Extracellular superoxide dismutase interaction with endothelial nitric oxide synthase in neonatal rat lung does not occur within the caveolin.

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Extracellular superoxide dismutase interaction with endothelial nitric oxide synthase in neonatal rat lung does not occur within the caveolin

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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ABSTRACT

Extracellular Superoxide Dismutase (ECSOD), the only extracellular scavenger of the free radical superoxide ($O_2^{\cdot -}$) is developmentally regulated. ECSOD is intracellular therefore inactive in fetal and neonatal rabbit lung. Secretion of active ECSOD to the extracellular compartment occurs with age after birth[1]. ECSOD is believed to protect nitric oxide ($NO^{\cdot}$) and preserve its function at the cellular and tissue level in newborn lung[2, 3]. ECSOD is modulated by oxygen tension in a similar fashion as endothelial NOS (eNOS) in neonatal lung. ECSOD co- localizes with eNOS in the endothelium in adult ferret heart[4]. We co-localized ECSOD and eNOS in rat lung tissues by immunohistochemistry (IHC) and immunohistofluorescent (IF) techniques. ECSOD localizes to epithelial cells in neonatal and two day old rat lung and the staining for eNOS is the same. By IF double labeling, co-localization of ECSOD and eNOS was strongly present in neonatal rat lung, less apparent in 2day rat lung and showed limited co-localization in adult rat lung.

Endothelial NOS is a membrane bound protein located in caveolae that binds with caveolin-1 when inactive[5]. We studied co-localization of ECSOD with caveolin protein in rat lung. There was no co-localization of ECSOD with caveolin protein in rat lung tissue by IF. For further studies of ECSOD and eNOS proteins, we immunoprecipitated ECSOD with eNOS using whole lung fresh tissue, ECSOD does not co-precipitate with eNOS. The converse was also found; in ECSOD

immunoprecipitated protein, eNOS was not detected. According to the data, these two proteins may not be in physical bond to each other. In Western blot analysis of sucrose gradient caveolin fractions, ECSOD was not detectable in the caveolin fractions where inactive eNOS is located. The fact that ECSOD was not present in caveolae, where eNOS is located, may imply that ECSOD co-localizes with active (phosphorylated) eNOS and not with inactive (non-phosphorylated) eNOS. We suggest that ECSOD may act as an intracellular carrier protein of $NO^{\cdot}$ before its secretion to the extracellular compartment. The interaction of ECSOD with eNOS in the phosphorylated state or via other mediators associated with eNOS, and its product, $NO^{\cdot}$ requires further study.

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List of Abbreviations

Antibiotic (Abx)

Antibody (AB)

Antimycotic (Am)

Balanced Salt Solution (HBSS)

Bronchopulmonary Dysplasia (BPD)

Canadian Council on Animal Care (CCAC)

Caveolin-1 protein (Cav1)

Column Buffer (CB buffer)

Copper Zinc SOD (CuZnSOD)

Dimethyl Sulphoxide (DMSO)

Disuccinimidyl Suberate (DSS)

Exhaled $NO^{\cdot}$ ($eNO^{\cdot}$)

Extracellular Superoxide Dismutase (ECSOD)

Fetal Bovine Serum FBS

Glutathione Peroxidases (GPxs)

Heparin Binding Domain (HBD)

Horseradish Peroxidase (HRP)

Hydrogen peroxide (H_2O_2)

Immunofluorescence (IF)

Immunohistochemistry (IHC)

Interleukin-8 (IL-8)

1.0 Introduction

Although oxygen is used by all aerobes for efficient production of energy, it is a toxic and mutagenic chemical [1]. Oxygen by-products are responsible for this "toxicity" which includes oxidants; compounds that are capable of providing oxygen and accepting an electron in chemical reactions known as an oxidation-reduction (re-dox) reaction [1]. Oxidants can directly damage DNA, can oxidize proteins and lipids or degrade enzymes essential for normal cellular function [1, 2]. All aerobic and aerotolerant organisms have some protection against oxygen and oxidants in order to survive in an oxygen abundant environment [1]. These protective mechanisms are collectively referred to as antioxidants [1, 2]. When there is an imbalance between antioxidants and oxidants in an organism, damage can occur with serious consequences for that organism [1-4].

Oxidative stress is responsible for a large group of diseases [2, 3, 5-12]. Oxidants have been found to contribute to the development of atherosclerosis, heart disease, diabetes, some degenerative neurologic diseases and aging [2, 5-12]. The lung is an organ with a huge epithelial surface area directly exposed to environmental oxygen. Oxygen is toxic to the lung and can result in both acute lung injury and chronic lung damage [2, 8, 13].

Developing lungs undergo a transition from a relatively low oxygen environment in the uterus to a higher oxygen tension of room air at the time of birth. Oxidant injury can occur in immature newborns that are exposed to this relatively

hyperoxic environment when their lungs are not yet fully prepared for this event. In addition, oxygen therapy is often used as treatment for lung diseases related to premature birth and can further injure the lung due to additional oxidant exposure [14, 15].

Bronchopulmonary Dysplasia (BPD) is a disease of immature lungs that occurs after exposure to oxygen and other injurious mechanisms [16]. Oxidative stress contributes in BPD pathology through different mechanisms. Oxidative stress interacts in cell signaling and adaptor proteins which are developmentally regulated in normal lung and their loss appears to correlate with mesenchymal expansion and the development of BPD [14]. BPD is characterized by impaired in alveologenesis [17-20]. Many key control-molecules have been identified in the process of alveologenesis, including various transcription factors, growth factors such as platelet-derived growth factor, fibroblast growth factors, and vascular endothelial growth factor, matrix-remodeling enzymes, and retinoids [21-26]. Matrix metalloproteins (MMP) are important in regulating fibrotic processes and degrade extracellular matrix proteins and fibrillar collagen [14]. MMP expression is regulated at the transcriptional level under oxidative stress [14]. Oxidative stress that increases both MMP and their inhibitors may damage lung by increasing collagenase activity causing disruption of extracellular matrix [27]. Extracellular matrix disruption is found in the lungs of children with BPD [17, 18, 20]. This lends support to the theory that oxidative stress may be partially responsible for BPD pathology. Also the role of oxidative stress in mitochondrial permeability transition and inflammation is known [14]. BPD is characterized by structural

abnormalities of the terminal pulmonary air spaces, which result in chronic lung disease [18]. Paradoxically with improved neonatal care of premature infants the incidence of BPD has increased albeit with reduced severity due to the advance of surfactant therapy [17]. Old BPD was known as infant respiratory distress syndrome and oxygen dependency in first 28 days of life and is characterized by radiography as hyperinflation and non-homogeneity of pulmonary tissue [17, 28]. Arrest of normal pulmonary development that occurs following birth is the hallmark of what is now referred to as the "new BPD" [17, 18].

Extracellular superoxide dismutase (ECSOD) is the only extracellular scavenger of superoxide and is anchored to the cell surface of airway epithelial cells, endothelial cells of pulmonary vasculature, and collagen macromolecules in the extracellular matrix by a heparin binding domain (HBD) [29, 30]. ECSOD, due to its proposed role as a regulator of nitric oxide ($NO^{\cdot}$) and its location on the cell surface, may be an important antioxidant in the protection of the lung and in the pathophysiology of BPD [31, 32].

Superoxide ($O_2^{\cdot -}$), an oxygen molecule with an extra electron, is produced in the mitochondria during respiration via the electron transport chain (NADPH oxidase enzymatic system in inflammatory cells and arachidonic acid metabolism in all cells)[1, 33]. Superoxide ($O_2^{\cdot -}$) can react with nitric oxide ($NO^{\cdot}$) to produce the highly reactive product, peroxinitrite ($OONO^-$) which itself is a toxic oxidant/reactive nitrogen species [34]. In the presence of superoxide dismutase (SOD), $O_2^{\cdot -}$ is dismutated to hydrogen peroxide (H_2O_2) then catalyzed to water (H_2O) resulting in availability of functional $NO^{\cdot}$ [1, 33, 35].

$NO^{\cdot}$ production by endothelial Nitric Oxide Synthase (eNOS) activity was first discovered in vascular endothelial cells [36]. Airway epithelial cells also contain eNOS [36, 37]. $NO^{\cdot}$, produced in the airway epithelial cell, diffuses out of the cell where it functions as a bronchodilator in airway smooth muscle [31, 35, 38]. Other roles of $NO^{\cdot}$ include vasodilation, cell signaling, and inflammation [12].

ECSOD, one of three known isoenzymes of SOD, is intracellular and inactive before birth [39-41]. Secretion of active protein occurs with age after birth [39]. ECSOD is modulated by oxygen tension in a similar fashion as eNOS in the neonatal lung [42]. After exposure to prenatal hypoxia, ECSOD expression is downregulated in rabbit lung at the transcriptional and post transcriptional levels [40]. Hyperoxia depletes active ECSOD from the alveolar parenchyma in adult mice by cleaving the heparin binding domain (HBD) resulting in an oxidant/antioxidant imbalance [43]. Conversely, ECSOD immunoreactive protein in neonatal rabbit lung increases after prenatal hyperoxic exposure and hastens its secretion to the extracellular compartment [44, 45]. ECSOD activity and mRNA do not change, implicating a post translational modification of the enzyme [44, 45]. When neonatal rats were exposed to hyperoxia (after birth), ECSOD protein in lung increased but activity level decreased [41, 46]. ECSOD protein in these hyperoxic neonatal rats underwent nitration (exposed to nitrosative stress) rendering the enzyme inactive [41].

Endothelial NOS is modulated by oxygen and is developmentally regulated [47]. It is a caveolin bound protein that is regulated by a number of factors within the cell [48, 49]. We know that in the inactive state, eNOS is bound to Caveolin-1

(Cav1) protein, which is anchored to the acetylcholine receptor and translocates once activated by phosphorylation [48, 50]. ECSOD and eNOS have been shown to co-localize in adult ferret heart but there have been no further studies to examine the role of eNOS in the regulation of ECSOD especially in the lung [51]. Additionally, the co-localization of ECSOD and eNOS has not been studied in the developing lung.

2.0 Review of the Literature

2.1 Normal Lung Development

2.1.1 Prenatal development

Epithelial cells of the primitive foregut endoderm form the proximal structures of the tracheobronchial tree [48, 52]. This first event in lung development is critically dependent on the surrounding mesenchyme [21, 53]. Thyroid transcription factor and hedgehog proteins (a group of signaling molecules) characterize lung branching morphogenesis [22, 54]. Without these vital signaling molecules, lung development would not proceed [52]. Tyrosine kinase linked receptors and their ligands positively modulate growth and branching morphogenesis, while transforming growth factor-β (TGF-β) family members have an inhibitory effect [55]. This allows for the forward growth of the lung in an organized fashion (branching morphogenesis) [55]. Epithelial-derived vascular endothelial growth factor (VEGF) may help to initiate vasculogenesis which coincides with branching morphogenesis [55]. Shear stress also induces endothelial Nitric Oxide Synthase (eNOS) phosphorylation and expression, the enzyme responsible for production of $NO^{\cdot}$ [56].

Prenatal human lung development is divided into five stages: Embryonic, Pseudoglandular, Canalicular, Saccular and Alveolar Stages [50].

2.1.1.1 The Embryonic Stage (up to 7 weeks gestation): The lungs appear on gestation day 26 (human) as a ventral outpouching of the foregut [50]. The laryngotracheal groove appears, deepens, and starts to separate the lung bud from

the prospective esophagus in a caudocranial direction [57]. At this stage, the mesenchyme is a loose arrangement of primitive cells [52]. The lung bud elongates, divides, and invades the surrounding mesenchyme [50, 52]. At age 4.5 weeks, future lobar bronchi are identified as five tiny saccules, two on the left and three on the right side [52]. Lung development is strongly determined by the interaction of epithelial tubules and the mesenchymal structures where autocrine and paracrine signaling governs differentiation steps [25, 57, 58]. Vascular connections are also established during this stage of lung development [52]. The pulmonary arteries are derived from the sixth pair of aortic arches and the pulmonary vein appears as a small tubule growing out from the left atrium of the heart [52, 57]. The first vascular plexus, which surrounds the foregut, is derived from the developing systemic circulation [57]. After 7 weeks, the lungs appear like a primitive small gland and enter the Pseudoglandular stage of development (Figure 1) [57].

2.1.1.2 The Pseudoglandular Stage (5 to 17 weeks gestation): At the beginning of this stage, the lung consists of epithelial tubules surrounded by a relatively thick mesenchyme [50]. The rate of cellular proliferation increases significantly at the beginning of the Pseudoglandular stage [57]. Sequential branching of the airways and vascular structures continues, with the formation of the conducting airways, terminal bronchioles, and primitive acinar structures [57]. Approximately, half of the epithelial cell mass of the rat lung parenchyma present in the saccular stage just before birth (day 23) was already there in the late pseudoglandular stage (day 20) [58]. In a study by Kitaoka and co-workers, in the human lung, all airway

divisions to the level of the alveolar ducts were present towards the end of the pseudoglandular stage [59]. Smooth muscle develops in the newly formed airway wall during this stage and the epithelium is ladened with glycogen, which represents the fuel for cytodifferentiation [50]. Epithelial cell differentiation is apparent in the primitive acinar structures with the expression of a number of genes (e.g. fibroblast growth factor, *sonic hedgehog* and Gli genes, Wnt genes, *β-catenin* & *BMP4*) that are expressed selectively in the distal respiratory epithelium [25]. For example, surfactant proteins are first detected in these structures between 12-14 weeks of gestation [23]. At 24 weeks of gestation (human), the airway wall has the same structure as adults [55]. The first ciliated cells, along with goblet and basal cells appear in the central airways and spread with time to the more peripheral tubules [50]. However, the epithelial lining of the outermost periphery is maintained, at least in part, in an undifferentiated state until the alveolar stage [58]. Cartilage formation is found centrally prior to 10 weeks [50]. Arteries and veins develop (branching) during this stage [50]. These structures, in particular the bronchial tree, are abundantly innervated by autonomic nerves from the very early stages (Figure 2) [60].

2.1.1.3 The Canalicular Stage (17 th to 23rd weeks of gestation): The transition of the pseudoglandular to canalicular stage is marked by the appearance of the acinus, the prospective gas-exchanging tissue becoming visible under the light microscope [52]. During this stage, preacinar airways continue to increase in size, while the peripheral airways continue to divide [52]. This stage is called canalicular because the future lung parenchyma becomes canalized by the

multiplication of capillaries [52, 57]. The cuboidal, glycogen-rich epithelial cells lining the tubules begin to flatten out, and develop a region with a thin barrier (future alveolar-capillary membrane) [52]. Due to the interactions between the mesodermally derived endothelium and the endodermally derived epithelium, differentiation of alveolar Type I and Type II epithelial cells begins [58]. All saccular air spaces are lined by elongated Type I and cubical Type II alveolar cells by 20-22 weeks of gestation [23]. Four-5 weeks before surfactant can be detected in the amniotic fluid; lamellar bodies developed in Type II alveolar cells [55]. The undifferentiated epithelial cells contain few small lamellar bodies before they really start to differentiate into either Type I or Type II alveolar cells [58, 61]. This process has been used to claim that Type II cells are stem cells for both alveolar Type I and Type II cell lines (Figure 3) [58].

2.1.1.4 The Saccular stage (24 weeks to 36 weeks gestation): During the transition from the canalicular stage to the saccular stage, the peripheral airways form typical terminal clusters of widened air spaces called saccules [57]. Thinning of the epithelium occurs in the lung periphery that leads to the formation of the blood-gas barrier that at this stage is as thin as in an adult lung [57]. The most important characterization of this stage in lung development is the maturation of surfactant proteins within the laminar bodies [52, 57]. The pulmonary parenchyma continues to grow during the saccular stage (Figure 4) [52] .

2.1.1.5 The Alveolar stage (36 weeks to post term - age 8 years): At the beginning of this stage in lung development, the edges of the saccules contain discrete bundles of elastin and muscle, which form small crests subdividing the walls [52,

57]. These crests elongate to produce primitive alveoli, which have a double capillary supply within mesenchymal tissue between the two layers of epithelial cells [55]. Mature alveoli with a single capillary eventually line the saccules, now called alveolar ducts [52, 57]. At birth (40 weeks gestation), the number of alveoli consists of one-third to one-half of the adult number of alveoli (300 million alveoli) (Figures 5 & 6) [55].

2.1.2 Adaptation to extrauterine life

Neonatal lung undergoes a transition from a relative low oxygen tension within the uterine compartment to a comparably high oxygen tension of room air at the time of birth. Pulmonary vascular resistance falls and blood flow increases immediately [62]. The initial rapid dilation of the pulmonary vasculature is stimulated by mechanical ventilation and increase in oxygen tension [35]. There is a surge in both nitric oxide synthase (NOS) protein and enzyme activity at birth [47]. Shear stress regulates vascular tone and diameter by production of $NO^{\cdot}$ by endothelial nitric-oxide synthase (eNOS), the rate-limiting enzyme essential for $NO^{\cdot}$ synthesis [56]. Shear-dependent activation of eNOS includes phosphorylation, acylation, and translocation of enzyme as well as its interaction with other molecules such as heat shock protein 90 (Hsp90) [48].

2.1.3 Postnatal development

The pulmonary arterial thickness falls to mature levels by 3 months of age [55]. The proliferation of the number of alveoli continues till 2-3 years of age and some

argue that this process occurs until 8 years of age (human)[55]. The lung can only "grow" or increase in size as the rib cage expands [55]. Alveolar size and surface area continue to increase during adolescence due to hypertrophy rather than proliferation of cells [50, 55]. The amount of muscle relative to airway size increases during the first year of life [55]. The sub mucosal glands mass is greater in children than adults [55]. By adulthood, males have relatively large airways and lung volume size is dependent on height attained [55]. Boys, in general, have more alveoli than girls for a given height [55].

2.1.4 Human *vs.* non-human lung development

Although there are similarities between human and non-human lung development, the timing of protein expression and developmental stages differ amongst species [52]. Rodents are born during the saccular stage and start breathing in room air [52]. They begin alveolarization by the fifth day of postnatal life. A 19th day fetal rat (term = 22 days gestation) is at the end of the canalicular stage and beginning of the saccular stage and is comparable to an immature (24-26 weeks) human fetus (term 38-40 weeks gestation) (Table 1) [52].

2.2 Bronchopulmonary dysplasia (BPD)

2.2.1 General overview

Preterm neonates with respiratory distress syndrome (RDS) often develop a chronic form of lung disease called Bronchopulmonary Dysplasia (BPD), which is characterized by decreased alveolar and vascular development [21]. BPD develops

when an infant's lung, which has not fully developed, is ventilated and has to adjust to room air conditions outside the uterus [20, 63]. Development of BPD is not limited to preterm newborns; it can happen among full-term babies with hypoplastic lungs or other forms of lung injury [16, 28, 63]. Oxidant injury is one of many factors relevant to BPD development in the premature infant [64]. An increase in extracellular interstitial fibrosis in children with BPD and the correlation of severe BPD pathology with pulmonary hypertension and abnormal pulmonary vascular development, suggests that antioxidant imbalance may lead to disrupted $NO^{\cdot}$ function and hence the importance of antioxidant function, especially in the extracellular space in the lung [14, 65]. In patients diagnosed with BPD, interstitial cells staining for ECSOD were often localized to the same sites as the cells expressing α-smooth muscle actin, which is known to correspond to interstitial myofibroblast-type cells [64]. Since fibrosis is one of the main pathologic features seen in BPD, ECSOD may have a role in development of this disease [8, 29].

2.2.2 Definition

The different diagnostic criteria for BPD have largely been based on prolonged dependency on oxygen therapy [17].

2.2.2.1 Northway: BPD was first described by William Northway in 1967 [17]. He described a disease in surviving premature infants in which there was oxygen dependency during the first month of life in addition to compatible clinical and radiologic findings [17]. Northway described 4 stages for BPD [28]. During stage 1

(days 1 to 3) marked alveolar and interstitial edema with hyaline membranes, atelectasis, and necrosis of bronchial mucosa is noted [28]. The chest radiograph is consistent with hyaline membrane disease [28]. During stage 2 (days 4 to 10), atelectasis becomes more extensive, alternating with areas of emphysema, widespread necrosis and repair of bronchial mucosa [28]. On chest x-ray study, the lung fields are opaque with air bronchograms [28]. During stage 3 (days 11 to 30), extensive bronchial and bronchiolar metaplasia and hyperplasia evolve [28]. Areas of emphysema are surrounded by areas of atelectasis, accompanied by massive interstitial edema with thickening of basement membranes [28]. The lung appears cystic with areas of hyperinflation and areas of atelectasis in x-ray [28]. During stage 4 (after 30 days) there is massive fibrosis of the lung with destruction of alveoli and airways, hypertrophy of bronchial smooth muscle and metaplasia of airway mucosa [28]. Finally, there is actual loss of pulmonary arterioles and capillaries and medial muscular hypertrophy of remaining vessels [28]. The chest x-ray study reveals massive fibrosis and edema with areas of overinflation [28].

