Efficient Formation of Nitric Oxide from Selective Oxidation of *N*-Aryl *N*′-Hydroxyguanidines by Inducible Nitric Oxide Synthase

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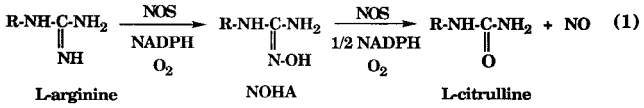
Received December 14, 1998; Revised Manuscript Received February 16, 1999

ABSTRACT: Inducible nitric oxide synthase (NOS II) efficiently catalyzes the oxidation of *N*-(4-chlorophenyl)*N*′-hydroxyguanidine **1** by NADPH and O_2, with concomitant formation of the corresponding urea and NO. The characteristics of this reaction are very similar to those of the NOS-dependent oxidation of endogenous N$^{\omega}$-hydroxy-L-arginine (NOHA), i.e., (i) the formation of products resulting from an oxidation of the substrate C═N(OH) bond, the corresponding urea and NO, in a 1:1 molar ratio, (ii) the absolute requirement of the tetrahydrobiopterin (BH_4) cofactor for NO formation, and (iii) the strong inhibitory effects of L-arginine (L-arg) and classical inhibitors of NOSs. *N*-Hydroxyguanidine **1** is not as good a substrate for NOS II as is NOHA (K_m = 500 μM versus 15 μM for NOHA). However, it leads to relatively high rates of NO formation which are only 4-fold lower than those obtained with NOHA (V_m = 390 ± 50 nmol NO min^{-1} mg protein^{-1}, corresponding roughly to 100 turnovers min^{-1}). Preliminary results indicate that some other *N*-aryl *N*′-hydroxyguanidines exhibit a similar behavior. These results show for the first time that simple exogenous compounds may act as NO donors after oxidative activation by NOSs. They also suggest a possible implication of NOSs in the oxidative metabolism of certain classes of xenobiotics.

Nitric oxide (NO)[1] is an important mediator of a number of important physiological functions including maintenance of vascular tone, neuronal signaling, and host response to infection (*1*, *2*). The synthesis of NO is catalyzed by neuronal, inducible, and endothelial isoforms of nitric oxide synthases (NOSs) (*3*−*5*). Each of the isoforms catalyzes the stepwise formation of NO and L-citrulline from the oxidation of L-arginine (L-arg) by O_2 and NADPH, with the intermediate formation of *N*$^{\omega}$-hydroxy-L-arginine (NOHA) (eq 1) (*6*, *7*).



(1)

All of the isoforms are homodimeric proteins that involve an oxygenase domain containing iron protoporphyrin IX, tetrahydrobiopterin (BH_4) and the substrate binding site (*8*, *9*), and a reductase domain containing FAD, FMN, and the binding site for NADPH (*10*).

Up to now, very few compounds have been clearly shown to act as NOS substrates with formation of NO (*11*). Homo-L-arginine (*12*, *13*) and *N*$^{\omega}$-hydroxy-homo-L-arginine (*12*) have been recently reported to be oxidized into homo-L-citrulline and NO by neuronal and inducible NOSs. However, nor-L-arginine (*11*, *13*) and nor-NOHA (*12*) are very poor substrates of NOSs. In a more general manner, slight modifications of the α-amino acid function of L-arg led to derivatives such as L-arginine methyl ester, *N*$^{\alpha}$-acetyl-L-arginine, and D-arginine that are not substrates of NOSs (*11*). *N*$^{\omega}$-Methyl-L-arginine, a widely used inhibitor of NOSs, is oxidized by NOSs with a very limited formation of NO (*14*, *15*). L-Indospicine (*16*) and *N*$^{\omega}$-hydroxy-L-indospicine (*17*), in which the δ-NH group of L-arg (or NOHA) has been replaced by a CH_2 moiety, fail to act as NOS substrates and do not seem to interact with the NOS active site.

