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Differential effects of substrate-analogue inhibitors on nitric oxide synthase dimerization

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ABSTRACT

Nitric oxide synthase (NOS) isoforms are hemoenzymes that are only active as homodimers. We have examined the effect of the substrate-analogue inhibitors, N^G-monomethyl-L-arginine (L-NMA), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME), N^5-(1-iminoethyl)-L-ornithine (L-NIO), and N^6-(1-iminoethyl)-L-lysine (L-NIL), the guanidine-containing inhibitor aminoguanidine (AG), and the amidine moiety-containing iNOS-specific inhibitor 1400 W, on the formation of NOS dimer. Of these inhibitors, L-NMA effectively not only inhibited iNOS dimerization, but also destabilized its dimeric form in RAW264.7 cells stimulated with lipopolysaccharide plus interferon-γ, but not eNOS dimerization in endothelial cells. Importantly, this inhibition was highly correlated with NO production. These inhibitory effects were significantly reversed by addition of L-arginine. However, L-NNA, L-NAME, and AG in part or significantly increased dimerization of iNOS and eNOS in intact cells, and the other inhibitors assessed did not alter dimerization of iNOS and eNOS. These data taken together suggest that substituted groups of an arginine guanidino moiety play an important role in NOS dimerization as well as its catalytic activity. Our results indicate that L-NMA can inhibit iNOS-dependent NO production by preventing iNOS dimerization and destabilizing its dimeric form.

1. Introduction

Nitric oxide (NO) generated from L-arginine by three isoforms of nitric oxide synthase (NOS) plays an important role in both physiological and pathological conditions. Small amounts of NO produced by the constitutive Ca^{2+}-dependent NOS isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), acts as a physiological signal molecule for neurotransmission and vasorelaxation, respectively [1]. However, a large amount of NO produced by inducible NOS (iNOS) is associated with immune reactions such as microbial killing and tumor cell death, whereas inappropriate or overproduction of NO has been implicated in the pathophysiology of a wide range of disorders [1]. The suppression of NO production in endothelial cells causes hypertension and atherosclerosis since it results in elevated systemic blood pressure and vascular inflammation. Many studies have demonstrated that inhibition of iNOS may function as a therapeutic strategy in the treatment of variety of diseases states, to include septic shock [1,2]. As such, many chemical inhibitors for iNOS have been developed as therapeutic drugs aimed at selective modulation of iNOS activity.

NOS isoforms are flavoheme enzymes that are only active as homodimers. Each NOS monomer contains a C-terminal electron-supplying reductase domain that binds FMN, FDA, and NADPH and a catalytic N-terminal oxygenase domain that binds heme, tetrahydrobiopterin (H_4B), and substrate [1,3]. In addition, a calmodulin-binding region is a structural and functional linker between reductase and oxidase domains [3,4]. Extensive studies have defined many biochemical and structural requirements for NOS dimerization. Studies with iNOS demonstrate that heme, H_4B, and L-arginine promote subunit dimerization, which correlates with NO synthesis [5,6]. In contrast, the iNOS subunit incubations that omitted arginine, heme or H_4B did not induce the formation of dimeric NOS, leading to effective suppression of NO synthesis. Thus, chemical compounds that regulate iNOS dimerization have broad therapeutic potential in iNOS-mediated pathologies.

Several imidazole-containing compounds that act as heme ligands block NO formation by inhibiting iNOS dimerization [8].

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On the other hand, the quinolinone-based NOS inhibitor KLYP956, which is a non-arginine and non-imidazole-based compound inhibit and/or destabilize iNOS dimerization, resulting in the inhibition of NO synthesis [7]. Many classic substrate-competitive inhibitors share structural similarities with L-arginine and are thus sensitive to local arginine concentrations and competition with the substrate. However, the effect of substrate-analogue NOS inhibitors on iNOS dimerization has not been examined. In this report, we found that arginine-analogue NOS inhibitor L-NMA, but not other inhibitors, inhibited and/or destabilized iNOS dimerization, but not eNOS, resulting in the inhibition of its catalytic activity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), HEPES, penicillin–streptomycin solution were purchased from Hyclone Laboratories (Logan, Utah). Recombinant mouse IFN-γ was purchased from R&D systems (Minneapolis, MN, USA). N^G-Methyl-L-arginine (L-NMA), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME), N^5-(1-iminoethyl)-L-ornithine (L-NIO), and N^6-(1-iminoethyl)-L-lysine (L-NIL), aminoguanidine (AG), and 1400W were purchased from Cayman Chemical (Ann Arbor, MI). MG132 and 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM/DA) were obtained from Calbiochem (San Diego, CA) and Molecular Probes (Eugene, OR), respectively. Antibodies for iNOS and eNOS were purchased from BD Transduction Laboratories (Lexington, CA). Other chemicals were obtained from Sigma (St. Louis, MO) unless indicated otherwise.

