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Anti-pterins as Tools to Characterize the Function of Tetrahydrobiopterin in NO Synthase*

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Nitric oxide synthases (NOS) are homodimeric enzymes that NADPH-dependently convert L-arginine to nitric oxide and L-citrulline. Interestingly, all NOS also require (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4Bip) for maximal activity although the mechanism is not fully understood. Basal NOS activity, *i.e.* that in the absence of exogenous H_4Bip, has been attributed to enzyme-associated H_4Bip. To elucidate further H_4Bip function in purified NOS, we developed two types of pterin-based NOS inhibitors, termed anti-pterins. In contrast to type II anti-pterins, type I anti-pterins specifically displaced enzyme-associated H_4Bip and inhibited H_4Bip-stimulated NOS activity in a fully competitive manner but, surprisingly, had no effect on basal NOS activity. Moreover, for a number of different NOS preparations basal activity (percent of V_{max}) was frequently higher than the percentage of pterin saturation and was not affected by preincubation of enzyme with H_4Bip. Thus, basal NOS activity appeared to be independent of enzyme-associated H_4Bip. The lack of intrinsic 4*a*-pterincarbinolamine dehydratase activity argued against classical H_4Bip redox cycling in NOS. Rather, H_4Bip was required for both maximal activity and stability of NOS by binding to the oxygenase/dimerization domain and preventing monomerization and inactivation during L-arginine turnover. Since anti-pterins were also effective in intact cells, they may become useful in modulating states of pathologically high nitric oxide formation.

Pteridines are widespread cofactors in mammalian cells (1) and in lower life forms (2), where they generally function as co-substrates or allosteric regulator molecules. For example, the biosynthesis of nitric oxide (NO),[1] an important messenger molecule with multiple biological functions in blood vessels, neurons, macrophages, and other cells (3), is catalyzed by a family of NO synthases (NOS). So far, three NOS isoforms have been isolated, cloned, and characterized as follows: a neuronal (NOS-I), an inducible (NOS-II), and an endothelial (NOS-III) isoform (4). In view of the multifaceted signaling roles of NO in biology, the molecular mechanisms by which NOS activity is regulated is of primary interest. The different NOS isoforms primarily differ in their mechanisms of activation and expressional regulation (4). Despite these differences, all three NOS isoforms are self-sufficient cytochrome P_{450}-type enzymes containing an intramolecular P_{450} reductase domain in the C terminus. The latter enables the calmodulin-triggered electron transfer from NADPH via enzyme-bound flavins (FAD and FMN) to the N-terminal heme of the NOS oxygenase domain where the oxidation of L-arginine to L-citrulline and NO, or a derivative, takes place (5).

In contrast to other cytochrome P_{450}/cytochrome P_{450} reductase systems, all NOS isoforms require in addition to NADPH the presence of another redox-active cofactor, H_4Bip (6, 7). The reason for this has remained unclear (8) and has prevented a full understanding of the molecular mechanisms of NO synthesis (9). Clues pertaining to the role of H_4Bip may be derived from the catalytic and allosteric roles H_4Bip and other pterins play in nature. For example, H_4Bip acts catalytically as the sole oxygen acceptor for the aromatic amino acid hydroxylases (1); for NOS, this role is already fulfilled by the cytochrome P_{450}-type heme (10, 11). In a recent study (12) it was shown that heme iron reduction can take place independently of the pterin ring oxidation state. Although this would indicate a requirement for fully reduced pterin at a reaction step beyond heme iron reduction, recent evidence (13) suggests that the reduction of the oxyferroheme complex may be the main function of H_4Bip in NOS catalysis. However, if H_4Bip participates in the NOS reaction mechanism similar to aromatic amino acid hydroxylases, namely as an electron donating cofactor, one would expect accessory pterin-4*a*-carbinolamine dehydratase (PCD) to augment the reaction of H_4Bip to quinoid-H_2Bip (q-H_2Bip) or H_4Bip to be recycled by accessory dihydropteridine reductase. Alternatively, NOS may contain intrinsic PCD and dihydropteridine reductase activity. Indeed, some dihydropteridine re-

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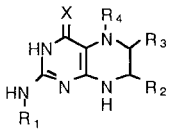
[1] The abbreviations used are: NO, nitric oxide; CHAPSO, 3-[(3-cholamidopropyl)-imethylammonio]-2-hydroxy-1-propansulfonate; H_2Bip, dihydrobiopterin; H_4Bip, 5,6,7,8-L-tetrahydro-L-biopterin; H_2Pte, dihydro-pterin; H_4Pte, tetrahydro-pterin; N+ NOS_{ox}, NOS-I N terminus + oxygenase domain; NOS, NO synthase; NOS*, basally active NOS; NOS°, basally inactive NOS; NOS_{ox}, NOS-I oxygenase domain; NOS_{red}, NOS-I reductase domain; PCD, pterin-4a-carbinolamine dehydratase; PHS, anti-pterin code number; Pte, pterin; q-H_2Bip, quinoid-H_2Bip; TEA, triethanolamine.

TABLE 1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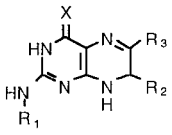
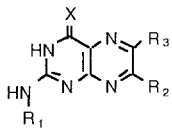
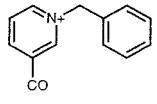
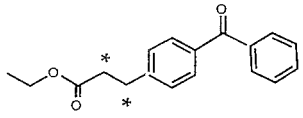

2, H_2Pte


3, Pte

Anti-Pterin (100 μM)		Pterin scaffold and substituents					NOS activity (% of V_{max})		IC_{50}
Type		R_1	R_2	R_3	R_4	X	- H_4Bip	+ 2 μM H_4Bip	(μM)
Control							16.9 ± 0.9	100.0 ± 0.8	
H_2Bip	I **2**	H	H			O	25.8 ± 0.6	18.0 ± 1.2	25
PHS-32	I **1**	H	H	CH_2-O-CO (cyclic R_3/R_4)		O	26.7 ± 0.1	31.4 ± 5.5	35
PHS-203	I **1**	H	H			NH	30.0 ± 0.3	28.0 ± 1.1	9
PHS-52	II **1**	H	H			O	4.8 ± 0.3	10.1 ± 3.5	50
PHS-72	II **1**	$COCH(CH_3)_2$	Ph	H	CSNHPh	O	0.0 ± 0.2	0.1 ± 0.9	16
PHS-176	II **3**	H	H	Ph		O	5.8 ± 3.6	2.3 ± 1.2	50
									

ductase activity of NOS has been described but requires unphysiologically high concentrations of q-H_2Bip, has a low yield (14), and is therefore unlikely to take place under physiological conditions.