2.2.2.2 Modern: The "New definition of BPD" is based on immaturity of the lung, prenatal infection and inflammation [18]. The duration of oxygen dependency has been modified to reflect the severity of BPD that was initially described [18].

Increasing survival of low birth weight babies has also had an impact on BPD diagnosis, necessitating the change in criteria used to describe this disease [18]. The duration of oxygen dependency based on corrected gestational age is critical to this definition of BPD in current times [18].

Although the importance of a definite criterion for the diagnosis of BPD has little impact on clinical practice, it is important for quality control and when the development of BPD is used as an end point in clinical research trials [17].

According to the most current definition, treatment with oxygen of more than 21% (FiO_2 0.21) for at least 28 days (following birth) is considered a criterion for the diagnosis of BPD in an infant [20]. Mild BPD is defined as an infant who is breathing room air at 36 weeks (corrected gestational age; CGA) or upon discharge but who exhibited; moderate BPD is defined as an infant requiring supplemental oxygen (FiO_2 0.30 or 30%) at 36 weeks CGA or upon discharge; and the need for oxygen (> 30%) and/or positive pressure mechanical ventilation at 36 weeks CGA is classified as severe BPD (Table 2) [20].

In a study by Walsh and colleges, a more "physiologic" definition for BPD was proposed [63]. They defined BPD by a standardized oxygen saturation monitoring test at 36 weeks corrected gestational age, and compared this physiological definition with the standard definition of BPD based solely on oxygen administration [63]. Infants were tested at 36 weeks CGA (range 35-37 weeks) [63]. Infants treated with mechanical ventilation, continuous positive airway pressure, or with supplemental oxygen exceeding 30% (FiO_2 0.30) were diagnosed with BPD [63]. Infants receiving supplemental oxygen < 30% underwent a timed stepwise reduction in oxygen to room air (21%) [63]. Those who failed the test were diagnosed with BPD [63]. An absence of BPD diagnosis was defined as an infant who tolerated room air oxygen (21%) while maintaining oxygen saturation ≥ 88% as measured by pulse oximetry (SpO_2) [63]. According

to this clinical definition, 36% of the study population was diagnosed with BPD. The previous physiologic definition identified 24% of those infants studied as having a diagnosis of BPD [63]. They concluded that the physiologic definition was valid and reliable compared to a definition of BPD based solely on oxygen administration [63].

2.2.3 Epidemiology

Bronchopulmonary Dysplasia is a disease predominantly of low birth weight infants (less than 1000 g) [17-21]. The incidence of BPD is increasing because of improved survival of extreme low birth weight infants at 24-26 week gestational age [17]. However, the true incidence of BPD is undetermined due to a lack of uniformity and inconsistencies in the definition of the disease [17]. BPD is uncommon in babies born after 32 weeks of gestation (late saccular stage of lung development) when surfactant protein maturation is occurring and along with the lung matrix [18, 21]. Genetic factors and gender (male) are important risk factors for the development of BPD [66]. Genes encoding multifunctional proteins in the distal lung are prime candidates for causing susceptibility to neonatal lung disease, including bronchopulmonary dysplasia [66].

2.2.4 Theories of BPD pathogenesis

2.2.4.1 Barotrauma: Mechanical ventilation, considered an essential therapy in the treatment for Respiratory Distress Syndrome due to immature lungs in preterm babies, is largely responsible for barotrauma in the lungs [18]. A normal, or non-

mechanically ventilated breath occurs by diaphragm contraction (main breathing muscle), which enlarges the chest cage resulting in a pressure difference between the intrathoracic cavity and the environment [18, 67]. This, results in a higher pressure in the environment than inside the lungs and the air flows downstream to the area of lower pressure [65, 67]. Therefore, a normal breath is a "negative pressure breath"[65, 67]. Mechanical ventilation uses positive pressure to drive air ± oxygen into the lungs and is non-physiologic and therefore can cause injury [65, 67]. Barotrauma (ventilation induced lung injury) causes endothelial and epithelial disruption and leakage of fluid [67]. This results in a process that is similar to the pathology seen in other lung disease processes (e.g. ARDS and tension pneumothorax)[67]. These prematurely born infants are more susceptible to injury with mechanical ventilation because the lungs are exposed to air, surfactant is deficient, and the lung matrix is not fully developed [67]. In addition, air spaces may also contain residual fetal lung fluid which leaves the lungs susceptible to injury [67]. Preterm infants may be artificially hyperventilated (increased amount of air entering the pulmonary alveoli resulting in reduction of carbon dioxide tension) which results in hypocapnia (low $PaCO_2$) after birth which correlates with an increased in inflammation [19]. Severe BPD pathologically is defined as having an altered inflation pattern of atelectasis coupled with over inflation, severe airway epithelial lesions (hyperplasia and squamous metaplasia), airway smooth muscle hyperplasia, extensive fibroproliferation, prominent vascular hypertensive lesions, and decreased internal

surface area and number of alveoli [18]. With technological advances the current pathologic definition of BPD is defined as decreased numbers with large and simplified alveoli (alveolar hypoplasia, decreased acinar complexity), decreased number and dysmorphic capillaries, variable interstitial fibroproliferation, arterial/arteriolar vascular lesions, negligible airway epithelial lesions, and variable airway smooth muscle hyperplasia [18]. Observations over time (1987 to 2003) suggest that CPAP (Continuous Positive Airway Pressure) through a nasal tube, instead of invasive mechanical ventilation (endotracheal tube) may decrease barotrauma (lower "swings of pressure") and result in less injury to the lung [19].

2.2.4.2 Inflammation/infection: Low-birth weight infants may have exposure to chorioamnionitis (infections of the amniotic fluid) who initially have no signs or symptoms of RDS, but may go onto develop respiratory failure with subsequent requirement for oxygen supplementation and mechanical ventilation [18]. Inflammation is considered a key risk factor for the development of BPD in premature infants. In general, an imbalance between the pro- and anti-inflammatory factors may considerably affect normal alveolization and pulmonary vascular development in preterm infant that later develop BPD [68]. Chemokinetic protein factors (e.g. interleukin-8; IL-8) are responsible for the recruitment of inflammatory cells into the lung and are expressed in infants who later develop BPD [18, 69]. In addition, upregulation of various adhesion proteins (e.g. E-selectin), increased concentration of elastase (a powerful proteinase in neutrophils), and low α1-proteinase inhibitors (which inhibits elastase) has important roles in the inflammatory response and subsequent development of

BPD [69]. Mechanical ventilation results in a shift of neutrophils from the circulation to the gas exchange areas of the lung and this increases the risk of developing lung injury and permanent lung damage as seen in BPD [69]. The highest levels of inflammatory cytokines are noted in infants receiving mechanical ventilation where high inflation pressures are used with an absence of continuous inflation (positive end-expiratory pressure) [69, 70] .

2.2.4.3 Oxidative stress/oxidant injury

Accumulating data have suggested that oxidative stress may be involved in the pathogenesis of BPD [14, 71, 72]. Markers of increased peroxidation have been found in tracheal lavage fluid and in the urine in infants who later developed BPD [14]. Oxygen toxicity through reactive oxygen species that interact with and alter essential cell components is most likely responsible [6]. Hyperoxia ($FiO_2 > 0.21$) may interfere with nitric oxide synthase activity and $NO^{\cdot}$ production (vasodilator and bronchodilator), and increase cell nitrotyrosine content [73]. The major reactive species include the superoxide radical $O_2^{\cdot}$ [73]$^{-}$.

2.2.5 Effects on lung development

2.2.5.1 Immediate: Preterm infants who are likely to develop Bronchopulmonary Dysplasia are born during the Canalicular phase of lung development (24-26 gestational weeks), when alveolar and distal vasculature structures are not developed [18, 57]. Premature birth and the beginning of pulmonary gas exchange interrupts this normal alveolar and distal vasculature development, and results in what is now recognized as the major features of BPD (lack of normal

alveolarization and vascular hypertonicity)[17]. Infants with BPD, regardless of whether steroids or surfactant therapies were used, show a lack of increased complexity in lung development (a decrease in alveolarization), an abnormal capillary morphology (increase in vascular tone and reactivity), and an interstitium with variable cellularity / fibroproliferation [17].

2.2.5.2 Long term: Lung pathology studies in children with BPD > 10 years old revealed decreased numbers of alveoli and in total surface area [18]. Abnormalities of the pulmonary circulation are recognized as major contributor to the high morbidity and mortality of Bronchopulmonary Dysplasia because of altered growth and structure of the vasculature resulting in pulmonary hypertension [74]. Vascular injury may be a secondary effect of direct epithelial cell injury, with final disruption of critical signaling between the developing airspace and vasculature [65].

Additionally, primary injury to either the airspace or to the lung circulation might result in detrimental secondary effects on the other systems (heart and kidney) [65].

2.2.6 Animal models of BPD

2.2.6.1 Baboon: The baboon is a primate that has been used as a model of BPD by many investigators since lung maturity and morphometry are similar to human lung and the conditions of premature birth with the development of Respiratory Distress Syndrome can be fully mimicked [72, 75]. The baboon is most often used *in vivo* studies [72, 76]. BPD histological changes in baboon also mimic the

histology noted in human BPD [76]. This model is more expensive than other animal models (e.g. rat or rabbit) that make it unrealistic to many researchers.

2.2.6.2 Rat: The rat is an alternative animal model to the baboon for BPD research [65]. The rat term pup is born during saccular stage of lung development and therefore can be considered to be similar to the human premature infant [52]. This model has been used for both *in vivo* and *in vitro* studies of BPD [46, 65]. Lung injury is produced in this animal model by subjecting the rat pups to hyperoxia ($O_2 \geq$ 95%)[41]. This complex process involves using 2 rat dams (mothers) with their litters (pups) [41]. At the time of birth, the pups are mixed and then fostered to a new dam [41]. The dams are then placed, along with their new litter, into a hyperoxic environment ($FiO_2 \geq 0.95$) or room air (FiO_2 0.21) [41]. The mothers are switched between hyperoxia and room air conditions every 24 hours to maintain their health [41]. After one week, the resulting histologic changes in the rat pup lungs resemble human BPD [41]. The short period of gestation (23days) in rat makes this animal model ideal for lung developmental researchers to use in their studies.

2.3 Oxidant injury

2.3.1 Oxygen

Over time, the percentage of oxygen gas in the earth's atmosphere has changed [1]. Currently, oxygen consists of 20.99% of atmospheric air (nitrogen 79% and minimal other gases (< 0.01%)[1]. Most cells and organs in the body are never

exposed to the full concentration of atmospheric oxygen except for the skin, lungs, conjunctiva, and aural tympanum (Figure 7)[1].

Except for anaerobic and aerotolerant organisms which can survive without oxygen, all animals, plants and bacteria require oxygen to efficiently produce energy [1]. The electron transport chain in the mitochondria, is the fuel source for the cell and organism, and is O_2 dependent (Figure 8)[1]. A series of metalloproteins bound to the inner membrane of the mitochondria act, in essence, as a molecular wire, carrying electrons from the strongly reducing products of the citric acid cycle (NADH, succinate) to highly oxidizing dioxygen (Figure 9) [1, 77]. The *direct* oxidation of these substrates by O_2 would release a great deal of energy as useless heat [1]. Instead, indirect oxidation occurs, whereby the electrons are passed through a series of metal clusters and cofactors, including flavins, iron-sulphur clusters, cytochromes, and copper centers [1]. These groups are embedded in large membrane-bound proteins, and the passage of electrons through them is coupled to the transport of protons across the membrane, from the interior matrix space of the mitochondria to the intermembrane space [1, 78]. In effect, the energy released by the electron flow is used to do work, work that creates a proton concentration gradient across the membrane [1]. The potential energy inherent in that gradient is then used to drive the synthesis of ATP when protons flow back across the membrane through another enzyme complex, ATP synthase [1].

Although oxygen is a toxic and mutagenic chemical, aerobes have antioxidant defenses to protect against it [1].

2.3.2 Superoxide theory of oxygen toxicity

Superoxide ($O_2^{\cdot -}$) is produced during cellular respiration in the mitochondria. In addition, other sources of $O_2^{\cdot -}$ include the NADPH oxidase enzymatic system in inflammatory cells, and the arachidonic acid metabolism in all cells [73]. Superoxide is the major contributor to oxygen toxicity and results in protein oxidation, lipid oxidation (loss of inequity of cellular lipid bi-layer membrane), enzyme inactivation and mutates DNA [1].

2.3.3 Reactive species

Reactive species is a collective term often used to include not only free radicals, but also some non-radical derivatives from re-dox reactions [1]. Radicals can be formed when a covalent bond is broken and the relative compounds are capable of accepting an electron [1]. The energy to break a covalent bound can be provided by heat, electromagnetic radiation or other factors [1]. Wide ranges of free radicals are made in living systems [1]. Thiol radicals, chlorine radicals, carbon-centered radicals, transition metal ions, and reactive nitrogen species are examples of other common biologically active reactive species (Table 3)[1].

Reactive oxygen species (ROS) are capable of providing oxygen and accepting an electron during a chemical reaction include not only the oxygen radicals (superoxide and hydroxyl radical) but also some non-radical derivatives of oxygen (do not donate / accept electrons e.g. hydrogen peroxide, hydrochlorous acid and ozone) (Table 4) [1].

Hydroxyl radical ($OH^{\bullet}$) can be generated by multiple reactions; UV induced hemolytic fission of the hydrogen peroxide in sunlight-exposed skin [1].

($H\text{-}O\text{-}O\text{-}H \rightarrow 2OH^{\bullet}$) from ozone (through reaction with unsaturated compounds and production of molozonide), during ethanol metabolism, and during peroxynitrous acid decomposition, and other sources [1].

$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$[1]

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$[1]

As soon as $OH^{\bullet}$ is formed, it reacts with molecules in its immediate vicinity (almost every type of molecule in the living cell) produces injury and is considered to be most damaging of all the ROS [1].

Superoxide radical ($O_2^{\bullet -}$) is far less reactive than the hydroxyl radical [1]. However it reacts quickly with $NO^{\bullet}$ ($NO^{\bullet} + O_2 \rightarrow ONOO^-$) to make peroxinitrite which is more injurious [12, 33]. This reaction is comparable to the rate at which $O_2^{\bullet -}$ is denatured to hydrogen peroxidase (H_2O_2) by superoxide dismutase (Figure 10)[1].

$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ ($T_1 = <0.3\ M^{-1}s^{-1}$)[1]

The dismutation of superoxide under physiological conditions usually proceeds by reactions that have high time constants (T_1):

$HO_2^{\bullet} + O_2^{\bullet -} + H^+ \rightarrow H_2O_2 + O_2$ ($T_1 = 9.7 \times 10^7\ M^{-1}s^{-1}$)[1]

$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$ ($T_1 = 8.3 \times 10^5\ M^{-1}s^{-1}$)[1]

Other factors that affect the T_1 of these reactions include the pH of the solution [1, 73]. The protonated form of $O_2^{\bullet -}$, $HO_2^{\bullet}$ is more reactive than $O_2^{\bullet -}$ itself and is

formed in a low pH environment similar to pH near the cell membranes [1, 73]. $O_2^{\cdot -}$ in cells is derived from membrane bound systems [1, 73]. As $O_2^{\cdot -}$ is highly reactive in biologic solvents, the $O_2^{\cdot -}$ that is formed near the hydrophobic membranes results in injury [1, 73]. $O_2^{\cdot -}$ also reacts with certain iron-sulphur clusters in enzymes, and certain phenoxyl radicals [73]. Most activated phagocytic cells produce superoxide; several enzymes (e.g. xanthine oxidase) can reduce oxygen to the superoxide radical [1]. Many biologically important molecules oxidize in the presence of oxygen also produce the superoxide radical (e.g. auto-oxidation metal ions) [73]. About 3% of hemoglobin in human erythrocytes undergoes oxidation and produce superoxide radicals every day [1].

Haem - Fe^{2+} - $O_2 \rightarrow O_2^{\cdot -}$ + haem – Fe (III)[1]

At physiological $O_2^{\cdot -}$ levels, about 1-3% of oxygen reduced in the mitochondria forms $O_2^{\cdot -}$[1]. This increases in the presence of hyperoxia and mitochondrial injury (due to leakage of electrons from the electron transport chain) and contributes to mitochondrial DNA damage [79]. Bacterial superoxide production is also a source of this free radical in the body [1]. Endoplasmic reticulum (via oxidase activity of p450) and also in the membrane surrounding the nucleus is considered as an additional exogenous source of superoxide production [73]. Overall, in an adult at rest, 1.7Kg of superoxide is produced each year [1].

Hydrogen peroxide (H_2O_2), which mixes rapidly with water *in vivo*, can pass through water channels and diffuse across the lipid bilayer of cell membranes [1].

H_2O_2 is a poorly reactive agent that is produced by many enzymes but any biological system that generates $O_2^{\cdot -}$ will produce H_2O_2 [73]. Despite its poor reactivity, H_2O_2 can be cytotoxic and at high concentrations is often used as a disinfectant [1]. Hydroxyl radicals account for most of the damage seen in H_2O_2 treated cells [73, 80]. Regardless of H_2O_2 toxicity, this important compound may be responsible for intracellular signaling and as such, is important at lower concentrations in the cell [1]. Generation of H_2O_2 leads to the activation of protein tyrosine kinases followed by the stimulation of downstream signaling systems [80]. For example, H_2O_2 leads to mitogen-activated protein kinase, phospholipase C, and phosphatidylinositol 3- kinase activation [80, 81]. These biochemical reactions have different intracellular effects from modulating gene expression to altering Ca^{2+} flux from the intracellular pool [81].

2.3.4 Oxidant injury in the lung

The lung is an organ with a large epithelial area exposed to environmental oxygen and therefore is exposed to the by- products of normal oxygen metabolism [73]. Although production of reactive oxygen radicals and reactive nitrogen radicals in highly localized domains is essential for normal physiologic function of the cell and important in cellular signaling, airway diseases are generally the result of an imbalance between oxidants and antioxidants (mechanisms that protect from the harmful effects of reactive oxygen species [3, 5, 82-84]. The products of oxidant species and damaged proteins and lipids are used as the "footprint" that oxidant injury has occurred [73]. An increase in extracellular superoxide is a prominent

feature in many inflammatory lung diseases [73]. Oxygen is a toxic chemical even for the lungs and therefore requires greater defenses than other tissues [79].

2.4 Antioxidant protection

2.4.1 Definition

Aerobes, despite their need for oxygen to produce energy, need defenses to survive in an oxygen environment [1]. Every mechanism that has been developed to protect cells organisms against oxygen toxicity is considered an antioxidant [1].

In some bacteria like *E-coli*, there is an intracellular redox sensor which transmits a signal to the flagella involved in moving. *E. coli* can then escapes and move away from regions of high oxygen tension [85].

Electron transport chains that minimize $O_2^{\cdot -}$ production by packing redox constituents together, is another method of antioxidant defense [1]. Cytochrome oxidase, catalyses the stepwise four electron reduction of $O_2^{\cdot -}$ to H_2O without releasing reactive oxygen species [1].

Ambient oxygen tension (150 mm Hg) falls in a stepwise fashion from the air to the lung (100 mm Hg) to the blood and then to tissue and cells. In fact, measured oxygen tension at the cellular level ranges from 1 mm Hg to 10 mm Hg. (Figure 7)[1]. There are enzymatic and non-enzymatic antioxidant proteins that protect the cell against oxidative stress [4]. The composition of cellular antioxidants differs by cell type and by tissue, which reflects the type of exposure to oxidants [1, 64].

Airways are unique in both their exposure to high levels of environmental oxidants and their unusually high concentration of extracellular antioxidants [79].

