SUPEROXIDE DISMUTASE AND CATALASE ARE REQUIRED TO DETECT *NO FROM BOTH COUPLED AND UNCOUPLED NEURONAL NO SYNTHASE

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Abstract—Despite numerous approaches to measuring nitric oxide (*NO) formation from purified NO synthase (NOS), it is still not clear whether *NO is a direct or indirect product of the NO synthase reaction. The direct detection of catalytically formed *NO is complicated by side reactions with reactive oxide species like H2O2 and superoxide. The aim of the present study was therefore to reinvestigate these reactions both electrochemically and by chemiluminescence detection with particular emphasis on the requirement for cofactors and their interference with *NO detection. Flavins were found to generate large amounts of H2O2 and were therefore excluded from subsequent incubations. Under conditions of both coupled and uncoupled catalysis, SOD was absolutely required to detect *NO from NOS. H2O2 formation took place also in the presence of SOD and gave a smaller yet significant interfering signal. Similar data were obtained when the proposed intermediate N⁶-hydroxy-L-arginine was utilized as substrate. In conclusion, standard Clark-type *NO electrodes are cross-sensitive to H2O2 and therefore both SOD and catalase are absolutely required to specifically detect *NO from NOS. © 2004 Elsevier Inc. All rights reserved.

Keywords—Nitric oxide, NOS, Superoxide, Catalase, SOD, Flavins, NO electrode, Free radicals

INTRODUCTION

Nitric oxide synthases (NOS) catalyze the oxidation of a terminal guanidino nitrogen of L-arginine [1]. All three known NOS isoforms represent multifunctional oxidoreductases that require tetrahydrobiopterin (H4Bip), Ca²⁺/calmodulin, FAD, and FMN as cofactors or stabilizing molecules and L-arginine, molecular oxygen, and NADPH as substrates [2,3]. For activity measurements, the by-product of the NOS reaction, L-citrulline, is commonly determined utilizing cation-exchange chromatography. In contrast, to date *NO formation has mostly been measured indirectly (e.g., via the oxyhemoglobin assay or the Griess reaction [4]), as the direct measurement of NOS-derived *NO is difficult and the correct conditions for its detection remain controversial [5–7]. There is considerable evidence that in vivo NOS is not saturated with L-arginine and H4Bip. Under these conditions, the electron flow within the enzyme is uncoupled and neuronal NOS (NOS-I) also catalyzes the generation of hydrogen peroxide (H2O2) [8]. Furthermore, especially L-arginine levels are thought to regulate superoxide production from NOS-I [9–13]. Autoxidation of reagent H4Bip [14] was suggested as an alternative mechanism of O₂⁻ formation. Superoxide, of either origin, could rapidly inactivate *NO to peroxynitrite and hence complicate direct *NO measurements, especially when the short half-life of *NO (<5 s) is taken into account.

In this context, intracellular H4Bip seems to play an ambivalent role in NOS catalysis; on the one hand, it is apparently required as an activator and stabilizer of NOS; at the same time, it was suggested to be a source for O₂⁻, which immediately scavenges NOS-derived *NO.
at diffusion-limited rates [14]. However, other groups did not detect relevant amounts of \( \text{O}_2^- \) from H\(_2\)Bip by electron paramagnetic resonance (EPR) [11,15] and no autoxidation products from H\(_2\)Bip could be found in HPLC measurements, but it seemed to scavenge reactive nitrogen species [16]. Therefore, rather than H\(_2\)Bip autoxidation [14], flavin-derived reactive oxygen species (ROS) may prevent the detection of NOS-derived \(^\bullet\)NO in vitro [5].

