Nitric oxide-induced autoinhibition of neuronal nitric oxide synthase in the presence of the autooxidation-resistant pteridine 5-methyltetrahydrobiopterin

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Nitric oxide synthase (NOS) catalysis results in formation of NO or superoxide ($O_2^{\cdot-}$) depending on the presence or absence of the cofactor tetrahydrobiopterin (BH4). In the absence of $O_2^{\cdot-}$ scavengers, net NO formation cannot be detected even at saturating BH4 concentrations, which is thought to be due to $O_2^{\cdot-}$ production by BH4 autooxidation. Because the *N*-5-methylated analogue of BH4 (5-Me-BH4) sustains NOS catalysis and is autooxidation-resistant, net NO formation by the neuronal isoform of NOS (nNOS) can be observed at saturating 5-Me-BH4 concentrations. Here we compare the effects of 5-Me-BH4 on L-citrulline formation, NADPH oxidation, H_2O_2 production and soluble guanylate cyclase (sGC) stimulation. All activities were stimulated biphasically (EC_{50} approx. 0.2 μ M and more than 1 mM), with an intermediate inhibitory phase at the same pterin concentration as that required for net NO generation and sGC stimulation (4 μ M). Concomitantly with inhibition, the NADPH/L-citrulline stoichiometry decreased from 2.0 to 1.6. Inhibition occurred only at high enzyme concentrations (IC_{50}

approx. 10 nM nNOS) and was antagonized by oxyhaemoglobin and by BH4. We ascribe the first stimulatory phase to high-affinity binding of 5-Me-BH4. The inhibitory phase is due to low-affinity binding, resulting in fully coupled catalysis, complete inhibition of $O_2^{\cdot-}$ production and net NO formation. At high enzyme concentrations and thus high NO levels, this causes autoinhibition. NO scavenging by 5-Me-BH4 at concentrations above 1 mM, resulting in the antagonization of inhibition of NOS, explains the second stimulatory phase. In agreement with these assignments 5-Me-BH4 was found to stimulate formation of a haem-NO complex during NOS catalysis. The observation of inhibition with 5-Me-BH4 but not with BH4 implies that, unless $O_2^{\cdot-}$ scavengers are present, a physiological role for NO-induced autoinhibition is unlikely.

Key words: oxyhaemoglobin, stoichiometry, superoxide, (un)-coupling.

INTRODUCTION

Nitric oxide synthase (NOS; EC 1.14.13.39) generates NO from L-arginine, O_2 and NADPH-derived electrons [1–4]. The enzyme consists of a FAD- and FMN-containing reductase domain, an oxygenase domain that accommodates haem and tetrahydrobiopterin [BH4; (6*R*)-5,6,7,8-tetrahydro-6-(*L*-erythro-1',2'-dihydroxypropyl)pterin] as prosthetic groups [5] and a calmodulin (CaM)-binding region. Oxidation of L-arginine and reduction of O_2 take place at the haem, while the flavins shuttle the required electrons from NADPH to the oxygenase domain. This internal electron transfer occurs only in the presence of Ca^{2+} /CaM. As purified, the neuronal isoform of NOS (nNOS) contains two haems and one BH4 per dimer [6], but the enzyme binds a second pterin equivalent in the presence of excess BH4. The odd stoichiometry has been ascribed to anti-co-operativity between the binding of two BH4 molecules to the two subunits of dimeric NOS [7]. The formation of NO occurs when both arginine and BH4 are present. In the absence of either of these compounds, nNOS-catalysed NADPH oxidation is uncoupled from NO synthesis and results in the reduction of O_2 to superoxide ($O_2^{\cdot-}$) [8–10].

In our efforts to elucidate the function of BH4 in NO synthesis, we found that 5-methyl-6,7,8-trihydrobiopterin (5-Me-BH4) can substitute BH4 as a cofactor [11]. This BH4 analogue proved to be a valuable tool for studying the catalytic mechanism. First,

the results were relevant to the continuing debate about whether BH4 is redox-active in NOS [3–5]. The mere observation of NO synthesis stimulated by 5-Me-BH4 ruled out a redox cycle between BH4 and quinonoid BH2 [6,7-dihydro(8*H*)biopterin] as an essential element in the catalytic mechanism, because 5-Me-BH4 cannot be reversibly oxidized to a quinonoid dihydro species [11]. Secondly, the stimulation of L-citrulline production by BH4-free nNOS [nNOS(BH4–)] depended in a biphasic fashion on the pterin concentration, which seemingly provided the first direct evidence for anti-co-operative binding of pterin. Lastly, with 5-Me-BH4 we were able to detect NO synthesis polarographically [11]. NO has so far been detected only with the use of NO scavengers such as haemoglobin [12,13] or in the presence of superoxide dismutase (SOD) [14,15], because at low BH4 levels NOS is partly uncoupled and produces NO and $O_2^{\cdot-}$ simultaneously [9], whereas at high levels $O_2^{\cdot-}$ is formed by the autooxidation of BH4 [14]. In either case, NO accumulation is prevented by the rapid reaction between $O_2^{\cdot-}$ and NO [16]. The resistance of 5-Me-BH4 to autooxidation [11,17,18] permitted the direct demonstration of NO synthesis by NOS in the absence of scavengers of NO and $O_2^{\cdot-}$. However, the EC_{50} for NO formation did not coincide with either of the two stimulatory phases observed for L-citrulline formation [11].

In the present study we set out to establish the effects of 5-Me-BH4 on NADPH oxidation and $O_2^{\cdot-}$ formation by nNOS as isolated, containing 1 equiv. of BH4 per dimer [nNOS(BH4+)],

Abbreviations used: BH4, tetrahydrobiopterin [(6*R*)-5,6,7,8-tetrahydro-6-(*L*-erythro-1',2'-dihydroxypropyl)pterin]; CaM, calmodulin; HbO₂, oxyhaemoglobin; 5-Me-BH4, 5-methyl-6,7,8-trihydrobiopterin; NOS, nitric oxide synthase; nNOS, neuronal isoform of NOS; nNOS(BH4–), BH4-free nNOS; nNOS(BH4+), nNOS as isolated, containing 1 equiv. of BH4 per dimer; sGC, soluble guanylate cyclase; SIN-1, 3-morpholino-sydnonimine; SOD, superoxide dismutase; Sper-NO, spermine NONate (*N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine).

