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MACROPHAGE AND ENDOTHELIAL CELL NITRIC OXIDE SYNTHESIS: CELL-TYPE SELECTIVE INHIBITION BY N^G-AMINOARGININE, N^G-NITROARGININE AND N^G-METHYLARGININE

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SUMMARY. Many cell types are known to synthesize nitric oxide (NO·) from L-arginine. There appear to be at least two forms of NO· synthase: an inducible, tetrahydrobiopterin- and flavin-dependent activity exemplified by the macrophage enzyme and a constitutive, Ca^{++}-dependent activity exemplified by the endothelial cell enzyme. L-N^G-methylarginine inhibits NO· synthesis by both cell types. We now report that L-N^G-aminoarginine and L-N^G-nitroarginine are about 100-fold more potent than N^G-methylarginine in blocking endothelial cell NO· synthesis. In contrast, N^G-aminoarginine and N^G-methylarginine are about equipotent with macrophages whereas N^G-nitroarginine is much less potent. Since macrophage and endothelial cell NO· synthesis are differentially sensitive to the inhibitors, the panel of inhibitors can be used in complex biological systems to determine if macrophage-like or endothelial-like cells are the predominant source of NO·. Indeed, all three inhibitors elicit a strong pressor response in the anesthetized guinea pig, a result consistent with the view that endothelial cells continually produce vasodilatory NO·.

In 1987 Hibbs and coworkers reported that the antitumor activity of macrophages depends on the metabolism of arginine to nitrate, nitrite and citrulline (1,2). Subsequent studies established that nitrate and nitrite are derived from nitric oxide (NO·), a short-lived, lipophilic free radical that is the primary nitrogen oxide formed by macrophages (3-6) and some tumor cells (7) in response to lymphokines and immuno-stimulants. The cytotoxicity of NO· is due in part to inactivation of iron-sulfur centers in aconitase and mitochondrial complexes I and II (1,8,9).

Nitric oxide is also produced by endothelial cells. Initially characterized as endothelium-derived relaxing factor (EDRF) (10), endothelium-derived nitric oxide (EDNO) activates the soluble guanylate cyclase of vascular smooth muscle cells; increasing levels of cGMP cause a decrease in intracellu-

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Abbreviations: EDRF, endothelium-derived reaxing factor; EDNO, endothelium-derived nitric oxide; NMA, L-N^G-methylarginine; NAA, L-N^G-aminoarginine; NNA, L-N^G-nitroarginine; BAEC, bovine aortic endothelial cell.

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lar calcium with consequent vascular relaxation (11,12). EDNO is also a potent inhibitor of platelet aggregation and adhesion (13).

In both macrophages and endothelial cells NO· is derived from a guanidino nitrogen of L-arginine (14-16). Studies with partially purified macrophage NO· synthase show that arginine oxidation is stimulated by or dependent on NADPH, tetrahydrobiopterin (17,18), FAD, and thiol (19). Although the endothelial cell synthase has not been purified, studies with cell-free homogenates indicate the reaction is dependent on NADPH and stimulated by thiol and Ca^{++}/calmodulin (20; S.S. Gross, unpublished); stimulation by tetrahydrobiopterin has not been shown. Nitric oxide synthesis by both macrophages and endothelial cells is inhibited by L-N^G-methylarginine (NMA) (1,15,16). To further elucidate similarities and differences characterizing the macrophage and endothelial cell NO· synthases, we have examined inhibition by additional N^G-substituted arginine analogues; the findings demonstrate that cell-type selective inhibition of NO· synthesis is possible and suggest that the arginine binding sites of the macrophage and endothelial cell synthases differ [2].

MATERIALS AND METHODS

Chemicals: L-N^G-Methylarginine (NMA) was synthesized as described (23). L-N^G-Aminoarginine (NAA) was synthesized by catalytic reduction of L-N^G-nitroarginine (NNA) in 15 % acetic acid at room temperature; the crude reaction product was treated with arginase to destroy contaminating arginine, and NAA was then crystallized as a pure monoflavianate salt. Flavianic acid was removed with Dowex 1 (OH^-) (23). Details of the synthesis will be presented elsewhere. NNA and all other reagents were from Sigma or Fisher.

Cells: Bovine aortic endothelial cells (BAEC) were obtained and cloned by limiting dilution (24) and stored in liquid N_2. As needed, cells were grown to confluence (1-2 x 10^5/well) on 0.2% gelatin-coated 24-well plates containing 0.8 ml of a medium composed of RPMI (Mediatech, Washington, D.C.), 20 % iron-supplemented fetal calf serum (Hyclone, Logan, UT), 1.6 mM glutamine, 80 U/ml penicillin and 80 μg/ml streptomycin. Cells were used at passages 4-9.

