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A Novel Nonenzymatic Pathway for the Generation of Nitric Oxide by the Reaction of Hydrogen Peroxide and D- or L-Arginine

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Nitric oxide (NO) is a biologically active molecule known to be enzymatically synthesized from L-arginine in the presence of NO synthetase (NOS). In this study, we demonstrate a novel non-enzymatic pathway for NO synthesis involving hydrogen peroxide and D- or L-arginine. We employed two measures of NO generation. The first consists in the demonstration of the oxidative metabolites of NO (NO_2 + NO_3 = NO_X) and the second is the confirmatory finding of chemiluminescence derived from NO. The results show that NO_X increases in the incubation mixture containing hydrogen peroxide coupled with D-arginine, L-arginine, L-canavanine, and even the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). However, chemiluminescence was detected only from the reactions of hydrogen peroxide and D- or L-arginine and was diminished by the addition of carboxy-2-phenyl-4, 4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), a specific scavenger of NO, confirming NO generation in the reaction.

Nitric oxide (NO) is an important cell messenger which is enzymatically synthesized from the guanidino nitrogen of L-arginine (1). It is known for a variety of physiologic effects such as the regulation of vascular tone, neurotransmission and several host defense mechanisms (2-6). Additionally, there are a number of toxic effects as well, most of which derive from the generation of the hydroxyl radical via formation of its peroxidative product, peroxynitrite (7,8).

In the course of studies of guanidino compounds as uremic toxins, we discovered that reactive oxygen species (ROS) play an important role in the non-enzymatic synthesis of both methylguanidine (9) and guanidine (10). The synthesis of NO, however, is largely felt to be enzymatic requiring the enzyme nitric oxide synthetase (NOS) in its production. Moreover, it is reported that the urinary excretion of the oxidation products of NO (NO_2 + NO_3 = NO_X) actually increases during administration of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), in the anesthetized rat (11) and in the long term incubation of isolated glomeruli (12). In addition, it is reported that the increase in urinary excretion of NO_X in conscious rats is the same whether the substrate administered in D-or L-arginine (13). These findings raise a question whether NO is synthesized only by the action of NOS. In this study, we investigated the effect of hydrogen peroxide, a relatively stable ROS on NO synthesis, a non-enzymatic pathway, by detecting NO_X in the Griess reaction (14) and chemiluminescence (15) derived from NO.

METHODS

Chemicals. L-NAME, carboxy-2-phenyl-4, 4, 5, 5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO) and NOR-1 ($C_8H_{13}N_3O_5$) were purchased from the Wako Pure Chemical Co., Osaka, Japan. All other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO, USA. All the chemicals were of the best available quality.

Measurement of nitrite and nitrate in the reactions of hydrogen peroxide and various amino acids or guanidino compounds. The effect of hydrogen peroxide on the synthesis of NO from potential precursors of NO synthesis such as L-arginine, D-arginine, L-canavanine, L-NAME, L-glycine, L-citrulline, L-ornithine, guanidine, methylguanidine, guanidinosuccinic acid, guanidinoacetic acid, creatine, creatinine, agmatine or aminoguanidine in the long term incubation was examined. Nitrite and nitrate, the oxidative metabolites of NO, were measured by the Griess reaction (14).

The reaction mixture consisted of 1.0 ml of 20 mM potassium phosphate buffer (pH 7.4 at 37°C) containing 10 mM hydrogen peroxide and each compound at a concentration of 10 mM was incubated for 0, 1, 2, 3, 4, 5 days in a 10 ml glass test tube with shaking at 60 cycle/min and temperature was kept at 37°C in room air. To evaluate dose dependency in the case of L-arginine, the reaction mixture con-

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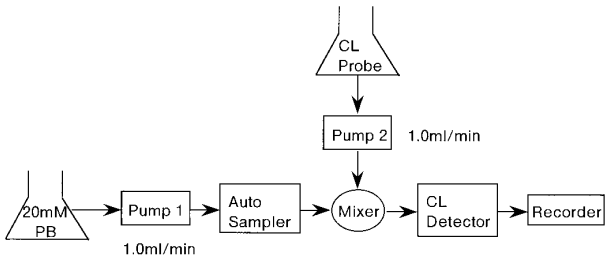

FIG. 1. Diagram of the device employed to measure NO generation. Real time measurement of NO was performed by the detection of chemiluminescence derived from peroxynitrite generated by the reaction of NO and hydrogen peroxide. Abbreviations: PB, potassium phosphate buffer; X, xanthine; XO, xanthine oxidase; CL, chemiluminescence.

taining 2, 5,10, 20 mM L-arginine and 0, 5, 10, 20, 30, 40, 50 mM hydrogen peroxide was incubated for 3 days.

