PULMONARY NEUROEPITHELIAL BODIES
AS AIRWAY OXYGEN CHEMORECEPTORS

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate department of Laboratory Medicine and Pathobiology
University of Toronto

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Pulmonary Neuroepithelial Bodies as Lung Oxygen Chemoreceptors

Masters of Science, 2000

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ABSTRACT

We conducted respiratory measurements under normoxic and hypoxic conditions in neonatal knock out mice with non-functional NADPH oxidase. Five-day-old OD mice were faster and shallower breathers during normoxia as well as hypoxia and their maximum hypoxic ventilatory response was lower compared to the WT control group. The observed differences in ventilatory responses implicate NADPH oxidase as an O₂ sensor involved in neonatal ventilatory control, possibly modulated via pulmonary NEBs. We also assessed respiratory responses to drug-induced inhibition of NEB cell oxygen sensory mechanism by administering NADPH oxidase blocker into the airways under hypoxic or normoxic conditions before and after bilateral vagotomy in newborn rabbits. No apparent effects were observed possibly due to low dose and limited access of the drug to NEBs through the lungs. These studies provide preliminary evidence on a whole animal level for the involvement of NEBs as airway oxygen sensors in the control of respiration.
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Pulmonary Neuroepithelial Bodies: Morphology, histology, anatomy, development and alteration in association with disease

Pulmonary neuroendocrine cells (PNEC) are a group of morphologically and histochemically distinct group of cells that are distributed throughout the tracheobronchial epithelium. A subgroup of PNECs that are restricted to the intrapulmonary airways and form innervated clusters of usually 3 to 20 cells, are called Neuroepithelial Bodies (NEB), a term coined by Lauweryns and Peuskens almost 30 years ago (Fig. 1) [1]. Several authors have reviewed the morphology, development and functional studies of NEBs [2-4]. Originally believed to be derived from the neural crest, the bulk of evidence now suggests that NEBs differentiate from pluripotent stem cells of endodermal origin [5]. In addition to a variety of mammals including humans [1,6], PNECs have also been described in the gas exchange organs of the amphibians [7-9], reptiles [10], birds [11] and fish [12,13]. Since NEBs have been conserved during evolution, it is believed that they play an important role in the respiratory organs. It should be mentioned that different species have somewhat different NEB morphology. In frog, for example, NEBs are not directly exposed to the airway lumen and are covered by a thin cytoplasmic process from the surrounding non-endocrine cells [9] while in the mouse and rat, NEBs protrude into the airway lumen [14].

Histologically, NEBs are characterized by a clear cytoplasm with normal H&E staining, and hence their original nomenclature as clear cells or “Helle Zellen”. In fact “Helle-Zellen” was the name used by F. Feyrter, an Austrian pathologist who first described the presence of these cells, over 50 years ago [15]. They are also strongly argyrophilic when stained with silver-containing compounds. Another morphological characteristic of the NEB cells is the presence of numerous cytoplasmic dense core vesicles (DCV) usually at their basal pole [16]. Based on these morphological characteristics, respectively, these cells are also referred to as argyrophilic cells [6] and small granule cells [17]. The NEB morphology at the
luminal pole is varied among different species and also changes during different stages of life; however, in human, NEBs are directly exposed to the lumen of the airways surrounded by non-ciliated Clara cells [18]. Moreover, in most cases especially in fetal stages, they are more prominently located at the airway branch points [19] as, for example, in fetal rabbits, 60% of NEBs were located at or near airway branch points [20] (Fig. 3).

As a member of the APUD (amine precursor uptake and decarboxylation) cell family, NEBs contain numerous biologically active amine and peptides, which are widely used as neuroendocrine markers (Table 1). Once again, the expression of each of these markers is varied among different species and different developmental stages. For instance, neural cell adhesion molecule (NCAM)–a membrane protein involved in cell-cell adhesion within the central and peripheral nervous systems–was demonstrated to be present in NEB cells of fetal and neonatal cat lung but was absent in the mature animal [21]. In NEB cells, the most predominant biogenic amine is serotonin (5-HT), which is detectable by immunohistochemical methods. Its level peaks at the fetal stages and decreases postnatally [20]. Serotonin is released from NEBs upon exposure to hypoxia both in vivo [22] and in vitro [23]. Since serotonin has several pharmacological activities in the lung including vasoconstriction and increased vascular permeability in addition to its role as a neurotransmitter, NEBs may be involved in neurotransmitter-mediated neural or local paracrine modulation of ventilation/perfusion ratio in the lung. Several immunomarkers have been identified that are restricted to the plasma membrane of the NEB cells including Leu7/NHK (CD57) [24] and MOC-1 (monoclonal-1) antigen, which belongs to a class of neural adhesion molecules (NCAM). A murine antibody raised against MOC-1 has been shown to react specifically with rabbit fetal NEBs and human PNEC cultures which has been successfully used for separation of NEB cells through immunomagnetic separation method [25]. Other important NEB cell membrane proteins are the membrane-associated domains of the NADPH oxidase complex, namely gp91phox and p22phox, which are thought to function as the O2 sensor protein in NEB cells and will be discussed in more detail [26].
One interesting characteristic of NEB cells is their innervation by two types of nerve endings, namely afferent-like (sensory) and efferent-like (motor) endings as was demonstrated ultrastructurally in rabbit fetuses and neonates [16]. In rabbits, it has been shown that the sensory axons originate from the nodose ganglion and travel via the vagus nerve [27]. This is suggestive of a possible role NEBs might play in the control of ventilation by being a sensory and/or effector organ as a part of a central or local neural reflex.

As mentioned previously, distribution and frequency of NEBs change during different stages of life. In general, the highest densities of NEBs are found at birth, especially in species with very immature neonates [28]. The first appearance of NEB cells occurs around day 21 in the rabbit fetus and between gestational weeks 10 and 12 in human [29]. In human, rabbit and hamster lungs, it has been shown that the relative frequency of NEBs peaks at birth and declines afterward [29, 30]. In experiments done in rabbits, it was shown that the size of NEBs peaks at day 11 postnatally [20]. Since NEB differentiation precedes the appearance of other pulmonary epithelial cells, NEBs may play an important role in the initiation and control of lung morphogenesis [29]. A study of mouse model of naphthalene-induced airway injury, characterized by extensive airway epithelial damage and particularly Clara cell ablation, has suggested that NEBs are a reservoir of pollutant-resistant progenitor cells that respond to depletion of airway progenitors such as Clara cells [31].

There are several clinical situations that have been associated with changes in NEB morphology or frequency. Among these conditions are meconium aspiration with persistent fetal circulation [32], apnea of prematurity, Sudden Infant Death Syndrome (SIDS) [33], and Congenital Central Hypoventilation Syndrome [34] where NEBs appear abnormally enlarged. There is an increase in the number of PNECs in children with bronchopulmonary dysplasia [35], victims of SIDS [33], asthma [36] and cystic fibrosis [37]. In some other pediatric disorders such as hyaline membrane disease, a reduction in PNEC/NEBs has been reported [35]. Similar changes have been associated with adult pulmonary diseases such as pneumonitis [38], chronic obstructive pulmonary diseases [39] and bronchial
asthma [36] and smoke-related lung disorders [40]. It has also been shown that maternal smoking potentiates hyperplasia of the PNEC system in the lungs of infants who die of SIDS and a dysfunction of these cells may contribute to the pathophysiology of SIDS [41]. Furthermore, small-cell lung carcinoma, the most common malignant form of lung cancer, is believed to derive from PNECs [42, 43].

**Control of ventilation and NEBs**

**Control of ventilation, an overview**

In spite of a constantly varying demand for $O_2$ uptake and $CO_2$ output by the body, partial pressures of arterial gases are normally kept within remarkably close limits. The oxygen homeostasis is achieved because of an elaborate control system that governs ventilatory functions. Control of respiration is based on feedback and feedforward mechanisms involving central and peripheral components that sense or predict changes in supply and demand for oxygen (Fig. 4). Like other control mechanisms, respiratory control system is composed of three major elements: respiratory centers, sensors and effectors.

**Respiratory centers**

Anatomically, respiratory centers are located in the medulla and pons regions of the brain. They can be divided into three major groups: medullary respiratory center, apneustic center and pneumotaxic center. Medullary respiratory center consists of dorsal and ventral respiratory groups that are associated with inspiration and expiration, respectively [44]. Since expiration is generally achieved by passive relaxation of respiratory muscles, ventral respiratory groups are generally silent during normal breathing and are only activated during rigorous breathing. The intrinsic respiratory pattern generator is widely believed to be located in the dorsal respiratory group. The respiratory rhythm is continuously modulated by inputs from other brain centers such as nuclei of tractus solitarius, which are located in the region of dorsal respiratory group and receive impulses from vagus and glossopharyngeal nerves [45].
Apneustic center is located in the lower pons. Impulses from this center have excitatory effects on dorsal respiratory group and therefore prolong the inspiratory cycle. Its function in normal breathing is still unknown [46]. Pneumotaxic center is located in the upper pons. Activation of this center inhibits inspiration and regulates inspiratory volume and respiratory rate. The destruction of pneumotaxic center does not disrupt the normal respiratory rhythm and therefore it is believed that its function is the fine tuning of respiratory pattern, for example in setting the end expiratory lung volume.

The afferent or sensory part of the respiratory control system is composed of central chemoreceptors, arterial (peripheral) chemoreceptors and lung receptors. There are other receptors that have minor contribution to the ventilatory control system, such as arterial baroreceptors that are activated by increased blood pressure and cause hypoventilation or apnea and gamma system of the intercostal and diaphragm muscle which sense the strength of contraction of respiratory muscle.