2.2. Cell culture

The macrophage RAW264.7 cells were grown in DMEM media supplemented with 5% inactivated fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 25 mM HEPES at 37 °C under a humidified 5% CO_2/95% air. RAW264.7 cells were treated with LPS (1 μg/ml)/IFN-γ (100 units/ml) in the presence or absence of NOS inhibitors. Human umbilical endothelial cells (HUVECs) were isolated and cultured in the presence or absence of NOS inhibitors as described previously [9].

2.3. Measurements of NO production

The levels of nitrite, a stable oxidized product of NO, in the culture media from RAW264.7 cells treated with LPS (1 μg/ml)/ IFN-γ (100 units/ml) were measured using Griess reagents [10]. The levels of NO produced in HUVECs were measured using the DAF-FM/DA detection system as previously described [9].

2.4. Gel-based dimer assay for NOS

NOS dimerization was determined by low-temperature SDS–PAGE as described previously [11]. In brief, cells harvested from plates were lysed with lysis buffer (100 mM Tris–HCl, pH 7.6, 0.5% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1 mM PMSF protease inhibitor). Lysates were centrifuged at

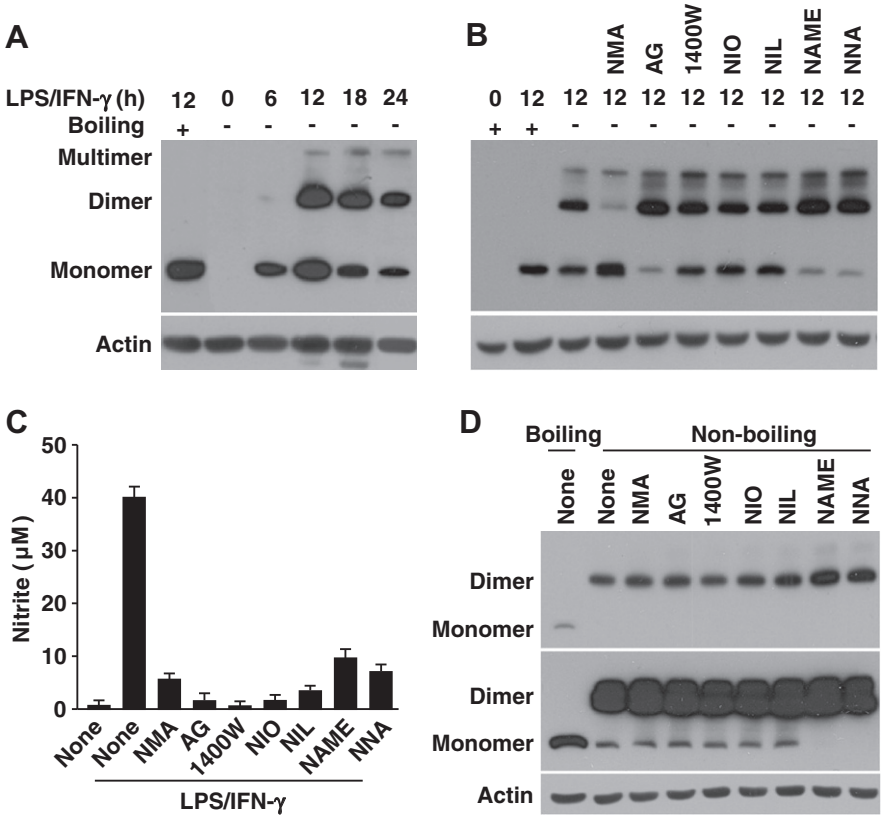

Fig. 1. The effects of NOS inhibitors on NOS dimerization. (A) RAW264.7 cells were stimulated with LPS/IFN-γ for the indicated time periods. iNOS protein was determined in cell lysates by Western blot analysis. (B and C) RAW264.7 cells were stimulated with LPS/IFN-γ in the presence or absence of 1 mM NOS inhibitors for 12 h. (B) iNOS protein was determined by Western blot analysis. (C) The level of nitrite was determined in the culture media by Griess reaction. (D) HUVECs were incubated with or without NOS inhibitors for 12 h, and eNOS was determined by Western blotting.

