First Non-α-Amino Acid Guanidines Acting as Efficient NO Precursors upon Oxidation by NO-Synthase II or Activated Mouse Macrophages

Sylvie Dijols,[‡] Jean-Luc Boucher,[‡] Michel Lepoivre,[§] David Lefevre-Groboillot,[‡] Magali Moreau,[‡] Yves Frapart,[‡] Eleni Rekka,[∥] Abigail L. Meade,[⊥] Dennis J. Stuehr,[⊥] and Daniel Mansuy*,[‡]

UMR 8601 CNRS, Université Paris V R. Descartes, 45 Rue des Saints-Pères, 75270 Paris Cedex 06, France, URA 1116 CNRS, Université Paris Sud-Orsay, Bat 430, 91105 Orsay Cedex, France, Department of Pharmaceutical Chemistry, Aristotelian University of Thessaloniki, Thessaloniki 54006, Greece, and Department of Immunology, Lerner Research Foundation, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195

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ABSTRACT: A study of the oxidation of a series of guanidines related to L-arginine (L-Arg) and of various alkyl- and arylguanidines, by recombinant NO-synthase II (NOS II), led us to the discovery of the first non-α-amino acid guanidine substrate of NOS, acting as an efficient NO precursor. This compound, 3-(trifluoromethyl)propylguanidine, **4**, led to a rate of NO formation ($k_{cat} = 220 \pm 50$ min^{-1}) only 2 times lower than that of L-Arg. Formation of 1 mol of NO upon NOS II-catalyzed oxidation of **4** occurred with consumption of 2.9 mol of NADPH, which corresponds to a 52% coupling between electron transfer and oxygenation of its guanidine function. Its oxidation by activated mouse macrophages in an L-Arg-free medium resulted in NO_2^- formation that was inhibited by classical NOS inhibitors with a rate only 2−3 times lower than that observed with L-Arg itself. These results open the way toward the research of selective, stable guanidine substrates of NOS that could be interesting, new NO donors after in situ oxidation by a given NOS isoform.

Nitric oxide (NO) is a key inter- and intracellular messenger molecule involved in the maintenance of vascular tone, neuronal signaling, and host response to infection (*1*, *2*). The biosynthesis of NO in mammals is catalyzed by constitutive neuronal and endothelial nitric oxide synthases (NOS[1] I and III, respectively) and by inducible NOS (NOS II), which is expressed in macrophages following induction by inflammatory mediators (*3*, *4*). All three NOSs produce NO and L-citrulline from the two-step oxidation of L-arginine (L-Arg) by NADPH and O_2 with formation of N^{ω}-hydroxy-L-arginine (NOHA) as an intermediate (eqs 1 and 2) (*5*−*7*).

$$RNHC(NH_2){=}NH + O_2 + 2e^- + 2H^+ \xrightarrow{NOS} RNHC(NH_2){=}NOH + H_2O \quad (1)$$

$$RNHC(NH_2){=}NOH + O_2 + e^- + H^+ \xrightarrow{NOS} RNHC(NH_2){=}O + NO + H_2O \quad (2)$$

Since many physiopathological situations are characterized either by an overproduction of NO or by a deficit in NO formation, many groups have worked during these last years to find either selective NOS inhibitors or selective NOS substrates acting as NO precursors. This research has led to many powerful NOS inhibitors (*8*); however, only a few NOS substrates have been reported so far (*9*). Most of them are α-amino acids closely related to L-Arg or NOHA, such as homo-L-Arg and homo-NOHA (*10*, *11*), or (*E*)-dehydro-L-Arg with a C=C double bond in the L-Arg side chain (*12*). In fact, small changes of the L-Arg or NOHA structure completely abolish the NOS-dependent formation of NO. Such examples include the decrease of the L-Arg chain length, as in nor-L-Arg (*13*) or nor-NOHA (*10*), the esterification of the α-COOH function of L-Arg (*9*), or the replacement of the δ-NH group of L-Arg or NOHA with a CH_2 group as in indospicine or N^{ω}-hydroxyindospicine (*14*, *15*). However, more recently, it has been reported that some selected non-α-amino acid compounds bearing an *N*-hydroxyguanidine function act as substrates for the second step of NOS (eq 2) with formation of NO. For instance, the removal of either the α-NH_2 group or the COOH group of NOHA leads to a dramatic decrease in the level of NO formation, whereas the removal of both groups leads to a simple, non-α-amino acid compound, *N*-butyl-*N*′-hydroxyguanidine, that acts as a substrate almost as efficient as NOHA itself for NO production (*16*). Moreover, some *N*-aryl-*N*′-hydroxy-

* To whom correspondence should be addressed. Fax: 33 (0)1 42 86 83 87. E-mail: Daniel.Mansuy@biomedicale.univ-paris5.fr.
[‡] UMR 8601 CNRS.
[§] URA 1116 CNRS.
[∥] Aristotelian University of Thessaloniki.
[⊥] Cleveland Clinic.