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and on the stoichiometry of NADP⁺ and L-citrulline formation. In the course of these studies we found the source of the discrepancy between the stimulation of NO formation and L-citrulline production. True low-affinity binding of 5-Me-BH4 occurs in the micromolar range and results in the net formation of NO. It hardly affects L-citrulline formation under standard assay conditions and causes a pronounced decrease in activity at higher enzyme concentrations. We show that the latter effect is due to autoinhibition by NO. The stimulation of L-citrulline formation by 5-Me-BH4 concentrations exceeding 1 mM, which we originally ascribed to low-affinity binding, turned out to be due to a reversal of autoinhibition, resulting from NO consumption at very high concentrations of 5-Me-BH4.

NO-induced inhibition of NOS, its mechanism, and its supposed regulatory role *in vivo* are contentious issues, with several groups reporting inhibition that others fail to see under similar conditions [4,5,14,19,20], and references therein). The present observation of inhibition with the autoxidation-resistant pterin 5-Me-BH4, but not with the autoxidizable natural cofactor BH4, is relevant to that debate. In the light of these new results we discuss the likelihood of NO-induced inhibitions occurring *in vivo*.

EXPERIMENTAL

Materials

Recombinant rat nNOS was purified from baculovirus-infected insect cells, as described previously [21,22]. Enzyme concentrations are expressed as the concentration of the monomer, assuming a molecular mass of 160 kDa. Soluble guanylate cyclase (sGC) was purified from bovine lung as described [23]. L-[2,3,4,5-³H]Arginine hydrochloride (57 Ci/mmol) and [α -³²P]GTP (400 Ci/mmol) were purchased from MedPro (Amersham, Vienna, Austria). BH4 and 5-Me-BH4 were from Dr B. Schircks Laboratories (Jona, Switzerland). NO solutions were prepared as described [24]. 3-Morpholino-sydnnonimine (SIN-1) was a gift from Dr K. Schönafinger (Höchst Marion Roussel, Frankfurt, Germany); peroxynitrite (ONOO⁻) was synthesized as described [25]. Spermine NONOate (Sper-NO; *N*-{4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl}-1,3-propanediamine) was obtained from Alexis Corporation (Laufen, Switzerland). Sper-NO stock solutions were prepared in 10 mM NaOH. Other chemicals were from Sigma (Vienna, Austria).

Determinations of NOS activity

The simultaneous determination of NADPH oxidation, citrulline formation and H₂O₂ production was performed as described previously [26]. NADPH oxidation was monitored spectrophotometrically at 340 nm and 37 °C in a total volume of 250 μ l [8,27]. After 10 min, two 100 μ l aliquots were taken for duplicate determinations of [³H]citrulline [28] and H₂O₂ [9]. Unless indicated otherwise, the measuring cuvette contained 0.14 μ M nNOS, 50 mM potassium phosphate, pH 7.4, 0.2 mM [³H]arginine (approx. 133 000 c.p.m.), 0.5 mM CaCl₂, 40 μ g/ml CaM, 2.4 mM 2-mercaptoethanol and 0.2 mM CHAPS, with other reagents as required. The reaction was started by the addition of 0.2 mM NADPH. The observed rates were corrected by the subtraction of blank rates obtained in the absence of CaM. When the effect of oxyhaemoglobin (HbO₂) on citrulline formation was studied, the same reaction conditions were maintained, except that the reaction volume was 100 μ l, less labelled arginine was

added (approx. 50 000 c.p.m.) and the incubation time was decreased to 3 min. The indirect detection of NO formation by the transformation of HbO₂ into methaemoglobin was determined as described previously [29,30] but with the same experimental conditions as above plus 50 μ M HbO₂.

The effect of the NOS reaction product NO/O₂⁻ on enzyme activity was determined by measuring the production of L-[³H]citrulline in the presence of various concentrations of SIN-1 under standard assay conditions [28] but in the absence of added pterins or flavins. Specifically, the reaction mixture contained 12.5 nM nNOS, 50 mM potassium phosphate, pH 7.4, 0.1 mM [³H]arginine (approx. 50 000 c.p.m.), 0.2 mM NADPH, 0.5 mM CaCl₂, 10 μ g/ml CaM, 2.4 mM 2-mercaptoethanol, 0.2 mM CHAPS and 0–30 mM SIN-1. The same experimental conditions were applied to study the effect of ONOO⁻ (0–1 mM). SIN-1 and ONOO⁻ dilutions were made in freshly prepared 10 mM NaOH at 10 $\times$ final concentrations, and added to the reaction mixture immediately after the initiation of catalysis by the addition of NOS.

Stimulation of sGC

The activation of sGC by nNOS was determined as described previously [31]. The complete assay mixture contained 1 μ g/ml sGC, 5 μ g/ml nNOS, 50 mM potassium phosphate, pH 7.4, 3 mM MgCl₂, 1 mM cGMP, 0.5 mM [α -³²P]GTP (approx. 200 000 c.p.m.), 50 μ g/ml BSA, 0.1 mM L-arginine, 10 μ M CaCl₂, 10 μ g/ml CaM, 50 μ M NADPH, 40 μ M CHAPS and various concentrations of 5-Me-BH4.

