Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3049–3055

BIOORGANIC & MEDICINAL CHEMISTRY

Novel Substrates for Nitric Oxide Synthases

Ming Xian,[a] Noriko Fujiwara,[b] Zhong Wen,[a] Tingwei Cai,[a] Satoshi Kazuma,[b] Adam J. Janczuk,[a] Xiaoping Tang,[a] Vladislav V. Telyatnikov,[b] Yingxin Zhang,[a] Xinchao Chen,[a] Yasuhide Miyamoto,[b] Naoyuki Taniguchi[b,*] and Peng George Wang[a,*]

[a]*Department of Chemistry, Wayne State University, Detroit, MI 48202, USA*
[b]*Department of Biochemistry, Osaka University Medical School, Osaka 565-0871, Japan*

Received 20 December 2001; accepted 30 January 2002

Abstract—Enzymatic generation of nitric oxide (NO) by nitric oxide synthase (NOS) consists of two oxidation steps. The first step converts L-arginine to N^G-hydroxy-L-arginine (NOHA), a key intermediate, and the second step converts NOHA to NO and L-citrulline. To fully probe the substrate specificity of the second enzymatic step, an extensive structural screening was carried out using a series of *N*-alkyl (and *N*-aryl) substituted-*N*′-hydroxyguanidines (**1**–**14**). Among the eleven *N*-alkyl-*N*′-hydroxyguanidines evaluated, *N*-*n*-propyl (**2**), *N*-*iso*-propyl (**3**), *N*-*n*-butyl (**4**), *N*-*s*-butyl (**5**), *N*-*iso*-butyl (**6**), *N*-pentyl (**8**) and *N*-*iso*-pentyl (**9**) derivatives were efficiently oxidized by the three isoenzymes of NOS (nNOS, iNOS and eNOS) to generate NO. *N*-Butyl-*N*′-hydroxyguanidine (**4**) was the best substrate for iNOS ($K_m = 33\ \mu M$) and *N*-*iso*-propyl-*N*′-hydroxyguanidine (**3**) was the best substrate for nNOS ($K_m = 56\ \mu M$). When the alkyl substituents were too small (such as ethyl **1**) or too large (such as hexyl **10** and cyclohexyl **11**), the activity decreased significantly. This suggests that the van der Waals interaction between the alkyl group and the hydrophobic cavity in the NOS active site contributes significantly to the relative reactivity of compounds **3**–**11**. Moreover, five *N*-aryl-*N*′-hydroxyguanidines were found to be good substrates for iNOS, but not substrates for eNOS and nNOS. *N*-phenyl-*N*′-hydroxyguanidine was the best substrate among them ($K_m = 243\ \mu M$). This work demonstrates that *N*-alkyl substituted hydroxyguanidine compounds are novel NOS substrates which 'short-circuit' the first oxidation step of NOS, and *N*-aryl substituted hydroxyguanidine compounds are isoform selective NOS substrate. © 2002 Published by Elsevier Science Ltd.

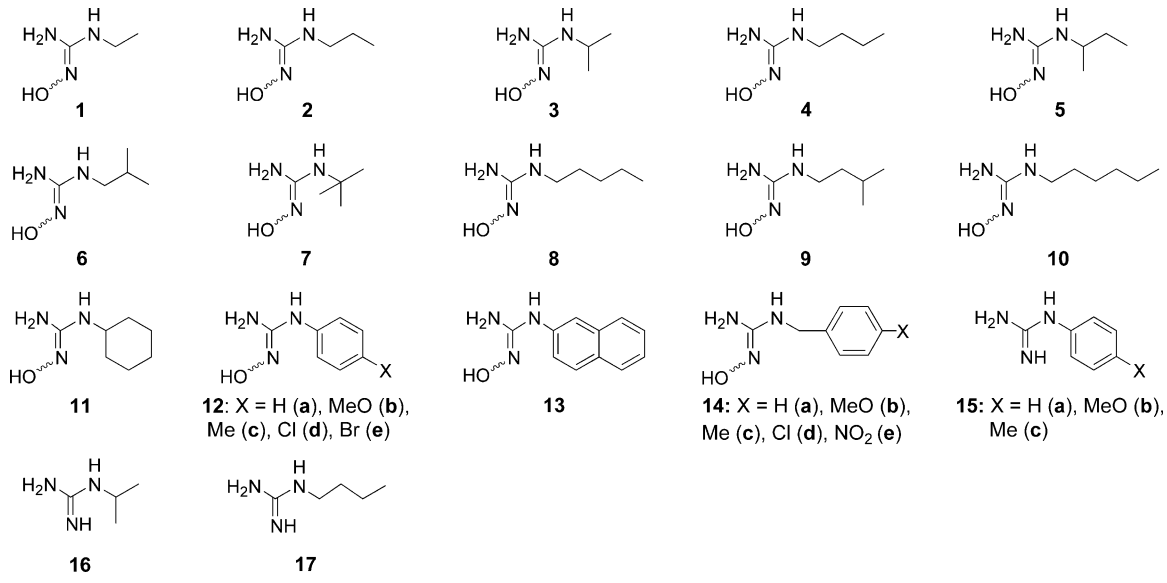
Introduction

Nitric oxide (NO) plays an important role in numerous physiological and pathological processes.[1,2] The biochemical production of NO starts from the initial oxidation of L-arginine to produce N^G-hydroxy-L-arginine (NOHA), followed by a second oxidation forming NO and L-citrulline.[3,4] This conversion is catalyzed by three distinct mammalian nitric oxide synthases (NOS) [i.e., endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)].[5,6]