Methods to directly quantify \(^\bullet\)NO formation from purified NOS include electrochemical detection using a Clark-type electrode with reversed polarity [14,17], chemiluminescent detection (CLD) [17], and EPR spectroscopy by scavenging \(^\bullet\)NO with spin trap compounds such as N-methyl-d-glucamine dithiocarbonate (MGD) and subsequent formation of a paramagnetic complex, NO–Fe–MGD [18]. Previous studies [7,14,17] demonstrated that \(^\bullet\)NO could not be detected directly unless high concentrations of superoxide dismutase (SOD; 5 kU ml\(^{-1}\)) were also present. It therefore was suggested that \(^\bullet\)NO may not be the primary NOS product. One possible candidate, or at least model compound, for an alternative product would be nitroxyl (HNO). SOD catalyzes the conversion of HNO to \(^\bullet\)NO [19,20] and this reaction could well account for the SOD dependence of NOS-derived \(^\bullet\)NO signals [17]. Conversely, in a different study [5] using Fe–MGD EPR spectroscopy, a NOS-derived signal could be observed in the absence of both SOD and flavins (FAD and FMN). However, recent evidence suggested that this method is not selective for \(^\bullet\)NO and therefore not applicable to discriminate between \(^\bullet\)NO and related N-oxides, especially nitroxyl [18,21], although this was questioned [22].

The present study was therefore conducted to further elaborate the experimental conditions for the electrochemical detection of \(^\bullet\)NO during uncoupled as well as coupled NOS-I catalysis and to investigate the possible interference of H\(_2\)Bip or NOS-derived ROS with \(^\bullet\)NO measurements.

**EXPERIMENTAL PROCEDURES**

**Materials**

\( \text{N}^\text{\textbeta} \)-Hydroxy-L-arginine (OH-Arg) was obtained from Calbiochem (La Jolla, CA, USA); CPTIO and spermine NONOate were from Cayman Chemical Company (Ann Arbor, MI, USA); FAD, GSH, SOD, and catalase were from Boehringer Mannheim (Mannheim, Germany); FMN was from Fluka (Buchs, Switzerland); H\(_2\)Bip was from Dr. Schirks Laboratories (Jona, Switzerland). All other reagents were of the highest analytical grade available and from either Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

**Expression and purification of human recombinant NOS-I and purification of native porcine cerebellum NOS-I**

Human recombinant NOS-I was expressed in a baculovirus/Sf9 cell system and purified to apparent homogeneity by subsequent 2’,5’-ADP–Sepharose and calmodulin affinity chromatography [6]. The purity of the preparation was determined densitometrically from Coomassie-stained SDS/PAGE gels. The protein concentration was determined according to the method of Bradford using bovine serum albumin as a standard [23]. Two enzyme preparations with similar specific activities (400 nmol mg\(^{-1}\) min\(^{-1}\)) and purity (\(\geq95\%\)) were used. For comparison, native porcine cerebellum NOS-I, purified as published [17], was used.

**Electrochemical detection**

The electrochemical detection was performed as previously described [17]. The reversed polarity Clark-type electrode detects \(^\bullet\)NO, but is also sensitive for H\(_2\)O\(_2\) in a ratio of 1:5 [24]. In order to quantify both \(^\bullet\)NO and H\(_2\)O\(_2\), the electrode was calibrated with authentic \(^\bullet\)NO and H\(_2\)O\(_2\). The detection limits for \(^\bullet\)NO and H\(_2\)O\(_2\) were 10 and 50 nM, respectively, based on a signal-to-noise ratio of 3:1. All experiments were carried out at room temperature. Each electrode reading given in the figures is representative of three to six independent experiments.

**Nonenzymatic electrode signals**

To determine whether the observed electrode signals were of nonenzymatic origin, i.e., derived from flavins or other assay components, some experiments were performed by incubating FAD or FMN in a total volume of 300 \(\mu\)l Tris·HCl (50 mM, pH 7.4) containing 1 mM NADPH. The reaction was started by addition of 5 \(\mu\)M FAD or 5 \(\mu\)M FMN. In some of the experiments, 10 \(\mu\)M H\(_2\)Bip, 5 kU ml\(^{-1}\) SOD, and 5.2 kU ml\(^{-1}\) catalase were also included in the incubation mixture.

