Autoinhibition of neuronal nitric oxide synthase: distinct effects of reactive nitrogen and oxygen species on enzyme activity

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Nitric oxide (NO) synthases (NOSs), which catalyse the oxidation of L-arginine to L-citrulline and an oxide of nitrogen, possibly NO or nitroxyl (NO⁻), are subject to autoinhibition by a mechanism that has yet to be fully elucidated. In the present study we investigated the actions of NO and other NOS-derived products as possible autoregulators of enzyme activity. With the use of purified NOS-I, L-arginine turnover was found to operate initially at $V_{\max}$ (0–15 min, phase I) although, despite the presence of excess substrate and cofactors, prolonged catalysis (15–90 min, phase II) was associated with a rapid decline in L-arginine turnover. Taken together, these observations suggested that one or more NOS products inactivate NOS. Indeed, exogenously applied reactive nitrogen oxide species (RNSs) decreased $V_{\max}$ during phase I, although with different potencies (NO⁻ > NO > ONOO⁻) and efficacies (NO > NO⁻ = ONOO⁻). The NO scavengers oxyhaemoglobin (HbO₂; 100 μ M) and 1*H*-imidazol-1-yloxy-2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-

oxide (CPTIO; 10 μ M) and the ONOO⁻ scavenger GSH (7 mM) had no effect on NOS activity during phase I, except for an endogenous autoinhibitory influence of NO and ONOO⁻. However, superoxide dismutase (SOD; 300 units/ml), which is thought either to increase the half-life of NO or to convert NO⁻ to NO, lowered $V_{\max}$ in an NO-dependent manner because this effect was selectively antagonized by HbO₂ (100 μ M). This latter observation demonstrated the requirement of SOD to reveal endogenous NO-mediated autoinhibition. Importantly, during phase II of catalysis, NOS became uncoupled and began to form H₂O₂ because catalase, which metabolizes H₂O₂, increased enzyme activity. Consistent with this, exogenous H₂O₂ also inhibited NOS activity during phase I. Thus during catalysis NOS is subject to complex autoinhibition by both enzyme-derived RNS and H₂O₂, differentially affecting enzyme activity.

Key words: autoregulation, hydrogen peroxide, NOS catalysis.

INTRODUCTION

Nitric oxide (NO) is an important intercellular and intracellular signalling molecule synthesized from L-arginine [1–3] by NO synthase (NOS; L-arginine, NADPH:oxygen oxidoreductase, nitric oxide-forming; EC 1.14.13.39), a self-sufficient cytochrome P450-type haem-protein family with an intramolecular cytochrome P450 reductase domain. Importantly, NO is a mediator of a variety of biological processes including vascular relaxation, modulation of platelet aggregation and cytotoxicity of activated macrophages. The synthesis of NO involves NADPH-dependent electron flow to O₂ from the reductase domain of NOS and depends on the interaction of NOS with the Ca²⁺-binding protein calmodulin (CaM) [4]. However, in the absence of the substrate, L-arginine, NOS has been demonstrated to reduce O₂ at the expense of NADPH to form superoxide (O₂⁻) [5,6] and H₂O₂ [7]. Finally, all NOS isoforms bind and require GSH to protect essential NOS protein thiols [8] and, for unknown reasons, tetrahydrobiopterin cofactor [(6*R*)-5,6,7,8-tetrahydro-L-biopterin (H₄Bip)] [9,10].

NO is a ubiquitous signalling molecule that seems to be important in a variety of cellular functions. However, in concert with reactive oxygen species (ROSs), NO can be transformed

into a highly potent and effective cytotoxic entity of pathophysiological significance [11,12]. Therefore a detailed understanding of the molecular mechanism(s) regulating NO synthesis is paramount for an insight into the function of NO at the cellular level. Indeed, several additional mechanisms that might modulate NO synthesis have been postulated. These include endogenous inhibitory *N*^ω-methylated L-arginines [13], protein phosphorylation [14,15], protein–protein interactions [16], acylation [17,18], changes in substrate supply [19], subcellular localization [17] and subunit dimerization [20,21]. An attractive hypothesis for regulating NOS activity is autoinhibition or a biological feedback control mechanism by which excessively generated NO can regulate the amount of subsequent NO synthesis. This hypothesis was consistent with the observation that exogenous NO, and in particular donors of NO, diminish NOS activity in a concentration-dependent manner [22,23]. Further evidence for an autoinhibition mechanism was rather indirect, inconsistent and mainly based on the use of HbO₂ and superoxide dismutase (SOD) as pharmacological tools with both positive [22–24] and negative [8,25,26] results, even in the same laboratory [22,23,26]. Moreover, it remains controversial under which assay conditions NOS produces detectable levels of NO when measured either by electrochemical means or by EPR spec-

