PERKIN 2

Investigations into the spin trapping of nitric oxide and superoxide: models to explore free radical generation by nitric oxide synthase

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Nitric oxide synthase catalyzes the oxidative metabolism of L-arginine to L-citrulline and $NO^•$. During cycling, this enzyme, besides generating $NO^•$, also secretes $O_2^{•-}$. These free radicals react at diffusion controlled rates to produce $ONOO^-$, which has been shown to decompose to give, among a variety of products, small amounts of $HO^•$. Thus, during oxidation of L-arginine by nitric oxide synthase, $NO^•$ and $O_2^{•-}$ will be secreted, and as a result, produce H_2O_2 from the dismutation of $O_2^{•-}$, and $ONOO^-$ from the reaction of $NO^•$ and $O_2^{•-}$. Hydroxyl radical can then be formed either by the reaction of H_2O_2 with transition metal ions or decomposition of $ONOO^-$. Using different spin traps unique to each of these free radicals, EPR spectroscopy has been used to identify $NO^•$, $O_2^{•-}$ and $HO^•$.

Introduction

Nitric oxide ($NO^•$) is synthesized during the oxidative metabolism of L-arginine by a family of enzymes known as nitric oxide synthases: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III).[1] In the absence of substrate, NOS I secretes $O_2^{•-}$.[2] More recent findings demonstrate that all isozymes of NOS secrete $O_2^{•-}$ and $NO^•$ at concentrations of L-arginine below its K_m.[3] Under these experimental conditions, it is remarkable that $O_2^{•-}$ and $NO^•$ were spin trapped and identified by EPR spectroscopy,[2,3a–g] considering these free radicals react to produce $ONOO^-$,[4] with a second order rate constant of 3.8 to 19×10^9 M^{-1} s^{-1}.[5] In contrast at pH 7.4, $O_2^{•-}$ is spin trapped by 5,5-dimethyl-1-pyrroline 1-oxide **1** and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide **3** (Scheme 1) at only 12 M^{-1} s^{-1} and 60 M^{-1} s^{-1}, respectively.[6] Given the enormous difference in rate constants between the competing reactions noted above, we hypothesized[3f] that the ability to spin trap $O_2^{•-}$ under the experimental conditions described therein[3d–f] may rest on the fact that $O_2^{•-}$ and $NO^•$ are generated sequentially at the same site, the heme. After $O_2^{•-}$ is produced, NOS must cycle twice before $NO^•$ is secreted.[3f] For NOS II, it has been reported that $O_2^{•-}$ and $NO^•$ are produced at two different sites, the heme and the flavin domains.[3c] A recent paper, however, suggested that NOS I behaves similarly to NOS II.[3g] One may infer from these studies[3c,3g] that during the NOS metabolism of L-arginine both free radicals are produced nearly simultaneously, just separated by the distance between the reductase and oxidase domains. This may not be sufficient, however, to overcome the enormous disparity in rate constants between the reaction of $O_2^{•-}$ with nitrones[6] or $NO^•$.[5] Thus, it was surprising to find that $O_2^{•-}$ was spin trapped during NOS metabolism of L-arginine.[3c,3g]

While no study, to date, has reported on the concurrent spin trapping of $NO^•$ and $O_2^{•-}$, there are obvious advantages in identifying these free radicals in one EPR spectroscopic measurement. One only needs to be cognizant of the potential role spin trapping has already played in unraveling the enzymology of NOS to appreciate the need to undertake such studies. First, however, we needed to choose spin traps that would initially react with each of these free radicals and second exhibit EPR spectra that were distinguishable. Even though the spin traps for $O_2^{•-}$ are well defined and limited to a specific group of compounds, namely nitrones (Scheme 1), there are, nevertheless, three different classes of spin traps for $NO^•$: "activated" *cis*-conjugated dienes,[7] nitronyl aminoxyls[8] and ferro-chelates[9] (Scheme 2). Each was considered in the light of experiments proposed herein. We initially eliminated the *cis*-conjugated diene **8** based on its poor solubility in H_2O,[7a,7b] small second order rate constant with $NO^•$[8c] and an EPR spectrum of the corresponding aminoxyl **9** that overlaps with spin trapped adducts derived from reaction of $O_2^{•-}$ with **1**, **3**, or **5**. We likewise excluded nitronyl aminoxyls such as **10**. Here, this class of spin traps for $NO^•$ was discarded as there is too much overlap between nitronyl aminoxyl **10**, imino aminoxyl **11** and aminoxyls derived from the spin trapping of $O_2^{•-}$ by **1**, **3**, or **5** to allow an accurate assessment of the conversion of **10** to **11** by $NO^•$. In contrast, ferro-chelates, such as **12**, react with $NO^•$, forming NO–Fe(DTCS)$_2$ **13**. In this case, however, the nitrosyl–iron chelate exhibits a broad three-lined EPR spectrum with a *g*-value of 2.04, considerably different from spin trapped adducts such as **2**, **4**, or **6** with *g*-values of ≅2.00. Thus, experiments described below use spin traps **1**, **3**, or **5** for $O_2^{•-}$ and **12** for $NO^•$ (Scheme 1 and Scheme 2). Herein, we report on experiments pertinent to spin trapping $NO^•$ and $O_2^{•-}$ using Fe^{2+}(DTCS)$_2$ **12** and either **1**, **3** or 1,3,3-trimethyl-6-azabicyclo-[3.2.1.]oct-6-ene-*N*-oxide **5** (Scheme 1 and Scheme 2).[6,10]