**Central and arterial chemoreceptors**

Central chemoreceptors are located in the ventral surface of the medulla in the vicinity of nuclei of ventral respiratory group. They can be activated by increased $P_{CO_2}$ or $[H^+]$ in local blood circulation or cerebrospinal fluid (CSF). Central chemoreceptors account for more than 80% of the ventilatory response due to increased arterial $P_{CO_2}$ [47]. Arterial chemoreceptors are located in the carotid body at the bifurcation of the common carotid artery, and in the aortic bodies around the aortic arch. Carotid bodies are the principal $O_2$ censor and their afferent signal travels via the carotid sinus nerve to the pertosal ganglion of the glossopharyngeal nerve. At the cellular level carotid bodies are composed of type I cells (glomus cells), which contain various neurotransmitters, including dopamine and serotonin, and type II cells (sheath cells). The branches of the carotid sinus nerve are synaptically connected to the glomus cells that are believed to be oxygen chemoreceptors. At least 5% of the nerve endings on the glomus cells are efferent, which modulate
chemoreceptor sensitivity, and originate from the superior cervical ganglion. Both carotid and aortic bodies respond to reduced arterial $P_{O_2}$ and pH and increased arterial $P_{CO_2}$. Although carotid bodies are extremely sensitive to changes in arterial oxygen partial pressure, their response to hypoxemia is non-linear and a steep increase in activity starts only when arterial $P_{O_2}$ drops below 50 mmHg. Therefore, under normoxic conditions, arterial $O_2$ sensors account for only a small part of the chemical drive for breathing ($\approx 15\%$ in awake animals) [48].

**Pulmonary receptors**

A large number of different types of receptors have been identified in the lungs that are sensitive to a variety of physical and chemical stimuli [49]. Although a small portion of them might have connection with the sympathetic nervous system, afferents from almost all of these receptors are conducted via the vagus nerve. There are three major lung receptors whose physiological functions have been identified so far: pulmonary stretch receptors (slowly adapting receptors), C-fiber endings (J receptors) and irritant receptors (rapidly adapting receptors) [50].

Pulmonary stretch receptors are believed to lie within airway smooth muscle and their impulses travel via large myelinated fibers in vagus nerve [51, 52]. They respond with a slowly adapting and regular discharge to sustained stimulus [53]. The inflation reflex (Hering-Breuer reflex), deflation reflex and Head’s paradoxical reflex are known to arise from pulmonary stretch receptors. The more important Hering-Breuer reflex consists of inhibition of inspiration in response to sustained inflation of the lung [54].

C-fiber endings or Paintal’s juxtapulmonary capillary receptors (J receptors) are believed to be localized within alveolar wall in close proximity to the pulmonary capillaries. Afferents travel via nonmyelinated fibers in vagus nerve, which comprise the vast majority of vagal afferents [55]. Unlike other lung receptors, signals from nonmyelinated endings do not show any clear correlation with the respiratory movement. Perineural capsaicin treatment of the vagus nerve, which selectively blocks C-fibers, does not result in significant alteration of breathing
pattern [56], whereas stimulation of C-fibers result in increased pharyngeal constrictor activity [57] and apnea followed by rapid, shallow breathing [58]. J receptors appear to be nociceptive and are activated by congestion of pulmonary capillaries, increase in alveolar interstitial fluid volume, tissue damage and release of various mediators. Irritant receptors (rapidly adapting receptors) are thought to lie in the epithelial layer of the airways. They respond with a rapidly adapting, irregular response to a maintained stimulus [53]. Irritant receptors are stimulated by inhalation of noxious gases, smoke, dust and cold air. Their signal is transmitted via myelinated fibers of vagus nerve. Their stimulation causes bronchoconstriction and hyperpnea [59].

The identification of lung receptors has been based on physiological studies and their morphological counterparts have not yet been identified. As will be explained in the next section, pulmonary NEBs have morphological characteristics that make them a good candidate for lung receptors. Although our present study is focused on the oxygen sensing properties of NEBs, their possible involvement as chemo-, mechano- or irritant receptors has not yet been investigated.

**NEBs as pulmonary oxygen sensors**

There are many similarities between the morphology of NEB cells and other oxygen sensing cells such as the glomus cells of the carotid bodies that strongly suggest a similar functionality between the two groups of cells. The morphological characteristics that support an airway oxygen receptor function for NEBs are innervation, location and neurotransmitter content. Moreover, there has been many in vivo and vitro studies that directly or indirectly support this hypothesis.

The association of pulmonary neuroepithelial bodies with nerve fibers has long suggested a receptor role for these cells [1]. As far back as 1949, Frohlich, who first described the innervation of NEBs, suggested a sensory receptor function for NEBs that monitor certain chemical changes in the lung and initiate a respiratory
reflex via neural connections [60]. The ultrastructural studies of synaptic contacts between NEB cells and terminal branches of vagus suggest that NEB cells are the presynaptic element of the neuro-receptor complex [61]. The study of nerve fiber degeneration after vagotomy in rabbit has demonstrated that more that 70% of nerve endings are sensory that originated form cell bodies in nodose ganglia [62]. More recently, with the use of anterograde tracing with Dil (1,1'-dioleyl-3.3.3’.3’-tetramethylindo-carbocyanine methanosulfonate) and confocal microscopy, it has been confirmed that, in rats, almost all of the intrapulmonary sensory branches of vagus that originate from nodose ganglion are associated with NEB cells [63]. An intact innervation is essential for hypoxia-induced degranulation of NEB cell. However, short-term infranodose and long-term supranodose vagotomy that disrupt NEBs connection to CNS without resulting in degeneration of intracorpuscular innervation does not affect hypoxia-induced degranulation of NEBs [27, 62, 64, 65]. This demonstrates that what is essential for an intact NEB hypoxic responsiveness is an intact local synaptic connection and not necessarily the connection to CNS.

Similar to glomus cells of carotid bodies, NEB cells contain different neurotransmitters stored within dense core vesicles that are released upon exposure to hypoxia [18, 23]. In a cross-circulation study, Lauweryns et al. (1978) have shown that only airway hypoxia, and not hypoxemia, stimulated NEBs. In a unilateral lung hypoxia experiment in rabbits, only the hypoxic lung (right lung) exhibited an increased exocytosis and a lower serotonin content in comparison with the NEBs in the left lung. Therefore the hypoxia-induced degranulation of NEBs was caused only by exposure to hypoxic air and that their activation was a local phenomenon that was not propagated to other NEBs by blood steam or neural connections [66].

Morphological studies have shown that NEBs are predominantly located at or near large airway bifurcation sites [20, 29, 67]. This location may be ideal for rapid sensing of changes of lung gases and provides additional evidence favoring a chemoreceptor function for NEBs.
Analysis of NEB cells in culture has offered the most direct evidence in support of NEB chemoreceptor hypothesis. Using fetal rabbit lung cultures, it was shown that exposure of NEB cells to hypoxia caused depletion of serotonin content of the cells and the extent of 5-HT depletion was in direct correlation with the duration and severity of hypoxia [23]. Using whole-cell patch clamp analysis in rabbit NEB cultures, Youngson et al. (1993) demonstrated that the membrane of NEB cells possess voltage-activated potassium, calcium and sodium currents and that hypoxia reversibly reduced the outward potassium current without altering the inward currents. Similar hypoxia-sensitive K⁺ currents have been shown in NEB cells in fresh lung slices from neonatal rabbit [68] and mice [69] as well as in small-cell lung carcinoma cell lines (H-146) [70] that are believed to derive from NEB cells [43].

In summary, it is evident that pulmonary NEBs are hypoxia sensitive cell clusters and that intact intrapulmonary vagal innervation is essential for their local and/or systemic role in the control of respiration. Based on the developmental changes in the distribution and frequency, which shows a prominence during fetal and neonatal periods followed by a decline with increasing age, NEBs could play a more important role in the control of breathing during neonatal adaptation.

**Molecular mechanisms of oxygen sensing in NEBs**

Although the physiological responses to hypoxia at cellular and molecular levels have been extensively studied, the precise transduction mechanisms are still a matter of debate. In general, there are two competing views with regard to the location of the oxygen sensor namely, cell membrane and intracellular. The intracellular model for O₂ sensors propose mitochondrial respiratory chain proteins [71] and HIF-1 (Hypoxia Inducible Factor-1) [72]. The so-called "membrane model", is represented by a hypoxia sensitive ion channel linked to a membrane-bound NADPH oxidase [71]. The presence of heme-like protein of NADPH oxidase has been identified in the carotid body type 1 cells [73], pulmonary neuroepithelial bodies [74], phagocytes, endothelial cells [75], and pulmonary artery smooth muscle cells [76].
NADPH oxidase is a multi-component enzyme consisting of at least 6 subunits: membrane-bound cytochrome b$_{558}$ (a heterodimer comprised of gp91$^{phox}$ and p22$^{phox}$), and cytosolic p47$^{phox}$, p67$^{phox}$, p40$^{phox}$, rac, Rap1A and Rap 2 (Fig. 5). This complex accepts an electron at the cytosolic side of the membrane and reduces a molecule of oxygen at the extracellular side to produce superoxide anion. In phagocytic cells, the superoxide is subsequently converted to hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HClO) and other microbicidal products [77]. Using in situ hybridization method and Northern blot analysis, the presence and expression of the mRNA encoding gp91$^{phox}$ and p22$^{phox}$ proteins have been documented in NEB cells of fetal rabbit and neonatal human lung while the adjacent cells did not show any reactivity [74]. Using microfluorometry with dihydrorhodamine 123 as a molecular probe for H$_2$O$_2$ generation, NADPH oxidase in NEB cells, unlike neutrophils, has been shown to exhibit basal activity which could be altered by known oxidase inhibitor (diphenyleneiodonium; DPI) and stimulator (phorbol 12-myristate 13-acetate) [74]. Moreover, administration of DPI, an inhibitor of NADPH oxidase, caused a reduction in membrane K$_+^+$ current in NEB cell cultures that was similar in magnitude to that observed in hypoxia and rendered them unresponsive to superimposed hypoxia [68, 78].