Abbreviations used: Angeli's salt, Na₂N₂O₃; sodium trioxodinitrate; CPTIO, 1*H*-imidazol-1-yloxy-2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide sodium salt; tempone-H, 1-hydroxy-2,2,6,6-tetramethylpyrrolidine hydrochloride; CaM, calmodulin; H₄Bip, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; MetHb, methaemoglobin; NOS, NO synthase; RNS, reactive nitrogen oxide species; ROS, reactive oxygen species; SOD, superoxide dismutase; spermine NONOate, *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propane diamine.

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troscopy [27–29]. If published results are taken together, there is some uncertainty with respect to a straightforward, NO-mediated autoinhibition of NOS. The effects on NOS activity of other possible NOS-derived products (in addition to NO) have not been considered in this context. These include reactive nitrogen oxide species (RNSs) such as NO^- or ONOO^- (stemming from the interaction of NO and $\text{O}_2^{\cdot-}$ or NO^- and O_2) and ROSs such as $\text{O}_2^{\cdot-}$ and H_2O_2 .

The molecular mechanism by which endogenously produced NO can inhibit NOS is yet to be fully understood. The inhibitory effect of NO can be partly antagonized by NO scavengers such as HbO_2 [22,30], suggesting the direct involvement of NO. It seems unlikely that NO interferes with the availability of essential cofactors for catalysis such as NADPH, CaM or H_4Bip because these are usually present in excess [22,30]. Moreover, NO does not compete with L-arginine for the substrate-binding site within the catalytic centre because the Michaelis constants for L-arginine of both NOS-I [22] and NOS-III [30] were not altered during NO-mediated inhibition. However, NO is known to interact with other free radicals, thiol groups and transition metals such as haem iron [31] and this might provide clues. In support of the latter, NO has a high affinity for haem-containing proteins such as Hb, myoglobin, cytochrome P450, soluble guanylate cyclase and cyclo-oxygenase; a likely mechanism of NO-mediated autoinhibition might therefore involve direct interaction with the haem prosthetic group of NOS. This is in agreement with the formation of a ferrous-nitrosyl NOS complex during catalysis [22,26].

The aim of the present study was therefore to investigate NOS autoinhibition further by taking into account the recent findings that (1) free NO can be detected only under certain assay conditions in NOS incubates [27,28,29] and (2) NOS-derived ROS can accumulate [32,33] and contribute to the enzyme's autoregulation.

EXPERIMENTAL

Materials

Sodium trioxodinitrate ($\text{Na}_2\text{N}_2\text{O}_3$; Angeli's salt), was prepared as described [34]; 1-hydroxy-2,2,6,6-tetramethylpyrrolidine hydrochloride (tempone-H) was obtained from Alexis Deutschland (Grünberg, Germany); L-[2,3,4,5- ^3H]arginine hydrochloride from Amersham (Braunschweig, Germany); NADPH from AppliChem (Darmstadt, Germany); catalase (beef liver, 20 mg/ml), Cu-containing SOD (bovine erythrocytes, 5 k-units/mg), GSH and xanthine oxidase (cow milk, 1 unit/mg) from Boehringer Mannheim (Mannheim, Germany); 1*H*-imidazol-1-yl-2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide (CPTIO) and *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propane diamine (spermine NONOate) from Cayman (Ann Arbor, MI, U.S.A.); H_4Bip from Dr. Schirks Laboratories (Jona, Switzerland); H_2O_2 solution (30%) and L-methionine from Merck AG (Darmstadt, Germany); FAD, FMN, L-arginine hydrochloride, methaemoglobin (MetHb, bovine), *N*^ω-methyl-L-arginine, *N*^ω-nitro-L-arginine, *N*^ω-nitro-L-arginine methyl ester, phosphodiesterase 3',5'-cyclic nucleotide activator (CaM) and uric acid sodium salt from Sigma Chemicals (Deisenhofen, Germany). All other chemicals, reagents and solvents were of the highest purity available from either Merck AG or Sigma Chemicals. Water was deionized to 18 M Ω (Milli-Q; Millipore, Eschborn, Germany). Unless indicated otherwise, all chemicals were dissolved in degassed, argon-gassed water except for Angeli's salt and spermine NONOate, which were dissolved in 10 mM NaOH and the corresponding vehicle was used as a control.

