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Review Article

iNOS homodimerization as a target for potential new therapeutic agents

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CONTENTS

Abstract

Nitric oxide (NO) plays an important role in various physiological processes. Nitric oxide is synthesized by a family of NO synthases (NOS). Of the three isoforms of NOS, inducible NOS (iNOS) is closely linked to inflammatory and autoimmune diseases. The suppression of excess NO production in participating cells may be helpful in improving disease status. This review describes a new mechanism of inhibition of NO production by targeting iNOS dimerization. Nitric oxide synthase isoforms are only active as homodimers. The oxygenase domains of two NOS monomers interact to form the dimer. Certain imidazole derivatives, including PPA250, inhibit this step and suppress NO production. Crystallographic studies have shown that an iNOS dimerization inhibitor blocks dimer formation by coordinating the heme in the monomer. Oral administration of PPA250 suppressed the development of arthritis after clinical symptoms had appeared in animal models. PPA250 also decreased the serum concentration of NO in mice treated with lipopolysaccharide (LPS). These results indicate that inhibitors of iNOS homodimerization could be useful therapeutic agents for rheumatoid arthritis, septic shock and other diseases in which NO is involved.

Introduction

Nitric oxide (NO) is a small reactive molecule with an important role in various physiological processes, including modulation of inflammatory responses and regulation of vessel tone (1, 2). Nitric oxide synthase (NOS) catalyzes the formation of NO and L-citrulline from L-arginine (Arg) and oxygen. The NOS family consists of three known mammalian isoforms (3). The neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed under noninflammatory conditions, and their activity is tightly regulated by Ca^{2+}-dependent calmodulin (4-6). The third, inducible isoform (iNOS) is a key mediator of inflammation and host defense systems (7).

Expression of iNOS is induced at a transcriptional level by inflammatory stimuli, including interferon (IFN), interleukin (IL)-1, tumor necrosis factor (TNF) and bacterial lipopolysaccharide (LPS) (8). Production of excess NO and prolonged induction of iNOS have been observed in various inflammatory and autoimmune diseases, including septic shock, hemorrhagic shock, systemic lupus erythematosus, Sjögren's syndrome, vasculitis, rheumatoid arthritis (RA) and osteoarthritis (OA) (7, 9-11). Several iNOS inhibitors have been shown to lead to improvements in both patients and animal models (12-15). Accordingly, NO has been implicated in the pathology of these diseases.

Suppression of excess NO formation in participating cells may be helpful in improving disease status. Inhibitors of NO production have been widely studied over the last few decades, with the aim of developing therapeutic agents for these diseases. So far, despite enormous efforts in design and testing of inhibitors, only a very limited number of compounds are in late clinical development. Changing the strategy for inhibiting NO production could therefore be therapeutically advantageous. This review highlights a novel target, the NOS dimerization step essential for enzyme activation and NO production.

Inhibitors of NO production

Many compounds that inhibit NO production have been developed as potential new therapeutic agents for

the diseases mentioned above. Hobbs (1) grouped compounds able to prevent the biological activity of NO into six main categories according to their mechanism of action: 1) drugs preventing uptake of Arg into cells, thus denying NOS its substrate; 2) agents that reduce the supply of cofactors required for NOS-catalyzed oxidation of Arg, including Ca^{2+} sequesterers, tetrahydrobiopterin (H_4B) synthesis inhibitors and calmodulin (CaM) antagonists; 3) inhibitors of electron flow via NADPH/flavins and agents interfering with the heme moiety; 4) inhibitors of the expression of NOS; 5) drugs preventing the binding of substrate to NOS; and 6) scavengers of NO. Of these classes, the substrate-mimetic inhibitors such as aminoguanidine and N^G-monomethyl-L-arginine (L-NMMA) have been most extensively researched. However, recent advances in NOS research reveal that there could be another class of agents with a new mechanism of action —the NOS dimerization inhibitors, the efficacy of which resides in the fact that NOS acquires the capacity to synthesize NO only through forming homodimers.

