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The Protective Effect of Tetrahydrobiopterin on the Nitric Oxide-Mediated Inhibition of Purified Nitric Oxide Synthase

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SUMMARY: The nitric oxide synthases (NOS) are a class of enzymes responsible for the generation of NO *via* an oxygen and NADPH dependent oxidation of the amino acid arginine. These enzymes are ironheme proteins which contain FAD and FMN and, enigmatically, require tetrahydrobiopterin (BH_4). NOS has recently been shown to be subject to inhibition by its product, NO. Preliminary data by us indicate that a possible role for BH_4 is to prevent and/or reverse the NO-mediated inhibition of NOS. The objective of this study was to elucidate the mechanism by which BH_4 protects NOS against NO inhibition. Protection of NOS from NO inhibition was observed by both BH_4 and the BH_4 regeneration system, dihydropteridine reductase (DHPR)/NADH. NO, rather than an oxidation product, appears to be the inhibitory species. Protection by BH_4 is not likely due to a simple chemical reaction between BH_4 and NO or its oxidation product, NO_2. The results are consistent with a protective mechanism by which BH_4 may act as a nonstoichiometric reducing agent for a redox active enzyme component, such as the ironheme, to prevent NO ligation.

The catalytic conversion of arginine to nitric oxide (NO) and citrulline is performed by a class of enzymes generally referred to as the nitric oxide synthases (NOS). The physiological relevance of this process has been reviewed [for example, see 1, 2]. Analogous to the more well characterized cytochrome P450 enzyme systems (P450), NOS is an ironheme protein [3, 4, 5, 6] which utilizes NADPH and molecular oxygen and requires FAD and FMN [7]. One of the outstanding features of NOS which distinguishes it from P450, however, is its requirement for tetrahydrobiopterin (BH_4) [for example, 8, 9, 10]. Although the requirement for BH_4 for maximal catalytic activity is well established, the specific role of BH_4 in NOS catalysis is not very clear. Giovanelli and coworkers suggested that BH_4 may not have a stoichiometric role in NOS catalysis and may be acting as an allosteric modulator or may function to protect NOS by keeping a crucial catalytic component in the reduced state [11]. Hevel and Marletta have proposed a likely redox role for BH_4 in macrophage NOS catalysis and did not rule out the

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Abbreviations: NOS, nitric oxide synthase; NO, nitric oxide; BH_4, tetrahydrobiopterin; DHPR, dihydropteridine reductase; P450, cytochrome P450.

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possibility that BH_4 may reactivate the NOS after an occasional inactivation event during turnover [10]. More recently, we have proposed that BH_4 may be involved in the protection of NOS from inhibition by its product, NO [12] and thus may be utilized in a redox and nonstoichiometric fashion. The purpose for this study was to further evaluate the protective action of BH4 on NOS inhibition by NO and to elucidate the mechanism of this protective effect.

MATERIALS AND METHODS

Chemicals and Solutions: EDTA, EGTA, L-ascorbic acid, glycerol, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride, dithiothreitol, FAD, $NADP^+$, NADPH, L-arginine, calmodulin, L-citrulline, NADH, dihydropteridine reductase, DEAE Sephacel, and Trizma Base were purchased from Sigma Chemical Co. (St. Louis, MO). Tetrahydrobiopterin was purchased from Schircks laboratories (Jona, Switzerland). 2',5'-ADP Sepharose 4B was obtained from Pharmacia (Milwaukee, WI). NO gas (Matheson Gas Products, Cucamunga, CA) was passed through basic water before use to trap any nitrogen dioxide (NO_2) impurity. NO_2 gas (Matheson) was used directly from the tank. Aqueous solutions of NO were obtained by bubbling 70-100 mL of pure NO gas through 5 mL of degassed buffer contained in a Schlenk tube sealed with a rubber septum. The concentration of the NO solution was approximately 2 mM as determined by sparging the gaseous contents of a 1 mL sample into a chemiluminescence detector (Antek 720) with a nitrogen stream. Quantitation of the sparged NO was accomplished by comparison to a standard curve based on the detector response from injections of authentic NO. Dowex AG50W-X8 (H^+ form), 100-200 mesh and Dowex AG 1-X8 (acetate form), 100-200 mesh were purchased from Bio-Rad Laboratories. Aquasol-2 was purchased from Du Pont Company/NEN Research Products.

