Inhibition of inducible nitric oxide synthase in respiratory diseases

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Abstract
Nitric oxide (NO) is a key physiological mediator and disturbed regulation of NO release is associated with the pathophysiology of almost all inflammatory diseases. A multitude of inhibitors of NOSs (nitric oxide synthases) have been developed, initially with low or even no selectivity against the constitutively expressed NOS isoenzymes, eNOS (endothelial NOS) and nNOS (neuronal NOS). In the meanwhile these efforts yielded potent and highly selective INOS (inducible NOS) inhibitors. Moreover, INOS inhibitors have been shown to exert beneficial anti-inflammatory effects in a wide variety of acute and chronic animal models of inflammation. In the present mini-review, we summarize some of our current knowledge of inhibitors of the INOS isoenzyme, their biochemical properties and efficacy in animal models of pulmonary diseases and in human disease itself. Moreover, the potential benefit of INOS inhibition in animal models of COPD (chronic obstructive pulmonary disease), such as cigarette smoke-induced pulmonary inflammation, has not been explicitly studied so far. In this context, we demonstrated recently that both a semi-selective INOS inhibitor \{\text{L-NIL} (N\text{6}-(1-iminoethyl)-L-lysine hydrochloride)\} and highly selective INOS inhibitors (GW274150 and BYK402750) potently diminished inflammation in a cigarette smoke mouse model mimicking certain aspects of human COPD. Therefore, despite the disappointing results from recent asthma trials, INOS inhibition could still be of therapeutic utility in COPD, a concept which needs to be challenged and validated in human disease.

Introduction
Nitric oxide (NO), a gaseous free radical, is a key physiological mediator of pulmonary function, and excessive NO synthesis, mostly by INOS [inducible NOS (nitric oxide synthase)], has been implicated in the pathophysiology of almost all pulmonary diseases. Furthermore, peroxynitrite, a highly reactive radical derived from NO and O$_2^{-}$, may be the ‘ugly guy’ in various lung diseases mediating tissue destruction, inflammation and vasoconstriction.

Two classes of enzymes exist and they differ in their activation profile and their capacity to generate NO. iNOS expression is up-regulated by various pro-inflammatory signals resulting in micromolar NO concentrations and is thereby part of immediate immune defence reactions. In contrast, the constitutively expressed eNOS (endothelial NOS) and nNOS (neuronal NOS) enzymes are involved in blood pressure regulation and neurotransmission respectively. They generate nanomolar concentrations of NO regulated by changes of enzymatic activity upon changes of intracellular Ca$^{2+}$ concentrations [1].

iNOS has been linked to many inflammatory diseases including septic shock, asthma, rheumatoid arthritis and COPD (chronic obstructive pulmonary disease), and selective inhibition of iNOS seems to be a promising approach for the treatment of different inflammatory diseases. A pathological role of iNOS-derived excessive NO production was shown in a variety of animal models of inflammation using different iNOS inhibitors, although many of these compounds have only limited selectivity.

On the other hand, it is well accepted that inhibition or dysfunction of eNOS can be detrimental, leading to systemic and pulmonary hypertension, endothelial dysfunction and vessel remodelling, although some publications also suggested a contribution of eNOS-derived NO under acute inflammatory conditions [2].

The transformation of animal data into a clinical response in humans is hampered by a large difference in the expression pattern of iNOS between species: whereas rodent cells respond with a rapid, strong and sustained up-regulation of iNOS to stimulation by LPS (lipopolysaccharide) and pro-inflammatory cytokines, human immune cells only marginally produce NO from iNOS.

Biochemistry of iNOS inhibitors
A great deal of efforts have been directed towards discovering potent and iNOS-selective inhibitors in order to overcome
potential side effects linked with the inhibition of the constitutively eNOS and nNOS. Comparison between published X-ray crystal structures of NOS isoforms shows that the structure of the active sites are highly conserved in the l-arginine binding region, suggesting that the development of isoform-selective inhibitors would be rather difficult [3].