2.4.2 Antioxidants (human)

2.4.2.1 Non-enzymatic antioxidants: The non-enzymatic antioxidants include low molecular weigh compounds such as glutathione, ascorbate (Vitamin C), urate, α-tocopherol (Vitamin E), bilirubin, Zinc and lipoic acid [86, 87]. The concentration of these antioxidants varies in dependent on cell type [64]. Other high molecular weight molecules that might be considered antioxidants include proteins that have oxidizable thiol groups such as albumin or proteins that bind free metals such as transferrin [79].

2.4.2.2 Enzymatic antioxidants: Superoxide dismutases, glutathione peroxidase, catalase, and thioredoxin are all enzymatic antioxidants known to exist in mammals [1, 14]. The distribution of antioxidant enzymes differ depending on cell type [73]. Thioredoxin is a polypeptide concentrated in the endoplasmic reticulum, but is also found on the cell surface [1]. It contains two adjacent thiol groups in its reduced form that are converted to a disulphide in the oxidized form [1]. Thioredoxin is capable of undergoing redox reactions with multiple proteins. Oxidized thioredoxine can be re-reduced by the thioredoxin reductase enzyme [1]. Thioredoxin acts as a hydrogen donor for ribonucleotide reductase, supplies electrons to methionine sulphoxide reductase (an enzyme that repairs oxidative damage to methionine residues in proteins), reactivates dehydrogenases in cells

subjected to oxidative stress, and is a component of the thiol- specific antioxidant system [1].

2.4.3 Antioxidants in the lung

A feature of antioxidant defense in the lung is a high concentration of glutathione in the epithelial lining fluid compared to other compartments in the body [64]. Various lung cells are different in resistance to oxidant stress that is partly associated with the cell specific expression of antioxidant enzymes [88]. For example, alveolar Type II epithelial cells highly express Copper Zinc SOD (CuZnSOD or SOD1), Manganese SOD (MnSOD or SOD2), and catalase (CAT) and are quite resistant to oxidant stress [89]. In contrast, alveolar Type I cells have a low concentration of these antioxidant enzymes and are more sensitive to injury and death under oxidative stress [89]. Alveolar macrophages have high expression of catalase [64]. Also there are sources of peroxidase, glutathione, and non enzymatic antioxidants in the lung [13].

2.4.3.1 Catalase and Glutathione: Catalase directly catalyzes the decomposition of H_2O_2 to H_2O [1]. Increased ROS production following mitochondrial GSH depletion represents a crucial event [90].

$2\ H2O2 \rightarrow 2\ H_2O + O_2$ [1]

There are two known different isozymes of catalase; one contains a haem containing group and the other manganese [1].

Catalase activity is largely located in peroxisomes, which contain many of the cellular enzymes that generate H_2O_2 [1]. However, some non-peroxisomal catalase

exists in mitochondria depending on organ type (e.g. liver of guinea-pigs and heart of rat)[1].

Glutathione peroxidases remove H_2O_2 to H_2O by a coupled reduction with oxidized reduced glutathione (GSH) [1].

$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$ [1]

Although glutathione peroxidases (GPxs) are specific for GSH, they can act on other peroxides. The peroxide group is then reduced to an alcohol [1]. GPxs consist of four protein subunits, each of which contains one atom of the element selenium at its active site [1].

Glutathione reductase enzymes reduce GSSG back to GSH [1].

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ [1]

Glutathione S-transferases (GST) act by conjugating xenobiotics to glutathione [1]. The liver is rich in GST and the resulting glutathione conjugates are often excreted into bile through the same pumps that are involved in the export of GSSG when liver is subjected to oxidative stress [1].

Some GSTs show glutathione-peroxidase activity with organic hydroperoxides. In addition to their catalytic function, many GSTs appear to serve as intracellular carrier proteins for haem, bilirubin, bile pigments, and steroids, which bind non-enzymatcally to the proteins [1, 73].

2.4.3.2 Superoxide dismutases: Superoxide dismutases are enzymes that dismutate superoxide to H_2O_2 [1]. These are metal containing enzymes that differ in structure, location, folding, and metal binding [1, 64].

2.5 Superoxide Dismutase

2.5.1 General overview

2.5.1.1 History: Superoxide dismutase was first described in the literature by Joe M. McCord and Irwin Fridovich (1969) and led to the superoxide theory of oxygen toxicity [91, 92]. This, in turn opened the oxidative research field. They purified an enzyme and identified it as an erythrocuprein [92]. The fact that a protein existed which was a competitive inhibitor of the reduction of cytochrome c by xanthine oxidase was first observed in 1962 [92].

SOD exists in aerobic organisms, and in many cases, multiple forms exist in a single cell that highlights the specialization and importance of this family of antioxidant enzymes [1]. Different isoenzymes of SOD have been identified, distinguished by protein folding and the nature of the catalytic metal ion [1].

The discovery of superoxide dismutase enzymes led to the superoxide theory of oxygen toxicity, which proposes that superoxide is a major factor in oxygen toxicity; SOD is an essential defense against O_2 toxicity, and is the only enzymatic system that decomposes superoxide radicals to H_2O_2 [13, 93]

There are three isozymes of SODs recognized in human cells; CuZnSOD, MnSOD, and extracellular SOD (ECSOD) [1]. In other organisms there are other isoforms of SOD, for example Iron SOD (Fe-SOD) which is found in E-Coli and Nickel SOD

(NiSOD) in *Streptomyces griseus* [1]. Studies on these isoforms of SOD with different metal ions bring new insights into the role of metal ions on the active sites of the enzymes [94], [93].

2.5.1.2 Superoxide dismutases in the lung: SOD is the primary enzyme targeted to the superoxide radical in the normal lung, as well as the lung exposed to oxidant related lung diseases [13]. SODs have multiple functions in regulating intracellular and extracellular levels of superoxide, hydrogen peroxide, and nitrogen metabolites and participate in normal cell homeostasis [13].

Different cell types in the lung vary in their distribution of SOD enzymes [88]

CuZnSOD is mostly located in ciliated epithelial cells and alveolar cells [1, 13, 64]. Hepatocytes have the highest concentration of CuZnSOD in the body [1]. CuZnSOD is expressed in the cytosol, the nucleus, and the mitochondrial intermembrane [1].

MnSOD is in high concentration in alveolar Type II cells, macrophages, and cells with high metabolic capacity [64]. Mitochondria, the organelles responsible for ATP generation, have the most abundant amount of MnSOD [88]. To date, MnSOD has only been located in the mitochondria [1].

ECSOD, located in extracellular matrix, is heavily expressed in airway epithelial cell junctions and around the surface of vascular and airway smooth muscle cells [95, 96]. It is the main SOD in aorta and other blood vessels, where it is located around smooth muscle cells, and adventitia [13]. ECSOD has an affinity for negatively charged molecules such as heparin; in the extracellular matrix it is located with collagen type I fibers [13].

2.5.1.3 Superoxide dismutases in BPD: To date, the expression of superoxide dismutases and other lung antioxidants have been found to be similar in normal and diseased lung [64]. However, oxidant stress is considered an important factor in the development of lung disease in premature infants [6]. Some studies examining SOD, the primary defense against free radicals in the lung, that indicate that there is a tendency towards increased CuZnSOD, MnSOD immunoreactive protein in alveolar macrophages and ECSOD protein in the interstitium, arteries, and metaplastic alveolar epithelial cells in BPD [97]. Recombinant human CuZnSOD instilled into airways of premature infants may reduce early pulmonary injury, resulting in improved clinical status when measured at one year corrected age [98]. These studies have shown some promise in preventing lung injury but they need to be validated [6].

2.5.2 CuZnSOD (SOD1) and MnSOD (SOD2)

CuZnSOD (SOD1) is expressed in alveolar cells and prominently in ciliated epithelial cells [64]. It is located in the cytosol and in lysosymes within the cytosol [1]. SOD1 is also the predominant SOD in blood vessels [99]. SOD1 role in the prevention of superoxide mediated vascular hypertrophy and enhancing $NO^{\cdot}$ mediated mechanism is known [99]. Female gender, aging, shear stress, and exercise result have all been found to associate with increased SOD1 expression in cultured human aortic endothelial cells [13].

Mitochondrial electron transport chain is the major source for superoxide production in the body [1]. MnSOD or the mitochondrial SOD is the first line of

defense against oxidative stress in the mitochondria [1, 13, 100]. In fact, complete absence of MnSOD is incompatible with life (mouse knock-out studies)[100]. There is relatively high expression of SOD2 in the endothelial cells of vessels [99]. Its effective role in prevention of many vascular diseases has been studied [99]. In the lung, SOD2 has been found in airways, alveolar Type II cells, alveolar macrophages, and interstitial fibroblasts [99].

2.5.3 ECSOD (SOD3)

Extracellular superoxide dismutase is the only extracellular scavenger of superoxide [43, 46]. It is the predominant SOD in the pulmonary artery and is produced by alveolar Type II cells, airway epithelial cells, and vascular endothelial cells [32, 75]. ECSOD is believed to modulate $NO^{\cdot}$ function and availability in the pulmonary vasculature and airway [31].

2.6 Extracellular superoxide dismutase (ECSOD)

2.6.1 ECSOD isoenzyme

ECSOD is an enzyme containing copper and zinc that had previously been isolated from bovine and human erythrocytes as hemocuprein and erythrocuprein [101]. ECSOD is a proteoglycan with 222 amino acids [29]. It is the only extracellular scavenger of superoxide and has been found to be a tetrameric protein of approximately 135 kDa [101].

The N-terminal structure of ECSOD is important for its stability. This region forms a tertiary structure with α-helical content [29, 43]. This structure mediates the tetramerization of the ECSOD subunits [29]. The hydrophobic region of this amphiphatic helix is essential for the interaction, which is very strong [29, 43]. Thus, the quaternary structure of ECSOD is maintained in the presence of strong denaturants as urea or guanidinium chloride [29].

The structure of the central region of ECSOD protein is approximately identical to the structure of the final two-thirds of CuZnSOD [29]. This region is the metal bound part of ECSOD [29].

The C-terminal is essential for enzyme bioavailability [29]. This region consists of a flexible extension with a helical structure which can easily be cleaved [29, 43]. This has been identified as the Heparin Binding Domain (HBD)[102]. ECSOD is synthesized and secreted in the intact form and then slowly and continuously modified to generate the C- terminal cleaved form [29]. A high content of positive charge residues in the C- terminal binds to the negative charged heparin protein and is responsible for anchoring of this protein in the extracellular matrix bound to collagen [29]. The affinity for collagen appears to be heterogeneous [29].

Two different forms of ECSOD have been discovered to date, each with a distinct disulfide pattern [29]. Free (unbound) cystein at amino acid 195 is required to generate inactive ECSOD [29]. The generation of inactive ECSOD, is the result of a highly regulated folding event during protein synthesis [103]. Depending on the ratio of intact and cleaved subunits in the ECSOD tetramer, a heterogeneous population of enzymes can be secreted [29]. The ECSOD protein is separated into

three distinct fractions with no (type A), intermediate (type B), and high affinity for heparin (type C) [29]. The intracellular machinery has the capacity to generate intact and cleaved subunits [29]. As the binding to collagen is mediated by the extracellular matrix region, the affinity for collagen is also likely to be heterogeneous [29, 79].

2.6.2 ECSOD in the developing lung

ECSOD activity increases with age in the developing lung but the protein remains stable [40]. The ECSOD protein is located intracellular and inactive in preterm and term rabbit lung [44]. Secretion of active enzyme into extracellular compartment increases with age [45].

The infant, at the time of birth undergoes a transition from its intrauterine environment to a relatively hyperoxic environment when the lungs are exposed to room air [44]. Oxidative stress has been implicated in many neonatal lung diseases especially in the premature infant with exposure to oxygen [104]. Injury to the lungs occurs when oxidative stress exceeds the antioxidant capabilities of the infant [104]. Developmentally determined localization of ECSOD might be related to its role as the only extracellular scavenger of superoxide in developing lung.

2.6.3 Proposed function for ECSOD in the developing lung

ECSOD not only protects against high oxygen tension in newborn lung, but may also preserve $NO^{\cdot}$ dependent mechanisms in the developing lung [105]. In the

presence of ECSOD there is less superoxide available to react with $NO^{\cdot}$ [31]. Peroxinitrite ($OONO^{-}$), the product of superoxide and nitric oxide is a highly reactive radical and responsible for carcinogenic and mutagenic effects (Figure 10) [106].

2.6.4 Oxygen effects on ECSOD

ECSOD is regulated by oxygen tension [40, 43, 89, 105]. According to the study by Giles et al, pregnant rabbits exposed to prenatal hypobaric hypoxia showed a decrease in ECSOD mRNA expression in lungs of full-term kits, whereas ECSOD protein expression was decreased at all ages studied [40]. Hypoxia delayed the secretion of ECSOD isoenzyme into the airways and pulmonary vasculature [40]. ECSOD activity was decreased in the 1-week-old kits exposed to prenatal hypoxia [40]. The authors concluded that hypoxia downregulates ECSOD expression at both the transcriptional and posttranscriptional levels [40]. Prenatal hypoxia delays secretion of active ECSOD enzyme [40, 55].

In adult and fetal hyperoxic animal models, a significant increase in ECSOD enzyme activity and protein secretion in Type II alveolar cells was more obvious in fetal cells than in adults [46, 89].

In another study, adult rats exposed to 100% oxygen for 72 hours had significantly depleted ECSOD from the alveolar parenchyma [43]. ECSOD mRNA expression was unaffected and there was an increase in the ratio of proteolyzed (cleaved) to intact ECSOD after hyperoxia [43]. The study concluded that hyperoxia depletes ECSOD from the alveolar parenchyma by cleaving the heparin-

binding domain [43, 107] In another study, a doubling of lung ECSOD protein was noted in newborn rats that were exposed to 95% oxygen for 1 week [41]. ECSOD protein secretion increased, but ECSOD enzyme activity did not change with oxygen exposure [41]. In addition, there was no change due to hyperoxia in ECSOD mRNA levels [41]. However, in this study the authors found that ECSOD was a target of nitration in hyperoxia and offered an explanation for low ECSOD activity despite its increase in protein levels in oxygen exposed neonatal lung [41].

2.7 Nitric Oxide Synthase (NOS)

2.7.1 General overview

Nitric oxide synthase (NOS) is an enzyme that converts the amino acid L- arginine to L- citrulline and releases NO˙ [1, 48, 108]. In the absence of L- arginine, NOS and its co-factors may lead to $O_2^{\bullet -}$ generation [109]. There are three isoforms of NOS, neuronal NOS (nNOS) originally identified in nervous system tissue, inducible NOS (iNOS) first identified in macrophages, inflammatory cells, and liver cells after treatment with endotoxin or certain cytokines and endothelial NOS (eNOS) expressed constitutively in endothelial cells [37]. All these three isoforms of NOS have been detected in airway epithelial cells and they are developmentally regulated [110].

The three NOS isoforms are expressed in proximal lung epithelium and are differentially expressed in distal lung epithelium in sheep model [37]. In fetal lung, eNOS expression was evident in bronchial and proximal bronchiolar epithelia but

was absent in terminal and respiratory bronchioles and alveolar epithelium[37, 48] . iNOS was available not only in the same areas as eNOS, but also in terminal and respiratory bronchioles [37, 48]. nNOS was found in epithelium at all levels including the alveolar wall [37, 48]. iNOS and nNOS were also detected in airway and vascular smooth muscle [37].

2.7.2 Endothelial NOS in the lung

Endothelial nitric oxide synthase (eNOS) is produced in endothelial vascular cells and epithelial airway cells in the lung [48]. The principle source of exhaled NO$^{\cdot}$ (eNO$^{\cdot}$) is epithelial cells where it has an important role in smooth muscle relaxation, modulation of ciliary motility, and mucin secretion [36]. eNOS expression peaks near birth and is implicated in lung angiogenesis and airway development [111].

2.7.3 Active and inactive forms of eNOS

Endothelial nitric oxide is highly reactive and its regulation is tightly controlled [48]. Active eNOS is distributed on the membrane of endothelial and airway epithelial cells [48]. Dual acylation of eNOS translocates it to the caveolae, where the caveolin protein, Cav-1 inhibits eNOS [48, 112]. Myristolation (addition of the myristoyl group which is one of the less common fatty acyl residues of phospholipid in the biological membrane) of eNOS targets the protein to the Golgi apparatus, where it undergoes palmitoylation (addition of palmitoyl group, the palmitoyl residue is one of the common acyl residues of phospholipids membrane)[48]. The myristolated and palmitoylated protein is then targeted to

the caveolae. eNOS activity requires binding of both calmodulin and tetrahydrobiopterin [48]. Calmodulin binding is trigged by intracellular Ca^{2+} [48].

2.7.4 NOS product: Nitric oxide ($NO^{\cdot}$)

There are three primary functions of $NO^{\cdot}$: vasodilatation, bronchodilation, and cell signaling [109]. $NO^{\cdot}$ is involved in the regulation of gastrointestinal and genitor-urinary smooth muscle contraction and secretion of water and salt of epithelial cells [109]. $NO^{\cdot}$ functions as a vasodilator in the male reproductive system . Interestingly endothelial cells of vessels contain both eNOS and ECSOD [109]. Nitric oxide is produced in the epithelial cells of airways and it moves out of the cell to modulate smooth muscle tone [109]. In the presence of superoxide, nitric oxide turns into the highly reactive free radical peroxinitrite ($OONO^{-}$)[113]. Nitration of peroxinitrite can produce nitrotyrosine that is a potential biomarker of nitrosative stress [73]. After nitrosation of ($OONO^{-}$), mutagenic products of $NO^{\cdot}$ are produced [73]. Thiyl radicals ($RS^{\cdot}$) are the by-product of peroxinitrite oxidation [1, 73].

2.7.5 $NO^{\cdot}$ in the developing lung

$NO^{\cdot}$ is an important, integral chemical in the transition from fetal to adult type pulmonary vessel and airway physiology [114]. Failure of this transition results in persistently high pulmonary vascular pressures and persistent pulmonary hypertension of the newborn (PPHN)[115]. $NO^{\cdot}$ readily binds certain transitional metal ions, to exert its physiological effects [109]. For example smooth muscle

binding of $NO^{\cdot}$ with Fe^{2+} haem groups in the enzyme guanylate cyclase, can lead to production of more cyclic GMP, depletion of intracellular free Ca^{2+}, and relaxation of the smooth muscle [1].

2.7.6 Functional interaction of ECSOD and eNOS:

ECSOD and eNOS are both developmentally regulated [46, 109]. Both are modulated by oxygen tension [46, 109]. One of the physiologic functions proposed for ECSOD is enhancing $NO^{\cdot}$ dependent mechanisms in the lung [31, 32]. Superoxide produced from a one-electron reduction of oxygen can undergo either spontaneous or enzyme-catalyzed dismutation to hydrogen peroxide or can react with nitric oxide to form the toxic product peroxinitrite which finally can produce the highly toxic hydroxyl radical (Figure 10) [1]. ECSOD is likely to play an important role in mediating nitric-induced signaling events, since the reaction of superoxide and nitric oxide can interfere with nitric oxide signaling [30]. Superoxide and hydrogen peroxide seem to play a significant role in promoting endothelial cell proliferation and migration, possibly through regulation of eNOS activity [108].

ECSOD which is an inactive protein when it is intracellular, is secreted from the cell after birth where is become active [39]. $NO^{\cdot}$ also moves from the cytosol through the membrane out of the cell [32]. The interactions of ECSOD and eNOS either in the cytosol or on the membrane needs to be further clarified.

2.8 Caveolin

The caveolin family proteins are typically associated with microdomains that are found in the plasma membrane of numerous cells [112]. These microdomains are referred to caveolae [112]. Caveolins are small proteins (18-24kDa) that have a hairpin loop conformation with both N and C termini exposed to the cytoplasm [112]. These proteins have the capacity to bind cholesterol and a variety of proteins, such as receptors, Src-like kinases, G-proteins, H-Ras, MEK/ERK kinases and nitric oxide synthases, which are involved in signal transduction processes [112]. Caveolin-1, the resident integral membrane protein of caveolae, directly interacts with and inhibits in a dynamic fashion eNOS in vitro and in endothelial cells in vivo (Figure 11) [48].

2.9 Objective

The overall objective of this study was to examine the physical interaction of ECSOD and eNOS in airway epithelial cells of developing rat lung.

2.10 Hypothesis

Since we believe that ECSOD and NO˙ interact with each other functionally, I hypothesized that, *"Extracellular Superoxide Dismutase is modulated by endothelial Nitric Oxide synthase and this is developmentally regulated".*

The first aim was to localize ECSOD and eNOS in developing rat lung.