Assuming long-term administration of the inhibitor for therapeutic purposes, high isoform specificity is important, as other isoforms also play major physiological roles. It is difficult to design Arg-mimetic competitive inhibitors with high selectivity, as this mechanism targets the common substrate. On the other hand, although dimerization is essential for enzyme activation of all NOSs (3, 16, 17), only iNOS is induced during inflammation. The stable active homodimer is rapidly formed from newly synthesized NOS monomers in the presence of sufficient cofactors. Thus, compounds present in the inflammatory sites that inhibit NOS dimerization will prevent NO production by iNOS, but not constitutive NOSs, which are regulated by Ca^{2+}. However, a few recent reports show that eNOS and nNOS are also inducible to some extent (18, 19). Therefore, it is preferable that iNOS dimerization inhibitor candidates for medical use not inhibit other isoforms. Distinct dimer interactions and regulation between isoforms, as described below, may render this mechanism beneficial as an isoform-specific drug target.

iNOS homodimerization and its catalytic action

As shown in Figure 1, NOSs have a bidomain structure in which a C-terminal reductase domain containing binding sites for NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and CaM is linked to an N-terminal oxygenase domain that contains binding sites for Arg, H_4B and heme (16, 20, 21). The newly synthesized NOS protein first binds FAD, FMN and CaM to form a monomer that contains a functional reductase domain but can not synthesize NO. Dimer formation begins with the insertion of a heme, which induces rapid conformational change (16, 20, 21). Two NOS monomers with hemes form an intermediate of dimerization, which is stabilized to the active homodimer in the presence of H4B (16, 22). Dimer formation is essential for catalytic activity

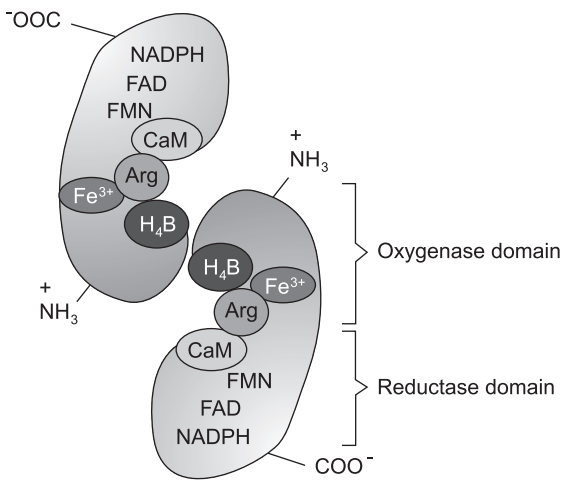


Fig. 1. The NOS homodimer.

because the catalytically critical flavin-to-heme electron transfer step occurs across groups located in adjacent subunits in the homodimer (23).

Homodimer formation activates all NOSs. A yeast two-hybrid study with oxygenase and reductase domains shows that iNOS subunit association involves only oxygenase domain interactions (24). However, the association of eNOS and nNOS involves three kinds of interaction, between oxygenase domains (head to head), reductase domains (tail to tail) and across both domains (head to tail) (25). Moreover, urea dissociation studies and analysis of crystallographic structures of NOSs indicate that NOS isoforms, in spite of their general structural similarity, differ markedly in their association strengths, interfaces, and in how Arg and H_4B influence their formation and stability (26). Accordingly, it can be expected that inhibitors targeting this step will have high isoform specificity.

Known iNOS dimerization inhibitors

To date, several groups of researchers have reported compounds that inhibit homodimer formation of iNOS. As shown in Figure 2, the antifungal imidazoles clotrimazole and miconazole were first reported to block assembly of iNOS into an active dimer in 1999 (27). Combinatorial chemistry over the ensuing years revealed BBS-1 and related compounds with pyrimidine-imidazole cores to be potent and selective iNOS dimerization inhibitors (28), and in 2002, BBS-2 was found to have similar effects (29). Moreover PPA250, or 3-(2,4-difluorophenyl)-6-[2-[4-(1*H*-imidazol-1-ylmethyl)phenoxy]ethoxy]-2-phenyl-pyridine, an iNOS dimerization inhibitor, was the first such compound demonstrated to have therapeutic effects by oral administration in animal models of NO-related disease, as described in the following section (30). This