The earliest inhibitors of NOSs were simple analogues of l-arginine such as l-NMMA (N\(^\text{G}\)-monomethyl-l-arginine), l-NAME (N\(^\text{G}\)-nitro-l-arginine methyl ester), thiocitrullines or l-NIL [N\(^\text{6}\)-l-arginine (L)-lysine hydrochloride]. Many of them show less than 10-fold selectivity and should be regarded as non-selective. For example, l-NAME, commonly used as a tool compound for studying the effects of NOS inhibition, has no iNOS selectivity (Table 1). A large number of isothiourea derivates were described as NOS inhibitors. EIT (5-ethylisothiourea) in particular has been shown to be highly potent in the low nanomolar range; however, it also lacks isoform specificity [3,4].

Haem-liganding compounds such as BBS-1 and BBS-2 were identified as inhibitors preventing the assembly of the newly synthesized monomeric NOS protein into the functional homodimeric form [5]. The high turnover of iNOS protein compared with eNOS or nNOS in the cell is thought to lead to isoform selectivity of these compounds. However, although eNOS and nNOS protein might be turned over slowly, newly synthesized protein will still need to dimerize, which might lead to potential selectivity problems during chronic treatment.

In the meantime, a variety of structurally different iNOS-selective inhibitors have been described, such as 1400W [6], GW274150 [7], AR-C102222 [8], BYK191023 [9,10] and the recently presented BYK402750 [11]. These inhibitors demonstrate high iNOS selectivity and in vivo efficacy (Table 1), making them ideal tools to study the pathophysiological role of iNOS in inflammatory diseases.

However, despite the progress in the development of potent and selective iNOS inhibitors none of them has yet reached the market.

### Asthma

Initially, in mice, Feder et al. [12] demonstrated that ovalbumin sensitization and challenge did not induce iNOS in B6D2F1/J mice and the partially iNOS-selective inhibitor l-NIL did not reduce eosinophilia, which was decreased by non-selective inhibitors such as l-NAME, l-NMMA and aminoguanidine. In contrast, in Balb/c mice, Trifilieff et al. [13] showed both iNOS induction in inflammatory infiltrate and inhibition of eosinophilia and neutrophilia by l-NAME, EIT and AMT (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride) in ovalbumin-sensitized and -challenged animals, suggesting that iNOS regulation in allergic inflammation is variable within different mouse strains.

In ovalbumin-sensitized Brown-Norway rats, Eynott et al. [14] demonstrated iNOS induction and increased exhaled NO shortly after ovalbumin challenge and SC-51, the prodrug of l-NIL, did reduce neutrophil influx into BALF (bronchoalveolar lavage fluid) and bronchial hyperresponsiveness. In repeatedly ovalbumin-challenged rats, SC-51 additionally reduced eosinophil- and CD4-positive T-cell influx into the lung as measured by BALF [15].

Conflicting results were reported from iNOS-knockout mice compared with mice with targeted deletion of nNOS or eNOS. While iNOS was up-regulated in ovalbumin-sensitized and -challenged wild-type C57BL/6J mice and absent in iNOS-knockout mice, methacholine-mediated airway hyperresponsiveness was only reduced in nNOS-knockout and in nNOS/eNOS double deficient mice, but was not significantly different in iNOS-knockout animals [16]. Similarly, Landgraf et al. [17] demonstrated significant inhibition of inflammatory cell influx into BALF and decreased airway hyperreactivity and mucus secretion by acute l-NAME and aminoguanidine treatment in C57BL/6J mice respectively, effects that were absent in mice with targeted deletion of the iNOS gene. One explanation could be a compensatory up-regulation of non-deleted NOS isoforms within the knockout animals or effects on lung perfusion due to eNOS inhibition.

iNOS expression is increased in human asthmatic airway epithelial and inflammatory cells. Hamid et al. [18] and Lane et al. [19] showed a clear correlation between iNOS expression in bronchial epithelium and elevated exhaled NO levels in asthmatic children. Substantially increased levels of exhaled NO in human steroid-naive asthmatics were described in a seminal study by Kharitonov et al. [20]. Today, the measurement of exhaled NO in asthmatics is an established non-invasive technique, especially in children with mild to moderate asthma, and has been shown to be sensitive to glucocorticoid treatment. In fact, the measurement of exhaled NO in asthmatics can guide the treatment with inhaled glucocorticoids [21]. Moreover, peroxynitrite in exhaled breath condensate seems to be elevated in human asthma.

