The Role of Tetrahydrobiopterin in the Regulation of Neuronal Nitric-oxide Synthase-generated Superoxide

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Tetrahydrobiopterin (H4B) is a critical element in the nitric-oxide synthase (NOS) metabolism of L-arginine to L-citrulline and NO. It has been hypothesized that in the absence of or under nonsaturating levels of L-arginine where O2 reduction is the primary outcome of NOS activation, H4B promotes the generation of H2O2 at the expense of O2. The experiments were designed to test this hypothesis. To test this theory, two different enzyme preparations, H4B-bound NOS I and H4B-free NOS I, were used. Initial rates of NADPH turnover and O2 utilization were found to be considerably greater in the H4B-bound NOS I preparation than in the H4B-free NOS I preparation. In contrast, the initial generation of O2 from the H4B-free NOS I preparation was found to be substantially greater than that measured using the H4B-bound NOS I preparation. Finally, by spin trapping nearly all of the NOS I produced O2 we found that the initial rate of H2O2 production by H4B-bound NOS I was considerably greater than that for H4B-free NOS I.

Neuronal nitric-oxide synthase (NOS I), a member of a family of heme-containing monoxygenases that metabolize L-arginine to NO and L-citrulline (1, 2), contains an N-terminal oxidase domain with binding sites for L-arginine and (6R)-5,6,7,8-tetrahydro-L-biopterin (H4B) and a C-terminal reductase domain with binding sites for FMN, FAD, and NADPH. The domains are connected by a Ca2+/calmodulin-binding region that allows electron transport through the enzyme (3). One rather interesting finding is that H4B, although not essential for heme reduction (4), is nevertheless necessary for significant production of NO from L-arginine (5). This co-factor stabilizes the dimeric structure of NOS (6–8), enhances substrate affinity (9, 10), increases the rate of NADPH consumption (4, 11), promotes destabilization of the NOS-Fe2+O2 species (12), and regulates the mid-point potential of NOS heme (4).

In 1992, we discovered that NOS I generates O2 in the absence of L-arginine (13). More recently, NOS II and NOS III, like NOS I, have been found to generate O2 during enzymic cycling (14–16). In the presence of L-arginine, NOS I generates NO and O2; the ratio of these free radicals is dependent upon the concentration of L-arginine (17, 18). Thus, L-arginine is one of the controlling factors that dictate the selectivity of free radicals produced by NOS. However, in the absence of substrate, NOS uses O2 as the terminal electron acceptor, generating O2 and H2O2 by sequential one-electron reductive steps (see Fig. 1). Under these conditions, there is undoubtedly an alternative mechanism by which NOS regulates the formation of each of these cell-signaling products of O2 reduction. One possibility is that H4B controls production of O2 by increasing the reduction rate of the NOS-Fe2+O2 species (Refs. 19–22 and Fig. 1). Evidence to support this theory comes from experiments where the addition of H4B to purified NOS I diminished the spin trapping of O2 (17, 18, 23). However, these findings must be viewed with caution, because H4B in aqueous solution has been reported to scavenge O2 (24–26) with a rate constant of 3.9 × 105 M−1 s−1 (26). Thus, the conclusion drawn from the earlier studies (17, 18, 23) that H4B regulates NO production of O2 is unsubstantiated, because free H4B and the spin trap compete for NO-producing O2 (26). In contrast, when H4B is bound to NOS at the heme near the dimer interface (27, 28), this pterin is thought not to undergo such reactions. This paper describes a series of experiments designed to test whether H4B bound to NOS is pivotal in shifting the rate of electron flow to the NOS-Fe2+O2 species, thereby promoting formation of H2O2 at the expense of O2.

EXPERIMENTAL PROCEDURES

Materials—NADPH, calmodulin, L-arginine, calcium chloride, H4B, and horseradish peroxidase were purchased from Sigma-Aldrich. Superoxide dismutase was obtained from Roche Diagnostics (Mannheim, Germany). H4B was obtained from Schircks Laboratories (Jona, Switzerland). L-[U-14C]Arginine monohydrochloride ([14C]L-arginine) was purchased from Biosolve (Valkenswaard, The Netherlands). Amplex Red (10-acetyl-3,7-dihydroxyphenoxazin) was purchased from Molecular Probes (Eugene, OR). Amplex Red was dissolved in Me2SO and stored under N2 at −80 °C until use. The spin trap for O2, 5-tert-butoxy carbonyl-5-methyl-1-pyrrolinyl-N-oxide (BMP0) was prepared according to the method described by Zhao et al. (29). All other chemicals were used as purchased without further purification.