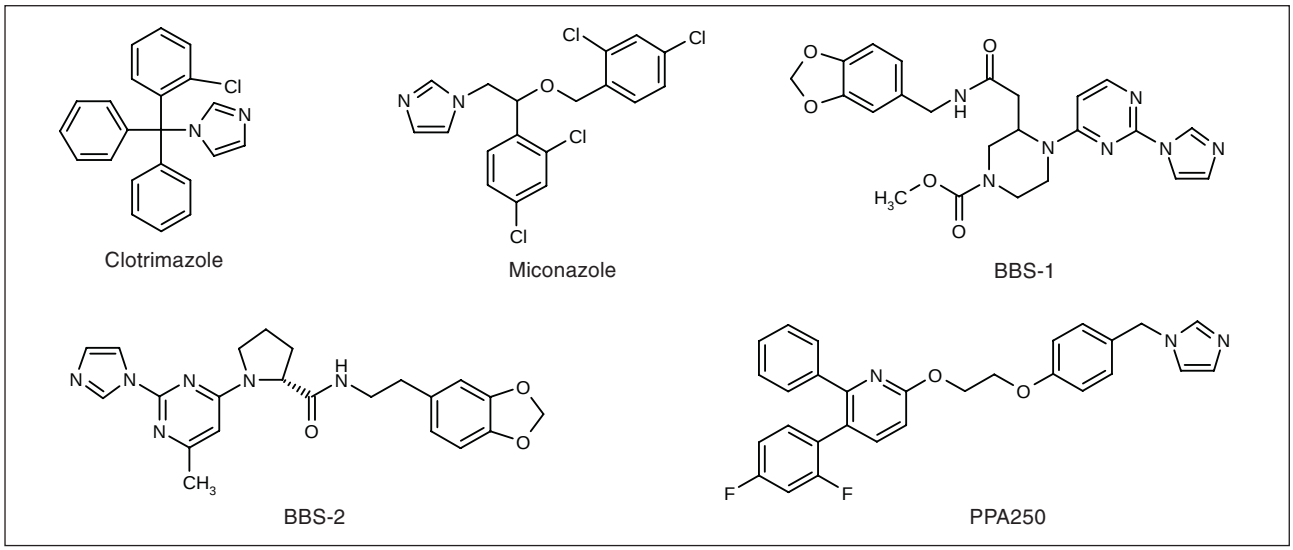


Fig. 2. Chemical structures of iNOS dimerization inhibitors.

compound is also believed not to inhibit eNOS, as its administration for 7 days did not increase blood pressure in normal rats *in vivo* (unpublished data).

All of the dimerization inhibitors mentioned above are imidazole-containing structures, and no compounds based on another skeleton have been discovered so far. It is established that imidazole compounds inhibit various heme-containing enzymes, including cytochrome P-450 (CYP), by binding the heme group to varying degrees. Indeed, CYP and the C-terminal portion of the NOS protein closely resemble one another. The IC_{50} values for BBS-2 (29) and PPA250 (unpublished data) against CYP3A4 are about 150 and 100 nM, respectively. Thus, cross-reactivity with CYPs, in particular CYP3A4, a major drug-metabolizing enzyme, might be potentially problematic. However, this new target might signal a breakthrough in the development of NO inhibitors, none of which have been approved for use as therapeutic agents despite numerous studies.

Molecular mechanism of inhibition of iNOS homodimerization

Regarding the molecular mechanism of interference with the homodimerization of iNOS, X-ray crystallography of BBS-1 revealed that the bulky imidazole residue coordinates the heme in the iNOS monomer, leading to irreversible allosteric disruption of protein-protein interactions at the dimer interface (28, 29). Moreover, spectroscopy and competitive binding tests with imidazole show that the inhibitor binds to a heme-containing iNOS monomer, forming an inactive iNOS monomer-heme-inhibitor complex in an H_4B- and Arg-independent manner (29). A general model for iNOS dimer assembly and its inhibition by imidazoles is proposed in Figure 3 (27, 29). Heme insertion into monomeric iNOS, the first step in dimer formation, gives a loosely bound dimer intermediate (DIMER-Fe), which then binds H_4B and Arg to become stable and enzymatically active (DIMER-Fe-H_4B-Arg). There is little reversible equilibrium toward iNOS monomer once the iNOS dimer has formed. In the presence of inhibitors, the transient heme-containing monomer species (MONOMER-Fe) are bound to form inactive monomer-heme-inhibitor complexes (MONOMER-Fe-INHIBITOR).