**NOS-derived signals**

Measurements of NOS-derived electrochemical signals (0.14 \(\mu\)M NOS) were performed in a total volume of 300 \(\mu\)l Tris·HCl (50 mM, pH 7.4) containing 1 mM NADPH, 1 mM L-arginine or OH-Arg, and 0.05 or 1 \(\mu\)M CaM for uncoupled and coupled conditions, respectively. The enzymatic reaction was started by addition of 1 mM CaCl\(_2\). Where indicated, 10 \(\mu\)M H\(_2\)Bip, 5 kU ml\(^{-1}\) SOD, up to 3.9 kU ml\(^{-1}\) catalase, and 100 or 300 \(\mu\)M CPTIO were also included in the incubation mixture. In all experiments with NOS-I, 15 \(\mu\)g of enzyme was used.
Chemiluminometric NO detection

A microreaction chamber containing 50 mM Mops buffer (pH 7.0), 5 μM FAD, 10 μM FMN, 1 mM NADPH, 50 nM CaM, 7 mM GSH, 1 mM CaCl2, and 500 μM either L-arginine or OH-Arg was connected to a CLD 780 TR chemiluminescence measurement device (eco Physics, Dürnten, Switzerland). The reaction mixture, which also contained 5 kU ml⁻¹ SOD for some experiments, was equilibrated with NOx-free air passed over the continuously stirred sample. The reaction was started by the addition of NOS (10 μg) and carried out at room temperature in a total volume of 1.0 ml. Calibrations, measurements, and data analysis were performed as described [17]. The detection limit for NO was 5 pmol min⁻¹ based on a signal-to-noise ratio of 3:1. Neither H₂O₂ nor O₂⁻ cannot be detected with this method, as verified by authentic H₂O₂ or a superoxide-generating system (xanthine oxidase/hypoxanthine; not shown). Results are expressed as means ± SEM of at least three independent experiments.

RESULTS

NO reacts with both O₂⁻ and H₂O₂ leading to a considerable quenching of the NO electrode signal. Both ROS are supposed to be formed during uncoupled, enzymatic L-arginine turnover but also nonenzymatically from assay components. In order to optimize the assay conditions for the electrochemical NO detection, we investigated possible nonenzymatic sources of O₂⁻ and H₂O₂.

Both FAD and FMN generate electrode signals due to H₂O₂ formation

The presence of FAD (5 μM) in Tris HCl buffer (pH 7.4) resulted in a strong NADPH-dependent electrochemical signal (Fig. 1a) not affected by up to 5 kU ml⁻¹ SOD (Fig. 1c). However, the presence of catalase (5.2 kU ml⁻¹) completely abolished this signal (Fig. 1e), suggesting that the source of this signal was H₂O₂. Because only uncharged molecules, and not O₂⁻, can be detected by the electrode used, SOD should have increased the signal if concomitant O₂⁻ formation took place, as it catalyzes the dismutation of O₂⁻ to H₂O₂ (compare Fig. 1a with 1c). Therefore concomitant superoxide formation, as suggested by a recent study [5], seems to be minor compared to H₂O₂ generation. Similar data were obtained with FMN (5 μM) (Fig. 1b). Again, the electrochemical signal was not altered upon addition of SOD (Fig. 1d), but was abolished by catalase (5.2 kU ml⁻¹) (Fig. 1f). These data suggest that predominantly H₂O₂ is formed from flavins. However, the simultaneous formation of small amounts of O₂⁻ remains a possibility [5].

![Fig. 1. Reagent flavins generate H₂O₂ during their nonenzymatic reduction by 1 mM NADPH. The reaction was started (▲) by addition of 5 μM FAD (a, c, e) or FMN (b, d, f); thereafter, electrochemical measurements were performed as described under Experimental Procedures. In the absence of SOD or catalase (a, b), a prominent signal was recorded that was not affected by the presence of 5 kU ml⁻¹ SOD (c, d). The simultaneous application of 5 kU ml⁻¹ SOD and 5.2 kU ml⁻¹ catalase (c, f) totally abolished the signals, indicating predominant H₂O₂ formation from both flavins during their reduction by NADPH. Original recordings are representative of three independent experiments yielding similar results.](image)

No electrochemical signals can be obtained from H₄Bip

It has been postulated that H₄Bip produces O₂⁻ upon autoxidation [14]. In contrast, we did not detect any electrochemical signal from 10 μM H₄Bip, neither in the absence nor in the presence of 1 mM NADPH or SOD (5 kU ml⁻¹; data not shown). In agreement with this, a recent study utilizing EPR technology [5] revealed that
H4Bip (10 μM), in aqueous oxygenated solution, did not generate O2•−, which was confirmed by another study examining the influence of cofactors on superoxide production from NOS [11].

