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Electrochemical and Peroxidase Oxidation Study of *N*′-Hydroxyguanidine Derivatives as NO Donors

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Abstract—The electrochemical properties of a series of *N*-substituted-*N*′-hydroxyguanidines were studied. Two oxidation potentials of each compound were obtained by cyclic voltammetry. The E_{ox1} values were from 0.51 to 0.62 V, while the E_{ox2} values were from 1.14 to 1.81 V in acetonitrile solution. Next, their enzymatic controlled NO release abilities were evaluated. All *N*′-hydroxyguanidines exhibited efficient NO release abilities under the oxidation by horseradish peroxidase in the presence of H_2O_2.

Nitric oxide (NO), a messenger molecule, plays an important role in numerous physiological and pathophysiological processes, such as neuronal communication, blood vessel modulation, and immune response.[1,2] Endogenous NO is produced almost exclusively by L-arginine catabolism to L-citrulline in a reaction catalyzed by a family of three NO synthases (NOSs) (Scheme 1).[3,4] The three isoforms of NOSs share structural similarities and have nearly identical catalytic mechanisms.[5–9] Each of the isoforms catalyzes the stepwise formation of NO and L-citrulline from L-arginine, O_2 and NADPH-derived electrons. During the 5 electron oxidation process, *N*-hydroxy-L-arginine (NHA) serves as a key intermediate.

The guanidine fragment is distinct for the NO biosynthesis. It is the guanidine group that undergoes oxidation to the *N*-hydroxyguanidine and further to urea and NO by NOS. The binding of the substrates with the heme of NOSs is largely due to the guanidine or hydroxyguanidine groups. So it is rational to expect that other *N*′-hydroxyguanidine derivatives can be used as enzymatic NO donors. Up to now, several hydroxyguanidines have been demonstrated to be substrates of NOS, such as homo-NHA (**1**),[10,11] *N*-butyl-*N*′-hydroxyguanidine (**2**),[12] and *N*-(*para*-hydroxyphenyl) *N*′-hydroxyguanidine (**3**).[13] But they are all poorer substrates than NHA. Moreover, some substituted hydroxy guanidines,[14,15] such as *N*-hydroxy-*N*′-aminoguanidine derivative,[16] have been shown to exhibit cytotoxicity and antitumor activity. Even unsubstituted *N*-hydroxyguanidine has anticancer activity.[17] NO formation from these compounds was suggested to be the mechanistic basis for the cytotoxic effects (Fig. 1).[18]

Since the mechanism of NO formation in vivo was disclosed, scientists have found that a series of heme proteins could catalyze the oxidation of physiological and non-physiological hydroxyguanidine-containing compounds to release NO.[19] For example, *N*-hydroxydebrisoquine could be oxidized by liver microsomes to release NO. Recently, we reported *N*-hydroxyl derivatives of guanidine based cardiovascular drugs shown good NO-releasing ability under the oxidation by horseradish peroxidase (HRP) in the presence of H_2O_2.[20] Studies indicated that non-physiological guanidines and *N*-hydroxyguanidines could be regarded as nitric oxide donors. These results may explain the cardiovascular activity of some therapeutic reagents.[21] It has been shown that *N*-alkyl-*N*′-hydroxyguanidines can release NO by oxidation with some chemical oxidants, such as $Pb(OAc)_4$ and K_3FeCN_6/H_2O_2.[22] To study this new type of enzymatic NO donors, we prepared a series of substituted *N*′-hydroxyguanidines. Their electrochemical properties and NO-release abilities by horseradish peroxidase were studied, so as to find some relationship between oxidation potentials and NO releasing ability.

