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Nitric oxide synthases catalyze superoxide formation

Bernd Mayer*

During the last decade, dozens of studies from at least 10 independent laboratories have demonstrated enzymatic superoxide formation by nitric oxide synthases (NOS). However, in a recent article published in *FEBS Letters* Dr. Xu suggested that the observed superoxide generation resulted from autoxidation of redox-active cofactors and not from an enzymatic reaction [1]. In the following I wish to summarize the overwhelming experimental evidence in favor of uncoupled superoxide formation as an intrinsic catalytic function of all three NOS isoforms. In addition, I will provide a possible explanation for Dr. Xu's negative findings.

In 1991, neuronal NOS was identified as a complex oxidoreductase with multiple enzymatic functions [2]. One of the reactions catalyzed by the enzyme purified from pig brain was the oxidation of NADPH in the absence of the substrate L-arginine. This reaction, resembling uncoupled substrate oxidation by cytochrome P450s, was accompanied by consumption of molecular oxygen and strictly dependent on the presence of Ca^{2+}/calmodulin. In subsequent studies it was shown that subsaturating concentrations of either the substrate L-arginine or the cofactor tetrahydrobiopterin (BH_4) provoke the uncoupling of NADPH-dependent reductive oxygen activation from L-arginine oxidation, resulting in the generation of superoxide and hydrogen peroxide instead of NO [3,4]. The transfer of electrons from NADPH to the heme iron, where the reductive oxygen activation takes place, requires bound calmodulin (for review see [5]). Thus, based on current knowledge it is no surprise that peroxide formation by neuronal NOS was found to be strictly dependent on the presence of Ca^{2+}/calmodulin and to be blocked by the heme site inhibitor N^G-nitro-L-arginine [3,4]. More recently, similar data were obtained with the inducible [6] and endothelial [7] isoforms, demonstrating that enzymatic formation of superoxide/hydrogen peroxide is a general feature of NOS and that these reduced oxygen species are certainly not 'non-specific by-products' of the reaction, as suggested by Dr. Xu [1].

Dr. Xu is correct in stating that some of the reducing cofactors essential to NOS catalysis, especially BH_4 and flavin mononucleotide, produce superoxide in the course of autoxidation reactions. Albeit this non-enzymatic superoxide production results in the inactivation of enzymatically produced NO and interferes with the measurement of free NO in vitro [8], it must not be confused with the highly specific and tightly regulated NOS-catalyzed reaction. Thus, Ca^{2+}/calmodulin-dependent superoxide formation by BH_4-deficient neuronal NOS (saturated with L-arginine) is completely inhibited by excess BH_4, demonstrating the essential role of the pterin for the coupling of NADPH-dependent oxygen reduction to L-arginine oxidation [3]. In other words, the non-enzymatic autoxidation reactions stressed by Dr. Xu are not relevant when peroxide formation is measured against Ca^{2+}- or calmodulin-deficient blanks.

The EPR data Dr. Xu shows in Fig. 1 of the report [1] are in striking disagreement with all previous EPR spin trapping studies performed with NOS (e.g. [4,6,7]). It is a difficult task to explain this discrepancy with the limited amount of information given in the paper. Dr. Xu observed that addition of purified neuronal NOS to a mixture of cofactors did not increase the EPR signal obtained with the cofactors alone. However, it remains unclear how much NOS-derived superoxide was expected and whether the experimental design would have made it possible to detect the difference. Unfortunately, neither the source nor the specific activity of the enzyme preparation is given. In fact, Dr. Xu has not even tested whether the added enzyme was active at all under the conditions of superoxide detection. In this context it is puzzling that, according to the figure legend, the EPR measurements were performed in the absence of added Ca^{2+} ions. Although sufficient (micromolar) Ca^{2+} is usually present in buffer solutions, enzyme preparations often contain chelators to inhibit tissue proteases. Considering the fairly high final NOS concentration of 12 μg/ml, chelators possibly present in the enzyme stock solutions could have reduced the free Ca^{2+} concentration in the final assay mixture below the threshold level of $\sim$0.5 μM required for calmodulin binding to neuronal NOS. I wish to emphasize that this is only one out of many possible reasons for the apparent lack of activity of neuronal NOS in Dr. Xu's experiments. In any case, a careful experimenter is expected to perform appropriate positive controls before questioning a well established scientific concept with preliminary negative data.

