Allosteric regulation of neuronal nitric oxide synthase by tetrahydrobiopterin and suppression of auto-damaging superoxide

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The underlying mechanisms regulating the activity of the family of homodimeric nitric oxide synthases (NOSs) and, in particular, the requirement for (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H₄Bip) are not fully understood. Here we have investigated possible allosteric and stabilizing effects of H₄Bip on neuronal NOS (NOS-I) during the conversion of substrate, L-arginine, into L-citrulline and nitric oxide. Indeed, in kinetic studies dual allosteric interactions between L-arginine and H₄Bip activated recombinant human NOS-I to increase L-arginine turnover. Consistent with this was the observation that H₄Bip, but not the pterin-based NOS inhibitor 2-amino-4,6-dioxo-3,4,5,6,8,8a,9,10-octahydro-oxazolo[1,2-*f*]-pteridine (PHS-32), caused an L-arginine-dependent increase in the haem Soret band, indicating an increase in substrate binding to recombinant human NOS-I. Conversely, L-arginine was observed to increase in a concentration-dependent manner H₄Bip binding to pig brain NOS-I. Secondly, we

investigated the stabilization of NOS quaternary structure by H₄Bip in relation to uncoupled catalysis. Under catalytic assay conditions and in the absence of H₄Bip, dimeric recombinant human NOS-I dissociated into inactive monomers. Monomerization was related to the uncoupling of reductive oxygen activation, because it was inhibited by both superoxide dismutase and the inhibitor *N*^ω-nitro-L-arginine. Importantly, H₄Bip was found to react chemically with superoxide (O₂^{•−}) and enzyme-bound H₄Bip was consumed under O₂^{•−}-generating conditions in the absence of substrate. These results suggest that H₄Bip allosterically activates NOS-I and stabilizes quaternary structure by a novel mechanism involving the direct interception of auto-damaging O₂^{•−}.

Key words: H4Bip modulation, monomerization, nitric oxide synthase catalysis, reactive oxygen species.

INTRODUCTION

Nitric oxide (NO) is an important physiological and pathophysiological mediator [1,2] synthesized by a family of homodimeric enzymes termed NO synthases, types I to III (NOS I–III; EC 1.14.13.39), which NADPH-dependently oxidize L-arginine to form L-citrulline and NO [3,4]. In this reaction, O₂ serves as the co-substrate and NADPH donates the reducing equivalents [4,5]. At the molecular level, all isoforms of NOS contain FAD, FMN, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H₄Bip) and iron protoporphyrin IX (haem) within a two-domain system consisting of an N-terminal oxygenase domain and a C-terminal reductase domain connected by a calmodulin (CaM)-binding site [6,7]. Binding of CaM promotes electron flow from the flavins, FAD and FMN, within the reductase domain to the oxygenase haem [8]. Biochemical and functional parallels suggest that all NOS isoforms are members of the mammalian cytochrome P450 superfamily because they contain an intrinsic P450 reductase domain fused with a P450 haem domain within a single polypeptide [7,9]. However, the NOS isoforms differ primarily with respect to their expressional regulation, subcellular localization and activation mechanisms.

The observation that additional H₄Bip is required for maximal activation of NOS [10,11] led to the suggestion that H₄Bip might have an essential function in NO formation similar to that for the

aromatic amino acid hydroxylases [12], i.e. as an oxygen acceptor and electron donor catalysing the initial N-hydroxylation of L-arginine. However, the current consensus in the literature does not support such a classical catalytic role [13–17], although some studies can be found to the contrary [18]. Alternatively, protective and stabilizing effects of H₄Bip have been described, including (1) the stabilization of the NOS mRNA product [19], (2) protection of the NOS catalytic centre from oxidative damage [16,20], (3) prevention of the formation of superoxide (O₂^{•−}) [21] and H₂O₂ [22] by coupling reductive oxygen activation to L-arginine oxidation, and (4) promotion of subunit assembly from monomers and stabilization of dimers in the presence of chemical denaturants [23,24] or under native conditions during L-arginine turnover [17,25]. Interestingly, the stabilizing effects of H₄Bip might be isoform-dependent. For NOS-I, H₄Bip is required for dimer stabilization [17,24,25] but not for subunit assembly [26,27]; for NOS-II and III, the current evidence that supports a role for H₄Bip in subunit assembly is inconsistent [23,28–33] even when the enzymes have been solved at the crystal level [16,34].

Allosteric modulation within the catalytic centre of NOS by H₄Bip has also been reported [35–40]. In a radioligand binding study, Mayer and co-workers demonstrated a 6-fold increase in the affinity of NOS-I for [³H]H₄Bip in the presence of L-arginine [35]. For NOS-II, both positive [37] and negative [38] findings have been reported, again suggesting isoform specificity. In

Abbreviations used: CaM, calmodulin; H₄Bip, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; HX, hypoxanthine; NO₂Arg, *N*^ω-nitro-L-arginine; NOS-I, NO synthase type I (neuronal NOS); PHS-32, 2-amino-4,6-dioxo-3,4,5,6,8,8a,9,10-octahydro-oxazolo[1,2-*f*]-pteridine; S_{0.5}, concentration for half-maximal stimulation; SOD, superoxide dismutase; XOD, xanthine oxidase.

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addition, H_4Bip increased the binding of [3H]N $^{\omega}$ -nitro-L-arginine ([3H]NO $_2$ Arg) to NOS-I [35,39] 2-fold, revealing a dual allosteric interaction between these binding sites. However, in functional studies with H_4Bip -depleted NOS-II, H_4Bip failed to alter the IC $_{50}$ for enzyme inhibition by the L-arginine-based inhibitor NO $_2$ Arg [40]. Moreover, at the crystal level H_4Bip did not affect substrate binding by creating an open channel [34] as had previously been demonstrated [16]. Consequently, it remains to be established whether this binding-site co-operativity is a general phenomenon for all NOS isoforms and of relevance during catalysis. Moreover, possible allosteric interactions outside the oxygenase domain of NOS have not been explored in any great detail.