Structure-activity relationships (SAR) and design of iNOS dimerization inhibitors

The finding that dimerization is essential for iNOS activity prompted us to synthesize a series of compounds with the aim of developing an iNOS dimerization inhibitor. We have screened for compounds targeting NOS homodimerization with a cell-based iNOS assay using a mouse macrophage-like cell line, RAW264.7, stimulated with interferon gamma (IFNγ) and LPS (31).

To find a lead compound, diphenylpyrazine compounds containing various linkers and imidazole moieties were screened for inhibitory activity on NO production (Table I). As a result, -OCH_2CH_2O- was identified as being the best linker, and compound **1**, bearing a 1-imidazolylmethyl moiety, showed the most potent inhibitory activity. Interestingly, NO-inhibitory activity decreased upon the introduction of substituents to the imidazole (compound **10**), and when the substitution position on the imidazole was altered (compounds **8** and **9**). These results suggested that the steric environment of the nitrogen atom at the 3-position of the imidazole ring was related to the ability to bind iNOS protein.

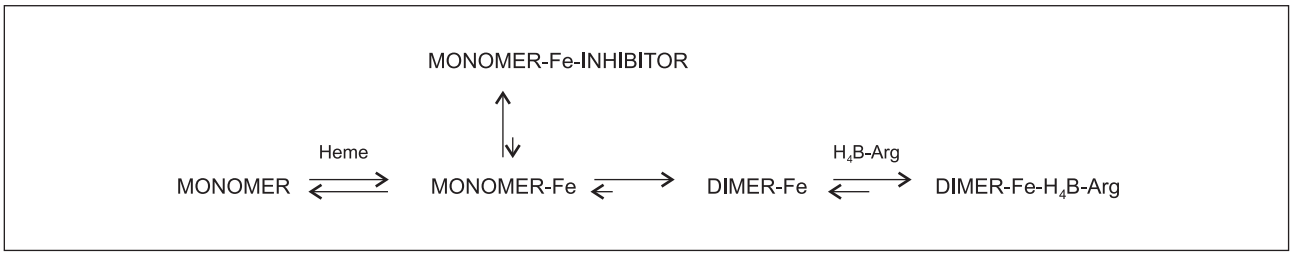
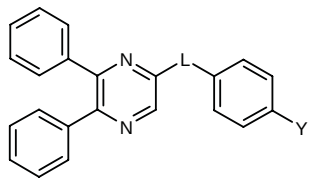
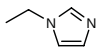
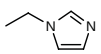
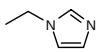
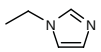
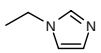
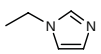
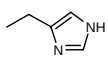
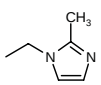


Fig. 3. Model for inhibition of iNOS dimer assembly by iNOS dimerization inhibitors.

Table I: Inhibition of NO production by stimulated RAW264.7 cells.

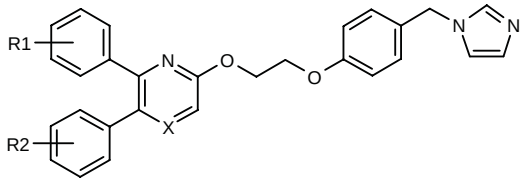


Compounds	L	Y	% of Control (20 μg/ml)
1	-O(CH$_2$)$_2$O-		2.8
2	-O(CH$_2$)$_2$CH$_2$-		91.9
3	-CH$_2$(CH$_2$)$_2$O-		31.7
4	-CH$_2$(CH$_2$)$_2$CH$_2$-		75.1
5	-S(CH$_2$)$_2$O-		37.2
6	-N(Me)(CH$_2$)$_2$O-		29.0
7	-O(CH$_2$)$_2$O-		58.3
8	-O(CH$_2$)$_2$O-		78.7
9	-O(CH$_2$)$_2$O-		72.5
10	-O(CH$_2$)$_2$O-		96.2