Preparation of NOS-I

NOS-I was isolated and purified from pig cerebellum in accordance with methods described previously [8,21,35] by DEAE ion-exchange chromatography and 2',5'-ADP-Sepharose affinity chromatography. The yield of this purification method was 1.0–1.4 mg from 1 kg of tissue with greater than 30% purity and a specific activity of up to 463 nmol L-citrulline/min per mg. Purified NOS-I was stored frozen at -80°C in 50 μl aliquots containing 5 μl of glycerol until the day of use. Protein concentrations were determined spectrophotometrically by a modified Bradford method [36], with BSA as a standard, on 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 an enhanced chemiluminescence kit (ECL[®]; Amersham, Braunschweig, Germany) and a NOS-I-specific antibody (Transduction Laboratories, Hamburg, Germany).

Determination of NOS activity

The catalytic activity of NOS-I was assayed by the calcium CaM-dependent conversion of L-[^3H]arginine to L-[^3H]citrulline [8,37] at 37°C during a standard incubation period of 15 min unless indicated otherwise. If not stated otherwise, reaction mixtures contained 0.25 μg of NOS-I, 50 nM CaM, 1 mM CaCl_2 , 250 μM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulphonic acid, 5 μM FAD, 5 μM FMN, 5 μM H_4Bip and 80 μM L-arginine, including 5.55 kBq of L-[2,3,4,5- ^3H]arginine and 1 mM NADPH in a total volume of 100 μl , 50 mM triethanolamine buffer, pH 7.5. The L-citrulline formed was separated by cation-exchange chromatography and measured by liquid-scintillation counting [8,37]. A unit of enzyme activity is defined as the production of 1 nmol of citrulline/min per mg of NOS enzyme.

NO, ONOO⁻, HbO₂, H₂O₂ and O₂^{·-}-generating system solutions

Aqueous stock solutions of NO, ONOO⁻ and HbO₂ were prepared as described [28,38]. The $\text{O}_2^{\cdot-}$ -generating system consisted of 250 μM hypoxanthine and up to 100 m-units/ml xanthine oxidase resulting in, for example, an $\text{O}_2^{\cdot-}$ flux at 0.75 m-units/ml xanthine oxidase of 0.5 $\mu\text{M}/\text{min}$. The accumulation of H_2O_2 during the NOS activity assay was determined spectrophotometrically by the formation of ferric thiocyanate, as described [7].

Calculation of NO and NO⁻ concentrations

Spermine NONOate-derived NO concentrations were calculated as described [39]. NO⁻ concentrations were based on the disproportionation reaction of 1 mol of Angeli's salt (at neutral pH) into 1 mol each of nitrite and NO⁻ [40]. This stoichiometry has recently been confirmed experimentally (M. Feelisch and H. H. H. W. Schmidt, unpublished work).

Statistics

Unless indicated otherwise, results shown are means $\pm$ S.E.M. Statistical analyses were performed either by an analysis of variance (ANOVA) or by Student's unpaired *t* test (two-tailed). *P* < 0.05 was taken to indicate statistical significance.

RESULTS

Time course of NOS-catalysed L-arginine turnover and the effect of NO, RNS and ROS on NOS activity

NOS catalysis was examined over a 90 min incubation period in the presence of excess substrate and cofactors (see also the Experimental section) to ensure the saturation of all enzyme-binding sites. During the initial 15 min period of L-arginine turnover, NOS activity operated at $V_{\max}$; this period was termed phase I (Figure 1; see also Figure 3B). However, the reaction velocity gradually declined during a second phase (termed phase II) and approached zero after approx. 60 min (Figure 1).