Functional production of oxygen radicals is hypothesized to initiate transduction of hypoxia stimulus via modulation of a redox-sensitive K$_+^+$ channel in pulmonary NEB cells (Fig. 6) [78]. Patch-clamp studies in our laboratory have demonstrated that intact NEBs in lung slice preparations from mice lacking the gp91phox subunit of NADPH oxidase, known as oxidase deficient (OD) or X-CGD (X-linked Chronic Granulomatous Disease) mice, with disrupted oxidase function, fail to respond to hypoxia [69]. These studies provide strong evidence indicating that the dysfunction of NADPH oxidase disrupts the O$_2$ sensing capability of the NEBs.
Fig. 1: NEB (arrow) and solitary PNEC (arrowheads) within airway epithelium of fetal rabbit lung (26 day gestation) using immunohistochemical staining for serotonin (5-hydroxytryptamine, 5-HT) (×99). At higher magnification (inset), NEB cells are extending into the airway lumen (arrowhead) (×231).
Fig. 2: Transmission electron micrograph of NEB cells (×2400) from neonatal rabbit lung. NEB cells contain numerous cytoplasmic dense core vesicles (DCVs) (arrowheads). Penetrating between NEB cells are afferent-like nerve terminals (arrows) with an abundance of mitochondria. Electron micrograph of a neuroepithelial body consisting of more than 80 neuroepithelial cells (white arrowheads) obtained from fetal rabbit lung (×750). At the basal side, NEBs are limited to the basement membrane (arrow). At the apical surface, which is exposed to airway lumen, they contain microvilli (black arrowhead) and are surrounded by Clara cells (CL).
Fig. 3: Distribution map of NEBs obtained from 20 serial sections immunostained for serotonin in a 6 day old (A) and 56 day old (B) rabbit lung. The NEBs (solid dots) are more numerous at day 6 of age and are mostly localized around the point of airway bifurcation. (Cho et al., Cell Tissue Res 1989, 255:353)
Table 1: Immunohistochemical and molecular markers of NEB cells in Mammalian lung [1].

<table>
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<tr>
<th>Amine</th>
<th>Amine Metabolizing Enzymes</th>
<th>Regulatory Peptides</th>
<th>Neuroendocrine Markers</th>
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<tr>
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<td>Aromatic amino acid decarboxylase [82]</td>
<td>Calcitonin [84]</td>
<td>Protein gene product 9.5 [82]</td>
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<td>Calcitonin gene related peptide [85]</td>
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<td>Calbindin-D28K (Calcium Binding Protein) [86]</td>
<td>Synaptophysin [93]</td>
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<td>Cholecystokinin [87]</td>
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Fig. 4: Schematic representation of three elements of the respiratory control system: respiratory centers, sensors and effectors. The output of the respiratory rhythm generator is constantly influenced by the feedback and feedforward signals from peripheral receptors and central brain centers. (modified from Feldman, 1995)
Mechano- & Chemoreception

Respiratory Center

Vagus N.

Glossopharyngeal N.

Arterial Chemoreception

Blood PO2, PCO2 & pH

Ventilation

Pump Motoneurons (Cervical)

Resistance Motoneurons (Cranial)

Airway Muscles

Pump Muscles (Diaphragm, Intercostal, Abdominal) (Cervical)

Bronchial Smooth Muscles

NEB

Mechanoreceptors, Irritant receptors, ...

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Fig. 5: The structure of NADPH oxidase in neutrophils. It is composed of membrane (Rap1A, p22phox and gp91phox) and cytosolic (p40phox, p47phox, P67phox and Rac2) components. Rac2 and Rap1A are G-proteins and function in other cell processes beside oxidase activation. Upon stimulation, p47phox becomes phosphorylated which result in the assembly of membrane and cytosolic components and activation of NADPH oxidase [94].
Fig. 6: Schematic representation of the proposed O2 transduction pathway in NEB cells. NADPH oxidase is the first step in the transduction cascade and is responsible for reducing O$_2$ to O$_2^-$, which in turn produce other reduced species such as hydrogen peroxide (1). Disruption of NADPH oxidase function would mimic hypoxic or anoxic conditions with reduced production of O$_2^-$ from O2 molecules. Decrease in the production of reduced species such as O$_2^-$ would result in the closure of O$_2$-sensitive K$^+$ channels thereby suppressing the outward K$^+$ current (2), leading to membrane depolarization (3), and increased influx of Ca$^{2+}$ (through voltage-gated Ca$^{2+}$ channels (4)). Increased intracellular Ca$^{2+}$ concentration (5) in turn causes release of neurotransmitters (such as 5-HT) into the synaptic cleft (6). The signal will be transmitted via afferent nerve endings, originating from vagus nerve, to central respiratory centers (modified from Gonzalez et al., Trend Neurosci 1992; 15:146).
HYPOTHESIS

The general hypothesis postulates that NEBs are airway O2 sensors involved in the control of breathing. Specifically, we hypothesize that inactivation or inhibition of hypoxic transduction mechanism in pulmonary NEBs by alteration (i.e. dysfunction or inhibition) of membrane NADPH oxidase will affect the normoxic and/or acute hypoxic ventilatory response during neonatal period.

OBJECTIVES AND AIMS

The present work had two major objectives:

1. The mechanism of oxygen sensing is modulated at the plasma membrane level through the oxygen sensor protein, NADPH oxidase.
2. Alteration (i.e. dysfunction or inhibition) of specific components of the O2 sensor molecular complex induce functional changes in airway O2 sensor demonstrable in relevant animal model during the perinatal period.

The aims of this study were as follow:

1. To develop a system for measurement of respiratory parameters in small animals to allow the study of ventilatory control mechanisms in desired animal models, i.e. knock-out mice.
2. To assess possible effects of NADPH oxidase deficiency on the control of ventilation at the whole animal level by obtaining respiratory measurements under normoxic and hypoxic conditions in neonatal oxidase deficient mice (OD or X-CGD mice, a knock-out mouse model lacking functional NADPH oxidase) and comparing that with the wild-type (WT) control group.
3. To characterize the possible effects of NADPH oxidase deficiency on NEB frequency or distribution in OD mice.
4. To test whether inhibition of NEB function by administration of NADPH oxidase blocker (DPI) into the airways of neonatal rabbits affects respiration.
CHAPTER 1: RESPIRATORY CONTROL IN NEONATAL MICE WITH NADPH OXIDASE DEFICIENCY

We hypothesized that the OD mice with altered NADPH oxidase function, and therefore abnormal NEB responses, would have hypoxic and/or normoxic ventilatory parameters that differ from those of the control WT mice. To assess the effects of oxidase deficiency on the control of ventilation at the whole animal level, we conducted respiratory measurements under normoxic and hypoxic conditions in neonatal OD mice and compared that with the WT control group.

Oxidase deficient (OD) or X-CGD mice

OD mice were originally developed to serve as an animal model for the study of a disease called X-linked chronic granulomatous disease (X-CGD or CGD, for short). CGD is a group of recessive inherited disorders, which result from mutation in any one of the four subunits of the respiratory burst oxidase (NADPH oxidase) [95]. In 2/3 of the patients, the mutations have been located in the gp91phox subunit of the enzyme [96]. Due to lack of membrane burst oxidase activity in phagocytic leukocytes and hence microbicidal activity, CGD patients are susceptible to recurrent suppurative microbial infection and chronic inflammation with granuloma formation [97].

Gene targeting technique was used to generate OD mice with a null allele for gp91phox protein located on X chromosome. The targeting vector contained an expression cassette for neomycin-resistance gene, which was inserted into the third exon of gp91phox using electroporation method. The original founder mice were derived from a cross between E129 and C57 BL/6 strains. Using immunoblot analysis, it was shown that cells obtained from OD mice had no detectable gp91phox protein [98]. Respiratory burst oxidase activity, as measured by nitroblue tetrazolium (NBT) test, was also absent in OD mice. OD mice display
no apparent phenotypic abnormality but they have an increased susceptibility to bacterial and fungal infections such as *Staphylococcus aureus*, *Listeria monocytogen* [99] and *Aspergillus fumigatus* [98]. OD mice have been used for the study of several other pathologic conditions including ischemic stroke, complement induced lung injury and neuronal apoptosis.

In an animal model for the study of brain ischemia-reperfusion injury in which cerebral arteries of animals were transiently occluded, OD mice showed a significantly lower brain infarct volume compared to wild-type control group [100]. This result suggested a pivotal role for the NADPH oxidase in the pathogenesis of brain strokes.

Production of reactive oxygen species (ROS) has been implicated in sympathetic neuron apoptosis [101]. NGF-deprived sympathetic nerve cell cultures from OD mice had considerably lower frequency of apoptosis than the control culture and the rate of apoptosis was similar to NGF-maintained neurons [102]. These results strongly implicated NADPH oxidase as contributing to neuronal apoptosis.

OD mouse model has also been used to study the role of reactive oxygen species in the pathogenesis of lung injury. In a complement-induced lung injury model created by intravenous infusion of cobra venom factor (CVF), the production of oxygen radicals, particularly $\text{H}_2\text{O}_2$ generated form $\text{O}_2^\cdot$, was shown to be an essential factor in the creation of lung injury in WT mice, as was demonstrated by the protective effects of catalase. However, after CVF injection, OD mice developed a catalase-insensitive form of lung injury that was otherwise similar to that of the WT mice. These observations indicated that, in OD mice, oxygen radical production and lung injury in response to injection of CVF occurred through NADPH oxidase-independent pathways [103].

In oxygen responsive cells such as pulmonary artery smooth muscle cells (PASMC), type I cells of the carotid bodies and pulmonary NEBs, hypoxia causes the closure of membrane $K^+$ channels resulting in membrane depolarization and increase in intracellular $\text{Ca}^{2+}$ concentration [71, 78, 104]. It has been postulated that inhibition of $K^+$ channels is mediated by hypoxia-induced changes in ROS
production by NADPH oxidase. Therefore, OD mice have been used in several in vitro studies regarding hypoxia transduction in these cells. In an isolated mouse lung model, it was shown that OD mice produced significantly less ROS than wild-type mice. Despite the virtual absence of ROS in OD mice, PASMCs from OD mice had intact hypoxia-sensitive membrane K⁺ current and hypoxic pulmonary vasoconstriction. These observations, although demonstrate that NADPH oxidase is the main source of ROS generation in the lung, does not support the hypothesis that NADPH oxidase serves as the pulmonary vascular O₂ sensor [105]. However, data obtained from patch-clamp experiments on intact NEBs in fresh lung slices from OD mice have shown that, unlike wild-type (WT) control, hypoxia had no effect on membrane K⁺ current. H₂O₂, a ROS produced by NADPH oxidase, increased membrane K⁺ current in NEB cells from both WT and OD mice. Therefore, it was concluded that NADPH oxidase acts as the O₂ sensor in NEBs [69]. Despite these in vitro experiments, the present study is the first to use OD mice as a whole animal model for the study of ventilatory control mechanisms. Other knock-out mice models, such as NOS-1/NOS-3 deficient mice [106], have recently been used for the in vivo study of hypoxic ventilatory response (HVR). There are a few potential problems associated with the use of mouse models, especially transgenic mice, which require careful consideration when interpreting experimental results. A comparative study of HVR in numerous inbred strains of mice has demonstrated a significant inter-strain variability. In particular, it was found that C3H/HeJ and C57BL/6J strains, the latter being the same strain as OD mice, appear least responsive to acute hypoxic challenge [107]. Therefore, it would be difficult to detect differences in HVR between groups of mice that belong to strains with weak hypoxic response such as between OD and WT control mice. The other complication is associated with knock-out mouse models. The viability of such transgenic animals lacking molecules that are involved in vital physiological functions, such as O₂ homeostasis in the case of OD mice, is
strongly suggestive of the presence of alternative pathways. For example, in the case of OD mice, it has been suggested that a "low-output" NADPH oxidase may use p22\textsuperscript{phox} to generate superoxide radicals without requiring gp91\textsuperscript{phox} component and therefore remain active even in the absence of functional gp91\textsuperscript{phox} subunit [108].

