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Invited Review

The Role of Nitric Oxide in the Regulation of Ion Channels in Airway Epithelium: Implications for Diseases of the Lung

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The human respiratory tract is covered with airway surface liquid (ASL) that is essential for lung defense and normal airway function. The quantity and composition of ASL is regulated by active ion transport across the airway epithelium. Abnormal electrolyte transport produces changes in ASL volume and composition, inhibits mucociliary clearance and leads to chronic infection of airway surfaces, as is evident in cystic fibrosis. Agonists that induce intracellular increases in cAMP or Ca^{2+} are generally associated with electrolyte secretion. While these mechanisms have been studied in detail for many years, modulation of ion channels by nitric oxide (NO) has emerged only recently as a significant determinant of ion channel function. NO is a physiological regulator of transepithelial ion movement and alterations of its generation and action may play an important role in the pathogenesis of lung disorders characterized by hypersecretion of ASL. This review presents the current understanding of regulation of airway epithelial ion channels by NO and attempts to highlight the importance of this regulation for lung defense.

Keywords: airway surface liquid; cyclic GMP; transepithelial transport; cystic fibrosis

INTRODUCTION

Recent progress in our understanding of the metabolic and biochemical functions of airway epithelial cells has changed the traditional view of the epithelium as a relative passive physical barrier that protects sensory nerves and smooth muscle from stimulation by inhaled irritants. The current view of airway epithelium is focused on the central role played by epithelial cells in the pulmonary host defense. The human respiratory tract is covered with a thin layer of airway surface liquid (ASL), which is essential for lung defense and normal airway function. The quantity and composition of ASL is thought to be regulated by active ion transport across the airway epithelium.[1] Ion movement towards the airway lumen promotes fluid secretion and ASL hydration, whereas ion movement towards the submucosa permits fluid absorption and dehydration of ASL. Abnormal electrolyte transport

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produces changes in ASL volume and composition, depresses mucociliary clearance and leads to chronic infection of airway surfaces, as is evident in cystic fibrosis (CF).[2]

Regulation of cellular ion transport processes depends on the availability of ATP and is frequently categorized on the basis of potential second messengers involved in its activation.[3] While the role of cAMP and Ca^{2+} in regulation of Cl^-secretion in human airways is well established, much less is known about the contribution of nitric oxide to ASL homeostasis.

Nitric oxide (NO) is present in the exhaled air of humans but its exact origin and a relative contribution to NO synthesis by various cellular sources within the respiratory tract are unknown. Some studies have suggested that exhaled NO is produced in the lung, whereas other showed that exhaled NO is produced in the terminal region of the bronchial tree (for review see[4]). Recently, it has been proposed that NO production in the lung can be divided into contributions from the alveoli and the conducting airways.[5]

NO is synthesized via the oxidation of L-arginine to L-citrulline by the Ca^{2+}-dependent nitric oxide synthases (NOS), endothelial NOS (eNOS) and neuronal NOS (nNOS).[6] These enzymes generate small quantities of NO that participate in physiological functions via activation of the soluble guanylyl cyclase (sGC). NO is also synthesized by an inducible, Ca^{2+}-independent form of NOS (iNOS), which generates large and sustained amounts of NO that may be beneficial or harmful for the cells that produce it, as well as for other cells in the vicinity. All of these isoforms of NOS have been identified in human respiratory tract and are thought to contribute to NO production.[7]

There are several important observations that suggest the involvement of NO in a variety of physiological and pathological processes in the airways. Endogenously produced NO regulates mucociliary activity in the upper respiratory tract by modulating cilia beat frequency, thus contributing to efficient mucociliary clearance.[8] The concentrations of NO in nasal passages greatly exceed those that are inhibitory to some bacteria, indicating a role for NO in host defense.[4] Increased production of NO at sites of inflammation has been shown to exert both harmful and protective effects. Proinflammatory actions of NO include activation of enzymes, such as metalloproteinases, or production of the highly oxidizing agent peroxynitrite ($ONOO^-$), which may directly cause tissue damage.[9] A protective role of iNOS in inflammation is suggested by experiments with iNOS knockout mice, which indicate that the absence of iNOS leads to more severe inflammatory response than in the wild type mice.[10] More recently, it has been found that the airway epithelium from patients with cystic fibrosis (CF) expresses much lower levels of iNOS mRNA and protein than normal lung.[11] Since upregulation of iNOS expression is thought to enhance anti-microbial effects and reduce neutrophil sequestration in the lung, its relative lack in CF may be responsible for a significant neutrophilic infiltration that characterizes the disease.[11]