To increase activity, pyrazine and pyridine ring substituents were introduced to the phenyl group at the C2- and C3-position (Table II). Substitution with pyrazine compounds did not affect inhibitory activity, but the pyridine compounds showed clear effects: the 2,4-difluoro [**16**], 3-nitro [**17**] and 4-methylthio [**19**] compounds were more active than the unsubstituted compound **15**. Among these, compound **16** (PPA250) showed the most potent inhibitory activity.

Some of the compounds described above were confirmed to modify iNOS activity by reducing dimerization in RAW264.7 cells stimulated with IFNγ and LPS. The results for PPA250 are shown in Figure 4 (30). Inducible nitric oxide synthase is induced in RAW264.7 cells stimulated with IFNγ and LPS. Monomers and dimers may be separated by SDS-polyacrylamide gel electrophoresis (PAGE) run in an ice bath without heat denaturation. PPA250 decreased the amount of the dimer concentration-

Table II: Inhibition of NO production by stimulated RAW264.7 cells.



Compounds	R^1	R^2	X	IC_{50} (μM)
1	H	H	N	0.34
11	4-F	4-F	N	0.22
12	4-CF_3	4-CF_3	N	0.24
13	3-OMe	3-OMe	N	1.4
14	4-CF_3	H	N	0.36
15	H	H	CH	0.94
16 (PPA250)	H	2,4-diF	CH	0.082
17	H	3-NO_2	CH	0.15
18	H	3-NH_2	CH	2.4
19	H	4-SMe	CH	0.32

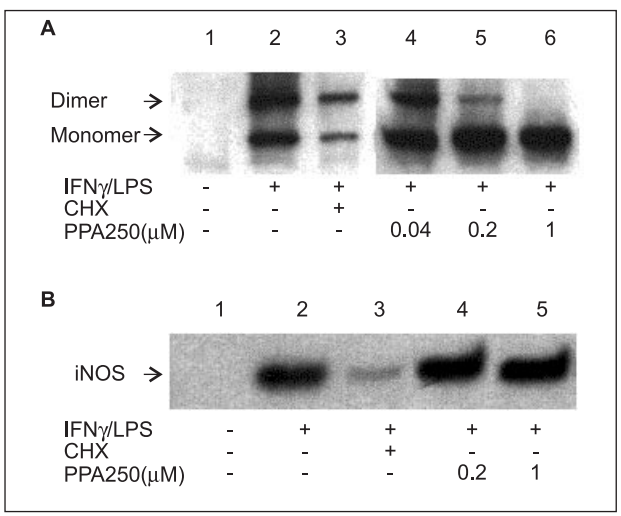


Fig. 4. Western blot analysis of iNOS. RAW264.7 cells were harvested at 18 h after stimulation and the cell lysates were subjected to SDS-PAGE without (A) or with (B) heat denaturation. CHX: cycloheximide.

dependently (Fig. 4A), but had no effect on the total amount of iNOS protein present, as determined by SDS-PAGE after heat denaturation (Fig. 4B). This shows that dimerization of iNOS is blocked by concentrations of PPA250 similar to those that inhibit NO production. However, PPA250 failed to inhibit iNOS activity in a crude cell lysate prepared from stimulated cells. These results indicate that inhibition of NO production by PPA250 in stimulated RAW264.7 cells is due to the prevention of iNOS dimerization, and not to a decrease in its *de novo* synthesis or inhibition of enzyme activity of the iNOS active dimer (30).

Therapeutic potential of PPA250

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology. However, concentrations of nitrite, a stable degradation product of NO, are known to be increased in synovial fluid and serum from patients with RA (9, 10). In addition, induced iNOS mRNA and protein or increased NO production have been observed in cultures of synovium and cartilage from RA or osteoarthritis (OA) patients (9). Based on these data, it is speculated that iNOS-derived NO is involved in the inflammation associated with RA.