H_4Bip can also allosterically regulate the activity of other enzymes (15). In radioligand binding studies, H_4Bip was shown to lower the K_D (16, 17) and, in enzyme kinetic experiments, the K_m of NOS for L-arginine.[2] H_4Bip was shown to stabilize the axial ligand geometry of the NOS heme iron in NOS-I and NOS-II (17). In addition, NOS-II requires the presence of H_4Bip, heme, and L-arginine for dimer assembly in recombinant protein expression systems (18). However, in the case of recombinant NOS-I and NOS-III, dimer formation appears to be pterin-independent and regulated solely by the intracellular availability of heme (19, 20). Related structural effects of H_4Bip that have been described include an increase in temperature stability of NOS-I dimers in the presence of the protein denaturant SDS (16) and reassembly of urea-dissociated dimers (12).

Nevertheless, the function of H_4Bip in NOS catalysis has remained unclear. In order to address this issue, we have developed a photoaffinity label for the NOS pterin-binding site and pharmacologically characterized novel pterin-derived inhibitors of NOS both *in vitro* and in intact cells (anti-pterins; see Ref. 21). NOS-associated H_4Bip and the dimerization state of NOS was compared with basal NOS activity. In addition, we investigated the possible PCD dependence or intrinsic PCD activity of NOS.

EXPERIMENTAL PROCEDURES

Materials—[2,3,4,5-^{3}H]L-Arginine hydrochloride (2.85 TBq/mmol) was purchased from Amersham Pharmacia Biotech (Freiburg, Germany); H_4Bip and H_2Bip were from Dr. B. Schircks Laboratories (Jona, Switzerland); NADPH was from Applichem (Darmstadt, Germany); FAD, FMN, and GSH were from Boehringer Mannheim (Mannheim, Germany); all high pressure liquid chromatography solvents were obtained from Roth (Karlsruhe, Germany); all cell culture materials were from Life Technologies, Inc. (Eggenstein, Germany). 6-Methyl-7,8-dihydropterin-4*a*-carbinolamine was prepared as described previously (22). Purified human PCD (23) was a generous gift from Dr B. Thöny (Zürich, Switzerland). All other chemicals were of the highest purity grade available and obtained from either Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Water was deionized to 18 MΩ cm (Milli-Q; Millipore, Eschborn, Germany), and de-oxygenated with argon.

Enzyme Purification—NOS-I was isolated from porcine cerebellum by ammonium sulfate precipitation and 2′,5′-ADP-Sepharose affinity chromatography as described previously (24). To exclude the possibility that this purification method influenced the inhibitor profile of the anti-pterins, NOS-I was alternatively purified by DEAE-ion exchange chromatography (200 mM NaCl, elution buffer; 20–25 ml min^{-1}, flow rate) instead of ammonium sulfate precipitation. Native enzyme had a specific activity of up to 670.0 nmol mg^{-1} min^{-1}. Recombinant NOS-I was expressed in a baculovirus/Sf9 cell system, purified by 2′,5′-ADP-Sepharose and calmodulin affinity chromatography to a specific activity of up to 217.1 nmol mg^{-1} min^{-1}.

Protein Determination—Protein concentrations were determined spectrophotometrically according to the method of Bradford (25).

Enzyme Kinetic Analysis—NOS activity was determined as formation of [^{3}H]L-citrulline from [^{3}H]L-arginine as described previously (24). Unless otherwise indicated NOS-I was incubated at pH 7.2 for 15 min at 37 °C in a total volume of 100 μl of 50 mM triethanolamine HCl (TEA) buffer containing 25 μM L-arginine (5.55 kBq), 1 mM NADPH, 5 μM FAD, 10 μM FMN, 50 nM calmodulin, 1 mM $CaCl_2$, 250 μM CHAPSO, and in the presence (maximal activity) or absence of 2 μM H_4Bip (basal activity). EC_{50} and K_m values were estimated by non-linear regression analysis (Prism; GraphPad, San Diego, CA). The results shown represent mean values ± S.E. of 3–4 experiments, each performed in triplicate.

Determination of NOS-associated Flavins—To determine the total amount of NOS-associated flavins (26), NOS was incubated at 37 °C with 4 M urea (in 50 mM HEPES, pH 7.5) for 4 h; to determine the

[2] P. Kotsonis, M. La, A. Reif, and H. H. H. W. Schmidt, unpublished observations.

spontaneous release of flavins NOS was incubated at 37 °C for 15 min (50 mM HEPES, pH 7.5) in the absence or presence of anti-pterins. The low molecular mass fraction, containing flavins that dissociated from NOS, was separated by centrifugation (10,000 × *g*, 4 °C, 20 min) using filters with a 10-kDa molecular mass cut-off (Ultrafree MC; Millipore, Eschborn, Germany). Samples were analyzed by reverse-phase high pressure liquid chromatography with fluorometric detection (λ_{ex} = 460 nm, λ_{em} = 530 nm) with a LiChroCart 250/4 LiChrospher 100 RP18 (5 μM) columns (Merck, Darmstadt, Germany) at a flow rate of 1 ml min^{-1} (linear gradient from 100% 5 mM NH_4OAc, pH 6, and 0% CH_3CN at 0 min to 96% NH_4OAc and 4% CH_3CN at 30 min). FAD and FMN content was 1.0 (FAD) and 0.7 (FMN) mol per subunit of NOS.