The role of NO in regulation of ion channels and transporters is now well established (Fig. 1). NO increases the activity of chloride channels[12], cyclic nucleotide-gated channels[13], several classes of K^+channels[14], but inhibits epithelial Na^+ channels[15–17]. In addition, NO has been shown to inhibit Na^+/K^+-ATPase[18], the Na^+/H^+ exchanger[19], and to regulate permeability of the paracellular pathway in airway inflammation[20]. Most of these effects were shown to involve activation of the NO/cGMP-dependent pathway, but there is also some evidence for cGMP-independent NO action.[15] These studies suggest that NO in human airways plays an important role in the control of transepithelial electrolyte and mucus secretion, especially under inflammatory conditions characterized by increased levels of NO generated by iNOS.

The aim of this review is to examine the effects of NO on the physiological regulation of transepithelial electrolyte secretion in human air-

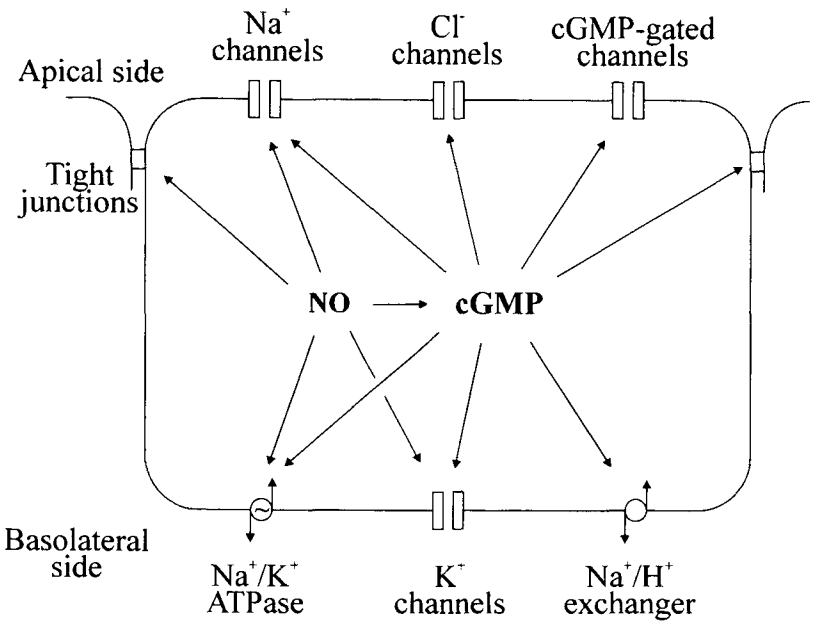

FIGURE 1 Molecular targets for NO action in human airway epithelial cells. NO regulates the activities of K^+ channels, Na^+ channels, Cl^-channels, the basolateral Na^+/K^+-ATPase and the Na^+/H^+ exchanger. NO may also affect ionic permeability of tight junctions, modifying the paracellular pathway. While most of these effects were shown to involve activation of the NO/cGMP-dependent pathway, there is also significant evidence for cGMP-independent action of NO on ion channels and transporters

ways. We will briefly describe the molecular mechanisms of ion movement in human airways and discuss evidence showing a crucial role of NO in this process. We will also discuss pathological alterations of transepithelial ion movements and the clinical relevance of these changes.