Purification of NOS: The purification of rat brain NOS was accomplished using a modification of the procedure of Bredt and Snyder [13] and has been described previously [14].

Protein Determination: Protein concentrations were determined using the Bradford, Coomassie blue method described by Bio Rad laboratories (Hercules, CA). Bovine serum albumin (Pierce, Rockford, IL) was used as the standard.

Assay of NOS Activity: NOS activity was measured using the method of Bredt and Snyder [15] which monitors the conversion of [^{3}H]arginine to [^{3}H]citrulline and has been utilized and described previously by us [14]. Enzymatic reactions were initiated by addition of the reaction cofactors and substrates to buffer solution containing NOS. Incubations carried out in the presence of exogenously added NO or NO_2 were performed in the usual way with the following modifications. NO was added to the incubation by simply adding an NO-buffer solution, of known concentration (see above), to the mixture containing NOS immediately after the addition of the reaction cofactors (including the appropriate concentration of BH_4) and substrates. NO_2 was added to the incubation by adding a diluted mixture of NO_2 in air, *via* a gastight syringe, into the incubation mixture containing NOS immediately after the addition of the reaction cofactors and substrates. In studies examining the effects of ascorbate or DHPR, addition of ascorbate or DHPR/NADH (100 μM final concentration) solutions to the incubation mixture was performed just after the addition of NOS. In all cases, control incubations contained only vehicle.

Reaction of BH_4 with NO and NO_2: A possible reaction between NO and BH_4 was examined by both observing possible changes in the optical spectrum of BH_4 in the presence of NO and by monitoring NO loss in the presence of BH_4 by chemiluminescence detection. In the chemiluminescence experiment, 100 μL of NO gas (4.5 μmol) was injected, using a gastight syringe, into each of two 10 mL round bottomed flasks which had been degassed with nitrogen and sealed with a gastight rubber septum. Into one flask was then injected 2 mL of degassed 100 mM phosphate buffer, pH 7.4. Into the other flask was injected 2 mL of degassed buffer containing 2 mg of BH_4 (6.4 μmol). Both solutions were then stirred for 20 minutes. 100 μL aliquots of the headspace from these reactions were then examined for NO using the chemiluminescence detector. Samples were reexamined after 1 hr. In experiments using UV spectroscopy to monitor BH_4, degassed solutions of BH_4 under nitrogen were made up in septum sealed quartz cuvettes. Spectra were then recorded on a Uvicon 810 (San Diego, CA) double beam spectrophotometer with buffer in the reference cuvette. Samples were scanned from 240-340 nm. Introduction of NO, NO_2 and other gasses was accomplished by injection through the

septum using gastight syringes. Rate data for the loss of BH_4 was obtained by monitoring the decrease in absorbance at 297 nm.

RESULTS

Protection of NO-mediated inhibition of NOS by BH_4: As previously reported, NO was capable of inhibiting NOS activity [12, 16-20]. The activity of purified rat cerebellar NOS was typically inhibited 70-90% by 10-50 μM NO in the presence of 10 μM BH_4 in reaction mixture (data not shown). However, the addition of 50-500 μM BH_4 to the incubation mixture protected, in a concentration dependent manner, against inhibition by NO. In fact, in the presence of 500 μM BH_4, NO inhibition of NOS was almost abolished. Also, under these conditions, BH_4 had no significant effect on control NOS activity (**Figure 1**). Significantly, another reducing agent, ascorbate, did not offer any protection against NOS inhibition by NO even when used at 2 mM concentrations (data not shown).