Other L-arg analogues, such as canavanine (*18*), ϵ-guanidino-caproic acid (*13*, *18*), agmatine (*13*) and *N*$^{\omega}$-hydroxy-agmatine (*19*), and L-tyrosyl-L-arginine (*20*), have been proposed to be NOS substrates as they caused endothelium-dependent vasorelaxation or accumulation of nitrite in cell cultures. However, only some of these results have been confirmed with purified NOSs, and the precise nature of the transformations of these compounds is not yet known (*21*).

The very limited number of substrates for NOSs known so far suggests that highly specific structural features are required for NO generation by these enzymes. However, simple guanidines and alkyl-*iso*-thioureas, such as aminoguanidine, *S*-ethyl-*iso*-thiourea (SEITU), and *N*-phenyl-*S*-methyl-*iso*-thiourea, are strong inhibitors of NOSs (*22*−

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; NOS II, recombinant inducible NOS; L-arg, L-arginine; BH_4, (6R)-5,6,7,8-tetrahydro-L-biopterin; NOHA, *N*$^{\omega}$-hydroxy-L-arginine; SEITU, *S*-ethyl-*iso*-thiourea; NO_2 L-arg, *N*$^{\omega}$-nitro-L-arginine; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane sulfonic acid; SOD, superoxide dismutase; RP-HPLC, reverse phase high performance liquid chromatography.

10.1021/bi982930p CCC: $18.00
Published on Web 03/20/1999

25). Visible spectroscopy and EPR experiments have shown that some of these compounds are bound in the active site of NOS, in close proximity to the heme (*24*, *26*−*28*). These results suggest that simple compounds, not directly derived from L-arg, can interact with the NOSs' active sites, and that some of them bearing a guanidine or *N*-hydroxyguanidine function could be oxidized by NOSs with formation of NO. In fact, we have recently reported that various amidoximes and *N*-hydroxyguanidines are oxidized by hepatic cytochromes P450 with formation of the corresponding amides and ureas as well as of nitrogen oxides including NO (*29*).

In an effort to find new exogenous substrates of NOSs that could lead to NO after selective oxidation by NOSs and to better know the structural requirements for NO biosynthesis by NOSs, we have synthesized a series of compounds bearing a guanidine or a *N*-hydroxyguanidine function and studied their oxidation by NADPH and O_2 in the presence of a purified recombinant inducible NOS (NOS II). In the present communication, we report preliminary results showing that some *N*-aryl *N*′-hydroxyguanidines are very efficiently oxidized by NOS II into the corresponding *N*-aryl ureas with concomitant formation of NO.

MATERIALS AND METHODS

Materials. (6R)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Alexis (Coger, Paris, France). NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (from yeast) came from Boehringer Mannheim Biochemicals. L-Arg, L-citrulline, N^{ω}-nitro-L-arginine (NO_2 L-arg), *S*-ethyl-*iso*-thiourea (SEITU), bovine erythrocyte superoxide dismutase (SOD), bovine liver catalase, bovine hemoglobin, bovine serum albumin, *Aspergillus* nitrate reductase, and 2′,5′-ADP-agarose resin were purchased from Sigma. NOHA was prepared by a modification of the procedure of Wallace and Fukuto with N^{δ}(benzyloxycarbonyl)-L-ornithine as starting material (*17*, *30*). Sephadex G50-medium and Ni-nitrilotriacetate TSepharose CL4B resins were products of Pharmacia. Other reagents for colorimetric assays, HPLC, and syntheses were purchased from Aldrich, Sigma, or Janssen unless otherwise indicated.

Syntheses. N-(4-Chlorophenyl)*N*′-hydroxyguanidine **1** was prepared in 65% yield by reaction of cyanamide **3** with hydroxylamine hydrochloride in anhydrous ethanol according to the procedure of Schantl and Türk (*31*). mp 129 °C (lit. (*31*) 129−131 °C); ^{1}H NMR (d_6 DMSO) 8.42 (s, 1H), 7.76 (s, 1H), 7.30 (d, 2H, $J = 8.7$), 7.16 (d, 2H, $J = 8.7$), 5.08 (s, 2H); IR (KBr): 3480, 3340, 1660, 1090, 820 cm^{-1}.