[1] Abbreviations: L-Arg, L-arginine; NOHA, N^{ω}-hydroxy-L-arginine; NO_2-Arg, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NMMA, N^{ω}-methyl-L-arginine; AG, aminoguanidine hydrochloride; SEITU, *S*-ethylisothiourea; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; BSA, bovine serum albumin; SOD, superoxide dismutase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LPS, lipopolysaccharide; γ-INF, γ-interferon; NOS, nitric oxide synthase; EPR, electron paramagnetic resonance; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DETC, diethyl dithiocarbamate; Fe−$DETC_2$, ferrous diethyldithiocarbamate complex; Fe−$DETC_2$−NO, ferrous mononitrosyl diethyldithiocarbamate complex; TLC, thin-layer chromatography; HRMS, high-resolution mass spectrometry.

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guanidines are oxidized by NOS II, in the presence of NADPH and O_2, with concomitant formation of the corresponding *N*-arylurea and NO in a 1:1 molar ratio (*17*, *18*).

A similar study of the oxidation of compounds bearing a guanidine function by NOS showed that it is much more difficult to find guanidine substrates that are able to be oxidized by NOS with NO formation, as this requires the two steps indicated in eqs 1 and 2. Here, we report results comparing the oxidation of a series of L-arginine-related compounds and of various alkyl- and arylguanidines by recombinant NOS II. These results show for the first time that some non-α-amino acid guanidines are efficiently oxidized by NOS II with formation of NO, not only in a cell-free system based on recombinant NOS II but also in activated murine macrophages. The most efficient one, 3-(trifluoromethyl)propylguanidine, leads to a rate of NO formation only 2 times lower than that of L-Arg itself.

EXPERIMENTAL PROCEDURES

General Chemistry. Pyrazole-1-carboxamidine hydrochloride was purchased from Aldrich. Other chemicals and reagents were purchased from Fluka or Acros and were used without further purification. Dioxane and diethyl ether were distilled over sodium benzophenone. Chemical reactions were monitored by thin-layer chromatography (TLC) using Merck precoated silica gel $60F_{254}$ (0.25 mm thickness) plates. The guanidines on the plates were visualized using the Sakaguchi reagent (spray of a 0.1% solution of 8-hydroxyquinoline in acetone followed by a spray of a mixture of 0.2 mL of bromine in 100 mL of 0.5 N NaOH). Matrex Silica (Millipore, 35−70 μm) was utilized for flash chromatography. Melting points were determined on a Kofler apparatus and are uncorrected. Proton (^{1}H) NMR spectra were recorded on a Bruker ARX 250 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane with peak multiplicities abbreviated as follows: singlet, s; broad singlet, br s; doublet, d; triplet, t; and multiplet, m. Coupling constants (*J*) are reported in hertz. Mass spectra were recorded at Ecole Normale Superieure (Paris, France) on a RiberMag system with chemical ionization (CI) capabilities. High-resolution mass spectrometry is indicated as HRMS. Elemental analyses were performed at Service de Microanalyze, Pierre and Marie Curie University (Paris, France).

General Procedures for the Synthesis of Guanidines. Procedure A. This procedure was used for guanylation of reactive aliphatic amines (*19*). The amine, pyrazole-1-carboxamidine hydrochloride, and diisopropylethylamine (DIEA) (3.0 mmol each) were dissolved in 1.5 mL of dry *N*,*N*-dimethylformamide (DMF), and the reaction mixture was stirred at room temperature under nitrogen while being monitored by TLC. After a few hours, dry ether was added to complete precipitation of the crude product which was collected, washed with ether, and purified on a silica gel column. The products were separated by a stepwise gradient elution from 0 to 20% methanol in ethyl acetate. Evaporation of the solvents gave the pure guanidines.

Procedure B. This procedure was used for guanylation of less reactive amines (*20*). The amine and *N*,*N*′-bis(*tert*-butyloxycarbonyl)pyrazole-1-carboxamidine (*20*) (2.5 mmol each) were dissolved in 5 mL of dry chloroform, and the reaction mixture was stirred at room temperature under nitrogen while being monitored by TLC. The reaction mixtures were concentrated, diluted with cyclohexane, and directly applied to a short silica gel column. The products were separated from unreacted starting compounds by a stepwise gradient elution from 0 to 50% ethyl acetate in cyclohexane. Evaporation of the solvents and drying in vacuo gave the expected *N*,*N*′-bis(*tert*-butyloxycarbonyl)guanidines. These intermediate products were dissolved in methanol containing 5 N aqueous HCl, and the reaction mixture was stirred at room temperature for a few hours. The solvents were evaporated in vacuo, and the residue was redissolved in a small amount of water and lyophilized, leaving pure guanidines.

