Origin of superoxide production by endothelial nitric oxide synthase

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Received 26 September 1998

Abstract Using fluorescence optical and electron spin resonance spectroscopy, we have investigated the production of superoxide by bovine endothelial nitric oxide synthase (NOS). In contrast to neuronal NOS, the heme moiety is identified as the exclusive source of superoxide production by endothelial NOS. Thus, calmodulin-mediated enzyme regulation affects production of nitric oxide and superoxide simultaneously and inseparably. The balance between the nitric oxide/superoxide reaction pathways may be shifted by addition of exogenous heme-specific agents, such as tetrahydrobiopterin. Our results have direct relevance for the pathophysiology of atherosclerosis.

Key words: Nitric oxide synthase; Superoxide; Tetrahydrobiopterin; Electron spin resonance; Flavin

1. Introduction

The dualistic nature of nitric oxide synthases (NOS), producing both nitric oxide (NO) and superoxide, has stimulated research with regard to the exact (patho)physiological role of NOS [1]. In the literature, neuronal NOS (nNOS) has clearly been identified as a source of superoxide production, where both the flavin and heme domains proved capable of oxygen radical production [2]. For endothelial NOS (eNOS) the situation is less clear. Of note, eNOS differs from nNOS in several ways. Endothelial NOS is unique among the NOS isoforms in being dually acylated. This property is required for eNOS binding and targeting to caveolae in the endothelial plasma membranes [3]. Activity of eNOS is regulated by a balanced interaction of eNOS with either the activating calmodulin or the inhibiting caveolin-1 [4].

In view of the role of eNOS in atherogenesis, the question whether superoxide can be generated by the flavin domain of eNOS remains of crucial importance. Since the calmodulin-binding site and the heme-containing oxygenase domain are the only places accessible for therapeutic manipulation of eNOS, exact location of the superoxide-producing site has direct therapeutic consequences. In the present study we have addressed the following questions. First, does eNOS produce oxygen radicals? Second, what is the localization of oxygen radical production within the eNOS enzyme, consisting of a flavin reductase domain and a heme-containing oxygenase domain? Third, is it possible to modulate (pathological) shifts in NO/superoxide production by eNOS?

As a model for human eNOS we have used the baculovirus-expressed bovine eNOS.

2. Materials and methods

2.1. Materials

Recombinant bovine eNOS, expressed in a baculovirus/Sf9 system, was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Using gel electrophoresis, more than 90 weight % of the enzyme was in a dimeric state. The recombinant enzyme has been characterized as a functional analogue of native eNOS [5,6]. The spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was purchased from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). L-[2,3,4,5-^{3}H]Arginine HCl (57 Ci/mmol) was purchased from Amersham (Vienna, Austria). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH4) was from Alexis (Nottingham, UK). Diphenyl iodonium (DPI) was obtained from Aldrich (Munich, Germany). All other chemicals were from Sigma.

2.2. Electron spin resonance

Production of superoxide was detected indirectly by spin trapping (ESR) with the recently introduced spin trap DEPMPO. ESR experiments were carried out on a modified Bruker ESP 300 X band spectrometer equipped with a Bruker rectangular cavity operating in the TE102 mode. For EPR experiments a typical reaction mixture consisted of 20 μl DEPMPO (final concentration 50 mM), 5 μl eNOS (8 mg/ml) and various concentrations of cofactors, added with phosphate buffer (pH 7.4) to a final volume of 200 μl. The cofactors which were exogenously added to a standard reaction mixture were calcium chloride, calmodulin, NADPH and L-arginine. After thorough mixing samples (35 μl) were drawn by a syringe into a quartz capillary. This capillary was mounted on a teflon holder and placed within the cavity. Measurements were performed under aerobic conditions at room temperature with microwave power of 10 mW, microwave frequency of 9.44 GHz. The spectrum of DEPMPO was recorded at a magnetic field of 3370 Gauss with a sweep width of 200 Gauss. The magnetic field was modulated with a frequency of 100 kHz and amplitude 1 Gauss. The detector gain was 1.25×10^6, the time constant 41 ms and ADC conversion time 82 ms. Accumulating four scans enhanced the signal to noise ratio (S/N ratio). Quantitation of the free radical signal was performed by comparison of the S/N ratio. The measurements were performed in duplicate, 20 min after incubation of the enzyme.