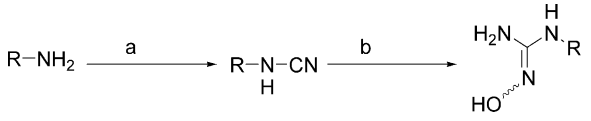
Since NO has been implicated in a wide variety of disease states, inhibitors and substrates of NOS could have great therapeutic potential in the treatment of these diseases. So far, identification of potent and selective inhibitors of NOS has been a subject of intense interest.[7,8] Among hundreds of candidates that have been tested, arginine competitors seem to be promising targets and numerous studies have been performed to determine the influence of substrate modifications on the interaction with the NOS active site. However, although it has been reported that the C=N–OH functional group of various *N*-hydroxyguanidines including NOHA, *N*-hydroxydebrisoquine and even some *N*-hydroxyguanidine containing drugs could be oxidatively cleaved by other enzymes such as cytochromes P450 and horseradish peroxidase with the formation of corresponding ureas and nitrogen oxides including NO,[9–11] only a few compounds have been clearly shown to be NOS substrates.[12–15] Besides L-arginine and NOHA, homo-L-arginine, N^{ω}-hydroxy-homo-L-arginine, and several L-arginine derivatives have been reported to be oxidized into corresponding ureas and NO by NOSs. But most other arginine derivatives such as D-arginine, L-arginine methyl ester, *N*-acetyl-L-arginine and agmatine

*Corresponding author. Tel.: +1-313-993-6759; fax: +1-313-577-2554; e-mail: pwang@chem.wayne.edu

0968-0896/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0968-0896(02)00155-4



Scheme 1.



Scheme 2. (a) BrCN, Et_3N, CH_2Cl_2; (b) $NH_2OH{\cdot}HCl$, K_2CO_3, EtOH.

Table 1. NO formation (nmol/min/mg protein) from oxidation of compounds **1**–**17** in the presence of NOS[a]

Compd	iNOS	nNOS	eNOS
NOHA	120 (100%)	595 (100%)	71 (100%)
L-Arg	55 (46%)	477 (79%)	27 (38%)
NHG	0 (0%)	0 (0%)	0 (0%)
1	3 (3%)	6 (1%)	4 (6%)
2	18 (15%)	418 (70%)	17 (24%)
3	38 (32%)	450 (76%)	27 (38%)
4	50 (42%)	380 (64%)	14 (20%)
5	14 (12%)	107 (18%)	3 (4%)
6	6 (5%)	14 (2%)	7 (10%)
7	0 (0%)	0 (0%)	0 (0%)
8	38 (32%)	185 (31%)	0 (0%)
9	29 (24%)	39 (6%)	0 (0%)
10	3 (3%)	24 (4%)	0 (0%)
11	4 (3%)	19 (3%)	0 (0%)
12a	39 (32%)	0 (0%)	0 (0%)
12b	35 (29%)	0 (0%)	0 (0%)
12c	29 (24%)	0 (0%)	0 (0%)
12d	16 (13%)	0 (0%)	0 (0%)
12e	18 (15%)	0 (0%)	0 (0%)
13	0 (0%)	0 (0%)	0 (0%)
14a–e	0 (0%)	0 (0%)	0 (0%)
15a–c	0 (0%)	0 (0%)	0 (0%)
16	0 (0%)	0 (0%)	0 (0%)
17	0 (0%)	0 (0%)	0 (0%)

[a]The initial rate of NO synthesis was determined at 37 °C using spectrophotometric oxyhemoglobin assay for NO. The concentration of substrate was 0.5 mM. The rates were also expressed as a percentage of those found for NOHA.

were found not to be substrates. N^{ω}-methyl-L-arginine is even a widely used NOS inhibitor.

The very limited number of substrates for NOSs suggests that highly specific structural features are required for NO generation from NOSs. Furthermore, because most substrates of NOSs are arginine or NOHA derivatives, it also suggests that the α-amino acid portions of those substrates play an important role in the catalysis.[16–18] However, since some simple guanidines and isothioureas such as aminoguanidine, *S*-ethyl-isothiourea, and *N*-phenyl-*S*-methyl-isothiourea are strong NOS inhibitors through binding at the active site,[19–23] it appears that the α-amino acid moiety of arginine can be removed without detrimental consequences, while the integrity of the guanidine function must be partially retained. It is logical then to assume that simple compounds bearing guanidine or hydroxyguanidine functions, not derived from L-Arg or NOHA, can interact with NOS active site and possibly serve as substrates of NOS.

In an effort to better understand the structural factors that are important for the substrates of NOS and to find a new type of enzymatic NO donors, we have synthesized a series of compounds bearing an *N*-hydroxyguanidine functional group and studied their oxidation by recombinant NOSs. Herein we report that several *N*-alkyl-*N*′-hydroxyguanidines can be efficiently oxidized by NOS to generate NO. Most interestingly, some *N*-aryl-*N*′-hydroxyguanidines have been found to be selective substrates for iNOS.