*1-Amino-4-guanidinobutane Dihydrochloride (Agmatine Dihydrochloride) **2***. Agmatine sulfate is commercially available (Sigma). However, because of its mode of preparation, it contains small amounts of L-Arg (*13*). Therefore, we have designed a synthetic procedure, allowing us to obtain agmatine without any trace of L-Arg. A solution of di-*tert*-butylpyrocarbonate (10 mmol) in 30 mL of dry dioxane was dropwise added at 0 °C to a solution of 1,4-diaminobutane (75 mmol) in 30 mL of dry dioxane, and the mixture was stirred for 24 h at room temperature. Dioxane was removed in vacuo, and H_2O (70 mL) was added to the residue. The precipitate was removed by filtration. The filtrate was extracted with dichloromethane, and the organic phase was dried over $MgSO_4$ and evaporated. Purification using silica gel column chromatography [80/15/5 (v/v) CH_2Cl_2/CH_3OH/NH_4OH] gave pure 1-amino-4-*tert*-butyloxycarbonylaminobutane as a colorless oil (89%) (*21*): ^{1}H NMR ($CDCl_3$) δ 4.62 (br s, 1H), 3.09 (m, 2H), 2.69 (m, 2H), 1.42 (br s, 13H). Treatment of 1-amino-4-*tert*-butyloxycarbonylaminobutane following procedure B gave pure 1-amino-4-guanidinobutane dihydrochloride as a white solid (79%): mp 174 °C; ^{1}H NMR (DMSO-d_6) δ 8.20 (m, 4H), 7.25 (m, 4H), 3.12 (m, 2H), 2.75 (t, 2H, J = 6.7), 1.55 (m, 4H). Anal. Calcd for $C_5H_{14}N_4$·2HCl: C, 29.57; H, 7.94; N, 27.58. Found: C, 29.58; H, 8.08; N, 27.39.

*n-Butylguanidine hydrochloride **3*** (*22*) was obtained as a colorless oil following procedure A (75%): ^{1}H NMR (DMSO-d_6) δ 7.69 (br s, 1H), 7.13 (br s, 4H), 3.09 (m, 2H, J = 6.6), 1.46 (m, 2H), 1.34 (m, 2H), 0.87 (t, 3H, J = 7.2). HRMS (CI/CH_4). Calcd for $C_5H_{14}N_3$: 116.1188 [$(M + H)^+$]. Found: 116.1187.

*3-(Trifluoromethyl)propylguanidine Hydrochloride **4***. A mixture of 1-bromo-4,4,4-trifluorobutane (1.3 mmol) and potassium phthalimide (1.8 mmol) in 12 mL of dry DMF was stirred for 1 day at room temperature. DMF was evaporated in vacuo, and the residue was redissolved in CH_2Cl_2. Crude *N*-(4,4,4-trifluorobutyl)phthalimide was purified by flash chromatography over SiO_2 using CH_2Cl_2 as an eluant and was obtained as white crystals (89%): mp 58−59 °C; ^{1}H NMR ($CDCl_3$) δ 7.83 (m, 2H), 7.72 (m, 2H), 3.74 (t, 2H, J = 6.8), 2.15 (m, 2H), 1.96 (m, 2H). *N*-(4,4,4-Trifluorobutyl)phthalimide (1.2 mmol) was dissolved in 2.5 mL of tetrahydrofurane, and a solution of hydrazine hydrate (7.8 mmol) in 1.5 mL of ethanol was added. The mixture was stirred for 15 h at room temperature. Twenty milliliters of 1 N HCl was added; the precipitate was filtered and washed with 1 N HCl. The pH of the aqueous phase was increased to 10 with NaOH, and the aqueous phase was

extracted 3-fold with CH_2Cl_2. The organic phase was dried over Na_2CO_3 and filtered. A solution of gaseous HCl in diethyl ether was added to the CH_2Cl_2 extracts, and the mixture was evaporated, leaving 4,4,4-trifluorobutylamine hydrochloride as an oil: ^{1}H NMR (DMSO-d_6) δ 8.18 (br s, 3H), 2.89 (m, 2H), 2.46 (m, 2H), 1.87 (m, 2H). Crude 4,4,4-trifluorobutylamine hydrochloride was mixed with Na_2CO_3 (7.0 mmol) and *N*,*N*′-bis(*tert*-butyloxycarbonyl)pyrazole-1-carboxamidine (1.2 mmol) in 2.5 mL of $CHCl_3$, and the reaction mixture was stirred for 1 day at room temperature. Filtration, evaporation in vacuo, and flash chromatography over SiO_2 using CH_2Cl_2 and cyclohexane (9/1) as an eluant gave *N*,*N*′-bis(*tert*-butyloxycarbonyl)-3-(trifluoromethyl)propylguanidine as a white solid (33% for the two steps): mp 111−112 °C; ^{1}H NMR ($CDCl_3$) δ 11.48 (br s, 1H), 8.36 (br s, 1H), 3.48 (q, 2H, J = 6.6), 2.13 (m, 2H), 1.83 (m, 2H), 1.49 (s, 18H). Treatment of the protected guanidine in methanol containing 5 N aqueous HCl at room temperature and evaporation in vacuo quantitatively yielded pure 3-(trifluoromethyl)propylguanidine hydrochloride as an oil: ^{1}H NMR (DMSO-d_6) δ 7.86 (br s, 1H), 7.22 (br s, 4H), 3.17 (m, 2H), 2.29 (m, 2H), 1.67 (m, 2H). HRMS (CI/CH_4). Calcd for $C_5H_{11}N_3F_3$: 170.0905 [$(M + H)^+$]. Found: 170.0899.