Thus, the only quantitatively relevant and nonenzymatic source of ROS under the investigated incubation conditions were flavins, which in the presence of NADPH formed H2O2, but no or only trace amounts of O2•−. As NOS is a flavoprotein, containing tightly bound endogenous FAD and FMN, it is only moderately or not at all dependent on exogenous flavins. This could be confirmed by measurement of the enzymatic con-

Fig. 2. NOS-I generates simultaneously an •NO precursor and hydrogen peroxide. As described under Experimental Procedures, 15 μg NOS-I was incubated in the presence of 1 mM L-arginine, 1 mM NADPH, and 0.05 (solid lines) or 1 μM (dashed lines) CaM and (a, c, e, g) in the absence and (b, d, f, h, i) presence of 10 μM H4Bip. Reactions were started by addition of 1 mM CaCl2 (▲). In the absence of both SOD and catalase, an electrochemical signal could be observed which was of similar height in the absence (a) and in the presence (b) of H4Bip. It could be totally abolished when 3.9 kU ml−1 catalase was present from the start on (c, d), indicating that the nature of this signal was H2O2. In the presence of 5 kU ml−1 SOD, but in the absence of catalase (e), a prominent signal was observed, which could be enlarged by addition of 10 μM H4Bip (f, solid line) and even more so when CaM-levels were saturating (f, dashed line). NOS under these conditions most likely produced •NO, generated by SOD from a yet unidentified •NO precursor molecule, and H2O2; when 3.9 kU ml−1 catalase was added (● in c and f), the signal was reduced, but this reduction was partly reversible. When both catalase and SOD were present from the start of the reaction on (g, h), the observed signals were more prominent (i), most likely due to initial H2O2 inactivation by catalase. The •NO scavenger CPTIO (○, 100 μM; □, 300 μM in f and h) abolished the signals dose-dependently and at higher concentrations completely. Original recordings are representative of nine independent experiments yielding similar results. (i) The bar graph represents means ± SEM (n = 9); the asterisk indicates a significant difference between both values (0.038 ± 0.005 and 0.06 ± 0.004 mV, respectively; p < .05; paired two-tailed t test).
version of L-arginine to L-citrulline (not shown). We therefore excluded flavins from the incubation mixture to minimize nonenzymatic H$_2$O$_2$ formation and its interference with enzymatic N-oxides.

Another source of ROS is uncoupled NOS catalysis [11]. There are two modes to induce uncoupling of NOS: submaximal saturation of either the L-arginine/H$_4$Bip or the calmodulin binding sites. As maximal levels of both L-arginine and H$_4$Bip are necessary to stabilize the NOS dimer during catalysis [25], uncoupling was induced in the present study by incubating NOS in the presence of reduced amounts of CaM. Under these conditions, electron flow to the oxygenase domain is reduced, which is thought to result in uncoupled superoxide or H$_2$O$_2$ formation from the flavins within the reductase domain. Uncoupled NOS catalysis then was compared to the coupled reaction, which was measured with enzyme fully saturated with calmodulin.

The specific detection of NOS-derived NO requires both SOD and catalase

Using these modified assay conditions, i.e., in the absence of both FAD and FMN, we examined NOS-derived electrode signals both in the absence (Fig. 2a) and in the presence (Fig. 2b) of 10 μM H$_4$Bip during L-arginine turnover. In both cases, the observed signal height was similar and the signals had similar kinetics. Moreover, NOS-derived electrode signals in the presence of H$_4$Bip but absence of SOD were similar for both coupled and uncoupled conditions of catalysis, i.e., saturating and subsaturating CaM concentrations (data not shown). In contrast, no electrode signal could be observed when catalase (3.9 kU ml$^{-1}$) was present in the incubation mixture from the beginning of the reaction, independent of the presence of H$_4$Bip (Figs. 2c and 2d). This remained unchanged by increasing CaM concentration (up to 1 μM) in the presence of H$_4$Bip. Thus, any electrode signal observed in the absence of SOD was entirely due to H$_2$O$_2$.