14,000×*g* for 10 min at 4 °C, and the supernatants were obtained for NOS dimerization assay. The concentration of protein was determined using a BCA method. Protein samples (20 μg) were mixed with an equal volume of ice-cold 2× loading buffer. Samples without boiling were ran on a 6% or 8% SDS–PAGE gel in pre-chilled 1× SDS running buffer in ice-cold room at 80 V. Protein bands of iNOS and eNOS were visualized by Western blotting using their specific antibodies. The intensity of target proteins was determined by densitometry.

2.5. Statistical analysis

The data are presented as means ± standard deviation (SD) of at least three separate experiments. Comparisons between two groups were analyzed using the Student's *t*-test, and significance was established at a *P* value <0.05.

3. Results

3.1. Differential effects of NOS inhibitors on dimerization of iNOS and eNOS

When analyzing iNOS dimerization in LPS/IFN-γ-stimulated RAW264.7 cells, newly synthesized iNOS protein was present as a monomeric form at 6 h after simulation, and almost half amount of total iNOS protein remains dimeric at 12 h of stimulation, thereafter with an substantial decrease in total amount of iNOS protein (Fig. 1A). Exposure of membranes to X-ray film for longer time periods revealed multimeric forms of iNOS (Fig. 1A). The level of dimeric iNOS was significantly reduced by treatment with 1 mM L-NMA, resulting in an increase in the level of monomeric iNOS, whereas AG, L-NAME, and L-NNA increased dimerization of iNOS (Fig. 1B). However, the inhibitors, 1400W, L-NIO, and L-NIL did not affect iNOS dimerization (Fig. 1B). Under these experimental conditions, all inhibitors effectively inhibited NO production as compared with control. To examine the effects of these inhibitors on eNOS dimerization, HUVECs were cultured with these inhibitors and analyzed for eNOS dimerization. L-NAME or L-NNA partially increased eNOS dimerization, whereas the other inhibitors did not affect eNOS dimerization at 12 h of incubation (Fig. 1D), and similar effects were observed in HUVECs incubated with L-NMA at 24 h and 36 h (data not shown).

3.2. L-NMA inhibits iNOS dimerization, but not eNOS, in a dose-dependent manner

Treatment with L-NMA decreased the level of dimeric iNOS in a concentration-dependent manner, resulting in an increase in monomeric iNOS levels (Fig. 2A and B). The half-inhibitory effect of L-NMA on iNOS dimerization appeared at about 15–20 μM, in which these values highly correlated with the IC_{50} for NO production (Fig. 2C). However, treatment with L-NMA did not affect the level of dimeric eNOS in HUVECs, but slightly increased the level of multimeric eNOS at a concentration of 0.5 mM, with no further increase observed at higher concentrations (Fig. 2D). Moreover, incubation with L-NMA dose-dependently inhibited NO production in HUVECs. These results suggest that L-NMA can suppress NO production in immune-activated macrophages by inhibiting and destabilizing iNOS dimerization, as well as inhibit NO synthesis in endothelial cells by blocking eNOS activity without altering its dimerization.