4-Hydroxybutylguanidine hydrochloride ***5*** was obtained as a white solid following procedure A (89%): mp 106−108 °C; ^{1}H NMR (DMSO-d_6) δ 7.77 (br s, 1H), 7.17 (br s, 4H), 4.48 (br s, 1H), 3.39 (m, 2H), 3.07 (m, 2H), 1.45 (m, 4H); Anal. Calcd for $C_5H_{13}N_3O$ HCl: C, 35.82; H, 8.42; N, 25.07. Found: C, 36.09; H, 8.51; N, 25.16.

n-Hexylguanidine hydrochloride ***6*** was obtained as a colorless oil following procedure A (89%): ^{1}H NMR (DMSO-d_6) δ 7.70 (br s, 1H), 7.14 (br s, 4H), 3.07 (m, 2H, J = 6.6), 1.44 (m, 2H), 1.26 (m, 6H), 0.86 (t, 3H, J = 6.7). HRMS (CI/CH_4). Calcd for $C_7H_{18}N_3$: 144.1501 [$(M + H)^+$]. Found: 144.1501.

2-Methylbutylguanidine hydrochloride ***7*** was obtained as a colorless oil following procedure B (62%): ^{1}H NMR (DMSO-d_6) δ 7.74 (br s, 1H), 7.15 (br s, 4H), 2.96 (m, 2H), 1.52 (m, 1H), 1.37 (m, 1H), 1.11 (m, 1H), 0.85 (m, 6H); ^{1}H NMR (D_2O) δ 3.10 (m, 2H), 1.72 (m, 1H), 1.45 (m, 1H), 1.24 (m, 1H), 0.96 (d, 6H, J = 6.5). Anal. Calcd for $C_6H_{15}N_3$·HCl·0.3H_2O: C, 42.13; H, 9.78; N, 24.56. Found: C, 41.97; H, 9.77; N, 24.53.

3,3-Dimethylbutylguanidine hydrochloride ***8*** was obtained as a white solid following procedure A (81%): mp 203−204 °C; ^{1}H NMR (DMSO-d_6) δ 7.65 (br s, 1H), 7.15 (br s, 4H), 3.08 (br s, 2H), 1.38 (t, 2H, J = 8.0), 0.89 (s, 9H). Anal. Calcd for $C_7H_{17}N_3$·HCl: C, 46.79; H, 10.10; N, 23.38. Found: C, 46.78; H, 10.18; N, 23.20.

(Cyclopropyl)methylguanidine hydrochloride ***9*** was obtained as a colorless oil following procedure B (80%): ^{1}H NMR (DMSO-d_6) δ 7.74 (br s, 1H), 7.17 (br s, 4H), 2.99 (t, 2H, J = 6.3), 0.96 (m, 1H), 0.47 (m, 2H), 0.20 (m, 2H). Anal. Calcd for $C_5H_{11}N_3$·HCl·0.9H_2O: C, 36.21; H, 8.39; N, 25.34. Found: C, 36.44; H, 8.39; N, 25.41.

Benzylguanidine hydrochloride ***10*** was obtained as a white solid following procedure B (56%): mp 173 °C; ^{1}H NMR (DMSO-d_6) δ 8.14 (br s, 1H), 7.37 (m, 9H), 4.37 (br s, 2H). Anal. Calcd for $C_8H_{11}N_3$·HCl·0.1H_2O: C, 51.26; H, 6.56; N, 22.42. Found: C, 51.36; H, 6.70; N, 22.17.

Cyclohexylguanidine hydrochloride ***11*** was obtained as a white solid following procedure A (80%): mp 227 °C [lit. (*19*) mp 228−229 °C]; ^{1}H NMR (DMSO-d_6) δ 7.74 (br s, 1H), 7.14 (br s, 4H), 1.75 (m, 5H), 1.21 (m, 6H). The data are in agreement with those of the literature (*19*).

4-Fluorophenylguanidine hydrochloride ***12*** was obtained as a white solid following procedure B (60%): mp 130−131 °C; ^{1}H NMR (DMSO-d_6) δ 9.93 (br s, 1H), 7.48 (br s, 4H), 7.26 (m, 4H). Anal. Calcd for $C_7H_8N_3F$·HCl: C, 44.34; H, 4.78; N, 22.16. Found: C, 44.17; H, 4.96; N, 22.01.

4-Chlorophenylguanidine hydrochloride ***13*** was obtained as a white solid following procedure B (68%): mp 167−168 °C [lit. (*23*) mp 166−167 °C]; ^{1}H NMR (DMSO-d_6) δ 10.00 (br s, 1H), 7.53 (br s, 4H), 7.48 (d, 2H, J = 8.7), 7.25 (d, 2H, J = 8.7). Anal. Calcd for $C_7H_8N_3Cl$·HCl: C, 40.80; H, 4.40; N, 20.39. Found: C, 40.62; H, 4.44; N, 20.26.

4-Methoxyphenylguanidine hydrochloride ***14*** was obtained as a white solid following procedure B (71%): mp 143−144 °C [lit. (*19*) mp 144−146 °C]; ^{1}H NMR (DMSO-d_6) δ 9.62 (br s, 1H), 7.31 (br s, 4H), 7.15 (d, 2H, J = 8.9), 6.99 (d, 2H, J = 8.9), 3.76 (s, 3H). Anal. Calcd for $C_8H_{11}N_3O$·HCl: C, 47.65; H, 6.00; N, 20.84. Found: C, 47.62; H, 6.08; N, 20.73.