Results and discussion

Our initial series of experiments was designed to optimize conditions that might allow the concurrent spin trapping of $O_2^{•-}$ and $NO^•$. For these studies, SPER-NO ((*Z*)-1-{*N*-(3-aminopropyl)-*N*-[4-(3-aminopropylammonio)butyl]amino}diazen-1-ium-1,2-diolate) was the source of $NO^•$, generating this free radical at a flux of 2 μM min^{-1} while the concentration of **12** was 800 μM (Fig. 1A). In an independent series of experiments, $O_2^{•-}$ at 2 μM min^{-1}, from the action of xanthine oxidase on hypoxanthine, reacted with either **3** (50 mM, Fig. 1B), **1** (100 mM, Fig. 1C) or **5** (50 mM, Fig. 1D). From these data, it is apparent that the EPR spectrum of **13** is sufficiently separated

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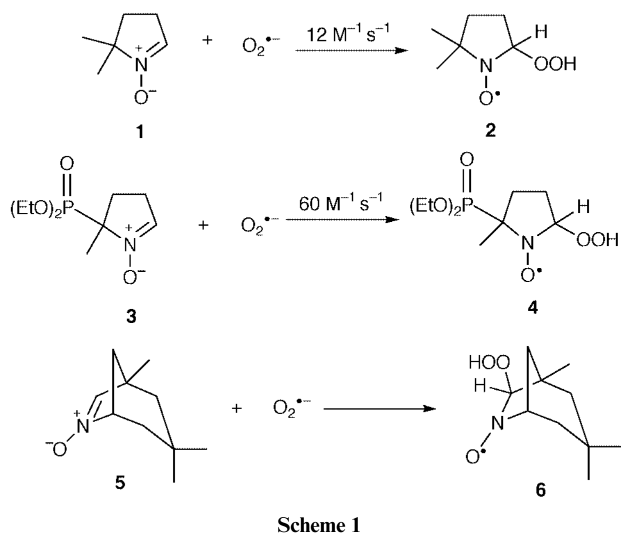
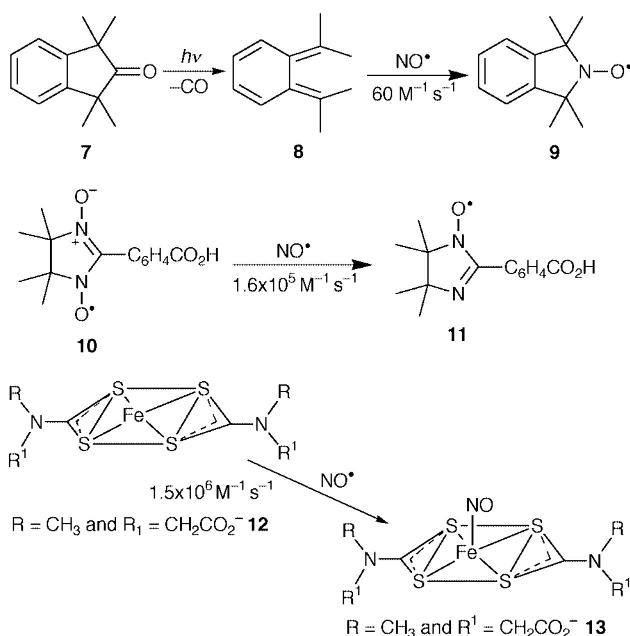


Scheme 1

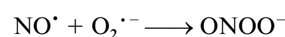


Scheme 2

from those of **2** (Fig. 1C) and **6** (Fig. 1D) that if we were to be able to spin trap NO$^•$ and $O_2^{•-}$, we could readily observe the unique EPR spectrum of each spin trapped adduct. There is, however, some overlap in the EPR spectrum of **13** and **4** (Fig. 1B), making data interpretation a little more difficult.

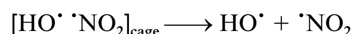
When we incubated **12** (800 µM) and **3** (50 mM) with the $O_2^{•-}$ and NO$^•$ generating systems described above (1 µM min^{-1} of each free radical), we only recorded an EPR spectrum corresponding to **13** (Fig. 2A). Similar results were obtained when **1** (100 mM) (data not shown) or **5** (50 mM, data not shown) was substituted for **3** in the above reaction. While these findings were not unexpected, we were, however, optimistic that experimental conditions might allow the concurrent spin trapping of $O_2^{•-}$ and NO$^•$. These results suggest that any remaining $O_2^{•-}$ not scavenged by NO$^•$ disproportionated to H_2O_2. Thus, there was no $O_2^{•-}$ available to be spin trapped by either **1**, **3**, or **5**. Evidence in support of this thesis comes from data shown in Fig. 2. Here, **5** (50 mM), in the absence of **12**, was incubated with $O_2^{•-}$ and NO$^•$ at 2 µM min^{-1} of each free radical. As shown in Fig. 2B, there was no EPR spectrum recorded. Yet, at a higher flux of $O_2^{•-}$ (8 µM min^{-1}) than that of NO$^•$ (2 µM min^{-1}), an EPR spectrum corresponding to **6** was obtained (Fig. 2C).