\textbf{Methods:}

\textbf{Experimental design for respiratory measurements:}
OD mice (at day 1 and 5 of age) with a non-functional allele for the gp91\textsuperscript{phox} subunit of the NADPH oxidase cytochrome b were obtained from Dr. M.C. Dinauer (Indiana University, Indianapolis, IND) [98] and the animal colony was bred and maintained at the animal facility of the Hospital for Sick Children. The first generation has been backcrossed with C57 BL/6 strain for 11 generations and therefore a C57 BL/6 strain has been used as the wild type (WT) control group as also used in our previous study [69].

The experimental apparatus was designed to allow the measurement of ventilatory parameters, the rate of oxygen consumption, and body temperature (Fig. 1.1). The apparatus consisted of two chambers. The animal was placed inside the apparatus so that its head was positioned inside chamber A and its body in chamber B. The two chambers were separated by an air-tight seal in the form of a latex collar fitted around the neck of the animal. To measure lung ventilation, the body chamber was connected to a pneumatograph and a differential pressure transducer (Validyne, Model DP103) detected airflow in and out of chamber B. A MacLab 2/e data acquisition system (Chart Software, ADInstrument, CA) acquired breathing flow rate data and integrated flow measurements in real time to yield tidal volume. Calibration was achieved by injecting known volumes of air with a syringe into chamber A. The inlet air and the plethysmograph were submerged in a water bath maintained at 34°C, the thermo-neutral temperature for neonatal mice [109]. To measure the
rate of oxygen consumption, at specific intervals, the chamber A was closed and connected to the pressure transducer. CO$_2$ was absorbed inside the chamber A using sodium hydroxide pellets (Ascarite). The rate of change in pressure corresponded to the rate of oxygen consumption (V$_{O2}$), which was corrected to STPD. The pressure-volume relationship was calibrated using injection of known volumes of air into chamber A. At all times, a thermocouple probe was used to monitor the animal's body temperature. The peak hypoxic ventilatory responses and the time to reach the peak values were measured in each individual animal and then averaged for group comparison.

Experimental protocol:

Body size, weight and sex were recorded before each experiment. We used 10 animals from each age group of which five were the WT control mice and the other five, the OD mice. Animals were placed inside the chamber and the whole apparatus submerged in a warm bath at about 34°C. Circulation of water-saturated normoxic air with the CO$_2$ absorbed was initiated and the O$_2$ concentration monitored. Animals were kept in the plethysmograph for one hour before measurements to permit acclimatization and attainment of thermal equilibrium. Subsequently, recordings were initiated in a sequence of 4 minutes of ventilatory measurement and 8 minutes of O$_2$ consumption rate measurement. This cycle was repeated two more times before initiation of the hypoxic stimulus. Following the normoxic phase, the air was switched to hypoxia at 15% O$_2$ concentration (P$_{O2}$=114mmHg) balanced with nitrogen and immediately continued with measurement cycles of respiratory and O$_2$ consumption for 56 min before reverting the system back to normoxia. Throughout the 3-hour experiment, body temperature was regularly recorded. At the end of the experiment, animals were sacrificed and the lungs removed and fixed in 10% neutral buffered formalin for microscopic studies.
Immunohistochemistry and quantitative analysis of NEBs:

We used an immunohistochemical approach for mapping the distribution of NEBs in whole-mount preparations of the intrapulmonary airways of 5-day-old OD and WT control mice. Tissue preparation and micro-dissection was performed as described by Avadhanam et al. (1997). The micro-dissected right infra-cardiac lobe of the lung was dehydrated, cleared and rehydrated in graded series of ethanol (70, 95 and 100%), xylene (3 changes) and ethanol (100, 95 and 70%), respectively, for 15 minutes each. Tissues were permeabilized by incubating the airway preparation in 0.3% Triton-X-100 for 1 hour. Endogenous peroxidase activity was blocked by incubating the tissue in 5 μl/ml of 30% H₂O₂ in methanol at room temperature for 1 hour. Tissues were washed with phosphate-buffered saline (PBS) and non-specific binding blocked by 20% goat serum and then permeabilized in 0.5% Triton-X-100 in PBS for 1 hour. The tissues were then incubated for 48 hours at 4°C in CGRP (Calcitonin-Gene Related Peptide) antiserum (Sigma, St. Louis, MO) at a dilution of 1:500 in 20% goat serum/PBS blocking solution. Between the incubations, tissue preparations were washed with several changes of 0.01 mol/l PBS, pH 7.4. Subsequently, samples were incubated in biotinylated goat anti-rabbit antibody (1:200) (Vector Laboratories, Burlingame, CA) in 20% goat serum/PBS for 5 hours at 4°C followed by 1 hour at room temperature. Samples were washed in PBS and incubated overnight at 4°C in ABC (Avidin Biotinylated enzyme Complex) reagent (Vector Laboratories, Burlingame, CA). They were washed in PBS and subsequently immersed in a solution of 0.5% diaminobenzidine (DAB), 0.005% NiCl and 0.075% H₂O₂ for 1 min and the reactions were stopped by two changes of PBS. Stained specimens were kept in PBS at 4°C prior to analysis.

Branching of the minor airways from the main airway tract was most commonly found to occur in groups of 3 or 4. The branching pattern of the main airway tract from an infra-cardiac lobe of a 5-day-old OD mouse is illustrated in Figure 1.2. In order to analyze the distribution of NEBs, a diagrammatic map of neuroepithelial cell bodies along the main axial airway tract was prepared for each specimen.
(Fig. 1.2). As was previously described by Avadhanam et al., we used the nomenclature system based on branching history as the anatomical hallmark for classification and comparison of NEB distribution [110]. According to this system, the axial airway is divided into repeating zones of bifurcation and airway segments [110]. To standardize the comparison of NEB distribution between the control and OD mice, NEBs present in the bifurcation zones and airway segments 1 and 2 were counted and their frequency compared.

**Statistical Analysis:**

The overall design was a two-factor experiment with repeated measures on one of the factors. The factors are control versus experimental groups (c/e) and time (sequence), which has the repeated measurements. The model that we use for a two-factor experiment comparing 2 levels of factor A (c/e), having n subjects per levels of factor A, and b levels of factor B (sequence) is

\[ y_{ijk} = \mu + \alpha_i + \beta_j + \alpha \beta_{ij} + \epsilon_{ijk} \]

where \( \alpha, \beta, \) and \( \alpha \beta \) are fixed effects corresponding to main effects for factor A (c/e), factor B (sequence), and their interaction, respectively [111]. Based on this model, data were evaluated with a two-way ANOVA test with repeated measures on factor B (sequence). Since the validity of this model is based on the normality and equality of variance of data, logarithmic transformation of data was performed to satisfy the requirements of the statistical tests.

A student t-test was used to compare the peak response time and the maximum responses between the two groups. For age and group comparisons of normoxic variables, a two-way ANOVA was used.

For the NEB morphometry analysis, the average number of NEBs per airway segment and bifurcation zone was determined. Comparisons of NEB number of various airway segments between the age-matched OD and WT control group were made using one-way analysis of variance. Unless otherwise stated, all the values are reported as the mean±standard deviation.
Results:

The average body and lung weights were, respectively, 1.38±0.19 g and 0.035±0.003 g for the 1-day-old WT control and 1.25±0.058 g and 0.032±0.005 g for the 1-day-old OD mice (P-value for the differences between the groups: $P_{\text{Body weight}}=0.33$, $P_{\text{Lung weight}}=0.13$). The average body and lung weights for the 5-day-old animals were, respectively, 2.57±0.35 g and 0.0682±0.0172 g for the control and 2.34±0.16 g and 0.0651±0.0092 g for the OD mice (P-value for the differences between the groups: $P_{\text{Body weight}}=0.14$, $P_{\text{Lung weight}}=0.65$); thus in both age groups, lungs constituted approximately 2.6% of body weight.

Frequency of breathing:

Both OD and WT control 1-day-old mice had a characteristic intermittent breathing pattern consisting of periods of regular breathing followed by apnea of varying duration both in hypoxia and normoxia (Fig. 1.3). In our study of breathing frequency, we measured the instantaneous frequency ($f$) during the bouts of breathing and the long-term average frequency ($f_{\text{avg}}$). The $f_{\text{avg}}$ was calculated by dividing the number of breaths by the total duration of recording in each section, which included both breathing and apneic periods. In the 1-day-old mice, the normoxic values of $f_{\text{avg}}$ and $f$ were 1.38±0.12 breath.sec$^{-1}$ and 1.58±0.12 breath.sec$^{-1}$ for WT control and 1.62±0.24 breath.sec$^{-1}$ and 1.83±0.23 breath.sec$^{-1}$ for OD mice, respectively. There was no significant difference between the one-day-old WT control and OD mice in the values of $f$ or $f_{\text{avg}}$ in normoxia or in the maximum response level in hypoxia. In 5-day-old mice, the breathing pattern was fairly regular shorter periods of apnea (Fig. 1.3). 5-day-old OD mice showed a small but significantly higher $f_{\text{avg}}$ in normoxia (Table 1.1) and hypoxia than WT mice ($P=0.037$).