My second aim was to determine if ECSOD is a caveolin bound protein before its secretion to the extracellular matrix in rat lung epithelial cells.

2.11 Specific aims

1. To localize ECSOD and eNOS in developing rat lung.
2. To determine if ECSOD is a caveolin bound protein before its secretion to the extracellular matrix in rat lung epithelial cells.

3.0 Materials and methods

Animal studies were approved by the University of Manitoba Animal Ethics Protocol Management and Review Committee. Animals were housed according to the Canadian Council on Animal Care (CCAC) guidelines. Fetal (day 19 gestational age), neonatal (day 0), neonatal (2 day, day 4, and 7 day postnatal age), and adult Sprague Dawley rats were used to study ECSOD and eNOS in the developing lung (Table 1).

Lung tissue was harvested after euthanization of the pregnant mother (fetal), or the rat pup. An intraperitoneal injection of Euthanyl was used at a dose of 200 mg/kg. Pinch testing of the rats' back paw and the loss of tail reflexes were used to confirm that euthanization had occurred. The abdomen was opened using a scalpel and the diaphragm was punctured inferiorly by blunt scissors to produce a pneumothorax to collapse the lung in the thorax. Following this, a mediastinotomy was performed and the lungs removed with mediastinal and hilar structures stripped from the lungs.

Protein Isolation

Following dissection, lungs were placed in a cold E-selectin lysis buffer (50 mM Tris PH 7.6, 3% Igepal 15 ml, 150 mM NaCl 4.33 g, 1mM Mg Cl2 0.102 g, and 5mM EDTA 0.931g in 500 ml/buffer) to which the Anti-Protease cocktail has been added in the ratio of 50 µl to 1ml of buffer. The Anti-Protease cocktail contains; 1, 10 Phenanthroline, 3-4 Dichloroisocoumaren, and trans-epoxysuccinyl-L-

leucylamido butane. The tissue was manually minced in the lysis buffer, homogenized physically in a homogenizer tube, and centrifuged for 30 minutes at 10000g at 4°C. The concentration of proteins was detected by BCA (Sigma, St Louis, Missouri) protein assay (4% of tissue isolated as protein).

Monolayer epithelial cell culturing [61]

Lungs were washed in Balanced Salt Solution – HBSS; Penicillin 100 U/ml-Streptomycin 100 mcg/ml-Amphotericin 0.25 mcg/ml – Antibiotic (Abx) – Antimycotic (Am) and Gentamicin 50 mcg/ml) and then dissociated by manual mincing and incubation (HBSS/Ab-Am/Gentamicin, and 1%Trypsin) at 37°C x 20 minutes. To stop digestion, dissociated cells were washed (Dulbecco's Modified Eagles' Medium – DMEM; 10% Fetal Bovine Serum (FBS), (Gibco, Grand Island, NY); Penicillin 100 U/ml; Streptomycin 100 mcg/ml) before undergoing a second dissociation (HBSS/Abx-Am/Gentamicin/1%Trypsin at 37°C x 20 minutes. Cells were rewashed (DMEM/FBS/Penicillin/Streptomycin) then filtered (50 μm mesh). After diluting in trypan blue stain and counting using a hemacytometer, they were divided in two groups, pelleted (48550 g x5 minutes), and plated with either normal fetal bovine serum or carbon stripped fetal bovine serum (to prevent differentiation and maturation in fetal and neonate cells)[61]. Cells were re-suspended in DMEM/FBS and plated at 2 x 10^7 per 100 mm dishes. After one hour, the non-attached cells, (epithelial cells), were plated. Cells were "fed" every two days with DMEM/FBS and harvested when cell cultures were confluent (determined by light microscopy) in the dishes. The cells were washed in PBS and

after adding 1 ml of NaHCO3 with PMSF (phenyl methane sulfonyl fluoride, from SIGMA), scrapped with a cell scraper (Costar # 3010) to harvest for study.

Sucrose Gradient Caveolin Fractioning [116]

Tissue (scraped in NaHCO3 plus PMSF) was sonicated on ice (3 ×3 seconds). The homogenized tissue was added to the same volume of 90% sucrose (final solution of 45% sucrose). 12 ml Beckman Ultra-Clear centrifuge tubes were loaded very carefully to prevent from mixing of different percentages of sucrose, in a way that 4 ml of 45% sucrose containing the sample was at the bottom, 4 ml of 35% sucrose was in the middle and 4 ml of 5% sucrose was at the top. Tubes were balanced, and centrifuged for 18 hours in 200000 g. After this period of time, caveolin fractions can be seen as a cloudy ring in the middle part of tube [116].

Analysis

Immunohistochemistry: After dissection, the tissue was fixed in 10% formalin for less than 24 hours in order to prevent antigen degradation. Placing the tissue in degraded ethanol and washing in 3 x 20 minutes washes in histological grader xylene (Fisher, Fairlawn, NJ) (not more than one hour), prepared in for placement in liquid paraffin (3 changes of one hour at 57°C). Paraffinized tissue was brought to room temperature. After the paraffin was softened at 60°C for one hour, paraffin embedded tissue sections were deparaffinized in xylene (3 x 10 minutes). An endogenous peroxidase block for 60 minutes was done in a solution of (4ml H_2O_2 in 60 ml EtOH). After gradually bringing the slides to ddH_2O, they

were washed with PBS. Tissue was blocked in 5% normal serum, 1% bovine serum in 3% milk in phosphate buffer saline (PBS).

The first antibody (primary antibody) incubation was with monoclonal mouse anti-rat ECSOD AB (locally produced in partnership with Dr. J. Wilkins, Immunology, University of Manitoba) and rabbit anti-bovine eNOS AB 1:250 (BD Transduction Laboratories, Mississauga, ON) overnight. After warming up to room temperature and washing with PBS (phosphate buffered saline) (Sigma, St. Louis, Missouri) slides were incubated for one hour with goat anti-mouse biotinylated secondary antibody followed by another set of washing and then incubated in Horseradish peroxidase enzyme-streptavidin conjugate (Inno Genex, San Ramon, CA).

Slides were washed with PBS and proteins were detected with Diaminobenzine (DAB) Developing Reagent and Meyer Hematoxylin counterstain for 10 seconds.

Pictures were taken by Olympus microscope (model # ULH10HGAPO) at the magnification of 40 x.

Immunofluorescence: After dissection, the tissue was placed in Optimal Tissue Temperature embedding tissue medium (OCT) (Tissue Tek, Torrance, CA). The plastic cubes contained tissue embedded in OCT, were flash frozen in liquid nitrogen, and kept at -80° C. Tissue cubes were cut with a cold microtome, onto Fisher microscopic slides. Cryo sections were then fixed in 3% paraformaldehyde after washing in cold PBS and equilibrated in cold Column Buffer (CB buffer) for 10 minutes.

Sections were then incubated in 0.3 % Triton-X-100 in Cyto- Tris buffered serum (TBS) for 40 minutes. They were stored at 4° C for future staining after washing in Cyto-TBS.

Slides were blocked by Normal Donkey Serum (NDS) (Bio Can, Mississauga, ON) 10% in Cyto-TBS and incubated with rabbit anti-bovine eNOS Ab 1:250 (BD Transduction Laboratories), monoclonal mouse anti-rat ECSOD Ab 1:100 (locally produced), or rabbit caveolin Ab 1:100 (BD Transduction Laboratories, Mississauga, ON) at 4°C overnight. Slides were incubated with immunoflourescent secondary antibodies (280 μg of fluorescein donkey anti-mouse Cy3 antibody and 300 μg of fluorescein donkey anti-rabbit FITC, Conjugated Affinipure Donkey Anti-Mouse IgG, (Jackson Immuno Research) for one hour in the room temperature, cover slipped with antifade (Cedarlane, Hornby, Ontario) and kept at 4°C. Pictures were taken at 60 x magnification.

Immunoprecipitation: Using Seize X protein G kit (Pierce, Rockford, IL), 0.2 ml of slurry protein G (for immunoprecipitation with anti-mouse eNOS Ab from BD Transduction Laboratories, Mississauga, ON) or 0.2 ml of slurry protein L (for immunoprecipitation with anti mouse monoclonal EC-SOD Ab (locally produced)) was bound to 50 mg of antibodies overnight at 4°C. After washing (Bup H Modified Dulbecco's PBS Pack), an appropriate amount of washing buffer (enough to dampen beads was added to the beads and mixed with Disuccinimidyl suberate (DSS) solute in Dimethyl Sulphoxide (DMSO) (Sigma, St Louis, Missouri) for one

hour at 4°C. Beads were washed with Immunopure IgG Elusion Buffer and kept for future precipitation.

Fresh tissues were immunoprecipitated overnight at 4°C with antibody bound beads. The samples were analyzed by Western blot. The same amount of tissue was precipitated with 50 mg of mouse IgG (Kappa) and mouse IgM (Lambda) proteins (Sigma, St Louis, Missouri) and used as the control.

Western blot analysis: Supernatants from homogenized samples underwent protein analysis. Twenty μg of total protein/sample were then loaded onto a Sodium Dodecyl Sulfate Poly Acrylamide (12% ECSOD, 7.5% eNOS, and 10% Cav1) gel (SDS-PAGE) (Biorad, Hercules, CA), then electrophoretically transferred to a millipore transfer membrane for one hour. The membrane was then incubated with primary mouse monoclonal ECSOD antibody (Ab) (locally made), anti-mouse eNOS Ab 1:1000, and mouse Cav1 Ab 1:1000 (BD Transduction Laboratories, Mississauga, ON) overnight at 4°C,then incubated with secondary antibody which was goat anti–mouse IgG Ab conjugated to HRP 1:1000 (ECL Streptavidin Horseradish Peroxidase conjugate, 1:20,000) x 1 hour. The blot was developed by using ECL reagent (Amersham Biosciences, Piscataway, NJ), for 2 minutes and developing on chemiluminescence film.

4.0 Results

In immunohistochemistry study of paraffin embedded neonate rat lung tissue, there is similar staining for ECSOD protein and eNOS protein. Airway epithelial cells are positively stained for ECSOD which is more localized intracellular (Figure 12). Neonate airway epithelial cells also stained for eNOS intracellularly (Figure 13).

In immunohistochemical staining of paraffin embedded 2 day rat lung tissue; there is similar staining for ECSOD protein and eNOS protein which is less than neonate rat lung tissue. Airway epithelial cells are positively stained for ECSOD which is distributed than neonate (Figure 14). These cells are also positively stained for eNOS (Figure 15).

In immunohistochemical staining of paraffin embedded adult rat lung tissue; airway epithelial cells are stained for ECSOD which is localized in the apical side of the cells (Figure 16). These cells also show positive staining for eNOS protein (Figure 17).

In immunofluorescent staining of frozen neonate rat lung tissue for ECSOD (in red) and eNOS (in green), these two proteins colocalize with strong yellow stain (Figure 18). By the same staining frozen neonate rat lung tissue staining for ECSOD (in red) and CAV (in green). Colocalization staining in yellow is negative for these two proteins (Figure 19).

In immunofluorescent staining of frozen 4day rat lung tissue for ECSOD (in red) and eNOS (in green), these two proteins colocalize with yellow stain which is less than neonate (Figure 20). In the same staining of frozen 4 day rat lung tissue for ECSOD (in red) and CAV (in green), yellow colocalization of these two proteins is negative (Figure 21).

In immunofluorescent staining of frozen 7day rat lung tissue for ECSOD (in red) and eNOS (in green), these two proteins fairly colocalize with yellow stain which is less than 4 day (Figure 22). In the same staining of frozen 7 day rat lung tissue for ECSOD (in red) and CAV (in green), yellow colocalization of these two proteins is negative (Figure 23).

In immunofluorescent staining of frozen adult rat lung tissue for ECSOD (in red) and eNOS (in green), yellow staining of colocalization of these two proteins is negative (Figure 24). In the same staining of frozen adult rat lung tissue for ECSOD (in red) and CAV (in green), yellow colocalization of these two proteins is negative (Figure 25).

Western blot analysis of immunoprecipitation with eNOS on fetal, neonate, and adult rat lung reveals no expression of ECSOD when membranes were incubated with ECSOD primary antibody. IgG was used as negative control and whole lysate was used as a positive control (Figure 26). Western blot analysis of immunoprecipitation with eNOS on rat lung tissue reveals eNOS bands when membranes were incubated with eNOS primary antibody as a positive control, while immunoprecipitation with IgG at the same blot was negative as a negative control (Figure 27).

Western blot analysis of sucrose gradient caveolin fractions in two groups of carbon stripped fetal bovine serum and normal fetal bovine serum incubated with ECSOD antibody. ECSOD is not expressed in caveolin fractions (Figure 28). Western blot analysis of sucrose gradient caveolin fractions in two groups of carbon stripped fetal bovine serum and normal fetal bovine serum incubated with Cav1 antibody as a positive control. Cav1 expresses in caveolin fractions (Figure 29).

5.0 Discussion

We examined the physical interaction of ECSOD and eNOS in rat lung epithelial cells. We found that there is similar staining for ECSOD and eNOS in fetal, neonatal and adult rat lung. This similar pattern of staining was also noted in rabbit lung [40]. In neonate rat lung, at the juncture of the canalicular stage and saccular stage of lung development, airway epithelial cells are positively stained for ECSOD on the apical surface of the cells. This staining is distributed to the extracellular matrix in older lungs. We also noted that airway epithelial cells and vascular endothelial cells stain for eNOS. Interestingly, in previous studies, the visible amount of staining for ECSOD and eNOS are similar with different oxygen tension exposures [40]. ECSOD immunoreactive protein abundance as measured by Western blot decreases with hypoxia and increases with hyperoxia; similar changes with eNOS immunoreactive protein abundance is noted (β-actin controls) [40]. The fact that these 2 proteins stain in a similar fashion and the apparent abundance of the proteins are changed in a similar fashion upon exposure to oxygen tension may imply that ECSOD and eNOS interact with each other. In fact, with immunofluorescent staining of frozen tissues ECSOD and eNOS co-localized in epithelial cells of immature lung which became less obvious with increased age.

The newborn lung undergoes a transition from low oxygen tension while in the uterus to a relative high oxygen tension in room air at the time of birth. The premature lung does not have an appropriate amount of active antioxidants to

protect against reactive metabolites of oxygen and therefore is more susceptible to oxidant toxicity [117]. When ECSOD is intracellular in the preterm lung, it is inactive; secretion of active ECSOD occurs predominantly after birth [39, 40]. This may have important consequences for the prematurely born infant who is exposed to significant oxidative stress without appropriate protection. Additionally, this may impact the transition of fetal to adult pulmonary circulation as the availability of NO· may be affected by the delay in secretion of active ECSOD.

An imbalance in the antioxidant enzymes and oxidative stress leads to injury as is noted in many lung diseases [6]. Bronchopulmonary Dysplasia (BPD) is a disease of the immature lung which is exposed to relatively high oxygen tensions in room air [20]. BPD is increasing in incidence and prevalence and the disease is characterized by underdeveloped simplified alveoli, decreased and dysmorphic capillaries, and variable interstitial fibroproliferation [17, 18]. Immature vascularity and pulmonary hypertension are often seen in severe BPD [18]. Oxidative stress is a known etiology for the development of BPD in infants [14]. In BPD, SOD has been shown to be an important enzyme; instillation of recombinant SOD into the airways of premature infants may be protective and pathologic studies reveal increased staining for ECSOD in interstitial cells [64, 115].

Superoxide is the predominant free radical in oxidative stress [1]. Superoxide can be dismutated to hydrogen peroxide which is then reduced to the hydroxyl radical and water, or it can react with NO· nitric oxide to make highly reactive radical, peroxinitrite (Figure 10) [1].

ECSOD does not co-localize with Cav1 in this study. We have shown this through IF staining and through staining for ECSOD in caveolin fractions. These findings are important as we assumed, albeit incorrectly that the caveolin might provide the route for ECSOD secretion from the cell. The fact that ECSOD was not a caveolin bound protein was a surprise. Since eNOS is a caveolin bound protein, in the inactive form, the assumption that ECSOD was also bound, while inactive, to the caveolin was reasonable. These findings imply that there may be an alternative mechanism for ECSOD secretion.

The fact that ECSOD and ENOS co-localize, but that ECSOD is not bound to caveolin support a new idea that ECSOD may co-localize solely with the active form of eNOS which disossiates from Cav1 protein when phosphorylated [48]. This lends more credibility to the theory that an important function of ECSOD is to protect $NO^{\cdot}$. $NO^{\cdot}$ diffuses from the cell to exert its action on neighboring cells [109]. If the role of ECSOD is to protect the functionality of $NO^{\cdot}$, then it would be reasonable to assume that ECSOD may travel out of the cell accompanying $NO^{\cdot}$ therefore acting as a carrier protein.

Carrier proteins for $NO^{\cdot}$ include a group of proteins collectively referred as S-nitrosothiols [118, 119]. The literature supports the idea that $NO^{\cdot}$ can function as a carrier protein, or in fact be carried by protein (e.g. hemoglobin) to exert its actions at another location [118, 119]. Therefore it is plausible that ECSOD may act as an intracellular carrier for $NO^{\cdot}$ prior to its secretion from the cell.

Potential pitfalls:

The difficulty with qualitative studies such as this, is that the results are implied rather than proven. To minimize this, all sections for IHC and IF were cut to the same thickness, underwent the same staining procedure, and at the same time (date) and each had its own negative control. The studies were done x 5 to confirm the results. Additionally, when the slides were examined under the microscope, the identity of the slide (fetal, neonatal, 2 day, etc) was not revealed until the interpretation of the findings were complete and agreed upon by 2 different observers. The slides were also examined on different days and the results compared to reduce inter- and intra- observer bias.

Morphometric and densitometry analysis of immunofluorescent pictures can be used to prove the localization of ECSOD and eNOS in airway epithelial cells. Since, we obtained staining consistent with co-localization, these analyses were not actively pursued.

Interestingly, when we studied the immunofluorescent localization of ECSOD and Cav1 protein in the same tissues, we discovered that there was no apparent interaction between these 2 proteins. However, eNOS, in its inactive form, is anchored to Cav1 protein in the caveolae. The same conditions (tissue thickness, staining procedure, and date of experiment, time processed and independent 2 observer analysis) were met for the ECSOD and Cav1 studies to ensure consistency.

ECSOD does not co localize with Cav1 in this study through IF staining and through staining for ECSOD in caveolin fractions. We did this with tissue, and with

epithelial cell cultures. Western blot analysis of caveolin fractions, incubated with Cav1 protein as positive control, did not stain for ECSOD. A quantitative method of measuring the amount of protein on Western blots involves re-probing the same membrane with a "housekeeping" protein to ensure that the blot was loaded properly and each lane contained the same amount of protein. Then the ratio of the protein studied with the housekeeping protein can be used to give an approximation of whether the protein was affected by the experimental conditions or not. For oxidative studies, indeed most experimental conditions, β-actin is an excellent housekeeping protein as it is relatively stable within the cell and is unaffected by oxygen tension. In our studies, we did not stain, at all, for ECSOD. We are able to use this particular antibody in other Western blot (immunoreactive studies) without difficulties. We were able to stain for other proteins (for example Cav1 in caveolin fractions) that were consistent over time. We also used increasing concentrations of antibody to ECSOD with the same results. We therefore elected to do immunoprecipitation studies for ECSOD with eNOS on fresh tissues. Using fresh tissue is the most sensitive method to gain meaningful results especially when working with enzyme proteins. IgG was used as the negative control and each lane was loaded with 20 μg of protein. Whole lysate was used as the positive control. Also each Western blot membrane was incubated with eNOS AB as a positive control (to ensure we precipitated eNOS from the sample). Protein assay were done on whole lysates and each lane was loaded with incremental increases in protein (5 – 100 x whole lysate lane). After each wash, the flow through effluent was detected by protein assay. We did not

stain for ECSOD when we immunoprecipated eNOS from the sample. This implies that ECSOD and eNOS might not be physically attached. This was repeated not only with whole lung tissue but also with monolayer cell cultures. Both tissue and cell culture stain for ECSOD under normal Western blot analysis/conditions.

