Review

Role of the nitric oxide–soluble guanylyl cyclase pathway in obstructive airway diseases

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ABSTRACT

Nitric oxide (NO) is a gasotransmitter, which is involved in many signaling processes in health and disease. Three enzymes generate NO from l-arginine, with citrulline formed as a by-product: neuronal NO synthase (nNOS or NOS1), endothelial NOS (eNOS or NOS3) and inducible NOS (iNOS or NOS2). NO is a ligand of soluble guanylyl cyclase (sGC), an intracellular heterodimer enzyme that catalyzes the conversion of guanosine triphosphate (GTP) to cyclic GMP (cGMP). cGMP further activates protein kinase G that eventually reduces the smooth muscle tone in bronchi or vessels. Phosphodiesterase 5 (PDE5) degrades cGMP to GMP. However, NO reacts with superoxide anion (O2/ C02), leading to formation of the pro-inflammatory molecule peroxynitrite.

Under physiological conditions, NO plays a homeostatic bronchoprotective role in healthy subjects. In obstructive airway diseases, NO can be beneficial by its bronchodilating effect, but could also be detrimental by the formation of peroxynitrite. Since asthma and COPD are associated with increased levels of exhaled NO, chronic inflammation and increased airway smooth muscle tone, the NO/sGC/cGMP pathway could be involved in these highly prevalent obstructive airway diseases. Here we review the involvement of NO, NO synthases, guanylyl cyclases, cGMP and phosphodiesterase-5 in asthma and COPD and potential therapeutic approaches to modulate this pathway.

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1. Nitric oxide (NO) and NO synthases

1.1. Introduction

Nitric oxide (NO) is the first identified gasotransmitter, which is involved in many diverse signaling processes including inflammation, smooth muscle tone and neurotransmission [12]. Within the respiratory tract, the main sources of NO are airway epithelial cells and endothelial cells [3,4]. Under physiological conditions, NO is present in the exhaled breath [5].

The synthesis of NO is catalyzed by 3 NO synthase (NOS) isoforms. Neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are constitutively expressed and their activity is regulated by intracellular calcium concentrations and calmodulin [6,7]. In an alternative pathway, eNOS can be activated by phosphorylation which renders it calcium-independent [8]. In conditions of oxidative stress and depletion of the co-factor BH4 (tetrahydrobiopterin), eNOS can become uncoupled resulting in the production of superoxide instead of NO [8]. Inducible NOS (iNOS or NOS2) is independent of calcium and is regulated by cytokines and pro-inflammatory stimuli [9]. However, this distinction is not that strict, since the activity of nNOS and eNOS, also referred to as “constitutive NOS (cNOS)”, can be induced by several cytokines (IL-1β, IFN-γ, TNF-α) [10,11]; while iNOS may be constitutively expressed at certain sites including the airway epithelium [12]. No can also be produced by various enzymes, starting from nitrite [13].

An overview of the expression of the 3 isoforms of NO synthase in human and murine lung tissue is shown in Table 1.
1.2. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is mainly caused by smoking cigarettes, which are an exogenous source of NO. Ansarin et al. [14] have analyzed the exhaled nitric oxide (eNO) levels in patients with COPD, patients with asthma and healthy controls. Patients with COPD had higher eNO levels than healthy controls, but lower levels than patients with asthma. Lung function parameters such as forced expiratory volume in 1 s (FEV$_1$) and carbon monoxide diffusing capacity (DLCO) were inversely correlated with eNO levels in patients with COPD. In contrast to patients with asthma, the use of inhaled corticosteroids had no significant influence on eNO levels in patients with COPD [14]. Brindacci et al. measured eNO at multiple expired flows to make a distinction between alveolar and bronchial NO and found that COPD is associated with elevated alveolar NO [15].