When SOD (5 kU/ml) was present from the beginning of experiment, an electrode signal could be detected during L-arginine turnover in the absence of both H$_4$Bip and catalase (Fig. 2e), which had kinetics different from that in the absence of SOD. The addition of exogenous H$_4$Bip now markedly increased the signal height (Fig. 2f, solid line) and even more so when CaM levels were saturating, i.e., under conditions of coupled NOS catalysis (Fig. 2f, dashed line).

To differentiate the source of this signal (\*NO, H$_2$O$_2$, or both), catalase was applied additionally 15 min after the start of the reaction (indicated by the filled circle in Figs. 2e and 2f). Almost immediately the signal was partially reduced to a value significantly above basal ($n = 6$; paired two-tailed t test; Figs. 2e and 2f; solid lines). Again, the signal had similar characteristics, but was more prominent when CaM levels were maximal (Fig. 2f, dashed line).

When catalase was present from the beginning of the experiment together with SOD, but in the absence of H$_4$Bip, the electrode signal from uncoupled NOS was lower than in the respective controls (Fig. 2g; compare with 2a and 2e). Again, when exogenous H$_4$Bip was included in the reaction mixture containing a subsaturating calmodulin concentration (0.05 μM CaM vs. 0.14 μM NOS), the electrode signal was increased to a small extent (Fig. 2h, solid line). Under fully coupled conditions (1 μM CaM), the electrode signals were more prominent but had similar kinetics (Fig. 2h, dashed line). The time point at which catalase was added seemed to be crucial: when catalase was added after 15 min of incubation, the height of the signal was about half of the signal measured upon the initial presence of catalase (Fig. 2i; compare 2h with 2f).

To examine whether the NOS-derived electrode signal was indeed \*NO, CPTIO, an irreversible and specific \*NO scavenger, was applied at concentrations of 100 and 300 μM. Independent of whether catalase was present from the beginning of experiment or added later, the addition of CPTIO completely abolished the signal (Figs. 2f and 2h). Moreover, when 100 μM CPTIO was present together with SOD and catalase from the beginning of the reaction, no signal was observed (data not shown). Therefore, signals observed in the presence of both SOD and catalase were considered to be \*NO.

Importantly, throughout all experiments the detection of authentic \*NO was absolutely dependent on both SOD and catalase: in the absence of SOD, the signals were entirely due to H$_2$O$_2$, in its presence, both \*NO and H$_2$O$_2$ were detected simultaneously (cf. Table 2).

Table 2. NOS-Derived Signals and Their Characteristics

<table>
<thead>
<tr>
<th>Additive</th>
<th>n</th>
<th>Signal height (mV) at t = 15 min</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>0.035 ± 0.002*</td>
<td>*NO precursor + H$_2$O$_2$</td>
</tr>
<tr>
<td>Catalase</td>
<td>7</td>
<td>n.d.</td>
<td>*NO precursor + H$_2$O</td>
</tr>
<tr>
<td>SOD</td>
<td>9</td>
<td>0.06 ± 0.004</td>
<td>H$_2$O$_2$ + *NO</td>
</tr>
<tr>
<td>SOD + catalase</td>
<td>9</td>
<td>0.038 ± 0.005*</td>
<td>H$_2$O + *NO</td>
</tr>
</tbody>
</table>

NOS (15 μg) was incubated in the absence of flavins, in the presence of 0.05 μM CaM (i.e., partially uncoupled conditions), as well as 10 μM H$_4$Bip, and otherwise under the conditions described under Experimental Procedures. When neither catalase nor SOD was present, NOS produced H$_2$O$_2$ as well as an undetectable N-oxide. SOD was absolutely required to detect specific \*NO signals, whereas catalase is important to eliminate H$_2$O$_2$ and thus protects \*NO from reacting with this ROS. \*NO precursor = currently not identified \*NO precursor molecule; n.d. = no signal detectable.

* Please note that in the absence of any additive, the signal is increasing constantly, whereas in the presence of SOD and catalase, it reaches a steady-state level at 15 min (cf. Fig. 2b with 2h).