Electrochemical detection of NO

Measurement of NO was performed as described previously [11]. To determine the effect of 5-Me-BH4 on NO autoxidation, a solution of authentic NO (approx. 1 μ M) was added to 50 mM potassium phosphate, pH 7.4, and its concentration was monitored continuously. The solution was then subjected to increasing concentrations of 5-Me-BH4. In another series of experiments the involvement of O₂⁻ in NO consumption by 5-Me-BH4 was investigated. In these experiments 10 μ M Sper-NO was used, instead of authentic NO, as a slow NO-releasing compound [32,33]. After a steady-state NO concentration had been established, 5-Me-BH4 (0.1–10 mM) and, 1–2 min later, SOD (1000 units/ml) were added.

Rapid-scan spectrophotometry

Rapid-scan spectra were measured with a Bio-Sequential SX-17MV Stopped-Flow ASVD spectrofluorimeter (Applied Photophysics, Leatherhead, Surrey, U.K.), equipped with the Photodiode Array Accessory. Samples were illuminated with an ozone-free 150 W xenon lamp and detected with a photodiode array, equipped with a 200–730 nm grating. Series of 40 spectra with evenly spaced time intervals were acquired over total reaction times of 1, 33 and 131 s. The final reaction mixture consisted of 1.5 μ M nNOS(BH4⁺), 50 mM potassium phosphate, pH 7.4, 0.05 mM NADPH, 0.5 mM CaCl₂, 40 μ g/ml CaM, 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol, with pterins as indicated and with or without 0.2 mM arginine. The enzyme was added to one syringe and NADPH was added to the other; all other compounds were present in both syringes. Reactions were performed at 25 °C.

RESULTS

Effects of 5-Me-BH₄ on NADPH oxidation, L-citrulline formation and H₂O₂ production by nNOS

In a previous paper [11] we showed that 5-Me-BH₄ stimulates the activity of nNOS(BH₄−) in a biphasic concentration-dependent fashion, whereas nNOS(BH₄+) is active in the absence of 5-Me-BH₄ and exhibits only the second stimulatory phase. We ascribed the two phases, which had EC₅₀ values of 0.2 μ M and more than 1 mM, to anti-co-operative binding of pterin to dimeric NOS. In the present study we set out to investigate the effect of 5-Me-BH₄ on the coupling between NADPH oxidation and NO synthesis. To that end we determined NADPH oxidation, L-citrulline formation and H₂O₂ production by nNOS(BH₄+) simultaneously in the presence of various concentrations of 5-Me-BH₄ (Figure 1). The experimental conditions in this assay differed from those used in standard ³H-citrulline determination in several respects, most notably by a 10–20-fold higher concentration of NOS. As expected, L-citrulline formation by nNOS(BH₄+) was hardly affected by the low pterin concentrations that were sufficient to induce the first stimulatory phase with nNOS(BH₄−) [11], although a slight increase in activity was sometimes observed (but not always; see Figure 2). This effect was probably due to variations in the BH₄ content of the enzyme, because it increased with aging of the enzyme preparations (results not shown). Surprisingly, L-citrulline formation was inhibited (40–50%) by

micromolar concentrations of 5-Me-BH₄. Simultaneously, NADPH oxidation decreased as well (50–60%) and H₂O₂ production was inhibited almost completely (Figure 1A); the NADP⁺/L-citrulline stoichiometry decreased from 2.0 to a nearly fully coupled value of 1.6 (Figure 1B). In agreement with our earlier results, stimulation of NOS activity was observed at 5-Me-BH₄ concentrations above 1 mM, an effect that was accompanied by increases in NADPH oxidation, H₂O₂ formation and NADP⁺/L-citrulline stoichiometry.

Although the results presented above are endpoint determinations, the NADPH concentration in those experiments was monitored continuously. The reaction usually proceeded linearly over the full 10 min but occasionally it tended to slow down. Typically, this effect was most prominent in those preparations that required the addition of low concentrations of pterin for optimal activity (the first stimulatory phase of Figure 1) and it was not observed in the presence of high (1 μ M or more) concentrations of 5-Me-BH₄. A possible cause of the phenomenon might have been a lower intrinsic stability of some preparations, resulting in loss of activity during catalysis, perhaps via the autoxidation of endogenous BH₄. NOS-produced H₂O₂ can be ruled out as the cause of the inactivation because catalase (1.8 or 5.7 μ g/ml) did not affect the NADPH oxidation kinetics (results not shown). To find out whether the time-dependent inhibition resulted in a change in coupling we determined the correlation between NADPH oxidation and L-citrulline formation by taking aliquots during the NADPH assay every 30 s for L-citrulline determination. Both in the absence and in the presence of 0.1 mM 5-Me-BH₄, the time course of L-citrulline production mirrored that of NADPH oxidation (results not shown). The time-dependent inactivation at low pterin concentrations did not significantly affect the results, except for a slight underestimation of the activities at pterin concentrations of 1 μ M or less. Consequently, a plot of the initial NADPH oxidation rates as a function of the pterin concentration closely resembled the profile shown in Figure 1, but with a somewhat more pronounced inhibitory phase (66% maximal inhibition; results not shown).

Figure 1 Effect of 5-Me-BH₄ on NADPH oxidation, L-citrulline formation and H₂O₂ production catalysed by nNOS(BH₄+))

(A) NADPH oxidation, L-citrulline formation and H₂O₂ production were determined in single samples at different 5-Me-BH₄ concentrations. NADPH oxidation (○) of a 0.25 ml reaction mixture was continuously monitored at 340 nm; after 10 min, four 50 μ l aliquots were taken for the determination of L-citrulline (●) and H₂O₂ (□). See the Experimental section for further details. To obtain the CaM-dependent NADPH oxidation rates, CaM(−) rates were subtracted from CaM(+) rates. (B) NADP⁺/L-citrulline stoichiometry (■) calculated from the same data. All data points are means $\pm$ S.E.M. for three or more independent experiments. Experimental conditions were: 0.14 μ M nNOS(BH₄), 50 mM potassium phosphate, pH 7.4, 0.2 mM NADPH, 0.5 mM CaCl₂, 0.2 mM [³H]arginine (approx. 133 000 c.p.m.), 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol, with or without 40 μ g/ml CaM; the temperature was 37 °C.