REGULATION OF EPITHELIAL ION TRANSPORT IN HUMAN AIRWAYS

It is generally accepted that epithelial cells are capable of both electrolyte absorption and secretion and that the net balance between these two processes controls the net fluid movement in the airways.[3] Active Na^+ absorption and anion (either Cl^- or HCO_3^-) secretion accounts entirely for transepithelial short circuit current (I_{sc}), suggesting that the contribution of other transport processes is negligible.[3] The relative contributions of absorptive and secretive processes to transepithelial ion movement appear to vary along the respiratory tract. In airway submucosal gland cells anion secretion prevails, whereas in distal lung epithelium, Na^+ absorption from the alveolus to the interstitial space is a dominant process. While anion secretion in submucosal glands is thought to play a key role in mucosal defenses by controlling the properties and amount of gland secretions, active Na^+ transport across the alveolar epithelium creates an osmotic gradient for fluid movement, thus helping to maintain the alveoli free of fluid.

Transepithelial ion secretion or absorption is made possible by segregation of ion channels, cotransporters and pumps to either the cell's apical or basolateral membrane. Figure 2 shows a model that describes anion secretion in human airway submucosal gland cells. Depending on

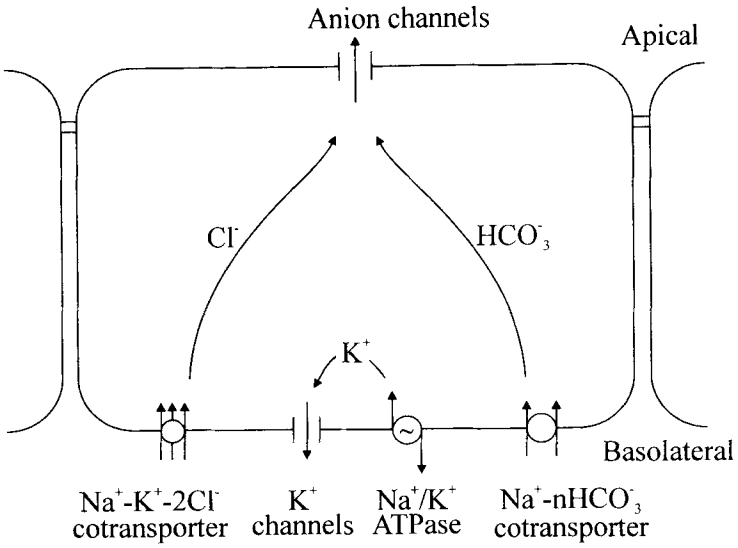

FIGURE 2 Model for anion secretion in human airway epithelial cells. Cl^- ions enter the cell via the Na^+-K^+-$2Cl^-$cotransporter, whereas HCO_3^- enters via the Na^+-$nHCO_3^-$ cotransporter, both of which are located in the basolateral membrane. The apical anion channel (CFTR) provides exit for both anions out of the cell (for details see[22]). The stoichiometry of the Na^+:HCO_3^- cotransporter in human airways (1:2 or 1:3) is unknown

the stimulus, Cl^- or HCO_3^- can enter at the basolateral membrane via electroneutral Na-K-2Cl or electrogenic Na-$n$$HCO_3^-$ cotransporters (n=2 or 3[21]), respectively.[22] The cotransporters use the energy in the transmembrane Na^+ gradient to accumulate anions within the cell above electrochemical equilibrium, which then leave the cell passively through apical membrane anion channels. The Na^+gradient that drives anion uptake is maintained by the Na^+-K^+-ATPase located in the basolateral membrane. In addition, basolateral K^+channels play important role in maintaining Cl^- secretion. The hyperpolarization of the apical membrane, which results from the activation of basolateral K^+ channels, increases the driving force for the exit of Cl^- and/or HCO_3^- ions.[23]

Regulation of anion secretion is frequently categorized according to the second messengers that are generated in the cell after exposure to a potential secretagogue.[23]Secretion can be mediated by elevation of $[cAMP]_i$, or elevation of $[Ca^{2+}]_i$. While the ion transport mechanisms regulated by these messengers have been studied for many years, the role of nitric oxide (NO) in the regulation of epithelial ion channels has emerged only recently as a significant determinant of ion channel function.[12,24] Nitric oxide has been shown to affect the function of several epithelial ion channels, and this regulation may provide a link between changes in the cell metabolic state and the permeability of the cell membrane.