2.3. Determination of NO production

NO production was determined as described previously [7]. In short, the formation of radioactive L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine was measured with 45 μg eNOS, incubated with 0.5 mM NADPH, 1 mM $CaCl_2$, 300 U/ml calmodulin, followed by separation on Dowex AG 50W-X4 and detection of [^{3}H]citrulline. All measurements were performed in triplicate. The reaction was performed in either the presence or the absence of tetrahydrobiopterin 15 μM, L-arginine 10 μM or the calcium chelator EDTA 10 mM.

2.4. Optical spectroscopy

Steady state UV-vis fluorescence emission and excitation spectra were measured under aerobic conditions at room temperature on a SPF 500 spectrofluorimeter (SLM-Aminco instruments) with 1 mm quartz cuvette. The fluorescence spectra were recorded as a function of emission wavelength, with the excitation wavelength fixed at $\lambda_{exc} = 443$ nm [8]. The excitation spectra were recorded as a function of excitation wavelength, with the observation wavelength fixed at $\lambda_{obs} = 525$ nm. The monochromator bandwidths were set at 2 nm.

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PII: S0014-5793(98)01292-7

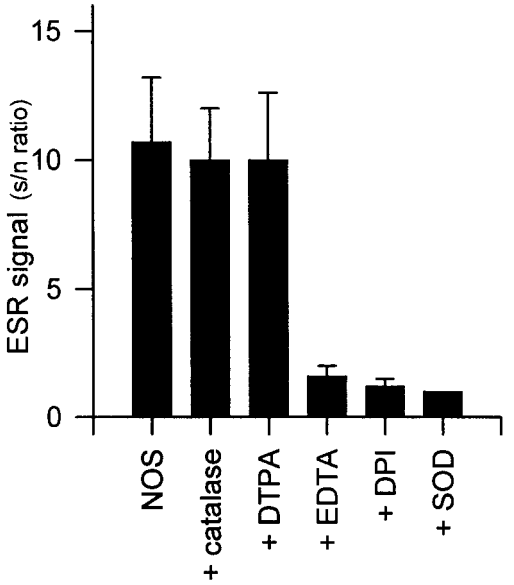

Fig. 1. Superoxide adduct formation by eNOS: effect of inhibitors.

3. Results

3.1. Oxygen radical adduct formation

In principle, a variety of different oxygen radicals may give rise to the formation of these adducts. We have identified the radical involved in eNOS-derived adduct formation as superoxide by addition of specific radical inhibitors. Thus, superoxide dismutase (SOD, 300 U/ml) completely abolished adduct formation, whereas catalase (10 U/ml) and the iron chelator DTPA (10 mM) did not reduce the DEPMPO-adduct signal (Fig. 1). In favorable cases the shape of the ESR spectrum also allows direct identification of the radical, which gives rise to the adduct formation. For DEPMPO, the spectra of the superoxide and hydroxyl radical adducts have been reported as characteristically different in the literature [9]. Direct visual comparison shows that our eNOS-induced ESR spectra have low intensity and a shape different from the DEPMPO-OOH adduct reported (Fig. 2) [9]. In order to test whether the adduct line shape depends on the rate of superoxide production, we have performed a series of DEPMPO trapping experiments on solutions with varying concentrations of the superoxide-generating system hypoxanthine-xanthine oxidase. At low hypoxanthine-xanthine oxidase concentrations (respectively 0.5 mM; 1 U/ml) we observed adduct spectra identical to those observed in our eNOS samples (Fig. 2). At higher hypoxanthine-xanthine oxidase concentrations (respectively 1 mM; 80 mU/ml) the shape of the adduct spectra changed to the form reported in the literature [9] as that of the DEPMPO-OOH adduct. For all trapping experiments on hypoxanthine-xanthine oxidase the adduct formation was inhibitable by SOD, whereas catalase and DTPA had no effect on adduct formation.