Evidence for autoinhibition of 5-Me-BH₄-saturated nNOS by NO

Because the decrease in activity caused by 10–100 μ M 5-Me-BH₄ coincided with the EC₅₀ of the pterin for NO synthesis [11], we suspected that NO might be the culprit. To investigate this we performed experiments in the presence of the NO scavenger HbO₂ (Figure 2). In agreement with our hypothesis, HbO₂ completely blocked the inhibition caused by 5-Me-BH₄. Similarly, increasing concentrations of 5-Me-BH₄ did not inhibit NOS activity when measured in the HbO₂ assay (results not shown).

In the absence of 5-Me-BH₄, HbO₂ itself was slightly inhibitory (Figure 2). One possible explanation is that O₂^{•−}, no longer scavenged by NO, inactivated the enzyme. To explore this possibility we repeated the experiments in the presence of SOD and catalase. In the absence of HbO₂, SOD/catalase decreased NOS activity at low 5-Me-BH₄ concentrations, so that the enzyme activity became independent of the pterin level. However, SOD/catalase did not affect activity in the presence of HbO₂, which implies that the HbO₂-induced decrease in L-citrulline production was not due to O₂^{•−}. The inhibition was possibly caused by contamination of HbO₂ with a small fraction of methaemoglobin. A recent report showed that methaemoglobin inhibited nNOS with an IC₅₀ of approx. 3 μ M [20]. At the high Hb concentration used in this study (0.12 mM), a contamination

Figure 2 Effects of HbO_2 and SOD/catalase on $\text{nNOS}(\text{BH}_4^+)$ activity at various 5-Me-BH4 concentrations

The formation of $\text{L-}[^3\text{H}]\text{citrulline}$ from $\text{L-}[^3\text{H}]\text{arginine}$ by $\text{nNOS}(\text{BH}_4^+)$ was measured at various concentrations of 5-Me-BH4 in a standard assay (see the Experimental section) in the absence ($\circ$) and presence ($\bullet$) of HbO_2 , and in the absence (**A**) and presence (**B**) of SOD/catalase. The reactions were stopped after 3 min. All data points are means $\pm$ S.E.M. for three or more independent experiments. Experimental conditions were: $0.14 \mu\text{M}$ $\text{nNOS}(\text{BH}_4^+)$, 50 mM potassium phosphate pH 7.4, 0.2 mM NADPH, 0.5 mM CaCl_2 , $40 \mu\text{g/ml}$ CaM, 0.2 mM $[\text{H}^3]\text{arginine}$ (approx. 50 000 c.p.m.), 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol, with or without 0.12 mM HbO_2 , with or without 1000 units/ml SOD, and with or without $0.36 \mu\text{g/ml}$ catalase; the temperature was 37°C .

with at most 1 % would suffice to explain the moderate inhibition that we observed. Looking at the results from a different angle, the inhibition by SOD/catalase in the absence of 5-Me-BH4 and its reversal by HbO_2 , provide further confirmation that NO inhibits NOS and that this autoinhibition is antagonized at low pterin levels by the simultaneous formation of $\text{O}_2^{\bullet-}$.

The pronounced autoinhibition by NO reported here was not observed under standard conditions of the $^3\text{H-L-citrulline}$ assay [11]. We ascribe this to the widely different enzyme concentrations in the two assays: under conditions of low NOS concentration and, consequently, low overall activity, less NO accumulation is expected to result in a lower degree of autoinhibition. To illustrate this we compared L-citrulline formation in the presence of 0.5 and $500 \mu\text{M}$ 5-Me-BH4 at various enzyme concentrations (Figure 3). At low NOS concentrations, activity was enhanced by saturating concentrations of 5-Me-BH4. Because of the low absolute activities, this stimulation was hard to quantify: a best fit ($r = 0.9984$) was obtained with a 50 % activity increase, but the theoretically expected 2-fold stimulation fitted the data almost as well ($r = 0.9975$). The maximal inhibition at high NOS concentrations was 50–60 %. In our previous paper on the effects of 5-Me-BH4, we applied NOS concentrations of approx. 10 nM [11]. As can be seen from Figure 3, at that enzyme concentration the NO-induced autoinhibition approximately counteracts the activity increase engendered by pterin saturation. Consequently, low-affinity pterin binding went unnoticed under those conditions.

To provide further evidence for the crucial roles of NO in causing, and of $\text{O}_2^{\bullet-}$ in preventing, NOS inhibition, we compared the effects of autooxidation-resistant 5-Me-BH4 and $\text{O}_2^{\bullet-}$ -producing BH4 on the initial rate of NADPH oxidation (Table 1).

Figure 3 Effect of enzyme concentration on the inhibition of L-citrulline formation by 5-Me-BH4

The formation of $\text{L-}[^3\text{H}]\text{citrulline}$ from $\text{L-}[^3\text{H}]\text{arginine}$ by $\text{nNOS}(\text{BH}_4^+)$ was determined in the presence of 0.5 and $500 \mu\text{M}$ 5-Me-BH4 at NOS concentrations between 1 nM and $1 \mu\text{M}$. The reactions were stopped after 10 min ($\circ$) or 3 min ($\bullet$). Plotted is the ratio of the activities at 0.5 and $500 \mu\text{M}$ 5-Me-BH4 as a function of the enzyme concentration. The activities varied between 60 and $35\,000 \text{ nM/min}$; catalytic-centre activities varied between 24 and 65 min^{-1} . All data points are means $\pm$ S.E.M. for at least three independent experiments. Fitting the data to the Hill equation yielded the following parameters: IC_{50} , $11 \pm 4 \text{ nM}$; Hill coefficient, 1.0 ± 0.2 ; $\text{ratio}_{[\text{NOS}] \rightarrow 0} = 1.4 \pm 0.1$; $\text{ratio}_{[\text{NOS}] \rightarrow \infty} = 0.43 \pm 0.03$; $r = 0.998$. The broken line drawn through the data points was fitted on the assumption that the ratio at low [NOS] approaches 2. Fitting parameters: IC_{50} , $2.6 \pm 0.3 \text{ nM}$; Hill coefficient, 0.61 ± 0.06 ; $\text{ratio}_{[\text{NOS}] \rightarrow \infty} = 0.37 \pm 0.03$; $r = 0.998$. Experimental conditions were: 50 mM potassium phosphate, pH 7.4, 0.2 mM NADPH, 0.5 mM CaCl_2 , $40 \mu\text{g/ml}$ CaM, 0.2 mM $[\text{H}^3]\text{arginine}$ (approx. 50 000 c.p.m.), 0.2 mM CHAPS, 2.4 mM 2-mercaptoethanol; the temperature was 37°C . The range of NOS concentrations applied in our previous study on the effects of 5-Me-BH4 [11] is indicated by hatching.

