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Review

Reactive nitrogen and oxygen species in airway inflammation

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Abstract

The free radical nitric oxide (NO) is an important mediator of many biological processes. Interestingly, the molecule appears to be a two-edged sword. Apart from NO having a function as a paracrine messenger, NO-derived oxidants are important weapons against invading pathogens. The role of NO in the airways is similarly ambiguous. Besides the task as a bronchodilator, NO and its derivatives play a role in the pathophysiology of asthma via their putative damaging effects on the airways. This deleterious effect can be increased by a nitrosative response to respiratory tract infections, since both the infectious agent and the host may suffer from the consequent nitrosative stress. Interestingly, respiratory infections can also compromise the beneficial (bronchodilator) effects of NO. This paper gives an overview on NO and its derivatives in the pathophysiology of airway inflammation.

Keywords: Radical; Airway; Lung; Nitric oxide (NO); Super oxide; Peroxynitrite

1. Introduction

The main function of the immune system is the defence of host organism against infectious agents. Phagocytic cells such as macrophages and neutrophils are essential for antimicrobial responses, because these cells release large amounts of highly toxic molecules, i.e. reactive nitrogen species and reactive oxygen species. There is evidence that not only inflammatory cells, but also non-inflammatory cells such as airway epithelium release these reactive molecules.

Besides beneficial effects in host defence mechanisms, reactive nitrogen species and reactive oxygen species have detrimental effects on the host itself, since these molecules are potentially as toxic to host cells as they are to microorganisms. The effects on host tissues may manifest themselves as (i) inflammatory diseases without the involvement of an intrinsically harmful pathogen, as in allergic and autoimmune disease, and (ii) in infections where a nitrosative response does not help to eliminate the infectious agent.

Reactive oxygen species are mainly derived from superoxide (O_2^{*-}), whereas reactive nitrogen species formation mostly starts with the synthesis of nitric oxide (NO). The formation and properties of these two important precursors are discussed below.

1.1. Nitric oxide

The free radical NO is well recognized as a mediator in a wide range of biological processes (Gaston et al., 1994; Moncada et al., 1991). With a molecular weight of 30 g mol^{-1}, NO is one of the smallest molecular mediators in biology. The diatomic molecule is formed in a variety of cells by the conversion of L-arginine to L-citrulline by NO synthase (NOS) (Moncada et al., 1989).

There are three isoforms of NOS, each with a distinct function. The isoforms are roughly divided into two classes: constitutive and inducible NOS. Neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are expressed constitutively, and inducible NOS (iNOS or NOS2) expression is upregulated, for example during inflammation (Moncada et al., 1991). nNOS and eNOS activity is dependent on calcium, whereas iNOS activity is little dependent on calcium fluxes. Generally, nNOS-derived NO is involved in neurotransmission (Kuriyama and Ohkuma, 1995; Snyder and Bredt, 1991). eNOS is important for the relaxation of vascular smooth muscle and is therefore involved in the regulation of blood pressure (Nathan, 1992). The inducible form, iNOS, is the main source of NO involved in inflammatory responses and pathogen killing and is expressed, in for example macrophages in response to inflammatory mediators, leading to the production of large amounts of NO (Fang, 1997a,b; Moncada et al., 1991; Rees et al., 1990). Studies with iNOS knockout mice clearly demonstrate an essential role

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for NO in immune responses to particular microorganisms (MacMicking et al., 1995; Wei et al., 1995).

The biology of NO is thus double-faced. In relatively low concentrations, NO is a paracrine messenger, but at high concentrations, for example released in an inflammatory context, it has putatively detrimental effects via the formation of reactive nitrogen species (Beckman and Koppenol, 1996). Both beneficial and deleterious effects of NO depend not only on the concentration, but also on the biological microenvironment in which NO is released (Muijsers et al., 1997). It must be stressed that the detrimental properties of NO appear not only when it is released from inducible NOS, but also when it is produced by the constitutive isoforms of the enzyme, for example during ischemia–reperfusion injury (Samdani et al., 1997).

1.2. Superoxide

The free radical, superoxide, is released during the respiratory burst of granulocytes and macrophages by NADPH oxidase activity, in response to several stimuli (Babior, 1978). Alternative sources of superoxide are the auto-oxidation of hemoglobin, myoglobin and cytochrome *c*. Furthermore, enzymes such as xanthine oxidase, aldehyde oxidase and a variety of flavin dehydrogenases are a source of superoxide. Superoxide is therefore released by virtually all aerobic cells (McCord and Fridovch, 1969). Interestingly, when L-arginine concentrations are relatively low, nNOS can produce both NO and superoxide, (Xia et al., 1996). Like that of NO, super oxide release during the respiratory burst of phagocytes is essential for the killing of invading pathogens (Mastroeni et al., 2000).

1.3. Peroxynitrite

NO and superoxide are free radicals (and therefore highly reactive) and both molecules react rapidly with many different molecules in a biological environment. Of particular interest is the interaction between the two molecules and their reactive downstream metabolites. Several metabolic routes are available when NO and superoxide are released simultaneously, which is a likely event during inflammatory responses. In fact, efficient killing of, for example, *Salmonella* by murine macrophages is dependent on both NADPH oxidase-derived superoxide and iNOS-derived NO (Mastroeni et al., 2000). Numerous products formed by the interaction of superoxide and NO are even more reactive than their precursors. The many possible metabolic routes that superoxide and NO can follow once released in vivo are now summarised briefly.