As noted above, the reaction of $O_2^{•-}$ and NO$^•$ results in the formation of ONOO$^-$.[4a] The fortunes of this peroxide have remained in doubt, although one compelling theory suggests the formation of HO$^•$ from decomposition of peroxynitrous acid.[11]



$$NO^• + O_2^{•-} \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \longrightarrow ONOOH \longrightarrow [HO^•\ {}^•NO_2]_{cage}$$



$$[HO^•\ {}^•NO_2]_{cage} \longrightarrow HO^• + {}^•NO_2$$

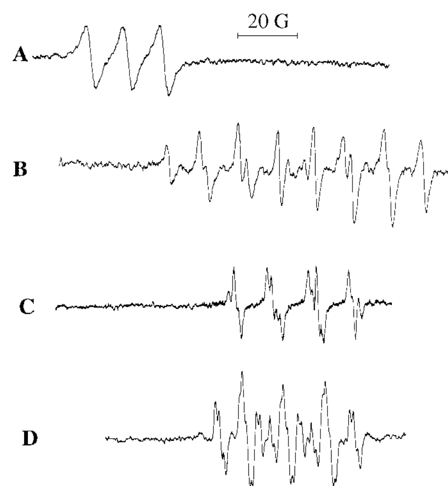


Fig. 1 Representative EPR spectra of spin-trapped adducts of NO$^•$ and $O_2^{•-}$. Spectra were recorded 2 min after commencement of the reactions of the free radical generation system with the spin trap, as described in the Experimental section. **A**. NO$^•$, generated from SPER-NO at a flux of 2 µM min^{-1}, was spin trapped by **12** (aqueous solution containing DTCS, 4 mM; ferrous sulfate, 800 µM, final concentrations). Hyperfine splitting constants for **13** are A_N = 14.9 G. **B**. $O_2^{•-}$, generated by the action of xanthine oxidase on hypoxanthine in sodium phosphate buffer pH 7.4 at an initial rate of 2 µM min^{-1}, was spin trapped by **3** (50 mM). Hyperfine splitting constants for **4** are A_N = 13.4 G, $A_{H\beta}$ = 11.9 G, A_P = 52.5 G. **C**. Same conditions as in **B**, except the spin trap was **1** (100 mM). Hyperfine splitting constants for **2** are A_N = 14.3 G, $A_{H\beta}$ = 11.4 G, $A_{H\gamma}$ = 1.3 G. **D**. Same conditions as in **B**, except the spin trap was **5** (50 mM) in the above reaction. Hyperfine splitting constants for **6** are A_N = 14.7 G, $A_{\beta\text{-H1}}$ = 10.4 G, $A_{\beta\text{-H2}}$ = 9.1 G, $A_{\gamma\text{-H1}}$ = 1.8 G, $A_{\gamma\text{-H2}}$ = 1.2 G.

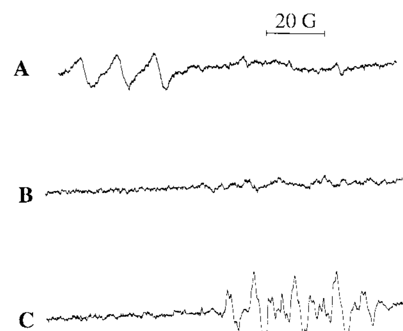


Fig. 2 **A**. Typical EPR spectra of **13** in the presence of spin traps for $O_2^{•-}$. NO$^•$, generated from SPER-NO at a flux of 1 µM min^{-1}, was spin trapped by **12** (aqueous solution containing DTCS, 4 mM; ferrous sulfate, 800 µM, final concentrations) in the presence of $O_2^{•-}$, generated by the action of xanthine oxidase on hypoxanthine in sodium phosphate buffer pH 7.4 at an initial rate of 1 µM min^{-1}, and **3** (50 mM). Hyperfine splitting constants for **13** are A_N = 14.9 G. EPR spectrum was recorded 2 min after the reaction commenced. **B**. Representative EPR spectra of **6**. NO$^•$, produced from SPER-NO at a flux of 2 µM min^{-1} and $O_2^{•-}$, generated by the action of xanthine oxidase on hypoxanthine at a flux of 2 µM min^{-1} in the presence of **5** (50 mM). **C**. Representative EPR spectra of **6**. NO$^•$, produced from SPER-NO at a flux of 2 µM min^{-1} and $O_2^{•-}$, generated by the action of xanthine oxidase on hypoxanthine at an initial rate of 8 µM min^{-1} in the presence of **5** (50 mM). EPR spectra were recorded 2 min after admixing the reagents. Hyperfine splitting constants for **6** are as reported in Fig. 1D.

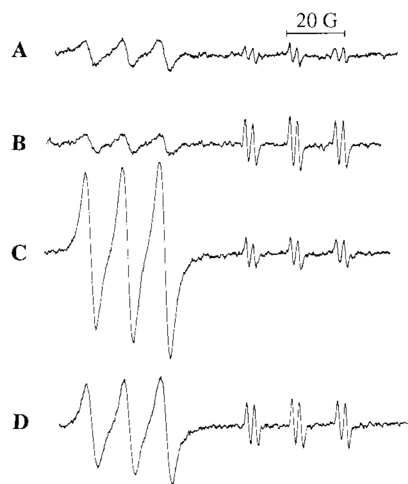
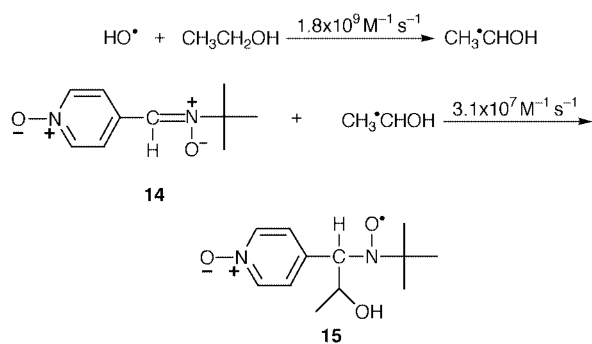


Fig. 3 Typical EPR spectra of **13** and **15**. **A**. NO$^•$, generated from SPER-NO at a flux of 1 μM min^{-1}, was spin trapped by **12** (aqueous solution containing DTCS, 4 mM; ferrous sulfate, 800 μM, final concentrations) in the presence of $O_2^{•-}$, generated by the action of xanthine oxidase on hypoxanthine in sodium phosphate buffer pH 7.4 at an initial rate of 1 μM min^{-1}, and **14** (60 mM)/EtOH (170 mM), detecting HO$^•$ as **15**. Hyperfine splitting constants for **13** are A_N = 14.9 G, whereas hyperfine splitting constants for **15** are A_N = 15.75 G, A_H = 2.4 G. **B**. Same conditions as in **A**, except the flux of NO$^•$ is 1 μM min^{-1} and the initial rate of $O_2^{•-}$ is 10 μM min^{-1}. **C**. Same conditions as in **A**, except the flux of NO$^•$ is 10 μM min^{-1} and the initial rate of $O_2^{•-}$ is 1 μM min^{-1}. **D**. Same conditions as in **A**, except the flux of NO$^•$ is 10 μM min^{-1} and the initial rate of $O_2^{•-}$ is 10 μM min^{-1}.