Results and Discussion

Starting from the corresponding amines, 22 *N*-substituted hydroxyguanidine derivatives (**1**–**14**) (Scheme 1) were synthesized using a general procedure previously reported[24] with some modification (Scheme 2) (see the experimental section for detail). These compounds were fully characteristized by ^1H, ^{13}C NMR, and high-resolution mass spectroscopy. Activity of these *N*-hydroxyguanidine compounds as substrates of NOS was evaluated by hemoglobin assay.[25] For our evaluation, NOHA was used as a control indicating 100% activity. The results were summarized in Table 1.

As expected, most of the synthetic hydroxyguanidine derivatives were not as active as L-Arg or NOHA. Without the α-amino acid moiety that allowed L-Arg or NOHA to be specifically recognized by NOS, these synthetic compounds may fail to interact most favorably with the corresponding binding site of NOS.[26,27] However, the results listed in Table 1 indicated that the activity of these compounds was related to the structure of substituents. Without any substituents, *N*-hydroxyguanidine (NHG) itself was not a substrate.[23] When a small alkyl group (such as ethyl, **1**) was attached to hydroxyguanidine, only low reactivity was observed. Surprisingly, one additional methylene group, *n*-propyl substitution in **2**, significantly enhanced the activity, especially for nNOS (up to 70% of NOHA). As the size of the alkyl chain increased to *iso*propyl or *n*-butyl group, **3** and **4** turned out to be good substrates. The K_m values of **3** was 77 μM for iNOS and $K_m = 56$ μM for nNOS, whereas the K_m values of **4** was 33 μM for iNOS and 67 μM for nNOS. However, if the alkyl substituents became too long or too bulky (such as *n*-hexyl in **10** and cyclohexyl in **11**), the activity decreased significantly. It was interesting to note that *n*-propyl in **2** permitted very good NOS activity but one more methyl group at its terminal carbon (**6**) significantly diminished its reactivity. A similar effect could also be found for *n*-butyl substituent (between **4** and **9**). Compared to its isopropyl counterpart (**3**), *N*-*tert*-butyl-*N*′-hydroxyguanidine (**7**) was not the substrate for all three NOSs.

The above results were consistent with a recent structure study,[28] which revealed the importance of nonpolar van der Waals interactions between inhibitor and NOS. As shown in crystal structures of NOS oxygenase domains,[27–32] NOHA is anchored at the catalytic site through H-bonds between *N*-hydroxyguanidino moiety and the conserved amino acid residues (Glu and Trp). A small hydrophobic cavity formed by three conserved amino acid residues (Pro, Phe, and Val) is located in close proximity to the catalytic site and is connected to the open substrate access channel. Specifically, according to the crystal structure study of substrate-bound NOS by Poulos' laboratory (University of California, Irvine, USA), *N*-alkyl-*N*′-hydroxyguanidine differs from NOHA in the orientation of *N*-substituent group (the results will be published elsewhere). Although the absence of H-bonding components in the *N*-substituent (e.g., amino and carboxylate groups in NOHA) leads to a distinct orientation of the *N*-hydroxyl group, yet substrates like **4** still retain fairly good NOS reactivity. This is because the hydroxyguanidino moiety is still anchored by multiple H-bonds and is orientated in a manner similar to NOHA at NOS active site, allowing efficient oxidation of hydroxyguanidino moiety by superoxoiron(III) heme intermediate.[19] Such orientation also allows an *N*-alkyl group of suitable size to be accommodated in the hydrophobic cavity via van der Waals interaction, including both the *n*-butyl chain in **4** and the *i*-propyl group in **3**, whereas such interaction would diminish for shorter alkyl chains (for NHG and **1**). On the other hand, the limited space in this cavity would hardly allow either long (in **10**), bulky (in **11**), or terminally branched alkyl chains (in **9**). The poor reactivity of **7** can be related to the bulky *t*-butyl group near the guanidino functionality. Steric hindrance between the *t*-butyl group and heme ring would disfavor the oxidation reaction by disrupting the orientation of guanidino group in the heme active site. This result indicates that the interaction between hydrophobic chains on substrates and the hydrophobic cavity in the enzyme active site could play a crucial role in determining the affinity of substrates. However, for *N*-hydroxyguanidines bearing an α-amino acid function, their binding site should be different from *N*-alkyl hydroxyguanidines. For example, NOHA and homo-NOHA bind well with NOS and serve as good substrates; whereas nor-NOHA, which is one methylene group shorter than NOHA, is not a NOS substrate. Moali et al. suggested that the strong binding between α-amino acid group and NOS makes it too short to position the *N*-hydroxyguanidine function in nor-NOHA close to the reaction center.[18] Overall, our experiments clearly demonstrated that, in the absence of the α-amino acid moiety, a favorable interaction between the hydrophobic patch of NOS and a suitable alkyl group of substrates contributed significantly to the binding.

Since *N*-isopropyl-*N*′-hydroxyguanidine (**3**) and *N*-*n*-butyl-*N*′-hydroxyguanidine (**4**) are excellent substrates for NOS, their corresponding derivatives, isopropylguanidine (**16**) and butylguanidine (**17**) have also been tested in our experiment. Unfortunately, these two compounds can not generate NO by NOSs even at high concentration (10 mM). This suggests that the two steps in the NO biosynthesis are distinct processes. The first oxidation step seems to require an exact 'natural' substrate such as L-arginine. Such substrate specificity can not be compromised by replacing L-arginine with simple *N*-substituted guanidine compounds. In contrast, the second oxidation step of NOS reaction is much more compromising. Some *N*-substituted hydroxyguanidines can be oxidized to produce NO in this step.