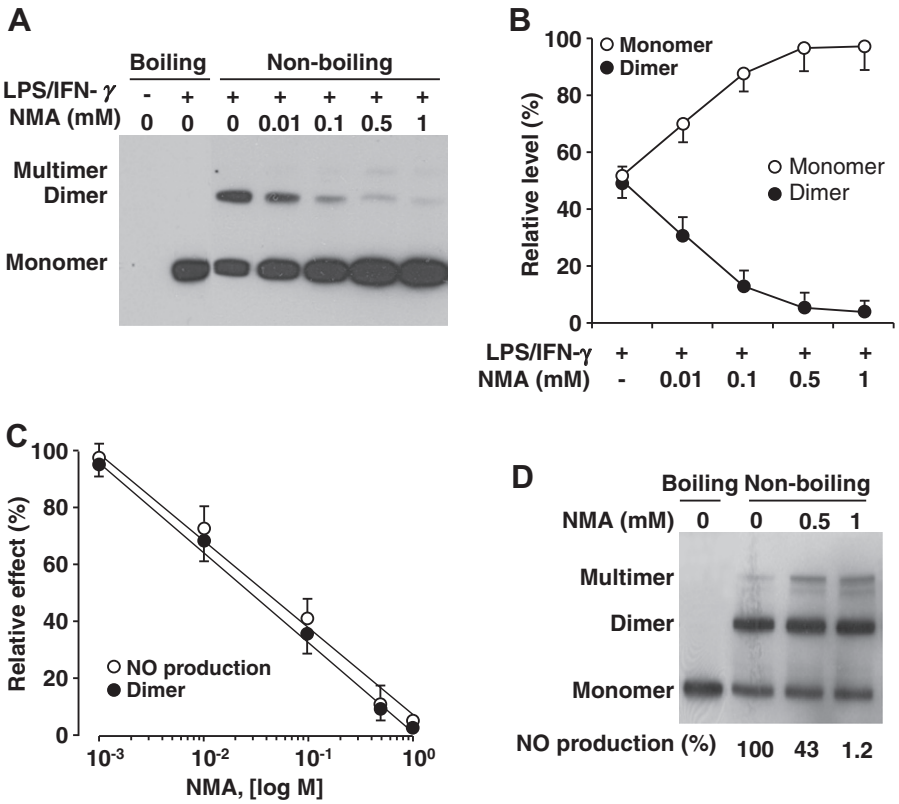

Fig. 2. L-NMA inhibits iNOS dimerization, but not eNOS. (A–C) RAW264.7 cells were stimulated with LPS/IFN-γ in the presence or absence of the indicated concentrations of L-NMA for 12 h. (A) iNOS was determined by Western blotting. (B) The level of iNOS was determined by densitometry. (C) The levels of nitrite were determined in the culture media by Griess reaction. (D) HUVECs were treated with or without L-NMA for 12 h. The protein band of eNOS was determined by Western blotting. The levels of NO were determined by confocal microscopy. Data shown in (B and C) are the mean ± SD ($n = 3$).