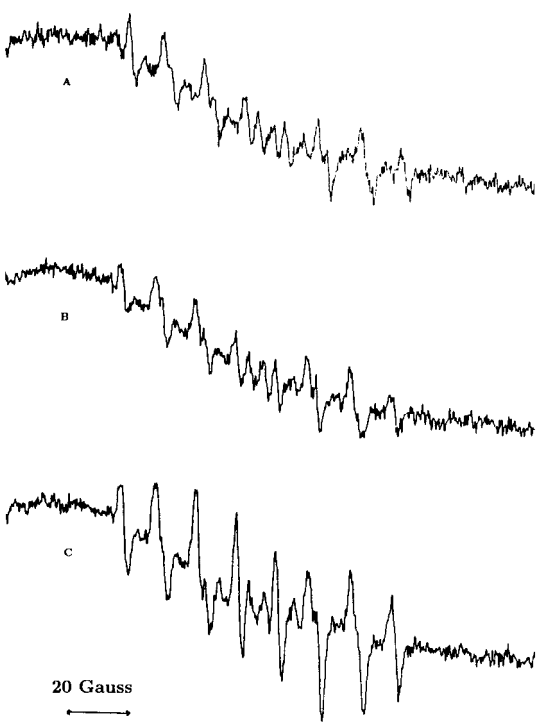

Fig. 2. Electron spin resonance spectra of spin trap adducts. A: eNOS. B: Xanthine oxidase (1 mU/ml) and hypoxanthine (0.5 mM). C: Xanthine oxidase (80 mU/ml) and hypoxanthine (1 mM). The broad background is caused by impurities in the quartz glassware and unrelated to the trapping experiments.

3.2. Localization of superoxide production within eNOS enzyme

Calcium chelation with EDTA (10 mM), which disrupts calmodulin-mediated electron transfer between the flavin reductase domain and the heme oxygenase domain, inhibits both superoxide and NO production (Figs. 1 and 4). We have used luminescence spectroscopy to observe the redox state of the flavin groups. Upon excitation at 443 nm the flavins have a characteristic fluorescence emission at 525 nm in the oxidized state, whereas the reduced form is not luminescent. eNOS without cofactors produced a clear fluores-

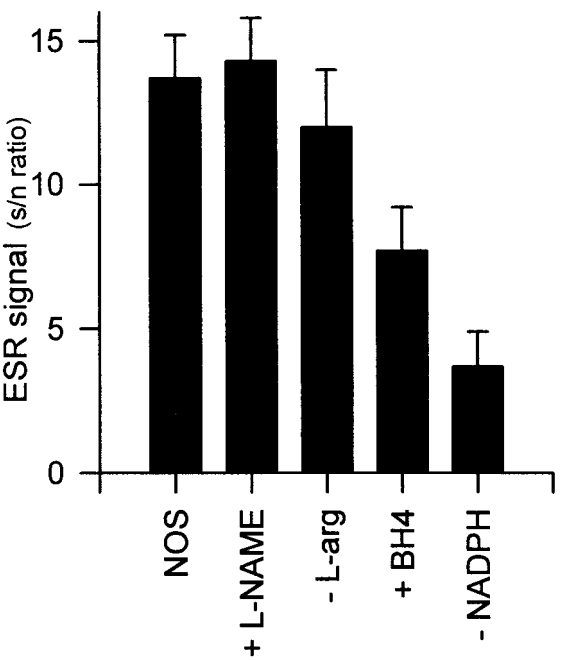

Fig. 3. Superoxide adduct formation by eNOS: effect of cofactors.

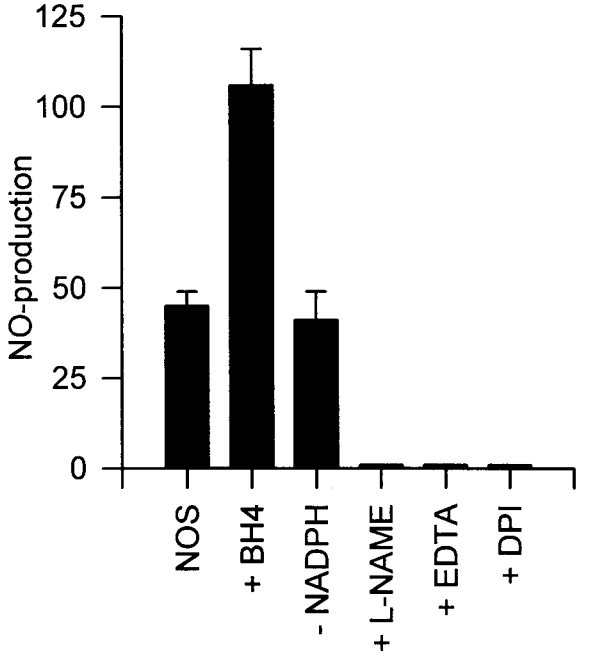

Fig. 4. NO production by eNOS. NO production expressed as pmol [^{3}H]citrulline/min/mg enzyme.

cence signal at a fixed detection wavelength of 525 nm. The corresponding excitation spectrum had a line shape characteristic of flavins. Upon addition of the cofactors calmodulin, calcium chloride and NADPH, the fluorescence signal remained unaffected, signifying very rapid restoration of the flavins to their oxidized states, presumably by rapid electron transfer towards the heme group (Fig. 5). After addition of the calcium chelator EDTA (10 mM), the flavin luminescence was strongly suppressed (Fig. 5). Suppletion of excess calcium chloride restored the luminescence intensity towards the original value, i.e. eNOS without cofactors (Fig. 5), accounting for a 10% dilution of the signal by adding the calcium chloride solution.