4-Chlorophenyl urea **2** was obtained in 90% yield by reacting 4-chloroaniline and potassium cyanate in 0.5 M HCl. mp 210 °C (lit. (*31*) 212 °C); ^{1}H NMR (d_6 DMSO) 8.65 (s, 1H), 7.41 (d, 2H, $J = 8.8$), 7.23 (d, 2H, $J = 8.8$), 5.89 (s, 2H); IR (KBr) 3420, 3310, 1650, 1540, 820 cm^{-1}; MS (CI_{NH_3}) *m*/*z* 190 and 188 ($M + NH_4^+$), 173 and 171 ($M + H^+$, 100%).

4-Chlorophenyl cyanamide **3** was obtained in 75% yield after treatment of 4-chloroaniline with cyanogen bromide according to the method of Schantl and Türk (*31*). mp 97 °C (lit. (*31*) 98−100 °C); ^{1}H NMR (d_6 DMSO) 10.35 (s, 1H), 7.37 (d, 2H, $J = 9.2$), 6.96 (d, 2H, $J = 9.2$); IR (KBr) 3150, 3080, 2230, 1600, 1500, 810 cm^{-1}; MS (CI_{NH3}) *m*/*z*: 170 ($M^+ + NH_3$), 154, 152 (M^+, 100%).

NOS II Expression and Purification. Recombinant NOS II was isolated and purified from *Escherichia coli* as described previously (*32*). *E. coli* was transformed with a plasmid containing mouse NOS II with a six-histidine tag on its N-terminus and a plasmid that contained human calmodulin. Purification was achieved through binding of the crude extract to nickel affinity resin, washing the resin to remove unbound proteins, and eluting with imidazole. Eluted protein was concentrated and subjected to 2′,5′-ADP affinity chromatography (*32*). NOS II was estimated to be more than 90% pure by SDS−PAGE electrophoresis.

Protein Determination. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard and the Bradford reagent from Biorad (*33*).

Hemoglobin Assay. The initial rate of NO synthesis was determined at 37 °C using the classical spectrophotometric oxyhemoglobin assay for NO (*34*, *35*). Briefly, 5−10 μL aliquots containing NOS II were added to a prewarmed cuvette that contained 50 mM HEPES buffer, pH 7.4, supplemented with 15 μM oxyhemoglobin, 100 units/mL SOD, 100 units/mL catalase, 1 mM NADPH, 100 μM BH_4, and substrate at the desired concentration to give a final volume of 150 μL. The reference cuvette had the same composition, except that 50 mM HEPES was added instead of NOS-containing solutions. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an increase in absorbance difference between 401 and 416 nm and quantitated using an extinction coefficient of 77 mM^{-1} cm^{-1} (*34*, *35*).

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 (*6*), except for the absence of oxyhemoglobin to avoid interferences. Initial NADPH concentration was 200 μM.

*Incubation of N-(4-Chlorophenyl)N′-hydroxyguanidine **1** in the Presence of NADPH, O_2, and NOS II.* Typical incubations contained 1 mM NADPH, 100 μM BH_4, 100 units/mL SOD, 100 units/mL catalase, and 0.5−1 μg NOS in 50 μL (final volume) of 50 mM HEPES buffer, pH 7.4. Incubations were shaken at 37 °C for the indicated time and were stopped either by heating 5 min at 95 °C for NO_2^- and NO_3^- measurements or by addition of 20 μL CH_3CN and 30 μL 5 mM H_3PO_4 containing 66.6 μM 4-chlorobenzamide (internal standard) for RP-HPLC analysis.