6.0 Conclusion

- In rat lung tissues, ECSOD and eNOS have similar staining by IHC and co-localize by IF. This implies that these two proteins might be physically interacting.
- In IHC and IF study of rat lung tissue in different ages, the expression of ECSOD and eNOS in premature lung is more pronounced than seen in neonatal lung or more mature lung. The apparent co-localization of ECSOD and eNOS diminishes with age.
- ECSOD does not co- localize with caveolin proteins
- No co-precipitation of ECSOD and eNOS was noted.
- ECSOD is not expressed in caveolin fractions where Cav1 is detectable

7.0 Speculation

The fact that ECSOD was not presented in caveolae, where eNOS is located, may imply that ECSOD co-localizes with active (phosphorylated) eNOS and not non-phosphorylated eNOS (inactive). Interaction of ECSOD with active or inactive eNOS and modulation of ECSOD either with eNOS or directly with its product, $NO^{\cdot}$, requires further study.

8.0 Future directions

ECSOD and eNOS co-localization in airway epithelial cells in lung may confer location of ECSOD protein to the cytosol, where active eNOS is located or the cell membrane. The inability to co-localize caveolin and ECSOD in airway epithelium may imply that caveolin protein is not important in ECSOD regulation or may act via other mediators (Figure 30). Therefore, ECSOD and eNOS have similar staining patterns and co-localize in a developmental fashion. ECSOD is not expressed in caveoli therefore the interactions of ECSOD with eNOS must occur after eNOS phosphorylation and activation. This may imply that ECSOD acts as an intracellular carrier protein of $NO^{\cdot}$ before secretion to the extracellular compartment. Proposed future directions are:

1. To study the interaction of ECSOD with the active form (phosphorylated) of eNOS (P-eNOS) by using immunohistochemistry (IHC), immuno-fluorescence (IF), and immuno-precipitation (IP). To study the relationship of ECSOD and P-eNOS by confocal microscopy, deconvolution microscopy and electron microscopy to understand the physical relationship of ECSOD with P-eNOS. In confocal microscopes there is a pinhole which is conjugate to the focal point of the lens. With this microscope the tissue can be scanned in images and prevent from haze background. Deconvoultion microscopy also work by removing image blur and divided in two typed of two dimensional and three dimensional microscopes.

2. To study whether ECSOD protects NO˙ in the epithelial cell or acts as its carrier protein by using monolayer cell culture techniques, manipulation of conditions in cell cultures and studying protein levels. To use cell cultures obtained from ECSOD knock out (no ECSOD activity), ECSOD transgenic mice (over express ECSOD) and wild type mice (normal ECSOD). To measure the amounts of eNOS and P-eNOS along with NO˙ levels (direct and indirect) in these cells. Stimulating eNOS to produce NO˙ using known phosphorylators of eNOS (platelet activating factor; PAF); Phosphoinositide-3 kinase- Protein Kinase B pathway (PI3K → Akt)) and other stimulators of eNOS (vascular endothelial growth factor (VEGF) via calcium/calmodulin) and measure the production of NO˙. Using a potent eNOS inhibitor to reverse the effects of phosphorylation (L-N^5-(1-Imionoethyl) ornithine-dihydro chloride; L-NIO, 2HCl) and measuring NO˙ production. To examine the physical location of these proteins using IHC, IF and IP. To investigate developing an in vivo model to test whether ECSOD acts as a carrier protein for NO˙.

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TABLE 1

Timing of morphological stages of lung development in human and rat

STAGE	HUMAN (Weeks)	RAT (Days)
	Term = 40	Term = 22 days
Embryonic	3-7	10-13
Pseudoglandular	5-17	13-18
Canalicular	16-26	18-20
Saccular	24-38	20-4 post natal (pn)
Alveolar	36-8 years	4pn-13pn

Timing of lung developmental stages is different between species

Adapted from Wert, S. Normal Lung Development. American Thoracic Society International Conference. 1998. Chicago, Illinois: American Thoracic Society.

TABLE 2

DEFINITION OF BRONCHOPULMONARY DYSPLASIA: DIAGNOSTIC CRITERIA

Gestational Age	< 32 wk	≥ 32 wk
Time point of assessment	36 wk PMA or discharge to home, whichever comes first	>28 d but < 56 d postnatal age or discharge to home, whichever comes first
Treatment with oxygen > 21% for at least 28 d plus		
Mild BPD	Breathing room air at 36 wk PMA or discharge, whichever comes first	Breathing room air by 56 d postnatal age or discharge, whichever comes first
Moderate BPD	Need for < 30% oxygen at 36 wk PMA or discharge, whichever comes first	Need for < 30% oxygen at 56 d postnatal age or discharge, whichever comes first
Severe BPD	Need for ≥ 30% oxygen and/or positive pressure, (PPV or NCPAP) at 36 wk PMA or discharge, whichever comes first	Need for ≥ 30% oxygen and/or positive pressure (PPV or NCPAP) at 56 d postnatal age or discharge, whichever comes first

Definition of abbreviations: BPD = bronchopulmonary dysplasia; NCPAP = nasal continuous positive airway pressure; PMA = postmenstrual age; PPV = positive-pressure ventilation.

Adapted from: Jobe, A. H. Am. J. Respir. Crit. Care Med, 2001;163(7):1723-1729

TABLE 3

Examples of free radicals

Name	Formula	Comments
Hydrogen atom	$H^{\bullet}$	The simplest free radical
Trichloromethyl	$CCl_3^{\bullet}$	A carbon-central radical
Superoxide	$O_2^{\bullet-}$	An oxygen- centered radical
Hydroxyl	$OH^{\bullet}$	A highly reactive oxygen- centered radical, attacks all biomolecules
Thiyl/perthiyl	$RS^{\bullet}/RSS^{\bullet}$	A group of radicals that have unpaired electrons residing on sulphur
Peroxyl, alkoxyl	$RO_2^{\bullet}$, $RO^{\bullet}$	Oxygen- centered radicals formed(among other routes) during the breakdown of organic peroxides and reaction of carbon radicals with O_2 ($RO_2^{\bullet}$)
Oxides of nitrogen	$NO^{\bullet}$, $NO_2^{\bullet}$	Nitric oxide is formed *in vivo* from the amino acid L- arginine, Nitrogen dioxide is made when $NO^{\bullet}$ reacts with O_2, both are found in polluted air and smoke from burning organic materials, e.g. cigarette smoke
Nitrogen- centered radicals	$C_6H_5N{=}N^{\bullet}$	Formed during oxidation of phenylhydrazine by erythrocytes e.g. phenyldiazine radical
Transition-metal ions	Fe, Cu, etc.	Ability to change oxidation numbers by one allows them to accept / donate single electrons, hence they are often powerful catalysts of free-radical reaction

Adapted from Halliwell & Gutteridge Free Radicals in Biology and Medicine 2000

TABLE 4

Reactive oxygen species

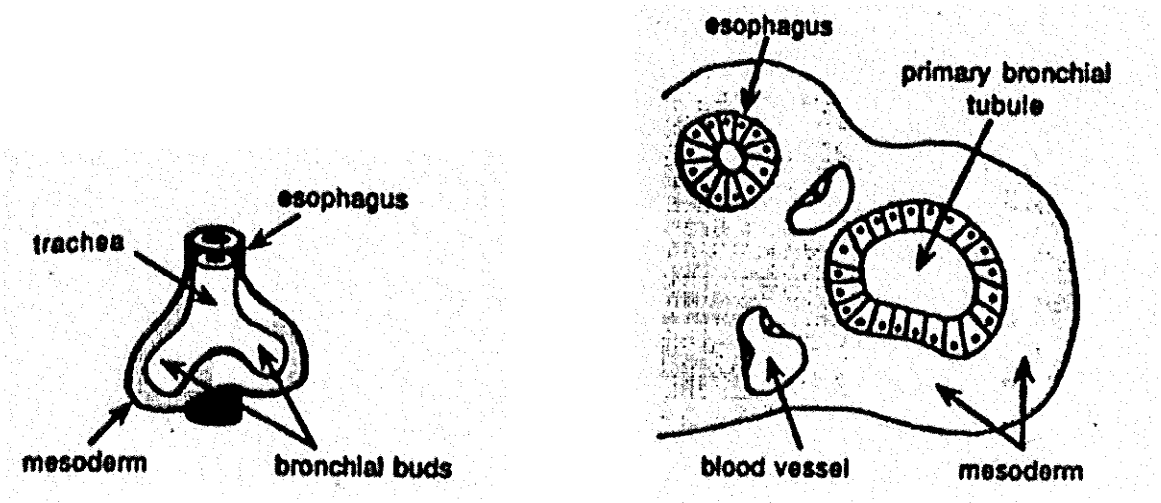
Radicals	Non- radicals
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
Peoxyl, $RO_2^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singelet oxygen
Hydroperoxyl, $HO_2^{\bullet}$	Peroxynitrite, $ONOO^-$

Adapted from Halliwell & Gutteridge Free Radicals in Biology and Medicine 2000

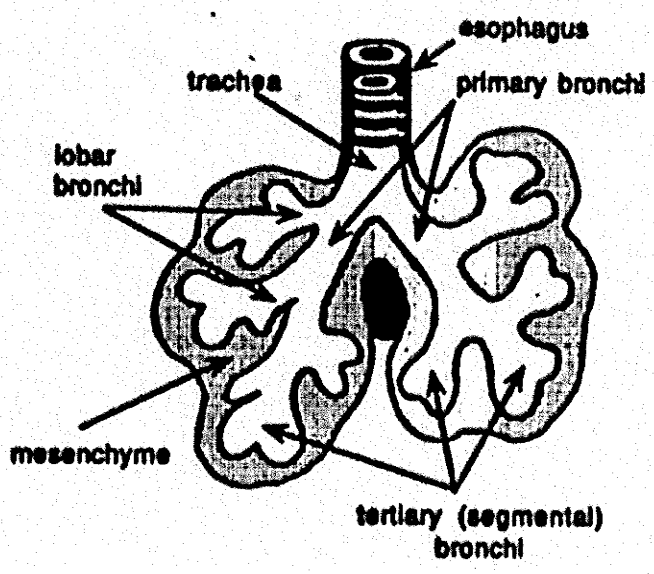
Reactive oxygen species (ROS): species which are capable of providing oxygen and accepting an electron during a chemical reaction

FIGURE 1

The Embryonic Stage of lung development



Development of lung buds, trachea, and bronchi happens at this stage.

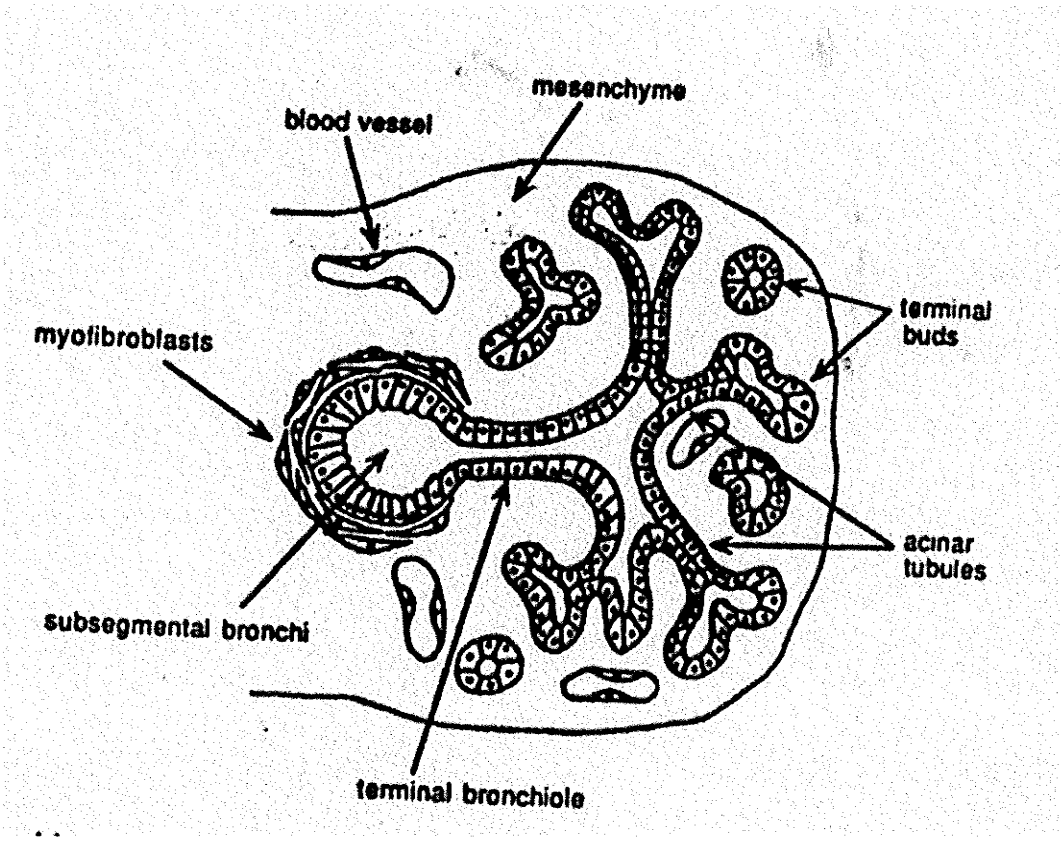


After the age of 7 weeks, the lung looks like a primitive small gland and has entered the pseudo glandular stage of development.

Whitsett, J. A. and S. Wert (1998). Molecular determinants of lung development. Kendig's Disorders of the Respiratory Tract in Children. V. Chernick, T. Boat and E. L. Kendig. Philadelphia, WB Saunders: 3-18.

FIGURE 2

The Pseudo glandular Stage of lung development

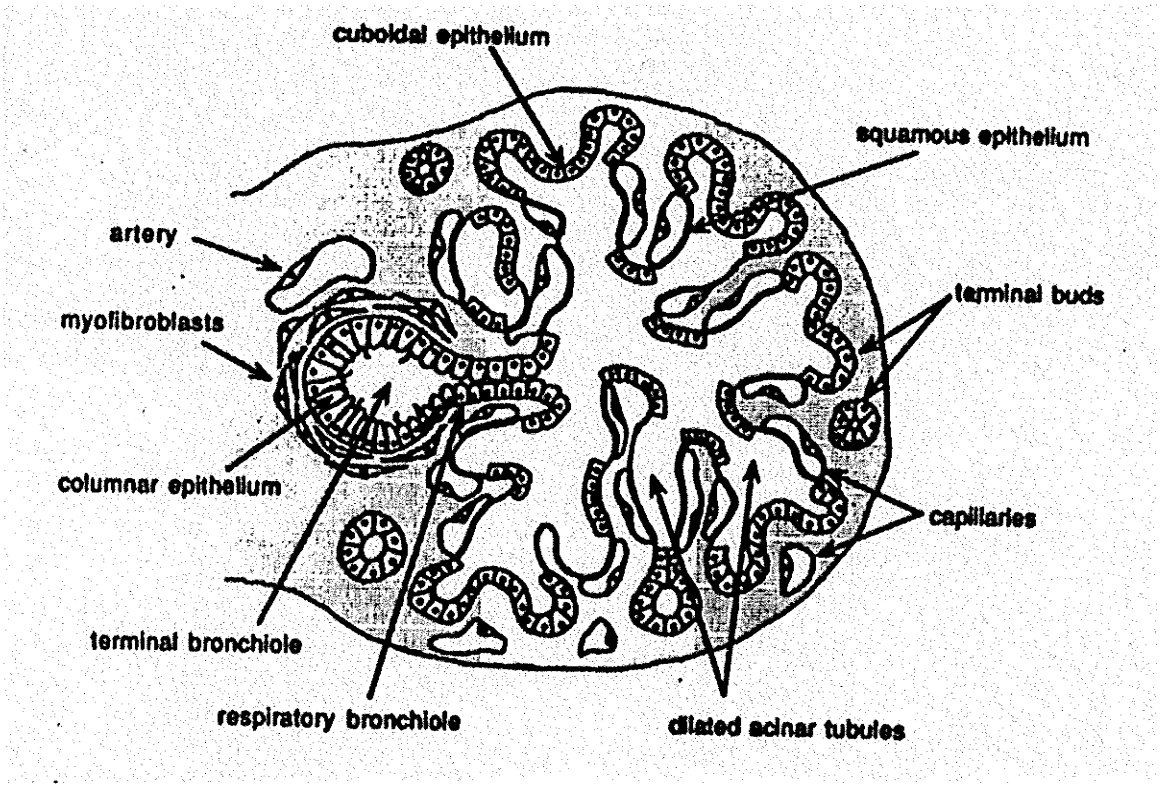


Development of subsegmental bronchi, terminal bronchioles, acinar tubules, mucous glands, cartilage, and smooth muscle happens at this stage.

Whitsett, J. A. and S. Wert (1998). Molecular determinants of lung development. Kendig's Disorders of the Respiratory Tract in Children. V. Chernick, T. Boat and E. L. Kendig. Philadelphia, WB Saunders: 3-18.

FIGURE 3

The Canalicular Stage of lung development

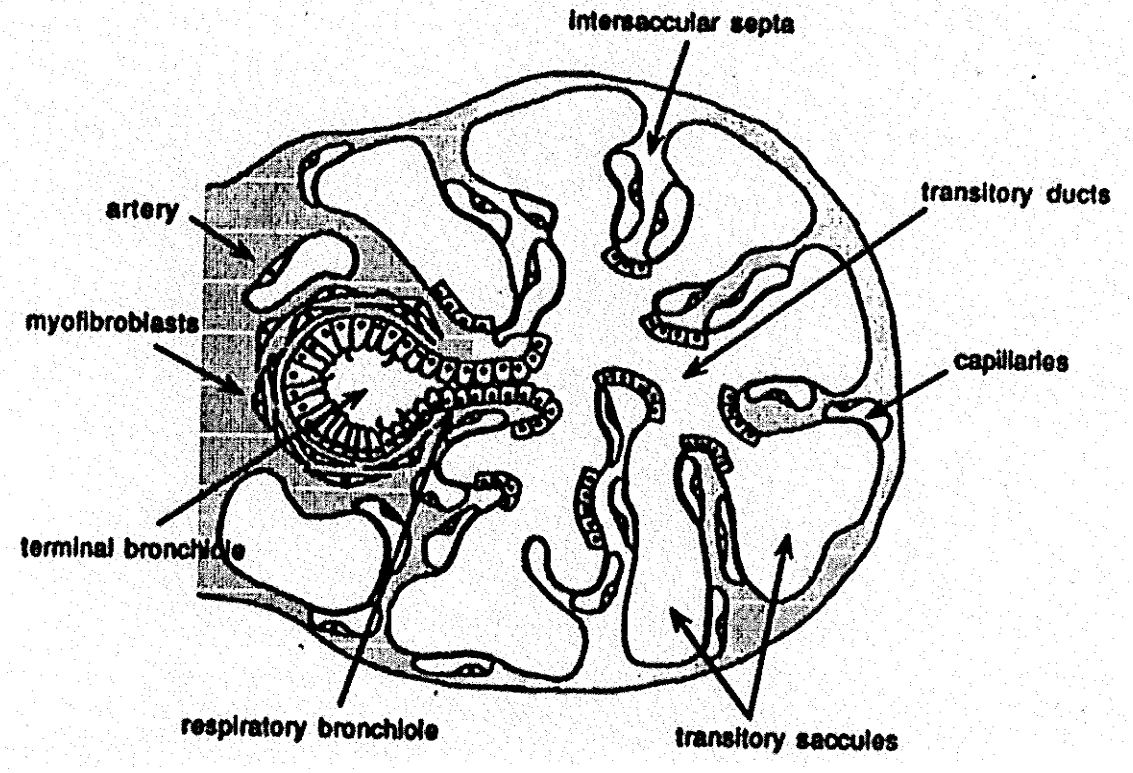


Development of respiratory bronchioles, acinus formation, vascularization, and epithelial cells type I and type II differentiation occurs at this stage.

Whitsett, J. A. and S. Wert (1998). Molecular determinants of lung development. Kendig's Disorders of the Respiratory Tract in Children. V. Chernick, T. Boat and E. L. Kendig. Philadelphia, WB Saunders: 3-18.