Protection of NO-mediated inhibition of NOS by DHPR/NADH: BH_4 is known to be unstable with respect to autoxidation by molecular O_2. That is, BH_4 can react with oxygen to give, initially, the quinoid dihydrobiopterin (q-BH_2) which tautomerizes to 7,8 dihydrobiopterin (BH_2) [21]. Thus, in the previous experiments, the concentration of BH_4 is likely to be decreasing throughout the incubation period with a subsequent buildup of oxidized BH_4 species such as q-BH_2 and BH_2. One way to keep BH_4 in the reduced state during the NOS incubation is to add dihydropteridine reductase (DHPR) and NADH to the incubation mixture. That is, any q-BH_2 formed from the autoxidation of BH_4 can be converted back to BH_4 thus maintaining a higher steady-state concentration of BH_4. The addition of DHPR/NADH with 10 μM BH_4 increased the activity of NOS to 115-157 % of control in the absence of NO (**Figure 2**). When 1

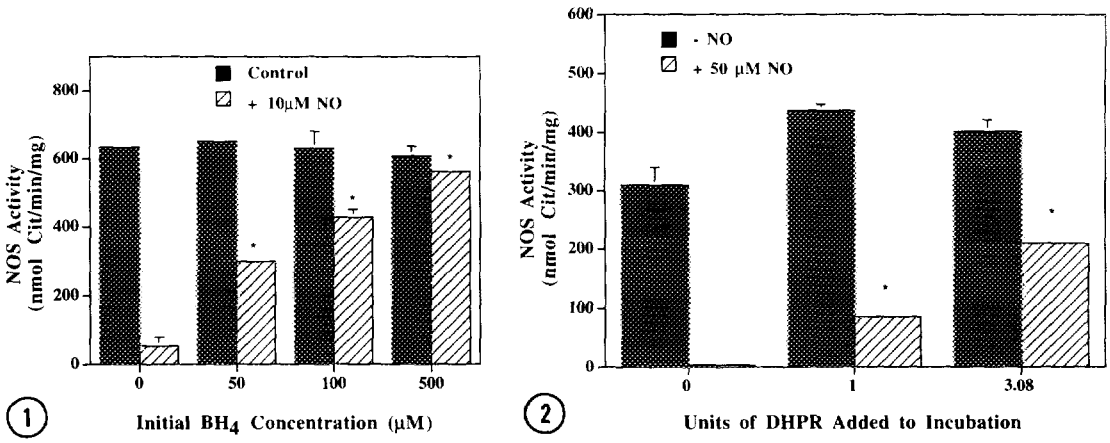

Figure 1. Protective effect of BH_4 on the inhibitory action of 10 μM NO on purified cerebellar NOS activity. Values represent the mean ± S.E. of duplicate experiments from two separate experiments. *$p < 0.001$ compared to the corresponding control.

Figure 2. Protective effect of dihydropteridine reductase (DHPR) on inhibitory action of 50 μM NO on NOS Activity. Values are expressed as the mean ± S.D. from 3 separate experiments. *$p < 0.001$ compared to the corresponding control.

unit of DHPR, 200 μM NADPH, and 10 μM BH_4 were added before the addition of NO, the inhibitory action of NO was reduced from 99% to 80%. 3.08 units of DHPR, added before the addition of NO, further reduced the NO-mediated inhibition to 48%. Thus, the addition of DHPR/NADH (to an incubation containing 10 μM BH_4) had a similar effect to adding high concentrations of BH_4 (50 μM).

Reaction between NO, NO_2 and BH_4: The possible reaction between NO and BH_4 was then examined. Using chemiluminescence detection for NO, it was found that addition of a degassed aqueous solution of BH_4 to a flask containing NO in nitrogen did not result in a decrease in measurable NO levels even after 1 hour. The possible reaction between BH_4 and NO (and NO-related species) was also examined by monitoring changes in the ultraviolet spectrum of BH_4 under a variety of conditions. Under anaerobic conditions, the spectrum of BH4 remained unchanged over 10 minutes. The addition of O_2, however, resulted in BH_4 oxidation as evidenced by a decrease in absorbance at 297 nm [22]. Further addition of approximately 4 equivalents of NO to the aerobic solution accelerated the rate of BH_4 loss by approximately 5-fold. However, BH_4 in degassed buffer remained unchanged when exposed to 4 equivalents of NO. The addition of 2 equivalents of O_2 to this system then resulted in a rapid loss of BH_4. These results indicate that BH_4 does not react directly with NO but will react with a product of NO autoxidation. Moreover, the addition of 8 equivalents of NO_2 to an anaerobic BH_4 solution also resulted in a rapid decline in BH_4 similar to that observed in the previous experiment with NO and oxygen (these results are summarized in **Table 1**).

