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Agmatine Enhances the NADPH Oxidase Activity of Neuronal NO Synthase and Leads to Oxidative Inactivation of the Enzyme

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ABSTRACT

It is established that agmatine, an endogenously formed decarboxylated arginine, is a weak competitive inhibitor of neuronal nitric-oxide synthase (nNOS) with an apparent K_i value of 660 μM [*Biochem J* **316:**247–249, 1996]. Although agmatine is known to bind to α-adrenergic and imidazoline receptors, it has been suggested that some of the pharmacological actions of agmatine, such as the prevention of morphine tolerance, may be due to the inhibition of nNOS. In the current study, we have discovered that agmatine, at concentrations much lower than the reported K_i value, leads to a time-, concentration-, NADPH-, and calmodulin-dependent irreversible inactivation of nNOS. The kinetics of inactivation could be described by an apparent dissociation constant for the initial reversible complex (K_i) and a pseudo first-order inactivation constant (k_{inact}) of 29 μM and 0.01 min^{-1}, respectively. As determined by high-performance liquid chromatography analysis, the mechanism of inactivation involves alteration of the prosthetic heme moiety of nNOS, in part to protein-bound products. Moreover, we discovered that agmatine causes a 3-fold increase in the NADPH oxidase activity of nNOS leading to the production of H_2O_2 and is a likely cause for the inactivation of the enzyme. Both the inactivation of nNOS and the oxidative stress produced should now be considered in the pharmacological actions of agmatine as well as provide insight into the potential biological effects of endogenously formed agmatine.

Agmatine is known to prevent tolerance to morphine analgesia (Kolesnikov et al., 1996; Fairbanks and Wilcox, 1997). Although this effect may be caused by interactions with the α-adrenergic receptors or imidazoline receptors, agmatine is also known to competitively inhibit nitric oxide synthase (Galea et al., 1996). Because morphine tolerance can be attenuated by the use of nitric-oxide synthase (NOS) inhibitors, such as 7-nitroindazole, N^G-nitro-L-arginine, and N^G-methyl-L-arginine (Kolesnikov et al., 1993; Majeed et al., 1994; Bhargava, 1995; Fairbanks and Wilcox, 1997), and that moxonidine, a putative imidazoline receptor agonist, does not modulate morphine tolerance (Fairbanks and Wilcox, 1997), it is possible that agmatine's pharmacological effect is due to inhibition of NOS. However, agmatine is a weak competitive inhibitor of NOS with K_i values of 660, 220, and 7500 μM, for the neuronal, macrophage, and endothelial isoforms of NOS, respectively. In one study (Gilad et al., 1996), an IC_{50} value of 160 μM for neuronal NOS (nNOS) was found, although another showed no inhibition of nNOS or the endothelial form (Auguet et al., 1995). An IC_{50} of 260 μM has been reported for the inducible isoform (Auguet et al., 1995). Although it has been argued that the local concentration of agmatine in NOS-containing cells may be high enough to competitively inhibit NOS (Galea et al., 1996), perhaps other mechanisms are responsible for the decrease in NO production. In fact, some of the pharmacologically active NOS inhibitors are not very potent competitive inhibitors but are actually time-dependent irreversible inactivators (Wolff and Lubeskie, 1995; Nakatsuka et al., 1998; Jianmongkol et al., 2000).

In the current study, we have discovered that agmatine causes the time-dependent irreversible inactivation of nNOS at concentrations approximately 10-fold lower than those observed for competitive inhibition. Agmatine causes a profound 3-fold increase in the NADPH oxidase activity of nNOS leading to the formation of hydrogen peroxide. This oxidase activity may be the mechanism for inactivation of the enzyme. Agmatine may be a useful tool in elucidating the mechanisms of regulation of the electron flux from NADPH to the heme of nNOS. Moreover, the inactivation of nNOS as

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ABBREVIATIONS: NOS, nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; HPLC, high-performance liquid chromatography.

well as the oxidative stress caused may play a role in the pharmacological actions of agmatine.