Next a series of *N*-aryl-*N*′-hydroxyguanidines (**12a**–**e**) were investigated. Most interestingly, it was found that these compounds were selective substrates for iNOS. Previously **12d** was shown to be a substrate for iNOS by Mansuy and co-workers.[12] In our study, *N*-phenyl-*N*′-hydroxyguanidine (**12a**) was the best substrate in this series ($K_m = 243$ μM). The rate of NO formation, when using 500 μM **12a**, was 39 nmol/min/mg protein, as high as 70% of L-Arg and 32% of NOHA. The NO formation rates of other substrates (**12b**–**e**) were in the range of 16–35 nmol/min/mg protein. When used in the assay for nNOS and eNOS, none of them produced detectable NO even at high concentrations (10 mM). This is in sharp contrast to that of their aliphatic counterparts (**3**–**10**), which exhibited good activity but hardly any isoform selectivity. These *N*-aryl-*N*′-hydroxyguanidine compounds are the first reported isoform selective NOS substrates so far. Given the high active-site conservation and high structural similarity among NOS isoforms, this observation deserves further mechanistic consideration.

Further study on the effects of incubation conditions on the formation of nitrite from these substrates indicated

that the characteristics of this reaction were very similar to those of the NOS-dependent oxidation of the endogenous NOHA. The reaction required the presence of both the active enzyme and other cofactors, and could be strongly inhibited by classical NOS inhibitors such as *N*-nitro-L-arginine (NNA) and *N*-methyl-L-arginine (NMA) (data not shown). It indicated that the oxidation of these substrates should occur in the active site of NOS and the mechanism was similar to NOHA.

Since **12a**–**e** were not substrates of nNOS and eNOS, we further examined them as inhibitors of these two enzymes. As shown in Table 2, **12a** and **12d** displayed the most efficient concentration-dependent inhibition of NOS-mediated nitrite formation in the presence of L-Arg (0.1 mM). Other compounds partially inhibited nitrite formation only at higher concentrations. The weak inhibition of these compounds suggested that their binding affinity to nNOS and eNOS was relatively weak compared with L-Arg.

To further explore the structural effect on the activity, some structure-related compounds (**13**–**15**) were prepared and assayed. When the phenyl ring was replaced by a naphthalene ring (**13**) or by a benzyl group (**14**), the activity of substrate was destroyed completely. This increase in size might over-grow the 'specific' phenyl-binding pocket in iNOS. As for *N*-alkyl substituted compounds, when the hydroxyguanidine group was changed to a guanidine group (**15**), none of them acted as a substrate of NOSs.

In summary, an extensive structural screening of *N*-hydroxyguanidine compounds revealed that relatively simple exogenous compounds, not bearing an amino acid function, could be oxidized by NOS in a manner similar to NOHA, with a significant rate of NO formation. The structure–activity relationship showed that a potent NOS substrate shared at least two characteristics: (1) an *N*-hydroxyguanidine functional group, capable of anchoring the substrate in the NOS active site and furnishing the second step of the NOS reaction; (2) a suitable hydrophobic chain that interacts favorably with the hydrophobic cavity in NOS active site. Currently, L-arginine supplementation has been studied in a variety of clinical situation where the increase of NO production is desired.[33,34] For example, L-arginine coated endovascular stents have been tested in controlling restoration after balloon angioplasty.[35] Findings in the present study indicated simple *N*-substituted hydroxyguanidine might also be used as NO donor supplementation. Moreover, to our best knowledge, *N*-aryl-*N*′-hydroxyguanidines (**12a**–**e**) are the first series of isoform selective substrates for NOS. This finding is significant not only for understanding the NOS mechanism, but also in using such compound as isoform-specific probe in biomedical experiments.

Table 2. IC_{50} (μM) of compounds **12a**–**e** to nNOS and eNOS

Compd	nNOS	eNOS
12a	900	1500
12b	3500	> 10,000
12c	4500	> 10,000
12d	1500	2000
12e	2600	5600

Experimental

Chemistry

General. All reagents were used as purchased from commercial suppliers without further processing. The NMR data were recorded on a Mercury-400 or -500 MHz spectrometer. MS spectra were obtained from a Kratos MS 80 spectrometer using electrospray ionization mode (ESI) or electronic ionization (EI). Silica gel F254 plates (Merck) and Silica Gel 60 (70–230 mesh) were used in analytical thin-layer chromatography (TLC) and column chromatography, respectively.

General procedure for synthesis of *N*-hydroxyguanidines

Synthesis of corresponding cyanamide. A solution of corresponding amine (10 mmol) and anhydrous Et_3N (1.512 g, 15.1 mmol) in dry CH_2Cl_2 (30 mL) was cooled to 0 °C under argon atmosphere. To the solution was added BrCN (3.5 mL 3.0 M in CH_2Cl_2, 10.5 mmol) dropwise. After stirring 10 h at rt, the mixture was filtrated through a short silicon gel column. The filtrate was concentrated. The crude product was purified by flash chromatography in 55–85% yield.