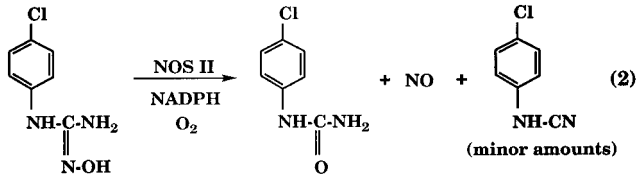
Measurement of NO_2^- and NO_3^- Formation. Measurements of NO_2^- and NO_3^- were performed following a previously described colorimetric method (*36*, *12*) using the Griess reagent (sulfanilamide 1% in HCl 0.5 N and *N*-(1-naphthyl) ethylenediamine 0.1% in HCl 0.5 N). When nitrate determination was necessary, reduction of nitrate to nitrite was performed in the presence of nitrate reductase and an NADPH-regenerating system, following a previously described protocol (*37*, *12*).

*Reverse-Phase HPLC Identification and Quantitation of Metabolites from NOS-II-Dependent Oxidation of **1**.* Separation of *N*-(4-chlorophenyl)*N*′-hydroxyguanidine metabolites was performed at 25 °C on a 250 × 4.6 mm Nucleosil ODS 5 μm column (SFCC−Shandon, France). Flow rate was 1 mL/min and the mobile phase was a gradient between solvent A (water containing 5 mM phosphoric acid, pH 2.6) and

solvent B (acetonitrile) using the following program: 0 min, 10% B; 5 min, linear gradient to 40% B in 15 min; 20 min, linear gradient to 100% B in 10 min; 30 min, linear gradient to 10% B in 5 min followed by 15 min reequilibration. The absorbance was monitored at 240 nm. The retention times for **1**, **2**, **3**, and 4-chlorobenzamide (internal standard) were 6.7, 19.5, 24.7, and 18.1 min, respectively. Calibration curves were made from identical mixtures containing various concentrations of **1**, **2**, and **3** but without NOS II.

RESULTS

*NOS II-Catalyzed Oxidation of N-(4-Chlorophenyl)N′-hydroxyguanidine **1** by NADPH and O_2.* Oxidation of *N*-(4-chlorophenyl)*N*′-hydroxyguanidine, **1**, by NADPH and O_2 in the presence of recombinant NOS II containing all its cofactors was found to lead to NO, which was detected by the conversion of oxyhemoglobin to methemoglobin, a classical test to detect and quantify NO formation (*34*, *35*). Linear increase in absorbance at 401 nm with a concomitant decrease of the absorbance at 416 nm was typical of the transformation of $HbFe^{II}$-O_2 to MetHb in the presence of NO (*34*, *35*). The assay was done, as usual (*34*, *35*), in the presence of superoxide dismutase and catalase; thus, the observed formation of methemoglobin was not due to any production of O_2^- or H_2O_2 by NOS II. Analysis of the reaction mixture by HPLC revealed the formation of two new products. The major product was found to be 4-chlorophenyl urea **2**, by comparison of its retention time with that of an authentic sample and by co-injection with the authentic compound. Minor amounts of a second metabolite that was found to be 4-chlorophenyl cyanamide **3** were also detected by HPLC (eq 2). Under the conditions of the hemoglobin assay for NO formation, it was checked that hemoglobin alone did not react with **1** and that formation of NO did not occur in the presence of all of the assay components except for NOS II (data not shown).



Similar incubations of **1** with NOS II were then performed in the absence of oxyhemoglobin and studied by HPLC for the formation of urea **2** and cyanamide **3** and by the classical colorimetric assays for NO_2^- and NO_3^- formation (*36*, *37*). These experiments clearly showed that the presence of oxyhemoglobin had no effect on the formation of **2** and **3** and that the amounts of $NO_2^- + NO_3^-$ formed in incubations without oxyhemoglobin well corresponded to the amounts of NO detected under the conditions of the hemoglobin assay.