Experimental Procedures

Materials

Agmatine was purchased from Janssen Chimica (Geel, Belgium). Aprotinin, L-arginine, D-arginine, asymmetric N^{ω},N^{ω}-dimethyl-L-arginine, calmodulin (bovine), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, hydrogen peroxide, leupeptin, myoglobin, $NADP^+$, NADPH, sodium cyanide, and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Nitroindazole was purchased from BIOMOL (Plymouth Meeting, PA). L-[^{14}C(U)]Arginine (330.0 mCi/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Dr. Schirck's Laboratory (Jona, Switzerland).

Methods

Purification of nNOS. nNOS was overexpressed in Sf9 insect cells as described previously (Bender et al., 1999). Oxyhemoglobin (25 μM) was added as a source of heme during expression. Cells were harvested and suspended in 1 volume of 10 mM HEPES, pH 7.5, containing 320 mM sucrose, 100 μM EDTA, 0.1 mM dithiothreitol, 10 μg/ml trypsin inhibitor, 100 μM leupeptin, 2 μg/ml aprotinin, 6 mM phenylmethylsulfonyl fluoride, and 10 μM BH_4, and the suspended cells were ruptured by Dounce homogenization. Lysates from infected Sf9 cells (8×10^9) were centrifuged at 100,000*g* for 1 h. The supernatant fraction was loaded onto a 2′,5′-ADP-Sepharose column (8 ml), and the nNOS was affinity-purified as described previously (Roman et al., 1995), except that 10 mM 2′-AMP in high salt buffer was used to elute the protein. The nNOS-containing fraction was concentrated and dialyzed against 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 μM BH_4 with the use of a ProDicon and a 100-kDa cut-off membrane. This ADP-Sepharose nNOS preparation had a specific activity of approximately 400 nmol/min/mg of protein, a heme content of 0.42 mol of heme/mol of monomer, and was stored at −80°C for use in inhibition studies. For some experiments this ADP-Sepharose nNOS preparation was further purified by loading onto a Sephacryl S-300 HR gel filtration column (2.6 × 100 cm, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10 μM BH_4. The proteins were eluted at a flow rate of 1.3 ml/min, and 1.5-ml fractions were collected and analyzed for protein content and NOS activity. The fractions containing NOS activity were pooled and supplemented with 10 μM FAD, 10 μM FMN, and 10 μM BH_4 before concentration with the use of a Centriplus concentrator (Amicon, Beverly, MA). This Sephacryl-purified nNOS preparation has a specific activity of 1000 nmol/min/mg of protein, a heme content of 0.88 mol of heme/mol of monomer, and was stored at −80°C.

Treatment of nNOS with Agmatine and NOS Activity Assay. The ADP-Sepharose nNOS preparation (73 μg/ml) was added to a "first reaction mixture" of 40 mM potassium phosphate, pH 7.4, containing 0.2 mM $CaCl_2$, 500 U/ml superoxide dismutase, 100 U/ml catalase, 40 μg/ml calmodulin, 100 μM BH_4, 1 mM dithiothreitol, the desired concentration of agmatine, and an NADPH-generating system composed of 0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 U of glucose-6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 180 μl. After incubation at 30°C, aliquots (20 μl) of the first reaction mixture were transferred to an "oxyhemoglobin assay mixture" containing 200 μM $CaCl_2$, 250 μM L-arginine, 100 μM BH_4, 100 U/ml catalase, 10 μg/ml calmodulin, 25 μM oxyhemoglobin, and an NADPH-generating system composed of 0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 U of glucose-6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 200 μl of 40 mM potassium phosphate, pH 7.4. The assay mixture was incubated at 37°C, and the rate of NO-mediated oxidation of oxyhemoglobin was monitored by measuring the difference in absorbance at λ401 and 411 nm with a microtiter plate reader (SpectraMax Plus; Molecular Devices, Sunnydale, CA) as previously described (Feelisch et al., 1996). The rate was determined from the linear portion of the time-dependent changes in absorbance. In studies where the NADPH oxidation was measured, the Sephacryl-purified nNOS (6 μg/ml) was added to a mixture containing 0.2 mM $CaCl_2$, 500 U/ml superoxide dismutase, 100 U/ml catalase, 20 μg/ml calmodulin, 100 μM BH_4, 1 mM dithiothreitol, 0.2 mM NADPH, and the desired amount of agmatine in a total volume of 180 μl of 40 mM potassium phosphate, pH 7.4, at 37°C. The NADPH oxidation was measured by the loss in absorbance at 340 nm. The initial rate of NADPH oxidation was determined from the data obtained in the first 60 s of the assay, because a straight line could be drawn from the observed data with a correlation of greater than 0.98. However, after this time, the rate of NADPH oxidation was found to decrease such that after 10 min, the rates were one tenth that of the initial rate. In some studies, an aliquot (75 μl) of the mixtures used for NADPH oxidation measurements was taken and the amount of hydrogen peroxide present was determined by the thiocyanate method (Vasquez-Vivar et al., 1999). In studies in which NO synthesis was measured by the use of radiolabeled arginine, aliquots (5 μl) of the first reaction mixture were transferred to a ^{14}C-arginine assay mixture containing 100 μM L-arginine (22 mCi/mmol), 200 μM $CaCl_2$, 100 μM BH_4, 100 U/ml catalase, 10 μg/ml calmodulin, and an NADPH-generating system composed of 0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 U of glucose-6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 100 μl of 40 mM potassium phosphate, pH 7.4. The assay mixture was incubated at 37°C for 5 min, and the amount of radiolabeled citrulline was quantified as described previously (Osawa et al., 1994a).