It has been demonstrated that oral administration of PPA250 suppresses the development of destructive polyarthritis in animal models (30). Mouse collagen-induced arthritis, a commonly used model of RA, was established by immunization with type II collagen. After clinical signs of arthritis had developed at day 28, mice received daily oral administration with 10 or 30 mg/kg of PPA250. PPA-250 reduced the inflammation score within 1 day in a dose-dependent manner (Fig. 5A). At a dose of 3 or 10 mg/kg, it also suppressed joint inflammation in rats with adjuvant-induced arthritis, another model of chronic inflammatory joint disease (30). These results suggest that oral administration of PPA250 has potential therapeutic value in the treatment of RA.

The development of arthritis is accompanied by the induction of iNOS and subsequent NO production in these models (14, 15, 32-35). Earlier studies in animal models demonstrated that although substrate-mimicking NO inhibitors such as L-NMMA, N^G-nitro-L-arginine methyl ester and N^G-methyl-L-arginine completely suppress the development of arthritis in these models when administered prophylactically (15, 32, 34, 35), they are much less effective when administered therapeutically (32, 33, 35). However, a curative effect of PPA250 was observed even when treatment was initiated after clinical symptoms had appeared in these animals (30). It is not clear what underlies these differences, but the mechanism of dimerization inhibitors might confer an advantage.

Septic shock

Septic shock, a major cause of death in intensive care units, is a severe systemic inflammatory response to a Gram-negative bacterial infection. In response to LPS, host cells, particularly macrophages, release inflammatory mediators such as TNF-α, IL-1β, IL-6, IFNγ and NO (8, 36). Considerable evidence suggests that excessive production of NO by iNOS contributes to the circulatory failure observed during septic shock (1, 37). Serum levels of NO oxidation products, such as nitrate, are elevated in both patients and experimental animals undergoing septic shock and sepsis syndrome (8, 38, 39), and iNOS inhibitors prevent LPS-induced mortality in mice (37, 39, 40). Also, mutant mice lacking iNOS are resistant to