As was mentioned before, the pattern of breathing in 1-day old group was characterized as intermittent breathing. The average durations of apnea were 1.92±0.51 sec for WT control and 2.35±1.57 sec for OD mice in 1-day-old group.
In the same group, the frequencies of occurrence of apnea were 5.00±2.32 min⁻¹ for WT control and 2.5±2.67 min⁻¹ for OD mice. In the 5-day-old group, the apneic durations for WT control and OD mice were 0.8±0.65 sec and 0.16±0.22 sec, respectively (P=0.165). The frequencies of occurrence of apnea in the 5-day-old group were 2.58±3.41 min⁻¹ and 0.25±0.29 min⁻¹ for WT control and OD mice, respectively (P<0.05). Although not statistically significant, 1-day-old OD mice showed a small increase in their total apneic duration in response to hypoxia. This response was due to an increase in the frequency of occurrence of apnea in OD mice that peaked at 11 min after the onset of hypoxia (WT=4.15±3.24 apneas.min⁻¹, OD=8.5±5.93 apneas.min⁻¹, df=8). There was no significant difference in apneic time between WT control and OD mice during hypoxia or normoxia (P=0.139, n=19) at both ages studied. Furthermore, 1-day-old mice showed significantly longer periods of apnea both in normoxia and in hypoxia than 5-day-old mice (P<0.001, n=19).

**Tidal volume:**
There was no significant difference in the average tidal volume (VT) per body weight between control and experimental group at either normoxia (Table 1.1) or hypoxia in 1-day-olds. In 5-day-olds, OD mice showed a significantly lower tidal volume per body weight at normoxia and hypoxia than the WT control group (P = 0.016). In response to hypoxia, the maximum response was also higher in WT control group (VTₘₐₓ =5.30±1.12 ml.Kg⁻¹) than OD group (VTₘₐₓ =3.07±0.75 ml.Kg⁻¹) (Fig. 1.4) but the response time to reach the maximum was not different (Tₚₐₓ =32.2±13.0 min, Tₚₐₓ =22.16±18.6 min, P=0.378, n=10).

**Minute Ventilation:**
Minute ventilation was calculated by multiplying the value of tidal volume per body weight by f_avg. Both age groups showed an increase in minute ventilation (VE) in response to hypoxia (Fig. 1.5).
In one-day-old mice, there was no statistically significant difference between the WT control and OD mice at any period during normoxia or hypoxia. In five-day olds there was no difference between the two groups in normoxia (P=0.279); however, the peak response in hypoxia was significantly higher in WT control group ($VE_{max} = 1056.4 \pm 233.2 \text{ ml.Kg}^{-1}.\text{min}^{-1}$) versus the OD mice ($VE_{max} = 683.6 \pm 126.0 \text{ ml.Kg}^{-1}.\text{min}^{-1}$; $P=0.020$, $n=10$) (Fig. 1.6). The peak increase in minute ventilation ($VE_{max} - VE_{normoxia}$) was $277.0 \pm 223.9\%$ greater in WT control than the OD mice.

**O2 consumption rate:**

There was no significant difference between the WT control and OD mice group in the rate of oxygen consumption ($V_{O2}$) in normoxia or hypoxia at either age group (Table 1.1). In addition, there was no significant difference in the ratio of ventilation over $V_{O2}$ between WT control and OD mice.

**NEB Morphometry:**

The total number of NEBs in bifurcation zones and airway segments 1 and 2 of the right infra-cardiac lobe of the 5-day-old OD and WT control mice was compared. There was no significant difference in the total number of NEBs present in these segments between the two groups (NWT=23.0±5.7, NOD=23.9±3.7, $P=0.70$, $n=21$). Moreover, there was no significant difference between the number of NEBs in each segment between the two groups (Fig. 1.7). The proportion of the total NEB at the bifurcation points in the studied segment of the infra-cardiac lobe was 42% in control and 45% in OD mice and the difference was not statistically significant ($P=0.5$, $n=21$).
**Discussion:**

The present study is the first to use the whole animal model to test the hypothesis that the disruption of O$_2$ sensing protein, NADPH oxidase, expressed in NEB cells affects the control of breathing. We report here three major observations. First of all, in 5-day-old mice we observed several differences in respiratory variables between the WT control and OD mice. These include reduced tidal volume per body weight and an increased breathing frequency in OD mice both under normoxia and hypoxia. However, there was no difference in normoxic minute ventilation, O$_2$ consumption or the minute ventilation/O$_2$ consumption ratio. Secondly, the 5-day-old OD mice had reduced maximum hypoxic minute ventilation per body weight compared to WT control mice. Thirdly, the distribution and frequency of NEBs in OD and WT control mice were similar, excluding abnormalities in NEB structure or lung histology as a cause of these functional differences.

The analysis of various ventilatory parameters examined suggests that OD mice respond differently to hypoxia than WT control mice and that these parameters change rapidly during the first week of life. Although the mechanistic basis for the observed differences in respiration between OD and WT control mice is not clear, several lines of evidence suggest possible involvement of pulmonary NEBs. It is well documented that NEBs express different components of NADPH oxidase as well as H$_2$O$_2$-sensitive K$^+$ channel (KV 3.3a) [74] and that hypoxia causes reduction in K$^+$ current as expected for an O$_2$ sensor [26, 78]. When cultures of NEB are exposed to DPI (Diphenyleneiodonium), a specific blocker of NADPH oxidase, a similar reduction in K$^+$ current was observed without further reduction upon subsequent hypoxia stimulus [78]. This suggests that the NADPH oxidase complex in NEB cells functions as an O$_2$ sensor as further substantiated by recent electrophysiological studies using OD mouse model [69].

During the neonatal period, when vagally innervated NEBs are most abundant, the vagus nerve is known to play an important role in the control of breathing by providing a positive feedback to respiration as part of the peripheral drive during adaptation to air breathing [112-114] and by maintaining the respiratory rhythm in
newborns [114]. It has also been shown that vagotomy in newborn animals results in a pronounced decrease in respiratory frequency, mostly due to lengthening of $T_E$, and an increase in tidal volume [112]. It is of interest to note that NEBs in neonatal rabbits subjected to infra-nodose vagotomy, showed degeneration of intra-corpuscular fibers indicating that the cell bodies innervating NEBs reside within the nodose ganglion [27]. Recent studies using neural tracer Dil (1,1'-dioleyl-3,3,3',3'-tetramethylindocarbocyanine methanosulfonate) have confirmed the vagal origin and derivation of NEB innervation from the nodose ganglion [115]. In addition, since most of the intra-mucosal Dil labeled nerve fibers were seen in association with NEBs, it was suggested that they might represent the predominant intra-pulmonary receptor population. Although the physiology of various pulmonary reflexes has been well characterized, their morphological counterparts have not been defined [116]. In fact, a majority (80-90%) of vagal sensory fibers from lower airways remains unclassified since no responses could be elicited with stimuli that invoke nociceptive or irritant reflexes [117]. Based on the above observation, it is tempting to speculate that these unclassified vagal afferents are linked to NEBs. This is also supported by indirect evidence that implicate vagal afferents in control of hypoxic breathing as demonstrated in chemo-denervated rabbits [118].

The observed differences between day 1 and day 5 mice could be explained by apparent peaking of NEB frequency in the lung at one-week postnatally as demonstrated in neonatal rabbits [20]. These quantitative differences could also reflect post-natal maturation of NEB functions.

In OD mice, the lack of a functional NADPH oxidase causes a reduced oxygen radical concentration in lung tissue [105]. According to a proposed transduction pathway (Fig. 5), the lack of a functional NADPH oxidase in OD mice may simulate chronic hypoxia and lead to NEB stimulation. This, in turn, could result in a vagally mediated shortening of breathing times and smaller tidal volume and a compensatory increased breathing frequency even at normoxia, a scenario consistent with our findings.
The inhibition of NADPH oxidase in carotid bodies, the peripheral arterial chemoreceptor, has been shown not to interfere with O₂ sensing [119]. Moreover, during the early perinatal period, the threshold resetting of arterial chemoreceptors diminishes their hypoxic responsiveness [120]. Thus, the cause of a lower hypoxic ventilatory peak response in OD mice could mostly be attributed to other peripheral chemoreceptors of which NEBs seem to be a good candidate.

The maintenance of oxygen homeostasis is of vital importance to organisms and oxygen-sensing pathways are an integral part of the oxygen homeostatic mechanisms. Therefore it seems reasonable to suggest that there could be alternate oxygen-sensing pathways, with NEBs being one of them that operate in parallel to provide a fail-safe system.
Fig. 1.1: Schematic representation of the experimental apparatus. The chambers (A and B) were submerged in a water bath (2) at 34°C of temperature. The flow of incoming air was controlled by a flowmeter after being passed over the H₂O/CO₂ scrubber. The air was then saturated (6) and warmed up to 34°C by going through a metallic coil (5) submerged in the water bath (2). The airflow into the chamber A was controlled by a switch box that consisted of four mechanically linked 3-way valves (4). Moving the handles to the right position connected chamber A to the pressure transducer and allowed for the measurement of the O₂ consumption rate. Switching the handles to the up position re-established the air circulation inside the chamber A and allowed the measurement of ventilatory parameters by connecting chamber B to the pressure transducer via pneumatograph. The two chambers were separated by an airtight seal in the form of a latex collar fitted around the neck of the animal (1). The pressure-volume relationship was calibrated using injection of known volumes of air by a syringe (3).
Fig. 1.2:  

A) A map of the distribution of NEBs in a whole-mount immunostained airway preparation from the right infracardiac lobe of a 5-day-old OD mouse. The axial pathway is divided into bifurcation zones and airway segments, providing an anatomical hallmark for NEB distribution. Green and red arrows mark the location of a NEB and a bifurcation point, respectively.