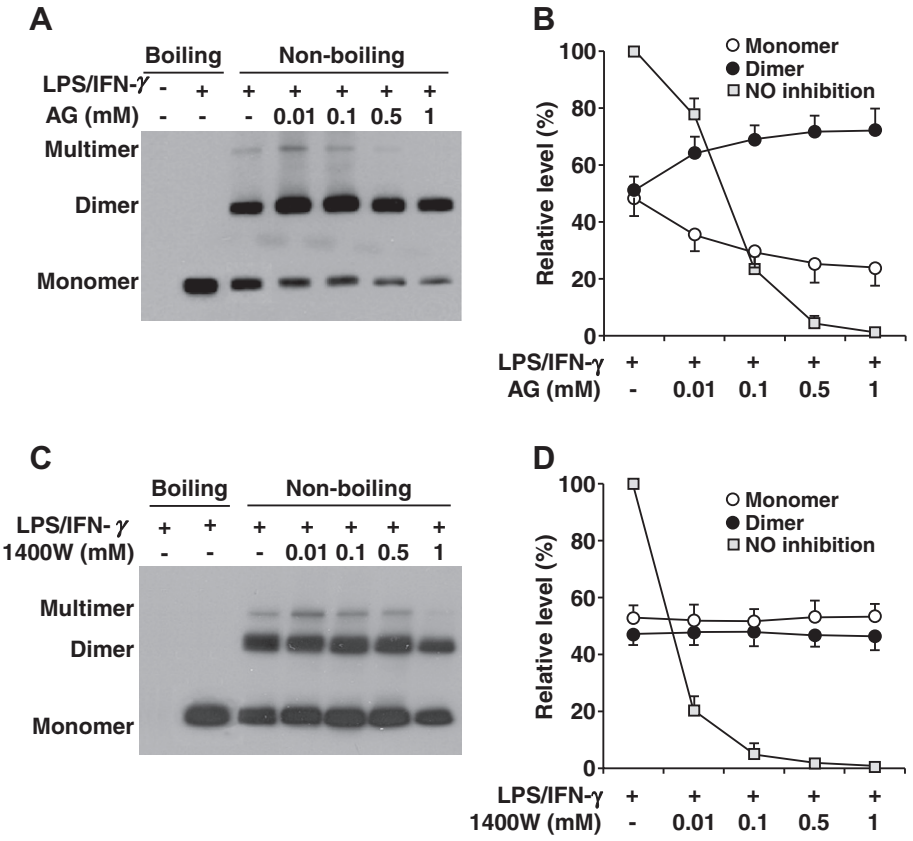

Fig. 3. The effects of iNOS-specific inhibitors AG and 1400 W on iNOS dimerization. RAW264.7 cells were stimulated with LPS/IFN-γ in the presence or absence of the indicated concentrations of AG or 1400 W for 12 h. (A and C) iNOS protein was determined by Western blotting. (B and D) The level of iNOS protein and nitrite were determined by Western blotting and Griess reaction. Data shown in (B and D) are the mean ± SD ($n = 4$).

3.3. AG, but not 1400W, increases iNOS dimerization

We next examined whether the iNOS-specific inhibitors, AG and 1400W, regulate iNOS dimerization. AG, a small compound containing the guanidino group of L-arginine linked to hydrazine (Supplementary Fig. 1), is equipotent to L-NMA as a selective inhibitor of iNOS activity but is 10 to 100-fold less potent as an inhibitor of the constitutive isoform [12]. Treatment with AG rapidly increased iNOS dimerization at a concentration of 10 μM, with a slightly further increase in iNOS dimerization at its higher concentrations up to 1 mM, resulting in a decrease in its monomer (Fig. 3A and B). As expected, treatment with AG inhibited NO production in LPS/IFNγ-stimulated macrophages in a concentration-dependent manner and almost completely suppressed NO synthesis at a concentration of 0.5 mM (Fig. 3 B). The amidine moiety-containing NOS inhibitor, 1400W is highly selective for iNOS compared with constitutive isoforms [13]. Therefore, we next examined the effect of 1400W on iNOS dimerization and NO production in LPS/IFN-γ-activated macrophages. Treatment with 1400W did not alter the ratio of dimeric and monomeric iNOS up to 1 mM (Fig. 3C and D). However, 1400W completely blocked NO synthesis in RAW264.7 cells treated with LPS/IFN-γ at 100 μM, and this effect (Fig. 3D) was more pronounced as compared with that of AG (Fig. 3B). Interestingly, both inhibitors slightly increased multimerization of iNOS at a concentration of 10 μM, and this form was gradually decreased at its higher concentrations (Fig. 3A and C). These results indicate that the inhibitory effects of AG and 1400W on iNOS activity were not directly associated with their abilities to inhibit or destabilize iNOS dimerization in LPS/IFN-γ-activated RAW264.7 cells.

3.4. L-NMA prevents iNOS dimerization and destabilizes its dimeric form, which are reversed by L-arginine

We further examined whether L-NMA inhibits iNOS dimerization or destabilizes dimeric iNOS to its monomeric form using a different cell culture system. Treatment with LPS/IFN-γ for a total of 13.5 h (pre-treatment for 11 h and post-treatment for 2.5 h) resulted in an increase in iNOS dimerization by 60–70% of total iNOS protein, and this increase was completely inhibited by post-treatment with L-NMA for 2.5 h (Fig. 4A). Post-treatment with L-NMA in the presence of cycloheximide and MG132, which block translational expression and proteosomal degradation of iNOS, also almost completely converted LPS/IFN-γ-induced dimeric iNOS to its monomeric form (Fig. 4A). These results suggest that L-NMA prevents not only iNOS dimerization, but also destabilizes the iNOS dimer. We further investigated whether L-arginine functions to regulate the effect of NOS inhibitors on iNOS dimerization and NO production. The addition of L-arginine (0.5 and 1.0 mM) partially reversed L-NMA-mediated suppressive effect of iNOS dimerization and NO production in RAW264.7 cells stimulated with LPS/IFN-γ (Fig. 4B and D). However, L-arginine did not affect AG- and 1400W-mediated dimeric status of iNOS and inhibition of NO production in RAW264.7 cells treated with LPS/IFN-γ (Fig. 4B and D). To further examine the effect of L-arginine on L-NMA-induced monomeric iNOS, cells were treated with LPS/IFN-γ and L-NMA for 11 h, followed by incubation with L-arginine for 2 h. Post-treatment with L-arginine partially reversed L-NMA-induced monomeric iNOS to its dimeric form (Fig. 4C), and this data was correlated with their NO production activity (data not shown). These results suggest that L-NMA containing a methylated