Determination of NOS-associated Pterin—Purified native porcine or recombinant human NOS-I (0.6–0.8 μg) was incubated in buffer (50 mM TEA, pH 7.2, 50 μM L-arginine, 1 mM $CaCl_2$, 5 μM FAD, 10 μM FMN, 250 μM CHAPSO, 1 mM NADPH) for 15 min at either 4 or 37 °C in the absence or presence of 100 μM PHS-32; where indicated, L-arginine was omitted. The reaction was stopped by adding ice-cold Tris/HCl, pH 6.7, and the samples were centrifuged (10,000 × *g*, 4 °C, 20 min) through 10-kDa cut-off filters. The low molecular mass filtrate contained pterin displaced from NOS-I. To determine enzyme-associated pterin, the NOS-containing residue was re-dissolved in ice-cold Tris/HCl, pH 6.7, and immediately oxidized with 0.2 M I_2 and 0.5 M KI in the presence of either 0.5 M HCl (acidic oxidation) or 0.5 M NaOH (alkaline oxidation) for 1 h at room temperature in the dark. The quantitative difference between the values obtained under acidic and alkaline conditions is a measure of H_4Bip (27). Samples oxidized under alkaline conditions were acidified with 1 M HCl followed by 0.1 M ascorbic acid; to those oxidized under acidic conditions, only water and ascorbic acid were added. All samples were analyzed by reverse-phase high pressure liquid chromatography using a LiChroCart 250/4 Purospher RP 18 (5 μm) column (Merck, Darmstadt, Germany) with fluorometric detection (λ_{ex} = 352 nm, λ_{em} = 438 nm), a flow rate of 1 ml min^{-1}, and an isocratic elution with 0.015 M KH_2PO_4/K_2HPO_4, pH 6.0.

Determination of NOS Activity in Intact Cells—N_1E-115 cells, a murine neuroblastoma cell line expressing NOS-I, were cultivated as monolayers in Dulbecco's modified Eagle's medium (1 g of glucose $liter^{-1}$) supplemented with 10% fetal calf serum, 2% newborn calf serum, 200 mM L-glutamine, non-essential amino acids, 5.5 mg of glucose $liter^{-1}$, 1% penicillin/streptomycin. To determine NOS activity, the cells were incubated in Krebs-Ringer buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin, 0.1 mM glucose). NO formation and release were quantified spectrophotometrically as the degradation product NO_2^- using the Griess reaction (28).

Fast Protein Liquid Chromatography Analysis of NOS Quaternary Structure—Purified recombinant human NOS-I (48 μg) was incubated for 15 min under conditions identical to the enzyme kinetic analysis in the absence or presence of 2 μM H_4Bip. The monomer/dimer ratio was subsequently determined by size-exclusion chromatography (Superose 6 HR 10/30, Amersham Pharmacia Biotech, Freiburg, Germany; 4 °C; 20 mM TEA, pH 7.5, 150 mM NaCl, 5% ethylene glycol; flow rate 0.25 ml min^{-1}). As standard, a gel filtration calibration kit (Sigma, Deisenhofen, Germany) was used: carboanhydratase (Stokes radius: 2.01 nm), albumin (3.55 nm), alcohol dehydrogenase (4.55 nm), apoferritin (6.1 nm), and thyroglobulin (8.5 nm). The void volume (V_0) was determined with dextran blue.

Dehydratation of 6-Methyl-4a-carbinolamine—The dehydration of 6-methyl-7,8-dihydropterin-4*a*-carbinolamine was monitored spectrophotometrically (245 nm) as described previously (22). Briefly, 12 μg of NOS or 4 μg of human wild-type PCD (11.9 kDa per subunit) were incubated for 23 or 10 min in a total volume of 1 ml of 10 mM Tris/HCl, pH 7.4, containing 55 μM 6-methyl-7,8-dihydropterin-4*a*-carbinolamine. Alternatively, 4 μg of NOS were incubated in the presence of 286 μM NADPH, 50 nM calmodulin, and 2 mM $CaCl_2$ but otherwise identical conditions.

Expression, Purification, and Analysis of NOS-I Domains—Three domains of human NOS-I (29), containing amino acid residues 1–708 (N terminus + oxygenase domain, N + NOS_{ox}; 83 kDa), 367–772 (oxygenase domain, NOS_{ox}; 44 kDa), or 782–1281 (reductase domain, NOS_{red}; 55 kDa), were subcloned into the expression plasmid pET29 (R & D Systems, Abingdon, UK) and expressed as S-tag fusion proteins. *Escherichia coli* (BL21, R & D Systems, Abingdon, UK) was grown at 37 °C in LB and supplemented with 10 μM δ-aminolevulinic acid and 5 μM riboflavin in the presence of kanamycin (30 μg l^{-1}) and chloromycin (20 μg l^{-1}) to an $A_{600} \approx 0.3$. After induction with 0.5 mM isopropyl-β-D-thiogalactoside, cells were grown for a further 2 h at 23 °C and lysed by sonification. Each NOS domain was expressed with equal efficiency as verified by anti-S-tag protein immunoblot (not shown). Purification of the S-tagged domains and immunodetection were performed according to the protocol of the supplier (R & D Systems, Abingdon, UK).

Photoaffinity Labeling with [^{3}H]PHS-176—For photoaffinity labeling, identical amounts of purified NOS-I domains were incubated for 30 min with 185 kBq of [^{3}H]PHS-176, a type II anti-pterin at 4 °C in the dark. As a control, untransfected *E. coli* cells were used. Photocovalent modifications with [^{3}H]PHS-176 were performed by irradiation at 360 nm for 120 min at 4 °C (30). Bound radioligand was separated from unbound radioligand by precipitation with ethanol and measured by liquid scintillation counting. The specificity of labeling was established by competition of [^{3}H]PHS-176 with 1 mM unlabeled anti-pterin. The level of nonspecific photoaffinity labeling was 31.2 ± 6.6% of total labeling as typical for this type of low affinity ligand (31–33). The concentration of unlabeled anti-pterins (1 mM; *i.e.* 20-fold over their IC_{50} values) used to show specificity and competition with respect of radiolabel incorporation was well in the range generally used to compete for [^{3}H]H_4Bip binding (34). Since there was no correlation between the molar extinction coefficient ε and inhibition of photoaffinity labeling, a nonspecific absorbance effect of the different anti-pterins can be excluded (data not shown).

RESULTS

Anti-pterins, a Novel Class of Pterin Site-specific NOS Inhibitors—From over 300 racemic pterin derivatives, 53 anti-pterins markedly inhibited NOS-I activity regardless of the oxygenation state of the pterin backbone: tetrahydro-, dihydro- (H_2Pte), or aromatic pterin (Pte). The six most potent inhibitors were chosen as lead compounds to investigate the function of H_4Bip within NOS-I catalysis (Table I). To exclude the possibility that the purification method for NOS-I influenced the anti-pterin structure-activity relationship, these anti-pterins were additionally assayed using native NOS-I purified by alternative methods (DEAE-ion exchange chromatography instead of ammonium sulfate precipitation). In both cases, similar inhibitor profiles were obtained (data not shown).