In peripheral lung tissue and bronchial submucosa of patients with COPD, iNOS expression was significantly increased, irrespective of the GOLD stage [10,16]. Patients with severe COPD had increased iNOS$^+$ cells in the alveolar wall and most of these cells were type II pneumocytes [17]. These results are in agreement with the observed increased alveolar NO in patients with COPD [15]. In bronchial submucosa, there was also an effect of smoking, since smokers without airflow limitation had increased iNOS levels [16]. The expression of iNOS was increased in bronchial smooth muscle cells of patients with COPD and was correlated with the degree of airflow limitation [18].

The expression levels of eNOS showed a discrepancy between the two localizations. In peripheral lung tissue, eNOS protein levels were similar between nonsmokers, smokers without airflow limitation and patients with COPD GOLD 1, 2 and 3. Remarkably, eNOS levels were significantly decreased in patients with severe COPD GOLD 4, probably caused by the destruction of the alveolar walls [10,19]. The levels of eNOS behaved similar to iNOS in bronchial submucosa [16].

The levels of nNOS were increased in peripheral lung tissue of patients with COPD GOLD stages 2, 3 and 4 compared with nonsmokers. Moreover, nNOS protein expression and disease severity (measured by FEV$_1$/% and FEV$_1$/FVC) were significantly correlated [10].

Similar to the results in human peripheral lung tissue [10], the levels of iNOS in murine lung homogenate were increased after 8 months cigarette smoke (CS) exposure, while the levels of eNOS were decreased [19]. In contrast to eNOS knockout mice, iNOS knockout mice were protected against the development of CS-induced emphysema. Treating wild-type mice with an iNOS inhibitor after 8 months CS exposure even reversed the lung damage and significantly downregulated the amount of granulocytes, macrophages and T-lymphocytes in the lung [19].

A chronic, iNOS-related inflammation, hypertrophy and hyperplasia of alveolar type II cells and several abnormalities in pulmonary structure and function develop in mice deficient in Surfactant Protein D (SP-D) [20]. SP-D and iNOS double knockout mice (DiNOS) maintained hyperplasia of alveolar type II cells, but they had a reduced inflammation, correction of the alveolar structural abnormalities and a restored lung function, compared with SP-D single KO mice. So by producing NO under pathological conditions, iNOS is involved in inflammation, development of structural abnormalities and lung function [20]. These findings and the observed increased iNOS expression in patients with COPD suggest that the inhibition of iNOS may be a potential therapy for patients with COPD [19,20]. However, in a murine model of elastase-induced emphysema [21], with increased expression of iNOS and eNOS, inhibition of iNOS decreased the amount of protein nitration, but had no effect on inflammation or development of emphysema. Inhibition of eNOS had overall no effect [21].

1.3. Asthma

In patients with asthma, especially in allergic asthmatics with eosinophilic airway inflammation, the levels of nitric oxide in exhaled air are significantly elevated compared with healthy subjects [22–24]. Moreover, lung function parameters such as FEV$_1$ and DLCO were inversely correlated with exhaled NO levels [14]. This increased flux of NO from the airways was significantly decreased after inhalation of aminoguanidine, a relatively selective iNOS inhibitor [25]. The expression of iNOS was increased in airway epithelial cells and inflammatory cells from patients with asthma compared with healthy subjects, suggesting that this enzyme plays an important role in the production of NO in pathological conditions [26,27]. Increased FeNO levels are a predictor of response to inhaled corticosteroids in patients with asthma [28]. iNOS levels in corticosteroid-treated asthmatics were significantly decreased compared to non-corticosteroid treated asthmatics [29]. Treatment of asthma patients with a selective iNOS inhibitor, GW274150, significantly reduced FeNO levels. However, this reduction in FeNO levels did not lead to a change in early or late responses to allergen challenge, or to a change in numbers of inflammatory cells in bronchoalveolar lavage (BAL) [30]. This suggests that therapeutic treatment with an iNOS inhibitor would not be beneficial in patients with asthma.