<table>
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<tr>
<th>IC(_{50}) (μM)</th>
<th>ED(_{50}) (μmol/kg per h)</th>
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<tr>
<td>iNOS</td>
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<tr>
<td>l-NMMA</td>
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<tr>
<td>l-NAME</td>
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<tr>
<td>l-NIL</td>
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<td>AMT</td>
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<td>1400W</td>
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<td>BYK402750</td>
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n.d., not determined.

Table 1 | Enzymatic potency of human NOS isoforms and in vivo efficacy (LPS-induced increase in plasma NO\(_\text{e}\), levels in rat) of standard NOS inhibitors
although to a lesser extent compared with COPD [22]. For a more detailed review, see, for instance, Ricciardolo et al. [23].

Although most of the animal studies using iNOS inhibitors showed beneficial effects in various models of asthma, a Phase II study in human mild asthmatics using the highly selective iNOS inhibitor GW274150 failed to show efficacy on early and late asthmatic response despite potent inhibition of exhaled NO (see below) [24]. On the other hand, this shows that iNOS-derived NO is not involved in bronchodilation, at least not in the study patient population.

**COPD**

So far, the effects of chronic cigarette smoke administration on iNOS induction and NO or peroxynitrite production in mice have not been analysed in depth. A combined smoke and burn model in sheep represents a model of ALI (acute lung injury) and is described below.

We demonstrated recently that administration of both a semi-selective NOS inhibitor (L-NIL) and highly selective iNOS inhibitors (GW274150 and BYK402750) potently diminished the influx of inflammatory cells and cytokine levels in BALF in an 11-day smoke mouse model mimicking certain aspects of human COPD [11]. In contrast, iNOS inhibition was ineffective in a 3-day smoke mouse model (C. Hesslinger, M.D. Lehner, A. Strub, R. Boer, W.R. Ulrich, R. Kuelzer, G. Lauer, D. Lin, D. Spicer, M. Fitzgerald, L. Wollin and C. Braun, unpublished work), suggesting that iNOS inhibitors preferentially affect subacute and chronic smoke-induced inflammation but are ineffective under acute inflammatory conditions. The question whether the anti-inflammatory activity of iNOS inhibition eventually results in a reduction of emphysema induction and airway remodelling has to be tested in long-term smoke models.

Furthermore, Anazawa et al. [25] showed increased arterial iNOS expression, superoxide production, serum NOx (nitrite and nitrate) production and intimal thickening in mice treated with cigarette smoke for 3, 7 and 21 days after placing a cuff around the carotid artery. Inhibition of iNOS by 30 mg/kg mercaptoethylguanidine twice a day or deletion of iNOS abrogated the intimal thickening in this combined model.

Human iNOS overexpression in COPD has been demonstrated in the alveolar wall, especially in ATII (alveolar type II) pneumocytes, within alveolar macrophages, neutrophils and eosinophils [23,26] and in skeletal muscle of COPD patients, especially in those with cachexia [27]. The levels of 3-nitrotyrosine and iNOS expression correlated well with severity of the disease as measured by FEV1 (forced expiratory volume in 1 s) [23,26].

In contrast with asthma, exhaled NO seems to only increase in stable COPD if measured at multiple expired flows to investigate peripheral lung contribution and this demonstrated that peripheral airways and the alveolar region are the predominant source of elevated exhaled NO in COPD, whereas in asthma it is the bronchial epithelium [28]. A correlation between COPD severity as measured by FEV1 percentage predicted, and an increase in exhaled NO levels could be demonstrated [28].

Furthermore, Agusti et al. [29] showed significantly increased exhaled NO levels in COPD patients during acute exacerbations and Maziak et al. [30] found elevated exhaled NO levels in patients with ‘unstable’ COPD, as defined by exacerbations or disease severity, which correlated with lung function as determined by FEV1 percentage predicted.