Contrary to this, other reports[12] argue, based on thermodynamic and kinetic considerations, that ONOOH decomposition does not lead to HO$^•$ and that the observed $^•$OH-like reactivity of ONOOH was derived from a vibrationally excited intermediate of the *trans*-peroxynitrous acid, ONOOH*. A more recent theoretical study has, however, suggested that the concerted pathway for rearrangement of ONOOH* involves a stepwise process that leads to hydrogen-bonded radical pairs, HO$^•$ and $^•NO_2$.[13] As these radical pairs are produced in a "cage," there is no barrier to recombination. Thus, any HO$^•$ that might be spin trapped would, therefore, have to be small. This is in line with experiments in which the amount of detectable HO$^•$ under physiological conditions was found to be low, ranging from 1 to 28% of the initial $ONOO^-$ concentration.[14]

With this background, we examined whether we could spin trap NO$^•$ and HO$^•$ during simultaneous generation of $O_2^{•-}$ and NO$^•$. For these experiments, **12** and **14** and EtOH were used to spin trap NO$^•$ and HO$^•$, respectively. Ethanol/**14** was chosen based on its specificity for HO$^•$, with a second order rate constant of 3.1×10^7 M^{-1} s^{-1} (Scheme 3).[15] The stability of **15** is



Scheme 3

remarkable, appearing to be relatively unaffected by the presence of a high flux of $O_2^{•-}$.[15] Finally, **14** was found not to be prone to $ONOO^-$ mediated oxidation.[14d] At equal fluxes of $O_2^{•-}$ and NO$^•$, either at 1 μM min^{-1}, Fig. 3A or at 10 μM

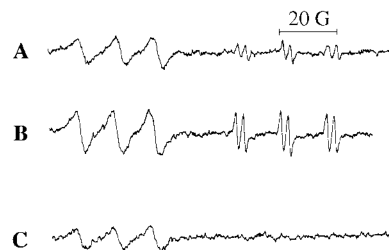


Fig. 4 Typical EPR spectra of **13** and **15**. **A**. NO$^•$, generated from SPER-NO at a flux of 1 μM min^{-1}, was spin trapped by **12** (aqueous solution containing DTCS, 4 mM; ferrous sulfate, 800 μM, final concentrations) in the presence of $O_2^{•-}$, generated by the action of xanthine oxidase on hypoxanthine in sodium phosphate buffer pH 7.4 at an initial rate of 1 μM min^{-1}, and **14** (60 mM)/EtOH (170 mM), detecting HO$^•$ as **15**. EPR spectra were recorded 2 min after commencing the reaction. Hyperfine splitting constants for **13** are A_N = 14.9 G, whereas hyperfine splitting constants for **15** are as reported in Fig. 3A. **B**. Same conditions as in **A**, except SOD (30 U mL^{-1}) was included in the reaction mixture. **C**. Same conditions as in **A**, except catalase (200 U mL^{-1}) was included in the reaction mixture.

min^{-1}, Fig. 3D, we were able to spin trap NO$^•$ and HO$^•$ as **13** and **15**, respectively.

As the flux ratio of NO$^•$ and $O_2^{•-}$, varied from 1 μM min^{-1} and 10 μM min^{-1} (Fig. 3B) to 10 μM min^{-1} and 1 μM min^{-1} (Fig. 3C), the peak heights of the corresponding spin trapped adduct reflected the ratio of free radicals generated. As **13** and **15** are very stable,[7a,15] given sufficient time, the accumulated spin trapped adducts should be detected at much lower fluxes of NO$^•$ and $O_2^{•-}$.

The source of HO$^•$, detected as **15**, can arise from the decomposition of $ONOO^-$ and/or **12**-catalyzed reduction of H_2O_2.[7a] To explore the importance of these pathways, we included **14** and EtOH in the NO$^•$ and $O_2^{•-}$ generating systems noted above at a flux of 1 μM min^{-1} of each free radical. We spin trapped a small amount of HO$^•$ (Fig. 4A). Inclusion of superoxide dismutase (SOD, 30 U mL^{-1}) in the reaction mixture enhanced the intensity of the EPR spectrum for **13** and **15** (Fig. 4B). Superoxide dismutase, by scavenging $O_2^{•-}$, suppressed the formation of $ONOO^-$ and enhanced the rate of H_2O_2 formation. This increased the spin trapping of both NO$^•$ and HO$^•$, the latter produced exclusively from **12**-catalyzed reduction of H_2O_2. In contrast, catalase by reacting with H_2O_2, but not $ONOO^-$,[16] quelled the spin trapping of HO$^•$ from **12** reduction of H_2O_2 (Fig. 4C).