In a murine model of allergic asthma, the pharmacological inhibition of iNOS by l-NAME or aminoguanidine significantly decreased the number of eosinophils and lymphocytes in BAL fluid in OVA-challenged mice compared with non-treated mice [31]. Also airway hyperreactivity (AHR) and mucus secretion were significantly decreased in OVA-challenged mice after treatment with an iNOS inhibitor. In the same study, number of BAL cells, AHR and mucus secretion were not different between iNOS knockout and wild-type mice, suggesting that the lack of iNOS in these constitutive knockout animals is probably compensated by other mechanisms. In another study using the OVA model of allergic asthma, no differences in AHR were observed in iNOS knockout

Table 1

<table>
<thead>
<tr>
<th>Patients/model</th>
<th>Tissue</th>
<th>nNOS</th>
<th>iNOS</th>
<th>eNOS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUMAN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with COPD/smokers/nonsmokers</td>
<td>Peripheral lung tissue</td>
<td>↑ GOLD 2,3,4 (M + P)</td>
<td>↓ GOLD 2,3,4 (M)</td>
<td>↓ GOLD 3, 4 (M)</td>
<td>[10]</td>
</tr>
<tr>
<td>Patients with COPD/smokers/nonsmokers</td>
<td>Bronchial submucosa</td>
<td>ND</td>
<td>↑ Smokers, COPD (P)</td>
<td>↑ Smokers, COPD (P)</td>
<td>[16]</td>
</tr>
<tr>
<td>Patients with severe COPD/smokers</td>
<td>Alveolar wall</td>
<td>ND</td>
<td>↑ Severe COPD (P)</td>
<td>ND</td>
<td>[17]</td>
</tr>
<tr>
<td>MICE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 8 months CS</td>
<td>Lung homogenate</td>
<td>ND</td>
<td>↑ (P)</td>
<td>↓ (M + P)</td>
<td>[19]</td>
</tr>
<tr>
<td>Elastase-induced emphysema</td>
<td>Lung homogenate</td>
<td>ND</td>
<td>↑ (P)</td>
<td>↓ (M + P)</td>
<td>[21]</td>
</tr>
</tbody>
</table>

Both transmembrane and soluble forms of guanylyl cyclases exist. The transmembrane, particulate GC (pGC) acts as a receptor for hormones such as atrial, brain (B-type) and C-type natriuretic peptides. For further information on this transmembrane form of GC, we refer the reader to an excellent review [39].

Soluble GC (sGC) is an intracellular receptor for gaseous ligands (NO and to a minor extent CO) and is able to associate with the plasma membrane through protein–protein interactions in a Ca²⁺- dependent manner [40]. sGC is a heterodimer, consisting of an α-subunit and a β-subunit. There are 2 forms of the α-subunit (α1 and α2) and of the β-subunit (β1 and β2), but only α1β1 and α2β1 are active. α1β1 and α2β1 are equally present in the brain, while α1β1 is the most prevalent form in other tissues such as the lung [41]. Both forms have a similar catalytic rate and sensitivity towards NO. The C-terminal catalytic domains of both isoforms are required to form a catalytic active centre. The β-subunit has an amino-terminal heme-binding domain. A heme moiety that interacts with the heme-binding domain, is essential for the sensing of NO, increasing the cGMP production from GTP [42]. The heme moiety is a large heterocyclic organic ring with a central metal ion (Fe). sGC is activated by nanomolar concentrations of NO in the presence of the reduced Fe²⁺ (ferrous) heme moiety, while oxidized, Fe³⁺ (ferric) heme is insensitive to NO (Fig. 2). Moreover, the oxidized heme rapidly released from the protein, resulting in the exposure of an ubiquitination site on the protein moiety, which leads to ubiquitination and proteolytic degradation of the enzyme. Similar to oxidized heme, heme-deficient sGC is unresponsive to NO. Oxidation is induced by exogenous molecules, such as ODQ (1H-[1,2,4] oxadiazolo-[4, 3-a]quinoxalin-1-one), and by endogenous molecules, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) [40].

Activation of sGC induces the generation of cyclic guanosine monophosphate (cGMP), phosphorylation of protein kinase G (PKG) and changes in activity of effector proteins such as phosphodiesterases (PDE), ion channels and ion pumps [43]. This pathway eventually leads to dilation of bronchi (bronchodilation) or vessels (vasodilation).