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Generation of superoxide from nitric oxide synthase

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The detection and quantification of superoxide anion in biological systems and from nitric oxide synthase (NOS) is one of the most challenging goals in the field of nitric oxide free radical research. Most of the available methodologies for detecting superoxide are indirect and unsuitable for detecting superoxide from NOS. In a recent contribution to this journal, Dr. Xu [1] questioned the formation of superoxide by endothelial NOS (eNOS) from electron paramagnetic resonance spin trapping experiments using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). The author concluded that superoxide is not formed by eNOS [1]. Electron paramagnetic resonance (EPR or ESR) is the only direct method to quantify superoxide and other free radicals. However, under physiological conditions the steady-state concentration of superoxide is low, which hinders its direct EPR detection. The EPR spin trapping technique enables detection of transient free radicals such as superoxide and hydroxyl radical [2,3]. This technique uses a nitrone compound that reacts with a free radical species to form a persistent radical adduct. For decades investigators used DMPO to detect superoxide. The first EPR spin trapping evidence for the calcium/calmodulin-dependent generation of superoxide from neuronal NOS was obtained using DMPO [4]. Although DMPO also detects superoxide from eNOS upon activation by calcium/calmodulin (Fig. 1, trace A; cf. trace C), the DMPO-superoxide adduct (DMPO-OOH) rapidly decays to form the DMPO-hydroxyl adduct (DMPO-OH) thus making superoxide quantification nearly impossible. Recently new and improved spin traps have been developed [5,6]. These are the carboxylated analogs of DMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO) and the phosphorylated analog 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO). Both EMPO and DEPMPO superoxide adducts do not decay to the corresponding hydroxyl adducts. Quantification of superoxide is, therefore, possible with these new spin traps [5]. As shown in Fig. 1, both EMPO (trace D) and DEPMPO (trace E) react with superoxide generated from eNOS to yield more persistent superoxide adducts than DMPO (trace F) as demonstrated by the higher signal-to-noise ratio of the EPR spectra (traces D and E) at the same rate of superoxide formation. It is important to note that increasing the concentration of DMPO in the incubation mixtures did not significantly improve superoxide detection, as shown in Fig. 1 (trace F). Superoxide was only marginally detected with resting enzyme and abolished by the addition of SOD (not shown). These data clearly demonstrate that DMPO is not an appropriate spin trap for detecting superoxide from NOS.

In view of the recent advancements in EPR spin trapping detection of superoxide, we believe that the use of a more suitable spin trap would be desirable before drawing conclu-

