalase in the patch pipette yielded similar results to NAC. Catalase caused an inhibition of outward current that occluded the hypoxic inhibition of outward current. Wt, weight.
To obtain further evidence that H$_2$O$_2$ plays a role in mediating the hypoxic sensitivity of H146 cells we monitored outward currents using conventional whole cell recording with catalase (1,000 U/ml) in the patch pipette. Catalase is a common enzyme used to catalyze the decomposition of H$_2$O$_2$. After generating an on-cell seal, the patch membrane was ruptured. Shortly after rupture of the patch, hypoxic inhibition of outward current was similar to that seen without catalase ($n = 8$; Fig. 3, A and E). At 10 min following breakthrough, outward current in normoxia in the catalase-dialyzed cells was reduced to a level comparable to that seen during the initial hypoxic exposure (Fig. 3E). Exposure of these cells to hypoxia at 10 min postrupture did not cause any inhibition of outward current (Fig. 3E), strongly suggesting that decreased H$_2$O$_2$ is a component of the hypoxia signaling pathway. This effect of catalase was prevented following prior incubation of the pipette solution with 50 mM aminotriazole, which inactivates the enzyme by binding to the peroxide binding site (data not shown).

**NOX2 mediates hypoxia sensing in H146 cells.** To further define the O$_2$-sensing mechanism in H146 cells, we next investigated the effects of NOX2 or NOX4 downregulation (siRNA knockdown) on the hypoxia response. In NOX2-D cells, bath application of hypoxia did not decrease outward current at more positive potentials as seen in control H146 cells ($n = 22$; Fig. 4A). Similarly, during current-clamp studies no significant increase in membrane potential was observed upon exposure to hypoxia in NOX2-D cells ($n = 22$; Fig. 4B). The response to bath application of TEA in NOX2-D cells was maintained, confirming that the expression of functional O$_2$-sensitive K$^+$ channels is unaffected under this experimental condition ($n = 22$; Fig. 4C). This also suggests that at a resting membrane potential, the TEA and O$_2$-sensitive K$^+$ channels are open and functional, but fail to respond to hypoxia due to the absence of H$_2$O$_2$ generated by NOX2 (16, 24).

In contrast, NOX4-D cells remained sensitive to changes in O$_2$ since exposure to hypoxia elicited a 20.3 ± 3.2% decrease in outward current at +30 mV ($P < 0.05$; $n = 26$; Fig. 4D). However, this inhibition of outward current was significantly less compared with control native H146 cells (30.1 ± 3.6% inhibition; $P < 0.05$). Similarly, during current-clamp studies, while hypoxia caused an increase in membrane potential, this decrease was significantly less compared with control native H146 cells (Fig. 4F). During current-clamp studies (F), hypoxia was able to cause an increase in membrane potential in NOX4 cells as was bath application of TEA (F).

![Fig. 4. Hypoxia sensing is dependent on NOX2 expression. Current voltage plots for cells exposed to NOX2 or NOX4 siRNA. A: in cells deficient in NOX2, exposure to hypoxia (Hyp) did not cause a decrease in outward current compared with normoxic control (C) or wash (W). Inset depicts steps at +30 mV during hypoxia from a resting membrane potential of ~60 mV. During current-clamp studies hypoxia did not elicit any membrane depolarization (B), however, application of TEA was able to cause an increase in membrane potential (C). In contrast, cells deficient in NOX4 still exhibited a hypoxic-sensitive outward current that was reversible by wash (D). Sample of outward current in inset. During current-clamp studies (E), hypoxia was able to cause an increase in membrane potential in NOX4 cells as was bath application of TEA (F).](http://ajplung.physiology.org/)

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00170.2012 • www.ajplung.org
Depolarization was significantly less than that of control H146 cells (4.2 ± 2.1 mV in NOX4-D cells compared with 8.2 ± 4.8 mV in control H146 cells; n = 26; Fig. 4E). During current-clamp studies, exposure of NOX4-D cells to TEA caused an increase in membrane potential (n = 26; 5.8 ± 3.1 mV; Fig. 4F), which was not significantly different from control H146 cells (6.4 ± 3.2 mV; Fig. 3A).