2. Metabolites of NO and superoxide

The most direct interaction between NO and superoxide is their rapid iso-stoichometric reaction to form the potent oxidant, peroxynitrite (Blough and Zafiriou, 1985; Huie and Padmaja, 1993). The rate constant of this reaction is near the diffusion controlled limit ($4–7 \times 10^9$ M^{-1} s^{-1}) (Huie and Padmaja, 1993; Goldstein and Czapski, 1995). The half-life of peroxynitrite at 37 °C and pH 7.4 is approximately 1 s. (Beckman et al., 1990; Pryor and Squadrito, 1995). When carbon dioxide is relatively abundant, the reaction of peroxynitrite with carbon dioxide is the most important breakdown route in biological environments (Uppu et al., 1996). The exact biochemical fate of peroxynitrite in biological systems is, however, very complex and is not yet completely clear (Muijsers et al., 1997).

Although peroxynitrite can nitrate tyrosine residues (Reiter et al., 2000), detection of 3-nitrotyrosine is not indisputable evidence for peroxynitrite formation, since there are alternative pathways leading to 3-nitrotyrosine formation (Eiserich et al., 1998a). This will be discussed in detail in a later section.

2.1. Reactive nitrogen species

In addition to the direct interaction of superoxide and NO forming peroxynitrite, the interaction yields several other reactive nitrogen. Besides peroxynitrite formation, NO-derived nitrite can be utilized in the myeloperoxidase pathway leading to NO_2Cl and NO_2^*. An overview of the metabolic routes of reactive nitrogen species is given in Fig. 1.

2.2. Measurement of reactive nitrogen species

As described above, several reactive species originate from the precursors, NO and superoxide. Due to the complex chemistry and often short half-lives of reactive nitrogen species, the exact metabolic fate in vivo remains unclear. Further, it is almost impossible to attribute a given effect in vivo to a certain reactive nitrogen intermediate. Nonetheless, some stable end products of reactive nitrogen species are detectable in body fluids and tissues. Firstly, nitrite and nitrate can be measured in plasma (Kelm, 1999; Ochoa et al., 1991). Furthermore, 3-nitrotyrosine residues can be found not only in tissue samples, using for example immunohistochemistry (Saleh et al., 1998), but also in biological fluids (Ohshima et al., 1999). NO can be measured in the exhaled air of asthmatic patients (Dupont et al., 1998; Massaro et al., 1996) and is thought to reflect the inflammatory state of the airways (Ten Hacken et al., 1998). Nonetheless, it is often difficult to interpret results from these techniques, since there is a high risk of artifacts. Nitrite and nitrate levels in plasma, for example, can reflect the dietary intake rather than NO metabolism in vivo (Ahren et al., 1999). Moreover, NO is also formed enzyme-independently from nitrite under acidic conditions (Zweier et al., 1999). Recently, Hunt et al. (2000) showed that the pH in the airways drops dramatically during an acute asthma attack, which facilitates the conversion of

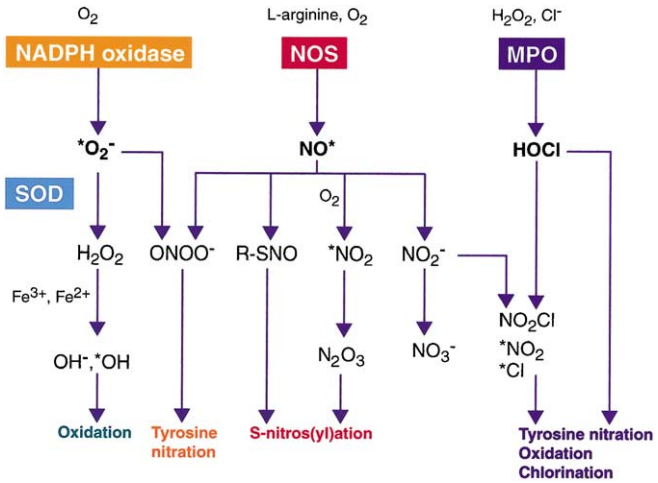

Fig. 1. Schematic overview of reactive nitrogen and oxygen species metabolism by inflammatory cells. NOS: NO synthase, MPO: myeloperoxidase, SOD: superoxide dismutase. See text for detail.

nitrite to NO. Hence, increased NO concentrations in the exhaled air of asthmatic patients may reflect nitrite conversion rather than NOS activity.

2.3. 3-Nitrotyrosine

An important, commonly used, marker of nitrosative stress is the formation of 3-nitrotyrosine residues. Although 3-nitrotyrosine is widely used as a specific marker of peroxynitrite formation, it is now clear that in vivo, there are more pathways leading to the formation of 3-nitrotyrosine (Eiserich et al., 1998a). 3-Nitrotyrosine is readily formed by a NO-independent process mediated by myeloperoxidase, with hydrogen peroxide and the NO metabolite, nitrite, as substrates (Eiserich et al., 1998a; Kettle et al., 1997). Moreover, eosinophil peroxidase is an even stronger promoter of 3-nitrotyrosine formation via this pathway (Wu et al., 1999). We recently found that, in a mouse model of allergy, 3-nitrotyrosine staining was clearly present in the airways of all ovalbumin challenged animals (Fig. 2B) and absent in those of all saline-challenged animals (Fig. 2A) (Muijsers et al., 2001(a)).