The aim of the present study was therefore to investigate possible allosteric effects of H_4Bip on neuronal NOS (NOS-I) and their functional relevance in the conversion of L-arginine into NO. In addition we investigated the stabilization of NOS-I quaternary structure by H_4Bip in relation to uncoupled catalysis and reactive oxygen species that have been recently shown to accumulate during catalysis and inactivate NOS-I [41].

EXPERIMENTAL

Materials

[6- 3H] H_4Bip was a gift from Dr R. G. Knowles (Glaxo Wellcome, Stevenage, Herts., U.K.); L-[2,3,4,5- 3H]arginine hydrochloride was purchased from Amersham (Braunschweig, Germany); NADPH was from AppliChem (Darmstadt, Germany); Cu-containing superoxide dismutase (SOD; bovine erythrocytes, 5 k-units/mg), xanthine oxidase (XOD; cow milk, 1 unit/mg) and reduced glutathione (GSH) were from Boehringer-Mannheim (Mannheim, Germany); H_4Bip was from Dr Schircks Laboratories (Jona, Switzerland); FAD, FMN, L-arginine hydrochloride, NO $_2$ Arg, hypoxanthine (HX) and phosphodiesterase 3',5'-cyclic nucleotide activator (CaM) were from Sigma Chemicals (Deisenhofen, Germany). The pterin-based analogue 2-amino-4,6-dioxo-3,4,5,6,8,8a,9,10-octahydro-oxazolo[1,2-f]-pteridine (PHS-32) was synthesized as described [17]. All other chemicals, reagents and solvents were of the highest purity available and were from Merck AG (Darmstadt, Germany), Sigma Chemicals (Deisenhofen, Germany) or vasopharm BIOTECH (Würzburg, Germany). Water was de-ionized to 18 M Ω (Milli-Q; Millipore, Eschborn, Germany). Unless otherwise indicated, all chemicals were dissolved in water deoxygenated with argon.

Preparation of recombinant human NOS-I

Sf9 cells were transfected with recombinant human NOS-I and the resulting enzyme was purified by 2',5'-ADP-Sepharose and CaM-Sepharose affinity chromatography [17,41]. The yield of this purification method was 25–35 mg of protein with a specific activity of up to 303 nmol of L-citrulline/min per mg. Purified NOS-I was stored at $-80^{\circ}C$ in 50 μ l aliquots containing 10% (v/v) glycerol until the day of use. Protein concentrations were determined spectrophotometrically by the Bradford method [42] with BSA as a standard and a SpectraMax 340 Microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The purity was established from densitometric scanning of Coomassie-stained SDS/PAGE gels with NIH Image software (National Institutes of Health, Bethesda, MD, U.S.A.). NOS-I immunoreactive bands were examined by Western blot analysis with a NOS-I specific antibody (Transduction Laboratories, Hamburg, Germany) and detection by enhanced chemiluminescence (ECL; Amersham, Braunschweig, Germany).

Determination of NOS activity

Catalytic activity of recombinant human NOS-I was assayed by the Ca $^{2+}$ /CaM-dependent conversion of L-[3H]arginine into L-[3H]citrulline [20,43] at $37^{\circ}C$ during a standard incubation period of 15 min. Unless stated otherwise, reaction mixtures of 100 μ l contained 0.40 μ g of NOS-I in triethanolamine buffer (50 mM), pH 7.2, consisting of 50 nM CaM, 1 mM CaCl $_2$, 250 μ M CHAPS, 7 mM reduced glutathione, 10 μ M FAD, 5 μ M FMN, H_4Bip (0–100 μ M), L-arginine (0–100 μ M) including 5.55 kBq of L-[2,3,4,5- 3H]arginine and 1 mM NADPH. The L-citrulline formed was separated by cation-exchange chromatography and measured by liquid-scintillation counting [20,43]. In kinetic experiments, non-cumulative concentration–response curves were constructed in triplicate for either H_4Bip (0–100 μ M) or L-arginine (0–100 μ M). Individual curves from each triplicate were subsequently fitted by non-linear regression analysis to sigmoidal concentration–response curves of variable slope, i.e. the Hill slope was not fixed equal to 1, using Prism software (Version 2a; GraphPAD, San Diego, CA, U.S.A.) and the 'built in' equation $Y = \text{bottom} + (\text{top} - \text{bottom}) / [1 + 10^{(\log EC_{50} - X) \times \text{Hill slope}}]$. The corresponding concentration for half-maximal stimulation ($S_{0.5}$) (for H_4Bip) or K_m (for L-arginine) value was then calculated from each curve by the software. These values were averaged (mean $\pm$ S.E.M.) for $n = 3$ –5 curves and then subjected to statistical analysis (Student's unpaired t test). A unit of enzyme activity is defined as the production of 1 nmol of L-citrulline/min per mg of NOS-I enzyme.

Binding of [3H] H_4Bip to NOS

Binding assays were performed in 96-well microfiltration plates containing PVDF membranes (Millipore, Bedford, MA, U.S.A.) as described previously [38,44]. In brief, recombinant human NOS-I (0.4 μ g) was incubated for 15 min at $25^{\circ}C$ in a total volume of 200 μ l containing 200 mM Tris/HCl buffer, pH 7.5, 4 mM dithiothreitol, 1 mM L-arginine and 10 nM [3H] H_4Bip (25 nCi per well). Under these conditions, NOS bound to the PVDF membrane by hydrophobic interactions and the separation of bound from free radioligand was performed as described [38] by filtration under reduced pressure with a microplate vacuum manifold (Millipore). The radioactivity on the filter membrane was determined by liquid-scintillation counting and results were corrected for non-specific binding, which was determined in the presence of 1 mM unlabelled H_4Bip . Competition studies were performed in the presence of increasing concentrations of PHS-32 (up to 100 μ M) and the corresponding K_i was calculated from displacement curves by non-linear least-squares curve fitting with GraphPAD PRISM software (Version 2a).