INTERACTIONS OF NITRIC OXIDE WITH ION CHANNELS

NO can react in biological systems with molecular oxygen (O_2), superoxide ($O_2^{\cdot-}$) and transition metals to form a variety of nitrogen oxides (NO_x), $ONOO^-$ and metal-NO adducts, respectively (Fig. 3).[25] The products of these reactions can have both stimulatory and inhibitory effects

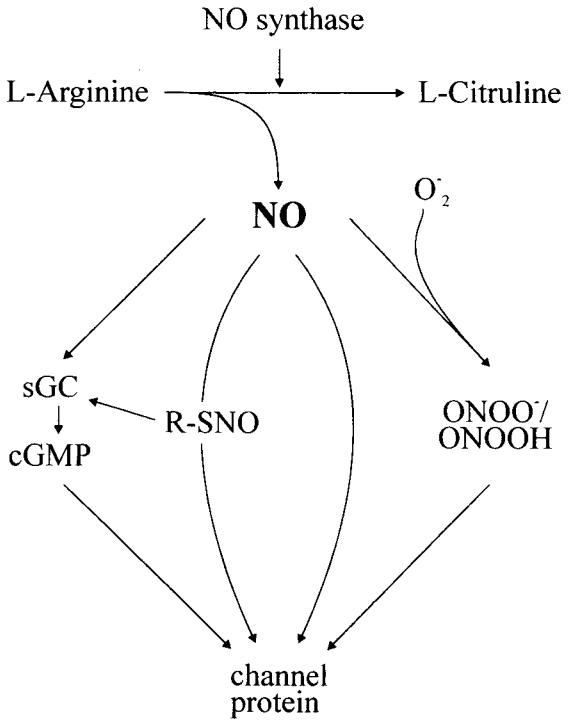

FIGURE 3 Pathways of NO interactions with ion channels. sGC – soluble guanylyl cyclase, R-SNO – S-nitrosothiols. Under physiological conditions the most likely pathway of NO action on ion channel involves activation of sGC and generation of cGMP. Under inflammatory conditions, increased production of nitrogen- and oxygen-related species may lead to excessive channel nitrosylation (R-SNO), oxidation ($ONOO^-$), or direct channel modification by NO

on ion channel function. The best-characterized mechanism of NO-mediated signal transduction is the activation of soluble guanylyl cyclase (sGC) and generation of cGMP. Activation of NO/cGMP pathway may affect ion channel function by at least four different mechanisms: 1) phosphorylation by cGMP-dependent protein kinase (PKG), 2) interaction with the cAMP-dependent pathway and inhibition of cyclic nucleotide-dependent phosphodiesterases, 3) direct activation of cyclic nucleotide gated channels, and 4) by modulation of $[Ca^{2+}]_i$ (Fig. 4).

Two isotypes of PKG have been described: PKG I (isoforms α and β) expressed in the lung, and PKG II expressed mainly in the intestine.[26] While PKG II has been shown to activate cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in intestine epithelial cells[26], neither the α nor β isoforms of PKG I activated CFTR in the lung[27]. However, PKG Iα has been shown to phosphorylate CFTR.[27] It is now well established that CFTR can function not only as anion channel, but also as a regulator of other ion and water channels.[28] Therefore, it is possible that PKG Iα phosphorylates sites that are not involved in CFTR channel function, but this modification could trigger regulation of other ion or water channels by CFTR.