B) Microdissected axial pathway of the infracardiac lung lobe of 5-day-old control mice using CGRP immunostaining. NEBs are indicated by arrows.
Fig. 1.3: A representative sample recording of ventilatory flow rate (ml.sec⁻¹) from each group at both ages at normoxia and at peak hypoxic response. At 1 day of age, the pattern of breathing of WT and OD mice was irregular and intermittent and was characterized by periods of apnea of varying lengths. Although not statistically significant, in response to hypoxia, 1-day-old OD mice showed an increase in the total apneic duration mainly due to an increase in the frequency of occurrence of apnea. In 5-day-old mice, the breathing was regular with only occasional short periods of apnea.
Fig. 1.4: The comparison of peak hypoxic VT in WT control and OD mice at 1 and 5 day of age (ml.Kg⁻¹). In 5-day-old group, the response was significantly higher in WT control mice compared to the OD mice. (Error bars represent standard deviation, *: denotes statistical significance at 95% confidence level.)
Maximum Hypoxic Tidal Volume (ml/Kg)

1 day old

5 day old

- OD mice
- WT Control mice
Fig. 1.5: Minute ventilation per body weight in 1- and 5-day-old WT control and OD group (ml.Kg$^{-1}$.min$^{-1}$). The x-axis represents the length of time after the introduction of hypoxia. In 5-day-old group, WT control mice shows a higher hypoxic response compared with OD group. (Error bars correspond to the standard deviation of the mean at each point.)
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Nomoxia $O_2$ 12 24 34 44 56 66 78 90

Time after hypoxia onset (min)

+ OD mice (1 day old)
+ WT control mice (1 day old)
+ OD mice (5 day old)
+ WT control mice (5 day old)

Minute Ventilation (ml/Kg.min)

Hypoxia
Normoxia

Time after hypoxia onset (min)
The comparison of peak minute ventilatory response to hypoxia in WT control and OD mice at 1- and 5- days of age (ml.Kg⁻¹.min⁻¹) The 5-day-old WT control mice show a significantly higher hypoxic response compared to the OD mice. (Error bars correspond to the standard deviations of the mean. *: denotes statistical significance at 95% confidence level.)
Fig. 1.7: Comparison of the number of NEBs in the first two bifurcation zones and airway segments of the intrapulmonary airways of right infra-cardiac lobe of 5-day-old WT control and OD mice. There was no statistically significant difference in NEB distribution between the two groups at each segment (error bars correspond to standard deviation of the mean).
Table 1.1: Summary of normoxic ventilatory parameters in WT control and OD mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 day old</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>favg</td>
<td>1.38±0.133 †</td>
<td>1.60±0.149 †</td>
</tr>
<tr>
<td>VT</td>
<td>2.95±0.344 †</td>
<td>2.72±0.385</td>
</tr>
<tr>
<td>VE</td>
<td>244.58±66.8 †</td>
<td>262.1±74.1 †</td>
</tr>
<tr>
<td>VO2</td>
<td>11.12±2.69 †</td>
<td>13.37±3.47 †</td>
</tr>
<tr>
<td><strong>5 day old</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>favg</td>
<td>2.40±0.122</td>
<td>3.18±0.149 *</td>
</tr>
<tr>
<td>VT</td>
<td>4.75±0.134</td>
<td>3.02±0.385 *</td>
</tr>
<tr>
<td>VE</td>
<td>705.44±90.96</td>
<td>556.9±74.7</td>
</tr>
<tr>
<td>VO2</td>
<td>27.25±2.46</td>
<td>22.92±3.00</td>
</tr>
</tbody>
</table>

favg, frequency of breathing including apneic periods (sec\(^{-1}\)); VT, tidal volume (ml.Kg\(^{-1}\)); VE, ventilation (ml.Kg\(^{-1}.min\(^{-1}\)); oxygen consumption at STPD (ml.Kg\(^{-1}.min\(^{-1}\)). These values are measured at normoxia and the error term corresponds to the standard error of the means in a 2-way ANOVA. (* and † indicate statistical significance at P<0.005 between groups and ages, respectively, n=19).
CHAPTER 2: EFFECTS OF INTRATRACHEAL ADMINISTRATION OF DPI, AN NADPH OXIDASE INHIBITOR, ON NEONATAL RABBIT RESPIRATION

Results presented in the previous chapter suggested that NADPH oxidase plays a role in the neonatal control of ventilation and hypoxic ventilatory response possibly at the level of pulmonary NEBs. In order to further define the contribution of NADPH oxidase of the NEB cells in the hypoxia transduction, we administered DPI, an inhibitor of the oxidase, intratracheally to anesthetized 4-day-old rabbits. The effects of DPI administration alone and in combination with vagotomy and hypoxia on cardiopulmonary parameters were recorded and compared with a control group. There were two major reasons for using rabbits instead of mice for the DPI experiment. Firstly, because of their small size, performing vagotorny, tracheostomy and intratracheal administration of DPI would have been technically difficult in neonatal mice. Secondly, pulmonary neuroendocrine system in rabbits has been extensively studied and most of our present data regarding NEBs have come from the study of rabbit models.

**Diphenylene iodonium:**

The bis-aryliodonium salt, Diphenylene iodonium (DPI) (Fig. 2.1A), is a strong arylating agent which reacts with nuceophiles to form covalent phenylated adducts [121]. DPI reacts with a number of redox systems such as NADPH oxidase in phagocytes [122], mitochondrial NADH oxidoreductase [123], nitric oxide synthase of macrophages and endothelial cells [124], bacterial nicotine oxidase [125], xanthine oxidase [126], quinone oxidoreductase and hepatic cytochrome P450 reductase [127]. In the case of NADPH oxidase, DPI exerts its inhibitory effects by binding to FAD and heme b redox centers of flavocytochrome b, which hinders the NADPH binding site and consequently
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prevents conversion of NADPH oxidase to its reduced configuration (Fig. 2.1B) [128] [104, 121]. DPI also inhibits the production of mitochondrial derived reactive oxygen species (ROS) at concentrations that also inhibit the plasma membrane NADPH oxidase [129]. Studies on carotid body type I cells and pulmonary artery smooth muscle cells of rats have also shown that DPI at 3-10 μM concentration is a non-selective blocker of Ca\textsuperscript{2+} and K\textsuperscript{+} channels through mechanisms that do not involve ROS production by NADPH oxidase [130, 131]. Although DPI is a non-specific inhibitor of flavoenzymes, decrease of cellular reactive oxygen species production in the presence of low concentrations of DPI is generally interpreted as resulting from inhibition of NADPH oxidase by DPI [132]. At 1 μM concentration, DPI would inhibit NADPH oxidase function by as much as 85% [129]. As a result, DPI has been extensively used in numerous studies to investigate various functions of NADPH oxidase including its possible role in hypoxia transduction in different tissues. Most notably, DPI has been used to study the role of NADPH oxidase in hypoxia transduction in carotid bodies, and pulmonary artery smooth muscle cells, both in vitro and in vivo, and in lung neuroepithelial bodies, in vitro.

In isolated type I cells from carotid bodies, DPI, at 3-10μM concentration, inhibited the hypoxic excitation. In the in vivo study, DPI (10μM) has also been shown to elevate carotid sinus nerve discharge in normoxia but to abolish further excitation in hypoxia [73]. DPI (0.5-4μM) selectively inhibits hypoxia-induced contraction in isolated pulmonary artery smooth muscle cells and hypoxic pulmonary vasoconstriction in isolated, perfused rat and rabbit lungs [104, 133, 134]. In cell cultures of fetal rabbit neuroepithelial bodies, DPI (0.4-4μM) caused a reduction in K\textsuperscript{+} current similar in magnitude to that seen with hypoxia. It also abolished hypoxic response of the NEB cells [78]. In a similar experiment, DPI (1μM) reversibly reduced K\textsuperscript{+} current and abolished hypoxic response in NEB cells in lung slice preparation from neonatal mice, the magnitude of which was comparable with the patch clamp data obtained from NEB cells of X-CGD mice lacking functional NADPH oxidase [69].
Based on these studies, it has been proposed that DPI and hypoxia have similar effects in terms of inhibiting the activity of oxidase [73].

**Methods:**

**Assessment of drug distribution in the lung after infusion**

We used iron-dextran to study the distribution and efficiency of different drug delivery methods namely nebulization and direct lung infusion methods. 4-day-old rabbits were anesthetized using Ketamine/Acepromazine drug combination at doses of 35 mg/Kg and 0.4 mg/Kg of body weight, respectively. Local anesthetic was also injected under the skin along the site of incision. Tracheostomy was performed by making a midline incision on the skin and subcutaneous tissues along the trachea in the neck, isolating and transecting trachea below the laryngeal cartilage and inserting an angiocatheter of the appropriate size inside the trachea as the endotracheal tube. For the nebulization study, the intratracheal tube of the animal was connected to a chamber that was linked to the outlet of the nebulizer and the scavenger system of the surgery room. The nebulizer was filled with 10% iron-dextran solution and connected to the air tank, which provided a source of normoxic airflow for nebulizing the solution. Animals were allowed to freely breath the iron-dextran saturated air through their intratracheal tube for duration of 15 minutes. They were then sacrificed and the lungs removed, fixed and stained as is explained below. For the lung infusion study, 0.04 ml of 10% iron-dextran solution in saline was infused into the lung through a very thin tube inserted inside the angiocatheter over a period of 2 minutes. Other volumes of iron-dextran solution, ranging from 0.02 to 0.06, were also tested to compare the effect of the volume of the infused solution on the drug distribution. The vital signs and respiratory pattern were continuously monitored to detect and avoid any possible blockage of the airways during the infusion. At the end of the infusion procedure, animals were sacrificed and the lungs removed and fixed in 10% formalin for 7 days. The lungs were then
embedded *en bloc* in paraffin. Transverse sections were cut through the lungs at several levels, stained for iron by Perl’s acid ferrocyanide technique and counterstained with nuclear fast red [135]. The distribution of the deposited iron on the lumenal surface of the epithelial layer of the lung slices was studied under a light microscope (Fig. 2.2).