3.3. External modulation of the superoxide-NO balance

In the presence of NAPDH, L-arginine, calcium and calmodulin a clear radical adduct was observed with ESR (Fig. 3). The adduct formation was completely abolished by SOD (300 U/ml) and the flavin reductase inhibitor DP (100 μM) (Fig. 2). Addition of the L-arginine analogue, L-NAME (0.1 M), did not alter the ESR signal intensity (Fig. 3). In contrast, upon exogenous addition of the cofactor BH4 (15 μM), the ESR intensity decreased (Fig. 3). As assessed by the production of L-[2,3,4,5-^{3}H]citrulline, NO production was totally inhibited by DPI (Fig. 4). Addition of L-NAME (0.1 M) practically annihilated the NO production. Addition of exogenous BH4 (15 μM) caused a more than twofold increase in NO production (Fig. 4).

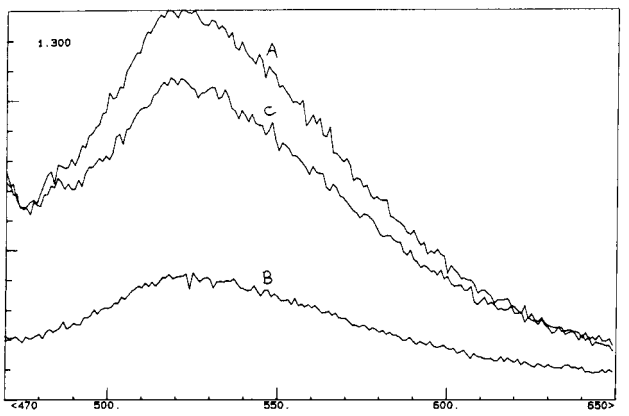

Fig. 5. Fluorescence emission spectrum of flavin groups in the reductase domain of eNOS. A: eNOS with cofactors. B: Addition of the calcium chelator EDTA (10 mM). C: Addition of excess calcium chloride (500 mM) on top of EDTA.

4. Discussion

4.1. Main conclusions

In the bovine eNOS model we clearly detect the formation of superoxide radicals with ESR after incubation with NADPH, calcium and calmodulin. Blockade of eNOS-mediated superoxide production, combined with disappearance of the specific fluorescence of the flavin groups in eNOS upon calcium chelation, shows that the heme moiety of eNOS is the exclusive source of superoxide production by eNOS. Addition of exogenous BH4 decreases eNOS-mediated superoxide production.

4.2. Origin of superoxide production within the eNOS enzyme

Arguments have been put forward favoring superoxide production by the flavin domain, by the heme domain or by both domains of eNOS [2,10,11]. In our eNOS model, removal of calcium by the calcium chelator EDTA completely prevented superoxide production. Since calmodulin serves as an electron shuttle between the reductase and oxygenase domains [12], this suggests the necessity of electron transfer from the flavin moiety to the heme moiety for superoxide production to occur. Since calmodulin binding has also been suggested to affect the affinity of the flavin reductase domain for NAPDH [2], one can consider two different mechanisms, which may account for the inhibitory effect of calcium depletion. First, calcium depletion detaches calmodulin from eNOS, somehow disabling the capacity of the reductase domain to receive the electrons from NADPH, thereby inactivating the eNOS enzyme as a whole. Alternatively, the reductase domain remains functional and accepts electrons from the NADPH. However, the flavin domain remains in its reduced state, since it cannot pass the electrons on to the heme group in the absence of calmodulin.