Table 1 Inhibition of NOS by 5-Me-BH4 requires the presence of arginine and is abolished by BH4

A comparison of the NADPH oxidation rates in the presence and absence of 5-Me-BH4, BH4 and arginine is shown. NADPH oxidation was determined by monitoring the decrease in A_{340} for 5 min. Rates in the absence of CaM were subtracted from those in the presence of CaM. Results are means $\pm$ S.E.M. for n experiments. Experimental conditions were: $0.45 \mu\text{M}$ $\text{nNOS}(\text{BH}_4^+)$, 50 mM potassium phosphate, pH 7.4, 0.2 mM NADPH, 0.5 mM CaCl_2 , 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol, in the presence or absence of $40 \mu\text{g/ml}$ CaM, with arginine, BH4 and 5-Me-BH4 as indicated; the temperature was 37°C . *Significant difference ($P < 0.01$ with Student's unpaired t test) from all other rates. All other differences were statistically insignificant ($P > 0.05$). Note that the [BH4] column does not take account of the fact that approx. $0.23 \mu\text{M}$ endogenous BH4 is introduced with the enzyme.

[Arginine] (mM)	[BH4] (mM)	[5-Me-BH4] (mM)	Activity ($\mu\text{M/min}$)	n
0.2	0	5×10^{-4}	20.5 ± 1.1	10
0.2	0.1	0	22.5 ± 1.2	4
0.2	0	0.1	$12.7 \pm 0.6^*$	6
0.2	0.1	0.1	20.4 ± 0.3	3
0	0	5×10^{-4}	21.7 ± 0.8	4
0	0	0.1	19.9 ± 2.1	3

Whereas 0.1 mM 5-Me-BH4 caused a highly significant inhibition (by 38 %, $P < 0.0005$), 0.1 mM BH4 had no effect ($P > 0.1$). Moreover, BH4 completely antagonized the inhibition by 5-Me-BH4. We also investigated how the omission of arginine affected

Figure 4 Effect of 5-Me-BH4 on NOS-induced stimulation of sGC

The stimulation by nNOS(BH4+) of cGMP formation from radioactively labelled GTP by sGC was measured in the presence of various concentrations of 5-Me-BH4. All data points are means $\pm$ S.E.M. for three independent experiments. The broken line is the best fit to the data, weighted according to their S.E.M., to the Hill equation. The result obtained at the highest 5-Me-BH4 concentration (1 mM) was not included in the fit. Fitting parameters: basal activity, 0.7 ± 0.2 μ mol of cGMP/min per mg; stimulated activity, 8.1 ± 0.8 μ mol of cGMP/min per mg; Hill coefficient, 1.7 ± 0.5 ; EC_{50} , 5.5 ± 1.3 μ M. Experimental conditions: 1 μ g/ml sGC, 5 μ g/ml nNOS, 50 mM potassium phosphate, pH 7.4, 3 mM $MgCl_2$, 1 mM cGMP, 0.5 mM [α - ^{32}P]GTP (approx. 200 000 c.p.m.), 50 μ g/ml BSA, 0.1 mM L-arginine, 10 μ M $CaCl_2$, 10 μ g/ml CaM, 50 μ M NADPH and 40 μ M CHAPS; the temperature was 37 $^{\circ}C$.

the activity. In the absence of arginine, and thus of NO production, 5-Me-BH4 did not significantly inhibit NADPH oxidation (Table 1).

SOD-independent stimulation of sGC by 5-Me-BH4-saturated nNOS

In agreement with the observation that NO synthesis by nNOS cannot be detected electrochemically unless SOD is present, it has been demonstrated that the stimulation *in vitro* by nNOS of sGC, the main physiological target of NO, also requires SOD [31]. The observation of SOD-independent NO accumulation in the presence of 5-Me-BH4 therefore suggests that in the presence of this pterin SOD is not necessary for sGC activation. To corroborate this, we determined how various concentrations of 5-Me-BH4 affected the stimulation by nNOS of the formation of cGMP by sGC. Indeed, we found that 5-Me-BH4 stimulated sGC from a basal level of 0.7 ± 0.2 μ mol/min per mg more than 10-fold to an activity of 8.1 ± 0.8 μ mol/min per mg (Figure 4). The EC_{50} for this stimulation (5.5 ± 1.3 μ M) matched the IC_{50} for inhibition of nNOS activity (3.9 ± 0.8 μ M, estimated from Figure 2) and the previously reported stimulation of NO production (approx. 5 μ M) [11].