Cyclic GMP and cAMP can exert opposite effects on ion secretion in some epithelial tissues.[29,30] However, other reports suggest that the effects of cGMP on Cl^- secretion could be similar to those induced by cAMP. In human airways cGMP has been shown to activate cAMP-dependent ion channels indirectly, by inhibiting cAMP phosphodiesterase (PDE III) and, consequently, increasing intracellular cAMP.[31,32] Moreover, cGMP could cross-activate protein kinase A and activate cAMP-dependent Cl^-channels.[33]

The cyclic nucleotide-gated (CNG) ion channels are thought to play a crucial role in sensory signal transduction pathways in retinal rods and olfactory neuroepithelial cells.[29,34] All CNG channels known today are cation selective and show structural similarities to voltage-gated K^+ channels and functional similarities to Ca^{2+} channels. Recently, it has been shown that airway epithelia express an ion channel that is highly homologous with the retinal CNG-1.[35] Activation of this channel by cGMP created a favorable electrochemical gradient for Cl^- movement, suggesting important role for CNG-1 channels in the regulation of transepithelial ion transport in the lung.

NO is an important mediator governing the overall control of intracellular Ca^{2+} homeostasis.[36] While regulation of $[Ca^{2+}]_i$ i is complex and involves many aspects of cell biochemistry,

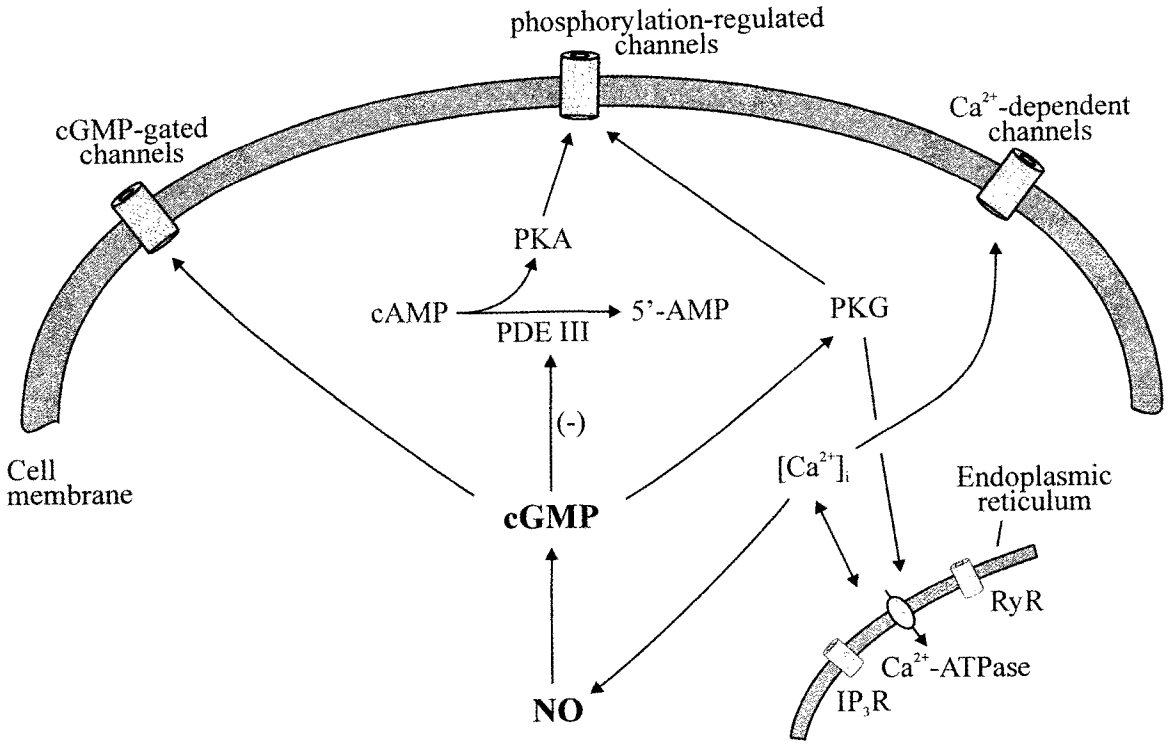

FIGURE 4 The NO-cGMP system in the regulation of ion channel function. NO regulates the activity of cGMP-dependent channels, phosphorylation-regulated channels and Ca^{2+}-dependent channels by exerting its effects on different second messenger systems (cGMP, cAMP, Ca^{2+}). The effect of NO on intracellular Ca^{2+} stores is likely to involve the cGMP/PKG/cADP ribose signaling pathway.[36] PKA – protein kinase A, PKG – protein kinase G, PDE III – phosphodiesterase III, RyR – ryanodine receptor, IP_3R – Inositol triphosphate receptor