Purification of NOS I—NOS I was expressed and purified essentially as described by Roman et al. (30), with the modification that the culture
volume was 500 ml rather than 1000 ml. The effluent from the ADP-Sepharose column was divided into two fractions: one that was reconstituted with H$_2$B (250 $\mu$M) at 4 °C and one that was not. Neither fraction was exposed to l-arginine at any point during purification. After overnight incubation on ice, both of the fractions were applied to an S-200 gel filtration column (Pharmacia Corp.) to remove excess H$_2$B and to further purify the enzyme. The dimer peak was collected and concentrated. The enzyme concentration was determined by its CO difference spectrum, as described in Ref. 30, using an extinction coefficient of 100 mm$^{-1}$ cm$^{-1}$ at $\Delta$e 444–475 nm. Neither fraction was ever frozen but was stored on ice and used the next day.

**NADPH Consumption**—Oxidation of NADPH was performed in a reaction using potassium phosphate buffer (50 mM, pH 7.4, 1 mM DTPA, 1 mM EDTA, 0.4 mg/ml Escherichia coli, 2 (1 mM) calmodulin (100 units/ml) and an extinction coefficient of 21 m M$^{-1}$ cm$^{-1}$ at 260 nm (32). The reaction mixture contained purified NOS I (0.40 mM) or H$_2$B-free NOS I (1.00 mM). The initial rate of NADPH oxidation was estimated using an extinction coefficient of 6.22 mm$^{-1}$ cm$^{-1}$.

**Oxygen Consumption**—Oxygen consumption was measured with a commercial oxygen monitoring system (Hansatech). The system was composed of a membrane-coated Clark-type electrode fitted in a glass body reaction chamber and equipped with a Teflon-coated stirring bar and an air-tight stopper. Data acquisition was performed with proprietary hardware and software (Hansatech). All of the measurements were performed at 25 °C. In a typical experiment, NADPH (150 $\mu$M) was added to either a solution of H$_2$B-bound NOS I (1.00 mM) or H$_2$B-free NOS I (0.40 mM). The initial rate of O$_2$ consumption was measured, assuming an initial O$_2$ concentration equal to 253.4 nmol/ml (31).

**Spin Trapping Superoxide from NOS**—Spin trapping of O$_2^-$ from purified H$_2$B-free NOS I and H$_2$B-bound NOS I was conducted by mixing all components described in the text to a final volume of 0.25 ml. The reaction mixture was then transferred to a flat quartz cell and placed into the cavity of an EPR spectrometer (model E-109; Varian Medical Systems, Inc., Palo Alto, CA). The EPR cell was open to the air, allowing O$_2$ to enter the quartz cell. EPR spectra were recorded at room temperature. Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; amplitude, 0.5 G; sweep time, 12.5 G/min; and response time, 0.5 s. The receiver gain is given in the legend to Fig. 2.

**Rate of Spin Trapping Constant of Superoxide**—The apparent rate constant for the spin trapping of O$_2^-$ by BMPO, generating the corresponding spin-trapped adduct of $\cdot$O$_2$, BMPO-OOH, was estimated using the model O$_2^-$ generating system of xanthine/xanthine oxidase. The reaction mixture contained BMPO (50 mM), hypoxanthine (400 $\mu$M), and sufficient xanthine oxidase to generate 1 $\mu$mol/min of O$_2$ as determined by the SOD-inhibitable reduction of ferriytochrome $c$ (80 $\mu$M) at 550 nm using an extinction coefficient of 21 m M$^{-1}$ cm$^{-1}$ (32). Purified NOS I (0.22–0.23 $\mu$M) was used as a competitive inhibitor (33). The reaction mixtures were immediately transferred to an EPR flat quartz cell and introduced into the cavity of the EPR spectrometer (model E-109; Varian Medical Systems, Inc.). EPR spectra were recorded at room temperature 3 min after the reaction was initiated by the addition of xanthine oxidase. Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; amplitude modulation, 0.5 G; sweep time, 12.5 G/min; and response time, 0.5 s.