Direct demonstration of the formation of a haem-NO complex during NOS catalysis

The results obtained so far suggest that during catalysis the NOS haem is present as an inhibitory NO complex. To investigate this we measured rapid-scan spectra with and without arginine, BH4, and 5-Me-BH4. In the absence of arginine, the main difference

Figure 5 Formation of a NO-haem complex during NOS catalysis in the presence of 5-Me-BH4 and arginine

Steady-state absorbance spectra during NOS catalysis were recorded by rapid-scan spectrophotometry. See the Experimental section for details. (A) Absorbance changes induced after a reaction time of 10.2 s in the presence of 2 μ M 5-Me-BH4 but without arginine. The absorbance difference spectrum was constructed by subtraction of the separately determined spectra of the reacting chromophores nNOS, NADPH and 5-Me-BH4. (B) Additional absorbance changes observed in the presence of 0.2 mM arginine and 0.1 mM 5-Me-BH4. The absorbance difference spectrum was obtained by subtracting the 0.3 s spectrum from the 5.0 s spectrum. (C) Comparison of the steady-state concentrations of the NO-haem complex monitored at 442 nm in the presence of 0.1 mM 5-Me-BH4 (●) and 0.1 mM BH4 (○). Experimental conditions were: 1.5 μ M nNOS(BH4+), 50 mM potassium phosphate, pH 7.4, 0.05 mM NADPH, 0.5 mM $CaCl_2$, 40 μ g/ml CaM, 0.2 mM arginine, 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol; the temperature was 25 $^{\circ}C$.

between the steady-state and resting NOS spectra consisted of the reduction of the flavins with an absorbance decrease centred at 460 nm, and a shoulder at 478 nm (Figure 5A). Flavin reduction was complete within 0.2 s (results not shown); the concentration of reduced flavin, calculated with a molar absorbance coefficient at 457 nm of 15.4 $mM^{-1} \cdot cm^{-1}$ [34], was 1.3 μ M. In the presence of arginine and 0.1 mM 5-Me-BH4, an additional absorbance change appeared that, after subtraction of the flavin-related changes, was characterized by an absorbance

increase at 442 nm, and decreases at 488 and below 420 nm (Figure 5B). The concentration of the NO complex as estimated from the absorbance changes at 444 and 436 nm was 1.1 μ M, indicating that approx. 73 % of the haem (85 % compared with the flavins) was present as the NO complex during steady state. In the presence of 2 μ M instead of 0.1 mM 5-Me-BH4, the NO complex also formed, but to a lesser extent (by 40–50 %; results not shown). Similarly, with 0.1 mM BH4 instead of 5-Me-BH4, NO complex formation was suppressed by approx. 50 % (Figure 5C). These results confirm that a haem–NO complex was formed during catalysis and that its steady-state concentration was enhanced by saturating 5-Me-BH4 concentrations but not by BH4.

NO scavenging in the presence of high 5-Me-BH4 concentrations

Because the inhibition by micromolar concentrations of 5-Me-BH4 was due to the accumulation of NO, we surmised that the activity increase in the millimolar range might be caused by NO consumption at very high concentrations of pterin. To verify this, we studied the effect of increasing the concentration of 5-Me-BH4 on the rate of NO autoxidation. As previously reported [11], 5-Me-BH4 did not affect the autoxidation rate at pterin concentrations of up to 3 mM; however, slight but significant NO consumption occurred at 10 mM 5-Me-BH4, which was inhibited by SOD (results not shown).

Effect of NO/O₂^{•−} on nNOS activity

At subsaturating pterin concentrations, the simultaneous production of NO and O₂^{•−} is expected to result in the rapid formation of ONOO[−] [16]. It was therefore of interest to assess the effect of ONOO[−] on NOS activity. We determined the effect of ONOO[−] in a standard 10 min citrulline assay, except that no flavins or BH4 were added. Peroxynitrite inhibited citrulline formation in a concentration-dependent manner (IC₅₀ 0.09 ± 0.01 mM, *n* = 3; results not shown). Because of the short lifetime of ONOO[−] at pH 7.5 [25,35], authentic ONOO[−] is a poor model for the continuous formation of NO/O₂^{•−} that occurs during NOS catalysis. In contrast, SIN-1, which like nNOS produces NO and O₂^{•−} simultaneously, has been demonstrated to mimic closely the actions of nNOS at subsaturating BH4 concentrations under a variety of conditions [31,36]. SIN-1 caused a concentration-dependent inhibition with an IC₅₀ of 5 ± 2 mM (results not shown). This concentration can be calculated [36] to correspond to 0.36 ± 0.14 mM NO/O₂^{•−} after 10 min.

DISCUSSION

Model describing the effects of 5-Me-BH4 on nNOS

From previous studies of the effects of 5-Me-BH4 on NOS activity we derived three main conclusions [11]: (1) the pterin cofactor does not cycle between the tetrahydro and quinonoid dihydro redox states during catalysis; (2) the lack of NO accumulation in the presence of saturating BH4 concentrations is caused by BH4 autoxidation; (3) the biphasic stimulation of nNOS(BH4−) by 5-Me-BH4 reflects anti-co-operative pterin binding. At the time we were unable to explain the discrepancy between the EC₅₀ values for NO formation and L-citrulline production. The present results allow us to resolve this issue by providing an alternative interpretation of the biphasic enzyme stimulation.

We observed inhibition of L-citrulline formation, NADPH oxidation and H₂O₂ production, as well as complete coupling of

NOS catalysis, at virtually the same 5-Me-BH4 concentration (4–6 μ M) as that previously shown to induce NO accumulation [11]. On the basis of the present results, which indicate that these phenomena, rather than the stimulation of activity that occurs above 1 mM, reflect pterin binding to the second, low-affinity site, we propose a model that fits all observations (Scheme 1). In the absence of pterin, NOS oxidizes NADPH and reduces O₂ to O₂^{•−} but produces no L-arginine-derived products. Binding of the first equivalent of 5-Me-BH4 (EC₅₀ approx. 0.2 μ M) generates an enzyme species, essentially identical with nNOS(BH4+), that produces NO and O₂^{•−} simultaneously. The formation of NADP⁺, L-citrulline and H₂O₂ can be observed but NO is not detected because of its rapid reaction with O₂^{•−}. In this state the enzyme is partly uncoupled (NADP⁺/L-citrulline stoichiometry approx. 2.0). Binding of the second equivalent of 5-Me-BH4 (EC₅₀ approx. 4 μ M) results in a species that produces only NO and no O₂^{•−}. As a result, H₂O₂ production ceases, NO accumulates to detectable levels and catalysis becomes fully coupled (NADP⁺/L-citrulline stoichiometry approx. 1.5). However, the rate of L-citrulline formation does not increase, because the binding of NO to the haem iron results in autoinhibition, completely offsetting the expected doubling in activity. At high enzyme concentrations, as used in the present experiments, the resulting relatively high steady-state concentrations of NO even cause a pronounced decrease in activity.