To establish the specificity of anti-pterins for the NOS-I pterin-binding site and to exclude any interference with other known cofactor/substrate-binding domains, PHS-32 was studied in greater detail. Although the V_{max} value of NOS was decreased by PHS-32 (Fig. 1), the EC_{50} values for L-arginine were not significantly changed and the EC_{50} values for NADPH even slightly increased (data not shown). In addition, there was no anti-pterin-induced loss of enzyme-bound flavins (FAD, FMN; Fig. 2). These data make a direct interaction of PHS-32 with the L-arginine, NADPH, and flavin-binding sites of NOS unlikely.

Enzyme kinetic experiments revealed distinct inhibitor profiles, and therefore anti-pterins were classified into two types. Type I anti-pterins antagonized the concentration-dependent stimulation of purified NOS-I by H_4Bip in a classical competitive manner (Fig. 1*A*). The dose-response curve to H_4Bip was shifted to the right in the presence of 100 μM PHS-32 from 0.15 ± 0.08 to 9.2 ± 5.15 μM. The inhibitory effect of PHS-32 on NOS was markedly although not fully reversed by H_4Bip. Interestingly, this was due to the fact that H_4Bip at high concentrations (>100 μM) itself had an inhibitory effect on NOS-I. In contrast, inhibition by type II anti-pterins was not fully competitive with respect to H_4Bip (Fig. 1*B*). This may be related to an interaction of the more complex chemical substituents of type II anti-pterins at residues R_3 or R_4 (Table I) with the recently identified hydrophobic exosite next to the NOS pterin-binding site (35, 36). Importantly, the inhibition by the prototypic anti-pterins PHS-32 and PHS-72 was fully reversible within 1 min when H_4Bip was added, and the inhibitor was diluted to a subthreshold concentration.[2] Interestingly, the inhibition of NOS by type I anti-pterins was always submaximal with no effect on basal activity (*i.e.* activity observed without addition of H_4Bip). Consistent with this, H_4Bip-stimulated enzyme activity could only be inhibited to the level of basal

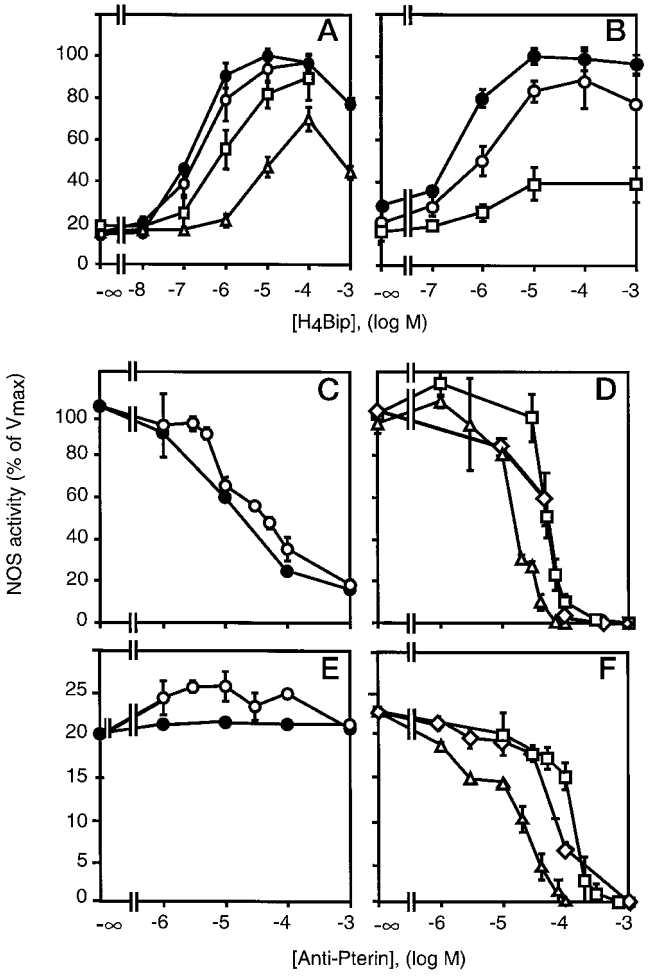


FIG. 1. **Enzyme kinetic analysis revealed distinct anti-pterin inhibitor profiles.** *A* and *B*, concentration-dependent stimulation of basal activity of purified porcine cerebellum NOS-I by H_4Bip in the absence and presence of the type I anti-pterin PHS-32 (*A*, ●, control; ○, 0.5 μM; □, 5 μM; △, 100 μM) and the type II anti-pterin PHS-72 (*B*, ●, control; ○, 10 μM; □, 30 μM). *C–F*, concentration-dependent inhibition of H_4Bip (2 μM)-stimulated (*C* and *D*) or basal (*E* and *F*) NOS activity by type I (*C* and *E*, ○, PHS-32; ●, H_2Bip) and type II anti-pterins (*D* and *F*, □, PHS-52; △, PHS-72; ◇, PHS-176).

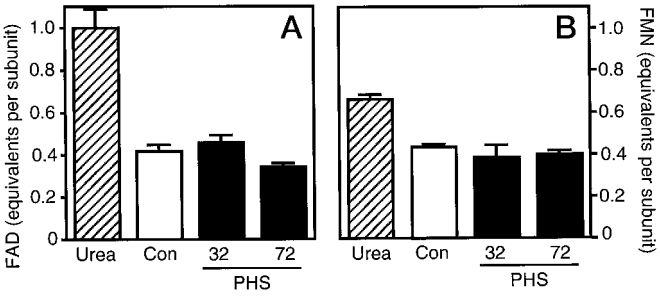


FIG. 2. **No interaction of anti-pterins with the NOS flavin-binding sites.** The displacement of flavins (*A*, FAD; *B*, FMN) from purified porcine NOS-I was determined in the absence (*Con*, control) or presence of 100 μM PHS-32 or PHS-72 as described under "Experimental Procedures." To determine the total amount of NOS-associated flavins, NOS was incubated in 4 M urea.

activity (Fig. 1, *C* and *E*). In contrast, type II anti-pterins abolished both basal and H_4Bip-stimulated NOS activity (Fig. 1, *D* and *F*).

Anti-pterins not only inhibited purified NOS-I but were also effective inhibitors of NO release from intact cells, *i.e.* in the neuronal cell line N_1E-115 which is known to express NOS-I.