**Estimation of the Half-life of BMPO-OOH**—The half-life of BMPO-OOH was determined by monitoring the decrease in the first line of the EPR spectrum of BMPO-OOH as a function of time. The reaction mixture contained BMPO (50 mM) and hypoxanthine (400 $\mu$M) in phosphate buffer (50 mM, pH 7.4, 1 mM DTPA, 1 mM EDTA, 0.1 mg/ml Escherichia coli, 2 (1 mM) calmodulin (100 units/ml, as defined in Ref. 34) was added. The reaction mixture was immediately transferred to an EPR flat quartz cell and introduced into the cavity of the EPR spectrometer (model E-109; Varian Medical Systems, Inc.). EPR spectra were recorded at various time intervals for 60 min.

**Hydrogen Peroxide Formation**—Estimation of H$_2$O$_2$ production was obtained by fluorometric analyses (fluorometer, Hitachi model F2500, High Technologies America, Inc., San Jose, CA). A modified method utilizing the dye Amplex Red was adopted (35–37). The incubation medium was supplemented with Amplex Red (1 $\mu$M) and horseradish peroxidase (5 units/ml) in sodium phosphate buffer (50 mM, 1 mM EDTA, pH 7.4). Reaction mixtures contained NADPH (160 $\mu$M), CaCl$_2$ (0.5 mM), calmodulin (100 units/ml), BMPO (100 mM), and SOD (0.04–80 units/ml). SOD (0.04 unit/ml) was added to each reaction to suppress initial fluorescence seen from the inclusion of NADPH, and SOD (0.14–80 units/ml) was used in control experiments described under "Results and Discussion." The reaction was initiated by the addition of purified H$_2$B-free NOS I (4 mM) or H$_2$B-bound NOS I (4 mM) into the reaction mixture. The initial rate of H$_2$O$_2$ generation was recorded as an increase in fluorescence of the dye at 585 nm with the excitation set at 550 nm. The fluorescence was calibrated by generating a standard curve with known concentrations of H$_2$O$_2$. The concentration of the commercial 30% H$_2$O$_2$ solution was calculated from light absorbance at 240 nm employing an extinction coefficient of 0.0436 mm$^{-1}$ cm$^{-1}$; the stock solution was diluted to 50 $\mu$l with water and used for calibration immediately. The specificity of horseradish peroxidase/Amplex Red toward H$_2$O$_2$ was confirmed, because tert-butyl hydroperoxide was not found to be a substrate.

**NOS I Activity by $[^{14}C]$-L-Citrulline Formation Assay**—The enzymatic activity was determined by its ability to catalyze the formation of L-citrulline from l-arginine as previously reported (17) with modifications. The reaction mixture contained purified NOS I (0.15 $\mu$M), CaCl$_2$ (2 mM), and mixture solution (15 mM-l-arginine (0.6 $\mu$Cl/ml) in the presence of NADPH (1 mM), l-arginine (50 $\mu$M), and calmodulin (100 units/ml) in HEPES buffer (50 mM HEPES, 0.5 mM EGTA, pH 7.4). The reaction was initiated by the addition of the mixture solution into the reaction mixture containing purified NOS I and CaCl$_2$ in the presence of various concentrations of BMP0 ranging from 50 to 150 mM. The reaction mixture was incubated at 23 °C for 10 min and terminated with 2 ml of stop solution (20 mM HEPES, 2 mM EDTA, pH 5.5). The product (15 mM-l-citrulline was separated by passing the reaction mixture through columns containing Dowex® 50W-X8 cation exchange resin precartivated with a NaOH solution (1 M), and radioactivity was counted using a scintillation counter (model LS 6500; Beckman Coulter Inc., Fullerton, CA).

**RESULTS AND DISCUSSION.**

In the absence of l-arginine, NOS I generates O$_2^-$ and H$_2$O$_2$; the latter was from either self-dismutation from O$_2^-$ or direct enzymic formation of H$_2$O$_2$. These reduction products of O$_2$ mediate different cell signaling pathways. Thus, it is important to understand how NOS I regulates formation of O$_2^-$ and H$_2$O$_2$. Clearly, one controlling factor is l-arginine, which upon oxidization by NOS to l-citrulline and NO$^-$ decreases the production of O$_2^-$ (13, 18, 23). Another limiting factor may be H$_2$B, because this pterin appears to be a critical element in shifting the competition between O$_2^-$ generation and direct production of H$_2$O$_2$. Here, the experiments are aimed at testing this hypothesis.