HPLC. HPLC was performed with the use of a Waters 600S controller, 717 plus autosampler, and 996 photodiode array detector (Waters Corp., Milford, MA). A portion of the first reaction mixture (115 μl) was injected onto an HPLC column (C4 Vydac; 5 μm, 0.21 × 15 cm) equilibrated with solvent A (0.1% trifluoroacetic acid) at a flow rate of 0.3 ml/min. A linear gradient was run to 75% solvent B (0.1% trifluoroacetic acid in acetonitrile) over 30 min followed by a linear gradient to 100% solvent B over 5 min. Absorbance at 220 and 400 nm was monitored. Myoglobin was used as a standard.

Results

Effect of Agmatine on Neuronal NOS Activity. As shown in Fig. 1A, agmatine caused a concentration- and time-dependent loss of nNOS activity. This process was saturable and could be described by an apparent dissociation constant for the initial reversible agmatine-enzyme complex (K_i) and an inactivation constant (k_{inact}) of 29 μM and 0.01 min^{-1}, respectively (Fig. 1B). As shown in Fig. 1C, the inactivation due to agmatine was dependent on the presence of calmodulin (compare ▲ and ♦), which is necessary for electron transfer from NADPH to the heme and indicates a metabolism-dependent step in the inactivation process. The omission of the NADPH-regenerating system did not have an effect in the first 20 min but greatly slowed the inactivation observed between 20 and 60 min, suggesting that the process is dependent on NADPH but that there are endogenous reducing equivalents present that are eventually exhausted after 20 min. As shown in Fig. 1D, the inactivation caused by 500 μM agmatine (▲) was slowed in the presence of 100 μM L-arginine (*) but not by 500 μM D-arginine (◇), indicating an active site-directed event. The inactivation of nNOS was verified in studies where the activity was measured by the

[^{14}C]arginine assay. Treatment of nNOS with 500 μM agmatine for 60 min gave 30 ± 1.2% residual activity, which is comparable with that found for the oxyhemoglobin method.

Agmatine Caused the Alteration of the Prosthetic Heme of nNOS. Figure 2A shows the HPLC profile of nNOS treated with agmatine in the absence of calmodulin. The major fraction with absorption at 400 nm corresponds to native heme (Heme), which dissociates from the nNOS protein (NOS) under the acidic conditions of the chromatography (Osawa et al., 1989). There are low levels of other 400-nm-absorbing compounds in this sample as well. As shown in Fig. 2B, treatment of nNOS with agmatine in the presence of calmodulin gave a loss in the peak area for native heme and the appearance of peaks with absorption at 400 nm (peaks 1 and 2). Peak 1 is a mixture of at least two peaks. Peak 2 coeluted with nNOS protein and may be an altered heme product irreversibly bound to the protein. The potential differences in absorptivity of the products preclude quantification by HPLC. As shown in Fig. 2C, the loss of heme observed on the HPLC profile at 400 nm was quantified and compared with the loss of nNOS activity. The loss of heme and activity occurred only when agmatine was incubated with nNOS in the presence of calmodulin (Fig. 2C, compare squares and circles). The loss in heme and nNOS activity were both time-dependent and of approximately the same extent. Thus, it seems that the heme alteration was responsible for the inactivation. In data not shown, nNOS treated with calmodulin for 30 min in the absence of agmatine gave a 45 ± 6% and 42 ± 4% decrease in heme and activity, respectively. Moreover, peaks 1 and 2 were observed, albeit to a lower amount than with agmatine, for nNOS treated with calmodulin alone. This suggests that calmodulin-dependent autoinactivation may be related to the agmatine-mediated inactivation.