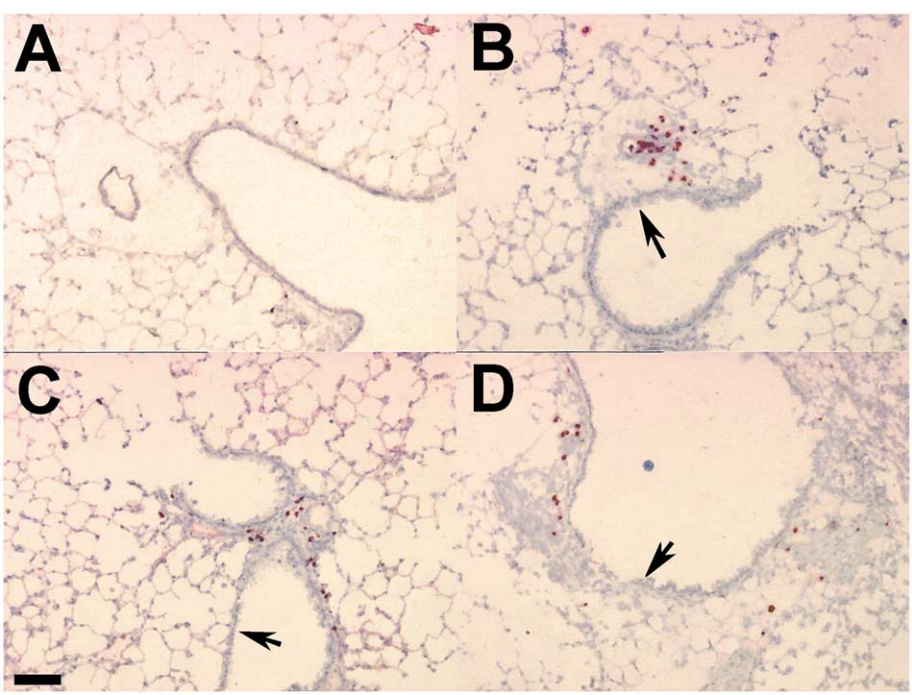

Fig. 2. Immunohistochemical demonstration of 3-nitrotyrsosine residues (red spots) in the airways of ovalbumin-sensitised mice challenged with either ovalbumin or saline. Arrowheads indicate airway epithelium. (A) Saline-challenged animal. (B) Ovalbumin-challenged animal. (C) Ovalbumin-challenged animal treated with apocynin and (D) ovalbumin-challenged animal treated with 1400W. Calibration bar in (C) represents 100 μM.

3-Nitrotyrosine staining was mainly concentrated in eosinophils throughout the airways. Positive eosinophils were found around blood vessels and in bronchus-associated lymphoid tissue (Fig. 2B). Pretreatment of the animals with either apocynin to block superoxide production or 1400W to block NO production by iNOS did not change eosinophil 3-nitrotyrosine staining in the airways (Fig. 2C and D, respectively). This finding indicates that the nitrotyrosine residues are formed by the action of peroxidases present in eosinophils and not by peroxynitrite formed by the interaction of superoxide and NO. The relative contribution of these peroxidase-mediated pathways and peroxynitrite to in vivo 3-nitrotyrosine formation are still unclear (Eiserich et al., 1998b; Reiter et al., 2000).

2.4. Pathophysiology of reactive nitrogen species

The effects of reactive nitrogen species, once they are formed in vivo, on tissues, cells and biomolecules are diverse. For example, important targets of reactive nitrogen species in proteins include tyrosine residues (Van der Vliet et al., 1999), thiols (Gaston, 1999) and heme groups (Fang, 1997b). Further, reactive nitrogen species alter lipid oxidation pathways (O'Donnell et al., 1999), cause DNA damage (Zingarelli et al., 1996) and inhibit mitochondrial respiration (Packer and Murphy, 1995). For detailed information about reactive nitrogen species mediated changes in biomolecules, the reader is referred to an extended review by Eiserich et al. (1998b). Although the exact mechanisms by which reactive nitrogen species affect the function of biological tissues remain unclear, the results of many studies indicate that reactive nitrogen species can compromise cell function. Exposure of cells to reactive nitrogen species leads to both apoptosis and necrosis, depending on the severity of cell damage (Murphy, 1999). Again, these detrimental effects may affect both an invading pathogen and the (infected) host.

2.5. Therapeutic approach

Because of the detrimental effects of reactive nitrogen species on host tissues, protection against these compounds is a promising therapeutic target, e.g. in inflammatory diseases (Hobbs et al., 1999). This protection can be achieved by scavenging reactive nitrogen species by, for example, anti-oxidants, by limiting NO synthesis and by limiting superoxide formation. A disadvantage of limiting the formation of reactive nitrogen species is, of course, a compromised defense against invading microorganisms. Moreover, non-specific NOS inhibition can lead to a compromised function of NO as paracrine messenger, leading for instance, to hypertension (Swislocki et al., 1995). The successful use of NOS inhibition therefore depends on the isoform of NOS involved, and on the selectivity of the inhibitor used. Nonetheless, limiting superoxide production by NADPH oxidase is of particular interest since superoxide release is also required for the formation of many reactive nitrogen species and inhibition of NADPH oxidase should not compromise other NO functions.