Inhibition of NOS by NO_2 and effect of BH_4: It is well known that NO reacts readily with molecular oxygen to, eventually, generate NO_2. Thus, the possible inhibition of NOS by NO_2 was examined. NO_2 was able to inhibit NOS activity at extremely high initial concentrations. However, at initial concentrations of NO_2 equivalent to those concentrations of NO which caused significant inhibition, no change in NOS activity was observed (**Figure 3**). For example, NO was

Table 1: Relative rates of BH_4 decomposition

	Conditions	Relative rate of BH_4 loss[1]
1.	0.5 mM BH_4 (anaerobic)	no reaction[2]
2.	0.5 mM BH_4 (anaerobic) + O_2 (2 equiv.)[3]	1
3.	(2) + NO (4 equiv.)[3]	5.4
4.	0.5 mM BH_4 (anaerobic) + NO (4 equiv.)[3]	no reaction[2]
5.	(4) + O_2 (2 equiv.)[3]	3.2
6.	0.5 mM BH_4 (anaerobic) + NO_2 (8 equiv.)[3]	4.5

[1] Rates of BH_4 loss determined by measuring the decrease in absorbance at 297 nm [22] after 10 minutes.
[2] No significant decrease in the absorbance at 297 nm observed after 10 minutes.
[3] Addition performed by introduction of the appropriate amount of the pure gas into the cuvette *via* gastight syringe. Equivalents based on BH_4.

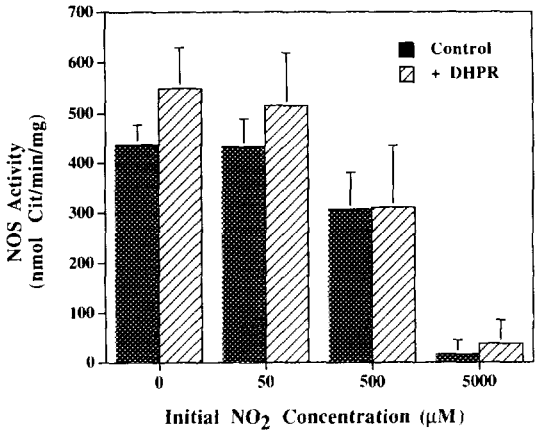

Figure 3. Effects of NO_2 and DHPR on NOS activity. Values represent the averages ± S.D. from 3 separate experiments.

found to inhibit NOS activity by 99% at a 50 μM initial concentration. However, at a 50 μM initial NO_2 concentration, no loss of activity, versus control, was observed. Moreover, at the initial concentrations of NO_2 that did result in inhibition of NOS activity (0.5 mM), no protection by DHPR/NADH was observed.

DISCUSSION

The inhibition of NOS by its product, NO, was first demonstrated by Rogers and Ignarro [16] and has been confirmed thereafter [17-20]. This inhibition may occur by a direct interaction between NO and the iron heme of NOS [12]. Significantly, cytochrome P450, a heme protein with reported similarity to NOS [for example see, 3], has also been shown to be inhibited by NO [23] (although the mechanism of inhibition has not been elucidated). It is also possible that NO may react with thiol groups or other amino acid side chains or prosthetic groups in NOS. For example, adenylyl cyclase was shown to lose calmodulin-mediated stimulation when NO was added, via a proposed oxidation of vicinal thiols in the enzyme [24].

In this study, 10 μM-50 μM NO inhibited the activity of purified rat cerebellar NOS by 70-99% in the presence of 10 μM BH_4 in the reaction mixture. However, the addition of 50-500 μM BH_4 produced concentration-dependent protection of the enzyme from NO-mediated inhibition. This protective effect does not appear to be a general phenomenon of reducing agents since ascorbate was unable to offer any protection and it was previously demonstrated that a large excess of NADPH also had no protective effect [12]. Since BH_4 is unstable with respect to oxygen, these results may indicate that the higher overall BH_4 concentrations, resulting from the higher initial concentrations, were responsible for the observed protection. If this were the case, it would be expected that a BH_4 regenerating system would also exhibit protective effects since it would maintain higher steady state BH_4 levels. This was indeed observed as a regenerating system consisting of DHPR/NADH was also able to attenuate the inhibitory action of NO in concentration-dependent manner.