Thioglycollate-elicited peritoneal macrophages were obtained from CD-1 mice and plated at 10^5 cells per well in 96 well microplates (9).

Nitrite Release: Endothelial cells were washed 3 times with 1 ml of Ca^{++}- and Mg^{++}-containing HEPES buffered saline (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 11.1 mM glucose, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, pH 7.5) and then incubated at 37° C. for the time indicated in RPMI with or without added arginine (select-Amine Kit, GIBCO Labs, Grand Island, N.Y.). NMA, NAA or NNA was added as indicated.

Macrophage NO· synthase activity was induced by incubating the cells with interferon-γ (500 units/ml) and 1 μg/ml E. coli lipopolysaccharide for 12 hr. The medium was then replaced with 100 μl of medium containing various concentrations of NMA, NAA, and NNA. After 24 hr., the nitrite concentration in the medium was measured; data were corrected for nitrite present in cell-free medium (≈5 μM).

Nitrite Assay: Nitrite was quantitated by an automated colorimetric assay based on the Griess reaction as described (25). Sample and Griess reagent (0.5% sulfanilamide, 0.05% napthylethylene diamine diHCl, 2.5% H_3PO_4) were

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[2] These studies were presented in part at the Washington FASEB meeting in April, 1990, and have are reported in abstracts (21,22).

reacted on-line using an automatic sample injector; nitrite concentration, proportional to OD_{543}, was determined using a flow-thru monitor by reference to a standard curve.

Measurement of Hemodynamic Parameters: Male Hartley guinea pigs (300-500 g) were anesthetized with sodium pentobarbital (45-60 mg/kg i.p.). A tracheal cannula was inserted, and the left carotid artery was cannulated and connected to a physiological pressure transducer (Statham, P23AA; Hato Bay, PR). Blood pressure tracings were displayed on a physiograph (Grass, P7; Quincy, Mass). NO· synthase inhibitors were given as bolus injections in the cannulated left jugular vein.

RESULTS

Characterization of NO· Synthesis by BAEC: Nitric oxide formation, quantitated on the basis of nitrite accumulating in the culture medium, was elicited by known stimulators of EDNO production; calcium ionophore A23187 typically increased nitrite release by cultured BAEC > 10-fold. Nitrite release was also stimulated by bradykinin, ADP, or ATP. Nitrite release in the presence of A23187 was linear with time for at least 4 hr. and was inhibited by NMA, a specific inhibitor of EDNO synthesis (Figure 1). These results strongly suggest that EDNO is the source of BAEC-derived nitrite.

In the absence of exogenous arginine, nitrite release after 4 hr. by A23187-stimulated BAEC was 0.875 ± 0.059 nmol/2x10^5 cells. Nitrite release increased about 2-fold when the concentration of arginine in the culture medium was increased; in media containing 1, 3, and 10 mM arginine, nitrite release after 4 hr was 1.63 ± 0.82, 1.68 ± 0.001, and 1.46 ± 0.082 nmol, respectively. As expected for a competitive inhibitor, inhibition by NMA

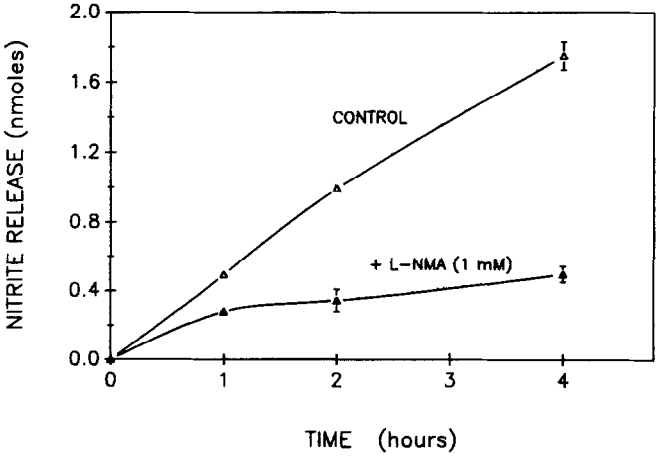

Figure 1. Time course of A23187-stimulated nitrite production by BAEC. Cells were incubated in media containing 1.15 mM arginine in the absence (Control) or presence of 1 mM NMA (NMA). At time zero, 5.6 μM A23187 was added, and the accumulation of nitrite in the culture medium was determined at the times indicated. Points are means ± S.E. of nitrite production in three individual culture wells.