General Biochemistry. L-Arginine, N^ω-nitro-L-arginine methyl ester (L-NAME), N^ω-methyl-L-arginine (NMMA), aminoguanidine hydrochloride (AG), *S*-ethylisothiourea hydrobromide (SEITU), L-lysine monohydrochloride, bovine erythrocyte superoxide dismutase (SOD), bovine liver catalase, bovine hemoglobin, and bovine serum albumin (BSA) were from Sigma Chemical Co. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Alexis Biochemicals (Coger, Paris, France). NADPH came from Boehringer Mannheim Biochemicals. Other reagents were purchased from Aldrich, Sigma, or Acros unless otherwise indicated.

NOS II Expression and Purification. Recombinant NOS II was isolated and purified from *Escherichia coli* in the absence of BH_4 and L-Arg as described previously (*24*). NOS II was estimated to be more than 95% pure by SDS−PAGE. Protein concentrations were determined by the method of Bradford using bovine serum albumin (BSA) as a standard and the Bradford reagent from Bio-Rad (*25*).

Preparation and Incubation of Murine Peritoneal Macrophages (26). Macrophages from specific pathogen free (C57Bl/6xDBA/2) F1 mice (Iffa Credo, L'Arbresle, France) were elicited by ip injection of thioglycollate broth (Institut Pasteur). Four days later, inflammatory macrophages were collected from the peritoneal cavity, centrifuged, washed, and then resuspended in RPMI 1640 medium (Life Technologies, Cergy-Pontoise, France) supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 5% heat-inactivated fetal calf serum (Life Technologies) at a concentration of 2×10^6 cells/mL. This suspension was plated in 96-well microplates in triplicate (100 μL per well) and incubated at 37 °C in a 5% CO_2 humidified atmosphere to allow macrophages to adhere for 2 h. Cell viability was assessed by trypan blue exclusion and was greater than 95%. Adherent macrophages were then incubated for 15 h (37 °C, 5% CO_2 atmosphere) in fresh RPMI 1640 medium in the presence of mouse γ-interferon (γ-INF) (20 units/mL, Ernst-Boehringer Institut für Arzneimittel Forschung, Vienna, Austria) and lipopolysaccharide (LPS) (100 ng/mL). After this period, the cells were washed with fresh RPMI medium without L-Arg to remove stimuli. Then, the cells were incubated for 30 min at 37 °C in L-Arg-free RPMI medium

to remove most of the intracellular L-Arg. The supernatants were then discarded and replaced with L-Arg-free RPMI either alone or in the presence of the studied compounds.

Assessment of NO Formation by the Hemoglobin Assay. The initial rates of NO formation were determined at 37 °C using the classical spectrophotometric oxyhemoglobin assay for NO (*27*, *28*) under conditions described previously (*16*, *17*).

Measurements of the Level of NO_2^- Formation. Measurements of the level of NO_2^- were performed following a previously described colorimetric method using the Griess reagent (*29*).

Measurement of the Level of NO Formation by EPR Spectroscopy. A stable solution of the ferrous Fe−$DETC_2$ complex was prepared following a previously described procedure (*30*, *31*). Fifty microliters of a 50 mM solution of DETC-Na and 50 μL of a 10 mM solution of $FeSO_4$ were added to 900 μL of a 20 mg/mL solution of BSA [all in 50 mM HEPES buffer (pH 7.4)] to give a final 500 μM solution of the Fe−$DETC_2$ complex. Formation of NO in the presence of the Fe−$DETC_2$ complex results in the formation of the paramagnetic ferrous mononitrosyl dithiocarbamate complex (Fe−$DETC_2$−NO) that exhibits a characteristic EPR signal (*30*, *31*). Incubation mixtures containing 1 mM NADPH, 100 μM BH_4, 100 units/mL superoxide dismutase, 100 units/mL catalase, and 1 mM L-Arg or guanidine **4** were added to the previously prepared 500 μM Fe−$DETC_2$ complex solution (final volume of 100 μL). In some experiments, 1 mM NO_2-Arg, NMMA, AG, or SEITU was added, or cofactors (NADPH or BH_4) were omitted. NOS II (20−100 nM) was added, and vials were incubated at 37 °C for the required periods of time. Reactions were stopped by ice cooling; the vials were immediately transferred to EPR tubes and the contents frozen by immersion in liquid nitrogen. Calculation of the amounts of NO generated in these incubation mixtures was carried out on the basis of a calibration curve obtained from the EPR signals of identical incubation mixtures (except that NOS II was omitted) in which known amounts of NO have been added. EPR spectra were recorded at 35 K on a Bruker EPR Elexsys 500 spectrometer operating at X-band frequency (9.44 GHz) equipped with a SHQ cavity fitted with an Oxford Instrument liquid helium probe. The following instrument settings were used: modulation frequency, 100 kHz; modulation amplitude, 1 mT; time constant, 0.04 s; field sweep, 30 mT; microwave power, 0.3 mW; sampling time, 81 s; and number of scans, 2. The amplitude of the peak at 329.5 mT ($g = 2.044$) was measured in the incubations containing NOS, and the amounts of NO were determined from the calibration curve. In independent experiments, it was also verified that the addition of the Fe−$DETC_2$ complex did not modify the activity of NOS II under those conditions (data not shown).

NADPH Consumption. NADPH consumption was followed by monitoring the decrease in absorbance at 340 nm and quantitated using an extinction coefficient of 6.2 mM^{-1} cm^{-1} under the conditions of the hemoglobin assay, except for the absence of oxyhemoglobin. The initial NADPH concentration was 200 μM.