3. Production and action of reactive nitrogen species in the airways

Reactive nitrogen species, which are readily formed during inflammatory responses, have beneficial as well as detrimental effects on biological tissues. Likewise, the role of NO and its metabolites in the airways is ambiguous. On one hand, eNOS- or nNOS-derived NO counteracts agonist-induced bronchoconstriction (Folkerts and Nijkamp, 1998; Lei et al., 1993). Furthermore, NO and NO donors relax human airway smooth muscle in vitro, presumably via activation of guanylate cyclase and the associated increase in cGMP (Ward et al., 1995). Interestingly, the concentration of NO in the air exhaled is decreased after acute bronchoconstriction, suggesting a bronchoprotective role for NO (De Gouw et al., 1998). For an extended review of the bronchodilator effects of NO, see Folkerts and Nijkamp (1998). In contrast, the molecule is implicated in the pathobiology of asthma, by way of putatively NO-derived reactive nitrogen species (Barnes, 1996; Barnes and Liew, 1995; Saleh et al., 1998; Van der Vliet et al., 1999).

3.1. Human alveolar macrophages

In contrast to murine macrophages, human mononuclear phagocytes do not release large amounts of NO in vivo, despite the presence of iNOS (Weinberg, 1998; Weinberg et al., 1995; Muijsers et al., 2001(b)). It is still unclear whether the lack of high output NO synthesis is an in vitro artifact. However, a vast number of human disease states are associated with increased iNOS expression in mononuclear phagocytes and consequent NO production (Weinberg, 1998).

Other NOS independent pathways leading to the formation of reactive nitrogen species may yet be of importance in human phagocytes. Firstly, the low pH in phagolysosomes could lead to the conversion of nitrite to NO in phagocytes (Harvey, 2000). Further, the formation of nitrating reactive nitrogen species is readily catalyzed by myeloperoxidase from nitrite and hydrogen peroxide (Eiserich et al., 1998a). Therefore, iNOS-derived NO as such may not be a crucial feature of human inflammatory cells in their nitrosative response to invading microorganisms. Consequently, limiting NO synthesis by means of specific iNOS inhibitors to protect host tissues from nitrosative stress may be a futile strategy in human inflammatory disease.

Important differences appear to exist between human and murine inflammatory cells with regard to the release

of NOS dependent reactive nitrogen species, which cannot be explained by a limited availability of the NOS substrate, L-arginine (Muijsers et al., 2001(b)). Despite low iNOS activity in human mononuclear cells, reactive nitrogen species are readily formed by, for example, human monocytes (Hazen et al., 1999) and human neutrophils (Lei et al., 1993), with nitrite instead of NO as a precursor.

3.2. T-cell differentiation

In addition to the involvement of reactive nitrogen species in the development of airway hyperresponsiveness, there are a number of reports of a putative role for NO as a mediator of T-cell response (Liew, 1995). NO can shift the Th_1/Th_2 balance in the direction of a Th_2-response (Chang et al., 1997). Moreover, exogenously applied NO decreases interleukin 4, interleukin 5 and interferon-γ production by human T-lymphocytes in vitro (Roozendaal et al., 1999). Interestingly, in such experiments, the inhibition of interleukins 4 and 5 production was dependent on cGMP, whereas the decreased interferon-γ production was more persistent and was dependent on additional mechanisms, presumably at the transcriptional level. Formation of reactive nitrogen species could therefore promote allergic responses by T-cells.

3.3. Cell migration

Data from Feder et al. (1997) who used the specific iNOS inhibitor, L-N6-(1-Iminoethyl) lysine (L-NIL), suggest that iNOS-derived NO does not contribute to the extravasation and subsequent migration of eosinophils into the airways of mice. In contrast, the selective NOS inhibitors, *S*-ethylisourea and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT), administered during the challenge phase do prevent both eosinophil and neutrophil influx into the airways (Trifilieff et al., 2000). Moreover, non-specific NOS inhibition by *N*(G)-monomethyl L-arginine (L-NMMA) or eNOS inhibition by *N*G-nitro-L-arginine methyl ester (L-NAME) results in decreased airway eosinophilia after allergen challenge in mice, rats and guinea pigs, suggesting that eNOS-derived NO, presumably released by the endothelium of the pulmonary vasculature, is involved in the extravasation of eosinophils (Trifilieff et al., 2000; Feder et al., 1997; Ferreira et al., 1998; Iijima et al., 1998). In contrast, knocking out either nNOS or eNOS does not change allergen-induced airway eosinophilia (De Sanctis et al., 1999), whereas conflicting data have been published for iNOS knockouts. There is one report of no change in airway eosinophilia (De Sanctis et al., 1999), while in another, airway eosinophilia was decreased in iNOS knockouts (Xiong et al., 1999). In the latter study, increased production of interferon-γ was offered as explanation for the decreased eosinophilia. Interestingly, Hofstra et al. (1998) showed that interferon-γ has a dual role in allergic airway disease. Endogenous interferon-γ is essential for the development of allergic airway inflammation, whereas administration of excess interferon-γ can inhibit the influx of eosinophils into the airway lumen (Hofstra et al., 1998). An overview of the results of different NOS inhibitor and NOS knockout studies on eosinophil recruitment in vivo in various species is given in Table 1. It is clear that further research is required to evaluate the relative contribution of the three NOS isoforms to the extravasation and migration of inflammatory cells during allergic airway inflammation.