The redox state of the flavin groups within the eNOS enzyme can be evaluated due to their specific absorbance and fluorescence properties [8]. Since eNOS concentrations were insufficient for direct detection of the optical absorption by eNOS, we used fluorescence spectroscopy, which allowed detection of fluorescence emission around $\lambda = 525$ nm in concentrated eNOS solutions (8 mg/ml) in the absence of cofactors. This fluorescence was attributed to flavins in the oxidized state, since both the absorption peaks (443 and 475 nm, as detected in the excitation spectrum) and the fluorescence emission spectra were characteristic for oxidized flavoproteins [8]. Since the flavins do not fluoresce in the reduced state, the fluorescence properties of eNOS provide a convenient and sensitive method to study the flavin redox state under experimental conditions. Upon addition of cofactors, the fluorescence behavior is unchanged. This shows that the flavins re-

main predominantly in the oxidized state. Since NADPH is being consumed, this indicates a rapid electron transfer from the reduced flavins to the heme group, thereby restoring the flavins to their oxidized state. Upon calcium chelation by EDTA, flavin fluorescence is strongly suppressed, demonstrating that the flavin reductase domain still extracts electrons from the cofactor NADPH. Despite the reduced state of the flavin reductase domain, no radical adduct formation could be demonstrated upon EDTA addition. Addition of excess calcium chloride restores electron flow to the heme moiety. Accordingly, the flavin fluorescence reappears. Simultaneously, NOS-derived radicals are again detected with ESR. These observations validate the second option and we therefore conclude that the flavin domain remains functional in the absence of calmodulin. The concomitant lack of oxygen radical formation shows that the heme domain is exclusively responsible for eNOS-mediated superoxide production. This property constitutes a clear difference with nNOS, where both the heme domain and the flavin domain contribute to oxygen radical production [2]. This finding in eNOS has two important implications. First, localized production of superoxide by the heme domain means that for eNOS superoxide production can be externally switched via calmodulin- and/or heme-specific agents. Second, a high priority should be given to the question whether and how the balance between the superoxide and nitric oxide production by eNOS may be influenced by exposure to external agents.

4.3. Shift in NO/superoxide balance by external agents

The substrate of eNOS, L-arginine, as well as an essential cofactor for NOS, tetrahydrobiopterin (BH4), have both been implicated to have an ameliorative effect during NOS dysfunction [13,14]. To evaluate whether these agents are able to cause a shift in the NO/superoxide balance of eNOS, we have tested the effect of both BH4 suppletion and L-arginine depletion. Addition of exogenous BH4 to eNOS resulted in a decrease in superoxide adduct formation, whereas NO production, assessed as conversion of [^{3}H]arginine to [^{3}H]citrulline, increased more than twofold. These data are in accordance with earlier findings, where depletion of BH4 has been shown to cause a shift from NO to superoxide and its superoxide dismutase product hydrogen peroxide by purified neuronal NOS [13,15]. Recently, we have extended these findings to a clinical study, where BH4 co-infusion reinforced NO activity in hypercholesterolemic patients in vivo [14]. With regard to the mechanism of BH4-induced changes, we note that the BH4-induced dimerization [2,14] of eNOS alone is not sufficient to explain the enhanced NO production, since the enzyme was used in the dimeric state, as assessed by gel electrophoresis [7].

Addition of excess L-NAME completely abolished NO production, whereas superoxide production by eNOS remained unaffected. In the presence of L-NAME, the production of superoxide could still be downregulated by subsequent addition of exogenous BH4. In this respect, eNOS (again) differs from neuronal and inducible NOS, where previous studies [10,15] have reported the abolition of superoxide formation by L-NAME. This shows that the heme moiety of eNOS remains capable of redox activity even in the presence of L-NAME. Further research will have to elucidate (a) to what extent the findings are affected by differences between isolated enzyme systems and the enzyme in viable cells and (b) the effect of additional risk factors, e.g. LDL cholesterol, on the kinetics of eNOS [16,17].

4.4. Implications for (patho)physiology

From the present study the following interesting findings may have implications for the relation between human vascular disease and eNOS. First, the simultaneous generation of both NO and superoxide, even in the presence of BH4 and L-arginine, proves that eNOS may serve as a peroxynitrite-producing enzyme under physiological conditions. Our data corroborate the data of Mayer et al. [18], who have argued that neuronal NOS produces ONOO rather than NO. Second, therapeutic interventions aimed at increasing NO production, such as eNOS transfection [19] and eNOS stimulation, will invariably also result in increased production of oxygen radicals. The ultimate effect depends on the local balance between oxygen radical stress and NO production. Hence, strategies for cardiovascular prevention should aim at restoration of the redox dysbalance and alteration of the NO/superoxide balance by eNOS, rather than focusing on increasing NO production per se [20].

Acknowledgement: We are grateful to Thea van Dam for laboratory assistance.

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