Rather than NO itself, peroxynitrite was shown by several groups to be elevated in COPD, indicating high oxidative stress and rapid reaction of NO (produced by iNOS) with superoxide, yielding peroxynitrite.

Recently, Osata et al. [31] showed significantly elevated peroxynitrite levels in the exhaled breath condensate of COPD patients when compared with healthy volunteers and smokers, correlating well with disease severity.

**IPF (idiopathic pulmonary fibrosis)**

The laboratory of Cuzzocrea and co-workers tested the iNOS-knockout mouse and the highly selective iNOS inhibitor GW274150 (5 mg/kg, once daily intraperitoneally) in the bleomycin-induced lung injury and fibrosis model [32] and demonstrated diminished lung collagen formation, lung fibrosis (histological score) and TGFβ (transforming growth factor β) expression both when the iNOS inhibitor was administrated in a prophylactic setting and when mice with a genetic iNOS deletion were used. Furthermore, iNOS inhibition or deletion caused a reduction in lung injury, oedema formation, pulmonary inflammation and mortality. However, there is no differentiation possible between an anti-inflammatory and a putative antifibrotic mode of action of iNOS inhibition under the conditions used. In this context, data from Jang et al. [33] suggest continuous iNOS expression during the initial inflammatory phase and the later fibrotic phase in bleomycin-treated Sprague-Dawley rats.

In contrast with asthma and COPD, little is known about iNOS expression and function in human lung fibrosis. A seminal paper by Saleh et al. [34] demonstrated elevated iNOS expression and 3-nitrotyrosine staining in inflammatory and epithelial cells of human IPF lungs.

**ALI and ARDS (acute respiratory distress syndrome)**

Today, LPS-mediated acute pulmonary inflammation is a standard model used in pulmonary pharmacology reflecting some aspects of ALI. There is a wide variety of examples showing pulmonary iNOS induction after systemic or local LPS administration. A potent reduction of lung inflammation and injury by NOS and iNOS inhibitors or in iNOS-knockout mice could be demonstrated.

McCluskie et al. [35] demonstrated potent inhibition of airway neutrophilia and exhaled NO in Wistar rats challenged with aerolized LPS both by the non-selective L-NAME and the highly selective 1400W.

Moreover, the induction of sepsis in mice by CLP (caecal ligation and puncture) revealed a prominent role of
pulmonary iNOS induction in ALI secondary to sepsis, and iNOS-knockout animals were completely protected, showing attenuated lung injury and inflammation [36].

The combined burn and smoke inhalation sheep model has been used as a classical model for ARDS, and Enkhbaatar et al. [37] showed, besides other effects, efficacy of the iNOS dimerization inhibitor BBS-2 in pulmonary gas exchange and oedema, airway obstruction and abnormal lung compliance among others. On the other hand, the same group presented evidence that a rather nNOS-selective inhibitor, 7-NI (7-nitroindazole), significantly reduced lung oedema and improved pulmonary gas exchange and shunt fraction in a similar ALI model [38].

Moreover, beneficial effects of iNOS inhibition or deletion have been shown in many different acute lung inflammation models, for example in hyperoxic lung injury in mice [39], in a surfactant protein D-deficient pulmonary inflammation model [40], in ozone-induced lung injury [41] and in a carrageenan-induced ALI model using GW274150 [42].

Ohsugi et al. [43] demonstrated that inhaled ONO-1714, a very potent but moderately selective iNOS inhibitor, diminished pulmonary inflammation, NOx and peroxynitrite production and prolonged survival in a Candida albicans-induced ALI model in mice.

Pulmonary hypertension

Although it is generally accepted that diminished eNOS-mediated NO production can lead to pulmonary hypertension that can be effectively treated for example by PDE5 (phosphodiesterase 5) inhibitors such as Sildenafil (Revatio™), the role of iNOS is largely unclear.