The total amounts of NO produced in the reaction mixture were determined just after the termination of the incubation using an autoanalyzer (TCI-NOX 1000, Tokyo Kasei Kogyo, Tokyo, Japan), which employs the technique of automated flow injection analysis (14) essentially based on the reaction of nitrite and the Griess reagent. Nitrate was determined by reducing it to nitrite using a Cd-Cu reduction column, then measuring the nitrite as above. Sodium nitrite and nitrate solution were used as standards.

Measurement of NO by detecting chemiluminescence. Real time detection of NO was required to rule out the possibility that hydrogen peroxide reacts with potential precursors to release NO_2 or NO_3 directly. We employed flow injection analysis for the detection of chemiluminescence derived from the reaction of luminol and peroxynitrite formed in the reaction of NO and hydrogen peroxide based on the previously reported method (15) as diagrammatically indicated in Figure 1. Fifty μl of the potential precursors listed above dissolved in 20 mM potassium phosphate buffer (pH 7.4 at 25°C) at the concentrations indicated in the results section were injected by autosampler. The carrier solution was 20 mM potassium phosphate buffer (pH 7.4 at 25°C). The chemiluminescence probe consisting of 7.2 μM luminol, 60 μM desferrioxamine, 0.8 mM potassium carbonate and hydrogen peroxide at the final concentrations indicated in the results section was mixed thoroughly using a rotating flow mixer (Kyowa Seimitsu Co., Tokyo, Japan) and finally introduced to a chemiluminescence detector connected to a chart recorder. The flow rate of pump 1 and 2 was 1.0 ml/min.

However, peroxynitrite cannot be distinguished from NO because the chemiluminescence is derived from peroxynitrite formed in the reaction of NO and hydrogen peroxide. For this purpose, the application of carboxy-PTIO (16), a recently developed specific scavenger of NO is useful. It does not affect NO generation but scavenges NO directly. We employed this substance to distinguish NO from peroxynitrite because the chemiluminescence disappears when NO is released in the presence of carboxy-PTIO. In this experiment, the concentration of L-arginine and hydrogen peroxide were fixed at 20 mM and 8 mM (final concentration), respectively, and carboxy-PTIO was added to the L-arginine solution at the concentrations indicated in the results section.

NOR-1, a derivative of NOR-3 (FK 409) and a known generator of NO (17) was dissolved in DMSO at a concentration of 1 μM and used as a standard and each value was expressed as its ratio to the chemiluminescence intensity derived from the injection of 50 μl of 1 μM NOR-1. The temperature of the laboratory was kept at 25°C for at least a half day before each experiment.

RESULTS

Measurement of nitrite and nitrate in the reactions of hydrogen peroxide and various amino acids or guanidino compounds. As shown in Figure 2, NO_X were detected in the reaction mixture containing 10 mM L-arginine, D-arginine, L-canavanine, or L-NAME and 10 mM hydrogen peroxide in amounts depending on the incubation period. However, there was no detection of NO_X in the reaction mixture containing 10 mM hydrogen peroxide and 10 mM L-glycine, L-citrulline, L-ornithine, guanidine, methylguanidine, guanidinosuccinic acid, guanidinoacetic acid, creatine, creatinine, agmatine or aminoguanidine. In the reaction mixtures containing 2, 5, 10, 20 mM L-arginine and 0, 5, 10, 20, 30, 40, 50 mM hydrogen peroxide incubated for 3 days, the amounts of NO_X increased as the concentrations of L-arginine and hydrogen peroxide were raised (Figure 3).

NO generation in the reactions of hydrogen peroxide and D-or L-arginine. We modified the original method and increased the concentration of hydrogen peroxide in the chemiluminescence probe. The signal intensity of chemiluminescence resulting from the injection of 50 μl of L-arginine increased as the concentrations of hydrogen peroxide and L-arginine were raised as shown in Figure 4. As shown in Figure 5, the addition of carboxy-PTIO to the reaction of L-arginine and hydrogen peroxide diminished the intensity of chemiluminescence in a dose dependent manner. Of the various reactants tested, NO was detected only from D-arginine in addition to L-arginine as shown in Figure 6, though NO_X was detected by the Griess reaction in the experiments combining L-canavanine or L-NAME and hydrogen peroxide. Although the data are not shown, the other amino acids and guanidino compounds listed in the meth-