3.4. Reactive nitrogen species in allergic airway diseases

It is evident that reactive nitrogen species are readily formed during allergic airway disease. Firstly, 3-nitrotyrosine formation is increased in the airways of asthmatic patients (Saleh et al., 1998). Furthermore, the concentration of NO in the exhaled air of asthmatic patients is increased (Dupont et al., 1998; Massaro et al., 1996; Ten Hacken et al., 1998), and this is reduced by anti-inflammatory therapy such as inhaled corticosteroids (Kharitonov et al., 1996a). The concentration of NO in the exhaled air is thought to reflect the inflammatory state of the airways (Kharitonov et al., 1996a). In any event, low doses of inhaled steroids decrease NO concentrations in the exhaled air, while to prevent airway hyperresponsiveness requires higher doses of steroids (Jatakanon et al., 1999). Lastly, inflammatory cells isolated from the airways of asthmatic patients after segmental allergen challenge release more superoxide than do those from control subjects (Calhoun et al., 1992). While reactive nitrogen species are formed during allergic airway disease, their exact role in the pathobiology of asthma remains unexplained.

Upregulation of iNOS has been reported in the airway epithelium and inflammatory cells from asthmatic airways (Guo et al., 2000; Hamid et al., 1993; Saleh et al., 1998) and after allergen challenge in rats (Liu et al., 1997). Moreover, iNOS expression (Trifilieff et al., 2000) and activity (De Sanctis et al., 1999; Trifilieff et al., 2000) is increased in the lungs of allergen-challenged mice. Lastly, allergen challenge results in increased levels of nitrate in the plasma, an effect which is not seen in iNOS knockout mice (Xiong et al., 1999). Interestingly, iNOS expression in human cells is inhibited by corticosteroids (Robbins et al., 1994; Saleh et al., 1998; Walker et al., 1997). This is important since inhaled corticosteroids prevent both iNOS expression in the airways (Saleh et al., 1998) and the increase in NO oxide concentration in the exhaled air (Kharitonov et al., 1996b) of asthmatic patients. An increased formation of reactive nitrogen species during allergic airway inflammation therefore seems to depend on iNOS.

The cellular and molecular mechanisms leading to increased NO synthesis and reactive nitrogen species forma-

Table 1
Overview of the effects of NOS inhibition or NOS knockout on allergen-induced airway hyperresponsiveness and eosinophil influx in different species in vivo compared to control or wildtype mice as described in literature

Species	Inhibitor	Target[a]	AHR	Eosinophils	Reference
Guinea pig	L-NAME	ENOS	↑	ND	Mehta et al. (1997)
	L-NAME	ENOS	↓	↓	Iijima et al. (1998)
	AG	INOS	↓	ND	Iijima et al. (1998)
Rat	L-NAME	ENOS	↑	=	Tulic et al. (2000)
	L-NAME	ENOS	ND	↓	Ferreira et al. (1998)
	L-NMMA	NS	↑	↑	Tulic et al. (2000)
	AG	INOS	=	↓	Tulic et al. (2000)
	SMLT	NNOS	=	↑	Tulic et al. (2000)
	SD3651	INOS	↓	=	Eynott et al. (2000)
Mouse	L-NMMA	NS	ND	↓	Feder et al. (1997)
	L-NIL	INOS	ND	=	Feder et al. (1997)
	AMT	INOS	ND	↓	Trifilieff et al. (2000)
	EIT	NS	ND	↓	Trifilieff et al. (2000)
	L-NAME	ENOS	ND	↓	Feder et al. (1997)
	L-NAME	ENOS	ND	↓	Trifilieff et al. (2000)
	AG	INOS	ND	↓	Feder et al. (1997)
	1400W	INOS	↓	=	Muijsers et al. (2001(a))
Mouse	KO[b]	INOS	=	↓	Xiong et al. (1999)
Knockouts	KO[b]	INOS	=	=	De Sanctis et al. (1999)
	KO[b]	ENOS	=	=	De Sanctis et al. (1999)
	KO[b]	NNOS	↓	=	De Sanctis et al. (1999)
	KO[b]	eNOS + nNOS	↓	=	De Sanctis et al. (1999)

AHR: airway hyperresponsiveness, eosinophils: number of eosinophils in the bronchoalveolar lavage fluid. = Unaltered, increase, decrease, ND not determined. L-NAME: *N*G-nitro-L-arginine methyl ester; L-NMMA: *N*G-mono-methyl-L-arginine; EIT: *S*-ethylisothiourea; AMT: 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine; AG: aminoguanidine; L-NIL: L-*N*6-(1-iminoethyl)lysine; SMLT: *S*-methyl-L-thiocitrulline; 1400W: *N*-(3-(Aminomethyl)-benzyl)acetamidine.
See text for detail.
[a]NOS isoform to which the inhibitor is relatively selective, NS: nonspecific (Garvey et al., 1997; Southan and Szabo, 1996).
[b]KO: knockout mice.