Hampl et al. [44] demonstrated transient iNOS induction in a rat model of hypoxia-induced pulmonary hypertension and a reduction of elevated pulmonary pressure by concomitant treatment with either l-NIL or l-NAME, suggesting at least an initial regulation of lung inflammation via iNOS. In contrast, ONO-1714 failed to reduce elevated pulmonary pressure and right heart hypertrophy in a hypobaric hypoxia-induced pulmonary hypertension model [45]. Differences between animal models or the NOS inhibitors may account for the contrary findings.

Treatment of human pulmonary diseases with iNOS inhibitors

Several non-selective NOS inhibitors and selective iNOS inhibitors have been tested in humans in investigator-initiated studies or Phase II trials using the non-selective inhibitors l-NMMA and aminoguanidine, SC-51 as a moderate selective l-NIL prodrug, as well as the highly selective iNOS inhibitor GW274150.

From human studies with l-NMMA in sepsis it was already known that concomitant inhibition of constitutive isoforms may be detrimental at least under acute inflammatory conditions, putatively due to increased pulmonary pressure and excessive vasoconstriction of pulmonary vessels by inhibiting eNOS [46].

Asthma

In the beginning, non-selective NOS inhibitors were used in human asthma patients e.g. by Taylor et al. [47]. Inhalation of nebulized l-NAME had no significant effects on early and late asthmatic response or on lung function measured as FEV1 in patients with mild allergic asthma despite a large reduction in exhaled NO. Furthermore, inhaled aminoguanidine, another non-selective NOS inhibitor, revealed similar results on exhaled NO levels when measured at different flows in asthmatics [48].

In another study using the semi-selective iNOS inhibitor l-NIL (administered orally in its prodrug form SC-51 at 20 and 200 mg per patient, single dose), Hansel et al. [49] could show a potent reduction in exhaled NO over a period of 72 h both in mild asthmatics and in healthy volunteers. In contrast, there was no effect on respiratory function after l-NIL administration.

A final study in steroid-naïve patients with asthma using the highly selective iNOS inhibitor GW274150 again could demonstrate a substantial and significant reduction in exhaled NO levels but failed to be effective on early and late asthmatic responses to allergen, on methacholine-induced airway hyperreactivity and inflammatory cell influx into BALF [24]. These disappointing results demonstrate the apparent differences between animal models and human disease (see the preceding section).

COPD

Brindicci et al. [50] tested the inhalation of the rather non-selective NOS inhibitor aminoguanidine in COPD patients, healthy smokers and healthy non-smoking subjects and found a significant but also incomplete reduction of exhaled NO produced from both the central and peripheral lungs and of NOx and peroxynitrite in exhaled breath condensate of COPD patients. 8-Isoprostane, a marker of oxidative stress, was not affected. These data suggest that at least a non-selective NOS inhibitor can indeed reduce the production of NO and peroxynitrite in human COPD but its incomplete efficacy reveals more questions than answers. Therefore a final proof of clinical efficacy by selective iNOS inhibition in COPD with concomitant functional effects on inflammation and lung function has still to be achieved, which is a challenge considering recent failures of iNOS inhibition in asthma trials.

Conclusions

Nitric oxide and, even more, peroxynitrite, both of which are produced under oxidative stress conditions, are central players in the pathophysiology of many pulmonary diseases such as asthma, COPD, IPF and ALI.

Selective inhibition of the inducible form of NOSs resulted in potent anti-inflammatory and tissue protective...
effects in almost all animal models tested. On the other hand, expression of iNOS is differentially regulated in humans and rodents and thus selective iNOS inhibition may also have functionally distinct consequences in humans and animals.

Dis appointing clinical trials in asthma patients using moderately and highly selective iNOS inhibitors suggest that data from animal models may not be directly transferred to the human situation. Nevertheless, iNOS inhibition may still be of therapeutic utility in COPD but the prominent anti-inflammatory effects of iNOS inhibition in subacute and long-term smoke mouse models has to be validated in human disease. Finally, iNOS inhibitors are still of interest for other inflammatory disease conditions; for instance, GW274150 has been tested in patients with rheumatoid arthritis (http://www.gsk.com, verified in February 2009).

References


Received 5 March 2009
doi:10.1042/BST0370886