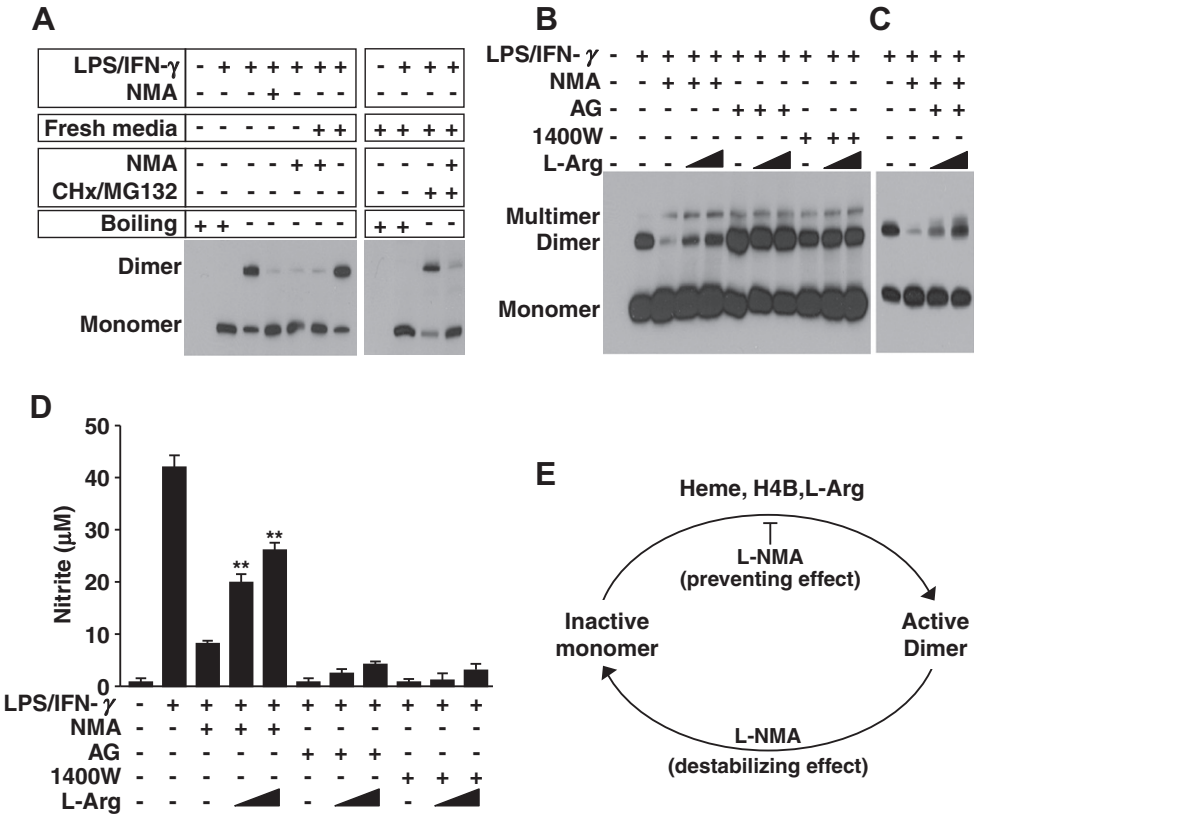

Fig. 4. L-NMA prevents iNOS dimerization and destabilizes its dimeric form, which are reversed by L-arginine. (A) RAW264.7 cells were stimulated with LPS/IFN-γ in the presence or absence of L-NMA for 11 h. Some cells were further cultured in the presence or absence of NMA without changing media for 2.5 h. Other cells were cultured in fresh media alone or media containing L-NMA and cycloheximide (100 μM) and MG132 (10 μM) for 2.5 h. The levels of iNOS protein were determined by Western blot analysis. (B and C) Cells were co-stimulated with LPS/IFN-γ and NOS inhibitors in the presence or absence of 0.5 or 1 mM L-arginine for 12 h. (B) iNOS protein were determined by Western blotting. (C) Cells pre-treated with LPS/IFN-γ and L-NMA for 11 h, followed by incubation with L-arginine for 2 h. The protein bands of iNOS were visualized by Western blotting. (D) The levels of nitrite were determined in the culture media by Griess reaction. Data shown are the mean ± SD ($n = 3$). $^{**}p < 0.01$ versus treatment with LPS/IFN-γ and L-NMA. (E) Possible mechanism by which L-NMA prevents dimerization of the *de novo* synthesized iNOS monomer and destabilizes active iNOS dimer to its inactive monomer.

guanidino group binds to the guanidine-binding pocket of the catalytic site of iNOS and inhibits NO synthesis via prevention of iNOS dimerization and destabilization of the iNOS dimer.

4. Discussion

Monomeric iNOS subunits form a homodimer, which is essential for their catalytic activity. Extensive studies have demonstrated that heme, H_4B, and L-arginine promote or stabilize iNOS dimerization, leading to an increase in NO synthesis from L-arginine [5,6]. For example, H_4B and L-arginine promoted dimerization of a purified heme-containing iNOS monomer [14–16] and an overexpressed iNOS in NIH3T3 cells [17]. These evidences suggest that the substrate analogue inhibitors can regulate iNOS dimerization; however, their regulatory effects on iNOS dimerization have not been elucidated. We here found that the substrate-analogue inhibitor L-NMA prevents iNOS dimerization and destabilizes iNOS dimer, as well as inhibits NO production in LPS/IFN-γ-activated RAW264.7 cells. These inhibitory effects were partially reversed by addition of L-arginine. However, this inhibitor did not alter eNOS dimerization in cultured HUVECs. These results suggest that L-NMA interferes with dimerization and stabilization, resulting in the inhibition of NO synthesis in immune-activated macrophages. Although the substituted guanidine moiety-containing inhibitors L-NNA, L-NAME, and AG slightly increased iNOS dimerization, they effectively blocked NO production in immune-macrophages, indicating that these inhibitors have a different mechanism of action from that of L-NMA.