Formation of urea and NO_2^- from **1** was clearly enzymatic as it required the presence of active NOS II and NADPH (Table 1). This oxidation of **1** to **2** and NO (and its stable oxidation products NO_2^- and NO_3^- under aerobic conditions) should occur in the active site of NOS II as it is strongly inhibited by classical inhibitors of NOSs, such as N^{ω}-nitro-L-arginine (*22*) and L-thiocitrulline (*38*), two analogues of L-arg, imidazole, a NOS heme ligand (*39*, *40*), and *S*-ethyl-*iso*-thiourea (SEITU), one of the most powerful inhibitors of NOSs (*22*, *24*) (Table 1). Accordingly, L-arg itself completely inhibited the formation of urea **2**. However, it led to an increased formation of NO_2^- (Table 1) as L-arg is a better substrate than **1** for NOS II as it will be shown below.

Table 1: Effects of Incubation Conditions on the Formation of Urea **2** and NO_2^- from Oxidation of *N*-hydroxyguanidine **1** in the Presence of Recombinant NOS II, NADPH, and O_2

conditions	% residual activity[a] urea **2**[b]	% residual activity[a] NO_2^-[c]
complete system	100	100
−NOS II	1 ± 0.5	1 ± 0.5
−NADPH	1 ± 0.5	10 ± 2
boiled NOS II[d]	<0.5	<0.5
+ imidazole[e]	7 ± 2	9 ± 2
+ SEITU[e]	1 ± 0.5	1 ± 0.5
+ NO_2 L-arg[e]	1 ± 0.5	10 ± 2
+ L-thiocitrulline[e]	1 ± 0.5	1 ± 0.5
+ L-arg[e]	1 ± 0.5	240 ± 20

[a] Expressed in % of the activity of the complete system containing 200 μM **1**, 1 mM NADPH, 100 μM BH_4, 100 units/mL SOD, 100 units/mL catalase, and 0.2 μM NOS II in 50 mM pH 7.4 HEPES buffer. Incubations were performed for 5 min at 37 °C; results are means ± SD from at least three experiments. [b] Incubations were stopped and injected onto RP-HPLC for detection and quantitation of **2**, as described under Materials and Methods. Initial rate of formation of urea **2** by the complete system: 155 ± 20 nmol (min mg)$^{-1}$. [c] Nitrite formation was quantitated by the addition of the Griess reagent to aliquots of the incubates as described under Materials and Methods: 145 ± 20 nmol (min mg)$^{-1}$ for the complete system. With the complete system, formation of NO_3^- is minor when compared to NO_2^- [166 ± 20 and 145 ± 20 nmol (min mg)$^{-1}$ for $NO_2^- + NO_3^-$ and NO_2^-, respectively]. Thus, for most control experiments reported in Table 1, NO_3^- formation could not be detected. [d] Incubations were performed in the presence of NOS II which was heated 10 min at 90 °C before incubation. [e] Addition of the indicated compounds (1 mM).

*Kinetic Analysis of NOS II-Dependent Oxidation of **1** into NO and Urea **2**.* Formation of urea **2** upon NOS II- and NADPH-dependent oxidation of **1** was linear as a function of time for about 5 min (Figure 1). It was also linearly dependent upon NOS II concentration, in the 0.025−0.2 μM range (data not shown). The initial rate of urea **2** formation, when using 200 μM **1**, was 155 ± 20 nmol urea **2** (min mg prot)$^{-1}$. A very similar rate of formation of $NO_2^- + NO_3^-$ [166 ± 20 nmol (min mg prot)$^{-1}$] was observed under identical conditions. Almost identical rates of NO formation were obtained in the presence of hemoglobin [175 ± 30 nmol (min mg prot)$^{-1}$]. These data clearly showed that NOS II-dependent oxidation of **1** leads to urea **2** and NO in a 1:1 molar ratio, as does NOS II-dependent oxidation of L-arg and NOHA to L-citrulline and NO (*1*, *2*, *6*−*8*).