Next, we explored the free radical profile of NOS I. We have previously demonstrated that in the absence of L-arginine purified NOS I generates $O_2^{•-}$,[2] whereas at saturating concentrations of L-arginine, no $O_2^{•-}$ was spin trapped by **1**.[2,3f] Implicit in these and similar studies[3] is the fact that in the presence of substrate, NOS transfers electrons to L-arginine, which would otherwise go to O_2 as the terminal electron acceptor. This results in the oxidation of L-arginine to L-citrulline and NO$^•$. Experiments were, therefore, designed in an attempt to spin trap $O_2^{•-}$ and NO$^•$ under a variety of experimental conditions. When **12** (2 mM) was added to purified NOS I in the presence of NADPH, Ca^{2+}/calmodulin, catalase (600 U mL^{-1}) and L-arginine (10 μM) NO$^•$ was spin trapped, as **13** (Fig. 5A), whose EPR spectrum increased in intensity with inclusion of SOD (100 U mL^{-1}) (Fig. 5B). In a parallel series of experiments, substituting **3** (50 mM) in place of **12** led to the spin trapping of $O_2^{•-}$, as **4** (Fig. 5C), which was inhibited by the presence of SOD (100 U mL^{-1}) in the reaction (Fig. 5D). Including **12** and **3** in the incubation mixture resulted only in spin trapping NO$^•$, as **13** (Fig. 5E). When Ca^{2+}/calmodulin was excluded from the reaction mixture, no EPR spectra were recorded, whether **12** (Fig. 5F) or **3** (data not shown) was present in the experiment. This latter observation points to NOS as the source of both NO$^•$ and $O_2^{•-}$. While these studies demon-

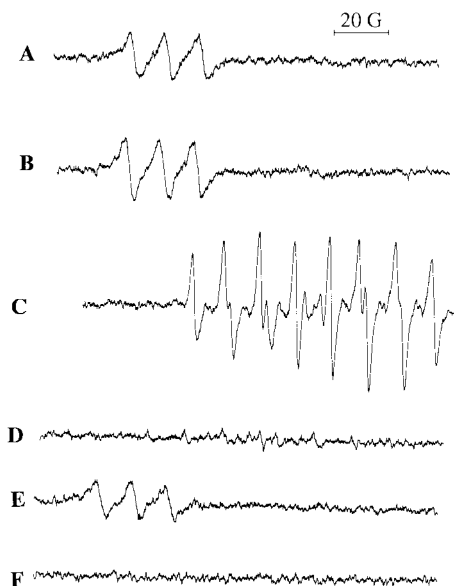


Fig. 5 Representative EPR spectra, derived from spin trapping of $NO^•$ and $O_2^{•-}$ by purified NOS I. Purified NOS I (34 μg protein) containing NADPH (123 μM) was incubated with $CaCl_2$ (2 mM), calmodulin (100 U mL^{-1}), L-arginine (10 μM), catalase (600 U mL^{-1}) in sodium phosphate buffer (50 mM, pH 7.4). **A**. In the presence of **12** (2 mM). Hyperfine splitting constants for **13** are $A_N = 14.9$ G. **B**. Same conditions as in **A**, except SOD (100 U mL^{-1}) was included in the reaction mixture. **C**. In the presence of **3** (100 mM). Hyperfine splitting constants for **4** are as reported in Fig. 1B. **D**. Same conditions as in **C**, except SOD (100 U mL^{-1}) was included in the reaction mixture. **E**. In the presence of **12** (2 mM) and **3** (100 mM). **F**. Same as **A**, except $CaCl_2$ and calmodulin were excluded from the reaction mixture. Receiver gain was 1.25×10^4.

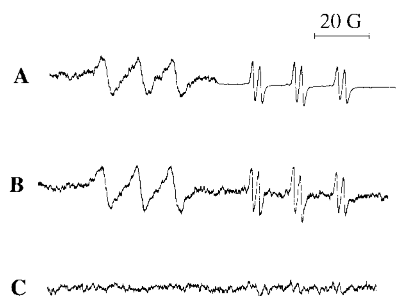


Fig. 6 Representative EPR spectra, derived from spin trapping of $NO^•$ and $HO^•$ as **15** by purified NOS I. Purified NOS I (74 μg protein) containing NADPH (270 μM) was incubated with $CaCl_2$ (2 mM), calmodulin (100 U mL^{-1}), L-arginine (10 μM) in sodium phosphate buffer (50 mM, pH 7.4). **A**. In the presence of **12** (2 mM) and **14** (50 mM), EtOH (170 mM). Hyperfine splitting constants for **13** are $A_N = 14.9$ G and for **15** are as reported in Fig. 3A. Receiver gain was 1.25×10^4 for **13** and 1.25×10^3 for **15**. **B**. The same as **A**. except catalase (600 U mL^{-1}) is included. Receiver gain was 1.25×10^4. **C**. Same conditions as in **A**, except $CaCl_2$ and calmodulin were not in the reaction mixture. Receiver gain was 1.25×10^4.

strated the ability to independently spin trap $NO^•$ and $O_2^{•-}$, under these experimental conditions it was not possible to simultaneously spin trap both free radicals, due primarily to the presence of **12** and secondarily, other reactions of $O_2^{•-}$ whose rate constants far exceed that with **3**. Similar findings were obtained with **1** (100 mM) (data not shown) or **5** (50 mM) (data not shown).

Based on favorable rate constants and our studies with model $O_2^{•-}$ and $NO^•$ generating systems, we felt that it might be possible to spin trap $HO^•$ and $NO^•$ during the oxidation of L-arginine by purified NOS I. When **12** (2 mM) and **14**/EtOH (50 mM/170 mM) were mixed with purified NOS I, NADPH, Ca^{2+}/calmodulin and L-arginine (10 μM), we obtained EPR spectra corresponding to **13** and **15** (Fig. 6A). Inclusion of catalase (600 U mL^{-1}) in the above reaction resulted in a marked inhibition of **15** (Fig. 6B). In the absence of Ca^{2+}/calmodulin, no EPR spectrum was recorded, even though **14**/EtOH was included in the reaction mixture (Fig. 6C). These data point to the fact that the primary source of **15** is from **12** catalyzed reduction of H_2O_2,[17] resulting from the dismutation of $O_2^{•-}$ generated by NOS I. Thus, in the presence of L-arginine (10 μM), we were able to concurrently monitor the formation of $NO^•$ and $HO^•$ from NOS I.