FIGURE 4

The Saccular stage of lung development

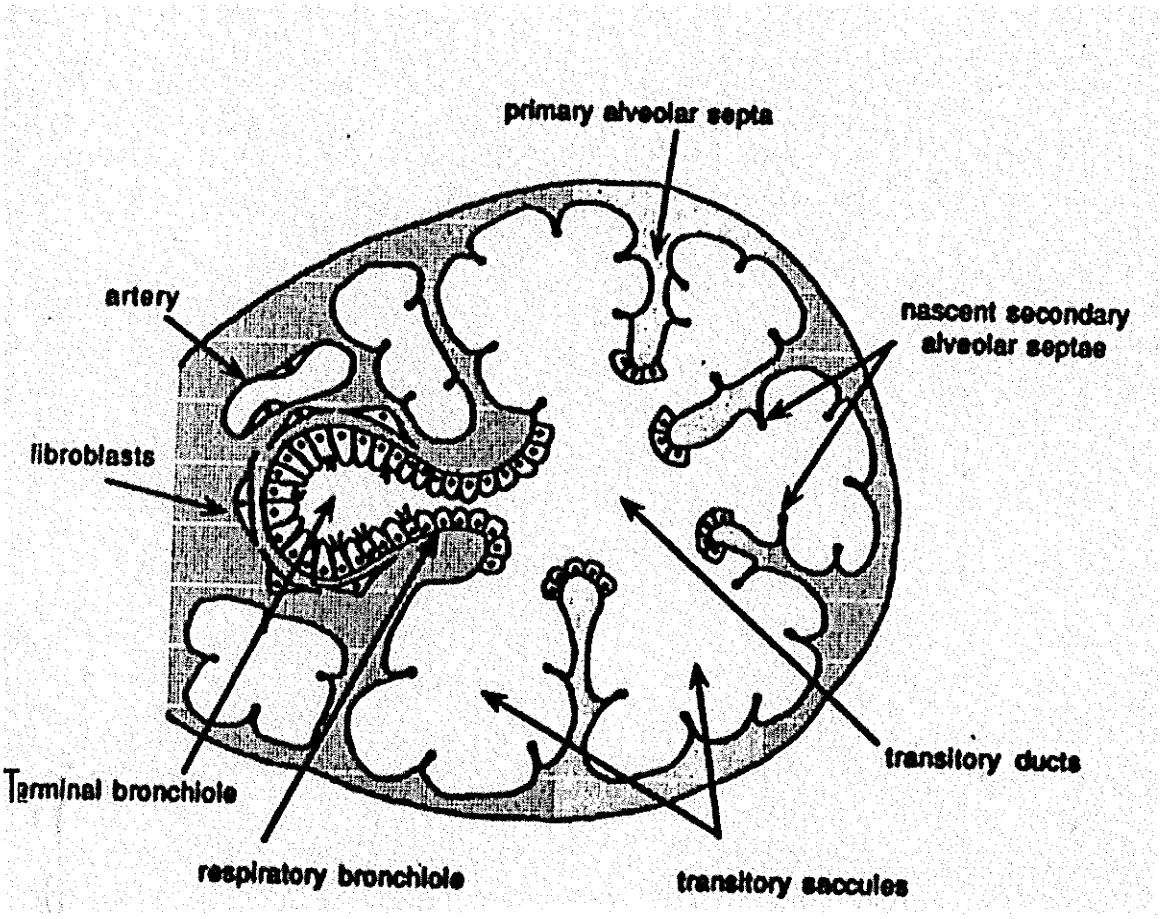


Dilation and subdivision of alveolar saccules and increase in gas exchange surface area occurs at this stage.

Whitsett, J. A. and S. Wert (1998). Molecular determinants of lung development. Kendig's Disorders of the Respiratory Tract in Children. V. Chernick, T. Boat and E. L. Kendig. Philadelphia, WB Saunders: 3-18.

FIGURE 5

Early alveolar stage of lung development



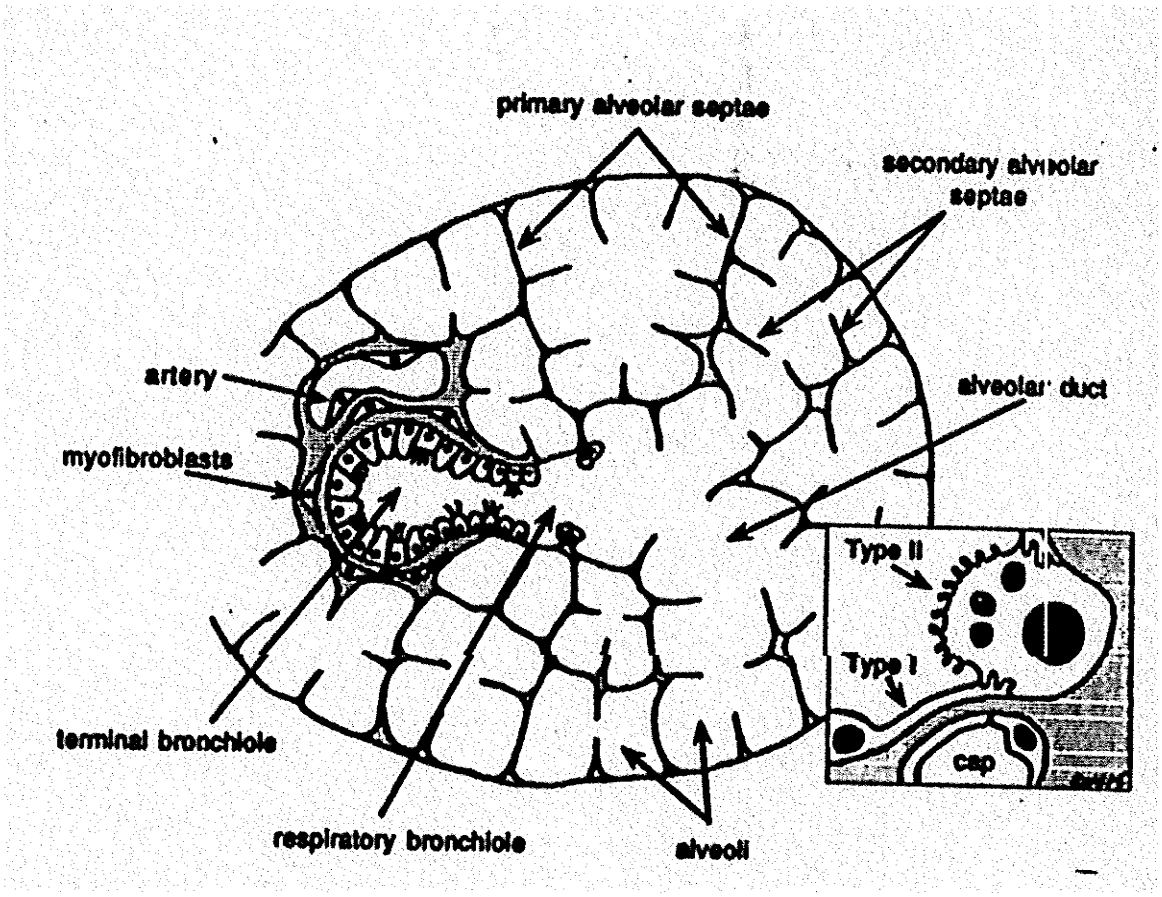
Further growth and alveolarization, and maturation of alveolar capillary network happen at this stage.

The edges of the saccules contain discrete bundles of elastin and muscle, which form small crests subdividing the walls. These crests elongate to produce primitive alveoli which have double capillary supply with mesenchymal tissue between the two layers of epithelial cells.

Whitsett, J. A. and S. Wert (1998). Molecular determinants of lung development. Kendig's Disorders of the Respiratory Tract in Children. V. Chernick, T. Boat and E. L. Kendig. Philadelphia, WB Saunders: 3-18.

FIGURE 6

Secondary alveolar stage of lung development



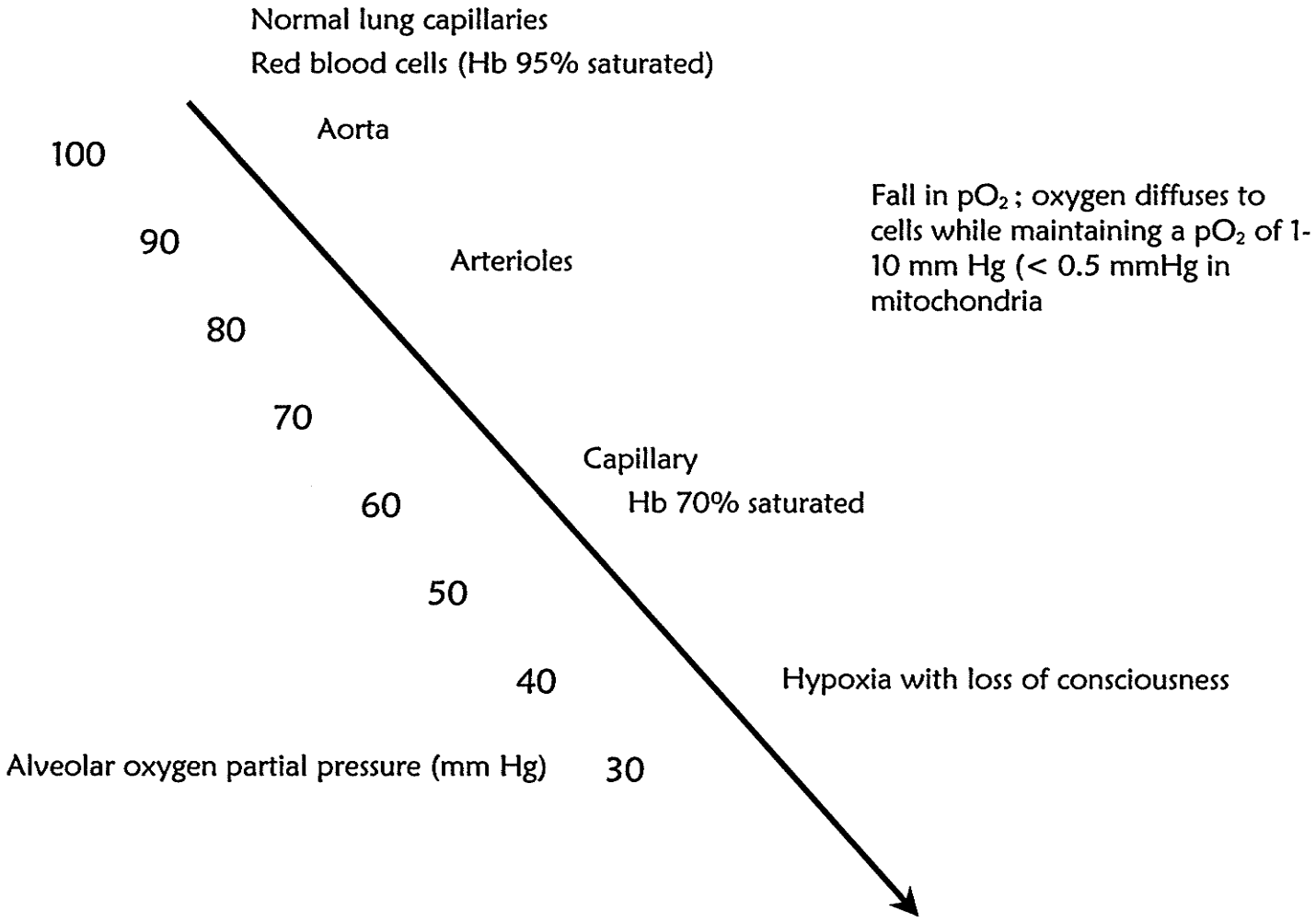
Further growth and alveolarization, and maturation of alveolar capillary network occur at this stage.

Mature alveoli with a single capillary eventually line the saccules, now called alveolar ducts.

Whitsett, J. A. and S. Wert (1998). Molecular determinants of lung development. Kendig's Disorders of the Respiratory Tract in Children. V. Chernick, T. Boat and E. L. Kendig. Philadelphia, WB Saunders: 3-18.

FIGURE 7

Oxygen concentration in the human body

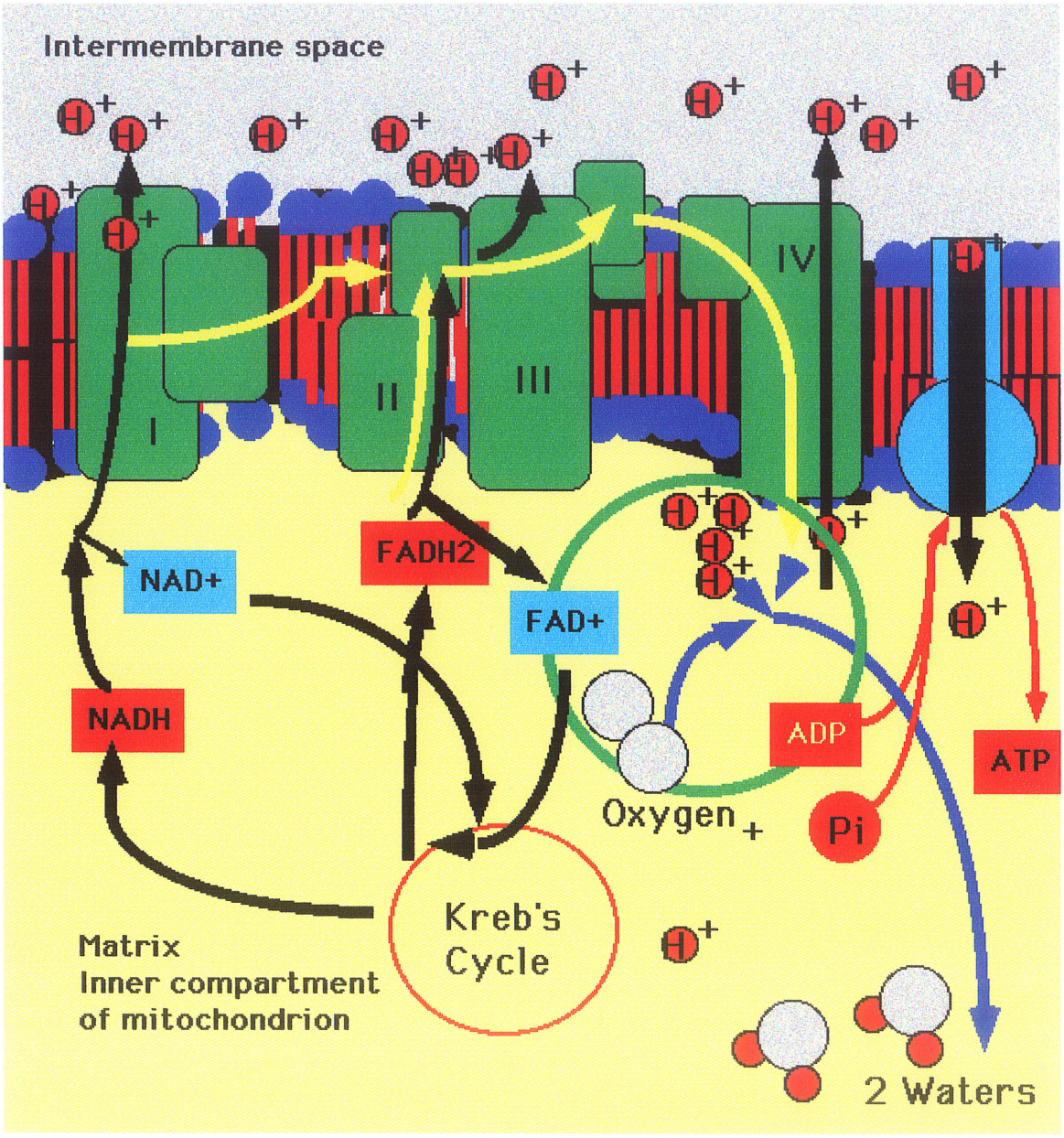


The approximate pO_2 in the human body. Most cells are exposed to a relatively low pO_2

Adapted from Halliwell & Gutteridge Free Radicals in Biology and Medicine 2000

FIGURE 8

Electron transport chain

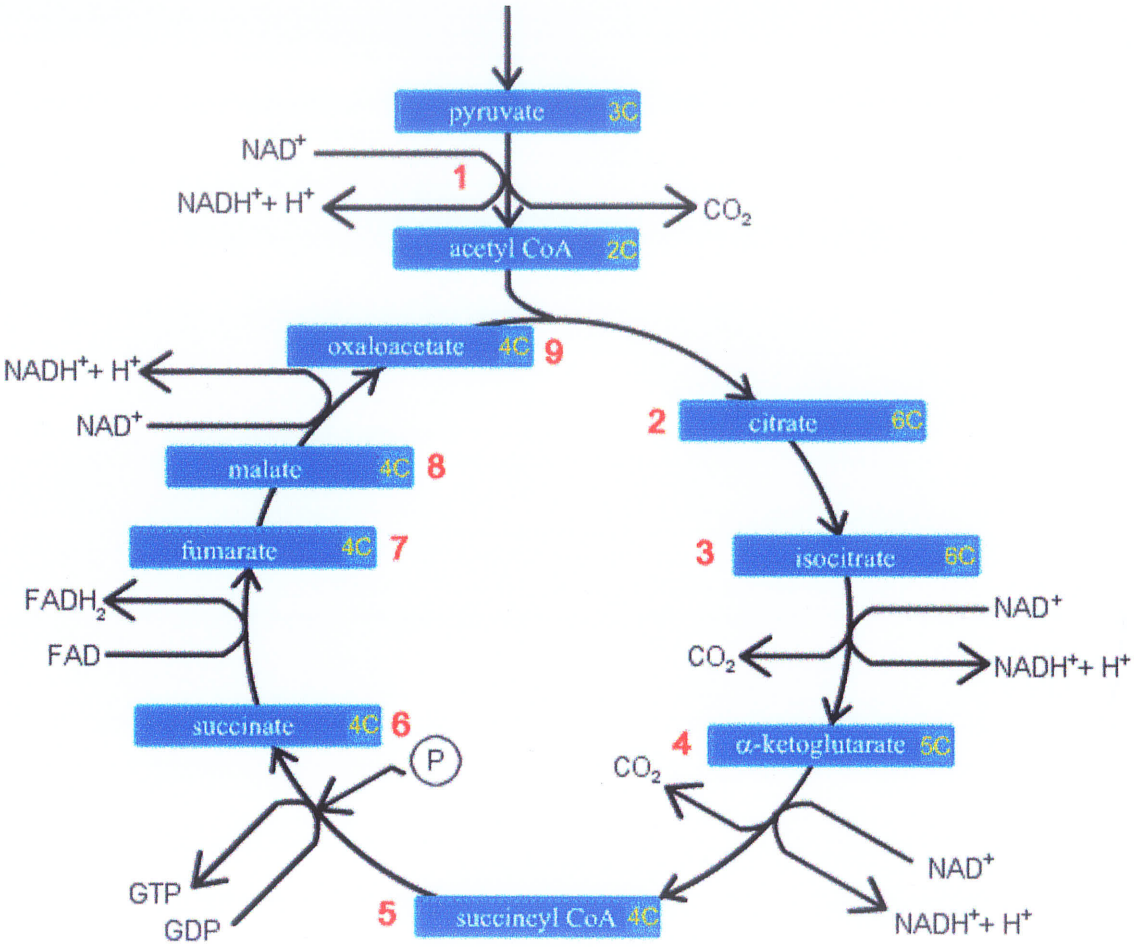


The presence of a series of electron transport molecules is a key component for the production of energy in the form of ATP in both aerobic respiration and the light-dependent reactions of photosynthesis.

http://staff.jccc.net/pdecell/cellresp/ets.html

FIGURE 9

Citric Acid Cycle

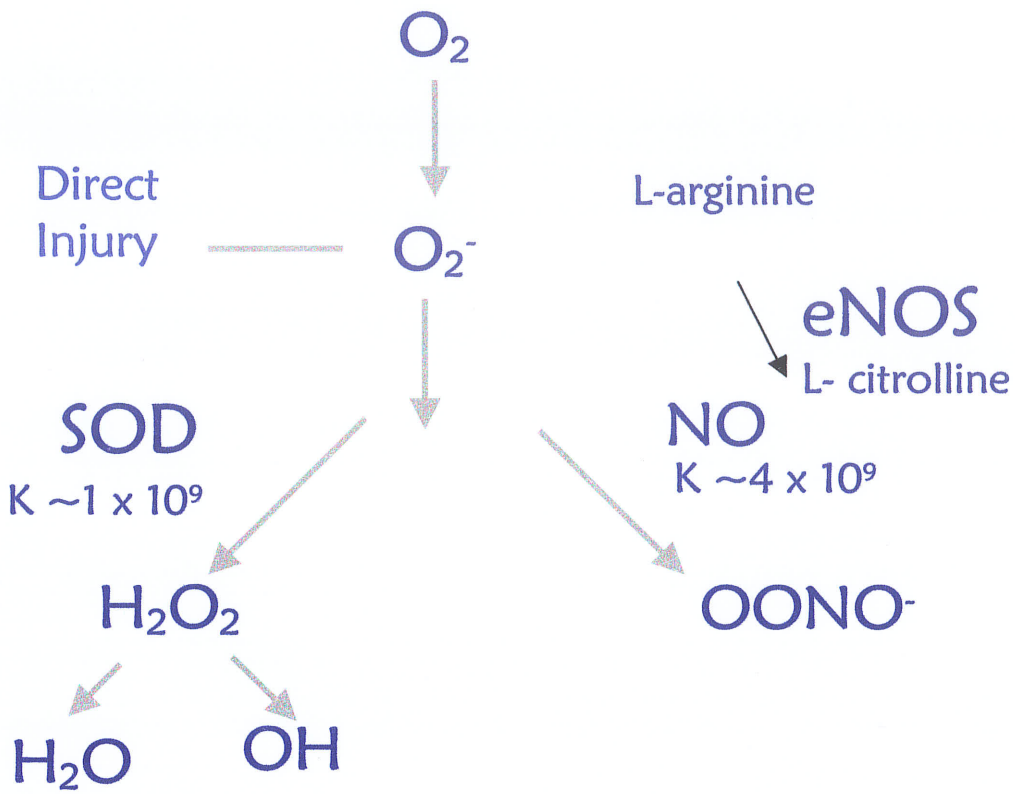


The pyruvate molecules produced during glycolysis contain energy in the bonds between their molecules. In order to use that energy, the cell must convert it to ATP. To do so, pyruvate molecules are processed through the Kreb Cycle, also known as the citric acid cycle.