Spectral determination of the binding of L-arginine to NOS

Optical absorption spectra (370–450 nm) were measured at $25^{\circ}C$ with a Beckman DU640 diode-array spectrophotometer (Beckman Instruments GmbH, Munich, Germany). Spectra were obtained for recombinant human NOS-I in an assay mixture added to a quartz cuvette (2.2 ml) consisting of 50 mM Tris/HCl buffer (pH 7.8), 2 mM MgCl $_2$, 10 μ M CaCl $_2$ and 1 mM NADPH, in the absence or presence of either H_4Bip (final concentration 50 μ M) or PHS-32 (300 μ M) before titration with L-arginine (final concentration of 100 or 200 μ M). Substrate binding affinities were studied by perturbation difference spectroscopy with published methods [6]. Changes in iron haem spin state (from low spin to high spin) on the addition of L-arginine were reflected as a shift in the Soret absorption peak at approx. 398 nm, indicating substrate binding to the active site [6].

Size-exclusion chromatography

Recombinant human NOS-I (20 μg) was incubated as described under the above assay conditions, except that in these experiments the concentrations of L-arginine, H₄Bip and CaM were increased to 2 mM, 50 μM and 2 μM respectively to ensure the saturation of all binding sites. The reaction was stopped by the addition of 20 μl of ice-cold EGTA (30 mM) and the samples were immediately snap-frozen in liquid nitrogen. After being thawed, the samples were centrifuged (10000 g at 4 °C for 10 min) and then a 100 μl aliquot (equivalent to 17 μg of NOS protein) was analysed by FPLC with a Superose 6 HR 10/30 gel-filtration column (Pharmacia Biotech, Freiburg, Germany) that had been equilibrated with 20 mM triethanolamine buffer (pH 7.5) containing 150 mM NaCl and 5% (v/v) ethylene glycol. Proteins were eluted at a flow rate of 0.25 ml/min and monitored by A_{280} . Under these conditions, the column provided a separation range from approx. 5 to 5000 kDa. Calibration curves were obtained by plotting the elution volume (V_e) of the standard proteins (carbonic anhydrase, albumin, alcohol dehydrogenase, apoferritin and thyroglobulin) against their known Stokes radii (2.01, 3.55, 4.56, 6.10 and 8.50 respectively). The void volume (V_0) was determined with Dextran Blue 2000.

Determination of NOS-bound H₄Bip

For measurements of NOS-I-bound H₄Bip, native pig NOS-I was used. Native enzyme was purified from pig cerebellum with previously described methods [17,20,45] by DEAE ion-exchange chromatography and 2',5'-ADP-Sepharose affinity chromatography (yield of 1.0–1.4 mg of protein from 1 kg of tissue, with a specific activity of up to 463 nmol of L-citrulline/min per mg). The amount of enzyme-bound H₄Bip was determined by reverse-phase HPLC coupled with fluorimetric detection (λ_{ex} = 352 nm, λ_{em} = 438 nm), with authentic reagent H₄Bip as a standard [17,25,42]. Native NOS-I (0.8–1.0 μg) was incubated in 50 mM Tris/HCl buffer, pH 7.2, containing 1 mM CaCl₂, 10 μM FAD, 5 μM FMN, 250 μM CHAPS, 1 mM NADPH for 15 min at either 4 or 37 °C. In some experiments, either L-arginine (10, 30 or 100 μM) or NO₂Arg (0.3 μM) was included in the incubation mixture as indicated. The reaction was stopped by the addition of ice-cold Tris/HCl buffer, pH 6.7. To determine H₄Bip, samples were then centrifuged (10000 g at 4 °C for 20 min) through 10 kDa cut-off filters (Millipore, Eschborn, Germany). The low-molecular-mass filtrate contained pterin displaced from NOS [17,25,42]. For determinations of enzyme-bound pterin, the NOS-containing residue was redissolved in Tris/HCl buffer, pH 6.7, and immediately oxidized to biopterin after treatment for 1 h in the dark with 0.2 M I₂ and 0.5 M KI in the presence of either 0.5 M HCl (acidic oxidation) or 0.5 M NaOH (alkaline oxidation). Samples oxidized under alkaline conditions were acidified with 1 M HCl followed by 0.1 M ascorbic acid; to samples oxidized under acidic conditions, only water and ascorbic acid were added. The quantitative difference between the two biopterin values obtained under the above conditions is a measure of H₄Bip [17,25]. All samples were analysed by reverse-phase HPLC with a LiChroCart 250/4 Purospher RP 18 column (Merck, Darmstadt, Germany) with isocratic elution and 0.015 M KH₂PO₄/K₂HPO₄ buffer (pH 6.0).

H₄Bip: chemical stability and co-incubation with O₂^{•-}

The O₂^{•-}-generating system consisted of 250 μM HX and 0.75 m-unit/ml XOD, which resulted in a flux rate of 0.05 μM

O₂^{•-}/min. H₄Bip (0.1 μM) was incubated for up to 20 min at 37 °C in the absence of NOS under otherwise catalytic assay conditions (i.e. 50 μM L-arginine, 1 mM NADPH, 10 μM FAD, 5 μM FMN, 50 nM CaM, 1 mM CaCl₂ and 250 μM CHAPS) and in the absence (control) or presence of the above O₂^{•-}-generating system.

Statistics

Unless indicated otherwise, results are means $\pm$ S.E.M. Statistical analysis was performed by Student's unpaired *t* test (two-tailed). *P* < 0.05 was taken as indicating statistical significance.