Dimerization of NOS monomers is initiated by the insertion of heme, which coordinates to the protein through a cysteine thiolate, resulting in rapid conformational changes [5,18]. The heme-containing iNOS monomer is an intermediate in the dimerization process and further forms a stable active dimer in the presence of H_4B and L-arginine [4,5]. Heme insertion into monomeric NOS appears to be mandatory for its dimerization, with H_4B and L-arginine promoting dimer formation and stabilizing dimer once formed. Limitation of intracellular heme level forms a loosely associated dimeric NOS [6,19], which readily dissociates in the presence of SDS. Without heme insertion, L-arginine or H_4B alone cannot promote assembly of monomeric iNOS to the dimeric form. A recent study suggests that once the iNOS dimer is formed in intact cells there is little or no significant return to the monomer [20,21]; however, it is not clear whether this will hold true in the presence of NOS inhibitors. We here found that, of several substrate-analogue inhibitors assessed in this study (Supplementary Fig. 1), L-NMA inhibited iNOS dimerization in immune-activated macrophages, which was highly correlated with the suppression in NO production. Because L-NMA inhibited NO production without affecting eNOS dimerization in HUVECs, the inhibitory effect of L-NMA on iNOS dimerization can be involved in inhibition of iNOS activity via dual mechanisms, such as direct inhibition of iNOS activity via competition with L-arginine in the catalytic pocket as previously demonstrated [21] and interference with iNOS dimerization.

Our results showed that IC_{50} values of L-NMA for iNOS dimerization and its enzyme activity were nearly identical at 15–20 μM (Fig. 2C). These data suggest that its inhibitory effect on iNOS activity is highly associated with the interference with iNOS dimerization, rather than direct inhibition of NO synthesis by competitively binding to the catalytic pocket of iNOS. However, this inhibitor inhibited eNOS-dependent NO production without affecting eNOS dimerization, suggesting that L-NMA inhibits eNOS activity by competitively binding to its catalytic pocket.