Kinetic experiments using either the hemoglobin assay or the HPLC detection of **2** (in the absence of hemoglobin) showed that formation of NO and urea **2** are saturable reactions with very similar K_m and V_m values [500 ± 50 μM and 390 ± 50 nmol (min mg prot)$^{-1}$ (Figure 2), respectively]. Formation of cyanamide **3**, in very minor amounts (Figure 1), was not a saturable reaction even at very high concentrations of **1** (up to 2 mM); this reaction was not further investigated.

*NADPH Consumption during NOS II-Catalyzed Oxidation of **1**.* To more deeply investigate the oxidation of **1** by NOS II, NADPH consumption was followed at the same time as formation of urea **2** and NO (or $NO_2^- + NO_3^-$). *N*-

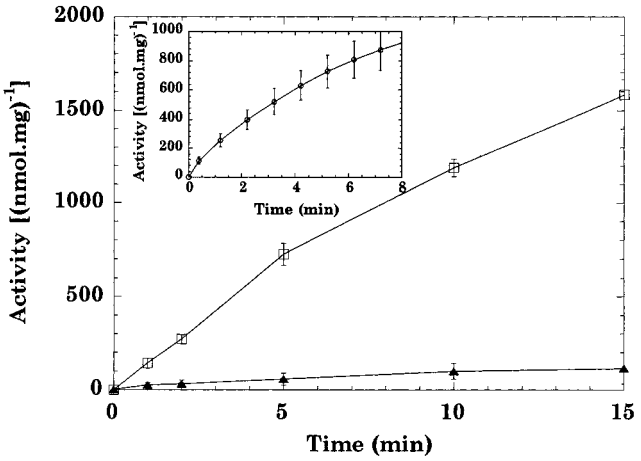


FIGURE 1: Time-dependent formation of urea **2** (□), cyanamide **3** (▲), and NO (○) upon NOS II-catalyzed oxidation of *N*-hydroxyguanidine **1** by NADPH and O_2. Purified NOS II (0.2 μM) was incubated at 37 °C in the presence of 200 μM **1**, 1 mM NADPH, 100 μM BH_4, 100 units/mL SOD, and 100 units/mL catalase in 50 mM HEPES buffer, pH 7.4. Formation of urea **2** was measured by HPLC analysis of the reaction mixtures as indicated in Materials and Methods. Inset: NO formation was monitored spectrophotometrically by following the increase in absorbance difference between 401 and 416 nm using similar experimental conditions, except for the presence of 15 μM oxyhemoglobin and NOS II concentration (0.03 μM). Values are means ± SD from three to five experiments.

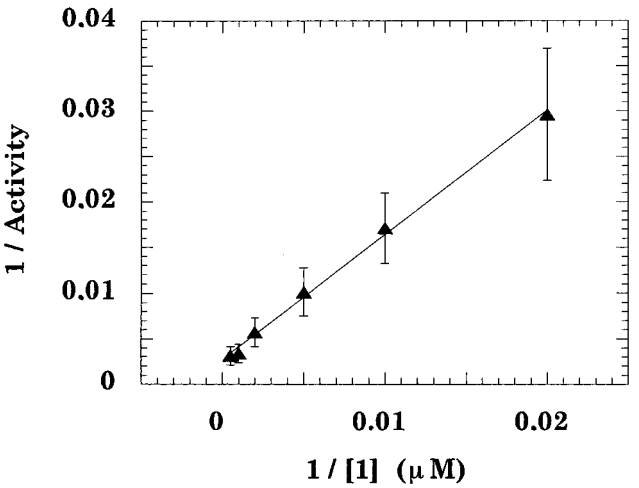


FIGURE 2: Lineweaver−Burk plot corresponding to the measurement of K_m and V_m values for the NOS II-dependent oxidation of *N*-hydroxyguanidine **1** to urea **2**. Conditions as in Figure 1. Activities are in nmol **2** (min mg protein)$^{-1}$. Values are means ± SD from three experiments.