To gain a better understanding of NOS autoinhibition during phases I and II, we investigated the effects of several exogenously added NOS products and candidate molecules for feedback inhibition. These included NO and other RNSs and ROSs, which had not been previously considered, in addition to the co-products L-citrulline and NADP⁺ (Table 1). In general, 0.25 μ g of NOS was used (equivalent to a 15.6 nM concentration of the monomer) and generated approx. 3.75 μ M L-citrulline during 15 min of catalysis. Therefore concentrations of potential NOS products above 10 μ M were not considered. Most notably, NO was the only compound tested that produced complete inhibition of NOS activity (Table 1). When NOS was co-incubated with different concentrations of the NO donor compound spermine NONOate, L-citrulline formation was inhibited in a concentration-dependent manner with an IC_{50} of 0.8 μ M NO (see Table 1). Importantly, the inhibition of NOS was complete in the presence of 4.2 μ M NO. Other NOS-derived products such as NO⁻, ONOO⁻ and H₂O₂ also inhibited NOS activity in a concentration-dependent manner. However, in contrast with NO, these compounds were less efficacious, inducing only partial inhibition even at the highest concentrations tested. For all these compounds, the maximal level of inhibition was similar (30–40% decrease in $V_{\max}$), although their potencies (IC_{50}) varied con-

Figure 1 Time course of NOS-catalysed L-citrulline formation

NOS-I (0.25 μ g) was incubated as described in the Experimental section for up to 90 min; enzyme activity was determined by measurement of the formation of L-citrulline from L-arginine. Total L-citrulline formation is shown as an index of NOS activity. I indicates phase I (0–15 min) of catalysis, during which NOS operates at $V_{\max}$ and product formation is linear; II indicates phase II (15–90 min), during which NOS activity declines rapidly. Results are means $\pm$ S.E.M. ($n = 3$).

Table 1 Candidates for negative feedback regulation of NOS

L-Citrulline formation was assayed for 15 min in the absence or presence of exogenously added NOS-derived products and is expressed as a percentage of control. NO was generated with spermine NONOate, NO⁻ with Angeli's salt, and O₂⁻ with hypoxanthine and xanthine oxidase (see the Experimental section). Results are means $\pm$ S.E.M. ($n = 3$ –12). IC_{50} values were determined by non-linear regression analysis.

Category	Species	IC_{50} (μ M)	Maximal inhibition of NOS (% of control)	<i>n</i>	<i>P</i>
RNS	NO	0.8	-98.8 ± 0.8	3	< 0.05
	NO ⁻	0.02	-40.7 ± 2.7	12	< 0.05
	ONOO ⁻	88	-39.3 ± 4.6	9	< 0.05
	NO ₂ ⁻	> 100	No inhibition	3	
ROS	NO ₃ ⁻	> 100	No inhibition	3	
	H ₂ O ₂	> 2	-31.4 ± 1.9	3	< 0.05
	O ₂ ⁻	> 10	No inhibition	6	
Co-products	L-Citrulline	> 10	No inhibition	3	
	NADP ⁺	> 1000	No inhibition	3

Figure 2 Effect of NO scavengers on NOS activity and kinetics

NOS-I was incubated as described in the Experimental section for up to 90 min in the absence (control, ○) or presence of the NO scavengers 100 μ M HbO₂ (A, ●) or 10 μ M CPTIO (B, ■). Total L-citrulline formation is shown as an index for NOS activity. Results are means $\pm$ S.E.M. ($n = 3$).

siderably (see Table 1). With regard to the threshold concentration for enzyme inhibition, the ranking of potency was NO⁻ > NO > H₂O₂ > ONOO⁻. Other NOS products, O₂⁻, NO₂⁻, NO₃⁻, L-citrulline and NADP⁺, did not have any signifi-

Figure 3 Inhibition of NOS activity by SOD

NOS-I was incubated as described in the Experimental section. (A) NOS-I was incubated during phase I in the absence (control, ○) or presence (●) of 5 k-units/ml SOD at different concentrations of NOS-I (up to 10 μg/ml). (B) NOS-I was incubated for up to 30 min in the absence (control, ○) or presence (●) of 300 units/ml SOD. In (A) and (B), total L-citrulline formation is shown in nmol. Results are means ± S.E.M. ($n = 3-9$).

cant inhibitory effect on NOS activity at concentrations of up to 10 μM or higher and were therefore not investigated further.