### 3. Soluble guanylyl cyclase

Guanylyl cyclases (GCs), members of the family of nucleotide cyclizing enzymes, are widely distributed signal-transduction enzymes that catalyze the conversion of GTP to cGMP.

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**Fig. 1.** The NO/sGC/cGMP signaling pathway in obstructive airway diseases. Bronchial and alveolar epithelial cells produce NO, which activates sGC under normal conditions. However, decreased sGC levels in COPD and asthma lead to an impaired downstream pathway; while upregulated PDE5 levels further decrease cGMP levels. Activated inflammatory cells such as macrophages and neutrophils also release NO and reactive oxygen species such as O₂⁻ and NO as ONOO⁻, leading to protein nitrination. NO: nitric oxide, GC: soluble guanylyl cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PDE5: phosphodiesterase 5, PKG: protein kinase G, O₂⁻: superoxide, ONOO⁻: peroxynitrite.
In patients with asthma, bronchodilation is impaired, despite the presence of large amounts of NO in the airways that could activate sGC and cause relaxation of the smooth muscle. In a murine model of allergic asthma, the levels of sGCα1, α2 and β1 were reduced in the lungs, both on mRNA and protein level [44]. Mice treated with the selective sGC inhibitor ODQ had increased airway reactivity to methacholine compared with sham-treated mice [44]. This finding suggests that sGC could be inhibited in patients with asthma, leading to the observed airway hyperresponsiveness. Also in patients with COPD, the lungs contain ample amounts of NO, but the airway tone remains elevated [14]. Patients with COPD have decreased pulmonary mRNA and protein levels of sGC which are correlated with disease severity [45]. The levels of sGC are also decreased in CS-exposed mice [45] CS-exposed mice deficient for the sGCα1 subunit had a significantly higher airway resistance compared with CS-exposed wild-type mice (Fig. 1). These results indicate that sGC downregulation due to CS exposure in humans and mice, emerges as an alternative pathophysiological mechanism of the airway hyperresponsiveness.

4. Phosphodiesterase 5 (PDE5)

Phosphodiesterase 5 (PDE5) degrades cGMP to GMP; thereby impairing the downstream effects of cGMP (Fig. 1). Sildenafil, a short-acting inhibitor of PDE5, is already on the market for erectile dysfunction and pulmonary arterial hypertension and induces smooth muscle relaxation. Tadalafil is a long-acting inhibitor of PDE5. The effect of PDE5 inhibition was analyzed in guinea pigs exposed to lipopolysaccharide (LPS) and in sensitized guinea pigs exposed to ovalbumin [46]. Pretreatment with sildenafil inhibited the LPS-induced airway hyperreactivity, influx of leukocytes and generation of NO. Exposure to ovalbumin caused early- and late-phase asthma responses which were not affected by sildenafil. However, AHR to histamine, leukocyte influx in BAL and increased NO metabolites in BAL were significantly attenuated in OVA-exposed mice after treatment with sildenafil [46].

In a rat model of acrolein (a component of cigarette smoke) exposure, sildenafil suppressed the acrolein-induced airway inflammation and mucus production [47]. CS-exposed mice have increased PDE5 protein levels in the lung compared with air-exposed mice [45]. These results suggest that PDE5 inhibitors have a therapeutic potential in airway diseases such as asthma and COPD. However, in contrast to OVA-challenged guinea pigs [46], OVA-challenged mice treated with sildenafil did not affect airway inflammation [48].

PDE5 inhibitors have not yet been tested in asthma or COPD for their anti-inflammatory properties. In contrast, PDE5 inhibitors have been investigated in patients with COPD and (concomitant) pulmonary hypertension. Treating patients with COPD-associated pulmonary hypertension with sildenafil or tadalafil did not improve exercise capacity or quality of life [49,50].