several steps in this process are likely to be affected by NO. In particular, NO has been shown to control Ca^{2+} release from intracellular stores via two families of receptors (channels), the inositol 1,4,5-triphosphate (IP_3) receptors and the ryanodine receptors (RyR) (Fig. 4). Inhibition of Ca^{2+} release by NO via IP_3 receptors is thought to involve cGMP-dependent activation of PKG and phosphorylation of IP_3 receptor.[36] The effect of NO on RyR is not completely understood, but it is likely to involve cGMP-dependent activation of ADP-ribosyl cyclase and production of cyclic ADP-ribose (cADPR), which can function as Ca^{2+} messenger or a modulator of RyR.[37] Similarly, activation of PKG via NO/cGMP dependent pathway regulates the activity of Ca^{2+}-ATPase, which controls the levels of $[Ca^{2+}]_i$.[36]

There is also significant evidence indicating cGMP-independent effects of NO on ion channel function. Under conditions when guanylyl cyclase was inhibited by methylene blue, NO donors produced considerable relaxation of rabbit aorta, which was blocked by charybdotoxin, an inhibitor of Ca^{2+}-activated K^+ channels.[38]Application of N-ethylmaleimide, which is known to covalently modify protein sulphydryl groups making them incapable of nitrosation, indicated that NO activation of K^+ channels is dependent on chemical modification of sulphydryl groups present on the channel protein. In addition, it has been shown that both

cGMP-dependent and cGMP-independent (S-nitrosation/thiol oxidation) effects are involved in regulation of L-type Ca^{2+} channels.[39] Whereas activation of the cGMP-dependent pathway inhibited channel function, S-nitrosation and/or oxidation of channel protein activated channel current. These experiments suggested that direct redox modulation of ion channels might be an important and physiologically relevant mechanism for modulation of channel function.

The cross talk between NO and intracellular Ca^{2+} has been suggested by several studies.[36] Increased levels of intracellular Ca^{2+} lead to increased production of NO by both eNOS and nNOS. In contrast, increased production of endogenous NO has been shown to reduce intracellular Ca^{2+} levels in smooth muscle cells via both cGMP-dependent and cGMP-independent pathways.[40] The results suggest that the interaction between Ca^{2+} and NO is a bidirectional cross talk between these two mediators. NO can inhibit Ca^{2+} release from intracellular stores via at least two families of channels, IP_3 receptors and ryanodine receptors, and its influx from the outside via the voltage-independent Ca^{2+} channels (Fig. 4). Inhibition of soluble guanylyl cyclase, PKG or phosphodiesterase has been shown to affect the decrease in intracellular Ca^{2+}, indicating the involvement of cGMP-dependent pathway.[40] However, when these mechanisms were blocked, cGMP-independent mechanisms were shown to mediate the decrease in intracellular $Ca^{2+}$to NO, suggesting multiple and redundant targets for NO action in smooth muscle cells.