Hydroxyguanidine **1** stimulated NADPH oxidase activity of NOS II, as 400 nmol of NADPH was consumed per min and mg protein instead of 285 nmol in the absence of **1**. With 200 μM **1**, formation of 1 mol of urea **2** and 1 mol of $NO_2^- + NO_3^-$ required the consumption of 2.2 ± 0.5 mol of NADPH.

It is well-known that during NOS-catalyzed oxidation of L-arg or NOHA by NADPH, the amounts of NADPH consumed for NO and L-citrulline formation are strongly dependent upon substrate concentrations (*6*, *26*, *41*, *42*). Measurement of the amounts of NADPH consumed for formation of urea **2** upon NOS II-catalyzed oxidation of **1** as a function of **1** concentration (Figure 3) clearly showed that the NADPH (consumed) to **2** molar ratio dramatically

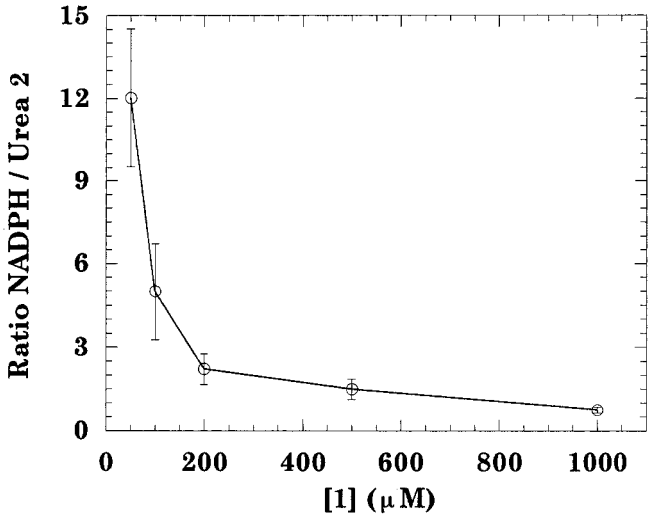


FIGURE 3: Effects of increasing *N*-hydroxyguanidine **1** concentration on the ratio of NADPH consumed to urea **2** formed. Purified NOS II (0.03 mM) was incubated for 5 min at 37 °C in 50 mM HEPES buffer, pH 7.4, containing 0.2 mM NADPH, 100 μM BH_4, 100 units/mL SOD, and 100 units/mL catalase in the presence of increasing concentrations of **1**. Rates of NADPH consumption were measured by following the decrease in absorbance at 340 nm and the formation of urea **2** by HPLC-analysis of the reaction mixture, as indicated in Materials and Methods. Values are means ± SD from three experiments.

decreased when increasing **1** concentration. With 1 mM **1**, only 0.8 ± 0.1 mol of NADPH were consumed for formation of 1 mol urea **2**.

*Key Role of BH_4 in the NOS II-Catalyzed Oxidation of **1** into **2** and NO.* In the absence of their cofactor BH_4, recombinant NOSs failed to catalyze the oxidation of L-arg to L-citrulline and NO (*32*, *43*, *44*). In a similar manner, oxidation of **1** in the presence of NADPH, O_2, and recombinant NOS II containing very low amounts of BH_4 (0.05 μM) led only to very low rates of formation of urea **2** [20 ± 10 nmol (min mg prot)$^{-1}$, instead of 155 ± 20 in the presence of BH_4]. Moreover, formation of NO could not be detected by the hemoglobin assay under those conditions. In fact, rates of formation of urea **2** and NO from oxidation of **1** by NADPH and O_2 in the presence of NOS II greatly depend on preincubation of recombinant NOS II with variable amounts of BH_4 (Figure 4). Maximal rates were obtained with 100 μM BH_4, and half-maximal effect was observed with 5 μM BH_4.