In these studies, in which the heme was measured by HPLC, we decreased the amount of catalase in the reaction mixtures from 100 to 10 U/ml to minimize the heme contribution from catalase. In the course of these studies, we noticed that the lower amounts of catalase caused a nearly complete inactivation of nNOS upon incubation with agmatine for 30 min (Fig. 2C), whereas at the higher concentrations of catalase only about half the activity was lost. We chose to further investigate the relationship of the catalase concentration with the extent of activity and heme loss. As shown in Table 1, in the absence of calmodulin, there was minimal activity or heme loss and this was not affected by the amount of catalase. The addition of calmodulin, which enabled redox reductions of nNOS to occur, caused the autoinactivation of nNOS and loss of heme. Moreover, there was greater autoinactivation when catalase was removed, suggesting that hydrogen peroxide is responsible (condition 6). This loss of heme and activity was greatly enhanced when agmatine was present. Catalase, even at a concentration of 100 U/ml, could not totally protect from the agmatine-mediated heme and activity loss. Under all conditions, the extent of heme loss approximated the activity loss, suggesting that heme alteration is intimately associated with the inactivation.

Agmatine Caused an Increase in the NADPH Oxidase Activity of nNOS. As shown in Fig. 3, we show with the use of a colorimetric assay (Vasquez-Vivar et al., 1999) that hydrogen peroxide (hatched bars) was being formed during a 3-min incubation of nNOS with agmatine and calmodulin. The peroxide formation was dependent on calmodulin whether agmatine was present or not. That calmodulin was required for peroxide formation is consistent with previous studies (Heinzel et al., 1992). To ensure that we were measuring hydrogen peroxide, the addition of catalase pre-

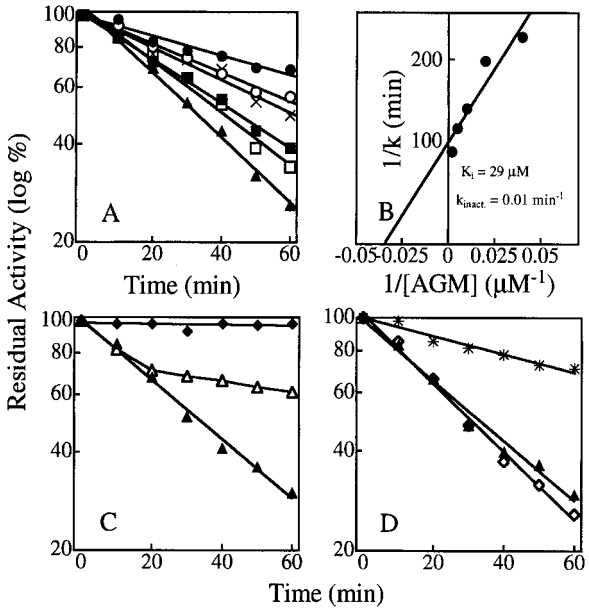

Fig. 1. Time-dependent inactivation of nNOS by agmatine. A, the extent of inhibition of nNOS activity of the ADP-Sepharose preparation at the following concentrations of agmatine in the first reaction mixture: ●, 0 μM; ○, 25 μM; ×, 50 μM; ■, 100 μM; □, 200 μM; ▲, 500 μM. The inactivation of nNOS activity was determined with the use of the first reaction mixture and the oxyhemoglobin assay mixture as described under *Experimental Procedures.* B, the replot of the data from A. C, the extent of inhibition of nNOS by 500 μM agmatine in the presence (▲) or absence (△) of the NADPH-regenerating system. The extent of inhibition in the presence of NADPH-regenerating system but in the absence of calmodulin is also shown (♦). D, the extent of inhibition of purified nNOS in the presence of: ▲, 500 μM agmatine; ∗, 500 μM agmatine and 100 μM L-arginine; ◇, 500 μM agmatine and 500 μM D-arginine. The specific activity at 100% for all samples was approximately 420 nmol/min/mg of protein.