Ten *N*-substituted-*N*′-hydroxyguanidines (**6a–j**)[23] were prepared according to the route in Scheme 2. The

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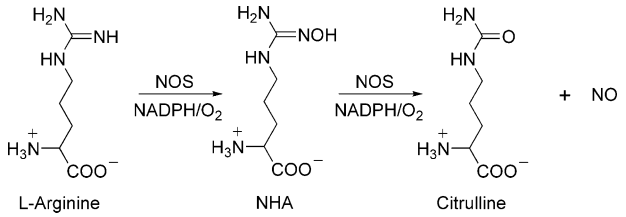


Scheme 1.

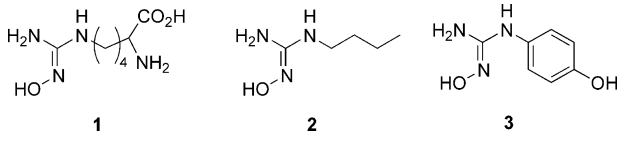
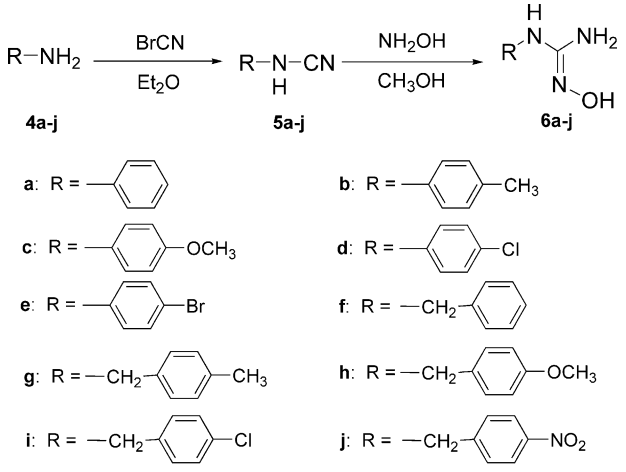


Figure 1. Unnatural NOS substrates.



Scheme 2.

cyanamide compounds (**5**) were obtained from the reaction of corresponding amines with cyanogen bromide.[24] Synthesis of phenyl substituted cyanamides (**5a–e**) was modified by adding triethylamine as a base to accelerate the reactions, because aromatic amines are relatively weak bases. Benzyl substituted cyanamides (**5f–j**) could be prepared quantitatively by reactions of two equivalent benzyl amines (**4f–j**) and one equivalent cyanogen bromide. Then they were successfully converted to the desired *N*-substituted-*N*′-hydroxyguanidines (**6a–j**) by treatment with hydroxyamine.[25] The free base forms of hydroxyguanidines are not stable, especially in basic solution, and decompose at room temperature. After purification of the final hydroxyguanidine products, we converted them to hydrochloride salts, which can be stored at room temperature for several days without decomposition.

The oxidation potentials of *N*-hydroxyguanidines were measured by cyclic voltammetry (Table 1).[26] A typical voltammogram obtained from compound **6a** is shown in Figure 2. At 100 mV/s, two irreversible oxidation peaks were observed at $E_{ox1} = +0.60$ V and $E_{ox2} = +1.61$ V. The oxidation peak of lower intensity at 1.10 V may be due to a decomposition product of the first oxidation. There was absolutely no sign of reversibility in either step. This indicates that the oxidation of

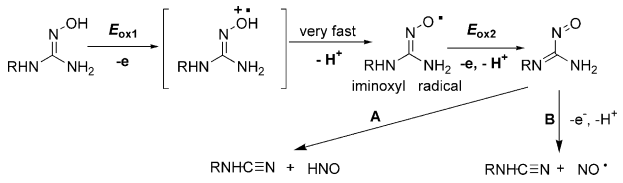
Table 1. The oxidation potentials of *N*-substituted-*N*′-hydroxyguanidines

Compd	E_{ox1} (V)	E_{ox2} (V)
6a	0.60	1.61
6b	0.57	1.48
6c	0.54	1.24
6d	0.61	1.72
6e	0.62	1.70
6f	0.65	1.44
6g	0.63	1.24
6h	Not obtained	1.27
6i	0.51	1.14
6j	0.60	1.45