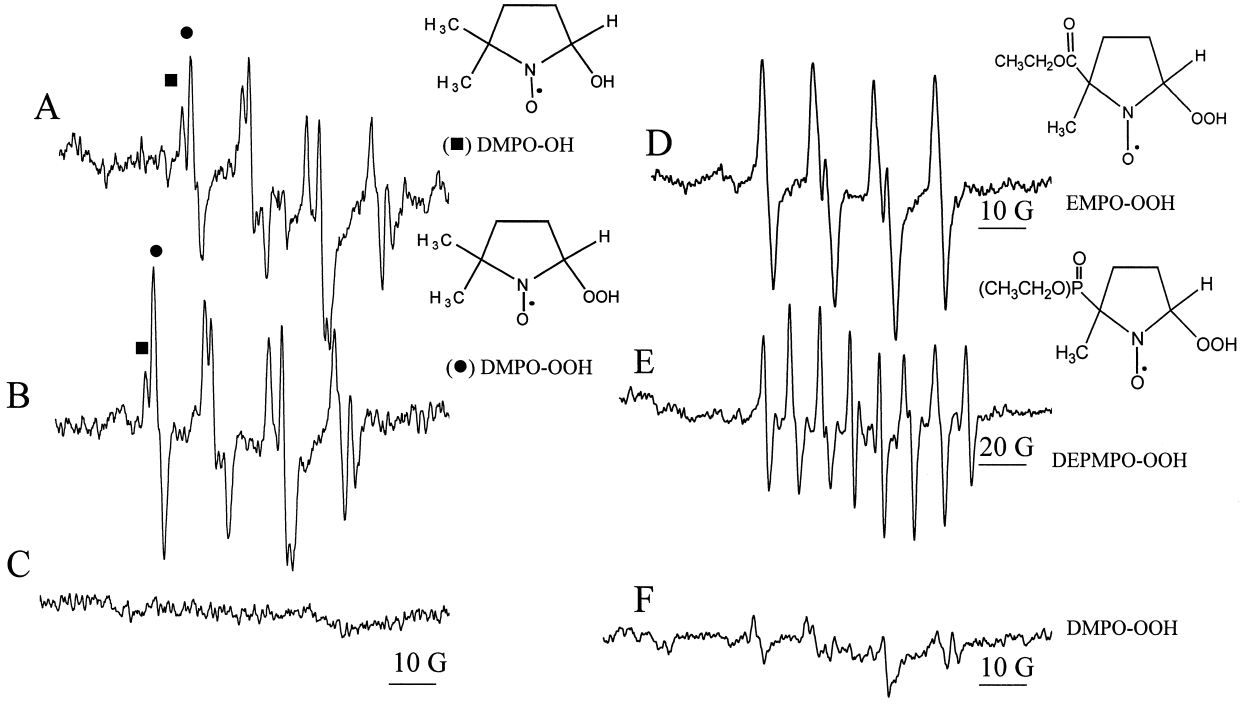

Fig. 1. EPR spin trapping of superoxide from eNOS. Incubation mixtures contained pterin-free eNOS (1 μg), NADPH (0.1 mM), calcium (0.2 mM), calmodulin (20 μg/ml), DTPA (0.1 mM) in HEPES buffer (50 mM, pH 7.4) and (A) DMPO (0.1 M). (B) As A in the presence of FAD (2 μM) and FMN (2 μM). (C) As A except calcium and calmodulin were omitted. (D) EMPO (0.05 M). (E) DEPMPO (0.05 M). (F) DMPO (0.1 M). Instrumental conditions: microwave power 2 mW, modulation amplitude 1 G, time constant 0.128 s, scan rate 1.6 G/s. Number of scans: 1 for A–C and 3 for D–F. The corresponding hyperfine splitting constants for the spectra are (in Gauss): DMPO-OH ($a^N = 14.9$; $a^H = 14.9$), DMPO-OOH ($a^N = 13.1$ G; $a^H = 11.6$; $a^H = 1.3$), EMPO-OOH ($a^N = 12.8$; $a^H = 11.4$; $a^H = 1.5$), DEPMPO-OOH (isomer 1) ($a^N = 13.0$; $a^P = 50.6$; $a^H = 11.7$), DEPMPO-OOH (isomer 2) ($a^N = 13.1$; $a^P = 48.5$; $a^H = 10$). Enzyme activity measured by the hemoglobin assay in the presence of 10 μM BH_4 was 178 nmol/min/mg protein.

sions about NOS biochemistry based on spin trapping experiments with DMPO. Previously, we and others have presented definitive evidence for the generation of superoxide from the oxygenase domain of endothelial and neuronal NOS by a calcium/calmodulin-dependent mechanism [7,8]. Thus, Dr. Xu's findings are dramatically opposite to those reported by us and others [4,7,8]. Using DEPMPO we estimated that 7 pmol eNOS presenting an enzyme activity of 133.3 nmol [^{14}C]citrulline/min/mg protein generated 6.7 ± 0.3 μM of superoxide adduct [7]. Current NOS research indicates that BH_4 critically controls one- and two-electron mechanism of oxygen activation by NOS [8]. It is likely that a comprehensive study on the mechanism of oxygen activation by NOS will reveal the role of BH_4 in superoxide, hydrogen peroxide and ultimately NOS turnover. This knowledge is critical to understanding the role of NOS in free radical pathology. All NOS isoforms (endothelial, neuronal and inducible) generate superoxide in the absence of BH_4 at different rates. Extrapolation of these findings to a cellular milieu is undoubtedly a challenging task.