Cyclic GMP-independent actions of NO on ion channels may involve redox modulation of channel proteins. These actions are especially likely to occur during inflammatory conditions associated with increased production of nitrogen- and oxygen-related species leading to excessive protein nitrosylation and/or oxidation. Some of these cytotoxic effects are mediated, in part, by the potent oxidant $ONOO^-$, that is produced by the near-diffusion limited reaction of NO with $O_2^{\cdot-}$.[41] The cytotoxicity of $ONOO^-$ depends upon its metabolism and reactions with various molecular targets including hydroxyl- and sulfhydryl-containing proteins. Peroxynitrite was shown to reduce the conductance of amiloride-sensitive Na^+ channels expressed in Xenopus oocytes.[42] The authors suggested that this effect was caused by oxidation of sulphydryl groups residing within the extracellular domain of the channel protein that control its activity. The basolateral K^+ channels in the kidney were shown to be activated by NO/cGMP pathway.[43] However, in the presence of $O_2^{\cdot-}$NO inhibited channel function, likely through the formation of $ONOO^-$ and oxidation of channel thiols. $ONOO^-$ is known to rapidly permeate phospholipid membranes[44] and may affect function of both intracellular and cell membrane ion channels. Application of 3-morpholinosydnonimine (SIN-1), which generates $ONOO^-$ by releasing $O_2^{\cdot-}$ and NO., was shown to modulate receptor-activated Ca^{2+}signaling in vascular endothelial cells.[45] The effect of $ONOO^-$ on intracellular Ca^{2+} could represent an early event in the process of foxidant-induced cell injury under inflammatory conditions.

THE EFFECTS OF NITRIC OXIDE ON AIRWAY EPITHELIAL ION CHANNELS

Nitric oxide exerts different effects on regulation of ion transport in the upper and lower sections of the respiratory tract. The alveolar epithelium, lined by the type I and II cells, regulates electrolyte and water transport to keep it moist while avoiding excessive buildup of the fluid. This process is mediated by the active salt transport that creates an osmotic gradient for fluid movement from the alveolar to the interstitial space. A number of observations have suggested that the vectorial Na^+ transport has been a main mechanism by which alveolar cells control fluid reabsorption and prevent pulmonary edema.[46] Na^+

ions enter the cell passively through the apical membrane, amiloride-sensitive Na^+ channels, and are then actively transported across the basolateral membrane by Na^+/K^+-ATPase.

Treatment of alveolar type II cells with NO donors has been shown to inhibit vectorial Na^+transport in several systems via both cGMP-dependent and -independent mechanisms.[15–17] Single channel patch clamp studies of Na^+ channels have indicated that NO decreased channel activity via cGMP-dependent pathway, since intracellular cGMP has increased after NO treatment, the permeant analog of cGMP, 8-Br-cGMP (1 mM), inhibited the channel in a manner similar to NO donors, and blockers of protein kinase G abolished the inhibitory effects of NO.[16] The inhibitory effect of NO on Na^+ absorption by alveolar type II cells has been also observed in the experiments with confluent monolayers in Ussing chamber studies.[15] However, this study did not demonstrate inhibition of transepithelial Na^+ transport by 8-Br-cGMP (0.4 mM), and the results suggested that NO exerted its effects via a cGMP-independent mechanism. In addition, NO has been shown to inhibit short circuit current by ~60% after permeabilization of the apical membrane with amphotericin B, indicating that NO affected function of Na^+/K^+-ATPase.[15] This conclusion is in agreement with a study showing that NO, generated in proximal tubule epithelial cells, inhibited Na^+/K^+-ATPase activity in an autocrine fashion, and that this inhibition was accompanied by reduced Na^+ transport.[18] However, in human corpus cavernosum smooth muscle cells NO has been shown to stimulate the activity of Na^+/K^+-ATPase.[47] These studies show that NO-dependent mechanisms may be tissue-specific. For these reasons the effects of NO on Na^+transport and fluid homeostasis in vivo are likely to be complex.

Over the past few years, inhaled NO gas has been widely used in intensive care medicine to treat respiratory insufficiency associated with variety of inflammatory lung disorders both in neonates and adults.[48] However, the effects of inhaled NO on Na^+ transport in distal lung epithelium remain unclear. One could expect that increased levels of NO in alveoli, either due to inhalation or due to activation of iNOS under inflammatory conditions, might promote lung edema formation by reducing the rate of alveolar fluid absorption. However, inhaled NO has been shown to prevent interleukin-1 induced edema formation in rat lungs[49], and it may be beneficial in high-altitude pulmonary edema by improving arterial oxygenation.[50]