Kinetic Constant Determinations. K_m and V_m values were determined by plotting the initial rate of NO formation versus substrate concentration. Curves were fitted to the equation $y = (V_m S)/(K_m + S)$ using Kaleidagraph, version 3.02.

Table 1: Formation of NO from Oxidation of R-NH-C(=NH)NH_2 Compounds, **1**−**14**, by NOS II

compound	R	initial rate[a]	% relative to L-Arg
1	$(CH_2)_3CH(NH_2)COOH$	1660 ± 200	100
2	$(CH_2)_3CH_2NH_2$	30 ± 10	2
3	$(CH_2)_3CH_3$	105 ± 20	6
4	$(CH_2)_3CF_3$	580 ± 100	35
5	$(CH_2)_3CH_2OH$	<5	<0.5
6	$(CH_2)_3CH_2CH_2CH_3$	<5	<0.5
7	$CH_2CH(CH_3)CH_2CH_3$	<5	<0.5
8	$CH_2CH_2C(CH_3)_2CH_3$	<5	<0.5
9	CH_2-cyclopropyl	<5	<0.5
10	benzyl	<5	<0.5
11	cyclohexyl	<5	<0.5
12	*p*-FC_6H_4	<5	<0.5
13	*p*-ClC_6H_4	<5	<0.5
14	*p*-$CH_3OC_6H_4$	<5	<0.5

[a] Mean values ± standard deviations, in nanomoles of NO per minute per milligram of protein, from five to eight experiments. NO formation was detected spectrophotometrically by following the transformation of oxyhemoglobin Fe^{II}-O_2 to methemoglobin Fe^{III} as described previously (*16*−*18*, *27*, *28*). Incubations were performed at 37 °C in 150 μL quartz cuvettes containing 1 mM NADPH, 100 μM BH_4, 100 units/mL SOD, 100 units/mL catalase, 12−15 μM oxyhemoglobin, 5 mM dithiothreitol, and the tested compound (1 mM) in 50 mM HEPES buffer (pH 7.4). Purified, recombinant NOS II was added to the sample cuvette, and the same volume of buffer was added to the reference cuvette. NOS II concentrations were 5−25 nM.

RESULTS AND DISCUSSION

Oxidation of a Series of Guanidines by Recombinant NOS II. A series of *N*-alkylguanidines related to L-Arg, $R(CH_2)_3$-$NHC(NH_2)$=NH, and various *N*-aryl- and *N*-alkylguanidines were synthesized by previously described procedures starting from the corresponding amines (*19*, *20*). Their structure and purity were established by ^{1}H NMR and mass spectrometry, and by elemental analysis.

Formation of NO upon oxidation of these guanidines by recombinant, purified NOS II, in the presence of NADPH and O_2, was followed by the standard hemoglobin assay that is based on the conversion of oxyhemoglobin to methemoglobin by NO (*27*, *28*). Although L-Arg was very efficiently oxidized by NOS II with formation of 1660 ± 200 nmol of NO min^{-1} (mg of protein)$^{-1}$, its analogue lacking the α-COOH function, agmatine **2**, was much less active, its rate of oxidation into NO being only 2% of that found for L-Arg (Table 1). This result is in agreement with the high substrate specificity of NOS and the great importance of the presence of both α-COOH and α-NH_2 functions of L-Arg or NOHA for their oxidation by NOS with NO formation (*1*−*5*, *9*, *13*). However, interestingly, oxidation by NOS II of the L-Arg analogue, butylguanidine **3**, in which both the α-NH_2 and α-COOH functions of L-Arg have been removed, led to a significant rate of NO formation [105 ± 20 nmol min^{-1} (mg of protein)$^{-1}$, 6% of that found for L-Arg]. This surprising ability of butylguanidine to act as a better NOS II substrate than the L-Arg analogue lacking the COOH function (agmatine) recalls the results recently reported for the corresponding series of NOHA analogues bearing an *N*-hydroxyguanidine function instead of a guanidine function (*16*). These results have been interpreted by the presence of a hydrophobic cavity that is close to the heme in the NOS II active site and that could be able to bind the hydrophobic, terminal part of the alkyl chain of the *N*-hydroxyguanidines

Table 2: Kinetic Constants Measured for the Oxidation of L-Arg and Alkylguanidines **3** and **4** by Recombinant Purified NOS II[a]

compound	K_m (μM)	k_{cat} (min^{-1})
L-arginine **1**	5 ± 1	400 ± 50
butylguanidine **3**	45 ± 10	23 ± 5
$CF_3(CH_2)_3$-guanidine **4**	275 ± 50	220 ± 50

[a] Mean values ± standard deviations from three to five experiments. NO formation was detected spectrophotometrically by following the transformation of oxyhemoglobin to methemoglobin as described in Table 1.

(*32*−*35*). However, the effect of the replacement of the $CH(NH_2)COOH$ group of NOHA with a CH_3 group was much more spectacular (67% of the activity of NO formation found with NOHA) (*16*) than the effect of the corresponding replacement in L-Arg (activity with **3** only 6% of that found for L-Arg, Table 1). This is presumably related to the more difficult two-step oxidation of a guanidine function than the one-step oxidation of the corresponding *N*-hydroxyguanidine function, which should require a more strict positioning of the substrate close to the heme.