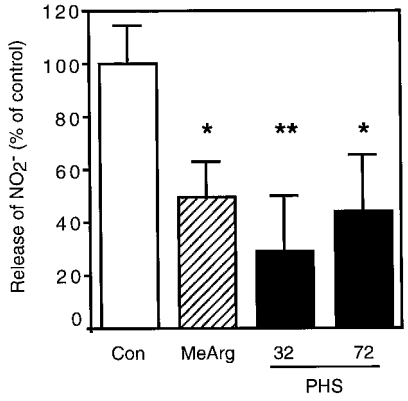


FIG. 3. **Anti-pterins are potent NOS inhibitors in intact cells.** N_1E cells (2×10^6 cells), a cell line expressing NOS-I, were incubated in Krebs-Ringer buffer in the absence (*Con*, control) or presence of 300 μM N^{ω}-methyl-L-arginine or 200 μM anti-pterin (PHS-32 or PHS-72) for 70 min. NOS activity was stimulated by the addition of the calcium ionophore A23187 (10 μM) 10 min after the addition of inhibitors. NO formation and release was measured as described under "Experimental Procedures." Cell viability, as determined by trypan blue exclusion, did not differ significantly between groups. Statistics were performed using Dunnett multiple comparison tests; *, $p < 0.05$; **, $p < 0.01$.

Ten minutes after the addition of inhibitors, NOS activity was stimulated by adding the Ca^{2+} ionophore A23187 (10 μM). Ca^{2+}-induced NO formation and release, determined as nitrite and nitrate accumulation over time (28), was markedly inhibited by both types of anti-pterins (Fig. 3) without affecting cell viability (data not shown). Moreover, the efficacy of anti-pterins was similar to that of N^{ω}-methyl-L-arginine, an arginine-based NOS inhibitor.

Basal NOS Activity Is Independent of Enzyme-associated H_4Bip—The type I anti-pterin PHS-32 had no effect on basal NOS activity (Fig. 1*E*) but significantly displaced endogenous NOS-associated H_4Bip (Fig. 4*A*). These data provided a first indication that basal NOS activity has no absolute requirement for H_4Bip. In contrast, the full inhibition of basal activity by the type II anti-pterins was not due to loss of endogenous H_4Bip from NOS (90 ± 7% of control, 100 μM PHS-72). It is possible that type II anti-pterins can only bind to the unoccupied, low affinity pterin-binding site of the NOS dimer (37). Nevertheless, inhibition by PHS-72 was fully reversible and inhibition was partially competitive with respect to H_4Bip (Fig. 1*B* and Fig. 6*A*). The exact mechanism by which type II anti-pterins inhibit NOS activity and where they position within NOS oxygenase domain (see below) is subject of further investigations.

We then examined a possible correlation between basal NOS activity (percent of V_{max}) and endogenous H_4Bip content (percent of NOS monomers). Importantly, basal NOS activity was measured during the initial 3 min of catalysis because only then L-arginine turnover was linear. Surprisingly, several NOS preparations had a higher basal activity (percent of V_{max}) than expected from their percentage of pterin saturation (percent molar pterin content per mol of NOS subunit), *e.g.* 52.7 *versus* 24.4, 41.3 *versus* 33.4, 49.6 *versus* 3.5, and 25.0 *versus* 18.0 for native enzyme, and 37.22 *versus* 6.2, 24.4 *versus* 8.3, 26.0 *versus* 0.17, and 39.9 *versus* 7.2 for recombinant enzyme. Among the different NOS-I preparations that were examined, there was one that was entirely independent of H_4Bip in the first 3 min of L-arginine turnover and yet only partially pterin-saturated. These data suggested that basal NOS activity is at least partially H_4Bip independent.

We then investigated whether basal NOS activity can be elevated by saturating all available high affinity pterin- bind-

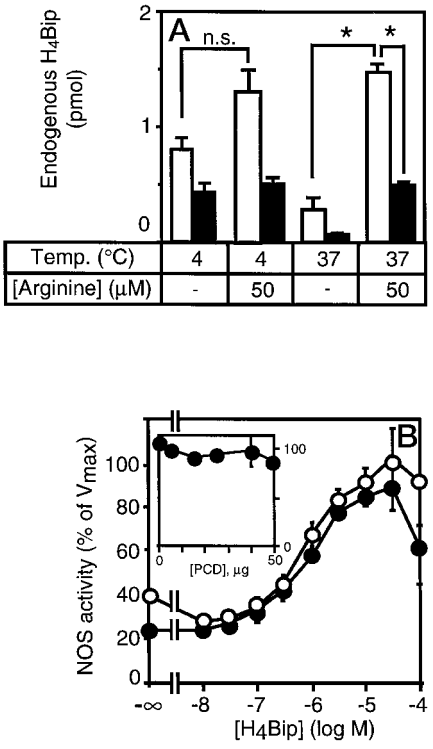


FIG. 4. **Basal NOS activity seems to be H_4Bip-independent.** *A*, purified porcine cerebellum NOS-I (0.6–0.8 μg) was preincubated for 15 min at 4 or 37 °C with or without L-arginine (50 μM) and in the absence (*open bar*) or presence (*closed bar*) of 100 μM PHS-32. NOS-I-derived pterin was determined as described under "Experimental Procedures." Similar results were obtained with recombinant human NOS-I. *B,* concentration-dependent stimulation of purified porcine NOS-I by H_4Bip in the absence (○) or presence (●) of 5 μg of PCD. Both V_{max} of NOS and the EC_{50} of H_4Bip were unchanged. The *inset* shows additionally that greater amounts of PCD (up to 50 μg) had no effect on V_{max} of NOS. *A* and *B*, one-way analysis of variance was followed by the Dunnett multiple comparison test. * indicates $p < 0.05$ and was accepted as level of significance between different treatments; *n.s.,* non-significant.