The role of NO in the regulation of ion transport in the upper airways has been explored in several studies. Elmer et al.[24] have examined the role of NO in regulating Na^+ and Cl^- transport in murine nasal epithelium and found that endogenously produced NO downregulates Na^+ absorption and leads to an increase in transepithelial Cl^- secretion. Activation of the cGMP-dependent pathway in Calu-3 cells, a human cell line derived from submucosal serous cells, has been shown to activate CFTR Cl^- channels.[51] This process is thought to be mediated via inhibition of cGMP-dependent phosphodiesterases and activation of a protein kinase A dependent pathway. Studies from our laboratories have shown that NO could activate non-CFTR Cl^- channels through the cGMP-dependent mechanism.[12]

The regulation of Cl^- channels by NO in human airways has attracted significant attention in the light of recent evidence that iNOS is constitutively expressed in epithelial cells[52,53], and that production of exhaled NO was significantly reduced in CF patients.[54] Cystic fibrosis results from a defect in the processing and/or activation of CFTR, resulting in diminished or absent Cl^- secretion in response to cAMP.[23] While the loss of cAMP-dependent Cl^- secretion through CFTR channels in CF is well established, the mechanisms underlying loss of basal Cl^- secretion and Na^+ hyperabsorption are unknown.[2]Recently, it has been shown that the presence of functional CFTR is required for

iNOS expression and that loss of NO production may be directly related to CF pathogenesis.[55] However, the exact mechanism of how loss of CFTR function alters inflammatory signaling pathways remains to be established.

While cyclic nucleotide-dependent regulation of CFTR channels has been clearly established, there is also a significant evidence for cyclic nucleotide-independent regulation of CFTR. The activity of CFTR is regulated by cellular redox potential[56] and via a covalent modification of CFTR Cys^{832} located in the R domain.[57] Since thiol-containing proteins serve as major target site for NO action, it is possible that reversible modification of Cys^{832} in CFTR might also control its activity under physiological conditions.

Increased expression of iNOS often leads to significant generation of $ONOO^-$. Indeed, increased formation of this oxidant has been detected in various inflammatory lung and cardiovascular disorders.[58] Peroxynitrite is known to oxidize cellular thiols, and this process has been shown to inhibit several ion channels.[42,45] Therefore, it is possible that $ONOO^-$ may also affect CFTR function via oxidation of thiols involved in channel function.

Potassium channels form a large and diverse gene family responsible for critical functions in numerous cell types, tissues and organs. Their functions include regulation of membrane potential, signal transduction, insulin secretion, hormone release, regulation of vascular tone, cell volume and immune response.[59] In many epithelial tissues, K^+ channels are found in the basolateral membrane of epithelial cells and are thought to be involved in hyperpolarization of resting cells, K^+ recycling, and modulation of NaCl transport. Present evidence suggests that the regulation of basolateral K^+ channels by NO is tissue dependent. For example, in the rat distal colon NO has been shown to inhibit K^+ transport[60], whereas in the kidney NO was shown to activate basolateral K^+ channels.[43]

Little is known about the effects of NO on the regulation of K^+ channels in human airway epithelial cells. Electrophysiological studies have identified several biophysically and pharmacologically different types of K^+ channels expressed in the basolateral membrane and some of them are likely to be a target for NO action. For example, the voltage-dependent, large-conductance, Ca^{2+}-activated K^+ channels (BK), consist of two dissimilar subunits, α and β, and there is presently strong evidence that the α subunit of BK channel is a substrate for PKG Iα phosphorylation *in vivo*.[61] This suggests that BK channels could be regulated by NO via cGMP-dependent pathway.

In conclusion, current evidence indicates that NO is a physiological regulator of transepithelial electrolyte and water movement in human airway epithelial cells. Pathological alterations of NO generation and action may play an important role in the pathogenesis of lung disorders characterized by hypersecretion of airway surface liquid. Understanding of these mechanisms is crucial to the development of novel therapeutic strategies related to the use of NO based technologies.

Acknowledgements

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