Conclusion

In this study, we have explored the spin trapping of $NO^•$, $O_2^{•-}$ and $HO^•$. In particular, we attempted to simultaneously spin trap $NO^•$ and $O_2^{•-}$ using **12** and either **1**, **3** or **5**. While we were able to spin trap each of these free radicals individually, we were unable to concurrently spin trap both. We believe this is the result of two factors. First, small rate constants for the spin trapping of $O_2^{•-}$ by **1**, **3** and undoubtedly **5** (Scheme 1). Second, the propensity of $O_2^{•-}$ to disproportionate, giving H_2O_2 as well as to react with **12**, which prevented the spin trapping of $O_2^{•-}$ with the current generation of nitrones. In contrast, we were able to spin trap $NO^•$ and $HO^•$, the latter primarily from the reduction of H_2O_2 and secondarily from the decomposition of $ONOO^-$. In this case, $HO^•$ was identified through its reaction with EtOH, generating the secondary free radical $CH_3^•CHOH$. Spin trapping of $CH_3^•CHOH$ by **14** yielded **15** (Scheme 3). Thus, we, in an indirect manner, detected $NO^•$ and $O_2^{•-}$ in the same EPR spectrum. Finally, it is worth noting that these studies can serve as a template for others to explore pathways by which enzymes, such as NOS, generate different free radicals under a variety of experimental paradigms.

Experimental

Reagents

Hypoxanthine, xanthine oxidase, ferricytochrome c, catalase and diethylenetriaminepentaacetic acid (DTPA), NADPH, calmodulin, L-arginine, ethyleneglycobis(aminoethyl ether)-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride and penicillin G/streptomycin solution were purchased from Sigma Chemical Company (St. Louis, MO). Chelex 100 ion exchange resin was purchased from Bio-Rad (Richmond, CA). 2′,5′-ADP-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Dulbecco's Modified Eagle Medium (DMEM), phosphate buffer saline (PBS) and fetal bovine serum were obtained from Gibco (Grand Island, NY). 2-Methyl-*N*-(4-pyridylmethylidene)propan-2-amine *N*,*N*′-dioxide (**14**) was obtained from Aldrich Chemical Company (Milwaukee, WI). Superoxide dismutase (SOD) was purchased from Boehringer Mannheim (Indianapolis, IN). SPER-NO was obtained from Midwest Research Institute (Kansas City, MO; now available from Alexis Biochemical, San Diego, CA). 5,5-Dimethyl-1-pyrroline 1-oxide (**1**), 1,3,3-trimethyl-6-azabicyclo[3.2.1.]oct-6-ene-*N*-oxide (**5**) and ammonium *N*-(dithiocarboxy)sarcosine (DTCS) were synthesized as described in the literature.[10a,10b,18] 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (**3**) was purchased from Calbiochem-Novabiochem, Corp. (La Jolla, CA).

EPR spectral measurements

EPR spectra of spin trapped adducts, derived from the reaction of $NO^•$, $O_2^{•-}$ and $HO^•$ with **12** for $NO^•$, **1**, **3** or **5** for $O_2^{•-}$ and **14**/EtOH for $HO^•$ were recorded using an EPR spectometer (Varian Associates E-9). Reaction mixtures were transferred to a flat quartz cell, fitted into the cavity of the spectrometer and spectra were recorded at room temperature. Spectrometer settings were: microwave power, 20 mW; modulation frequency,

100 kHz; modulation amplitude, 1.0 G; response time, 1 s; scan range, 200 G; and scan speed, 25 G min^{-1}. The receiver gain for each experiment is 8.0×10^3, unless otherwise given in the figure legend.

Source of free radicals

Superoxide was generated from the action of xanthine oxidase on hypoxanthine (400 μM, final concentration) in sodium phosphate buffer (50 mM, chelexed, pH 7.4). The initial rate of $O_2^{\bullet -}$ generation[19] was estimated by measuring the SOD-inhibitable reduction of ferricytochrome c (80 μM) at 550 nm using an extinction coefficient of 21 mM^{-1} cm^{-1}. Nitric oxide was generated from SPER-NO dissolved in NaOH (1 mM) and then added to a sodium phosphate buffer (50 mM, chelexed, pH 7.4). The rate of $NO^{\bullet}$ production was calculated based on the $t_{1/2} = 230$ min at 22 °C.[20]

Spin trapping of nitric oxide, superoxide and hydroxyl radical

Spin trapping of $O_2^{\bullet -}$ was performed by mixing the spin trap (**1**, 100 mM; **3**, 50 mM; **5**, 50 mM), hypoxanthine (400 μM), and sufficient xanthine oxidase in sodium phosphate buffer (50 mM, chelexed, pH 7.4) to reach the desired flux of $O_2^{\bullet -}$. Control experiments contained SOD (30 U mL^{-1}) and/or catalase (200 U mL^{-1}). The reaction mixtures were transferred to a flat quartz cell and fitted into the cavity of the spectrometer, and spectra were recorded at room temperature. Hyperfine splitting constants are presented in the figure legend.

For spin trapping $NO^{\bullet}$, a solution containing DTCS (4 mM) and ferrous sulfate (800 μM) was prepared in H_2O to which SPER-NO, prepared in sodium hydroxide (1 mM), was added in sodium phosphate buffer (50 mM, chelexed, pH 7.4) to achieve the desired flux of $NO^{\bullet}$. EPR spectra were recorded as detailed above.