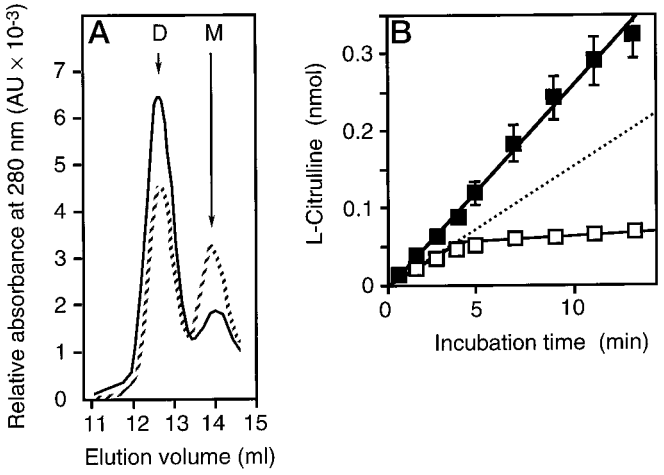


FIG. 5. **Tetrahydrobiopterin stabilizes NOS quaternary structure during L-arginine turnover.** *A*, formation of NOS-I monomers under catalytic active conditions is inhibited by exogenous H_4Bip. Purified recombinant human NOS-I (48 μg) was incubated for 15 min under conditions identical to the NOS activity assay in the absence (*dashed line*) or presence (*solid line*) of 2 μM H_4Bip. The chromatogram shown is representative of 4 independent experiments using two different NOS-I preparations (*D*, NOS-I dimer; *M*, NOS-I monomer). *B*, time course of L-citrulline formation in the absence (□) or presence of 2 μM H_4Bip (■). The *dashed line* would indicate a linear basal NOS activity over time.

ing sites of NOS-I. Purified porcine cerebellum NOS-I (6 μg ml^{-1} = 18.7 nM NOS dimer) was preincubated under non-catalytic conditions, *i.e.* in the absence of Ca^{2+}/calmodulin, for 5 min at 37 °C in a final volume of 6 μl of 25 mM TEA buffer, pH 7.2, containing 50 μM L-arginine either in the absence or presence of 100 nM H_4Bip. The concentration of H_4Bip used in the preincubation of 100 nM was in 5.3-fold molar excess over the NOS-I enzyme concentration and almost 2-fold above the EC_{50} value of H_4Bip for this preparation (66 ± 4 nM; n = 6). The direct addition of this concentration of H_4Bip to the assay mixture increased NOS-catalyzed L-arginine turnover from 23.40 ± 8.51% of V_{max} to 72.34 ± 4.26% of V_{max}. However, dilution of the preincubation mixture to a final concentration of 6 nM H_4Bip in the subsequent activity assay resulted in a complete loss of the stimulating effect of H_4Bip on NOS-catalyzed L-arginine turnover. In this case, NOS activity was identical to enzyme that had not been preincubated with 100 nM H_4Bip and was assayed only in the presence of 6 nM H_4Bip (17.02 ± 6.3% of V_{max}).

PCD and NOS—Evidence against a classical redox role of H_4Bip in stimulating NOS activity came from experiments investigating the possible PCD activity of NOS. In aromatic amino acid hydroxylases, the intermediate 4α-hydroxy derivative of H_4Bip is converted to q-H_2Bip (1). This conversion occurs spontaneously (with some side reactions to 7, 8-H_2Bip) and is augmented by pterin-4α-carbinolamine dehydratase (PCD). However, neither the basal NOS activity, V_{max}, nor the potency of H_4Bip to stimulate NOS was affected by co-incubations with PCD (5–50 μg; Fig. 4*B*), suggesting H_4Bip was either not cycling via a 4α-peroxy-H_4Bip/q-H_2Bip step or NOS was able to catalyze this step itself in a PCD-independent manner. The latter possibility was examined by measuring the PCD activity of NOS-I. However, up to 12 μg of NOS-I was devoid of any significant effect on the dehydration of 6-methyl-7,8-dihydropterin-4α-carbinolamine, a synthetic substrate for PCD, whereas 4 μg of PCD under identical conditions converted 11.5 nmol s^{-1}. Adding NOS-specific cofactors, NADPH, Ca^{2+}/calmodulin, had no effect. These data collectively exclude a classical redox cycling of H_4Bip.

H_4Bip, a NOS Stabilizing Cofactor—We also investigated a possible stabilizing role of H_4Bip on NOS enzyme structure during catalysis by size-exclusion chromatography in the absence and presence of H_4Bip (Fig. 5*A*). Purified NOS-I eluted as two protein peaks, a larger one at 12.82 ± 0.01 and a smaller one at 13.95 ± 0.05 ml corresponding to the known Stokes radii of 7.59 ± 0.01 and 6.24 ± 0.06 nm of the dimer (D) and monomer (M), respectively (27). The NOS monomer was inactive with respect to L-arginine conversion and contained no pterin[2]; it therefore behaved similarly to previously reported monomers of possibly incompletely dimerized NOS in heterologous expression systems or induced macrophages (34, 38). The addition of 2 μM H_4Bip significantly decreased the relative abundance of NOS monomers from 40.7 ± 1.7 to 27.5 ± 1.0% of total NOS-I, equivalent to a near doubling of the dimer/monomer ratio from 1.5 ± 0.1 to 2.7 ± 0.1.

We also analyzed the time course of NOS activation in the absence and presence of 5 μM exogenous H_4Bip (Fig. 5*B*). In the absence of H_4Bip, not only the initial L-citrulline formation was lower than in the presence of H_4Bip but the enzyme also inactivated more rapidly. The spontaneous inactivation of NOS-I in the absence of H_4Bip after 3 min of L-arginine turnover was prevented if H_4Bip was added at the start of the incubation. Then the rate of L-citrulline formation remained linear at V_{max} for at least 15 min. Interestingly, when the addition of H_4Bip was slightly delayed (at t = 4 or 6 min) the inactivation process

was prevented. At later time points, inactivation became increasingly irreversible.[2] These data indicate a stabilizing role for H_4Bip in NOS catalysis during L-arginine turnover. The requirement of H_4Bip as a stimulator of NOS activity at the onset of catalysis is in apparent conflict with a previous report (39) showing that H_4Bip is not essential during the first minutes of NOS incubation and enhances NO synthesis only after several minutes of incubation. However, the main finding of our kinetic experiments is the dramatic stabilizing effect of H_4Bip in the course of L-arginine turnover. This effect became evident and quantitatively important at later time points of incubation, *i.e.* >3 min and is in excellent agreement with most of the previous reports on the time course of L-arginine turnover by different NOS isoforms (5, 8, 39–42).