Several derivatives of butylguanidine **3** in which the terminal methyl group was replaced with different substituents such as CF_3, CH_2OH, and $(CH_2)_2CH_3$ or in which the terminal $CH_2CH_2CH_3$ group was replaced with $CH(CH_3)$-CH_2CH_3, $CH_2C(CH_3)_3$, or cyclopropyl substituents were synthesized and tested as potential substrates of NOS II (Table 1). The only compound of this series that was more active than **3** was 3-(trifluoromethyl)propylguanidine, **4**. Its oxidation by NOS II led to NO with a remarkable rate (35% of that found for L-Arg) for a non-α-amino acid guanidine (Table 1). It is noteworthy that other guanidines such as benzyl-, cyclohexyl-, and arylguanidines, **10**−**14**, failed to produce NO upon oxidation by NOS II (Table 1).

More detailed kinetic studies were performed on the NOS II-catalyzed oxidations of the most active compounds (**3** and **4**) by comparison with L-Arg. Formation of NO and of NO_2^- (using the Griess assay), from oxidation of **3** and **4**, exhibited characteristics very similar to those of NOS II-catalyzed oxidation of L-Arg (data not shown). (i) They absolutely required the presence of NOS II containing BH_4, NADPH, and O_2, and (ii) they were strongly inhibited by classical NOS inhibitors such as NO_2-Arg and SEITU. NOS II-catalyzed oxidation of compounds **3** and **4** exhibited classical saturation kinetics and Lineweaver−Burk plots. Table 2 shows that the k_{cat} value determined for **3** was much lower than that found for L-Arg (23 ± 5 vs 400 ± 50 min^{-1}), whereas the k_{cat} value found for **4** (220 ± 50 min^{-1}) was only 2-fold lower than that for L-Arg. However, the NOS II-catalyzed oxidations of **3** and **4** exhibited K_m values markedly higher than that of L-Arg oxidation (Table 2).

It is noteworthy that the rates of NO formation from **4** that were determined by the hemoglobin assay (*27*, *28*) always were in good agreement with those derived from the measurement of nitrite plus nitrate, using the Griess assay (*29*). Moreover, supplementary experiments were carried out to confirm the formation of NO itself in the NOS II-catalyzed oxidation of **4**. For that purpose, a method of detection and quantitation of NO by EPR spectroscopy, after trapping by the ferrous Fe−$DETC_2$ complex, was used (*31*). Binding of NO to that iron complex leads to a paramagnetic ferrous mononitrosyl complex, Fe−$DETC_2$−NO, that exhibits a characteristic EPR signal (*30*). Quantitation of the amounts of NO generated in incubation mixtures containing L-Arg or **4** and NOS II in the presence of all the necessary cofactors was carried out by measuring the amplitude of the $g = 2.044$ signal, on the basis of a calibration curve obtained from the EPR signals of identical incubation mixtures, not containing NOS II, in which known amounts of NO have been added. The amounts of NO detected by this EPR technique after oxidation of L-Arg and **4** by NOS II were in good agreement with those determined by the hemoglobin assay. Thus, NOS II-dependent oxidation of L-Arg led to ~3 times more NO than oxidation of **4**. Moreover, the extent of formation of the Fe−$DETC_2$−NO complex was strongly reduced in incubations containing NO_2-Arg, and formation was undetectable in incubations without either NADPH or NOS II (data not shown).

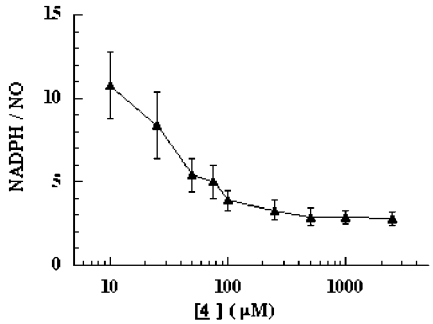

FIGURE 1: Ratio of NADPH consumption for NO formation during oxidation of compound **4** by NOS II as a function of the concentration of **4**. Rates of NADPH consumption were measured by following the decrease in absorbance at 340 nm under conditions identical to those described in Table 1, except for the absence of hemoglobin and the use of 0.2 mM NADPH. Values for the ratio of the number of moles of NADPH consumed per mole of NO formed are means ± standard deviations from three experiments.

An important parameter for the evaluation of the quality of a NOS substrate is the level of coupling between the electron transfer from NADPH and the transfer of oxygen atoms from O_2 to the substrate. This level is optimal if 1.5 mol of NADPH is consumed for the five-electron oxidation of the guanidine function to the corresponding urea and NO with consumption of 2 mol of O_2, in agreement with eqs 1 and 2. Figure 1 shows that NOS II-catalyzed oxidation of **4** to NO consumed 2.9 mol of NADPH per mole of NO produced, at saturating concentrations of **4**. When the levels of coupling between electron and oxygen atom transfer in NOS II-catalyzed oxidations of exogenous guanidine and *N*-hydroxyguanidine substrates are compared, it is not surprising that the best *N*-hydroxyguanidine, *N*-butyl-*N*′-hydroxyguanidine, is more efficiently oxidized (0.5 mol of NADPH consumed for 1 mol of NO formation as expected from eq 2, 100% coupling) (*16*) than the best non-amino acid guanidine, **4** (2.9 mol of NADPH consumed instead of 1.5 mol as expected from eqs 1 and 2, 52% coupling). This is expected for a guanidine function that is more difficult to oxidize and requires two steps for that. However, the level of coupling found for **4**, 52%, is quite remarkable for such a non-amino acid substrate.