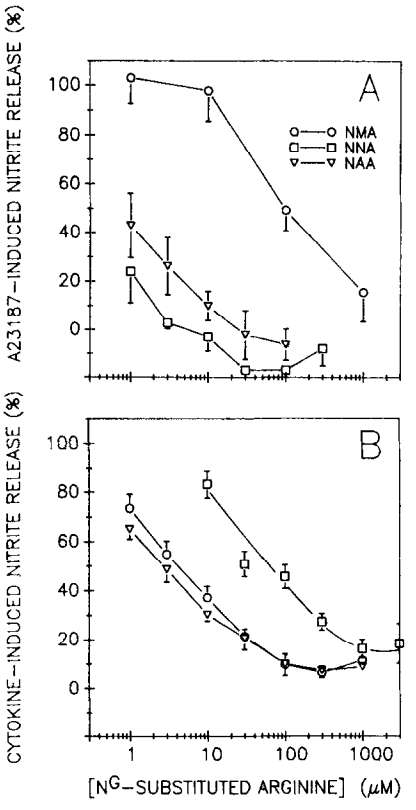

Figure 2. Comparison of concentration-response relationships for inhibition by NMA, NNA and NAA of nitrite production by A23187-stimulated BAEC (Panel A) or mouse peritoneal macrophages (Panel B). BAEC grown in normal media were incubated in arginine-free media alone or in the presence of the indicated concentration of inhibitor for 2 hr. Macrophage studies were carried out similarly in media containing 0.6 mM arginine. The points are means ± S.E. of the percent inhibition of nitrite production observed in three individual culture wells during this period.

decreased with increasing arginine concentration. In media containing 1, 3, and 10 mM arginine, 1 mM NMA inhibited nitrite release after 4 hr. by 91, 60, and 37 % of control values, respectively.

Inhibition of BAEC NO· Formation by NMA, NAA and NNA: Although NMA is the best characterized inhibitor of arginine-dependent NO· synthesis, the studies reported in Figure 2A demonstrate that both N^G-aminoarginine (NAA) and N^G-nitroarginine (NNA) are at least 100-fold more potent as inhibitors of BAEC NO· formation. Approximate ED_{50} values for NMA, NAA and NNA determined in the absence of exogenous arginine are 100 μM, 1 μM and ≤ 1 μM, respectively (Figure 2A); in media containing 1 mM arginine the ED_{50} of NMA was increased to about 300 μM (not shown). Although NNA appears to be slightly more potent than NAA in the BAEC studies shown, when the compounds were examined as inhibitors of acetylcholine-induced relaxation of rabbit aortic rings (an NO·-mediated effect) NAA was slightly more effective than NNA; both were about 30-fold more effective than NMA in this system (21).

Inhibition of Macrophage NO· Formation by NMA, NAA, and NNA: Thioglycollate-elicited mouse peritoneal macrophages produce NO· when stimulated by interferon-γ and lipopolysaccharide (9). As shown in Figure 2B, NMA and NAA are nearly equipotent as inhibitors of NO· synthesis by intact cells whereas NNA is significantly less effective. The ED_{50} values for NMA, NAA and NNA are about 2.6, 4.3 and 60 μM, respectively. The N^G-substituted arginines were also examined as inhibitors of partially purified macrophage NO· synthase under conditions similar to those reported previously (19). In the presence of 1 mM L-arginine the ED_{50} of NMA, NAA and NNA were about 180 μM, 40 μM, and 1.17 mM, respectively.

Endothelial Cell NO· Formation Affects Blood Pressure *In Vivo*: Bolus administration of NMA to anesthetized guinea pigs (26) and rabbits (27) has been previously shown to significantly increase blood pressure. Those studies demonstrated that vasorelaxation mediated by endogenous NO· is an important contributor to normal blood pressure homeostasis. As shown in Figure 3, NAA and NNA also elicit a dose-dependent increase in blood pressure in the guinea pig. At a dose of 1 mg/kg NAA has a hypertensive effect similar to that shown by NMA at 10 mg/kg; the greater activity of NAA is consistent with the endothelial cell origin of vasoregulatory NO·. The finding that NNA is also a potent hypertensive agent supports an endothelial cell origin of vasoregulatory NO·, but, in view of the BAEC studies, it was initially surprising that NNA was not equieffective with NAA *in vivo*. However, as indicated in the Figure 3