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Catalase is a reversible *NO scavenger

In the previous set of experiments we noted that catalase slightly, albeit reversibly, lowered the SOD-dependent NOS-derived *NO signal (Figs. 2f and 2h). We thus investigated the possibility of *NO binding and/or metabolism by catalase [26]. *NO signals derived from the *NO donor compound spermine NONOate (0.167 μM) were indeed attenuated by the addition of catalase (up to 10.4 kU ml⁻¹). However, similar to NOS-derived signals, this interaction was only transient and fully reversible within a few minutes. This effect could also be observed when catalase was added repetitively (Fig. 3a). Likewise, when SOD and catalase were present from the beginning of the experiment to ensure that only *NO and not H₂O₂ was recorded, repetitive addition of catalase (up to 3.9 kU ml⁻¹) had a transient effect on NOS-derived electrode signals (Fig. 3b). This indicated a reversible reaction of catalase with both NOS/SOD-derived and reagent *NO.

**OH-Arg as a substrate behaves similar to L-arginine**

In addition to L-arginine, the intermediate or by-product of NOS catalysis, OH-Arg, can be converted to L-citrulline by NOS. We therefore studied OH-Arg as an alternative substrate in equimolar concentrations as L-arginine. Under all investigated conditions, the observed signals had the same characteristics as those with L-arginine.

**Fig. 4.** Catalase has a reversible scavenging effect on NOS-derived signals with OH-Arg as substrate. Signals were registered electrochemically in the presence of 15 μg NOS-I, 1 mM OH-Arg, 10 μM H₄Bip, 5 kU ml⁻¹ SOD, and otherwise as described. 3.9 kU ml⁻¹ catalase was either (a) added after 15 min of incubation or (b) present from the onset of the incubation. Again, catalase reversibly attenuated the signal; notably, (c) when catalase was included from the start, the subsequently observed signal was higher than when catalase was added at t = 15 min. ▲ indicates the start of the reaction by addition of CaCl₂, ● the addition of catalase. Original recordings are representative of three to six independent experiments yielding similar results. The bar graph (c) represents means ± SEM (n = 6); the asterisk indicates a significant difference between both values (0.06 ± 0.006 and 0.109 ± 0.011 mV, respectively; p < .05, unpaired two-tailed t test).
Table 1. SOD Is Required to Detect *NO Formation from both L-Arginine and OH-Arg

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>SOD (5 kU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>0.61 ± 0.30ᵃ</td>
<td>7.81 ± 0.22ᵇ</td>
</tr>
<tr>
<td>OH-Arg</td>
<td>0.64 ± 0.25ᵃ</td>
<td>4.12 ± 0.61ᵇ</td>
</tr>
</tbody>
</table>

*NO from NOS (10 μg) was determined chemiluminometrically as described under Experimental Procedures in the absence or presence of SOD (5 kU ml⁻¹). For both substrates (500 μM), the presence of SOD was absolutely required to detect *NO.

ᵃ Not significantly different from baseline.
ᵇ Significantly different from baseline.

DISCUSSION

Only few reports claim to provide direct evidence for the formation of *NO as the main or sole product of L-arginine turnover by NOS, under both coupled and—presumably more physiological—uncoupled conditions. However, none of the *NO assays, including spin trapping, is direct. The lack of detectability of NOS-derived *NO is frequently attributed to the presence of ROS under the commonly used in vitro assay conditions. Moreover, ROS may derive from uncoupled NOS catalysis itself.

In this study, we demonstrate that the main non-enzymatic sources of ROS in the NOS incubation mixture are NADPH-dependently reduced flavins. Flavin-derived signals originated most likely from H₂O₂, as they were completely abolished by catalase, which is in accordance with previously published data [30]. It is unlikely that O₂⁻⁻ was the prevailing product of flavin reduction, as SOD, which converts O₂⁻⁻ to H₂O₂, had no effect on the signal height.

H₄Bip-derived O₂⁻⁻ has been suggested to be another significant source of ROS, thus reducing *NO recovery [14]. Surprisingly, no electrode signals could be detected from H₄Bip in the absence or in the presence of NADPH and/or SOD. Consistent with EPR spin trap data [5,11], our data exclude an interference of H₂Bip with the electrochemical signal. Based on these data, we consequently excluded exogenous FAD and FMN, but not H₂Bip, from all NOS incubations. Using this experimental protocol, all measured signals were considered to be of enzymatic origin, i.e., NOS-dependent.