http://library.thinkquest.org/C004535/aerobic_respiration.html

FIGURE 10

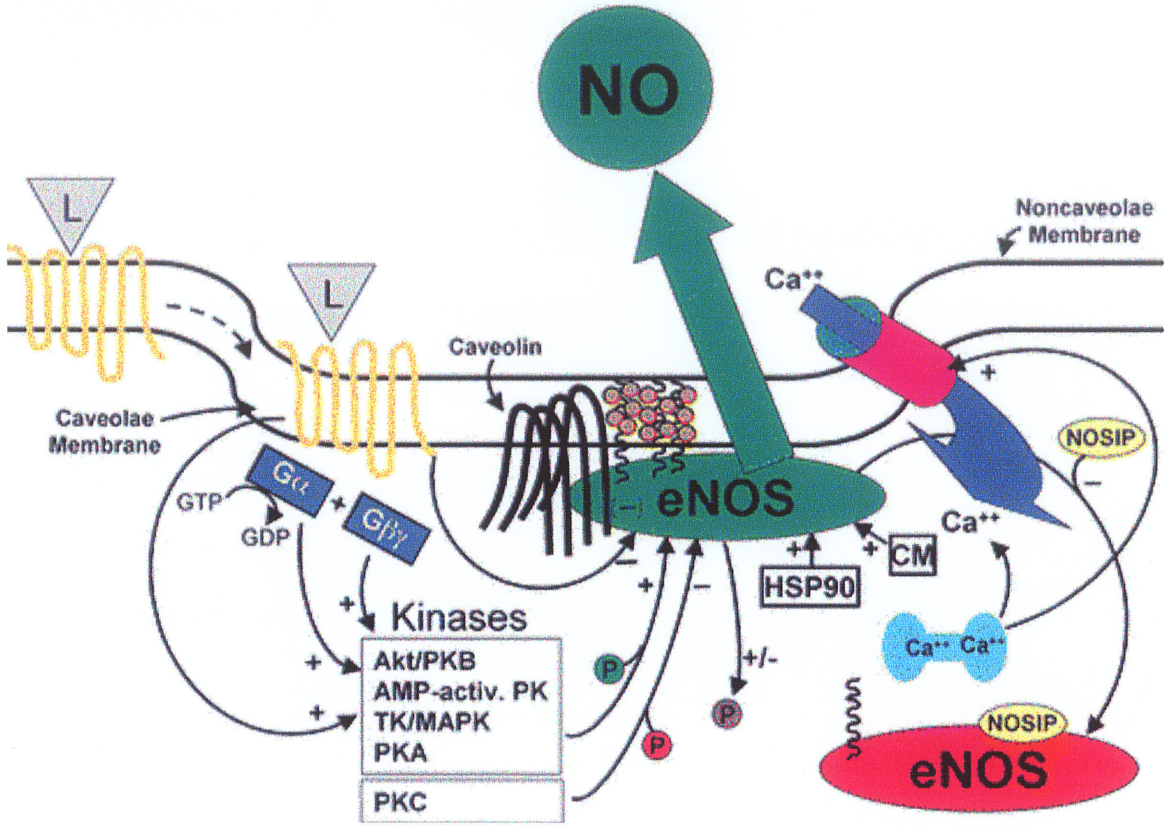
ECSOD preserves NO dependent mechanisms



The single electron reduction of oxygen produces superoxide. Superoxide, and its metabolites, is toxic to cells which leads to injury by lipid peroxidation of cell membranes, protein oxidation, DNA strand breakage, and enzyme inhibition. Superoxide is dismutated to hydrogen peroxide (spontaneously and enzymatically) which is then reduced to hydroxyl radical and water. Specific enzymes such as superoxide dismutase, catalase, and glutathione peroxidase scavenge these reactive oxygen species and prevent formation of the hydroxyl radical. Superoxide, in the presence of Nitric Oxide can form peroxinitrite which not only inactivates NO but causes nitrosative stress. Nitric oxide is formed in the presence of oxygen by the conversion of L-arginine to L- citrolline by the enzyme Nitric Oxide Synthase.

FIGURE 11

Mechanisms regulating eNOS activity in endothelial cell caveolae



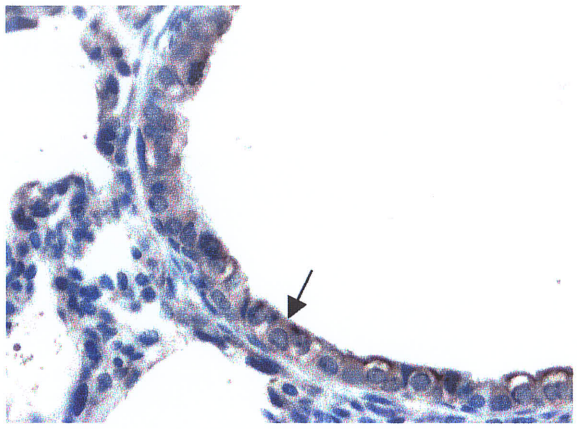
eNOS is localized to cholesterol-enriched (purple circles) caveolae, where interaction with caveolin attenuates the activity of the enzyme. Typical eNOS stimulation is initiated by ligand (L) binding to plasma membrane receptors then leads to the stimulation of multiple potential kinase pathways. Phosphorylation events result in eNOS stimulation (+) or eNOS inactivation (−). eNOS activity requires the binding of calcium-calmodulin. eNOS activity is enhanced by HSP90 binding. eNOS intracting protein (NOSIP) binds to eNOS to cause translocation of the enzyme from caveolae to intracellular sites, resulting in diminished NOS activity (change from green to red).

Shaul, P.W., Regulation of endothelial nitric oxide synthase: location, location, location. 2002. Annual Review of Physiology Vol. 64: 749-774

L: Ligand
NOSIP: NOS interacting protein

FIGURE 12

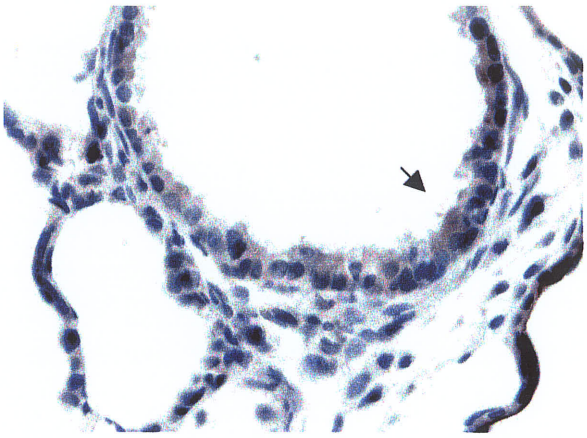
Immunohistochemical staining for ECSOD in neonate rat lung tissue



In immunohistochemical staining for ECSOD in neonate rat lung tissue, ECSOD is detected with brown Diaminobenzine developing reagent with Hematoxylin counterstain (blue). Similar staining of ECSOD and eNOS is seen especially in the immature lung. The arrow points to positive staining for ECSOD protein which is located intracellular at apical side of airway epithelial cell (40 x)

FIGURE 13

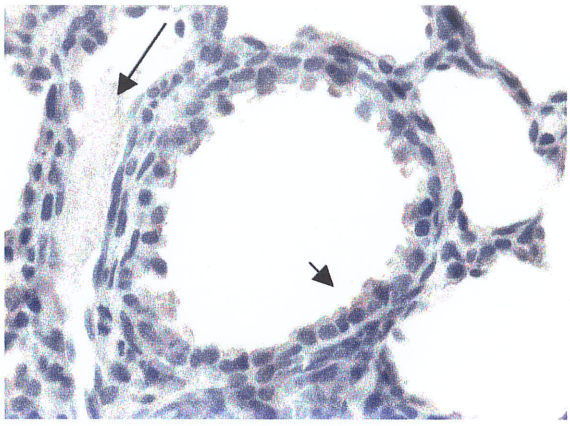
Immunohistochemical staining for eNOS in neonate rat lung tissue



In immunohistochemical staining for eNOS in neonate rat lung tissue, eNOS is detected with brown Diaminobenzine developing reagent with Hematoxylin counterstain (blue). Similar staining of ECSOD and eNOS is seen, especially in the immature lung. The arrow points to positive staining for eNOS protein in airway epithelial cell (40 x).

FIGURE 14

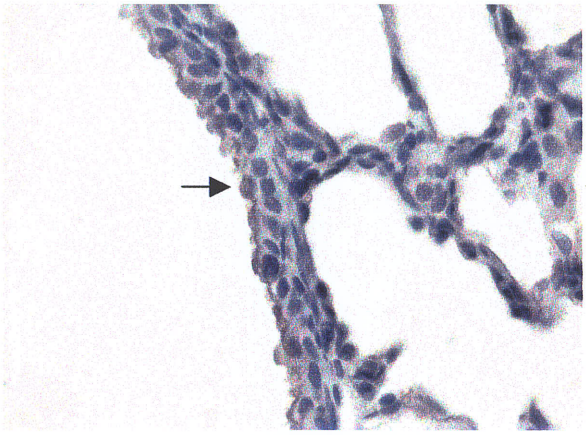
Immunohistochemical staining for ECSOD in 2 day rat lung tissue



In immunohistochemical staining for ECSOD in 2 day rat lung tissue, ECSOD is detected with brown Diaminobenzine developing reagent with Hematoxylin counterstain (blue). Similar staining of ECSOD and eNOS is seen especially in the immature lung. The arrows points to positive staining for ECSOD in airway epithelial cells and in the pulmonary vessels and red blood cells (40 x)

FIGURE 15

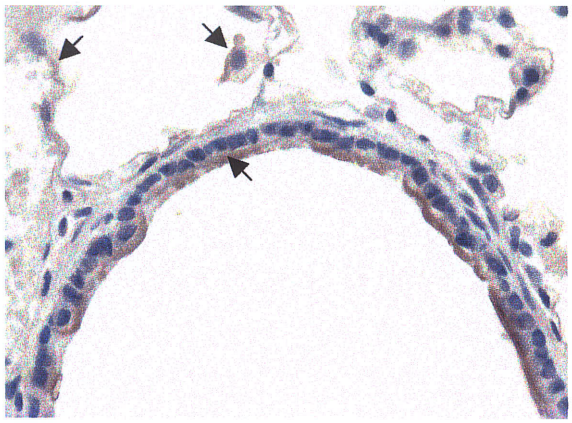
Immunohistochemical staining for eNOS in 2 day rat lung tissue



In immunohistochemical staining for eNOS in 2 day rat lung tissue, eNOS is detected with brown Diaminobenzine developing reagent with Hematoxylin counterstain (blue). Similar staining of ECSOD and eNOS is seen especially in the immature lung. The arrow points to positive staining for eNOS in airway epithelial cell (40 x)

FIGURE 16

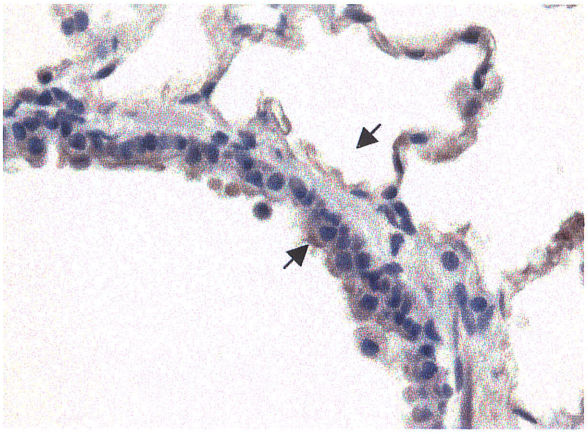
Immunohistochemical staining for ECSOD in adult rat lung tissue



In immunohistochemical staining for ECSOD in adult rat lung tissue, ECSOD is detected with brown Diaminobenzine developing reagent with Hematoxylin counterstain (blue). Similar staining of ECSOD and eNOS is seen especially in the immature lung. The arrows point to positive staining in airway epithelial cell and alveolar epithelial cell (40 x)

FIGURE 17

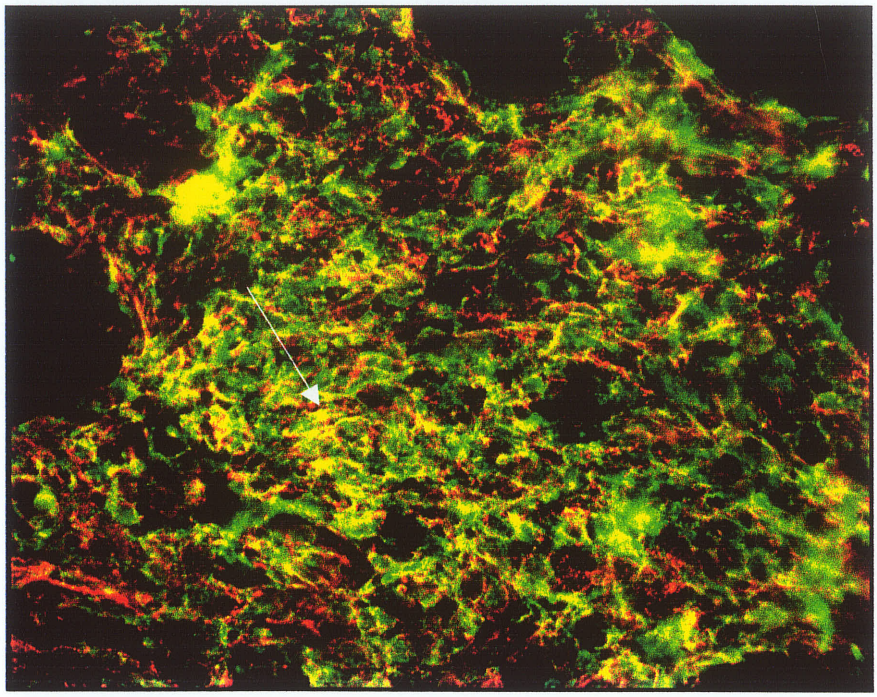
Immunohistochemical staining for eNOS in adult rat lung tissue



In immunohistochemical staining for eNOS in adult rat lung tissue, eNOS is detected with brown Diaminobenzine developing reagent with Hematoxylin counterstain (blue). Similar staining of ECSOD and eNOS is seen especially in the immature lung. The arrow point to positive staining for eNOS in airway epithelial cell. The upper arrow points to staining in the alveolar epithelial cell (40 x)

FIGURE 18

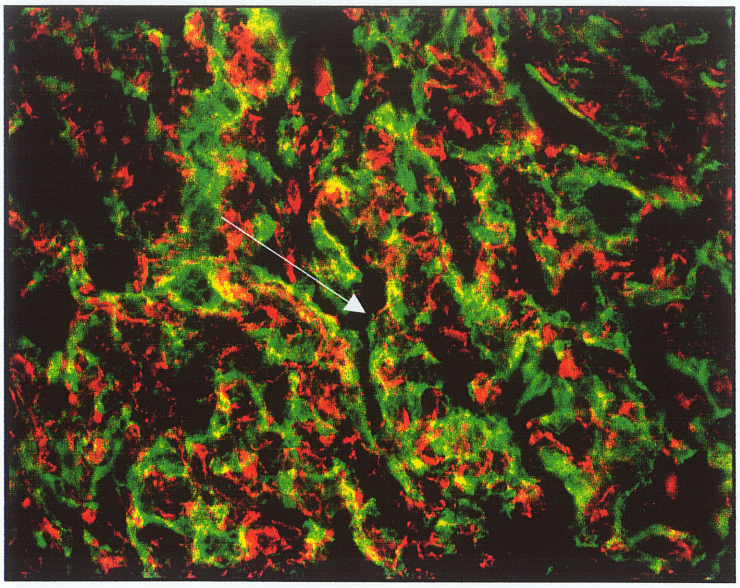
Immunofluorescent staining for ECSOD and eNOS in neonate rat lung



Frozen section of neonate rat lung labeled with immunoflourescent antibodies, anti mouse CY3 for ECSOD (red) and anti rabbit FITC for eNOS (green). Yellow staining suggests co-localization of ECSOD and eNOS. The arrow shows this co-localization of two proteins in lung (60x)

FIGURE 19

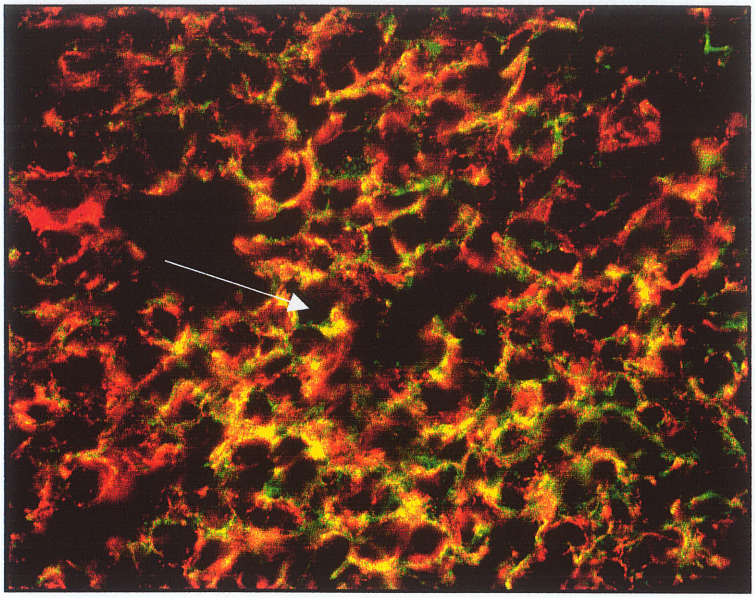
Immunofluorescent staining for ECSOD and Caveolin in neonate rat lung



Frozen section of neonate rat lung labeled with immunofluorescent antibodies, anti mouse CY3 for ECSOD (red) and anti rabbit FITC for Caveolin (Cav-1)(green). Yellow staining suggests co-localization of ECSOD and Caveolin. Cav-1 and ECSOD has minimal yellow staining. The arrow shows lung cells in which there is minimal/no yellow staining implying that there is no co-localization of these two proteins (60 x)

FIGURE 20

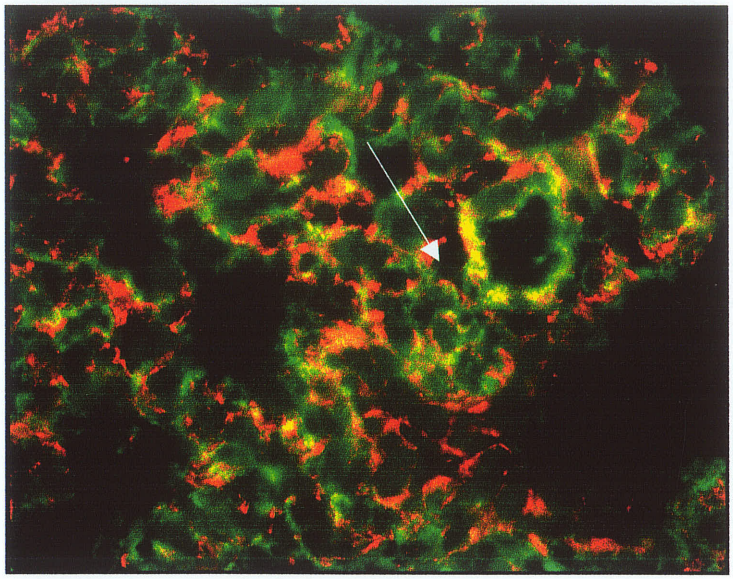
Immunofluorescent staining for ECSOD and eNOS in 4 day rat lung



Frozen section of 4 day rat lung labeled with immunofluorescent antibodies, anti mouse CY3 for ECSOD (red) and anti rabbit FITC for eNOS (green). Yellow staining suggests co-localization of ECSOD and eNOS. eNOS and ECSOD co-localize with strong yellow stain which diminishes with age. The arrow shows co-localization of these two proteins in lung which is less apparent than in neonatal lung (60 x).