tion upon allergen challenge are yet clear. In asthmatic subjects, iNOS protein is upregulated in the airway epithelium through transcriptional regulation (Guo et al., 1995, 2000). Interferon-γ is likely to play an important role in the induction of iNOS during asthma, since the cytokine is essential for iNOS expression in human airway epithelial cells in vitro (Guo et al., 1997; Punjabi et al., 1994; Robbins et al., 1994). Furthermore, the concentration of interferon-γ is increased in the epithelial lining fluid of asthmatics (Guo et al., 2000). Hence, the Th1 cytokine interferon-γ could be the link between allergic airway inflammation and iNOS in humans. Interestingly, the development of allergen-induced hyperresponsiveness in a murine model of allergic asthma is dependent on interferon-γ (Hessel et al., 1997). Therefore, interferon-γ could also be the cytokine responsible for iNOS induction during allergic airway inflammation in mice. A large population of the T-lymphocytes present in the airways after allergen challenge is non-antigen specific and releases interferon-γ (Ying et al., 1995). Indeed, thoracic lymph node cells from ovalbumin challenged animals release increased amounts of interferon-γ independently of in vitro ovalbumin stimulation (Hessel et al., 1997).

In contrast to human asthmatic airways, the airway epithelium of mice does not express iNOS during allergic airway inflammation (Trifilieff et al., 2000). Alveolar macrophages are a likely source of iNOS-dependent NO production in mice, since allergen-induced inflammatory infiltrates show iNOS expression (Trifilieff et al., 2000). Interferon-γ induces high output NO synthesis by murine macrophages in vitro (Muijsers et al., 2000). Moreover, iNOS expression is increased in macrophages after allergen challenge in rats (Liu et al., 1997).

Another possible mechanism leading to increased NO and reactive nitrogen species release is the activation of the low-affinity IgE receptor (CD23). Stimulation of CD23 on human mononuclear phagocytes enhances iNOS expression and NO release (Kolb et al., 1994, 1995). Interestingly, CD23 is also expressed on human airway epithelial cells (Atsuta et al., 1997). It is unclear whether CD23 contributes to the expression of iNOS in asthmatic airways (Saleh et al., 1998).

Calhoun et al. (1992) showed increased superoxide production by alveolar macrophages from asthmatic patients after segmental allergen challenge. Moreover, blood monocytes and neutrophils from asthmatic patients release more superoxide in vitro upon stimulation than do those from healthy controls (Demoly et al., 1995; Joseph et al., 1993). In addition to these inflammatory cells, alveolar type II cells (Van Klaveren et al., 1997) and the endothe-

lium of the pulmonary vasculature (Al-Mehdi et al., 1998) express NADPH oxidase. Moreover, administration of superoxide dismutase to allergen challenged guinea pigs prevents the development of airway hyperresponsiveness (Ikuta et al., 1992). Therefore, increased superoxide production during allergic airway inflammation is likely to contribute to the nitrosative stress and consequent damage to the airways via the interaction between reactive oxygen and nitrogen species.

Interferon-γ also is an important regulator of NADPH oxidase activity and expression in both human and murine phagocytes (Demoly et al., 1995; Rottenberg et al., 2000), and reactive nitrogen species formation upon interferon-γ/lipopolysaccharide stimulation is dependent on NADPH oxidase (Muijsers et al., 2000). Therefore, interferon-γ could be the connection between allergic airway inflammation and both superoxide and NO release, and consequently the formation of reactive nitrogen species. It is tempting to speculate that the formation of reactive nitrogen species during allergic airway inflammation depends on a Th1-like response rather than on a Th2 response.

These many observations make it clear that the role of NO in allergic airway inflammation is, to say the least, puzzling. Further research is required to elucidate the effects of NO on inflammatory cell migration and on T-helper cell responses. The effect of NO on these phenomena seems to be dependent on time, context and concentration. It is suggested that the in vivo methods available (i.e. systemic NOS inhibition or knocking out NOS isoforms) are not yet refined enough for proper investigation of the ambiguous and complex effects of NO on the development of allergic airway inflammation.

4. Reactive nitrogen species and airway hyperresponsiveness

Table 1 provides an overview of the effects of different NO synthase inhibitors on allergen-induced hyperresponsiveness in vivo in different species as described in the literature. As mentioned previously, the effects of the inhibitors depend on their relative selectivity to the three NOS isoforms. Although more or less selective inhibitors are available, at high concentrations even a selective inhibitor will inhibit the other isoforms. The iNOS inhibitor aminoguanidine, for example, is only about 10–100 times more potent against iNOS than against eNOS (Misko et al., 1993).

Since eNOS- or nNOS-derived NO counteracts agonist-induced bronchoconstriction, (Lei et al., 1993), inhibition of these enzymes may enhance airway hyperresponsiveness (Nijkamp et al., 1993; Schuiling et al., 1998b). On the other hand, iNOS inhibition putatively protects against the detrimental effects of high output NO release, thereby preventing the development of airway hyperresponsiveness. Hence, nonspecific NO synthase inhibition may antagonize the detrimental as well as the beneficial effects of NO.

This non-specificity may mostly account for the conflicting data summarized in Table 1.