For spin trapping $HO^{\bullet}$, as **15**, **14** (60 mM, final concentration) were EtOH (170 mM, final concentration) was added to the $NO^{\bullet}$ and $O_2^{\bullet -}$ generating systems described above at variable fluxes of each free radical, depending on the experimental design. In some experiments, **12** (800 μM, final concentration) was included in the reaction mixture.

NOS I purification

Stable nitric oxide synthase I-transfected human embryonic kidney 293 cells were cultured in Dulbecco's Modified Eagle's Medium, containing 10% fetal calf serum, penicillin G (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). Nitric oxide synthase I was purified from these cells by the method of Bredt and Snyder.[21] Briefly, cells were removed from the culture flasks and washed three times with phosphate-buffered saline and collected *via* centrifugation. The pellet was resuspended in buffer containing phenylmethylsulfonyl fluoride (PMSF) (100 mg mL^{-1}) and homogenized with a Polytron (Brinkmann Instruments, model PCU-2 at setting 2 for 10 s). The homogenate was centrifuged at 15 000 rpm for 20 min. The supernatant was applied to a 2′,5′-ADP-Sepharose affinity column. After washing the column three times with standard buffer containing 0.45 M NaCl, NOS was eluted with standard buffer containing 10 mM NADPH. Excess NADPH was removed by washing and the eluate was concentrated with CentriCell-30 (Polysciences, Warrington, PA) until the concentration of NADPH was approximately 1–1.5 mM as assessed spectrophotometrically at 340 nm ($\varepsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.[22]

Spin trapping experiments with purified NOS I

Spin trapping experiments with purified NOS I were conducted by mixing all the components described in the figure legends to a final volume of 0.30 mL. The experiment was initiated by adding freshly purified NOS I. Reaction mixtures were then transferred to a flat quartz cell, fitted into the cavity of the EPR spectrometer (Varian Associates, model E-9, Palo Alto, CA) and spectra were recorded at room temperature after addition of the enzyme. Microwave power was 20 mW; modulation frequency was 100 kHz with a scanning range of 200 G; modulation amplitude was 1 G; sweep time was 25 G min^{-1}; response time was 1 s and the receiver gain is presented in the figure legends.

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References

1 C. Nathan, *FASEB J.*, 1992, **6**, 3051.
2 S. Pou, W. S. Pou, D. S. Bredt, S. H. Snyder and G. M. Rosen, *J. Biol. Chem.*, 1992, **267**, 24 173.
3 (*a*) Y. Xia, V. L. Dawson, T. M. Dawson, S. H. Snyder and J. L. Zweier, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 6770; (*b*) R. M. F. Wever, T. van Dam, H. J. M. van Rijn, F. de Groot and T. J. Rabelink, *Biochem. Biophys. Res. Commun.*, 1997, **237**, 340; (*c*) Y. Xia, L. J. Roman, B. S. S. Masters and J. L. Zweier, *J. Biol. Chem.*, 1998, **273**, 22 635; (*d*) J. Vásquez-Vivar, B. Kalyanaraman, P. Martásek, N. Hogg, B. S. S. Masters, H. Karoui, P. Tordo and K. A. Pritchard, Jr., *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 9220; (*e*) Y. Xia, A.-L. Tsai, V. Berka and J. L. Zweier, *J. Biol. Chem.*, 1998, **273**, 25 804; (*f*) S. Pou, L. Keaton, W. Surichamorn and G. M. Rosen, *J. Biol. Chem.*, 1999, **274**, 9573; (*g*) J. Vásquez-Vivar, N. Hogg, P. Martásek, H. Karoui, K. A. Pritchard, Jr. and B. Kalyanaraman, *J. Biol. Chem.*, 1999, **274**, 26 736.
4 (*a*) N. V. Blough and O. C. Zafiriou, *Inorg. Chem.*, 1985, **24**, 3502; (*b*) Y. Xia, V. L. Dawson, T. M. Dawson, S. H. Snyder and J. L. Zweier, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 6770; (*c*) Y. Xia and J. L. Zweier, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 6954.
5 (*a*) R. E. Huie and S. Padmaja, *Free Radical Res. Commun.*, 1993, **18**, 195; (*b*) S. Goldstein and G. Czapski, *Free Radical Biol. Med.*, 1995, **19**, 505; (*c*) K. Kobayashi, M. Miki and S. Tagawa, *J. Chem. Soc., Dalton Trans.*, 1995, 2885; (*d*) R. Kissner, T. Nauser, P. Bugnon, P. G. Lye and W. H. Koppenol, *Chem. Res. Toxicol.*, 1997, **10**, 1285.
6 (*a*) E. Finkelstein, G. M. Rosen and E. J. Rauckman, *J. Am. Chem. Soc.*, 1980, **102**, 4994; (*b*) C. Fréjaville, H. Karoui, B. Tuccio, F. Le Moigne, M. Culcasi, S. Pietri, R. Lauricella and P. Tordo, *J. Chem. Soc., Chem. Commun.*, 1994, 1793.
7 (*a*) H.-G. Korth, K. U. Ingold, R. Sustmann, H. De Groot and H. Sies, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 891; (*b*) H. G. Korth, R. Sustmann, P. Lommes, T. Paul, H. de Groot, L. Hughes and K. U. Ingold, *J. Am. Chem. Soc.*, 1994, **116**, 2767; (*c*) T. Paul, M. A. Hassan, H.-G. Korth, R. Sustmann and D. V. Avila, *J. Org. Chem.*, 1996, **61**, 6835.
8 (*a*) T. Akaike, M. Yoshida, Y. Miyamoto, K. Sato, M. Kohno, K. Sasamoto, K. Miyazaki, S. Ueda and H. Maeda, *Biochemistry*, 1993, **32**, 827; (*b*) J. Joseph, B. Kalyanaraman and J. S. Hyde, *Biochem. Biophys. Res. Commun.*, 1993, **192**, 926; (*c*) N. Hogg, R. J. Singh, J. Joseph, F. Neese and B. Kalyanaraman, *Free Radical Res.*, 1995, **22**, 47.
9 (*a*) A. Komarov, D. Mattson, M. M. Jones, P. K. Singh and C.-S. Lai, *Biochem. Biophys. Res. Commun.*, 1993, **195**, 1191; (*b*) C.-S. Lai and A. M. Komarov, *FEBS Lett.*, 1994, **345**, 120; (*c*) C.-S. Lai and A. M. Komarov, *Biochim. Biophys. Acta*, 1995, **1272**, 29; (*d*) H. Fujii, J. Koscielniak and L. J. Berliner, *Magn. Reson. Med.*, 1997, **38**, 565; (*e*) T. Yoshimura, H. Yokoyama, S. Fujii, F. Takayama, K. Oikawa and H. Kamada, *Nature Biotechnol.*, 1996, **14**, 992; (*f*) H. Fujii, X. Wan, J. Zhong, L. J. Berliner and K. Yoshikawa, *Magn. Reson. Med.*, 1999, **42**, 235.
10 (*a*) S. Pou, P. Tsai, S. Porasuphatana, H. J. Halpern, G. V. R. Chandramouli, E. D. Barth and G. M. Rosen, *Biochim. Biophys. Acta*, 1999, **1427**, 216; (*b*) N. Sankurati and E. G. Janzen, *Tetrahedron Lett.*, 1996, **37**, 5313.
11 (*a*) E. Halfpenny and P. L. Robinson, *J. Chem. Soc.*, 1952, 928; (*b*) J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall and B. A. Freeman, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 1620; (*c*) N. Hogg, V. M. Darley-Usmar, M. T. Wilson and S. Moncada, *Biochem. J.*, 1992, **281**, 419; (*d*) J. P. Crow, C. Spruell, J. Chen, C. Gunn, H. Ischiropoulos, M. Tsai, C. D. Smith, R. Radi, W. H. Koppenol and J. S. Beckman, *Free Radical Biol. Med.*, 1994, **16**, 331;