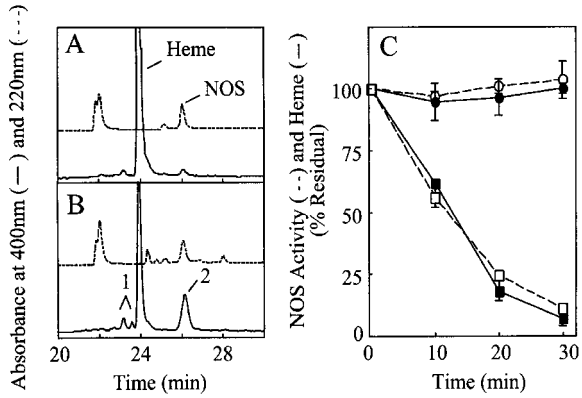

Fig. 2. Effect of agmatine on nNOS heme. A, HPLC profile of nNOS treated with 500 μM agmatine in the absence of calmodulin for 30 min. B, HPLC profile of nNOS treated with 500 μM agmatine in the presence of calmodulin for 30 min; C, time course of the loss of nNOS activity (dashed line, open symbols) or heme (solid line, closed symbols). The nNOS was treated with 500 μM agmatine in the presence (■,□) or absence (●,○) of calmodulin. The values in C are the mean ± S.E. (n = 3). ADP-Sepharose-purified nNOS was treated with agmatine as described in Fig. 1. HPLC analysis and NO synthase activity were determined as described under *Experimental Procedures.*

vented the detection of colorimetric product. We also examined the rate of NADPH oxidation catalyzed by nNOS (solid bars) to determine the magnitude of the total electron flux. As expected, there were very low levels of NADPH oxidation in the absence of calmodulin (Abu-Soud and Stuehr, 1993). The addition of calmodulin alone gave an increase in NADPH oxidation to a rate near 1600 nmol/min/mg of protein, which corresponds to that described earlier (Presta et al., 1997; Nishida and Ortiz de Montellano, 1998) but higher than others (List et al., 1997). This rate was attenuated somewhat in the presence of arginine, consistent with that previously reported (Presta et al., 1997; Nishida and Ortiz de Montellano, 1998). Agmatine was found to increase the rate of NADPH oxidation, in a manner that was dependent on the concentration of agmatine, to a maximum level of approximately 4500 nmol/min/mg. This suggests that the peroxide produced is caused in part by the greater electron flux through the enzyme and cannot be explained only by a partitioning to a more "leaky" pathway. The high level of NADPH oxidation was not maintained and this rate decreased over time, consistent with the inactivation of the enzyme.

Relationship between Inactivation and NADPH Oxidase Activity. As shown in Fig. 4, we found that the rate of inactivation determined at different concentrations of agmatine from Fig. 1 showed a correlation with the rate of NADPH oxidation elicited by agmatine. In addition, the rates of inactivation observed without agmatine or calmodulin were also plotted. This correlation suggests that the NADPH oxidase activity is involved in the inactivation process. Moreover, as shown in Fig. 5A, the agmatine-mediated enhancement in NADPH oxidation was inhibited by L-, but not D-, arginine (Fig. 5, compare D-ARG with L-ARG). This is consistent with the protection from inactivation by the same concentration of L-, but not D-, arginine determined above. In addition, we have found that 100 μM asymmetric dimethyl-L-arginine or 10 μM 7-nitroindazole also protected against agmatine-mediated inactivation (data not shown) as well as prevented the enhancement in NADPH oxidation (Fig. 5A). In each case, the NADPH oxidation correlated with the production of hydrogen peroxide (Fig. 5B). Thus, it seems that the NADPH oxidase activity is intimately associated with the inactivation process. Furthermore, 10 mM cyanide, which has been shown to interact at the heme site to inhibit heme-mediated redox cycling of nNOS (Pou et al., 1999), lead to the attenuation of the enhanced NADPH oxidation rate (Fig. 5A), suggesting that heme plays the major role in production of the reduced oxygen species.