***N*-Substituted-*N*′-hydroxyguanidines.** A mixture of cyanamide (0.6 mmol), hydroxyamine hydrochloride (43 mg, 0.61 mmol) and anhydrous K_2CO_3 (173 mg, 1.21 mmol) in anhydrous EtOH (5 mL) was stirred at rt for 5 h. Then the mixture was filtrated through Celite. After removing the solvent by rota-vap, the crude product was purified with flash chromatography to give *N*-hydroxyguanidine (40–78%).

***N*-Ethyl-*N*′-hydroxyguanidine 1.** ^{1}H NMR (500 MHz, CD_3OD) δ 3.27 (q, J = 7.0 Hz, 2H), 1.21 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.0, 36.2, 13.4. HRMS calcd for $C_3H_9N_3O$ 103.0746, found 103.0741.

***N*-*n*-Propyl-*N*′-hydroxyguanidine 2.** ^{1}H NMR (400 MHz, CD_3OD) δ 3.16 (t, J = 7.6 Hz, 2H), 1.57–1.65 (m, 2H), 0.97 (t, J = 7.6 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.6, 42.7, 22.0, 10.1. HRMS calcd for $C_4H_{11}N_3O$ 117.0902, found 117.0899.

***N*-*i*-Propyl-*N*′-hydroxyguanidine 3.** ^{1}H NMR (400 MHz, CD_3OD) δ 3.70–3.78 (m, 1H), 1.23 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.4, 43.9, 21.4. HRMS calcd for $C_4H_{11}N_3O$ 117.0902, found 117.0901.

***N*-*n*-Butyl-*N*′-hydroxyguanidine 4.** ^{1}H NMR (500 MHz, CD_3OD) δ 3.00 (t, J = 7.0 Hz, 2H), 1.45–1.53 (m, 2H), 1.34–1.38 (m, 2H), 0.993 (t, J = 7.5 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.9, 40.7, 31.7, 19.9, 13.0. HRMS calcd for $C_5H_{13}N_3O$ 131.1059, found 131.1060.

***N*-s-Butyl-*N*′-hydroxyguanidine 5.** ^{1}H NMR (400 MHz, CD_3OD) δ 3.52 (m, 1H), 1.56 (m, 2H), 1.20 (d, $J=6.4$ Hz, 2H), 0.95 (t, $J=7.2$ Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.95, 50.80, 30.40, 20.53, 10.67. HRMS calcd for $C_5H_{13}N_3O$ 131.1059, found 131.1059.

***N*-*iso*-Butyl-*N*′-hydroxyguanidine 6.** ^{1}H NMR (500 MHz, CD_3OD) δ 2.86 (t, $J=6.0$ Hz, 2H), 1.79 (m, 1H), 0.94 (d, $J=6.9$ Hz, 6H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.20, 49.74, 29.51, 20.48. HRMS calcd for $C_5H_{13}N_3O$ 131.1059, found 131.1060.

***N*-*t*-Butyl-*N*′-hydroxyguanidine 7.** ^{1}H NMR (500 MHz, CD_3OD) δ 1.40 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.35, 53.27, 29.17. HRMS calcd for $C_5H_{13}N_3O$ 131.1059, found 131.1062.

***N*-*n*-Pentyl-*N*′-hydroxyguanidine 8.** ^{1}H NMR (500 MHz, CD_3OD) δ 3.17 (t, $J=7.0$ Hz, 2H), 1.56–1.61 (m, 2H), 1.32–1.39 (m, 4H), 0.931 (t, $J=6.5$ Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.3, 41.1, 28.7, 28.5, 22.2, 13.1. HRMS calcd for $C_6H_{15}N_3O$ 145.1215, found 145.1217.

***N*-Isoamyl-*N*′-hydroxyguanidine 9.** ^{1}H NMR (500 MHz, CD_3OD) δ 3.02 (q, $J=7.5$ Hz, 2H), 1.65 (m, 1H), 1.40 (q, $J=7.0$ Hz, 2H), 0.92 (d, $J=6.5$ Hz, 6H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.10, 40.44, 39.65, 26.89, 22.88. HRMS calcd for $C_6H_{15}N_3O$ 145.1215, found 145.1216.

***N*-Hexyl-*N*′-hydroxyguanidine 10.** ^{1}H NMR (400 MHz, CD_3OD) δ 3.01 (t, $J=7.6$ Hz, 2H), 1.50–1.54 (m, 2H), 1.30–1.37 (m, 6H), 0.91 (t, $J=6.4$ Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.2, 42.2, 32.8, 30.6, 27.7, 23.7, 14.4. HRMS calcd for $C_7H_{17}N_3O$ 159.1372, found 159.1367.

***N*-Cyclohexyl-*N*′-hydroxyguanidine 11.** ^{1}H NMR (400 MHz, CD_3OD) δ 3.34–3.40 (m, 1H), 1.63–1.92 (m, 5H), 1.16–1.40 (m, 5H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.2, 50.8, 32.4, 25.0, 24.6. HRMS calcd for $C_7H_{15}N_3O$ 157.1215, found 157.1209.

***N*-Phenyl-*N*′-hydroxyguanidine 12a.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.30c7.20 (m, 4H), 7.03–6.98 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 155.4, 139.4, 129.0, 122.9, 120.4. HRMS calcd for $C_7H_9N_3O$ 151.0746, found 151.0747.