Role of endogenous NO and NOS-derived products on NOS activity: effect of NO scavengers and SOD

On the basis of the above observations with exogenous NOS products, endogenously generated NO was considered a likely candidate for NOS autoinhibition: if NOS was indeed subject to endogenous NO-mediated autoinhibition, then effective NO scavengers such as HbO₂ and CPTIO would be expected to increase NOS activity. However, each compound was without effect on V_{max} during phase I and, at longer incubation times during phase II, even tended to decrease total L-citrulline formation (Figure 2). As an alternative mechanistic tool in studying endogenously generated NO, SOD has been suggested to decrease NOS activity by increasing the apparent half-life of NO and thereby the degree of feedback inhibition [22]. Consistent with such a mechanism, in the present study SOD decreased V_{max} independently of NOS enzyme concentration (Figure 3A). Interestingly, the NOS product generated in the presence of SOD had all the characteristics of NO because the SOD-induced decrease in V_{max} could be reversed by the NO scavengers HbO₂ (Figure 4A) and CPTIO (results not shown). Taken together, these results demonstrate that SOD-dependently generated endogenous NO can autoinhibit NOS activity. These findings are in

Figure 4 Effects of SOD and MetHb on activity and kinetics of NOS

NOS-I was incubated as described in the Experimental section. (A) NOS-I was incubated during phase I in the absence (open column) or presence (filled columns) of 100 μM HbO₂, 10 μM MetHb or 5 k-units/ml SOD, either alone or in combination. (B) NOS-I was incubated for up to 90 min in the absence (control, ○) or presence of 300 units/ml SOD (●) or 10 μM MetHb (▲). (C) NOS-I was incubated for 60 min in the presence of different concentrations of MetHb (▲). L-citrulline formation is shown as a percentage of control. In (B), results are expressed as a percentage of the control value of L-citrulline formation after 90 min of incubation. Results are means ± S.E.M. [(A) $n = 12$; (B, C) $n = 3$]. Significance of differences: * $P < 0.05$ compared with control (ANOVA) (open bar); * $P < 0.05$ compared with HbO₂ (ANOVA).

accord with observations that NO formation from NOS can be detected only in the presence of SOD [27,28].

The SOD-dependent autoinhibition is unlikely to be due to the scavenging of endogenous O₂⁻ because exogenous O₂⁻ was without effect on NOS activity during phase I (see Table 1). Given that SOD can also catalyse the conversion of NOS-derived NO⁻ or a related NO precursor to NO [28], we considered the possibility that SOD might be acting by an alternative mechanism

Figure 5 Formation of H₂O₂ by NOS and the effects of catalase on L-citrulline formation

NOS-I was incubated as described in the Experimental section and the formation of H₂O₂ (A, B) or L-citrulline (A, C) was determined. (A) NOS-derived formation of H₂O₂ (■) and L-citrulline (●, ○) are shown under fully coupled reaction conditions (5 μM H₄Bip and 80 μM L-arginine) for incubation periods up to 90 min. L-citrulline formation was determined in the presence (●) or absence (○) of 5 k-units/ml catalase. (B) NOS-derived formation of H₂O₂ during a 45 min period of uncoupled catalysis in the absence (control, open column) or presence of different L-arginine-based NOS inhibitors, *N*^ω-nitro-L-arginine (NO₂Arg), *N*^ω-nitro-L-arginine methyl ester (L-NAME) or *N*^ω-methyl-L-arginine (MeArg) (all at 1 mM, filled columns). In (A), total L-citrulline formation is shown as an index of NOS activity. In (C), NOS-derived formation of L-citrulline is shown (expressed as fold increase) during a 45 min incubation in the absence or presence of different concentrations of catalase (●). Results are means ± S.E.M. (*n* = 3). Abbreviation: kU, k-units.

involving the conversion of NO⁻ to NO. To test this hypothesis, we examined the effect of MetHb, which has also been reported to convert NO⁻ to NO [41] analogously to SOD. Indeed, we have reproduced this observation although the yield is several-fold lower (M. Feelisch and H. H. W. Schmidt, unpublished work). In the present investigation MetHb mimicked SOD by decreasing

$V_{\max}$ (Figure 4B) in a concentration-dependent (Figure 4C) and HbO₂-reversible manner (Figure 4A). Furthermore, this effect was not additive with SOD and, despite similar effects on $V_{\max}$ (Figure 4A), the subsequent inhibition of L-citrulline formation during phase II was considerably more pronounced than that with SOD (Figure 4B). The exact reason for this remains unclear, as is the nature of the reaction of MetHb with the primary NOS product and its interaction with NOS. Collectively, these findings are highly suggestive of an autoinhibitory role for NO⁻ or a related product with a similar kinetic profile generated endogenously from NOS.