The mechanism by which BH_4 protects NOS from NO-mediated inhibition is not entirely clear. Several hypotheses for the observed effect can be put forth, however. For example, it is possible that free BH_4 is simply acting as a scavenger for NO. However, this seems unlikely since we have shown that a direct reaction between NO and BH_4 does not occur. It is also possible that an NO oxidation product is the actual NOS inhibitor and that BH_4 is simply scavenging that inhibitory species. For example, since NO is converted to NO_2, *via* air oxidation (reaction 1 and 2 below), it is possible that NO_2 is inhibitory and BH_4 protects NOS by scavenging this species.

1) $NO + O_2 \Leftrightarrow \cdot OONO$
2) $\cdot OONO + NO \Rightarrow 2NO_2$
3) $2NO_2\ (N_2O_4) + H_2O \Rightarrow NO_2^- + NO_3^- + 2H^+$
4) $NO_2 + NO \Leftrightarrow N_2O_3$
5) $N_2O_3 + H_2O \Rightarrow 2NO_2^- + 2H^+$

Also, since we have found that BH_4 will rapidly react with both NO_2 and air oxidized NO (which presumably produces NO_2 as an initial product), the chemistry for such a protective mechanism appears reasonable (**Table 1**). However, we have found that NO_2 is not a good inhibitor of NOS (requiring initial concentrations in excess of 100 μM) and whatever NOS inhibition does occur at high initial NO_2 concentrations was not protected against by DHPR. Thus, it is likely that the observed protection by BH_4 against NO-mediated NOS inhibition was not due to scavenging of NO_2. The inhibition by NO_2 observed at high concentrations was most likely due to nonspecific destruction of NOS and was unrelated to the observed inhibitory effect of NO. Other NO oxidation products such as NO_2^- and NO_3^- (from reactions 3, 4 and 5 above) have been shown not to inhibit NOS and are thus are not involved [12]. Certainly other transient NO oxidation products such as N_2O_3 (from reaction 4 above) may play a role in NOS inhibition. However, this appears unlikely since low concentrations of added NO_2, which would trap any NO generated by the enzyme and presumably raise N_2O_3 levels in the incubation mixture, did not inhibit NOS activity.

The possible role of BH_4 in NOS catalysis has been not been fully elucidated. It has been postulated that it could have a redox role [10] or serve as an allosteric modulator [11, 25]. Herein, we report that BH_4 is capable of protecting NOS against inhibition by its product, NO. Whether this phenomenon is physiologically relevant remains to be determined. The protection afforded NOS by BH_4 in these *in vitro* studies appears to be a direct result of an interaction between BH_4 and the inhibitory NOS-NO species. It has been postulated that NO can inhibit NOS by ligating the catalytic heme function forming an iron-nitrosyl complex. Thus, it is possible that BH_4 is capable of reducing off the inhibitory NO ligand which would result in a regeneration of active enzyme. This would therefore predict that BH_4 would have a non-stoichiometric redox role and that BH_4 was intimately coupled to the redox chemistry of the iron heme. Alternatively, as has been suggested previously, BH_4 may serve to keep a catalytic component of NOS reduced [11] and, possibly prevent the inhibitory actions of NO [12]. In this

scenario, BH_4 would be preventing NO inhibition as opposed to reversing it. Finally, BH_4 may be capable of reducing a nonheme NO-NOS species to regenerate the active enzyme. Again, this would be a non-stoichiometric redox role for BH_4. These and other possibilities are currently under investigation. In conclusion the present study confirms the previous observation that BH_4 is capable of protecting against NO-mediated inhibition of NOS. Moreover, we show that the observed protection is unique to BH_4 and a likely result of an action against NO itself rather than an action against a product from NO autoxidation.

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