TABLE 1

Effect of catalase on nNOS activity and heme content

The conditions and methods were as in Fig. 2. The activity and heme were measured after a 30-min incubation. The contribution of heme from catalase was less than 5% of that from nNOS. The specific activity of the nNOS at 100% was 440 nmol/min/mg of protein.

Component Omitted	Catalase	nNOS Activity	nNOS Heme
	U/ml	*% residual after 30 min*	
Calmodulin	100	100	100
Calmodulin	10	95	104
Calmodulin	0	94	104
Agmatine	100	89	99
Agmatine	10	77	76
Agmatine	0	43	52
None	100	64	67
None	10	21	31
None	0	4	18

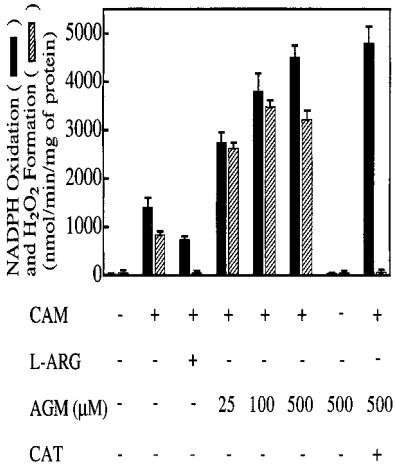

Fig. 3. Effect of agmatine on the NADPH oxidase activity of nNOS. The rate of NADPH oxidation (■) and H_2O_2 formation (▨) of the Sephacryl-purified preparation of nNOS was determined as described under *Experimental Procedures.* As indicated in the figure, samples contained 20 μg/ml of calmodulin (CAM), 100 μM L-arginine (L-ARG), agmatine (AGM) at the indicated concentrations, and 100 U/ml of catalase (CAT).

H_2O_2 Treatment of nNOS. As shown in Fig. 6, treatment of nNOS with a bolus of 100 μM H_2O_2, a level of peroxide that is lower than that found for nNOS treated with 500 μM agmatine, gave a loss in the peak area for native heme and the appearance of peaks with absorption at 400 nm (peaks 1 and 2). The altered heme products had highly similar retention times to those found for the agmatine-treated nNOS samples. After treatment with H_2O_2 for 30 min, there was 38 ± 1% residual heme present in the nNOS sample. Although a bolus of peroxide did not mimic the agmatine-mediated formation of peroxide, these studies confirm that peroxide can alter the heme prosthetic group of nNOS.

Discussion

We have shown for the first time that agmatine inactivates nNOS by a time-dependent process that requires calmodulin and NADPH and leads to the alteration of the heme prosthetic group. That heme alteration is involved in the inacti-

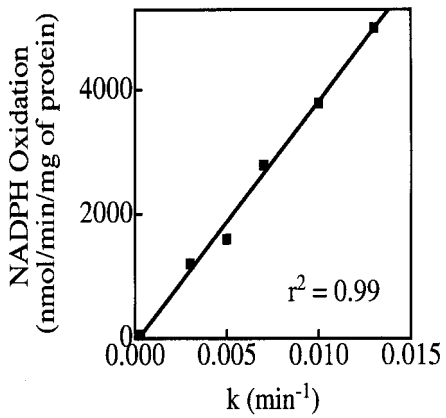

Fig. 4. Correlation of the NADPH oxidation rate with the rate of inactivation of nNOS. The NADPH oxidation was determined as in Fig. 3 at different agmatine concentrations (0–500 μM). The rate of inactivation at these agmatine concentrations was determined as in Fig. 1 and plotted against the rate of NADPH oxidation.