RESULTS

Allosteric modulation by H₄Bip and enzyme activation

We examined the functional significance of allosteric binding site interactions in the activation of recombinant human NOS-I and the conversion of L-arginine into L-citrulline and NO. The affinity

Figure 1 Allosteric effect of H₄Bip on the apparent affinity of NOS for L-arginine

(A) Recombinant human NOS-I was incubated as described in the Materials and methods section for 15 min and enzyme activity was determined from the formation of L-citrulline from L-arginine. NOS activity is shown as the formation of L-citrulline/min per mg of NOS-I enzyme. A concentration–response curve for L-arginine (0–100 μM) was constructed in the presence of one of the following concentrations of H₄Bip (μM): ●, 0; ○, 0.03; ▲, 0.1; △, 1.0. Each curve in (A) represents a typical experiment, performed in triplicate, with data that were subsequently fitted by non-linear regression analysis to sigmoidal concentration–response curves by using GraphPAD PRISM software. (B) The corresponding K_m values were calculated by non-linear regression analysis and are represented as means $\pm$ S.E.M. for three or four separate curves, each performed in triplicate. *Statistical difference from 0 μM H₄Bip (*P* < 0.05, Student's unpaired, two-tailed *t* test).

Figure 2 Effect of H₄Bip on spectral determinations of L-arginine binding to NOS

Recombinant human NOS-I was incubated as described in the Materials and methods section and changes in the spectral absorption of the haem Soret band after L-arginine binding were recorded. **(A)** Absorption spectra with L-arginine (100 or 200 μM) alone. **(B)** The addition of H₄Bip (50 μM) in the presence of L-arginine (100 or 200 μM) resulted in an absorbance change of the haem Soret band that had a peak (maximum) at approx. 398 nm, indicating an enzyme with bound L-arginine.

of NOS for substrate was investigated by constructing a concentration–response curve to L-arginine (0–100 μM) and examining the effect of exogenous H₄Bip on the corresponding $S_{0.5}$ or apparent K_m [46]. In the absence of H₄Bip, the K_m for L-arginine was 16.5 ± 2.8 μM, which is in the published range of the K_d (10 μM) for NOS-I from radioligand binding [39]. The addition of H₄Bip (up to 1 μM) to the incubation assay markedly increased V_{max} and decreased the K_m for L-arginine from 16.5 ± 2.8 to 7.1 ± 0.7 μM (Figure 1), revealing an allosteric mechanism: similar results were obtained with pig brain NOS-I (in the absence and the presence of 1 μM H₄Bip, the K_m values for L-arginine were 8.6 ± 1.3 or 4.9 ± 0.4 μM respectively). The Hill slope was not altered under the different conditions and was close to unity: 1.35 ± 0.17 (no H₄Bip), 1.21 ± 0.02 (0.03 μM H₄Bip), 1.18 ± 0.10 (0.1 μM H₄Bip) and 1.18 ± 0.05 (1 μM H₄Bip). Consistent with an allosteric role was the observation that in optical spectroscopy measurements H₄Bip caused an L-arginine-dependent absorbance change of the haem Soret band of recombinant human NOS-I, indicating an increase in L-arginine binding within the catalytic centre (compare Figures 2A and 2B).

Lack of allosteric modulation of the pterin-based inhibitor PHS-32

We also examined whether the pterin-based analogue PHS-32 (Figure 3) mimics the allosteric effects of H₄Bip. This derivative

Figure 3 Structure of H₄Bip and the pterin-based analogue PHS-32

Also shown is the numbering system of the pterin ring atoms for H₄Bip.

Figure 4 Displacement of [³H]H₄Bip binding and inhibition of enzyme activity by the pterin derivative PHS-32

(A) Recombinant human NOS-I was incubated as described in the Materials and methods section for 15 min with (6*R*)-[³H]H₄Bip (10 nM, 25 nCi) and increasing concentrations of PHS-32 (10 nM to 100 μM). The corresponding K_i was calculated from displacement curves by non-linear least-squares curve fitting with GraphPAD PRISM software. The binding data shown are means $\pm$ S.E.M. for one experiment performed in triplicate with one enzyme preparation. **(B)** Recombinant human NOS-I was incubated as described in the Materials and methods section for 15 min in the presence of 2 μM H₄Bip, 50 μM L-arginine and increasing concentrations of PHS-32 (3–300 μM). Enzyme activity was determined from the formation of L-citrulline from L-arginine. The corresponding IC_{50} was calculated from displacement curves by non-linear least-squares curve fitting with GraphPAD PRISM software.

seems to interact specifically with the H₄Bip site of recombinant human NOS-I, because (1) PHS-32 competitively displaced exogenous [³H]H₄Bip in radioligand binding studies ($K_i = 17$ nM; Figure 4A) and (2) PHS-32 inhibited H₄Bip-stimulated activity

Figure 5 Effect of the pterin-based analogue PHS-32 on spectral determinations of the binding of L-arginine to NOS

Recombinant human NOS-I was incubated as described in the Materials and methods section and the spectral changes of the haem Soret band after L-arginine binding were recorded. Absorption spectra are shown with L-arginine (100 or 200 μ M) in the presence of PHS-32 (300 μ M) (A), H₄Bip (50 μ M) (B) or a combination of PHS-32 (300 μ M) and H₄Bip (50 μ M) (C).

in a concentration-dependent manner ($IC_{50} = 22 \mu$ M; Figure 4B). However, in contrast with the spectral effects of H₄Bip, PHS-32 (300 μ M) did not cause an L-arginine-dependent change in absorbance of the haem Soret band (Figure 5A), indicating a lack of increase in substrate binding to recombinant human NOS-I. Taken together, these observations indicate that the 1,2-dihydroxypropyl side chain at C-6 of H₄Bip (Figure 3) might be important in promoting co-operativity within the pterin-binding pocket. Interestingly, the L-arginine-dependent absorbance change due to H₄Bip (50 μ M) could be effectively abolished by excess PHS-32 (300 μ M; Figure 5C), again confirming that this analogue interferes with H₄Bip function.