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A key negative control experiment provides evidence that nitric oxide synthase does not catalyze superoxide formation

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As described in my published article [1], I performed a simple negative control experiment, using denatured neuronal nitric oxide synthase (nNOS), to examine whether NOS catalyzes superoxide radical ($O_2^{\bullet -}$) generation. The idea of this experimental design is as follows: if native NOS catalyzes the $O_2^{\bullet -}$ radical generation, the denatured NOS would inhibit this process. Vice versa, if the denatured NOS system produces $O_2^{\bullet -}$ radicals under the same experimental condition as the native NOS system, this would suggest that $O_2^{\bullet -}$ generation is non-specific and irrelevant to NOS catalytic function. This independent control experiment has not previously been performed by other laboratories. Naturally, the positive results obtained from this negative control experiment would generate controversy and directly impact on a variety of hypotheses related to NOS function. Whether this negative control experiment is significant in the acquisition of our scientific knowledge, and whether the experimental data gained from this control would accurately represent the nature or essence of NOS function, are central questions that I would like to discuss.

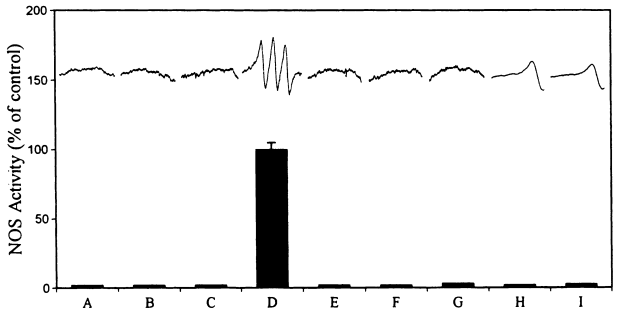

Fig. 1. Determination of nNOS activity and NO$^{\bullet}$ generation under various conditions. NOS activity (black bars) was measured by monitoring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline according to the NOSdetect Assay method of Stratagene [5]. NO$^{\bullet}$ formation (top insets, EPR spectra) was detected in the presence of L-arginine and 5 mM $Fe(MGD)_2$ complex. Total EPR scan time was 10 min. The final concentrations of nNOS, Ca^{2+}, NADPH, FMN, FAD, BH_4, CaM, L-arginine, [^{3}H]L-arginine, trypsin, L-NAME, TRIM, AMT, 7NI, and SOD were 12 μg/ml, 0.2 mM, 1 mM, 5 μM, 5 μM, 10 μM, 0.1 mg/ml, 0.64 mM, 0.64 mM, 1.2 μg/ml, 0.2 mM, 0.2 mM, 0.2 mM, 0.2 mM, and 1000 U/ml, respectively. Denatured nNOS was prepared by boiling native nNOS at 100°C for 10 min. Trypsin-digested nNOS was prepared by incubating native nNOS with trypsin overnight at 37°C. A: 50 mM Tris–HCl buffer, pH 7.4. B: Native nNOS. C: A mixture of NADPH+FAD+FMN+CaM+BH_4+Ca^{2+}+[^{3}H]L-arginine or L-arginine. D: B+C. E: Boiled nNOS+C. F: Trypsin-digested nNOS+C. G: D+L-NAME+TRIM+AMT+7NI. H: C+SOD. I: G+SOD. A triplet spectrum represents NO$^{\bullet}$ generation. The single peak (top insets) observed from conditions H and I represent $Cu(MGD)_2$. The data show that catalytic function of the enzyme was detected only from native nNOS in the presence of NADPH, NOS cofactors, Ca^{2+}, and [^{3}H]L-arginine or L-arginine.