As a source of NOS I, we used recombinant enzyme expressed in *Escherichia coli*. When isolated from this bacterium, NOS does not contain H$_2$B. For these studies, H$_2$B (250 $\mu$M) was added to a portion of the isolated NOS and incubated overnight at 4 °C, and this mixture was subsequently passed through an S-200 gel filtration column to remove excess H$_2$B. Nitric-oxide synthase I isolated in this manner is complemented with about 0.65 nmol H$_2$B/mol enzyme, i.e., 65% saturated (30). No excess, unbound H$_2$B is present. With these two preparations, H$_2$B-free NOS I and H$_2$B-bound NOS I, we investigated whether H$_2$B regulates O$_2^-$ generation.

In our first series of experiments, we estimated the initial rate of NADPH oxidation by incubating NADPH (150 $\mu$M) with either H$_2$B-free NOS I (0.40 $\mu$M) or H$_2$B-bound NOS I (0.40 $\mu$M) and CaCl$_2$ (2 mM/calmodulin (100 units/ml). These are important rates to measure, because both enzyme preparations generate O$_2^-$ in the absence of l-arginine. Thus, the flux of O$_2^-$ and the direct enzymic formation of H$_2$O$_2$ from H$_2$B-free NOS and H$_2$B-bound NOS is dependent on the initial rate of NADPH turnover. We found that the initial rate of NADPH oxidation with H$_2$B-bound NOS I was 164 ± 24 nmol/min/mg protein, whereas with H$_2$B-free NOS I, the initial rate of NADPH consumed was 59 ± 7 nmol/min/mg protein. This enhanced rate of NADPH oxidation when H$_2$B is bound to NOS I was similar to that previously reported (11).

In the absence of l-arginine, the final electron acceptor from the NADPH/NOS reduction is O$_2^-$ yielding O$_2^-$ and H$_2$O$_2$. Hydrogen peroxide can arise from the dismutation of O$_2^-$ as well as the one-electron reduction of Fe$^{3+}$/O$_2$. Followed by release.
of \( \text{H}_2\text{O}_2 \) (Fig. 1). Not surprisingly, according to \( \text{O}_2 \) consumption experiments, we found that independent of the NOS preparation, 1 mol of NADPH oxidized resulted in 1 mol of \( \text{O}_2 \) consumed (data not shown); the difference is in the ratio of the initial \( \text{O}_2 \) reduction products. The experiments were then designed to determine the onset of \( \text{O}_2^\cdot \) production and the initial rate of \( \text{H}_2\text{O}_2 \) formation at the expense of \( \text{O}_2^\cdot \).

Therefore, the relative concentration of the spin-trapped adduct, as determined by EPR spectral peak height, is directly related to \( k_{\text{app}} \) and \( k_{\text{cyto-Fe}^3}\), the rate constants for the spin trapping of \( \text{O}_2^\cdot \) by BMPO and ferricytochrome \( c \), respectively, at a given concentration of BMPO. Thus, Equation 5 can be represented as follows.

\[
A/A = 1 + k_{\text{cyto-Fe}^3}/[\text{cyto-Fe}^3]/k_{\text{app}}[\text{BMPO}]
\]  

(Eq. 6)

where \( A \) and \( A' \) represent the rate of spin trapping \( \text{O}_2^\cdot \) (EPR spectral peak height) in the absence and presence of ferricytochrome \( c \), respectively (33). If the concentration of the BMPO is fixed, then a plot of \( A/A' \) versus \([\text{cyto-Fe}^3] \) generates a straight line, and the slope is \( k_{\text{cyto-Fe}^3}/k_{\text{app}}[\text{BMPO}] \). (40). By using the known rate constant for the reduction of ferricytochrome \( c \) by \( \text{O}_2^\cdot \) to ferricytochrome \( c \), \( k_{\text{cyto-Fe}^3}/k_{\text{app}} = 1.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \) (39), the \( k_{\text{app}} \) for BMPO can be calculated. We found \( k_{\text{app}} = 77 \pm 5 \text{ M}^{-1} \text{s}^{-1} \) (n = 6).