N-hydroxyguanidines is a totally irreversible process. For phenyl substituted *N*-hydroxyguanidines (**6a–e**), the substituents on the phenyl ring may have an effect on the value of the peak potentials. Electron-donating groups lower the oxidation potentials, while electron-withdrawing groups have the opposite effect. But the differences are not pronounced. There is no obvious trend in oxidation potentials of benzyl *N*-hydroxyguanidines (**6f–j**), because the aromatic rings do not conjugate with the guanidine group. The oxidation mechanism of C=N-OH group of amidoximes has been proposed,[27] so *N*-hydroxyguanidines should have the same oxidation pattern (Scheme 3). It involves one electron oxidation with formation of an iminoxyl radical intermidiate (NHA$^{\bullet}$). The second step is the one electron oxidation of this radical leading to nitrosoimine. Elimination of HNO from the nitrosoimine would give the cyanamide (path A) whereas a further one-electron oxidation would generate the cyanamide and NO (path B). The appearance of two oxidation peaks supported the two-electron oxidation in agreement with previous report from Ingold,[28] that is, the removal of an electron from *N*-hydroxyguanidine at E_{ox1} is followed by a rapid, irreversible deprotonation. The iminoxyl radical formed is further oxidized at the higher potential and the resulting cation is again capable of loosing a proton irreversibly. The oxidation potentials of unnatural *N*-hydroxyguanidines are also comparable with *N*-hydroxyarginine ($E_{ox1} = +0.47$ V and $E_{ox2} = +1.05$ V).[28]

As shown in Scheme 1, the second step involved in the NOS-catalyzed oxidation of L-arginine is an oxidative cleavage of the C=NOH bond of *N*-hydroxyarginine with the formation of citrulline and NO. Besides NOS, cytochromes P450 have also been found to catalyze the oxidative cleavage of the C=NOH bond not only of *N*-hydroxyarginine but also of many other compounds such as ketoximes, amidoximes.[19,29]

Previous study showed that HRP catalyzed the oxidation of NHA by H_2O_2 with formation of citrulline and NO.[30] In this study, we found *N*-hydoxyguanidine derivatives, compounds **6a–j**, could be catalyzed by HRP with the formation of NO. HRP is one of the most studied peroxidases,[31] which has been shown to be an effective oxidzing enzyme for arylamidoximes by hydrogen peroxide. The products were *O*-(arylimidoyl) arylamidoximes and HNO/NO.[27,32] This peroxidase



Scheme 3.

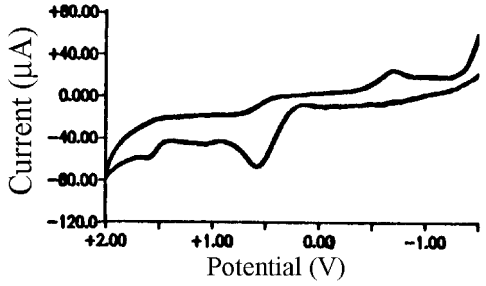


Figure 2. Cyclic voltammogram of **6a** in acetonitrite.

effects a one-electron oxidation of the substrate and is regenerated by transferring two electrons to a molecule of H_2O_2. It is also a model for in vivo systems, which can oxidize these types of compounds to generate NO. Compounds **6a**–**j** together with NHA and L-arginine were incubated with HRP and H_2O_2 separately. 20 min later, the concentration of NO_2^- generated in the solution was determined by Griess method.[33] The results are shown in Table 2.[34] The reaction product of **6c** was confirmed to be *p*-methoxyphenyl urea by comparison with standard sample.[30]