Figure 6 Allosteric effect of L-arginine on the apparent affinity of NOS for H₄Bip

(A) Recombinant human NOS-I was incubated as described in the Materials and methods section for 15 min and enzyme activity was determined from the formation of L-citrulline from L-arginine. NOS activity is shown as the formation of L-citrulline/min per mg of NOS-I enzyme. A concentration–response curve for H₄Bip (0–100 μ M) was constructed in the presence of one of the following concentrations of L-arginine (μ M): ●, 1; ○, 10; ▲, 30; △, 100. Each curve in (A) represents a typical experiment, performed in triplicate, with data that were subsequently fitted by non-linear regression analysis to sigmoidal concentration–response curves by using GraphPAD PRISM software. (B) The corresponding $S_{0.5}$ values were calculated by non-linear regression analysis and are represented as means $\pm$ S.E.M. for three separate experiments, each performed in triplicate. *Statistical difference from 1 μ M L-arginine ($P < 0.05$, Student's unpaired, two-tailed t test).

Allosteric effect of L-arginine on the H₄Bip-binding site

We examined whether L-arginine activates recombinant human NOS-I by modulating the affinity for H₄Bip cofactor during catalysis. In kinetic studies, the $S_{0.5}$ of NOS for H₄Bip was decreased in a concentration-dependent manner by L-arginine from 779 ± 133 nM at low (sub-saturating) L-arginine (1 μ M), to 124 ± 16 nM at saturating L-arginine (100 μ M; Figure 6), revealing an allosteric effect. The Hill slope was not altered under the different conditions and was close to unity: 0.88 ± 0.02 (1 μ M L-arginine), 0.83 ± 0.01 (10 μ M L-arginine), 0.84 ± 0.07 (30 μ M L-arginine) and 1.02 ± 0.12 (100 μ M L-arginine). Similar allosteric effects were observed with pig brain NOS-I (with 1 or 100 μ M L-arginine, $S_{0.5}$ values of H₄Bip were 389 ± 70 or 88 ± 10 nM respectively). This finding was confirmed biochemically when examining the effects of L-arginine on NOS-bound H₄Bip with HPLC coupled with fluorimetric detection [17,25]. We have shown previously that pterin bound to pig brain NOS-I is readily lost by an increase in temperature from 4 to 37 $^{\circ}$ C [17]. In the

Table 1 Effect of L-arginine on NOS-bound pterin

The pterin content of pig brain NOS-I was determined by HPLC coupled with fluorimetric detection as described in the Materials and methods section, with or without various treatments: L-arginine (10, 30 or 100 μ M), NO₂Arg (0.3 μ M) or a combination of L-arginine (30 μ M) and NO₂Arg (0.3 μ M). *,†Statistical difference from the absence of treatment at 4 and 37 °C respectively ($P < 0.05$, Student's unpaired, two-tailed t test). The ability of the pterin-based analogue PHS-32 (100 μ M) to displace NOS-bound pterin was also determined in either the absence or the presence of L-arginine (10 or 100 μ M). Results are means $\pm$ S.E.M. for three to seven separate experiments, each performed in triplicate with two to four different enzyme preparations.

Treatment	Temperature (°C)	NOS-bound pterin (pmol)
None	4	0.75 $\pm$ 0.09
None	37	0.23 $\pm$ 0.03*
L-Arginine (10 μ M)	37	0.47 $\pm$ 0.10†
L-Arginine (30 μ M)	37	1.10 $\pm$ 0.12†
L-Arginine (100 μ M)	37	0.76 $\pm$ 0.02†
NO ₂ Arg (0.3 μ M)	37	0.83 $\pm$ 0.14†
L-Arginine (30 μ M) + NO ₂ Arg (0.3 μ M)	37	0.81 $\pm$ 0.12†
PHS-32 (100 μ M)	37	0.05 $\pm$ 0.02
PHS-32 (100 μ M) + L-arginine (10 μ M)	37	0.03 $\pm$ 0.02
PHS-32 (100 μ M) + L-arginine (100 μ M)	37	0.36 $\pm$ 0.04

present study, L-arginine (10–100 μ M) increased the binding of pterin to pig brain NOS-I in a concentration-dependent manner (Table 1), providing direct evidence for the allosteric regulation of H₄Bip cofactor binding. Interestingly, the ability of the pterin derivative PHS-32 (100 μ M) to displace NOS-bound H₄Bip was considerably less pronounced in the presence of saturating L-arginine (100 μ M; Table 1) than with sub-saturating L-arginine (10 μ M; Table 1), providing additional evidence for allosterically induced changes in affinity.

Allosteric effect of the L-arginine-based inhibitor NO₂Arg

We investigated the structural basis for the allosteric effects of L-arginine and whether the L-arginine-based inhibitor NO₂Arg shares this property. The concentration of NO₂Arg (0.3 μ M) used was equivalent to the IC₅₀ for pig brain NOS-I [17]. Interestingly, NO₂Arg also increased NOS-bound pterin to an extent similar to that of L-arginine (30 and 100 μ M; Table 1), and, if anything, seemed to be even more potent than L-arginine. Taken together, these observations suggested that the allosteric effects of L-arginine are independent of the nature of the chemical composition of the terminal guanidino nitrogen and are not induced by changes in the oxidation state during L-arginine turnover.