Role of endogenous ONOO⁻: effect of ONOO⁻ scavengers

Given the lack of potency and efficacy of exogenous ONOO⁻ at inhibiting NOS activity (Table 1), we examined the possibility that NOS might already be subject to partial autoinhibition during phase I by endogenously formed ONOO⁻. A variety of putative ONOO⁻ scavengers were used as investigative tools. Initially, the specificities of these agents for ONOO⁻ were assessed by examining their abilities to antagonize the inhibitory effects of exogenous ONOO⁻ (100 μM) on NOS activity. Surprisingly, some of these agents (uric acid at 200 and 500 μM, and tempone-H at 40 μM) failed to antagonize the inhibitory effect of exogenous ONOO⁻ (100 μM), whereas others such as methionine (40 mM) were effective. However, methionine alone inhibited NOS activity during phase I in a concentration-dependent manner, revealing possibly non-specific actions and making interpretation difficult. Lower concentrations of methionine (20 mM) did not antagonize exogenous ONOO⁻ (100 μM). In contrast, GSH (7 mM), which effectively antagonized ONOO⁻ (100 μM), was without any effect during phase I, excluding an inhibitory influence of endogenous ONOO⁻ on NOS activity.

Effect of endogenously generated H₂O₂

In addition to the above RNSs, exogenous H₂O₂ was also observed to inhibit NOS activity (see Table 1). We therefore studied further a possible autoinhibitory role for H₂O₂ by measuring the amount of H₂O₂ generated by NOS under our assay conditions in the presence of excess L-arginine and H₄Bip. Interestingly, during phase I of catalysis no H₂O₂ formation from NOS was detected (Figure 5A). In contrast, after 15 min of catalysis and at the onset of phase II, the rate of H₂O₂ formation increased gradually until it reached a maximal rate of 0.2 μmol/min per mg, approx. 45 min after the beginning of incubation (Figure 5A). Thereafter, H₂O₂ formation continued for at least 180 min (results not shown). Thus the appearance of H₂O₂ formation coincided with the decrease in L-arginine turnover. To verify that the H₂O₂ measured under our conditions was derived from NOS catalysis, we co-incubated NOS in the presence and the absence of three different NOS inhibitors (all at 1 mM) that are thought to have distinct effects on uncoupled NOS catalysis [5,7]. Whereas *N*^ω-nitro-L-arginine and *N*^ω-nitro-L-arginine methyl ester inhibited H₂O₂ formation completely, the decrease was only partial in the presence of *N*^ω-methyl-L-arginine (Figure 5B). Thus the assay signal fulfilled all the criteria of NOS-derived H₂O₂ formation.

During phase II of catalysis and under standard incubation conditions, NOS generated approx. 7.2 μM H₂O₂ within 15 min, which is several-fold above its IC₅₀ and therefore in principle sufficient to exert an autoinhibitory effect on NOS. We then investigated further the mechanistic relevance of this H₂O₂

formation by using catalase, which inactivates H_2O_2 by forming O_2 and water. As expected for a selective role of endogenous H_2O_2 in phase II, catalase had no effect on $V_{\max}$ during phase I but in a concentration-dependent manner (Figure 5C) increased L-citrulline formation during phase II (Figure 5A). Interestingly, the onset of the effect of catalase coincided with the time point at which endogenous H_2O_2 formation was detected, presumably by scavenging autoinhibitory H_2O_2 .