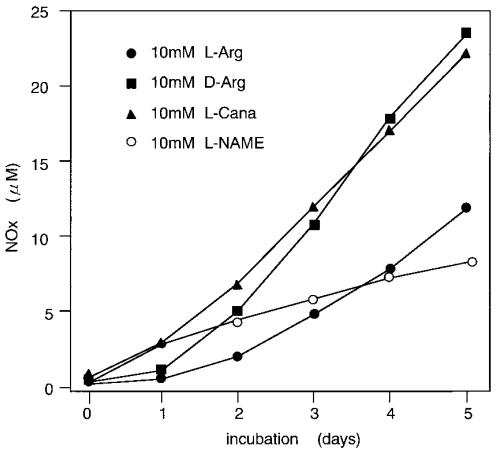

FIG. 2. Time course of NO_X production in the reaction mixture containing 10 mM L-arginine, D-arginine, L-canavanine, or L-NAME and 10 mM hydrogen peroxide. The amounts of NO_X increased depending on the incubation period. Values are expressed as final concentrations in the reaction mixture. Abbreviations: NO_X, nitrite and nitrate; Arg, arginine; Cana, canavanine; NAME, N^G-nitro arginine methyl ester.

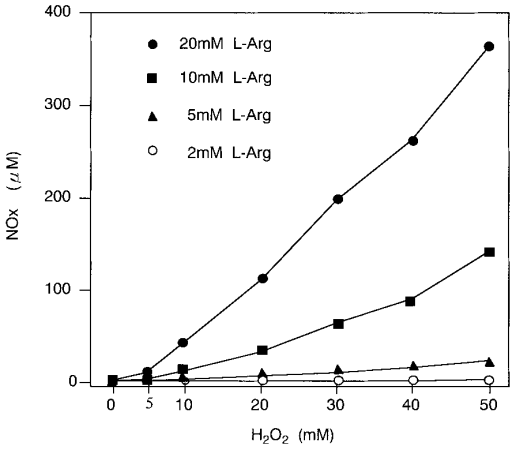

FIG. 3. Dependency of NO_X production from L-arginine on the concentration of L-arginine and hydrogen peroxide. The amounts of NO_X increased depending on the concentration of hydrogen peroxide and L-arginine. Incubation period was 3 days. Values were expressed as final concentrations in the reaction mixture. Abbreviations: NO_X, nitrite and nitrate; H_2O_2, hydrogen peroxide; Arg, arginine.

ods section even at a final concentration of 20 mM, did not serve as precursors of NO.

DISCUSSION

Heretofore, it has been universally accepted that the generation of NO from L-arginine occurs exclusively through the catalytic action of NOS (1). This study proposes a novel non-enzymatic pathway for NO synthesis from either D-or L-arginine. It impels, also, a reevaluation of NO for its dependence on the redox state and rationalizes several previously reported conflicting observations (11-13).

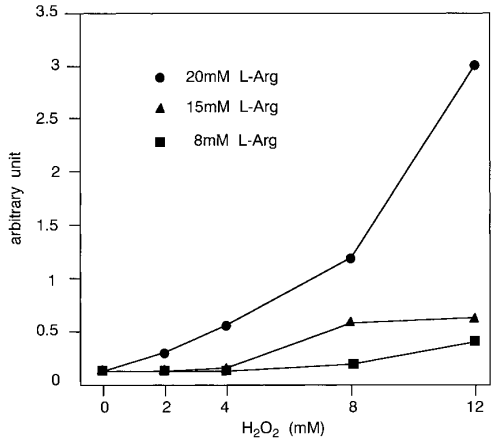

FIG. 4. NO generation by the reaction of L-arginine and hydrogen peroxide. The amount of NO generated increased depending on the concentration of L-arginine and hydrogen peroxide. The concentration of hydrogen peroxide indicated in the figure is the final concentration after mixing of the effluents of pumps 1 and 2. Abbreviations: H_2O_2, hydrogen peroxide; Arg, arginine.

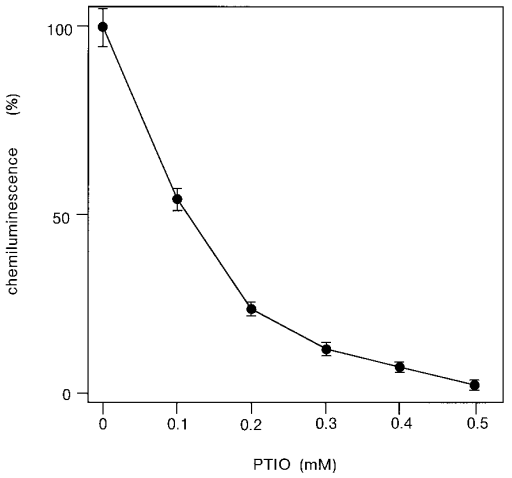

FIG. 5. Inhibitory effect of carboxy-PTIO on NO generation by the reaction of L-arginine and hydrogen peroxide. The addition of carboxy-PTIO to the L-arginine solution diminished the signal intensity of chemiluminescence in a dose dependent manner. The final concentration of L-arginine is 20 mM; that of hydrogen peroxide is 8 mM after mixing. The concentration of carboxy-PTIO indicated in the figure is the final concentration in the 20 mM L-arginine solution. Values are expressed as the mean ± the standard deviation of 5 determinations. Abbreviation: PTIO, carboxy-2-phenyl-4, 4, 5, 5-tetramethyl-imidazoline-1-oxyl-3-oxide.