We then determined the stability of BMPO-OOH. For these experiments, xanthine/xanthine oxidase was used as a continuous source of \( \text{O}_2^\cdot \) which in these studies afforded a low flux of 1 \( \mu\text{M} \text{O}_2^\cdot \)/min. After 10 min, SOD (30 units/ml) was added to quench the reaction. We found the half-life of BMPO-OOH to be 22.8 \( \pm \) 0.9 min (n = 6) at pH 7.4 (Scheme 1).

In choosing the best spin trap to estimate \( \text{O}_2^\cdot \) production from both enzyme preparations, we need a nitric oxide whose corresponding spin-trapped adduct of \( \text{O}_2^\cdot \) displays an EPR spectrum that is sufficiently robust to allow accurate measurements of spectral peak heights as a function of time. It appears that BMPO is the appropriate spin trap for these studies. We arrive at this decision based on three important findings. First, the rate constant for the reaction of BMPO with \( \text{O}_2^\cdot \) exceeded that of \( \text{H}_2\text{O}_2 \)-bound NOS (39). Further, ferricytochrome \( c \) does not interfere with the EPR spectrum of BMPO-OOH. The reaction of BMPO and ferricytochrome \( c \) with \( \text{O}_2^\cdot \) can be expressed as follows.

\[
\text{O}_2^\cdot + \text{BMPO} \rightarrow \text{BMPO-OOH}
\]  

(Eq. 1)

\[
\text{O}_2^\cdot + \text{cyto-Fe}^3 \rightarrow \text{cyto-Fe}^2 + \text{O}_2
\]  

(Eq. 2)

From Equations 1 and 2, the rate of \( \text{O}_2^\cdot \) elimination in the presence of ferricytochrome \( c \) can be represented as follows.

\[
-\frac{d[\text{O}_2^\cdot]}{dt} = k_{\text{app}}[\text{BMPO}][\text{O}_2^\cdot] + k_{\text{cyto-Fe}^3}[\text{cyto-Fe}^3][\text{O}_2^\cdot]
\]  

(Eq. 3)

In the absence of cytochrome \( c \), Equation 3 can be described as follows.

\[
\frac{d[\text{BMPO-OOH}]}{dt} = k_{\text{app}}[\text{BMPO}][\text{O}_2^\cdot]
\]  

(Eq. 4)

By dividing Equation 4 into Equation 3 and rearranging the terms, the competing reactions become the following.

\[
\frac{d[\text{O}_2^\cdot]}{dt}/d[\text{BMPO-OOH}] = 1 + k_{\text{cyto-Fe}^3}[\text{cyto-Fe}^3]/k_{\text{app}}[\text{BMPO}]
\]  

(Eq. 5)

Under our experimental conditions, the concentration of BMPO is in great excess with respect to the concentration of \( \text{O}_2^\cdot \).
spectral peak height as a function of time (see inset in Fig. 2). Fig. 2 reveals several novel findings. First, H4B-bound NOS I and H4B-free NOS I generated O2\textsuperscript{•−} in the absence of L-arginine. Second, at early time points, within the first several min after the reaction had commenced, H4B-free NOS I produced more O2\textsuperscript{•−} than that found for H4B-bound NOS. This result is remarkable when one considers that the turnover rate for H4B-bound NOS is 2.8 times faster than that for H4B-free NOS. Over time, the difference in the EPR spectral peak heights of BMPO-OOH from H4B-free NOS I and H4B-bound NOS I disappeared, approaching equality after only 4–5 min. When equilibrium had been reached, at ~8 min, the amount of O2\textsuperscript{•−} spin-trapped by BMPO from H4B-bound NOS I had far exceeded that from H4B-free NOS I.

We then investigated the source of H2O2 from H4B-bound NOS and H4B-free NOS. Hydrogen peroxide can be generated from the one-electron reduction of the NOS-Fe\textsuperscript{2+}O2 species followed by release of H2O2 (Fig. 1). Alternatively, this peroxide can arise from the dismutation of O2\textsuperscript{•−}, in which the rate constant at pH 7.4 is \(3.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) (45). It is therefore by no means a trivial task to separate these disparate pathways. After considering several options, we settled on an approach that required increasing the concentration of BMPO to a level so that this nitroxide would spin trap most, if not all, of the O2\textsuperscript{•−} produced (41). Thereupon, the only source of NOS-derived H2O2 would be from the one-electron reduction of the NOS-Fe\textsuperscript{2+}O2 species.