*NO rapidly reacts with ROS such as O₂⁻⁻, with intermediates in the Fenton or peroxidase mechanisms, and with iron-containing proteins. All of these reactions are relatively fast and lead to the formation of more or less stable intermediates, possibly causing subsequent oxidative cellular and protein damage [31–33]. Autoxidation of H₂Bip was proposed to generate O₂⁻⁻ [34], which, by instantly reacting with *NO to peroxynitrite [32,35], should account for the fast loss of *NO from NOS incubation mixtures [14]. However, under NOS assay conditions we found no H₂Bip autoxidation, neither electrochemically in the present study nor by HPLC measurements [16]. Thus, O₂⁻⁻ formation from H₂Bip in significant amounts seems unlikely and therefore cannot account for the absolute SOD dependence of NOS-derived *NO signals. Rather H₂Bip may react directly with *NO [16]. A recent study demonstrated by EPR measurements that NOS itself does not produce significant amounts of superoxide [36] (although there has been considerable controversy about this topic afterward [22,36–38]). This rules out superoxide production from fully coupled NOS, as used in the present study. Furthermore, no nonenzymatic ROS source was present. Why cannot NOS-derived *NO then be measured—both electrochemically and by CLD—under these circumstances, as it should be if *NO is indeed the primary reaction product of NOS?

In a recent study using EPR spectroscopy [5] a prominent EPR signal of NO–Fe–MGD was detected in the absence of SOD during NOS-I catalysis. This suggested that NOS-I, unlike our and others suggestions, is able to convert at least some L-arginine directly to *NO, avoiding any intermediate nitrogen species like HNO/NO⁻⁺. Importantly, Angeli’s salt was used as an HNO donor and gave no NO–Fe–MGD EPR signal. This is somehow surprising, as it implies a unique specificity of this assay for *NO over HNO, considering that it has
otherwise been shown for this iron complex to give an EPR signal with the NO' donor sodium nitroprusside and even to oxidize hydroxyurea to give an NO–Fe–MGD signal [39]. Furthermore, in these experiments no positive control was performed to show that Angeli’s salt, a hygroscopic compound that is highly susceptible to hydrolyzing to NO₂, was still intact. Additionally, no control experiments with SOD were reported. We have recently reinvestigated the specificity of this assay and, in contrast to Zweier et al., show that Fe–MGD is fully selective for arginine turnover. However, SOD (10 kU ml⁻¹) was still intact. Additionally, no control experiments with SOD were reported. We have recently reinvestigated the specificity of this assay and, in contrast to Zweier et al., show that Fe–MGD is fully 

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exogenous catalase was necessary to fully suppress H$_2$O$_2$ signals under all conditions examined. Consistent with this, in the aforementioned study a nonphysiological ratio of NOS/H$_2$O$_2$ was necessary to achieve a catalase-like activity of NOS. Taken together, a specific catalase activity of NOS seems unlikely and the observed effects might be ascribed to the general ability of heme proteins to catalyze redox reactions.

In conclusion, all studies investigating the direct chemical detection of a NOS-derived N-oxide [5,17,52] suggest at least the simultaneous appearance of HNO, NO$\cdot$, or related precursors as well as ‘NO. SOD, in this case, facilitates the transformation of the intermediates to ‘NO. Catalase is required to protect already formed ‘NO from reacting with both NOS-derived and exogenous H$_2$O$_2$. Both enzymes are therefore absolutely necessary to specifically quantify the ‘NO release from NO synthase.

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NOS-derived NO signals require both SOD

ABBREVIATIONS

CaM — calmodulin
CLD — chemiluminescence detection
CPTIO — carboxy-PTIO (1H-imidazol-1-yl oxy, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide, potassium salt)
EPR — electron paramagnetic resonance
H₂Bip — (6R)-tetrahydro-L-biopterin
*NO — nitric oxide
NOS — NO synthase
O₂⁺ — superoxide
OH-Arg — N⁷-hydroxy-L-arginine
PAGE — polyacrylamide gel electrophoresis
ROS — reactive oxygen species
SDS — sodium dodecyl sulfate
SOD — superoxide dismutase
spermine NONOate — N-[4-(1-aminopropyl)-2-hydroxy-2-nitrosohydro-zino]butyl]-1,3-propanediamine