*Oxidation of **4** by LPS and γ-INF-Activated Murine Macrophages.* To determine whether this oxidation of **4** with formation of NO also occurred in intact cells containing NOS, we performed experiments with **4** using mouse

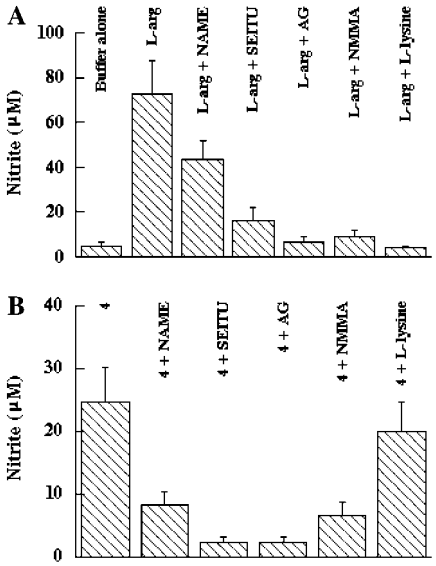

FIGURE 2: Nitrite accumulation from supernatants of activated murine macrophages incubated in the presence of either 1 mM L-Arg (A) or 2.5 mM alkylguanidine **4** (B). In some experiments, NOS inhibitors, NAME (1 mM), SEITU (100 μM), AG (2 mM), NMMA (1 mM), or L-lysine (20 mM), were also added to the medium containing either L-Arg (1 mM) or **4** (2.5 mM). Nitrite formation was assessed in the supernatants of the cells after 30 h at 37 °C using the Griess reagent, as described in Experimental Procedures. Values are means ± standard deviations from four experiments performed in triplicate.

macrophages previously treated with LPS and γ-INF, a mixture known to induce the expression of an inducible NOS in these cells (*26*). Accumulation of nitrite ions in the supernatant was used as an indication of formation of NO. As **4** displayed a lower affinity for NOS II than L-Arg (Table 2), activated mouse macrophages were first preincubated in L-Arg-free medium to decrease the level of L-Arg in the cells as much as possible. As expected, classical inhibitors of NOS such as SEITU, AG, and NMMA strongly decreased the level of NO_2^- accumulation in the supernatants of activated cells incubated in the presence of 1 mM L-Arg, whereas L-NAME was less efficient (Figure 2A). Furthermore, the addition of L-lysine, a known competitor for the y^+-amino acid transport system (*36*, *37*), also strongly inhibited NO_2^- formation in these supernatants. In control experiments, it was observed that supernatants of activated macrophages incubated in L-Arg-free RPMI medium contained low levels of NO_2^- in comparison to supernatants of cells incubated in RPMI medium containing 1 mM L-Arg (4 ± 2 and 70 ± 10 μM after 30 h, respectively). These results indicated that preincubation in L-Arg-free medium results in a strong decrease in intracellular L-Arg concentration and that these cells seem unable to synthesize L-Arg from other constituents of the medium under those conditions. Addition of 2.5 mM **4** to activated macrophages in L-Arg-free medium resulted in the accumulation of 25 ± 5 μM NO_2^- after 30 h. As previously observed when using L-Arg as a substrate, the addition of SEITU, AG, and NMMA to these incubations resulted in a strong decrease in NO_2^- levels, whereas L-NAME was a far less efficient inhibitor (Figure 2B). Interestingly, the addition of L-lysine was almost without effect on the accumulation of NO_2^- in these supernatants. These results suggest that guanidine **4** penetrates in mouse macrophages without significant need of the y^+-system. Further experiments showed that the amount of NO_2^- produced increased with an increase in the number of cells, the incubation times, or the concentration of **4** (in the 0.1−5 mM range) (data not shown). From these experiments, the concentration of **4** leading to the half-maximum rate of NO_2^- formation was found to be approximately 250 μM. Interestingly, this value is similar to the K_m value measured for the corresponding oxidation of **4** catalyzed by purified, recombinant NOS II (Table 2). The aforementioned results show that some non-α-amino acid guanidines act as substrates of NOS II with formation of NO. The best substrate found so far, compound **4**, is transformed to NO by NOS II with a maximum rate that is only 2 times lower than that observed with the natural substrate, L-Arg. When the results in Table 1 are considered, it is noteworthy that the arylguanidines **12**−**14** failed to act as substrates of NOS II whereas the corresponding *N*-hydroxyguanidines have been found to be good substrates of NOS II with NO formation (*17*, *18*). Experiments are currently being carried out to explain the particular efficiency of **4** as a non-α-amino acid NOS II substrate. Compound **4** penetrates into activated mouse macrophages without the help of the y^+-transport system and is oxidized by these cells with formation of NO_2^- in a manner similar to that of L-Arg. These results open the way toward the research of selective, stable guanidine substrates of each class of NOS that could be interesting new NO donors after in situ oxidation by a given NOS isoform.

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