(*e*) W. A. Pryor, X. Jin and G. L. Squadrito, *J. Am. Chem. Soc.*, 1996, **118**, 3125; (*f*) J. W. Coddington, J. K. Hurst and S. V. Lymar, *J. Am. Chem. Soc.*, 1999, **121**, 2438.
12 (*a*) W. H. Koppenol, J. J. Moreno, W. A. Pryor, H. Ischiropoulos and J. S. Beckman, *Chem. Res. Toxicol.*, 1992, **5**, 834; (*b*) D. Bartlett, D. F. Church, P. L. Bounds and W. H. Koppenol, *Free Radical Biol. Med.*, 1995, **18**, 85; (*c*) J.-N. Lemercier, G. L. Squadrito and W. A. Pryor, *Arch. Biochem. Biophys.*, 1995, **321**, 31; (*d*) S. Goldstein, G. L. Squadrito, W. A. Pryor and G. Czapski, *Free Radical Biol. Med.*, 1996, **21**, 965.
13 K. N. Houk, K. R. Condroski and W. A. Pryor, *J. Am. Chem. Soc.*, 1996, **118**, 13 002.
14 (*a*) G. Yang, T. E. G. Candy, M. Boaro, H. E. Wilkin, P. Jones, N. B. Nazhat, R. A. Saadalla-Nazhat and D. R. Blake, *Free Radical Biol. Med.*, 1992, **12**, 327; (*b*) O. Augusto, R. M. Gatti and R. Radi, *Arch. Biochem. Biophys.*, 1994, **310**, 118; (*c*) A. van der Vliet, C. H. O'Neill, B. Halliwell, C. E. Cross and H. Kaur, *FEBS Lett.*, 1994, **339**, 89; (*d*) S. Pou, S. Y. Nguyen, T. Gladwell and G. M. Rosen, *Biochim. Biophys. Acta*, 1995, **1244**, 62; (*e*) H. Kaur, M. Whiteman and B. Halliwell, *Free Radical Res.*, 1997, **26**, 71; (*f*) C. E. Richeson, P. Mulder, V. W. Bowry and K. U. Ingold, *J. Am. Chem. Soc.*, 1998, **120**, 7211; (*g*) G. R. Hodges and K. U. Ingold, *J. Am. Chem. Soc.*, 1999, **121**, 10 695.
15 S. Pou, C. L. Ramos, T. Gladwell, E. Renks, M. Centra, D. Young, M. S. Cohen and G. M. Rosen, *Anal. Biochem.*, 1994, **217**, 76.
16 R. Floris, S. R. Piersma, G. Yang, P. Jones and R. Wever, *Eur. J. Biochem.*, 1993, **215**, 767.
17 W. H. Koppenol, *Free Radical Biol. Med.*, 1993, **15**, 645.
18 R. Bonnett, R. F. C. Brown, V. M. Clark, I. D. Sutherland and A. Todd, *J. Chem. Soc.*, 1959, 2094.
19 H. Kuthan, V. Ullrich and R. Estabrook, *Biochem. J.*, 1982, **203**, 551.
20 C. M. Margos, D. Morley, D. A. Wink, T. M. Dunams, J. E. Saavedra, A. Hoffman, A. A. Bove, L. Isaac, J. A. Hrabie and L. K. Keefer, *J. Med. Chem.*, 1991, **34**, 3242.
21 D. S. Bredt and S. H. Snyder, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 682.
22 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.