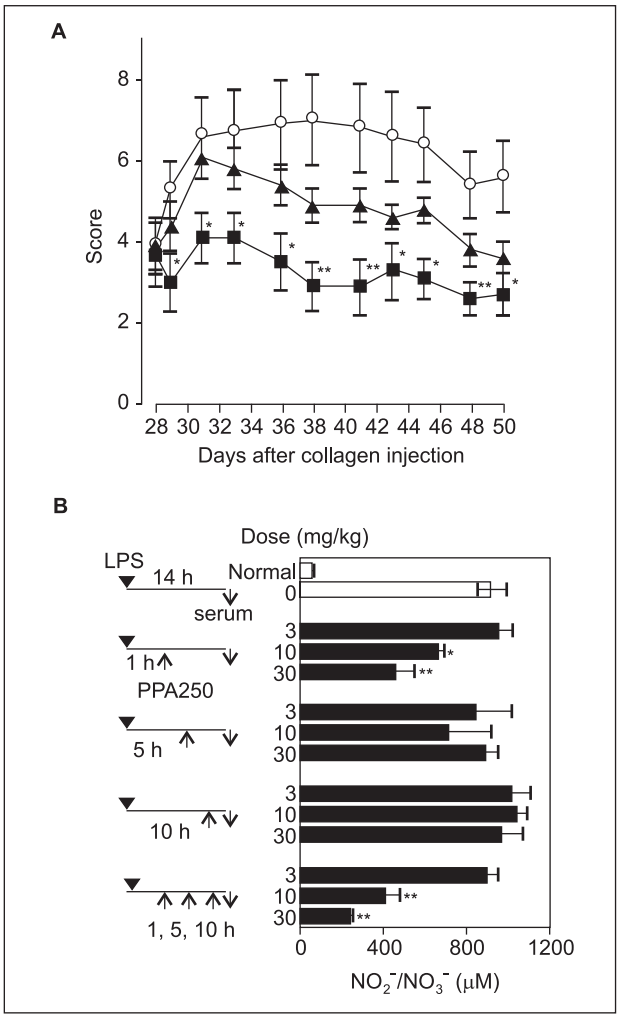


Fig. 5. Effects of PPA250 in animal models. (A) Effect of PPA250 on inflammation score in collagen-induced arthritis. DBA/1J mice were injected intradermally on day 0 with bovine collagen type II in complete Freund's adjuvant, followed at day 21 by a booster injection. PPA250 (10 or 30 mg/kg) was administered orally once a day for 22 days, starting on day 28. Open circles, vehicle control; closed triangles, PPA250 10 mg/kg; closed squares, PPA250 30 mg/kg. $^{**}p < 0.01$, $^{*}p < 0.05$ for PPA250 compared with vehicle. n=8 animals/group. (B) Nitrite/nitrate concentration in serum of septic mice treated with PPA250. BALB/c mice were administered 3, 10 or 30 mg/kg PPA250 orally at 1, 5 or 10 h after LPS injection. Mice were sacrificed 14 h after LPS injection and serum was collected. Data are means ± S.E. (n=3). $^{**}p < 0.01$, $^{*}p < 0.05$ compared to control.

LPS-induced mortality (41), establishing the crucial role of NO as a key agent in LPS-induced death.

Oral administration of PPA250 1 h after LPS injection significantly decreased the concentration of nitrite/nitrate, stable degradation products of NO, in a dose-dependent manner, as expected from the *in vitro* results (Fig. 5B) (30). However, administration at 5 or 10 h after LPS injection had no effect. Triple administration at these times was more effective than a single dose at 1 h. The reduction in alertness and piloerection observed in LPS-injected mice was dramatically recovered following doses of PPA250; it also decreased the serum concentration of NO. The results indicate that formation of iNOS dimers occurs soon after LPS injection, and that PPA250 does not effect the dissociation or degradation of the dimer once it has been formed. These observations are supported by a recent study in which i.p. administration of BBS-2 prevented endotoxin-induced systemic hypotension and attenuated myocardial dysfunction and impairment of hypoxic pulmonary vasoconstriction (42).

Other diseases

More recently, it has been reported that BBS-1 and BBS-2 prolonged survival and reduced myocardial inflammation and cardiomyocyte damage in acute cardiac allograft rejection (43). BBS-1 also prevented acute lung injury in sheep after burn and smoke inhalation injury, a model of acute respiratory distress syndrome, one of the major complications in fire victims with combined burn and smoke inhalation injuries (44).

We speculate that iNOS homodimerization appears to be a rational target for the development of novel therapeutic drugs. Notwithstanding, a more detailed understanding of inhibition of iNOS dimerization is necessary, as little is known about the ratio of dimerization of iNOS in inflammatory sites, the fate of monomeric iNOS accumulated by drug treatment, the rebound effect of the inhibitor, and differences of action when compared to classical iNOS inhibitors.

Conclusions

There are a great many reports regarding the overproduction of NO and overexpression of iNOS in various inflammatory and autoimmune diseases, including hemorrhagic shock, systemic lupus erythematosus, Sjögren's syndrome, vasculitis, OA, psoriasis and contact dermatitis (7, 9-11, 38), in addition to RA and septic shock. We have discussed iNOS homodimer formation in this review as a new target for decreasing NO production.

A newly synthesized imidazole compound, PPA250, inhibits the production of NO by stimulated RAW264.7 cells *in vitro* through prevention of iNOS dimerization, an essential step for enzyme activity. In two animal models of RA, oral administration of PPA250 suppressed the development of destructive polyarthritis after clinical symptoms had appeared. In addition, PPA250 and other iNOS dimerization inhibitors showed favorable effects in other models of NO-related diseases. Taken together, these data demonstrate that the novel class of NO production inhibitors targeting iNOS homodimerization could be useful therapeutic agents for diseases characterized by excessive NO production. In addition, these inhibitors may present new research tools for exploring iNOS dimerization processes.

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