**Respiratory measurement of ventilatory parameters after DPI injection**

Pregnant white New Zealand rabbits were obtained from the animal farm and shipped to the animal facility (McMaster Building, Hospital for Sick Children) two weeks prior to the expected time of delivery. Newborns were kept with their mother in maternal cages in the animal facility until day 4 when the young rabbits were collected for the experiment. The weight of animals were measured and recorded prior to each experiment. They were then anesthetized, shaved and prepared for the surgery. In order to measure respiratory parameters while administering experimental solutions into the lung, an apparatus was designed as illustrated in Fig. 2.3. The apparatus allowed for concurrent measurement and monitoring of respiratory parameters, heart rate, electrocardiogram and temperature. The intratracheal tube of the tracheostomized animal was connected to chamber A, which, in turn, was connected to the input air source. The small size of this chamber allowed for a short washout period as the input gas mixture was changed from normoxic to hypoxic and vice versa. To measure ventilatory parameters, an open plethysmography system was used in which chamber B, where the animal body was located, was connected to a pneumatograph linked to a pressure transducer (Validyne). Data acquisition software (ANADAT) acquired the flow rate data and, after correcting for the drifts in the signal, calculated the tidal volume and frequency of breathing. At all times during the experiment, the temperature inside the apparatus was monitored and maintained at 34°C using a heating lamp. Moreover, the heart
activity and respiratory movements of animals were monitored using an electrocardiogram with breathing movement-monitoring capability. During the experiment, animals were exposed to two different gas concentrations. The normoxic and hypoxic gas mixture contained 20% and 14% O₂ concentrations balanced with nitrogen, respectively. For anesthesia, a drug combination of Ketamine and Acepromazine at doses of 35 mg/Kg and 0.4 mg/Kg of body weight, respectively, was administered intraperitoneally. Ketamine produces a dissociative anesthesia while preserving respiratory drive. Monitoring the rabbit’s response to certain stimuli, such as pinching the toe and touching the eye, helped to assess the depth of anesthesia. In some cases the rabbit appeared to be unconscious, but positive reactions to these tests indicated that it could still feel pain, so the anesthetic was adjusted accordingly. To further ensure a pain-free procedure for the animal, local anesthetic was injected under the skin along the site of incision. Tracheostomy was performed by making a midline incision on the skin and subcutaneous tissues along the trachea in the neck, isolating and transecting trachea below the laryngeal cartilage and inserting an angiocatheter of the appropriate size inside the trachea as the endotracheal tube. During the procedure, vital signs were constantly monitored. Animals were incubated inside the chamber for 5 minutes prior to ventilatory measurements to ensure the acclimatization of animals to the chamber environment. All the sequences of the experiment are illustrated in Figure 2.4. All the following recordings were made for duration of 30 seconds. Initially, a baseline recording was made at normoxic condition. Subsequently, 0.04 ml of the control or DPI solution was slowly infused into the lung of the control and experimental groups, respectively, over a period of 2 minutes. The experimental group received the DPI solution, which contained DPI at 1 μM concentration dissolved in 0.1% DMSO (dimethyl sulfoxide)/water. The control group received the control solution that had the same concentration of ingredients, i.e. DMSO, as the DPI solution except that it did not contain DPI. Following the infusion, four consecutive recordings at 5-minute intervals were made under normoxic
conditions. At the end of the forth recording, animals were exposed to hypoxia for a period of 5 minutes and at the end of this period another recording was made and the gas mixture was switched to normoxic air. Three more recordings with 5-minute intervals were subsequently made.

In order to ensure the clearance of the effects of infused solutions and the hypoxia for the second part of the experiment, animals were incubated inside the chamber for a washout period of 20 minutes. Once again at this point in the experiment, the depth of anesthesia was assessed and, if necessary, the anesthetics were administered accordingly. At the end of the washout period another recording was made which was used as baseline level of respiratory parameters for the rest of the experiment. Animals were then removed from the chamber and a bilateral vagotomy below the nodose ganglia was performed through the incision that was made for tracheostomy. They were placed back inside the chamber and three recordings at 5-minute intervals were made subsequently. Following that, the DPI or control solution was infused into the lung over a 2-minute period and two more recordings separated by a 5-minute interval were made thereafter. Finally, animals were exposed to hypoxia for 5 minutes and the hypoxic ventilatory parameters were recorded at the end of this period. Animals were then removed and sacrificed by an overdose of barbiturate mixture (Euthanyl).

**Data analysis:**

The experimental model is a two-factor experiment with repeated measure on one factor. The factors are control versus experimental, i.e. DPI infused, groups (c/e) and time (sequence), which has the repeated measurements. The experiment is comprised of two separate sections: the first section being the DPI infusion/hypoxia experiment and the second section, separated from the first one by a washout period, being the vagotomy study. Changes in ventilatory parameters from the baseline levels measured at the beginning of each section were evaluated with a two-way ANOVA test. To assess the effects of vagotomy
before the infusion of drugs, a one-way ANOVA test was used. Unless otherwise stated, all the values are reported as the mean±standard deviation.

**Results:**

**The distribution of iron-dextran in the lung after intratracheal infusion**

By measuring the ratio of lung parenchyma that was stained for deposited iron to the total lung parenchyma in the mid-sagital sections of the lung, the iron-dextran study provided a semi-quantitative method for the assessment of drug delivery and distribution after nebulization or intratracheal infusion (Fig 2.2). Nebulization proved to be an inefficient method for drug delivery and consistently resulted in less than 15% lung coverage in mid-sagital sections. The efficiency of the nebulization method and hence the quantity of the delivered drug varied with changes in the minute ventilation of the animals. For these reasons, the use of nebulizer as a method for DPI delivery was not pursued any further.

Direct lung infusion, on the other hand, provided a more efficient and repeatable alternative for drug delivery. Infusion of different volumes of iron-dextran solution resulted in different levels of penetration into tracheobronchial tree. After intratracheal infusion of 0.1 ml, 0.2 ml and 0.4 ml of solution, 9.9%, 24.3% and 31.1% of the lung tissue in mid-sagital sections, respectively, showed positive staining for deposited iron (Fig. 2.2). The stained areas were always restricted to the central portions of the lung, containing the first few generations of the tracheobronchial tree. The infusion of more than 0.4 ml of solution would have resulted in severe respiratory distress and therefore were not used in this study.

**The effects of DPI infusion and hypoxia**

The anesthetized 4-day-old rabbits had a regular breathing pattern and stable vital signs during the experiment. Their average breathing and heart rate after the initial incubation period and before the start of experiment were 0.79±0.21
breath.sec\(^{-1}\) and 197.08±30.87 beats.min\(^{-1}\). The baseline minute ventilation per body weight was 0.0027±0.0008 ml.sec\(^{-1}\).g\(^{-1}\).

After the infusion of DPI solution, rabbits showed a small decrease in tidal volume and an increase in breathing frequency. However, when compared to the control group, these changes were not statistically significant (P\(_{\Delta VT}\)=0.088, P\(_{\Delta f}\)=0.943, n=14). \(\Delta VT_{s1}\), the change in tidal volume at sequence S1 from the pre-infusion level, for control and DPI group were \(-0.02±0.03\) ml and \(-0.03±0.03\) ml, respectively (Fig 2.5). \(\Delta f_{s1}\), the change in breathing frequency at sequence S1 from the pre-infusion level, for control and DPI group were 0.12±0.22 breath.sec\(^{-1}\) and 0.20±0.22 breath.sec\(^{-1}\), respectively (Fig. 2.6). The average minute ventilation per body weight, VE, remained almost unchanged after the infusion and there was no statistically significant difference between the control and DPI group (P\(_{\Delta VE}\)=0.330, n=14) (Fig. 2.7).

Hypoxia was introduced at the end of sequence S4 for duration of 5 minutes. In response to hypoxia, there was an increase in VT, f, and VE and a small decrease in heart rate in both control and DPI group. However, there was no statistically significant difference between the control and DPI group in any of the measured variables (P\(_{VT}\)=0.088, P\(_{f}\)=0.943, P\(_{VE}\)=0.33, P\(_{HR}\)=0.5, n=14).

Subsequently, normoxia was resumed and 3 more recordings were made (S6-S8) with no statistically detectable difference between the control and DPI group.

**The effects of vagotony, DPI and hypoxia on cardiopulmonary parameters**

To avoid the possible interference from the administered DPI and hypoxia in the first part of the experiment, the second part of the experiment started after a 20-minute washout period. In the pre-vagotony recording (S8), breathing pattern of rabbits were regular with respiratory parameters of \(VT_{s8}=0.305±0.12\) ml, \(f_{s8}=0.77±0.24\) breath.sec\(^{-1}\), \(VE_{s8}=0.00284±0.001\) ml.sec\(^{-1}\).g\(^{-1}\) and average heart rate of 161.33±73.22 beat.min\(^{-1}\). These pre-vagotony values were used as baseline levels for calculating changes (\(\Delta\)) in measured parameters in the second part of the experiment. Vagotomy profoundly affected breathing parameters resulting in statistically significant increase in VT (\(\Delta VT_{s8}=0.345±0.238\) ml,
P<0.001, n=14) (Fig 2.5) and a decrease in f ($\Delta f_{ss} = -0.42\pm0.17 \text{ breath. sec}^{-1}$, P<0.001, n=14) (Fig. 2.6). Although, the average minute ventilation per body weight did not significantly change immediately after vagotomy ($\Delta VE_{ss} = -0.000130\pm0.000875 \text{ ml. sec}^{-1}.g^{-1}$), it showed a reduction below the pre-vagotomy value 12 minutes after vagotomy ($\Delta VE_{s11} = -0.000662\pm0.000557 \text{ ml. sec}^{-1}.g^{-1}$, P=0.017, n=14) (Fig. 2.7). The breathing pattern was also affected by vagotomy. The plethysmography traces of tidal volume showed a change from a pattern similar to a sinusoid waveform before vagotomy to a more or less square waveform after vagotomy (Fig. 2.8). Heart rate was also increased after vagotomy, which was not statistically significant ($\Delta HR_{ss} = 45.917\pm68.719 \text{ beat. min}^{-1}$, P=0.141, n=14). The cardio-respiratory effects of vagotomy remained unchanged in the next two post-vagotomy recordings (S10, S11). At the end of sequence S11, rabbits received DPI or control solutions by lung infusion. Infusion did not result in any significant change in cardio-respiratory parameters ($P_{AVT}=0.999$, $P_{AV=0.491}$, $P_{AVE}=0.131$, $P_{AHR}=0.986$, n=14) or any statistically significant difference between the control and experimental group in post infusion recordings (S12, S13). Subsequently, hypoxia was introduced which did not have any effect on breathing frequency and, although not statistically significant, slightly increased the VT and VE. There was no statistically significant difference between the control and DPI group in response to hypoxia ($P_{AV}=0.387$, $P_{AVT}=0.767$, $P_{AVE}=0.150$, $P_{AHR}=0.254$, n=14).

**Discussion:**

The present study is the first to attempt to investigate NEB function directly at a whole animal level using an inhibitor of the $O_2$ sensor to assess the effects on ventilation. Our study showed three major findings. When compared to the control group, the administration of DPI by lung infusion neither caused any change in respiratory or cardiac parameters nor disrupted the acute hypoxic ventilatory response of the animals. Secondly, vagotomy profoundly changed breathing parameters by increasing the tidal volume, decreasing respiratory frequency and changing breathing pattern. Thirdly, after vagotomy, the
administration of DPI and, subsequently, hypoxia did not result in any change in cardio-respiratory parameters that was statistically different from the control group.