We then had to find a method that would meet the following criteria. First, the assay had to detect H2O2 in real time, not at some arbitrary time after the reaction had commenced. Second, the method must not interfere with the spin trapping of O2\textsuperscript{•−}. Third, given that NOS can easily transfer electrons to a wide variety of one-electron acceptors, such as ferricytochrome c (13), the assay had to be an oxidative process. Fourth, the method had to be sensitive and selective for H2O2. Given these limitations, we settled on a fluorometric assay developed by Zhou et al. (36, 37). The overall mechanism, shown below, involves three distinct reactions, as presented by Chance (46).

\[
\text{HRP-Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{HRP-Fe}^{4+} \quad (\text{Compound I})
\]
\[
\text{HRP-Fe}^{4+} (\text{Compound I}) + \text{Amplex Red-H}_2 \rightarrow \text{HRP-Fe}^{4+} \text{Amplex Red-H}^+
\]
\[
\text{HRP-Fe}^{4+} (\text{Compound II}) + \text{Amplex Red-H}^+ \rightarrow \text{HRP-Fe}^{4+} + \text{resorufin}
\]

**REACTIONS 1–3**

Although the rate constant for the reaction of H2O2 with horseradish peroxidase to form Compound I is 10 \(\times\) 10\(^6\) M\(^{-1}\) s\(^{-1}\) (46), the rate constants for the sequential one-electron reduction of Compounds I and II to the fluorescent resorufin from Amplex Red are unknown. However, based on similar reactions reported in the literature (46) involving Compound I and II with other donor molecules, we estimate that the rate constant would not be above 1 \(\times\) 10\(^5\) M\(^{-1}\) s\(^{-1}\), and most likely it is close to 1 \(\times\) 10\(^5\) M\(^{-1}\) s\(^{-1}\), too slow to allow quantitative estimates of the initial rate of H2O2 production. Therefore, the initial rate of NADPH consumption measured does not have a direct 1:1 relationship with the initial rate of H2O2 generated, as estimated by this fluorometric technique. Rather, only the ratio of initial rates of H2O2 formation can be used to draw conclusions about the relative fluxes of direct H2O2 generated by H4B-bound NOS and H4B-free NOS preparations.

Before we could measure the rate of H2O2 production from H4B-bound NOS I and H4B-free NOS I, however, a series of control experiments were conducted. Initially, we determined the appropriate concentration of BMPO for the fluorometric assay. Our goal was to find a concentration of BMPO that would maximally spin trap O2\textsuperscript{•−} without inhibiting NOS. For these studies, we added NADPH (1 mM) and CaCl\(_2\) (2 mM)/calmodulin (100 units/ml) to H4B-bound NOS I (0.40 \(\mu\)M) while we varied the concentration of BMPO from 50 to 150 mM. We found that at BMPO concentrations above 125 mM, there was no increase in the EPR spectral peak height of BMPO-OOH (data not shown). Under identical experimental conditions, NOS I activity was assessed by determining what effect, if any, BMPO had on enzymatic activity. We incubated NADPH (1 mM), CaCl\(_2\) (2 mM)/calmodulin (100 units/ml), \([1^{3}C]\)-L-arginine (0.6 \(\mu\)Ci/ml) with H4B-bound NOS I (0.15 \(\mu\)M), and BMPO (ranging from 0 to 150 mM). At concentrations of BMPO greater than 100 mM, significant inhibition in NOS activity was observed. Therefore, we chose a concentration of BMPO at 100 mM for the fluorometric experiments.

Next, we determined the effectiveness of BMPO (100 mM) to compete with SOD for NOS-derived O2\textsuperscript{•−} in the fluorometric assay. For these experiments, we mixed NADPH (160 \(\mu\)M) with H4B-bound NOS I (57 nM), CaCl\(_2\) (500 \(\mu\)M)/calmodulin (100 units/
ml), and SOD (ranging from 0.14 to 80 units/ml). We found that the rate of H$_2$O$_2$ production, as measured by an increase in fluorescence, was constant over this range of SOD used in the experiment (data not shown). Thus, where appropriate SOD (0.14 units/ml) was included in each reaction. When BMPO (100 mM) was added to the above reaction mixture, without and with SOD (0.14 units/ml), we found that BMPO spin-trapped 90% of O$_2^\bullet -$ generated by NOS.