We have described the role of ROS in the synthesis of methylguanidine (9) and guanidine (10) both of which are recognized uremic toxins and present in increased amounts in uremia. We demonstrated that several guanidino compounds such as creatinine or L-ca-

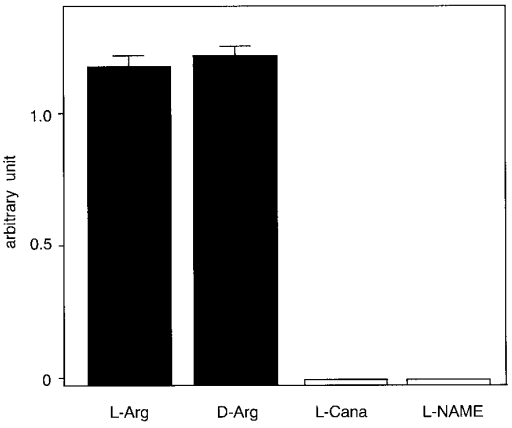

FIG. 6. The effect of various amino acids and guanidino compounds on NO generation by hydrogen peroxide. Chemiluminescence was apparent in the reaction of both D and L isomers of arginine reacting with hydrogen peroxide. However, NO release from L-canavanine or L-NAME could not be detected, though NO_X was detected by the Griess reaction. Other amino acids and guanidino compounds listed in the methods section did not serve as precursors of NO (not shown). Values are expressed as the mean ± the standard deviation of 5 determinations. Abbreviations: Arg, arginine; Cana, canavanine; NAME; N^G-nitro arginine methyl ester.

navanine release methylguanidine or guanidine when reacting with various ROS. Since the structure of NO is even simpler than that of these guanidines, the possibility is raised that it could be released from various amino acids or guanidino compounds.

Increased production of NO in addition to decreased renal clearance is suggested as a cause of the high plasma concentration of NO_X in dialysis patients (18). We have also found the production rate of NO in hemodialysis patients calculated from the increase of plasma NO_X levels during hemodialysis is significantly higher than that of healthy controls (19). Since the presence of a peroxidative state in patients with renal failure is commonly accepted (20), the current study suggests one possible mechanism for the elevated plasma level of NO_X.

These results indicate that evaluation of the amount of NO and its metabolites must be carefully interpreted from the view point of the redox state because NO is possibly released from D-or L-arginine non-enzymatically through the reaction with hydrogen peroxide, and NO_X measured by the Griess reaction does not always predict NO release. For example, in a study demonstrating that L-NAME causes glomerular NO_X production in long-term incubation (12), the authors speculate that the stimulated NO production is derived from enzymatic synthesis of L-arginine from L-NAME. In another report, there is no clear explanation concerning the fact that urinary excretion of NO_X in rats administered D-arginine did not differ from those administered L-arginine (13). It is possible and more reasonable to interpret the above findings to indicate that NO_X and not NO is released from the reaction of L-NAME and hydrogen peroxide generated under the peroxidative conditions of long-term incubation of glomeruli, and that NO is released from D-arginine reacting with hydrogen peroxide in vivo.

The breakthrough of the discovery of NOS and its physiological importance is so impressive that it has been hard to consider the synthetic pathway of NO without consideration of NOS. This study does not elucidate the mechanism for the generation of NO from D-or L-arginine by hydrogen peroxide. The nitrogen atom of NO could be derived from either the guanidino or the amino group. However, in addition to the variety of guanidino compounds other than the isomers of arginine which do not yield NO, it is also not generated by agmatine or aminoguanidine. Agmatine is a decarboxylated form of arginine and aminoguanidine is a combined amino and guanidino grouping. Together these negative results suggest that the reaction is not simply a cleavage of nitrogen from the guanidino or amino groups by hydrogen peroxide. Some specific reaction of hydrogen peroxide and D-or L-arginine similar to that seen in the synthesis of NO enzymatically through the action of NOS must be proposed. Further study is required to investigate the generation mechanism and the biological role of the NO generated non-enzymatically.

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