5. Therapy

Potential therapeutic approaches to modulate the NO/sGC/cGMP pathway are activation of sGC by NO-donors, sGC stimulators and sGC activators; or inhibition of the inflammation-induced formation of NO by iNOS inhibitors.

5.1. Nitric oxide donors

A reduced bioavailability and/or responsiveness to endogenously produced NO contributes to the development of several pathologies, including pulmonary diseases.

NO-donors, such as organic nitrates, release NO by spontaneous decomposition or bioconversion, thereby activating the enzyme sGC. However, the use of NO-donors is limited because of the potential lack of response, the development of tolerance and the non-specific interactions of NO with biomolecules (such as superoxide, leading to formation of peroxynitrite) [40]. Moreover, in the airways of patients with COPD or asthma, there are already large...
amounts of NO present that could activate sGC and induce smooth muscle relaxation.

5.2. Inducible NO synthase inhibitors

The research on the role of iNOS using animal models has given conflicting results. Given the species differences in the expression and regulation of iNOS, translational research is required to fully elucidate the function of iNOS in obstructive airway diseases. Treating asthma patients with the selective iNOS inhibitor GW274150 did not lead to a change in early or late responses to allergen challenge, or to a change in numbers of inflammatory cells in BAL [30].

In healthy subjects, physiological levels of NO are produced by eNOS and nNOS. In inflammatory conditions, NO levels increase mainly due to increased iNOS activity. By inhibiting iNOS, both the beneficial effects of NO through sGC and the pro-inflammatory effect of NO through formation of peroxynitrite are inhibited. The iNOS inhibitor can reduce the levels of NO, but ROS such as superoxide remain elevated. These ROS can react with the ‘constitutively’ produced NO, leading to the observed unchanged 3-nitrotyrosine (3-NT) levels in patients with asthma [30]. Indeed, an in vitro study using human alveolar epithelial cells of patients with severe asthma, has shown that not only nitrite, produced by iNOS, but also H2O2, produced by dual oxidases, are important in the formation of 3-NT [51].

Activation of the sGC/cGMP pathway on the one hand, and/or inhibition of the NO-induced formation of pro-inflammatory molecules on the other hand could be more beneficial.

5.3. Soluble guanylyl cyclase activators and stimulators

In several pathologies, including asthma and COPD, the NO/sGC/cGMP pathway can be compromised by the oxidized state of sGC, making it unresponsive to both endogenous and exogenous NO. Therefore, an NO-independent treatment could be recommended in these diseases [40]. Both sGC stimulators and sGC activators are potential therapies. sGC stimulators stimulate sGC directly and enhance the sensitivity of the reduced enzyme to low levels of bioavailable NO (Fig. 2). While sGC stimulators are heme-dependent, sGC activators activate the NO-unresponsive, heme-oxidized or heme-free enzyme (Fig. 2). The efficacy of the sGC stimulator riociguat has already been shown in patients with pulmonary arterial hypertension [52]. In the same line, a recent study demonstrated that treatment of two different Cs−exposed animal models with sGC stimulators riociguat (BAY 63-2521) or BAY 41-2272 prevent CS-induced pulmonary hypertension and emphysema [53].

BAY58-2667 (or cinaciguat) is a potent NO-independent sGC activator, replacing the weakly bound oxidized heme of sGC, leading to activation of the enzyme; while reduced hemes unresponsive to BAY58-2667 [40]. Treating CS-exposed mice with BAY58-2667 restored the sGC/cGMP pathway and significantly attenuated the CS-induced AHR compared with sham-treated mice [45], denoting sGC as a promising pharmaceutical target of obstructive airway diseases (Fig. 2).

6. Concluding remarks

The use of NO-donors and iNOS inhibitors as a treatment in obstructive airway diseases has not been successful. The specific activation of the sGC/cGMP pathway by treating patients with an sGC activator or stimulator may be a new therapeutic approach in obstructive lung diseases. The results of using sGC activators and sGC stimulators in animal models of asthma and COPD are promising, however further research is needed. Treatment by the inhaled route should be investigated to limit potential side-effects of systemic drug administration.

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