Based on these control experiments, we were confident that by inclusion of BMPO in the reaction mixture, most of the NOS-produced O$_2^\bullet -$ was spin-trapped. It is important to reiterate that although this fluorometric assay is very sensitive and selective for H$_2$O$_2$, the efficiency of this method is unknown. Nevertheless, when the reaction was conducted using only 4 mM of H$_4$B bound to NOS I preparation through a gel filtration column, resulting in H$_2$B-free NOS I (Fig. 2). This observation demonstrates that H$_4$B-bound NOS I generated more O$_2^\bullet -$ than the enzyme bound to NOS that scavenged O$_2$. The experiments conducted using excess H$_4$B, which may not have been under estimated.

The experiments presented here were designed to examine whether H$_4$B bound to NOS I, regulates O$_2^\bullet -$ production. In previous studies (17, 18, 23), H$_4$B was added to a NOS I preparation through a gel filtration column, containing NOS I preparation through a gel filtration column, containing only H$_4$B-bound NOS I.

The data presented here support our hypothesis that bound H$_4$B promoted the direct formation of O$_2^\bullet -$ at the expense of H$_2$O$_2$. Once the heme-O$_2$ intermediate NOS-I(Fe$^{3+}$) bound to NOS, that scavenged O$_2$, the efficiency of this method is unknown. Nevertheless, when the reaction was conducted using only 4 mM of H$_4$B bound to NOS I preparation through a gel filtration column, containing only H$_4$B-bound NOS I. The rate of H$_2$O$_2$ production, as measured by an increase in fluorescence, was constant over this range of SOD used in the experiment (data not shown). Thus, where appropriate SOD (0.14 units/ml) was included in each reaction. When BMPO (100 mM) was added to the above reaction mixture, without and with SOD (0.14 units/ml), we found that BMPO spin-trapped 90% of O$_2^\bullet -$ generated by NOS.

Finally, it is important to point out that H$_4$B plays a dual role in NOS reduction in the absence of l-arginine. First, this pterin enhances the rate of NADPH turnover. Second, H$_4$B shifts the ratio of O$_2$ reduction products by increasing the rate of direct H$_2$O$_2$ formation at the expense of O$_2^\bullet$.

Although it may be premature to speculate as to the physiologic significance of our findings, we offer one possible scenario. In the absence of or under low levels of L-arginine, where O$_2$ reduction is the primary end product of NOS activation, H$_4$B will undoubtedly play a critical role in regulating the generation of H$_2$O$_2$ and O$_2^\bullet$. Because each of these reduction products of O$_2$ activates a different cell signal pathway (47, 48), the importance of H$_2$O$_2$ in the regulation of NOS-derived O$_2$ and H$_2$O$_2$ and its diversity of physiological functions cannot be underestimated.

CONCLUSION

The experiments presented here were designed to examine whether H$_4$B, bound to NOS I, regulates O$_2^\bullet$ production. In previous studies (17, 18, 23), H$_4$B was added to a NOS I preparation through a gel filtration column, containing only H$_4$B-bound NOS I. The rate of H$_2$O$_2$ production, as measured by an increase in fluorescence, was constant over this range of SOD used in the experiment (data not shown). Thus, where appropriate SOD (0.14 units/ml) was included in each reaction. When BMPO (100 mM) was added to the above reaction mixture, without and with SOD (0.14 units/ml), we found that BMPO spin-trapped 90% of O$_2^\bullet$ generated by NOS.

Our findings point to the fact that NADPH/H$_4$B-bound NOS I yielded considerably more H$_2$O$_2$ than did NADPH/H$_2$B-free NOS I, even though both enzyme preparations reduce O$_2$ to O$_2^\bullet$. Given that NOS secretes O$_2^\bullet$ and H$_2$O$_2$, measuring H$_2$O$_2$ without eliminating O$_2^\bullet$ will undoubtedly lead to an overestimation of the direct NOS produced H$_2$O$_2$. It is worth noting that to our knowledge, this is the first report that unequivocally demonstrates that NOS I independently generates both O$_2^\bullet$ and H$_2$O$_2$.
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