Incubation the L-arginine or other substituted guanidines, such as phenylguanidine, with peroxidase did not result in the generation of nitrite, while incubation of all other *N*-hydroxyguanidines with the enzyme produced different amount of nitrite. **6i** was the best NO donor among these tested compounds, and it produced 151 μM nitrite in 20 min. Except **6b**, all other *N*-hydroxyguanidines were more potent NO donors than NHA under peroxidase oxidation. In control experiments, incubation of these compounds with H_2O_2 in the absence of HRP did not produce any nitrite. The differences of NO generation may be due to the difference of oxidation potentials or different binding affinity to the enzyme. Examination of the results in Tables 1 and 2, it seems that there is no direct correlation between oxidation potentials and NO release. Thus, oxidation potentials may not have a crucial effect on NO releasing. It has been found that classical peroxidases oxidize the substrate by electron transfer to heme center and bind the substrate near the heme edge.[35,36] The binding affinity of the substrate to the active site is speculated to be the main factor affecting NO generation.

HRP, cytochromes P450 and NOSs are similar in the types of reactions that they catalyze.[37] They have similar heme binding sites. Like P450s, HRP's oxidation of *N*-hydroxyguanidine is a rather general reaction.[38] P450s process needs NADPH to form the high valent

Table 2. Nitrite concentration in the reaction of substrate with peroxidase for 20 min (substrate: 600 μM, peroxidase: 80 μg/mL, H_2O_2: 600 μM)

Substrate	$[NO_2^-]$ μM
L-Arg	0
NHA	12
6a	28
6b	6
6c	24
6d	63
6e	54
6f	33
6g	113
6h	49
6i	151
6j	65

iron *oxo* heme intermediate, which contains the ferryl group $[Fe=O]^{+3}$, responsible for the oxidation of substrates.[39] In our study, H_2O_2 could substitute NADPH to generate the iron *oxo* form directly from the resting state of the heme. The NO releasing mechanism of NHGs by oxidation of HRP and H_2O_2 may be similar to NOSs', in that they generate the similar products.

Our study suggested a possible new way of endogenous NO formation different from the pathway, which only involves NOSs and NHA. This new way of NO formation is the oxidation of exogenous NHGs to NO by some hemeproteins in cells not containing NOSs. Using this strategy, it is possible to form NO in sites where it is needed but lacking NOSs. NHGs would act as a relatively stable and transportable precursor of NO.

In summary, a series of *N*-substituted-*N'*-hydroxyguanidine derivatives was prepared and their oxidation potentials were measured. Furthermore, NO releasing from these compounds under the catalysis HRP was demonstrated. These data clearly show that *N*-hydroxyguanidine derivatives are potential NO donors under the catalysis of peroxidase. There was no clear relationship between the oxidation potentials and enzymatic NO releasing ability. Such enzymatic controlled NO generating compounds have potential for use in a variety of biomedical applications. The biological activity and toxicity studies are currently in progress to further evaluate this class of NO donating compounds.

Acknowledgements

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23. Spectral data of compound **6a**–**e** and **6h** were reported in ref 13. Compound **6f**: ^{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. Compound **6g**: ^{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 ($M^+ + 1$); HRMS calcd for $C_9H_{13}N_3O$ (M^+-O) 163.1110, found 163.1107. Compound **6i**: ^{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. Compound **6j**: ^{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); ^{13}C NMR (100 MHz, CD_3OD) δ 158.6, 147.4, 145.8, 127.9, 123.5, 43.8; EIMS 211 ($M^+ + 1$); HRMS calcd for $C_8H_9N_3O_3$ (M^+-NH) 195.0644, found 195.0643.
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34. The enzymatic reactions were carried out in PBS buffer (pH 7.4) with 1 mM EDTA. Incubated mixtures containing 0.6 mM substrate, 0.6 mM H_2O_2, and 80 μg/mL HRP in a 500 μL final volume were shaken at 25 °C for 20 min. The concentration of NO_2^- generated in the system was determined by addition of the Griess reagent. Absorbances were measured at 548 nm. Calibration curves were made from identical incubated mixtures without the enzyme and containing various concentration of $NaNO_2$ to properly determine the amounts of NO_2^- formed in the enzymatic reactions.
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