Hypoxia-induced 5-HT release. We used carbon fiber amperometry to quantify hypoxia-induced 5-HT release from H146 cells to further examine the role of NOX enzymes in O2-sensing. As shown in Fig. 5A, acute hypoxia stimulated quantal release of 5-HT from H146 cells, as did exposure to the depolarizing stimulus, using high extracellular K+ (30 mM; Fig. 5A). The secretion of 5-HT was inhibited by the general blocker of voltage-gated Ca2+ channels, cadmium (Cd2+; 100 μM), suggesting that the voltage-gated Ca2+ channels play a key role in mediating secretory events in H146 cells (Fig. 5A). In NOX2-D cells, the hypoxic response was significantly reduced compared with normoxic control cells. While in control H146 cells, hypoxia-induced secretory events were 27.3 ± 6.4 events/min (n = 52; Fig. 5A), in NOX2-D cells, secretory events averaged 13.9 ± 4.8 events/min (n = 47), not significantly different from that of unstimulated control cells at 12.1 ± 5.9 events/min (Fig. 5B). In NOX4-D cells, the hypoxic response, although still present, was significantly less compared with control H146 cells [22.1 ± 4.2 events/min (Fig. 5C)]. Bath application of Cd2+ abolished hypoxia-induced 5-HT secretion in both normal control H146 cells, as well as in NOX2-D or NOX4-D cells.
NOX4-D cells. In all three cell lines, stimulation via 30 mM K+ elicited a robust secretory response, indicating that it was independent of NOX expression (Figs. 5, A–C).

**DISCUSSION**

Our data support the theory that the combination of NOX2/Kv is a predominant sensor in the H146 cell line, representative of human pulmonary neuroendocrine chemoreceptor cells. Gene profiling of both native NEB and H146 cells have shown expression of a wide range of mRNAs encoding NOX and O2-sensitive K+ channel proteins (10). Expression of these proteins and their mRNAs in native NEB and H146 cells is significantly different from that of adjacent airway epithelial cells that do not mount a hypoxic response (10). Although the O2-sensing mechanism by NOX enzymes is not completely understood, our studies suggest that the NOX2/O2-sensitive K+ channel (e.g., K,3.3) complexes may play a key role. Based on our studies, this model suggests that during hypoxia, a decrease in ROS production by NOX2 results in a decrease in outward current (closure of K+ channels) as also postulated by O’Kelly et al. (24). The subsequent closure of K+ channels leads to membrane depolarization, activation of voltage-gated Ca2+ channels and subsequent 5-HT release. The effects of released 5-HT likely include hypoxia-chemotransmission via vagal afferents innervating NEB or local effects with alterations in vascular blood flow to better ventilated portions of the lung (11, 35).

**Evidence for NOX2 as the predominant hypoxia sensor.** Our coimmunoprecipitation studies demonstrated that in H146 cells, NOX2 but not NOX4 formed molecular complexes with O2-sensitive K+ channel subunits that have been previously demonstrated to play a key role in mediating the hypoxic response in NEB cells (10, 37). In contrast, TASK1 was associated with NOX4 but not with NOX2. Our conclusion that NOX2 is the principal O2-sensor in human airway chemoreceptor cell line, and by inference in NEBs, is based on a number of observations. First, our previous immunolocalization studies have shown that these complexes colocalize to the plasma membrane as would be expected for an airway based sensor (10). Second, using siRNA, we demonstrate that NOX2 siRNA knockdown significantly reduced the hypoxic-response in H146 cells. The use of the siRNA approach provides a more specific means to study the O2-sensing mechanism. This is in contrast to the use of blockers of NADPH oxidase activity, such as diphenylene iodonium, which may have nonspecific effects in addition to being unable to preferentially alter activity of specific NOX isoforms. It should be noted that the specificity of diphenylene iodonium and its usefulness for studies on O2-sensing has been questioned since this compound also acts as a nonselective ion channel inhibitor in both pulmonary artery smooth muscle cells and carotid body glomus cells respectively (38, 39). This decrease of NOX2 expression affected the hypoxic response at the K+ channel level via loss of the mediating factor (presumably H2O2). In siRNA treated cells, expression of K+ channel was retained in addition to their being responsive to TEA (Fig. 5) and to changes in exogenous ROS modulation (data not shown). Using carbon fiber amperometry we were able to detect quantal release of 5-HT from H146 cells in response to hypoxia or depolarizing stimulus. In cells deficient in NOX2 mRNA, while hypoxia-induced 5-HT release was absent, the cells were able to release 5-HT in response to a depolarizing stimulus (30 mM K+) suggesting the secretory machinery was still intact in NOX2-D cells.

In contrast to the association between NOX2 and K,3.3/ K,4.3 ion channels, NOX4 was associated with TASK1. In siRNA knockdown studies, it appeared that NOX4-D cells retained a residual hypoxic response, although it was less robust compared with control H146 cells. According to our molecular studies, the decreased sensitivity to hypoxia in NOX4-D cells, compared with H146 cells, is unlikely due to a decrease in K+ channel expression, since the K+ current density profile was unaffected. It is therefore possible that the difference in hypoxic responses between these two cell models relates to reduced NOX4 activity, which may play a secondary role in the hypoxia-sensing mechanism in these cells. We conclude that NOX2 is the predominant O2-sensor in NEBs and related tumor cell model and that NOX4 may play a minor role in hypoxia sensing.