The Pterin-binding Site—Given that our findings suggested a role for H_4Bip in dimer stabilization during L-arginine turnover, we wanted to investigate whether the NOS pterin-binding site co-localizes within the oxygenase/dimerization domain. To identify the binding site of our anti-pterin compounds, we performed photoaffinity labeling of using the photolabile, tritiated type II anti-pterin PHS-176 and three domains of human NOS-I (29) as follows: the N terminus including the putative NOS-I oxygenase domain (N + NOS_{ox}; 83 kDa) containing amino acid residues 1–708, the oxygenase domain (NOS_{ox}; 44 kDa) containing amino acid residues 367–772, and the reductase domain (NOS_{red}; 55 kDa) containing amino acid residues 782–1281. All three domains were subcloned into the expression plasmid pET29 and expressed as S-tag fusion proteins. The binding of [^{3}H]PHS-176 occurred preferentially in the oxygenase/dimerization domain, whereas the C-terminal reductase domain of NOS-I was not labeled (Fig. 6*B*). Interestingly, the N terminus of NOS-I lowered anti-pterin binding to the oxygenase/dimerization domain. This may differentiate NOS-I from the other two NOS isoforms that have shorter N termini.

DISCUSSION

The present study investigates the function of H_4Bip in NO synthesis. We developed pterin-derived inhibitors of NOS to characterize the pterin-binding site of native NOS and the mechanism of activation by H_4Bip. Enzyme kinetic studies revealed two distinct inhibitor profiles. Type I anti-pterins inhibited the H_4Bip-stimulated NOS activity without inhibiting basal NOS activity, *i.e.* the activity that is observed in the absence of exogenous H_4Bip. Previously, basal NOS activity had been generally accepted to be due to tightly bound, endogenous H_4Bip that co-purifies with NOS and cannot be readily displaced (43). Surprisingly, the type I anti-pterin PHS-32 under different conditions effectively displaced endogenous pterin (>80%) without any effect on basal enzyme activity. The most likely explanation for this finding is that the basal activity of NOS is H_4Bip-independent. The possibility that PHS-32 (a non-aromatic pterin) acted as partial agonist was ruled out by recent studies with pterin-free NOS which is not activated by PHS-32.[2]

In several native and recombinant NOS-I preparations, no correlation between the endogenous H_4Bip content and basal initial L-citrulline formation was found. We propose that purified NOS-I is comprised of two conformational states as follows: one is responsible for a H_4Bip-independent, basally active enzyme (NOS*), the remaining enzyme (NOS°) is inactive or immediately inactivates in the absence of exogenous H_4Bip.

In a subsequent series of preincubation experiments, we observed that NOS° cannot be converted to NOS* by a short exposure to H_4Bip prior to the activity assay. This excluded again the possibility that binding of H_4Bip alone modifies the enzyme and is sufficient to induce basal activity or NOS*. Moreover, since NOS°-specific anti-pterins like PHS-32 were also effective inhibitors of NO synthesis in intact cells, NOS° is not a purification artifact and is physiologically co-expressed with NOS*. The cellular regulation of NOS* and NOS° may be an important mechanism of physiologically controlling NOS activity and may explain some clinical observations. For example, in some patients with phenylketonuria, the underlying cause of this disease is an impaired ability to synthesize H_4Bip (1). However, as these patients do not show symptoms that might be expected if H_4Bip deficiency led to decreased NOS activity with consequent impairment in immune function, blood pressure, and neuronal regulation, NOS in these patients might be at least partly independent of H_4Bip.

In addition, in all enzymes utilizing H_4Bip as an electron donor and oxygen acceptor, the intermediate 4*a*-hydroxypterin is converted to the q-H_2Bip. This conversion occurs spontaneously (with some side-reactions to 7,8-H_2Bip) and is augmented by PCD. In the present study using NOS-I, PCD failed to increase basal NOS activity, V_{max}, or the affinity for H_4Bip. Moreover, NOS-I does not contain PCD activity itself.

Since all our above findings collectively suggested that endogenous H_4Bip alone is not sufficient to account for basal NOS activity, we investigated other possible roles of H_4Bip. When we examined the effect of H_4Bip on the native quaternary structure of catalytically active NOS-I, a stabilizing function of H_4Bip was revealed. Purified NOS-I exists as a stable homodimer (27), and monomerization was only described to occur under denaturing conditions, *i.e.* in the presence of 4% SDS (16) or 5 M urea (12). However, we observed monomerization to occur also during L-arginine turnover, *i.e.* under physiological conditions. NOS-I was found to rapidly dissociate into inactive monomers in a time-dependent manner[2] correlating with a dramatic loss of activity after 3 min of incubation. The addition of H_4Bip prevented both the dissociation into inactive monomers as well as the corresponding loss in activity during turnover. Although dimer stabilization may not explain all of the actions of H_4Bip on NOS, it may contribute to the prevention of activity loss after several rounds of L-arginine turnover, and it is, thus, conceivable that H_4Bip is not essential from the start of NOS catalysis. A stabilizing effect of H_4Bip is further supported by a recent report showing that H_4Bip prevented monomerization of the NOS-II_{ox} domain (44). The dependence of NOS* on H_4Bip after 3 min of incubation may explain why expression of NOS under pterin-free conditions has resulted in less stable enzymes with reduced activity requiring H_4Bip from the start (17, 20, 37, 45). Most likely, the expression of NOS under these conditions predominantly yields NOS°.

In agreement with a stabilizing effect of H_4Bip within the catalytic center of NOS, we localized the NOS-I pterin-binding site to a 341-amino acid sequence of the oxygenase/dimerization domain (NOS_{ox}; see Ref. 46) using a ^{3}H-labeled type II anti-pterin ([^{3}H]PHS-176) as a photoaffinity probe. Interestingly, the N terminus of NOS-I, specific for this isoform, appears to modulate pterin binding. The inclusion of heme in this fragment agrees with an intimate H_4Bip-heme-arginine interaction. Recently, the crystal structure of the monomeric NOS-II oxygenase domain (35) and, while this manuscript was being prepared, additional structural information on the dimeric, pterin-containing oxygenase domain of NOS-II became available (36). The localization of the pterin-binding site in this domain is in excellent agreement with all our present findings. Taken together, a role of H_4Bip in electron transfer and classical H_4Bip cycling appears to be unlikely. However, a more recent study suggests that electron transfer, *i.e.* the reduction of the oxyferroheme complex, is indeed a main function of H_4Bip during NOS catalysis although direct heme to heme electron transfer remains a possibility (13). Clearly, further