NOS quaternary structure during L-arginine turnover

In addition to reciprocal interactions between the H₄Bip-binding site and the L-arginine-binding site, NOS-I dimer association and dissociation are thought to be regulated by H₄Bip (see the Introduction section). We therefore investigated the effects of L-arginine and H₄Bip on NOS-I quaternary structure during L-arginine turnover. For these structural investigations a greater quantity of recombinant human NOS-I (17 μ g) was incubated under identical assay conditions (see the Experimental section) for 15 min before size-exclusion chromatography. Dimeric NOS-I was found to be eluted as a main peak at 12.50 $\pm$ 0.01 ml (Figure 7), an elution volume equivalent to a Stokes radius of 7.80 $\pm$ 0.02 nm, which is in the published range [17,25,47]. A second smaller peak was also observed to be eluted at

Figure 7 Stabilizing effect of L-arginine and H₄Bip on NOS quaternary structure during L-arginine turnover

Typical chromatograms are shown after size-exclusion chromatography of recombinant human NOS-I incubated under catalytic assay conditions as described in the Materials and methods section for either $t = 0$ min (solid line) or $t = 15$ min (broken line) in the absence of L-arginine and H₄Bip (A) or $t = 15$ min in the absence of H₄Bip and either with (broken line) or without SOD (1 k-unit/ml; solid line) (B). Abbreviations: D, dimer; M, monomer.

13.97 $\pm$ 0.05 ml (Figure 7), corresponding to monomeric NOS-I with a Stokes radius of 6.06 $\pm$ 0.05 nm [17,25,26]. Both peaks contained NOS-I immunoreactive protein, as analysed by Western-blot analysis of the fractionated eluate; however, only the dimeric peak was active in L-arginine turnover and contained enzyme-bound H₄Bip (results not shown), in accord with published observations [17,25]. The addition of either L-arginine (2 mM) or H₄Bip (50 μ M) under catalytic assay conditions markedly inhibited monomerization (Table 2), revealing stabilizing effects on quaternary structure. Interestingly, only in the simultaneous presence of L-arginine (2 mM) and H₄Bip (50 μ M) could enzyme monomerization be completely inhibited (Table 2).

NOS monomerization and O₂^{•−}: effect of SOD

Because the monomerization of NOS seemed to be catalysis-dependent, the dependence on H₄Bip and L-arginine were investigated further with respect to possible NOS-derived products and changes in quaternary structure. It is known that in the absence of either L-arginine or H₄Bip, NOS becomes enzymically uncoupled and reduces molecular oxygen at the expense of NADPH to form reactive oxygen species such as O₂^{•−} [48–50] and H₂O₂ [22,41]. Moreover, we have recently shown that NOS-derived uncoupled catalysis products can accumulate during L-arginine turnover and influence enzyme activity [41]. Here we considered the possibility that the stabilizing effect of H₄Bip

Table 2 Stabilization of NOS quaternary structure during turnover of L-arginine

Human recombinant NOS-I was incubated as described in the Materials and methods section, with or without the following treatments: L-arginine (2 mM), H₄Bip (50 μ M), or combinations of L-arginine (2 mM) and H₄Bip (50 μ M), L-arginine (2 mM) and SOD (1 k-unit/ml) and L-arginine (2 mM) and NO₂Arg (1 mM). The effect of the pterin-based analogue PHS-32 (100 μ M) was also examined. Changes in enzyme structure were assessed from the peak heights of the corresponding dimeric and monomeric NOS for each individual chromatogram (see Figure 7). Results are expressed as a percentage of the respective control dimer and monomer peak heights at $t = 0$ min; results are means $\pm$ S.E.M. for three to five separate experiments. *,†Statistical difference from incubation time $t = 0$ min and $t = 15$ min respectively ($P < 0.05$, Student's unpaired, two-tailed t test); ‡statistical difference from incubation with L-arginine (2 mM) ($P < 0.05$, Student's unpaired, two-tailed t test).

Treatment	Incubation time (min)	Peak height (% of $t = 0$ min incubation)	
		Dimer	Monomer
None	0	100 $\pm$ 1	100 $\pm$ 9
None	15	40 $\pm$ 1*	385 $\pm$ 6*
L-Arginine (2 mM)	15	68 $\pm$ 3*†	225 $\pm$ 25*†
H ₄ Bip (50 μ M)	15	69 $\pm$ 2*†	267 $\pm$ 8*†
L-Arginine (2 mM) + H ₄ Bip (50 μ M)	15	95 $\pm$ 3†‡	107 $\pm$ 3†‡
L-Arginine (2 mM) + SOD (1 k-unit/ml)	15	87 $\pm$ 2*†‡	157 $\pm$ 9*†‡
L-Arginine (2 mM) + NO ₂ Arg (1 mM)	15	104 $\pm$ 4†‡	117 $\pm$ 4†‡
PHS-32 (100 μ M)	15	50 $\pm$ 4*†	365 $\pm$ 3*†

might involve an interference with auto-damaging uncoupled catalysis products. Consistent with this proposal was the observation that, in the absence of H₄Bip, NOS monomerization was inhibited by SOD (1 k-unit/ml; Figure 7B), implicating the involvement of destabilizing O₂^{•−}. Importantly, NOS seemed to be the source of O₂^{•−}, because NO₂Arg (1 mM), which inhibits the formation of O₂^{•−} from NOS-I [49,50], prevented monomerization (Table 2). Taken together, these observations suggest a protective role for H₄Bip by a novel mechanism involving the suppression of auto-damaging O₂^{•−}.