4.1. Inducible NOS

1400W is a highly selective, tightly binding iNOS inhibitor which is about a 1000-fold more selective for iNOS than for eNOS (Garvey et al., 1997). Hence, the fact that 1400W inhibited allergen-induced airway hyperresponsiveness (Muijsers et al., 2001(a)) suggests strongly that iNOS-derived NO release plays a causative role in the airway changes leading to hyperresponsiveness after allergen exposure in mice. Interestingly, similar results were obtained in rats when the novel selective iNOS inhibitor, SD3651 is used (Eynott et al., 2000).

iNOS knockout studies have not yet shown a role for iNOS-derived NO in the development of airway hyperresponsiveness in mice. Xiong et al. (1999) reported inhibition of allergic airway inflammation in iNOS-knockouts, but an unaltered development of airway hyperresponsiveness. De Sanctis et al. (1999) reported that knocking out the NOS2 gene affects neither allergic inflammation nor airway hyperresponsiveness. The fact that the production of interferon-γ by lung cells from iNOS knockout mice is increased is a likely explanation for the discrepancy between results of iNOS knockout and of inhibitor studies. High levels of interferon-γ have been demonstrated to inhibit the development of allergen-induced airway hyperresponsiveness in mice (Hofstra et al., 1998). Therefore, the effects of knocking out iNOS on allergic airway disease might reach further than simply abolish NO synthesis (Mashimo and Goyal, 1999), and could for example, enhanced interferon-γ release (Xiong et al., 1999). Likewise, the NOS-inhibitor studies have the important drawbacks that the selectivity of the inhibitors is hard to prove, and that it is impossible to block NO synthesis in vivo completely and continuously. Hence, it is impossible to draw valid conclusions about the NOS isoform involved in the development of airway hyperresponsiveness in mice. In spite of the iNOS knockout results, many other studies strongly suggest a role for iNOS in humans, rats, guinea pigs and mice, as mentioned above.

The NADPH oxidase inhibitor, apocynin, completely inhibits allergen-induced hyperresponsiveness in mice (Muijsers et al., 2001(a)). This suggests that the development of allergen-induced hyperresponsiveness is dependent on superoxide formation as well as on NO release. Since the release of reactive nitrogen species by murine macrophages is dependent on both superoxide and NO (Muijsers et al., 2000), these data suggest that during allergic airway disease, superoxide release enhances the detrimental effects of NO and vice versa. It is therefore

hypothesized that the development of allergen-induced hyperresponsiveness depends on the simultaneous release and consequent interaction of NO and superoxide. Thus, to limit superoxide release by apocynin may be preferable to limiting the release of reactive nitrogen species, since there is no risk of inhibiting bronchoprotective effects of NO on the airways.

4.2. Neuronal NOS

Interestingly, allergen-induced airway hyperresponsiveness is completely abolished in nNOS knockout mice (De Sanctis et al., 1999). The mechanism by which nNOS could be involved in airway hyperresponsiveness, however, is unclear. nNOS is directly involved in the relaxation of airway smooth muscle through counteraction of neuronal cholinergic responses (Kakuyama et al., 1999; Ward et al., 1993, 1995). Moreover, nNOS is expressed in human airway smooth muscle cells and has been suggested to play an antiproliferative role (Patel et al., 1999). Recently, we did immunostaining for nNOS, iNOS and eNOS in the airways of mice after allergen challenge and, surprisingly, found nNOS staining in the airway epithelium (Fig. 3).

Interferon-γ inhibits nNOS expression in human astrocytoma cells (Colasanti et al., 1999) and in rat rectum, spleen, brain and stomach as confirmed by Western blots (Bandyopadhyay et al., 1997). Hence, interferon-γ may not only promote the detrimental effects of NO by iNOS induction, but may also compromise the bronchorelaxing function of nNOS. Like iNOS, nNOS could be of importance because of the detrimental effects of high output NO synthesis and consequent formation of reactive nitrogen species. Again, further research is required to elucidate whether such high output NO synthesis by nNOS can occur in vivo.