**Role of NOX4 and TASK.** Our finding of an association of NOX4 with TASK1 channel is in agreement with recent studies by Lee et al. (22) showing that in HEK-293 cells, a renal cell carcinoma model that endogenously expresses NOX4, the activity of transfected TASK1 was inhibited by hypoxia. This hypoxia response was significantly augmented by cotransfection with NOX4, but not with NOX2/gp91phox. The O2-sensitivity of TASK1 was abolished by NOX4 siRNA and NADPH inhibitors, suggesting that NOX4 may represent the O2-sensor protein partner for TASK1 in this cell type. Other related studies suggest that it is O2 binding with the heme moiety of NOX4, that controls TASK1 activity and that the NADPH oxidase membrane subunit, gp22phox, might support the NOX4-TASK1 interaction. These observations, together with our findings, suggest a diversity of O2 sensors, even within the same cell type, matching specific NOX proteins with particular O2-sensitive K+ channel types (i.e., NOX2/ K,3.3; NOX4/ TASK1). It is therefore possible that NOX4/TASK1 molecular complex may constitute a secondary sensor that potentiates the activity of the NOX2/ K+-based sensor during hypoxia, or alternatively, NOX4/TASK1 molecular complex may be involved during asphyxial (hypoxia/ hypercapnia/acidosis) responses. Further studies are required to define the precise role of NOX4 in airway chemoreceptors.

**The hypoxic response is mediated via a decrease in ROS.** In other similar hypoxia-sensing systems, it has been demonstrated that exposure to reduced O2-levels leads to a decrease in ROS production, and this in turn triggers membrane depolarization and secretory events (5, 24, 32, 34). However, the precise nature and the location of the O2-sensor may differ. For example, in neonatal adrenal chromaffin cells, it has been suggested that complex I of the mitochondrial electron transport chain plays a key role in mediating the hypoxic response in these cells (5, 34). On the other hand, unlike neonatal adrenal chromaffin cells, it has been previously demonstrated that functional mitochondria are not required for O2-sensing in H146 cells (32). Related studies have also, indirectly, suggested that a decrease in ROS production may play a key role in mediating the hypoxia response (24). Here we show that by manipulating exogenous ROS levels, a decrease in ROS mediates the hypoxia response in H146 cells.
Thus our studies suggest that in H146 cells, and by inference in pulmonary NEBs, the hypoxia response is mediated predominantly via the activity of NOX2 proteins that produce ROS depending on ambient O2 level. The evidence for ROS/H2O2 modulation of K+ channels is derived from studies on voltage-activated K+ channels (Kv), where H2O2 is thought to act as a second messenger (36). The common feature of channels affected by H2O2 is a cysteine residue in the amino terminus shown to be highly redox sensitive (30). This amino acid terminus is believed to be intracellular and contains a site responsible for channel inactivation acting as a tethered ball and chain, which occludes the internal mouth of the channel. An O2 sensor model for NEBs, proposed by Patel and Honore (27a) incorporates our earlier model, where H2O2 producing NADPH oxidase is closely associated with O2-sensitive K+ channel (37). Similarly, BK channels contain a specific motif that confers sensitivity to ROS, similar to the large-conductance voltage and Ca2+-sensitive K+ channel (BK channel). The BK channel is known to play a role in mediating the hypoxic response in neonatal adrenomedullary chromaffin cells, likely via ROS modulation. Also part of the internal structural motif of the BK channel is known to be sensitive to changes in ROS (26, 33). Whether this is the case with the expressed K+ channels in H146 and other NEB cell related tumor cell lines remains to be elucidated.

ACKNOWLEDGMENT
Present address of J. Buttigieg: Department of Biology, University of Regina, Regina, Saskatchewan, Canada.

GRANTS
This work was supported by Grants MOP-12742 and MPG-15270 (to E. Cutz and H. Yeger) from Canadian Institute for Health Research.

DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: J.B. and J.P. performed experiments; J.B. and J.P. analyzed data; J.B. and E.C. interpreted results of experiments; J.B. and J.P. prepared figures; J.B. drafted manuscript; J.B. and E.C. edited and revised manuscript; H.Y. and E.C. conceived and designed of research; H.Y. and E.C. approved final version of manuscript.

REFERENCES