Reagent and NOS-bound H₄Bip: reaction with O₂^{•−}

Given the above structural observations, we examined further the mechanism(s) by which H₄Bip interferes with auto-damaging O₂^{•−}. One possibility is that H₄Bip reacts directly and inactivates O₂^{•−} generated within the catalytic centre of NOS. Indeed, under catalytic assay conditions (without enzyme) and in the presence of a low flux of O₂^{•−} (0.05 μ M/min generated from 250 μ M HX and 0.75 m-unit of XOD), H₄Bip (0.1 μ M) reacted chemically and decreased in a time-dependent manner (Figure 8A). The identity of the possible degradation product(s) is currently under investigation. The above experiment was also repeated by adding NOS to the incubation assay instead of an O₂^{•−}-generating system. Interestingly, when pig brain NOS-I was added in the absence of L-arginine, i.e. to uncouple catalysis fully and increase the endogenous generation of O₂^{•−} [49–51], total H₄Bip recovery (enzyme-bound and free in solution) became increasingly incomplete (Figure 8B). Most notably, H₄Bip (in the absence of NOS) was found to be chemically stable under these assay conditions (Figure 8A), in accord with previous observations [25]. Rather, these results collectively suggest that during uncoupled catalysis, NOS-bound H₄Bip might have a protective role by directly intercepting auto-damaging O₂^{•−}.

Figure 8 Chemical stability and loss of NOS-bound pterin during uncoupled catalysis

(A) H₄Bip (0.1 μ M) was incubated for up to 20 min in the absence (control, ○) or presence (▲) of a O₂^{•−}-generating system consisting of 250 μ M HX and 0.75 m-unit XOD, producing a flux of 0.05 μ M O₂^{•−}/min. Reagent H₄Bip was measured by HPLC at the incubation time points indicated and is shown as recovery. (B) Pig brain NOS-I was added to the incubation mixture (without a O₂^{•−}-generating system) under the above assay conditions for up to 20 min in the absence of L-arginine, to promote uncoupled catalysis and O₂^{•−} generation. In this situation, total H₄Bip recovery (enzyme-bound and free in solution) became increasingly incomplete (●) and was decreased in a time-dependent manner that was analogous to O₂^{•−} treatment in (A) (○). Results are means $\pm$ S.E.M. for three separate experiments.

DISCUSSION

The importance of allosteric interactions within the catalytic centre of NOS is indicated by an early report by the laboratory of Stuehr [23] that demonstrated a requirement for the simultaneous presence of L-arginine, H₄Bip and haem for the reassembly of inactive monomeric NOS-II (formed after treatment with urea) into dimeric, active enzyme. Consistent with this was the observation that radioligand binding with [³H]H₄Bip and [³H]NO₂Arg revealed a dual allosteric interaction between the H₄Bip-binding site and L-arginine-binding site of NOS-I [35], whereas for NOS-II this has still remained controversial [37,38]. However, at the crystal level H₄Bip was not found to affect substrate binding [34], in direct disagreement with an earlier report [16]. Moreover, in enzyme kinetic studies, H₄Bip did not alter the IC₅₀ of NO₂Arg for inhibiting the enzyme activity of human pterin-free NOS-II [40]. The reasons for this discrepancy are unclear but might reflect isoform differences or, alternatively, methodological differences, e.g. the use of H₄Bip-free NOS and measurements under catalytic [40] compared with non-catalytic conditions used in radioligand binding assays [35–39]. There is therefore uncertainty about the functional significance of

Scheme 1 H₄Bip allosterically activates NOS-I, couples catalysis and stabilizes dimeric structure

Binding-site interactions within the catalytic centre of NOS-I increase the enzyme's affinity for both L-arginine and H₄Bip and activate NOS-I. This results in optimal electron transfer within the catalytic centre and fully coupled catalysis from a stable dimeric enzyme (II). At lower L-arginine and/or H₄Bip concentrations, uncoupled catalysis occurs and O₂^{•-} is formed, which destabilizes the NOS-I quaternary structure (I) and promotes enzyme monomerization (III). In this situation, positive co-operativity is compromised, resulting in possibly sub-optimal electron transfer; H₄Bip has a protective function by directly intercepting auto-damaging O₂^{•-} from I.

allosteric binding site interactions in regulating NOS activity and NO generation.

Dual binding site interactions: functional implications

We examined the effect of H₄Bip on the apparent affinity of NOS-I for L-arginine and any possible functional consequences on NO synthesis. The addition of H₄Bip to the assay mixture not only increased enzyme activity but also decreased the apparent K_m of NOS for substrate. These kinetic observations provide direct evidence for an allosteric role of H₄Bip in the activation of NOS-I. This was supported by optical spectroscopy measurements in which H₄Bip caused an absorbance change in the NOS-I haem spectrum, indicating an increase in L-arginine binding within the catalytic centre. Interestingly, the pterin-based inhibitor PHS-32 [17], which differs structurally from H₄Bip in the nature of the C-6 chemical substituent, did not mimic the allosteric effects of H₄Bip. This latter finding provides an important insight into possible chemical interactions within the H₄Bip-binding pocket and a role for the 1,2-dihydroxypropyl side chain at the 6-position in promoting co-operativity. Indeed, in X-ray crystallographic studies with the oxygenase domain of NOS-II, chemical interactions within the pterin-binding pocket were observed between the 1,2-dihydroxypropyl side chain at C-6 of H₄Bip and Phe-470 and Ser-112 [16].

The mechanism by which H₄Bip activates NOS-I does not seem to extend to the adjacent CaM-binding site and the CaM-dependent autoinhibitory domain, because in radioligand binding studies CaM did not affect the increase in [³H]NO₂Arg binding due to H₄Bip [39]. However, there is some indication that the reductase domain of NOS-I might influence the oxygenase

domain. For example, the binding of [³H]NO₂Arg to NOS-I was inhibited by the flavoprotein inhibitor diphenyleneiodonium ('DPI'), which is thought to interfere with the reductase domain [39]. We also found an inhibitory effect of the N-terminal amino acid sequence (residues 1–366; 11 kDa) of NOS-I on the binding of a pterin-based photoaffinity label to the oxygenase domain [17]. Clearly, more work will be required for a better understanding of these interactions; a knowledge of the three-dimensional structure of the full-length dimeric NOS-I is desirable.