***N*-(4-Methoxyl)-phenyl-*N*′-hydroxyguanidine 12b.** ^{1}H NMR (500 MHz, CD_3OD) δ 7.12 (d, $J=9.0$ Hz, 2H), 6.80 (d, $J=9.0$ Hz, 2H), 3.73 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.5, 158.6, 127.5, 126.8, 115.0, 55.0. HRMS calcd for $C_8H_{11}N_3O_2$ 181.0851, found 181.0851.

***N*-(4-Methyl)-phenyl-*N*′-hydroxyguanidine 12c.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.09 (d, $J=8.0$ Hz, 2H), 7.02 (d, $J=8.0$ Hz, 2H), 2.24 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 154.6, 138.5, 131.2, 129.2, 119.3, 19.5. HRMS calcd for $C_8H_{11}N_3O$ 165.0902, found 165.0903.

***N*-(4-Chloro)-phenyl-*N*′-hydroxyguanidine 12d.** ^{1}H NMR (500 MHz, CD_3OD) δ 7.19 (d, $J=8.5$ Hz, 2H), 7.14 (d, $J=8.5$ Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 153.7, 140.4, 128.4, 125.3, 119.4. HRMS calcd for $C_7H_8ClN_3O$ 185.0356, found 185.0355.

***N*-(4-Bromo)-phenyl-*N*′-hydroxyguanidine 12e.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.28 (d, $J=9.0$ Hz, 2H), 7.14 (d, $J=9.0$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 153.5, 141.0, 131.4, 119.6, 112.3; HRMS calcd for $C_7H_8BrN_3O$ 228.9851, found 228.9854.

***N*-2-Naphthyl-*N*′-hydroxyguanidine 13.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.64–7.78 (m, 4H), 7.26–7.42 (m, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 153.3, 144.4, 134.6, 130.5, 128.6, 127.4, 126.8, 126.2, 123.9, 120.4, 117.0, HRMS calcd for $C_{11}H_{11}N_3O$ 201.0902, found 201.0899.

***N*-Benzyl-*N*′-hydroxyguanidine 14a.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.38–7.24 (m, 5H), 4.45 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.2, 136.6, 128.7, 127.8, 127.1, 44.4; HRMS calcd for $C_8H_{11}N_3O$ 165.0902, found 165.0908.

***N*-(4-Methoxyl)-benzyl-*N*′-hydroxyguanidine 14b.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.25 (d, $J=9.0$ Hz, 2H), 6.92 (d, $J=9.0$ Hz, 2H), 4.35 (s, 2H), 3.78 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.8, 159.2, 128.5, 128.3, 114.0, 54.3, 44.0. HRMS calcd for $C_9H_{13}O_2N_3$ 195.1008, found 195.1010.

***N*-(4-Methyl)-benzyl-*N*′-hydroxyguanidine 14c.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.19 (d, $J=8.0$ Hz, 2H), 7.11 (d, $J=8.0$ Hz, 2H), 4.13 (s, 2H), 2.29 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.8, 136.6, 136.4, 128.9, 127.3, 44.7, 20.0; EIMS 180.2 ($M^+ + 1$). HRMS calcd for $C_9H_{13}N_3O$ ($M^+ - O$) 163.1100, found 163.1107.

***N*-(4-Chloro)-benzyl-*N*′-hydroxyguanidine 14d.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.37(d, 2H, $J=8$ Hz), 7.33 (d, 2H, $J=8$ Hz), 4.93 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 154.5, 138.6, 128.8, 128.3, 118.6, 44.0; HRMS calcd for $C_8H_{10}ON_3Cl$ 199.0512, found 199.0513.

***N*-(4-Nitro)-benzyl-*N*′-hydroxyguanidine 14e.** ^{1}H NMR (400 MHz, CD_3OD) δ 8.20 (d, $J=9.0$ Hz, 2H), 7.59 (d, $J=9.0$ Hz, 2H), 4.99 (s, 2H); δ 8.20 (d, $J=9.0$ Hz, 2H), 7.59 (d, $J=9.0$ Hz, 2H), 4.99 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.6, 147.4, 145.8, 127.9, 123.5, 43.8; EIMS 211.0 ($M^+ + 1$). HRMS calcd for $C_8H_9N_3O_3$ ($M^+ - NH$) 195.0644, found 195.0643.

***N*-Phenylguanidine 15a.** ^{1}H NMR (400 MHz, CD_3OD) δ 7.45–7.52 (m, 2H), 7.32–7.38 (m, 1H), 7.25–7.32 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 152.1, 134.9, 129.9, 127.6, 125.6. HRMS calcd for $C_7H_9N_3$ 135.0796, found 135.0801.

***N*-(4-Methoxyl)-phenylguanidine 15b.** ^{1}H NMR (500 MHz, CD_3OD) δ 7.21 (d, $J=9.0$ Hz, 2H), 7.01 (d, $J=9.0$ Hz, 2H), 3.83 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.8, 157.6, 127.8, 127.0, 115.0, 55.1. HRMS calcd for $C_8H_{11}N_3O$ 165.0902, found 165.0904.

***N*-(4-Methyl)-phenylguanidine 15c.** ^{1}H NMR (500 MHz, CD_3OD) δ 7.23 (d, $J = 8.0$ Hz, 2H), 7.16 (d, $J = 8.0$ Hz, 2H), 2.34 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 156.9, 137.9, 132.0, 130.5, 125.6, 19.9. HRMS calcd for $C_8H_{11}N_3$ 149.0953, found 149.0951.