Before interpreting the results of this experiment, we need to address several issues regarding certain limitations in the design of our study that could restrict our conclusions.

Firstly, despite the fact that a great care was taken in the choice and dosage of the anesthetic drugs used in the experiment to minimize their cardiopulmonary side effects, anesthesia always introduces a complication, the extent of which can never be known. Therefore, the lack of any observable cardiopulmonary change due to the administration of DPI cannot be extended to normally breathing, unanesthetized animals.

Secondly, as was explained before, DPI has been shown to affect many cellular proteins including a host of oxidases and membrane channels. We used DPI at 1μM concentration in which it would be a more selective inhibitor of NADPH oxidase. Nevertheless, even at those low concentrations, DPI has a wide range of cellular targets that could modify the potential cardiopulmonary responses due to NADPH oxidase inhibition. Moreover, by possibly affecting the adjacent cells and disrupting the normal cellular interactions between NEBs and other lung cell populations, DPI could indirectly alter the function of NEB cells.

Thirdly, choosing the 1μM concentration for DPI was mainly based on in vitro studies, which used either cell cultures or thin fresh lung slices and that do not create an accessibility problem for administered DPI. On the other hand, intratracheal administration of DPI may result in somewhat reduced concentration in interstitial or alveolar compartments in company with varying concentration in NEB cells. In fact, in a recent experiment in which DPI was used to investigate the possible role of NEBs in the control of spontaneous tone in guinea pig tracheal preparations [136], only very high concentration of DPI (20μM) yielded positive results possibly because of overcoming the accessibility barrier. Therefore, unless we are able to measure the concentration of DPI after lung infusion at the cellular level, any negative result should be interpreted cautiously.
Finally, although lung infusion method was shown to be the most efficient and reliable choice for the delivery of DPI in our animal model, the drug distribution was mostly restricted to the central parts of the lung as was demonstrated by iron-dextran study. Therefore, only small portion of NEBs was affected leaving out the majority of alveolar NEBs that were not reached by the drug. Compared to the OD mice model in which all the NEBs were deficient in NADPH oxidase activity and hence unresponsive to hypoxia, DPI/rabbit model could not elicit the full response that is expected from the stimulation of pulmonary NEBs. Taking all of these issues into consideration, our finding that intra-tracheal administration of DPI (1μM) failed to affect cardiopulmonary parameters or hypoxic ventilatory response does not exclude the possible involvement of NADPH oxidase in cellular hypoxia transduction in pulmonary NEB cells.

In the second part of the experiment, vagotomy was shown to have a significant impact on respiration of newborn rabbits. This finding suggests an important role for vagus nerves in the control of ventilation. Since the majority of terminal intrapulmonary branches of vagus nerve have been shown to be associated with NEBs [63], it would strongly suggest the involvement of NEBs in the control of respiration.

Finally it should be mentioned that, although not statistically significant, the hypoxic ventilatory response was dramatically reduced after vagotomy, an interesting finding that merits further investigation.
Molecular structure of DPI [94].
Possible site of action of DPI on NADPH oxidase. DPI exerts its inhibitory effects by binding to FAD and heme b redox centers of flavocytochrome b, which hinders the NADPH binding site and consequently prevents conversion of NADPH oxidase to its reduced configuration.
Fig. 2.2: The distribution pattern of the deposited iron (blue stain indicated by arrows) on the lumenal surface of the epithelial layer of the lung slices after intratracheal infusion of 0.1 ml (1), 0.2 ml (2) and 0.4 ml (3) of Iron Dextran. Transverse sections were cut through the lungs and stained for iron by Perl’s acid ferrocyanide technique and counter stained with nuclear fast red.
Fig. 2.3: Schematic representation of the experimental apparatus for DPI/Vagotomy rabbit experiment.

(1) Pneumatograph (2) Pressure transducer (3) Data acquisition card and software (4) Syringe for the infusion of solutions.
Fig. 2.4: Schematic representation of the sequences of rabbit experiment. The duration of each recording was 30 seconds and except the 20-minute washout period between the first and the second part of the experiment, the recordings were separated by 5-minute intervals.
Fig. 2.5: The comparison of Tidal volumes in control- and DPI-infused 4-day-old rabbits in normoxia, hypoxia and post-vagotomy (ml). (Error bars represent standard deviation, *: denotes statistical significance at 95% confidence level.)
Sequence of Experiment

- Tidal Volume (Control group)
- Tidal Volume (DPI group)
Fig. 2.6: The comparison of breathing frequency in control- and DPI-infused 4-day-old rabbits in normoxia, hypoxia and post-vagotomy (Breath.sec$^{-1}$). (Error bars represent standard deviation, *: denotes statistical significance at 95% confidence level.)
Fig. 2.7: The comparison of minute ventilation corrected for body weight in control- and DPI-infused 4-day-old rabbits in normoxia, hypoxia and post-vagotomy (ml.sec\(^{-1}\).g\(^{-1}\)). (Error bars represent standard deviation.)
Sequence of Experiment

- Minute Ventilation (Control group)
- Minute Ventilation (DPI group)
Fig. 2.8: A representative sample recording of ventilatory tidal volume (ml.sec$^{-1}$) from 4-day-old rabbits before and after vagotomy. As a result of vagotomy, respiratory pattern changed from a sinusoid waveform to a more or less square waveform after vagotomy.
SUMMARY AND CONCLUSIONS

A) A sensitive and reproducible whole body plethysmography method for the measurement of respiratory parameters in newborn mice and other small laboratory animals was developed and used for the study of ventilation. The study of hypoxic ventilatory response in 1- and 5-day-old mice have shown several differences in respiratory variables between the WT control and OD mice. These changes were more pronounced in 5-day-old mice and included reduced tidal volume per body weight and increased breathing frequency in OD mice under both normoxic and hypoxic conditions. However, there was no difference in normoxic minute ventilation, \( O_2 \) consumption or the minute ventilation/\( O_2 \) consumption ratio.

In addition, the 5-day-old OD mice had reduced maximum hypoxic minute ventilation per body weight compared to WT control mice. There was no statistically significant difference between the 1-day-old control and OD mice in any of the measured respiratory parameters. The lack of measurable differences in hypoxic ventilatory response between OD and control group in 1-day-old mice may have been due to the weaker acute hypoxic response in newborn mice. In other words, the level of hypoxia used in this experiment, i.e. 15%, may not have been powerful enough to elicit a strong hypoxic ventilatory response to resolve statistically significant differences between the OD and control groups at 1 day of age. Indeed, in a recent study of the hypoxic ventilatory response in transgenic mice of C57BL/6J strain, the same strain that was used in our experiments, significantly lower concentrations of oxygen (8% and 10%) were used to induce acute hypoxic response [137]. On the other hand, the effect of age on the observed differences may stem from developmental changes in hypoxia sensitivity or mechanisms of hypoxia transduction during the perinatal period.
The distribution and frequency of NEBs in OD and WT control mice were similar, excluding abnormalities in NEB structure or lung histology as a cause of observed functional differences. The analysis of various ventilatory parameters examined suggests that OD mice respond differently to hypoxia than WT control mice and that these parameters change rapidly during the first week of life. These findings demonstrate a role for NADPH oxidase in the hypoxic transduction pathway and although the mechanistic basis for the observed differences in respiration between OD and WT control mice is not clear, several lines of evidence suggest possible involvement of pulmonary NEBs.

B) The effects of intratracheal administration of DPI (1μM) to 4-day old rabbits under normoxia and hypoxia and after vagotomy on ventilation were as follows: When compared to the control group, the administration of DPI by lung infusion neither caused any change in respiratory or cardiac parameters nor disrupted the acute hypoxic ventilatory response of the animals. Vagotomy profoundly changed breathing parameters by increasing the tidal volume, decreasing respiratory frequency and changing breathing pattern. After vagotomy, the administration of DPI and, subsequently, hypoxia did not result in any change in cardio-respiratory parameters that was statistically different from the control group. These studies provide preliminary evidence on a whole animal level for the involvement of NEBs as airway oxygen sensors in the control of respiration through a transduction mechanism involving NADPH oxidase. Thus pulmonary NEBs may play a critical role during normal neonatal adaptation to air breathing as well as various perinatal respiratory disorders. Due to the vital role of oxygen homeostasis in the survival of animals, it is reasonable to suggest that there could be alternate oxygen-sensing pathways that operate in parallel to provide a fail-safe system. Controlling the confounding effects of these parallel pathways will constitute the main challenge of the future studies aimed at elucidating the physiological role of NEBs in the hypoxic ventilatory response.
FUTURE STUDIES

The studies presented in this thesis just opened the first chapter in our investigation in the functional significance of pulmonary neuroepithelial cells at the whole animal level.

Demonstrating the transduction of hypoxia stimulus via pulmonary vagal afferents

Although our studies looked at the possible role of pulmonary NEBs in the control of breathing, the mechanisms and pathways of neural transmission of hypoxia-induced signal in the pulmonary NEBs to the central respiratory centers need to be elucidated. This can be achieved by using an isolated lung perfusion model combined with simultaneous vagal recording. Techniques of isolated lung perfusion have been widely used for detailed characterization of lung physiological and pathophysiological phenomena. The basic technique of rabbit lung perfusion has been described previously [137, 138]. Using this method we could easily answer some fundamental questions regarding the contribution of NEBs to hypoxic ventilatory response. Firstly, by recording from the pulmonary branches of the vagus nerve while reducing the inhaled concentration of O₂, we could demonstrate the transmission of hypoxic stimulus via vagus nerve. By maintaining the mechanics of ventilation at a steady state level and eliminating the possible interference from the lung mechano or irritant receptors we could causally link changes in the vagal afferent nerve signal to hypoxia. Since vagal afferents in intra-pulmonary airways seem to contact cells of neuroepithelial bodies [63], the hypoxia-induced changes in vagal nerve recording would provide a strong evidence for the involvement of NEBs in hypoxic ventilatory response. Secondly, an isolated lung model would allow us to more directly study the effects of different chemicals, such as DPI, and hypoxia on NEB cells in a model that precludes any possible interference from extrapulmonary sources, such as carotid bodies, that might have been affected by these stimuli. Finally, the
comparison of vagal recordings from OD mice, whose NEBs have been shown to be unresponsive to hypoxia, and WT mice using an isolated lung model would provide an insight into the function of NEBs in normoxic and hypoxic conditions.
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