vation stems from our finding that the extent of heme alteration is concomitant to the loss of activity; that is, the heme loss is agmatine-, time-, and calmodulin-dependent. The evidence for heme alteration was obtained by studies with the use of HPLC as established previously for myoglobin (Osawa et al., 1989; Sugiyama et al., 1997), hemoglobin (Kindt et al., 1992; Osawa et al., 1994b), and nNOS (Jianmongkol et al., 2000). We propose that the agmatine-mediated inactivation is caused by formation of hydrogen peroxide in the active site of nNOS and eventually leads to the oxidative alteration of the heme prosthetic group. That hydrogen peroxide is involved is based on the finding that agmatine-mediated heme loss and inactivation can be partially protected by catalase and that agmatine causes a profound enhancement in the production of hydrogen peroxide by nNOS. The increase in hydrogen peroxide was caused in large part by an increase in the rate of NADPH oxidation catalyzed by nNOS. Moreover, the initial rate of NADPH oxidation caused by agmatine was directly correlated with the rate of inactivation of nNOS. Interestingly, the rate of autoinactivation of nNOS and the NADPH oxidase activity observed with the enzyme in the absence of agmatine also falls on this linear correlation. Thus, it seems that agmatine-mediated inactivation may in fact be an enhancement of the natural autoinactivation process. In support of this notion, the autoinactivation of nNOS, similar to that found for the agmatine-mediated inactivation, is dependent on calmodulin and time and is attenuated by catalase.

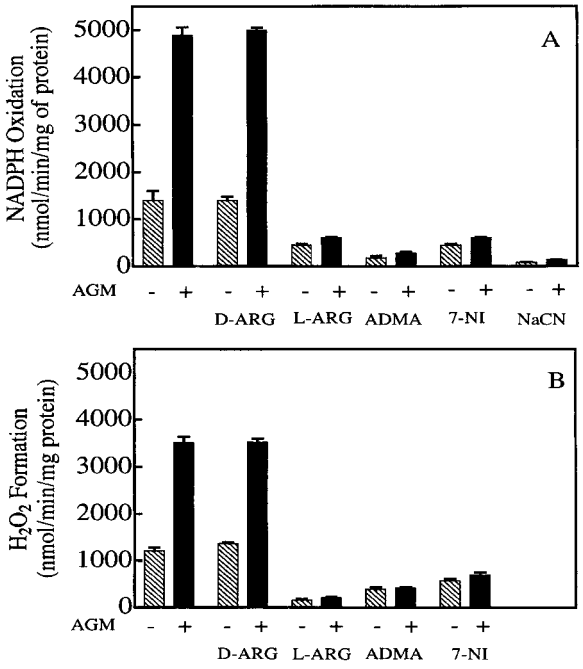

Fig. 5. Effect of substrates and competitive inhibitors on the NADPH oxidation of agmatine-treated nNOS. The NADPH oxidation (A) and hydrogen peroxide formation (B) catalyzed by the Sephacryl-purified nNOS were determined as described under *Experimental Procedures*. Samples were untreated (▨) or treated with 500 μM agmatine (■). As indicated, these samples were coincubated with 500 μM D-arginine (D-ARG), 100 μM L-arginine (L-ARG), 100 μM assymteric dimethyl L-arginine (ADMA), 10 μM 7-nitroindazole (7-NI), or 10 mM sodium cyanide (NaCN).

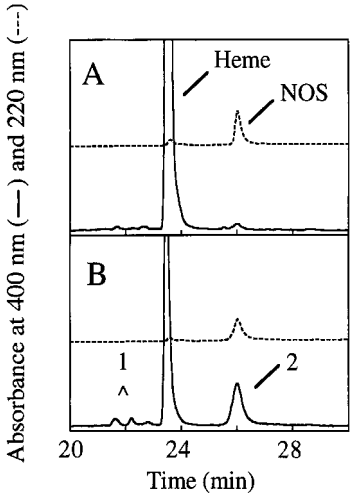

Fig. 6. HPLC profile of untreated and H_2O_2-treated nNOS. A, HPLC profile of untreated nNOS after 30 min. B, HPLC profile of nNOS treated with 100 μM H_2O_2 for 30 min. The Sephacryl-nNOS preparation (28 μg/ml) was treated with 100 μM H_2O_2 in a total volume of 250 μl of 40 mM potassium phosphate, pH 7.4. An aliquot (150 μl) was taken for heme determination by HPLC analysis as described under *Experimental Procedures*.