FIGURE 21

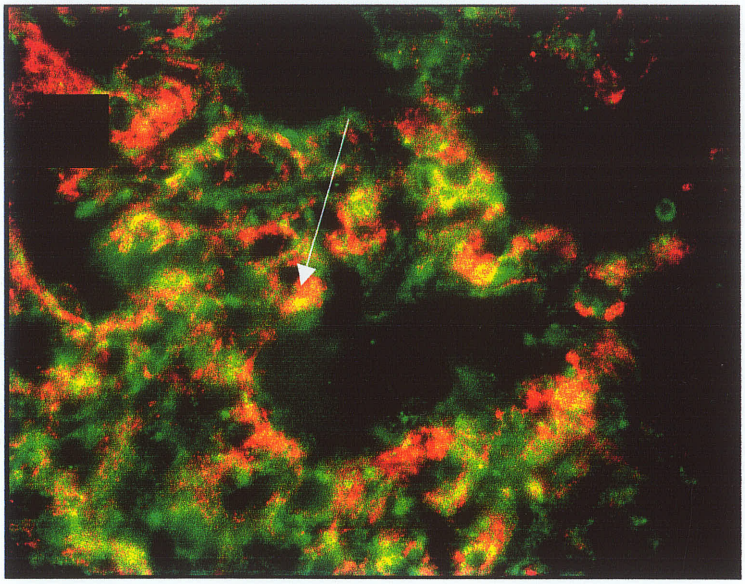
Immunofluorescent staining for ECSOD and Caveolin in 4 day rat lung



Frozen section of 4 day rat lung labeled with immunofluorescent antibodies, anti mouse CY3for ECSOD (red) and anti rabbit FITC for Caveolin (Cav-1)(green). Yellow staining suggests co-localization of ECSOD and Caveolin. Cav-1 and ECSOD has minimal yellow staining. The arrow shows lung cells in which there is minimal/no yellow staining implying that there is minimal/no co-localization of these two proteins in lung (60x)

FIGURE 22

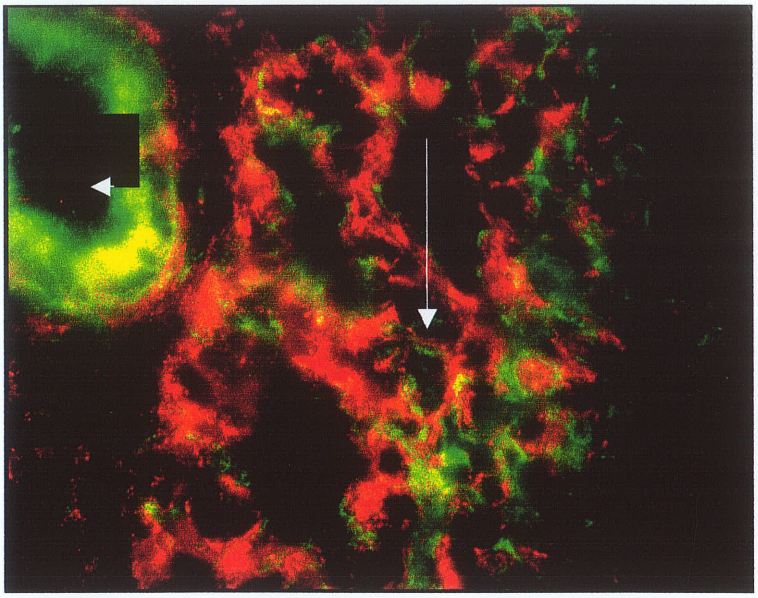
Immunofluorescent staining for ECSOD and eNOS in 7 day rat lung



Frozen section of 7 day rat lung labeled with immunofluorescent antibodies, anti mouse CY3for ECSOD (red) and anti rabbit FITC for eNOS (green). Yellow staining suggests co-localization of ECSOD and eNOS. eNOS and ECSOD co-localize with strong yellow stain which diminishes with age. The arrow points to yellow staining implying co-localization of these two proteins in the lung which is less apparent than in 4 day old rat (60x)

FIGURE 23

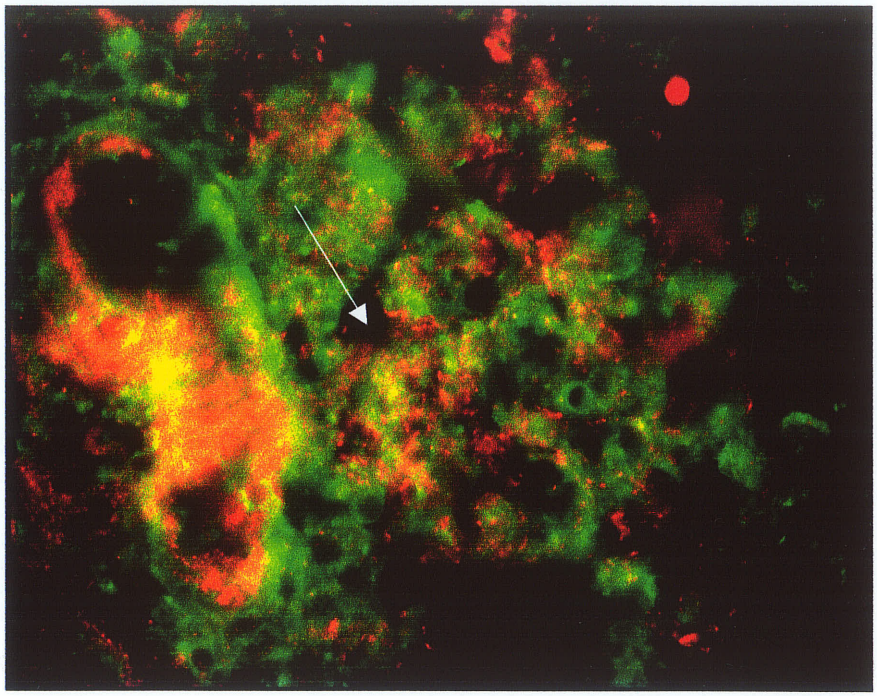
Immunofluorescent staining for ECSOD and Caveolin 7 day rat lung



Frozen section of 7 day rat lung labeled with immunofluorescent antibodies, anti mouse CY3for ECSOD (red) and anti rabbit FITC for Caveolin (Cav-1)(green). Yellow staining suggests co-localization of ECSOD and Caveolin. Cav-1 and ECSOD has minimal/no yellow staining. The arrow shows airway epithelial cell layer with no/minimal yellow staining suggesting that there is no co-localization of these two proteins in lung. The arrowhead points to a vessel lumen (60x)

FIGURE 24

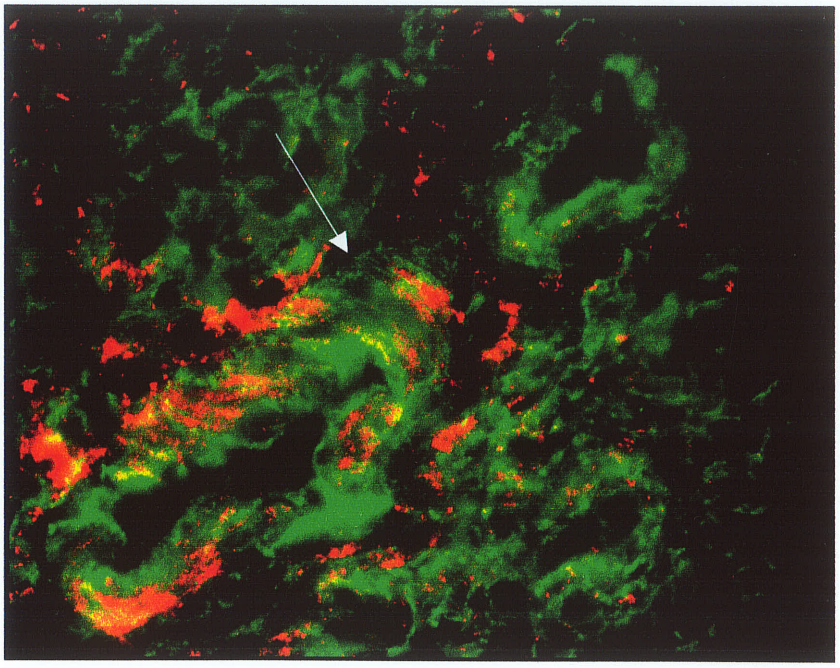
Immunofluorescent staining for ECSOD and eNOS in adult rat lung



Frozen section of Adult rat lung labeled with immunofluorescent antibodies, anti mouse CY3for ECSOD (red) and anti rabbit FITC for eNOS (green). Yellow staining suggest co-localization of ECSOD and eNOS. eNOS and ECSOD co-localize with strong yellow stain which diminishes with age. The arrow points to lung cells in which there is minimal/no yellow staining suggesting that there is no co-localization of these two proteins (60x)

FIGURE 25

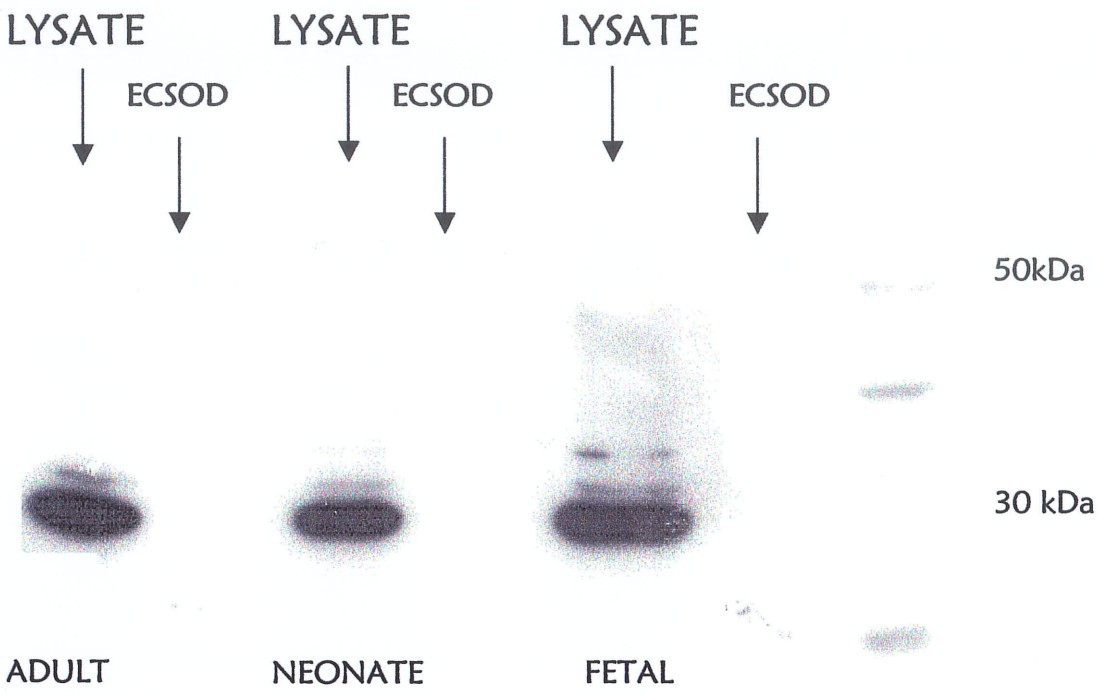
Immunofluorescent staining for ECSOD and Caveolin in adult rat lung



Frozen section of Adult rat lung labeled with immunofluorescent antibodies, anti mouse CY3for ECSOD (red) and anti rabbit FITC for Caveolin (Cav-1)(green). Yellow staining suggest co-localization of ECSOD and Caveolin. Cav-1 and ECSOD has minimal/no yellow staining. The arrow points to lung cells with minimal/no yellow staining suggesting no co-localization of these two proteins (60x)

FIGURE 26

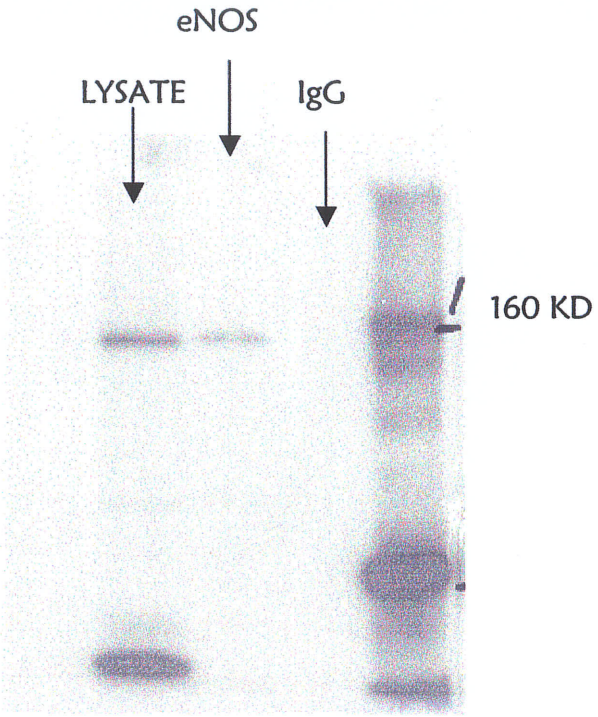
Immunoprecipitation of eNOS with probing for ECSOD



Immunoprecipitation of eNOS and probing for ECSOD (by Western blot) in fetal, neonate, and adult rat lung fresh tissue. This representative blot shows that there is no staining for ECSOD in eNOS precipitate. Whole lysate is used as the positive control (should note bands) and IgG is used as a negative control (no bands)(not shown).

FIGURE 27

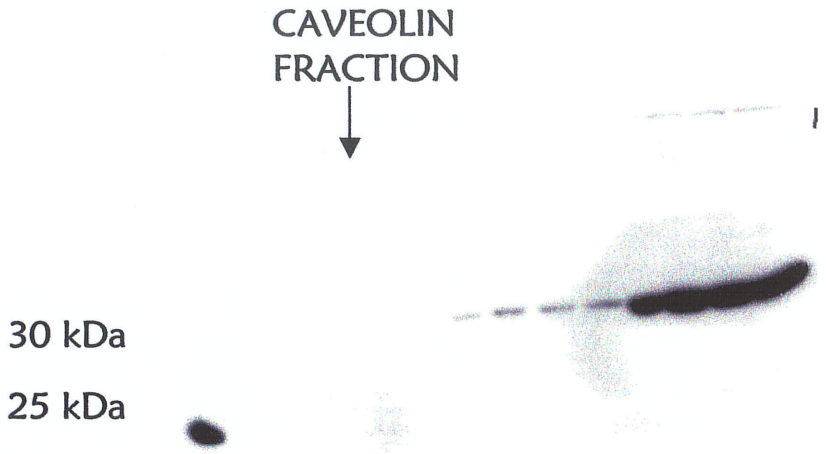
Immunoprecipitation with eNOS (control)



Immunoprecipitation with eNOS on fetal, neonate, and adult rat lung fresh tissue then probed for eNOS. This was done as a control measure for immunoprecipitation studies with ECSOD. The representative blot reveals binding of eNOS antibody to immobilized protein G.

FIGURE 28

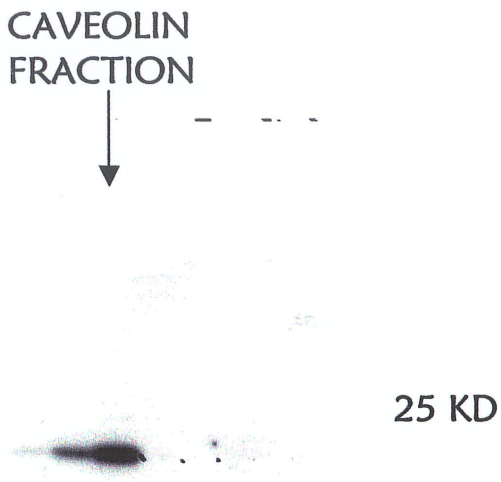
Analysis of sucrose gradient caveolin fractions (Western blot) probed for ECSOD



Western blot analysis of sucrose gradient caveolin fractions in fetal, neonate and adult lung epithelial cell cultures. The caveolin fractions were probed with ECSOD antibody. This representative blot shows that there is no staining for ECSOD in caveolin.

FIGURE 29

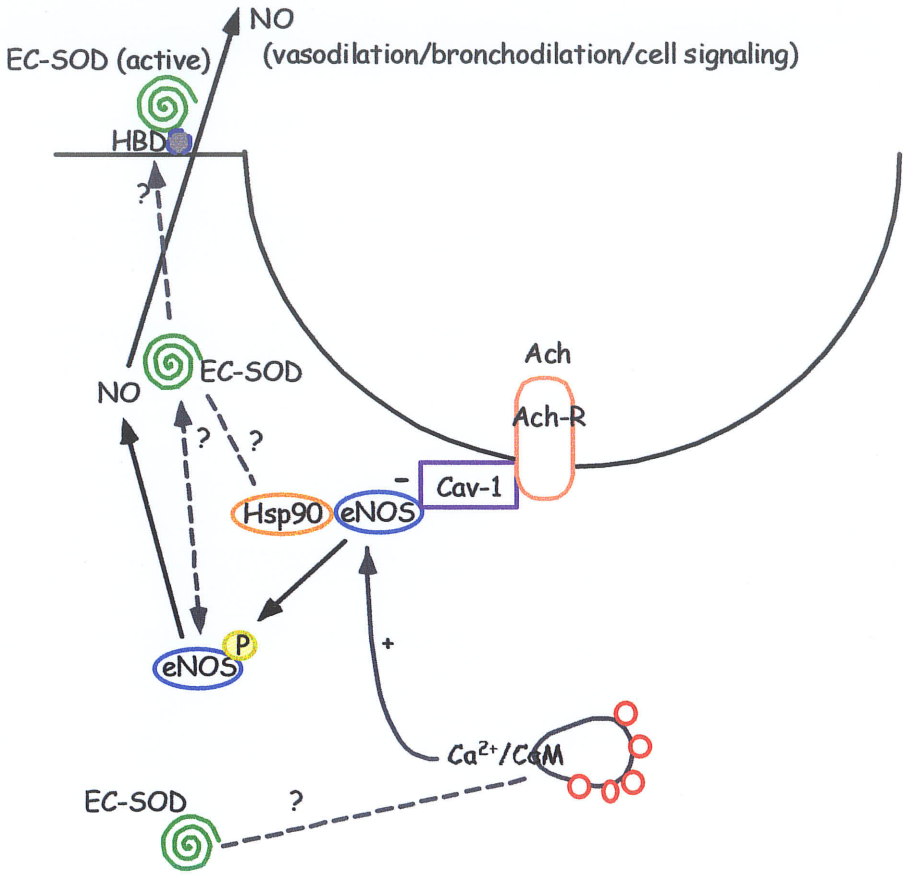
Analysis of sucrose gradient caveolin fractions (Western blot) probed for Cav-1



Western blot analysis of sucrose gradient caveolin fractions in fetal, neonate and adult lung epithelial cell cultures. The caveolin fractions were probed with Cav-1 antibody. This was done as a control measure for sucrose gradient caveolin fractions with ECSOD. This representative blot shows that there is staining for Cav-1 in caveolin fractions.

FIGURE 30

Potential pathways regulating ECSOD in the cell



ECSOD and eNOS co-localization in airway epithelial cells in lung may confer location of ECSOD protein to the cytosol where active eNOS is located or the cell membrane. The inability to co-localize caveolin and ECSOD in airway epithelium may imply that caveolin protein is not important in ECSOD regulation or may act via other mediators (which are shown with dotted line). We also propose that ECSOD may be secreted with NO˙ as a carrier protein to protect NO˙ from oxidative damage as it traverses the cell membrane to other cells.