The nNOS (Alexis Biochemicals, product number: 201-028-R050) was chosen for the study because it is highly purified ($\geq$98%, by SDS–PAGE) and has a specific activity of 0.7 μmol L-citrulline/mg/min. The $O_2^{\bullet -}$ radical production was detected from all forms of nNOS, including native, denatured, trypsin-digested, and inhibitor-inactivated NOS enzymes in the absence of L-arginine and presence of 0.2 mM Ca^{2+} (Ca^{2+} was omitted during the publication editing [1]), NOS coenzyme [reduced nicotinamide adenine dinucleotide phosphate (NADPH)] and cofactors, including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), and calmodulin (CaM) [1]. This experimental

evidence directly suggests that NOS does not catalyze $O_2^{\bullet-}$ formation, because the denatured nNOS [lack of enzymatic activity and $NO^{\bullet}$ generation (Fig. 1)] also showed a similar amount of $O_2^{\bullet-}$ radical generation compared with native nNOS. The evidence provided in the article [1] clearly indicates that the entire mixture of NOS coenzyme and cofactors generates $O_2^{\bullet-}$ radicals [1]. These results, in their own way, demonstrate that $O_2^{\bullet-}$ radicals generated from the NOS system are not functional products of NOS.

Dr. Mayer questioned that I have "not even tested whether the added enzyme was active at all under the conditions of superoxide detection." In fact, I did monitor both the nNOS activity and endogenous $NO^{\bullet}$ production under the same conditions as in the published article [1], except in the presence of either [^{3}H]L-arginine or L-arginine (Fig. 1). The results of these important studies (Fig. 1) were deleted from the published article [1] by the *FEBS Letters* editorial office due to limited space. Enzymatic conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline and the production of $NO^{\bullet}$ were detected only from native nNOS in the presence of Ca^{2+}, NADPH, NOS cofactors, and [^{3}H]L-arginine or L-arginine (Fig. 1D). In contrast, no NOS activity and $NO^{\bullet}$ generation were seen for the boiled (Fig. 1E), trypsin-digested (Fig. 1F), or inhibitor-inactivated (Fig. 1G) nNOS samples. These results indicate that boiled and trypsin-digested nNOS enzymes were denatured proteins, and that inhibitor-inactivated nNOS (Fig. 1G) completely lost its enzymatic function. Neither enzymatic activity nor $NO^{\bullet}$ generation was detected from boiled, trypsin-digested, or inhibitor-inactivated nNOS enzymes (Fig. 1), but a burst of $O_2^{\bullet-}$ radicals were formed [1]. Based on these data, I conclude that the $O_2^{\bullet-}$ radicals, generated from the NOS system, are purely chemical and non-specific by-products of the complex electron transfer and chemical interactions among the molecules. These results strongly indicate that $O_2^{\bullet-}$ radical generation is independent of NOS catalytic function.

Drs. Vasquez-Vivar and Kalyanaraman suggest that the spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), is "a more suitable spin trap" for detecting $O_2^{\bullet-}$. Indeed, I have used DEPMPO to determine the free radical generation from both native and denatured NOS systems under similar experimental conditions as described previously [1]. Non-specific $O_2^{\bullet-}$-dependent $OH^{\bullet}$ generation was observed from both the native and denatured NOS systems, and the results are consistent with the assumption that NOS does not catalyze $O_2^{\bullet-}$ radical formation. These results will be published in *Biochimica et Biophysica Acta* [2].

As a natural ubiquitous phenomenon, cellular non-specific enzyme-independent $O_2^{\bullet-}$ generation has been detected by many laboratories. For example, FAD and FMN oxidize, in the presence of oxygen, to yield $O_2^{\bullet-}$ radicals [3]. Autoxidation of BH_4 produces $O_2^{\bullet-}$ [4]. I have also observed that NADPH, alone, not only induces $O_2^{\bullet-}$ formation from both native and denatured NOS, but also from CaM, FMN, and FAD [2]. To distinguish between the enzyme-catalyzed and the enzyme function-independent $O_2^{\bullet-}$ radical generation from a reaction mixture, we must perform a key negative control experiment, that is the use of denatured enzyme to examine the alterations of enzymatic function. NOS requires the binding of its coenzyme (NADPH) and cofactors (FAD, FMN, CaM, and BH_4) in order to function. Yet, NOS coenzyme and cofactors induce $O_2^{\bullet-}$ generation. To scrutinize the catalytic function of NOS, we must not forget to compare NOS enzymatic properties under native and denatured conditions from an overwhelming background of non-specific free radical generation. This simple and rigorous scientific approach is necessary to avoid confusion in interpreting experimental data and guide us to explore the natural process of the subject in question. I believe a scientific concept can only become well established when subjected to appropriate positive and *negative* controls.

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