DISCUSSION

The aforementioned results show that NOS II efficiently catalyzes the oxidation of an exogenous aromatic *N*-hydroxyguanidine with formation of NO. The characteristics of this reaction are very similar to those of the NOS-dependent oxidation of the endogenous *N*-hydroxyguanidine NOHA, i.e., (1) the requirement of active NOS II and NADPH under aerobic conditions, (2) the formation of products resulting from an oxidation of the substrate C═N(OH) bond, the urea **2** (or L-citrulline), and NO in a 1:1 molar ratio (eq 2), (3) the absolute requirement of the presence of the BH_4 cofactor for NO formation, and (4) the strong inhibitory effects of L-arg and classical inhibitors of NOSs, such as N^{ω}-nitro-L-arginine, L-thiocitrulline, and SEITU.

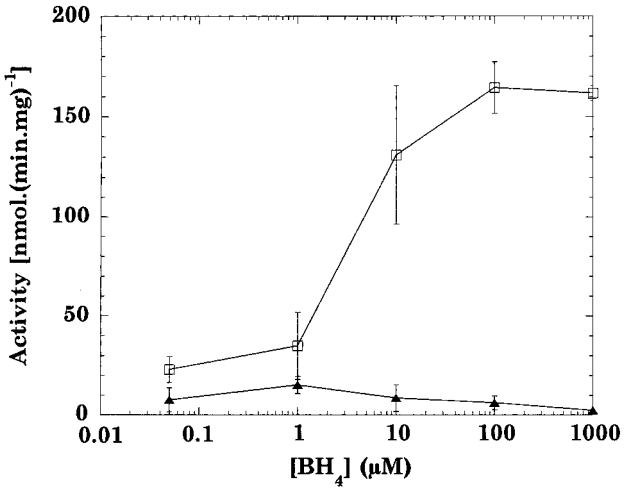


FIGURE 4: Effects of increasing BH_4 concentrations on the NOS II-catalyzed oxidation of *N*-hydroxyguanidine **1** by NADPH and O_2. Purified NOS II (0.2 μM) was preincubated 5 min at 37 °C in the presence of 100 units/mL SOD, 100 units/mL catalase, and increasing concentration of BH_4 in 50 mM HEPES buffer, pH 7.4. Reactions were started by the addition of 1 mM NADPH and 200 μM **1**. They were stopped after 5 min at 37 °C and analyzed by HPLC for urea **2** (□) and cyanamide **3** (▲) formation, as detailed in Materials and Methods. Values are means ± SD from three experiments.

N-Hydroxyguanidine **1** is not as good a substrate for NOS II as is NOHA, as it leads to a V_m for NO formation about 4-fold lower than NOHA [390 ± 50 versus 1690 ± 400 nmol NO (min mg protein)$^{-1}$] and to a K_m value markedly higher than NOHA (500 versus 15 μM) (*6*, *7*, *12*). This lower ability of **1** to act as substrate of NOS II, when compared to NOHA, is not surprising as **1** does not bear an α-amino acid function. Because of the absence of this function, **1** should fail to correctly interact with the residues of the NOS active site which specifically recognize the α-amino acid moiety (*8*, *9*) and should bind less strongly to the NOS active site.

However, the aforementioned results are interesting as they show for the first time that a relatively simple exogenous compound, not bearing an α-amino acid function, is oxidized by a NOS, in a manner similar to NOHA, with a significant rate of NO formation [390 ± 50 nmol (min mg protein)$^{-1}$] which corresponds roughly to 100 turnovers min^{-1}. Preliminary results showed us that some other *N*-aryl *N*′-hydroxyguanidines exhibit a similar behavior. Ongoing studies are currently done in order to find new NOS substrates with higher V_m and lower K_m values than **1**, as well as substrates that could be selectively oxidized by a given NOS isoform. These results should be important for the discovery of new NO donors selectively activated in situ by NOSs. They also suggest a possible implication of NOSs in the oxidative metabolism of certain classes of xenobiotics.

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BI982930P