The fact that the inhibition of L-citrulline formation by NO never exceeded 60 % shows that turnover can be maintained without the dissociation of NO from the haem. This is in agreement with the model proposed by Abu-Soud et al. [19,37], which has O₂ reacting with haem-bound NO.

Finally, at concentrations above 1 mM, 5-Me-BH4 consumes NO. The underlying mechanism is unclear but seems to involve O₂^{•−}. The reaction is manifested by higher H₂O₂ concentrations and lower net formation of NO. Consequently, autoinhibition diminishes, so that NADPH oxidation and L-citrulline formation are stimulated. The increasing NADP⁺/L-citrulline stoichiometry implies that the elevated NO consumption is somehow coupled to NADPH oxidation, probably by the re-reduction of oxidized pterin by NADPH [8].

NO-dependent autoinhibition of nNOS is observed only when O₂^{•−} formation is suppressed

The autoinhibition of NOS has been the topic of several previous investigations ([4,5,20], and references therein). Slow enzyme inactivation during NOS catalysis was reported under certain conditions, especially in the absence of saturating pterin concentrations. We sometimes observed a gradual loss of activity, which was more prominent in the absence of arginine and added pterin. The mechanism of this inactivation is unknown, and the involvement of NO is uncertain. Recently, H₂O₂ was found to be a major contributing factor [20]; however, the inactivation observed by us was not affected by catalase. Peroxynitrite was reported to inhibit NOS with IC₅₀ values ranging from 0.01 to 0.03 mM [38]. On the basis of our own observations with ONOO[−] and SIN-1, we predict only slight inhibition (at most 30 %) by NO/O₂^{•−} or ONOO[−] at subsaturating pterin concentrations under the experimental conditions applied in this study. Indeed, the observation that the combined presence of HbO₂ and SOD does not stimulate activity (see Figure 2) suggests that inhibition by NO/O₂^{•−} is negligible under these conditions. At the lower enzyme concentrations (approx. 10 nM) used in most standard activity assays no effects are expected at all.

Rapid reversible inhibition is expected to arise from the binding of NO to the NOS haem. Indeed, a correlation between inhibition

Scheme 1 Summary of postulated changes in the catalytic mechanism of nNOS dimers at various 5-Me-BH4 concentrations

In the absence of pterin, NOS catalyses the oxidation of NADPH and the reduction of O_2 to $O_2^{\cdot-}$. With 1 equiv. of 5-Me-BH4 bound per dimer (K_d approx. $0.2 \mu M$) the enzyme produces NO and $O_2^{\cdot-}$ simultaneously. We postulate that NO formation by the pterin-containing subunit is intrinsically faster but that NO remains bound to the haem until it reacts with $O_2^{\cdot-}$ produced by the other subunit in a slower reaction. This accounts for the observed NADPH/L-citrulline stoichiometry of 2.0 and for the fact that NO is not detected electrochemically under these conditions. When a second equivalent of 5-Me-BH4 is bound (K_d approx. $4 \mu M$), NOS becomes a true NO synthase that is autoinhibited to a considerable extent by the formation of a haem–NO complex. In this state the enzyme is fully coupled (NADPH/L-citrulline stoichiometry of 1.5) and NO can be detected electrochemically. Inhibition is relieved by NO scavengers such as HbO_2 , and by $O_2^{\cdot-}$ -producing compounds such as BH4. At very high concentrations (more than 1 mM) 5-Me-BH4 also antagonizes NO-induced inhibition. To explain the increasing NADPH/L-citrulline stoichiometry we postulate that autoxidized 5-Me-BH4 can be recycled by NADPH. In the maximally inhibited state the enzyme still has a catalytic-centre activity of $0.4 s^{-1}$, which is probably due to a reaction of O_2 with the NO–haem complex [19]. We propose that the same model describes the effects of BH4, except that the state with 1 equiv. of BH4 per dimer extends over a far wider concentration range, and NO formation and NOS autoinhibition are masked by the greater oxygen sensitivity of BH4. Abbreviations: Arg, arginine; Cit, L-citrulline; P, pterin (5-Me-BH4).

and the formation of a haem–nitrosyl complex was elegantly demonstrated with combined spectral and kinetic methods [19,37]. Subsequently, it was shown that in the presence of arginine, and therefore under conditions of NO synthesis, the apparent K_m for O_2 increased by an order of magnitude, which suggested that autoinhibition renders the enzyme sensitive to the O_2 concentration [37]. In our laboratory, autoinhibition, as well as electrochemically detectable NO, have so far been observed only in the presence of SOD, in agreement with the ability of BH4 to produce $O_2^{\cdot-}$, which should prevent NO accumulation at saturating pterin levels [14]. In confirmation of this, we now report inhibition in the absence of SOD with the autooxidation-resistant pterin 5-Me-BH4.

Our proposal that the 5-Me-BH4-induced inhibition is caused by NO is supported by the following observations: (1) inhibition coincides with the electrochemical detection of NO; (2) inhibition is antagonized by the NO scavenger HbO_2 ; (3) inhibition is enhanced at high NOS concentrations, and thus high steady-state NO levels; (4) inhibition requires the presence of arginine;

(5) under inhibitory conditions the formation of the NOS haem–NO complex is stimulated. The hypothesis that BH4 antagonizes inhibition by NO scavenging is supported by the following observations: (1) NOS inhibition is induced by 5-Me-BH4 but not by BH4 in the absence of SOD; (2) NO formation is observed with 5-Me-BH4 but not with BH4 in the absence of SOD; (3) sGC activation by NOS is stimulated by 5-Me-BH4 but not by BH4 in the absence of SOD; (4) BH4 antagonizes the inhibition induced by 5-Me-BH4; (5) BH4 lowers the steady-state concentration of the haem–NO complex. Similar observations also support the notion that, for NOS half-saturated with pterin, NO is scavenged by simultaneously produced $O_2^{\cdot-}$.