That agmatine-mediated inactivation alters the heme, in part, to protein-bound heme adducts is consistent with the oxidative modification of the heme (Catalano et al., 1989; Osawa and Williams, 1996). In addition, highly similar products were obtained, albeit to a much lower degree, for the autoinactivation reaction. Furthermore, hydrogen peroxide treatment of nNOS also gave highly similar heme products. This further supports the notion that peroxide is responsible for heme alteration and inactivation and that agmatine accelerates the natural autoinactivation process. In the future, the exact nature of the altered heme products should be elucidated and compared with that previously described for the reaction of peroxide with hemoproteins (Sugiyama et al., 1997).

The mechanism for the agmatine-mediated increase in NADPH oxidation is unknown. That all three major isoforms of NOS are NADPH oxidases and that nNOS in the presence of BH_4 is the most active is well established (Presta et al., 1997; Nishida and Ortiz de Montellano, 1998). The 3-fold increase in the nNOS catalyzed NADPH oxidation from 1600 to 4800 nmol/min/mg of protein is unprecedented. Arginine or various arginine derivatives have been shown to decrease the NADPH oxidation of nNOS (Abu-Soud et al., 1994; Nishida and Ortiz de Montellano, 1998). However, the binding of L-arginine to the inducible and endothelial isoforms does increase NADPH oxidation, with effects more prominent for the inducible isoform (Presta et al., 1997; Nishida and Ortiz de Montellano, 1998). It is possible that arginine does have an innate ability to facilitate electron transfer to the heme but this effect is masked for the nNOS isoform, perhaps by feedback inhibition by NO to form the ferrous-nitrosyl complex (Abu-Soud et al., 1995). We speculate that agmatine also has a similar innate ability to enhance NADPH oxidation, by virtue of its structural similarity with L-arginine, but because NO is not formed from agmatine (Galea et al., 1996; Gilad et al., 1996) there is no feedback inhibition. Therefore, the enhancement in NADPH oxidation is not masked by formation of a ferrous-nitrosyl complex. This speculation is supported by the finding that the rate of NADPH oxidation is 10-fold greater in the first second of metabolism of L-arginine by nNOS under conditions where

the formation of the ferrous-nitrosyl complex is minimized (Abu-Soud et al., 1995).

Agmatine is known to block tolerance to morphine in a manner similar to that of NOS inhibitors (Babey et al., 1994; Kolesnikov et al., 1996; Fairbanks and Wilcox, 1997). Thus, the interaction of agmatine with nNOS characterized here may be of pharmacological importance. Although it was known that agmatine is a weak competitive inhibitor of nNOS (Galea et al., 1996), the current study indicates that the time-dependent irreversible inactivation may be important, because it occurs at approximately 10-fold lower concentrations than the K_i value for competitive inhibition. Moreover, because the inhibition is irreversible, the duration of exposure to agmatine and the rate of synthesis of new nNOS protein may be important factors in determining the in vivo effects of agmatine. The large difference in the K_i value of 660 μM, which was determined over a 10-min incubation period (Galea et al., 1996), with the IC_{50} value of 160 μM, which was determined over a 3-h incubation period (Gilad et al., 1996), may reflect the time-dependent inactivation process described in the current study. Agmatine has been shown, in one study, to increase NO formation in endothelial cells by increasing cytosolic calcium most likely by a receptor-mediated mechanism (Morrissey and Klahr, 1997). This suggests that the effects of agmatine are complex and involve both direct and indirect effects on NO synthase activity.

The long term effects of agmatine on nNOS may be important, because it is formed endogenously by arginine decarboxylase as a precursor to biosynthesis of putrescine (Reis and Regunathan, 1999). Agmatine is produced in the brain and stored in neurons (Sastre et al., 1997; Reis and Regunathan, 1999), and it would likely interact with nNOS. The levels of agmatine are known to be elevated in depression (Halaris et al., 1999) and modulated by lipopolysaccharides and cytokines (Sastre et al., 1998). Thus, our findings on nNOS inactivation and activation of the NADPH oxidase activity has potential physiological or pathological consequences due to changes in the balance between reactive oxygen species and NO production.

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