DISCUSSION

NOS catalysis was examined in the present study over a 90 min incubation period: two distinct phases were evident. Initially, L-arginine turnover operated at the maximal reaction velocity during 15 min of catalysis (termed phase I) and thereafter declined progressively (phase II). We examined the possibility that endogenously generated NO might have a role because exogenously applied NO decreased $V_{\max}$ in a concentration-dependent manner during phase I. However, the NO scavengers HbO_2 and CPTIO were without effect on NOS activity during phase I and even tended to decrease activity during phase II. Taken together, these observations seemed to exclude any tonic influence of endogenously generated NO. Similar findings were reported for NOS-I by Stuehr and co-workers [26] with the use of HbO_2 , although Ignarro and co-workers [23] were able to show an increase and almost complete 'linearization' of NOS-I activity over a 10 min period. The exact reason for the discrepancy under similar assay conditions remains unclear, although it might be related to the purity of the NOS preparation and the presence of contaminant enzymes such as SOD or catalase. Stuehr and co-workers [26] reasoned that the failure of HbO_2 to increase activity was due to an inability to access and inhibit the formation of a ferrous-nitrosyl complex and showed that only NO generated in the vicinity of the catalytic centre can bind to the iron haem prosthetic group before diffusion and equilibration into solution. This might explain why the inhibitory effect of exogenously applied NO on NOS activity can only ever be partly antagonized by effective NO scavengers such as HbO_2 [30].

$\text{O}_2^{\cdot -}$ is known to react rapidly with NO to form ONOO^- ; it is possible that an extremely short half-life of endogenously generated NO prevents otherwise sufficient quantities from accumulating and exerting an autoinhibitory influence. In support of this, it was recently shown that the FAD-dependent consumption of NADPH is a significant source of $\text{O}_2^{\cdot -}$ in the reaction mixture and that NO from NOS-I can be detected by EPR spectroscopy only when FAD is omitted [29]. We investigated this proposal by repeating the above experiments with HbO_2 and CPTIO in the absence of FAD in the assay mixture. However, once again we failed to observe any endogenously mediated autoinhibition by NO (results not shown). Moreover, given that low concentrations of exogenous NO (less than $1 \mu\text{M}$) were also effective at inhibiting NOS activity, this argues against the suggestion that NO was being rapidly inactivated and scavenged by excessive $\text{O}_2^{\cdot -}$ in our reaction mixture. It also seems unlikely that endogenously generated ONOO^- (stemming from NO and $\text{O}_2^{\cdot -}$) exerts an inhibitory influence because (1) the ONOO^- scavenger GSH was without effect on enzyme activity and (2) the concentration of exogenous ONOO^- required to inhibit NOS was high (IC_{50} $88 \mu\text{M}$; see Table 1) and the degree of inhibition was modest. However, it is conceivable that ONOO^- might be generated under the present assay conditions, although most probably in insufficient quantities to influence NOS function.

It is noteworthy that the co-products of NOS catalysis, L-citrulline and NADP^+ , were without effect on NOS activity,

which excludes them as autoregulators of NOS function during catalysis. We observed marked differences in their ranking of potencies (IC_{50}) and efficacies by various RNSs including NO, NO^- and ONOO^- (see Table 1). This might be complicated by issues relating to the chemical stability and differences in the decomposition kinetics of these RNSs in solution, a point particularly pertinent for NO^- and ONOO^- at physiological pH. Interestingly, the potency of NO was found to be lower than the closely related RNS NO^- , despite NO being considerably more efficacious. Therefore we studied further the role of endogenously generated NO by using SOD as a pharmacological tool to increase the apparent half-life of NO. In the present study, SOD decreased NOS activity during phase I of catalysis, analogously to previous studies [23], and this effect was antagonized by HbO_2 and CPTIO. Taken together, these findings demonstrate the specific involvement of endogenous NO in mediating auto-inhibition and reveal that this phenomenon can be observed only under certain conditions with interventions. More importantly, in the physiological setting and in the presence of cellular SOD, excessively generated NO can become a prominent autoregulator of NOS function.

It is possible that the effect of SOD is due to the scavenging of endogenous $\text{O}_2^{\cdot -}$ that otherwise would react with NO and thereby decrease the steady-state NO concentrations in the assay solution. However, this seems unlikely given that exogenous $\text{O}_2^{\cdot -}$ was without effect on NOS activity. Rather, our results together suggest that SOD might be acting through an alternative mechanism by catalysing the conversion of NO^- to NO [28] because similar results were obtained with MetHb [41]. However, more work is required to implicate NO^- or a related product of NOS unequivocally as the labile precursor of NO or as a direct metabolite. Certainly, measurements with EPR spectroscopy argue against the generation of NO^- from NOS-I [29]. In this report, a spin trap signal from NOS-I was demonstrated during catalysis and was qualitatively similar to that of authentic NO, whereas the NO^- donor Angeli's salt produced no detectable signal [29]. However, these findings have recently been questioned because Angeli's salt was shown to generate a clear EPR signal analogous to that of NO, thus making any discrimination between NO^- and NO difficult (A. M. Komarov, D. A. Wink, M. Feelisch and H. H. H. W. Schmidt, unpublished work). Indeed, Angeli's salt is considered to be highly labile and can rapidly hydrolyse to nitrite [34]. Therefore the chemistry of commercially available Angeli's salt is thought to be problematic.