Isopropylguanidine (**16**) and butylguanidine (**17**) were prepared as previous report.[36,37]

Biochemistry

General. Dithiothreitol, NADPH, FAD, FMN, L-arginine, N^G-hydroxy-L-arginine (NOHA), hydroxyguanidine (NHG), manganese superoxide dismutase (SOD), *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES), and (6*R*)-5,6,7,8-tetrahydrobiopterin were purchased from Sigma. UV–vis spectra were recorded on a HP 8453 spectrophotometer.

Production and purification of human eNOS

Culture of *Spodoptera frugiperda* (Sf21) cells and manipulations of the baculovirus were performed according to the procedures described by Piwnica-Worms.[38] Sf21 cells were maintained in Grace's medium containing 3.3 mg/mL yeastolate, 3.3 mg/mL lactoalbumin hydrolysate, and 50 mg/mL gentamycin supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL) at 27 °C.

For the overproduction of human eNOS by the baculovirus/insect cells system, human eNOS cDNA cloned previously[39] was ligated into a baculovirus transfer vector pVL1392 (Invitrogen) at EcoRI site and cotransfected with Baculogold (Pharmingen) into Sf21 cells using lipofectin (GIBCO/BRL). 2×10^8 Sf21 cells were infected with baculovirus carrying human eNOS cDNA and were incubated for 3 days at 27 °C. Purification of the eNOS proteins which were overproduced in Sf21 cells was carried out essentially as described previously[40] with some modifications. Briefly, the Sf21 cells were homogenated in buffer A (50 mM Tris–HCl, 5 mM CHAPS, 1 mM DTT, 0.1 mM EDTA, 10% glycerol and protein inhibitor complex (Complete EDTA free, Roche). The homogenate was slowly stirred for 30 min at 4 °C and then centrifuged at 15,000*g* for 30 min. 4 M NaCl was added into the supernatant to a concentration of 0.1 M, and mixed with 2 mL of 2′,5′-ADP-sepharose. The slurry was stirred slowly for 1 h at 4 °C and subsequently poured into a column. The column was washed with buffer A, and buffer A containing 0.5 M NaCl, and then with buffer A alone. The human eNOS was eluted with buffer A containing 2 mM NADPH. The elute was concentrated using a Centricon YM30 microconcentrator (Amicon). The purity of the eNOS was assessed with SDS-PAGE.

Purification of iNOS. Supernatant of recombinant murine iNOS was purchased from Cayman Co. Ann Arbor, MI, USA. The purification of iNOS was performed by the same method as eNOS described above.

Purified nNOS (rat recombinant) was purchased from Cayman Co., Ann Arbor, MI, USA. It was used directly in the experiment without further purification.

Assay of the nitric oxide synthase catalyzed NO generation from *N*-hydroxyguanidines. The initial rate of NO synthesis was determined at 37 °C using the classical spectrophotometric oxyhemoglobin assay for NO. Briefly, 20–30 μL aliquots containing NOS, 5 μM BH_4, and 2–5 mM DTT were added to a prewarmed cuvette that contained 50 mM HEPES (pH 7.4), supplemented with 15 μM oxyhemoglobin, 100 units/mL SOD, 100 units/mL catalase, 200 μM NADPH, 4 μM FAD, 4 μM FMN, 5 μM BH_4, and 0.5 mM substrate at the desired concentration, to give a final volume of 0.9 mL. In the case of nNOS and eNOS, 1 mM $CaCl_2$ and 10 μg/mL CaM were present. For iNOS, 1 mM magnesium acetate was added. The reference cuvette had the same composition except that 50 mM HEPES, 5 μM BH_4, and 2–5 mM DTT were added instead of NOS-containing solutions. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an increase in absorbance at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1}.

Acknowledgements

This work is supported by National Institute of Health Grant GM54074 (P.G.W). We thank Dr. H. Li and Professor T. L. Poulos (University of California at Irvine) for their valuable discussions. P.G.W. also thanks a summer support from Osaka University which made the collaboration possible.