$O_2^{\cdot-}$ -insensitive NO-induced autoinhibition?

The results reported by Abu-Soud et al. [19,37] differ in several respects from our previous [14] and present observations. In contrast with our results, NO autoinhibition occurred in the presence of saturating BH4 levels. Furthermore, the inhibition

that we observed depended on the NOS concentration below 100 nM, whereas Abu-Soud et al. reported [NOS] independence down to 10 nM. In addition, inhibition was reported to be unaffected by $O_2^{\cdot-}$ and HbO_2 [19]. In contrast, we and others ([20], and references therein), observed SOD- and HbO_2 -sensitive inhibition. Although this indicates that NO inhibition, as observed by us, is affected by $O_2^{\cdot-}$, it does not exclude a $O_2^{\cdot-}$ -insensitive component. Such a component should manifest itself by an inhibitory effect of arginine on NADPH oxidation under standard assay conditions in the presence of excess BH4. Indeed, such inhibition has been reported consistently by Stuehr and co-workers [37,39,40] but not by us [8,41] (see also Table 1). It therefore seems that our NOS preparation does not show $O_2^{\cdot-}$ -insensitive inhibition. The concept of $O_2^{\cdot-}$ -insensitive NO-induced autoinhibition as postulated by Abu-Soud et al. is problematic because the proposed hypothesis requires the rate of NO removal from the haem complex by O_2 (0.06 s^{-1} at $250\text{ }\mu\text{M }O_2$) to be high compared with the NO dissociation rate [19,37]. Direct determination of the NO dissociation rate by the same group yielded a value for the rate constant of 128 s^{-1} [42]. It is unclear how this observation can be reconciled with the postulated mechanism.

At present we have no explanation for the discrepancies between our results and the observations made by others. The conflicting results might derive from intrinsic differences between the various enzyme preparations. Furthermore, our observations indicate that the enzyme concentrations, or, rather, the overall activities, at which previous experiments were performed might have affected the results. The dependence of autoinhibition on the enzyme concentration also calls for some caution when spectral data acquired in the micromolar range are compared directly with the results from activity assays obtained at nanomolar enzyme concentrations. A more fundamental explanation for the differences might perhaps be found in the salt dependence of NOS activity that was documented by us [43] and recently confirmed by others [44]. The latter study established a link between NO-induced autoinhibition and part of the salt effect. Abu-Soud et al. [19] found that, in contrast with nNOS, autoinhibition of the inducible isoform was affected by $O_2^{\cdot-}$ and HbO_2 . In view of this it might be significant that, unlike nNOS, inducible NOS is not stimulated by salts [43]. We found that the buffer triethanolamine mimicked the effects observed with simple salts [43]. It is therefore conceivable that autoinhibition will depend critically on choice of buffer, which differed between the studies of Abu-Soud et al. (Bis-Tris {1,3-bis[tris(hydroxymethyl)methylamino]propane}, Bis-Tris propane {1,3-bis[tris(hydroxymethyl)methylamino]propane}) [19,37] and ours (triethanolamine, potassium phosphate) [14]. The choice of buffer might also affect the outcome in a different fashion because some organic buffers react with ONOO $^-$, the reaction product of NO and $O_2^{\cdot-}$, to produce NO donors [45]. This will negate the NO-scavenging effect of $O_2^{\cdot-}$, and reinforce NO-induced autoinhibition.

Summarizing, although several questions remain to be answered, the results presented here suggest that $O_2^{\cdot-}$ -insensitive autoinhibition is not an intrinsic property of nNOS catalysis; this conclusion might be valid for the other isoforms as well.

Physiological significance of NO-induced autoinhibition

Current knowledge of the mechanism of NO-induced autoinhibition does not permit a full assessment of its physiological significance. Because of anti-co-operative pterin binding [7,46], NOS might contain only 1 equiv. of BH4 per dimer under most conditions. We proposed previously that this might predestine

the enzyme for the formation of NO/ $O_2^{\cdot-}$ and therefore of ONOO $^-$ [3,5,47]. Under those conditions, autoinhibition by NO will occur only in the presence of $O_2^{\cdot-}$ scavengers. Moreover, we recently presented evidence in support of an alternative mechanism, involving the formation of S-nitrosoglutathione, that allows NOS to stimulate sGC at subsaturating BH4 concentrations [31]. This pathway seems to be operative even when glutathione and SOD are both present, because glutathione overrode the effect of SOD on NOS activity [31].

When NOS is pterin-saturated, NO-induced autoinhibition is certainly relevant in the presence of SOD or other $O_2^{\cdot-}$ scavengers. In the absence of such compounds the physiological significance of inhibition by NO seems questionable in view of the high (more than $1\text{ }\mu\text{M}$) enzyme and pterin concentrations required. Previous studies suggest that the autooxidation of excess BH4 will prevent NO accumulation and sGC stimulation [14,15]. The present observation, that saturating concentrations of the autooxidation-resistant pterin 5-Me-BH4, but not of the natural cofactor BH4, allow NO detection and sGC stimulation, provide further support for this contention. Much therefore depends on whether the enzyme behaves as observed by us or by Stuehr and co-workers. If the link between NO-induced autoinhibition and the presence of salt [44] holds up, the high intracellular and extracellular ion concentrations *in vivo* will completely counteract the mechanism proposed by Abu-Soud et al. [19,37]. Although more work needs to be done to resolve these issues, the present study makes a physiological role of NO-induced inhibition in NOS catalysis seem less likely.

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