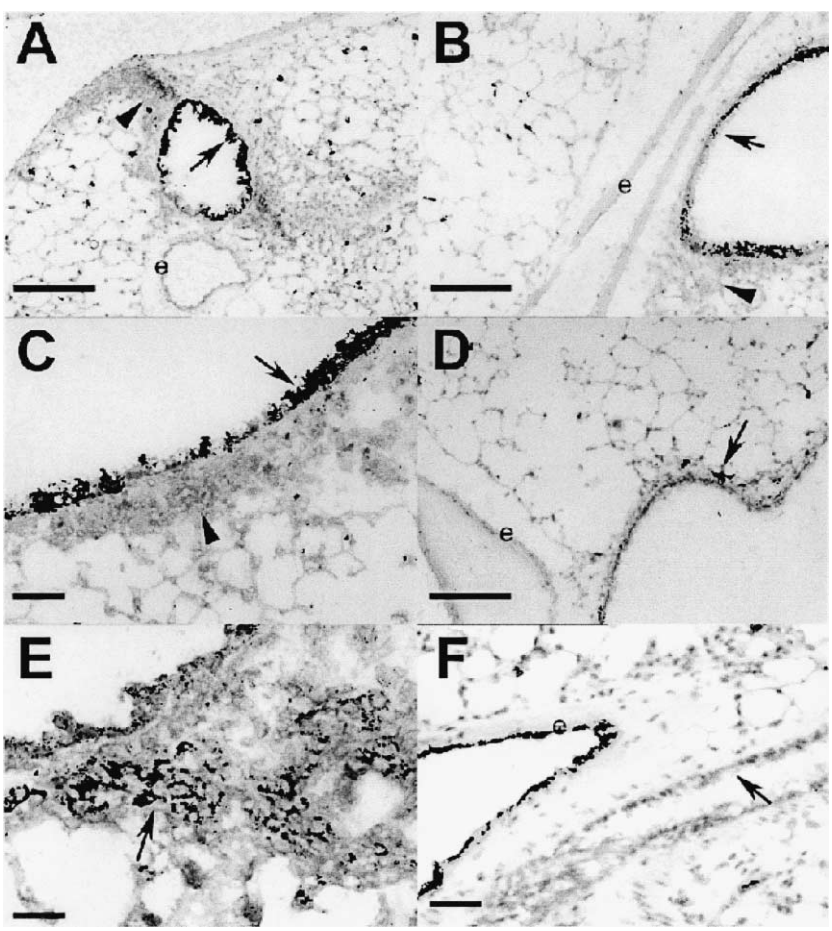

Fig. 3. nNOS , iNOS and eNOS immunohistochemical staining of the airways of ovalbumin sensitized mice challenged with either ovalbumin or saline, using polyclonal antibodies directed against nNOS, iNOS and eNOS (black staining). (A, B and C) Anti-nNOS (ABR) staining in the airway epithelium of ovalbumin challenged animals (arrows). Arrowheads indicate bronchus-associated lymphoid tissue. An 'e' indicates vascular tissue. (D) Section from saline-challenged animal stained with the nNOS antibody from ABR, showing focal staining (arrow). (E) Reactivity to the iNOS antibody (ABR) in bronchus-associated lymphoid tissue (arrow) of an ovalbumin-challenged animal. (F) eNOS staining (black) of the vascular endothelium (e) of an ovalbumin-challenged animal. No staining was found in the epithelium (arrow). Scale: section A, B and F: 40 × (calibration bars represent 200 μM), section C and F: 100 × (calibration bars represent 50 μM) and section E: 200 × (calibration bar represents 25 μM).

Interestingly, two independent studies have shown that variations in the nNOS gene are associated with human asthma (Gao et al., 2000; Grasemann et al., 1999), whereas such variations are not found for either iNOS or eNOS. The functional consequences of these variations are however, unknown.

5. Hypothesis and conclusions

At present, one can only speculate as to how reactive nitrogen species, once released in the airways, contribute to airway hyperresponsiveness. A likely target for reactive nitrogen species is the airway epithelium, which has two important functions in the control of airway responsiveness. Firstly, the epithelium forms a physical barrier protecting the airway interstitium against contractile agents, and secondly, it is an important source of bronchorelaxant compounds (Folkerts and Nijkamp, 1998). Epithelial shedding and damage have been reported in asthmatic subjects (Jeffery, 1998), and also occurs in mice after allergen challenge (Garlisi et al., 1997).

It is tempting to speculate that reactive nitrogen species contribute to the epithelial damage during allergic airway inflammation, considering the numerous detrimental effects of the compounds on cells (Eiserich et al., 1998b). In fact, reactive nitrogen species cause pulmonary cell death in vitro (Gow et al., 1998; Kampf et al., 1999). Moreover, iNOS-mediated damage to the airway epithelium contributes to airway hyperresponsiveness in guinea pigs (Schuiling et al., 1998a). In addition to cytotoxic effects, reactive nitrogen species are likely to induce more subtle changes in the epithelium, such as for example, changes in cyclo-oxygenase activity (Watkins et al., 1997) and eNOS inhibition (Sheehy et al., 1998). Interestingly, metalloproteases are activated by reactive nitrogen species (Maeda et al., 1998; Murrell et al., 1995), and the subsequent degradation of (extra)cellular proteins may further contribute to airway damage.

Kotsonis et al. (1999) showed that reactive nitrogen species are potent inhibitors of nNOS, which is an important source of NO involved in bronchorelaxation (Kakuyama et al., 1999; Ward et al., 1993, 1995). Superoxide formation could also contribute to airway hyperresponsiveness by scavenging the bronchorelaxant NO, as shown in guinea pigs by De Boer et al. (1998). Human asthmatic airways show clear staining for 3-nitrotyrosine and iNOS in the airway epithelium (Saleh et al., 1998), indicating that, in humans, the airway epithelium is exposed to nitrosative stress from inside the cells. In mice, however, allergen challenge does not induce iNOS (Trifilieff et al., 2000) or 3-nitrotyrosine staining in the airway epithelium (Fig. 2). Hence, other sources of reactive nitrogen species, for example alveolar macrophages, may be important in mice.

In conclusion, it appears that NO plays an important role in the pathophysiology of asthma. NO-derived reactive nitrogen species are readily formed during allergic airway inflammation and have been suggested to have detrimental effects on airway function. In contrast to these detrimental effects, endogenously released NO also has a bronchoprotective role, because it is able to antagonize agonist-induced bronchoconstriction. These phenomena open interesting new therapeutic approaches for the treatment of asthma exacerbation.

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