References and Notes

1. Balligand, J. L. In *Nitric Oxide*, *Handbook of Experimental Pharmacology*; Mayer, B., Ed.; Springer: Heidelberg, 2000; Vol. 143, p 1.
2. Kerwin, J. F.; Lancaster, J. R., Jr.; Feldman, P. L., Jr. *J. Med. Chem.* **1995**, *38*, 4243.
3. Fukuto, J. M.; Mayer, B. In *Methods in Nitric Oxide Research*; Feellisch, M., Stamler, J. S., Eds.; John Wiley & Sons: Chichester, England, 1996; p 147.
4. Andrew, P. J.; Mayer, B. *Cardiovas. Res.* **1999**, *43*, 521.
5. Groves, J. T.; Wang, C. C. Y. *Curr. Opin. Chem. Biol.* **2000**, *4*, 687.
6. Stuehr, D. J. *Biochim. Biophys. Acta* **1999**, *1411*, 217.
7. Marletta, M. A. *J. Med. Chem.* **1994**, *37*, 1899.
8. Chabrier, P. E.; Demerle-Pallardy, C.; Auguet, M. *Cell Mol. Life Sci.* **1999**, *55*, 1029.
9. Mansuy, D.; Boucher, J. L.; Clement, B. *Biochimie* **1995**, *77*, 661.
10. Jousserandot, A.; Boucher, J. L.; Henry, Y.; Niklaus, B.; Clement, B.; Mansuy, D. *Biochemistry* **1998**, *37*, 17179.
11. Xian, M.; Li, X.; Tang, X.; Chen, X.; Zheng, Z.; Galligan, J. J.; Kreulen, D. L.; Wang, P. G. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2377.
12. Renodon-Corniere, A.; Boucher, J. L.; Dijols, S.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **1999**, *38*, 4663.
13. Moali, C.; Boucher, J.-L.; Renodon-Corniere, A.; Stuehr, D. J.; Mansuy, D. *Chem. Res. Toxicol.* **2001**, *14*, 202.
14. Lee, Y.; Marletta, M. A.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *Bioorg. Med. Chem.* **1999**, *7*, 1097.
15. Dijols, S.; Perollier, C.; Lefevre-Groboillot, D.; Pethe, S.; Attias, R.; Boucher, J. L.; Stuehr, D. J.; Mansuy, D. *J. Med. Chem.* **2001**, *44*, 3199.

16. Grant, S. K.; Green, B. G.; Stiffey-Wilusz, J.; Durette, P. L.; Shah, S. K.; Kozarich, J. W. *Biochemistry* **1998**, *37*, 4174.
17. Moali, C.; Brollo, M.; Custot, J.; Sari, M. A.; Boucher, J. L.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **2000**, *39*, 8208.
18. Moali, C.; Boucher, J. L.; Sari, M. A.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **1998**, *37*, 10453.
19. Babu, B. R.; Griffith, O. W. *Curr. Opinion Chem. Biol.* **1998**, *2*, 491.
20. Shearer, B. G.; Lee, S.; Oplinger, J. A.; Frick, L. W.; Garvey, E. P.; Furfine, E. S. *J. Med. Chem.* **1997**, *40*, 1901.
21. Garvey, E. P.; Oplinger, J. A.; Tanoury, G. J.; Sherman, P. A.; Fowler, M.; Marshall, S.; Harmon, M. F.; Paith, J. E.; Furfine, E. S. *J. Biol. Chem.* **1994**, *269*, 26669.
22. Bryk, R.; Wolff, D. J. *Biochemistry* **1998**, *37*, 4844.
23. Sennequier, N.; Stuehr, D. J. *Biochemistry* **1996**, *35*, 5883.
24. Schantl, J. G.; Turk, W. *Sci. Pharm.* **1989**, *57*, 375.
25. Murphy, M. E.; Noack, E. *Methods Enzymol.* **1994**, *233*, 250.
26. Stuehr, D. J. *Annu. Rev. Pharmacol. Toxicol.* **1997**, *37*, 339.
27. Crane, B. R.; Arvai, A. S.; Ghosh, D. K.; Wu, C.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. *Science* **1998**, *279*, 2121.
28. Li, H.; Raman, C. S.; Martasek, P.; Kral, V.; Masters, B. S. S.; Poulos, T. L. *J. Inorg. Biochem.* **2000**, *81*, 133.
29. Crane, B. R.; Arvai, A. S.; Gachhui, R.; Wu, C. Q.; Ghosh, D. K.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. *Science* **1997**, *278*, 425.
30. Fischmann, T. O.; Hruza, A.; Niu, X. D.; Fossetta, J. D.; Lunn, C. A.; Dolphin, E.; Prongay, A. J.; Reichert, P. R.; Lundell, D. J.; Narula, S. K.; Weber, P. C. *Nat. Struct. Biol.* **1999**, *6*, 223.
31. Raman, C. S.; Li, H.; Martasek, P.; Kral, V.; Masters, B. S. S.; Poulos, T. L. *Cell* **1998**, *95*, 939.
32. Li, H. Y.; Raman, C. S.; Glaser, C. B.; Blasko, E.; Young, T. A.; Parkinson, J. F.; Whitlow, M.; Poulos, T. L. *J. Biol. Chem.* **1999**, *274*, 21276.
33. Sanders, D. B.; Kelley, T.; Larson, D. *Perfusion-UK* **2000**, *15*, 97.
34. Lonsdorfer, J.; Schaefer, A.; Mettauer, B.; Lampert, E.; Lonsdorfer-Wolf, E.; Uhlrich, J. *Semin. Hop. Paris* **1999**, *75*, 1255.
35. Janero, D. R.; Ewing, J. F. *Free Rad. Biol. Med.* **2000**, *29*, 1199.
36. Crowther, B. *J. Chem. Soc.* **1948**, 1641.
37. King, T. *J. Chem. Soc.* **1946**, 1065.
38. Piwnica-Worms, H. In *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds.; Greene/Wiley Interscience: New York, 1987; p 16.8.1.
39. Seo, H.-G.; Fujii, J.; Soejima, H.; Niikawa, N.; Taniguchi, N. *Biochem. Biophys. Res. Commun.* **1995**, *208*, 10.
40. Seo, H.-G.; Tatsumi, H.; Fujii, J.; Nishikawa, A.; Suzuki, K.; Kangawa, K.; Taniguchi, N. *J. Biochem.* **1994**, *115*, 602.