As an important addition to the above model, during prolonged catalysis ROSs become prominent and have a previously unrecognized role in regulating NOS function. At the beginning of phase II, NADPH-dependent activation of oxygen by NOS became, for unknown reasons, gradually uncoupled from L-arginine oxidation despite the presence of excess substrate, resulting in increasing rates of H_2O_2 formation. More importantly, during this phase the accumulation of H_2O_2 led to an inactivation of NOS with respect to L-arginine turnover, because catalase stabilized NOS during phase II without altering phase I. These results suggest that the transition from phase I to phase II is marked by a modification of the catalytic centre of NOS, possibly resulting in suboptimal electron transfer, a condition that self-perpetuates as it both leads to H_2O_2 formation and is further aggravated by it. Interestingly, evidence is emerging to suggest that the reductase domain of NOS-I might also be a significant source of $\text{O}_2^{\cdot -}$ production in a CaM-independent manner [42]. The above findings might have cellular implications for NOS-I, which is considered to have a role in brain injury associated with ischaemia and reperfusion, e.g. focal cere-

Scheme 1 Autoinhibition of NOS during phase I

Under defined conditions, NOS is subject to autoinhibition by endogenously generated molecules as indicated, in particular by NO or a NOS product that might be a labile NO precursor molecule sharing some characteristics of nitroxyl (NO⁻). Once generated, NO⁻ is converted to NO by SOD [28,41], decomposes to N₂O or reacts chemically with O₂ to produce ONOO⁻. Although ONOO⁻ can also be generated from NO by reaction with O₂⁻, it probably does not reach a sufficient concentration to exert an inhibitory influence on enzyme function. All three RNSs and putative NOS products NO⁻, NO and ONOO⁻ can inhibit NOS function during catalysis (phase I). In contrast, other co-products (NO₂⁻, NO₃⁻, L-citrulline and NADP⁺) are without effect.

Scheme 2 Autoinhibition of NOS during phase II

After 15 min of incubation and transition to phase II, NOS is inactivated by endogenous H₂O₂, stemming from uncoupled reduction of O₂. Catalase, by scavenging H₂O₂, stabilizes NOS during phase II of catalysis. Although the intra-assay source or sources of O₂⁻ are currently unclear, they might derive from the autooxidation of tetrahydrobiopterin (H₄Bip) [27,49], from the FAD-dependent consumption of NADPH [29] or from the reductase domain of NOS-I in a CaM-independent manner [42].

bral ischaemia [43]. Importantly, the underlying pathology of ischaemia also favours uncoupled NOS-I catalysis and ROS formation owing to a variety of prevailing intracellular conditions, including decreased substrate availability [6] and lowered pH [44]. However, the significance of these changes for NOS-I regulation and ROS production in intact neuronal cells has not been studied in any great detail.

In summary, here we show that during catalysis NOS is subject to complex autoinhibition by both enzyme-derived RNSs and H₂O₂ differentially affecting $V_{\max}$ and enzyme activity. Schemes 1 and 2 attempt to encompass all relevant autoinhibition pathways for regulating NOS function by both RNSs and ROSs during phases I and II respectively. However, it is also important to elucidate at the biochemical level the precise mechanism(s) by

which NOS is autoinhibited, in addition to previously described nitrosyl-haem formation [26]. Other mechanisms might include changes in post-translational haem insertion [45], auto-nitrosylation of essential thiols [8,46,47] or auto-nitration of essential protein tyrosine moieties [31,48]. Finally, a complete understanding of NOS autoinhibition is not only of mechanistic importance but is also relevant to cell biology and, in particular, pathophysiology, i.e. conditions typically associated with concomitant RNS and ROS formation such as ischaemia-reperfusion injury and inflammation.

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