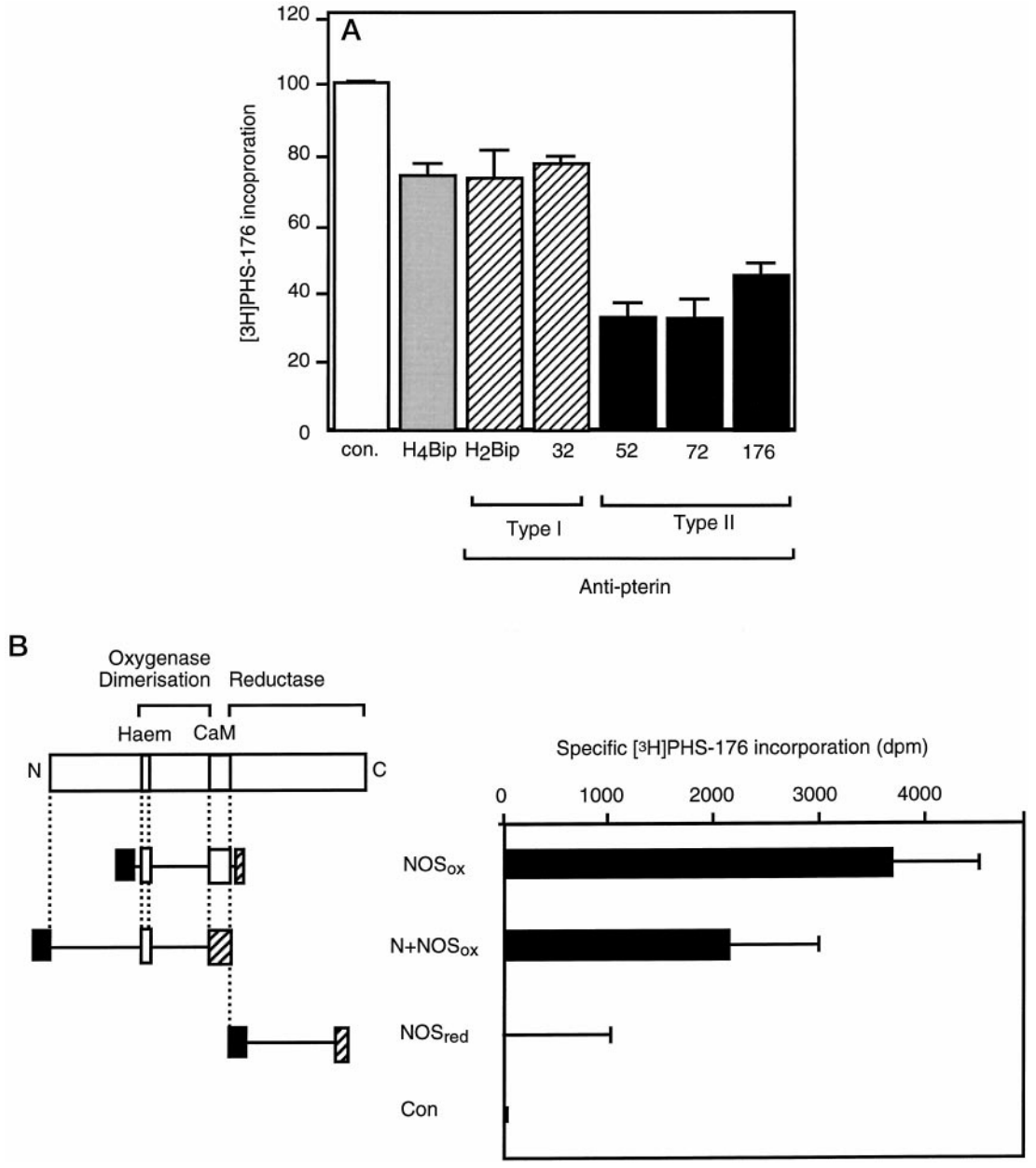


FIG. 6. **Photoaffinity labeling of the NOS pterin-binding site.** *A*, using a pterin site photoaffinity probe based on type II anti-pterins, NOS-I was specifically labeled. H_4Bip (*gray bar*) and type I anti-pterins (*hatched bars*), which failed to interact with an additional binding site (exosite) formed by type II anti-pterins, displaced the label only to a smaller extent. Unlike a radioligand binding assay, competition was expected to be incomplete. Type II anti-pterins (*solid bars*), which occupied both the pterin-binding site and the exosite site, competed more effectively with the photoaffinity label. This difference between types I and II anti-pterins was identical to their pharmacological classification. The molar extinction coefficient for each compound did not correlate with the respective degree of label displacement. Thus, it is unlikely that interference with light absorption during the photoactivation process interfered with photocovalent modification. Moreover, all combined NOS cofactors (5 mM L-arginine, 1 mM FAD, 1 mM FMN, 100 mM NADPH, 5 μM calmodulin) failed to influence the photoaffinity labeling (100 ± 4.5% control; 94 ± 9.7 in the presence of cofactors). *B*, the NOS-I pterin-binding domain was directly localized by photoaffinity labeling to a fragment between the heme and calmodulin-binding sites (Ile^{367}–Ser^{708}). For photoaffinity labeling identical amounts (verified by anti-S-tag protein immunoblot) of purified NOS-I domains (N + NOS_{ox}, 1–708; NOS_{ox}, 367–772; NOS_{red}, 782–1281) or as a control (*Con*), untransfected *E. coli* cells were used. Only specific labeling is shown. Results represent mean values ± S.E. (n = 3). Similar results were obtained in 3 independent expression experiments independent of whether the NOS domains were purified or whether total cell lysate was used. *Solid bar*, S-tag with thrombin site; *hatched bar*, C-terminal extension.

studies are needed to resolve this controversy.

In summary, H_4Bip may act by preventing monomerization during L-arginine turnover without having an essential role in basal NOS activity. This finding contrasts the previously well characterized function of H_4Bip as a redox-active cofactor and oxygen acceptor in aromatic amino acid hydroxylases and other enzymes. A PCD-like activity in NOS or PCD-augmented step in NOS catalysis is unlikely. In addition, we provide the first comprehensive report of anti-pterins as a new class of pharmacological lead compounds to modulate cellular NO formation with therapeutic potential in conditions of pathologically high NO formation.

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