In the present study, L-arginine was found to activate NOS-I by allosterically modulating the affinity for H₄Bip cofactor. In accord with this, L-arginine increased endogenous NOS-bound H₄Bip, providing direct evidence for dual co-operativity. This interaction has been previously demonstrated only for exogenous [³H]H₄Bip binding and can now be extended to endogenous H₄Bip modulation during enzyme catalysis. It is conceivable that this interaction might be of physiological significance in enzyme regulation, considering the relatively high levels of L-arginine in the cell (200–800 μ M) and its low K_m (2–10 μ M). Moreover, in pathophysiological situations associated with either decreased cellular pterin (e.g. in Down's syndrome or severe depression [52]), inherited H₄Bip deficiency (e.g. 'atypical phenylketonuria') or substrate deficiency (e.g. ischaemia), cofactor- or substrate-induced co-operativity might be compromised, resulting in decreased NO synthesis, uncoupled catalysis and oxidative stress.

The L-arginine-based NOS inhibitor NO₂Arg also positively modulated enzyme-bound H₄Bip and, if anything, seemed to be even more potent than the endogenous substrate. These findings reveal that the allosteric effects of L-arginine in NOS-I are most

probably independent of the nature of the terminal guanidino nitrogen and are not induced by changes in the oxidation state of substrate during catalysis. The increased potency of NO₂Arg compared with L-arginine is probably due to the fact that NO₂Arg binds to the L-arginine-binding site of NOS-I with 100-fold higher affinity and with a Hill coefficient close to unity [39].

Stabilizing effects of L-arginine and H₄Bip on NOS-I quaternary structure

During L-arginine turnover, NOS-I progressively monomerizes [17], resulting in enzyme inactivation due to the generation of reactive oxygen species such as H₂O₂ [41]. This contrasts with the situation in which the monomerization of NOS was induced chemically, either by using the protein inhibitor of neuronal NOS ('PIN'), which interferes with the PDZ site of NOS [53], by exposing dimeric NOS-II to 5 M urea [23,28] or by heat denaturation under reducing conditions [24]. [PDZ, a protein-binding module, is an acronym of the three proteins PSD-95 (a post-synaptic density protein of 95 kDa), Dlg (disk-large homology protein) and ZO-1 (zona occludens 1).] Here we show that the addition of either L-arginine or H₄Bip to the assay mixture stabilized dimeric NOS-I during catalysis and markedly inhibited monomerization. In agreement with this, Mayer and co-workers [24] demonstrated an increased resistance of NOS-I to monomerization by heat denaturation under reducing conditions when L-arginine and H₄Bip were present, although the mechanistic basis for this effect remains unclear. Interestingly, in the present study NOS-I monomerization could be prevented by the simultaneous presence of L-arginine and H₄Bip, indicating that during fully coupled reductive oxygen activation, dimeric enzyme is stable (II, Scheme 1) and is possibly less susceptible to monomerization and auto-inactivation (I, Scheme 1).

The pterin-based inhibitor, PHS-32, which effectively displaced both endogenous NOS-bound H₄Bip and, in binding studies, exogenous [³H]H₄Bip, also mimicked the stabilizing effect of H₄Bip. These findings reveal that the stabilizing effects of H₄Bip are probably independent of the composition of the C-6 pterin side chain. Similar conclusions were made by Presta et al. [54] for NOS-II on dimer reassembly by various analogues of pterin after urea-induced monomerization. In the present study, PHS-32 was less effective than H₄Bip at stabilizing dimeric NOS-I and this might be related to the inability of PHS-32 to support enzyme catalysis and allosterically regulate L-arginine binding.

During catalysis, NOS can produce significant quantities of reactive oxygen species (O₂^{•-} and H₂O₂) [21,22,41,50,51]; H₄Bip markedly attenuates this [21,22,37] by a largely unknown mechanism that might involve the haem domain [51]. Importantly, given that NOS-derived reactive oxygen species contribute to enzyme inactivation [41], we considered the possibility that H₄Bip stabilizes and protects NOS by interfering with auto-damaging O₂^{•-}. In agreement with this hypothesis, during enzyme catalysis and in the absence of H₄Bip, i.e. a situation in which O₂^{•-} is known to be formed [21,37,49], SOD was discovered to inhibit NOS monomerization. This finding provides a mechanistic insight into the stabilizing effects of H₄Bip. We propose that in the presence of saturating amounts of substrate, H₄Bip promotes binding-site co-operativity, which favours optimal electron transfer within the catalytic centre and coupled catalysis from a stable dimeric NOS complex (II, Scheme 1). Importantly, H₄Bip might have an additional protective role by directly 'scavenging' excessively generated O₂^{•-} from I (see Scheme 1) when L-arginine levels are limiting. This contention is supported by the observations that exogenous H₄Bip reacts chemically with O₂^{•-} to an as yet uncharacterized derivative and that NOS-bound

H₄Bip seemed to be consumed during fully uncoupled enzyme catalysis in the absence of substrate. There is an increasing evidence for a role of the reductase domain of NOS [21,55,56] as a source of O₂^{•-} production; H₄Bip might be involved in the regulation of these processes. In particular, the function of the recently identified ZnS₄ centre near the catalytic centre of NOS-III [34,57], NOS-II [57–59] and NOS-I [59] in relation to H₄Bip and the reductase domain remains to be elucidated.

In summary, we demonstrate here that allosteric interactions within the catalytic centre of NOS-I are functionally important in enzyme activation and NO synthesis. The present findings also reveal that NOS-derived O₂^{•-} is auto-damaging and that H₄Bip has a protective role by directly intercepting O₂^{•-}. This might be of pathophysiological relevance in conditions associated with L-arginine deficiency or during periods of uncoupled catalysis or oxidative stress, and might explain the unique feature of this cytochrome P450 enzyme in possessing another redox-active cofactor bound in the active centre. Scheme 1 attempts to encompass the relevance of allosteric mechanisms in activating NOS-I and stabilizing enzyme structure with respect to uncoupled catalysis.

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