Heme is an essential factor for NOS dimerization as well as a critical cofactor for the catalytic reaction in the oxygenase domain. The heme ligands, including imidazole-containing compounds, coordinate the heme iron within the catalytic domain of the enzyme and inhibit NO synthesis by blocking iNOS dimerization [21]. These inhibitors, which coordinate the heme in the iNOS monomer, disrupt helix 7a and helix 8, which are part of the dimer interface [21,22]. These evidences suggest that inhibitors binding to heme of the iNOS monomer prevent formation of a "dead-end" inhibitor-monomer complex which can not be prevented by L-arginine or H_4B. Because L-NMA is a structural analogue of the substrate L-arginine, this inhibitor competes with L-arginine in the substrate-binding cavity. Although L-NMA evokes a different mechanism of action from imidazole-containing inhibitors, we found that L-NMA interfered with iNOS dimerization, which is a new possible inhibitory mechanism for iNOS activity.

Both guanidine and amino acid moieties of L-arginine bind to the catalytic pocket of NOS via the formation of an extensive hydrogen bond network with surrounding protein residues, which are highly conserved among isoforms [18,22]. Interaction of L-arginine α-amino and carboxylate groups with hydrophilic side chains in NOS may be directly linked to structural elements involved in dimer formation; conversely interaction of the L-arginine guanidino group at the bottom of heme pocket may be involved in the oxidation of its guanidino nitrogen to produce NO [18]. We clearly demonstrate that L-NMA inhibits iNOS dimerization; however, L-NNA, L-NAME, and AG in part or significantly increased dimerization of iNOS and eNOS. Although the tautomerization behavior of the guanidino group is likely to be identical among L-arginine, L-NMA L-NNA, and L-NAME [23], hydrogen bond formation between their side chains and the surrounding protein residues of NOS is different between them. The nitro and amino groups of the guanidino moiety of L-NNA, L-NAME, and AG can be involved in the formation of hydrophilic interaction or hydrogen bond with the surrounding protein residues; however, the methyl group of L-NMA does not. These evidences indicate that modification of the guanidino moiety is thought to be important for dynamic monomer to dimer transition.

Assembly of the monomers to catalytically active dimers requires the coincident presence of heme, L-arginine, and pteridine, pointing to a role of these compounds in the post-translational processing of iNOS [5]. In contrast, a native constitutive NOS maintains its active dimeric conformation even in the absence of pteridine and L-arginine [11]. Three dimensional structures of the L-arginine-binding pocket in iNOS and eNOS are also partially different; for example, Asp in substrate-carboxylate binding pocket of iNOS is replaced by an Asn in eNOS [18]. This structural difference stands to reason how L-NMA specifically interferes with the dimerization of iNOS, but not eNOS. Moreover, the inhibitory effect of L-NMA on iNOS dimerization can be explained by two possible mechanisms (Fig. 4E). One possibility is the "preventive effect" in that L-NMA inhibits assembly of *de novo* synthesized monomeric iNOS to the dimeric form by binding to the iNOS monomer and subsequent prevention of heme insertion. An alternative possibility, the "destabilizing effect" is that L-NMA dissociates dimeric iNOS into its monomer by binding to the catalytic site of the active dimer. In this case, L-NMA can cause a significant loss of heme from the enzyme and lead to destabilization of the iNOS dimer to the monomer form [24].

In conclusion, we demonstrate that the common NOS inhibitor L-NMA prevents iNOS dimerization as well as destabilizes the iNOS dimer in immune-activated RAW264.7 cells; however, some other substrate-analogue inhibitors, such as L-NNA. L-NAME, and AG, in part or significantly promoted iNOS dimerization. These data indicate that the modified group of the L-arginine guanidino moiety is likely to be important for regulating iNOS dimerization. These results altogether suggest that L-NMA functions to inhibit iNOS, but not eNOS, dimerization and the catalytic activity of the enzyme, which provides important insight for both understanding its mechanism of action and the development of a selective inhibitor for iNOS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.123.

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