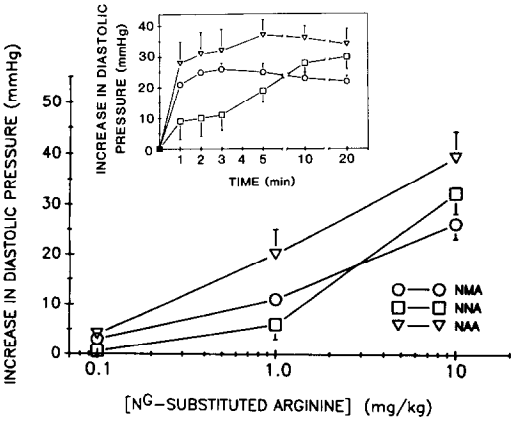

Figure 3. Effect of N^G-substituted arginines on diastolic blood pressure in the anesthetized guinea pig. Inhibitors were administered by bolus injection at the indicated dosages. Points are means ± S.E. (n=4) of the peak pressor response elicited. Inset: Time course of the pressor effect of N^G-substituted arginines (10 mg/kg). Conditions were as in main Figure. Two min. after infusion of L-arginine (30 mg/kg) the pressor effect of NMA and NAA was fully reversed, but that of NNA was decreased only 41 % (not shown).

inset, the onset of the hypertensive effect of NNA and its reversal by arginine is slow relative to that seen with NAA and NMA. It is thus possible that NNA, which has the most lipophilic N^G-substituent, attains a lower effective concentration than NAA and NMA after administration of an equivalent dose.

DISCUSSION

Arginine- and NADPH-dependent NO· synthesis has been reported for a variety of cell types including macrophages, neutrophils, Kupffer cells, endothelial cells, hepatocytes, neuroblastoma and adenocarcinoma cell lines, and cells of the cerebellum and adrenals (28). Although the activity has been purified to homogeneity only from cerebellum (29), studies with cell homogenates and semipurified protein fractions suggest the existence at least two isoforms of NO· synthase. Macrophages express a cytokine-inducible, tetrahydrobiopterin-dependent activity; cytokine-inducible enzyme has also been reported in Kupffer cells, hepatocytes and some tumor cells. Endothelial and cerebellar cells, on the other hand, express an agonist-responsive, Ca^{++}/calmodulin-dependent activity. The present studies indicate that NO· production by macrophages and endothelial cells can also be distinguished on the basis of the specificity of the arginine binding site for the inhibitors NMA, NAA and NNA. Although inhibitor specificity with intact cells may, in part, reflect transport differences, the studies with isolated macrophage NO· synthase suggest that the lesser activity of NNA is attributable to its poor binding by the enzyme and not to diminished cellular uptake. Notably, NNA has a bulkier N^G-substituent than NMA or NAA. Although the endothelial cell enzyme has not yet been purified, the observation that NNA and NAA are equieffective in BAEC indicates that the nitro group can be accommodated by the binding site of the endothelial cell isoform of NO· synthase. These findings are the first clear indication of a distinct difference in the catalytic binding sites of NO· synthase isoforms. The results suggest that macrophage and endothelial cell NO· synthases are fundamentally different enzymes and argue against the possibility that the isoforms represent identical catalytic domains or subunits that are differentially expressed or regulated.

As illustrated by the *in vivo* guinea pig studies, comparative studies using NAA, NNA and NMA can provide strong evidence that biological effects in complex systems are due to NO· produced by macrophage-like or endothelial cell-like enzymes. Although the finding that endothelial cells rather than macrophage (or similar cells) contribute to normal vasodilation is anticipated from physiological considerations, the source of the NO· accounting for the severe hypotension seen in animals exposed to tumor necrosis factor (23) or endotoxin (R. Kilbourn, *et al.*, unpublished) is less obvious. Studies with NAA, NNA and NMA are expected to elucidate the source of NO· in these and other physiological conditions.

With macrophage homogenates, with vascular rings (21), and in whole animals (21, present report) NAA is the most potent NO· synthesis inhibitor reported to date. Although any of several mechanistic considerations may account for the tight binding of NAA to NO· synthase, it is notable that the structure, charge distribution, and H-bonding potential of NAA is similar to N^G-hydroxyarginine, a possible intermediate in the synthesis of NO· from arginine (30). N^G-Aminoarginine may thus be tightly bound as a non-metabolizable analog of a normal reaction intermediate. Alternatively, it is likely that the unusual 5 electron oxidation mediated by NO· synthase requires the presence of metal ions in the vicinity of the catalytic site, and it is possible that the aminoguanidino moiety of NAA, which is expected to be a good metal ligand, is specifically and tightly bound via an aminoguanidino-metal bond. The latter possibility has been suggested by others (J.M. Fukuto and L.J. Ignarro, personal communication).

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