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NO accounts completely for the oxygenated nitrogen species generated by enzymic L-arginine oxygenation

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We have assessed the stoichiometry of the nitric oxide (NO) synthase reaction by using a novel e.p.r. technique. NO generated by crude and partially purified NO synthase from endothelial cells and *Escherichia coli*-lipopolysaccharide-activated macrophages was trapped by a ferrous diethyldithiocarbamate complex dispersed in yeast. The paramagnetic ferrous mononitrosyl dithiocarbamate complex formed exhibited a characteristic e.p.r. signal at $g_{\perp} = 2.035$ and $g_{\parallel} = 2.02$ with a triplet hyperfine structure (hfs) at $g_{\perp}$. NO, 3-morpholinosydnonimine and *S*-nitroso-L-cysteine, but not nitrite or hydroxylamine, generated a similar e.p.r. signal. NO generated by NO synthase and by SIN-1 accumulated at a constant rate for 1 h, as measured by continuous e.p.r. registration at 37 °C. The formation of e.p.r.-detectable NO by NO synthases was inhibited by N^G-nitro-L-arginine. Incubation with $[^{15}N]N^G$-L-arginine caused an e.p.r. signal with doublet hfs, indicating that the nitrosyl nitrogen derived exclusively from the guanidino nitrogen. The amount of NO generated by NO synthase as measured by e.p.r. technique was compared with formation of L-[^{3}H]citrulline from L-[^{3}H]arginine. NO and L-citrulline were detected at a 1:1 ratio with both NO synthase preparations. GSH and thiol depletion did not significantly affect NO synthase activity, excluding *S*-nitrosothiols as intermediates in the NO synthase reaction. We conclude that NO fully accounts for the immediate oxygenated nitrogen species derived from the enzymic oxygenation of L-arginine.

INTRODUCTION

Many mammalian cells express 'NO synthases' that convert L-arginine into L-citrulline and an oxygenated form of nitrogen (NO_x) [1]. This NO_x, or a labile derivative thereof, is an intra- and inter-cellular signal that influences intracellular calcium homoeostasis, thereby counteracting Ca^{2+}-dependent cell responses such as contraction of vascular smooth muscle and aggregation of platelets [1]. NO_x from macrophages is regarded as a cytotoxic effector molecule mediating the unspecific cellular defence against both invading pathogens and tumour cells [2].

Although it is generally accepted that the vasoactive NO_x released from endothelial cells (the endothelium-derived relaxing factor, EDRF) [3] and the L-arginine-derived cytotoxic effector molecule released from macrophages and other cells is identical with NO [1–8], other investigators have proposed that hydroxylamine [9,10], a nitrosothiol [11] or a mixture of these [10] can also account for the activity of this NO_x. This uncertainty derives mainly from the fact that the techniques used to quantify NO in oxygen-containing aqueous solutions are unable to discriminate between NO and labile NO donors. NO_x is frequently detected by its biological activity on target cells (smooth-muscle cells [1,3] or platelets [12]) and target enzymes (soluble guanylyl cyclase [13–15]), by formation of methaemoglobin from HbO_2 [16,17] and by the stable NO_x metabolite nitrite via a diazotization reaction [18,19]). After transfer into the gas phase, NO can be detected by the chemiluminescence emitted from the reaction with ozone [5,20,21] and by g.l.c.–m.s. [22,23]. As shown by other investigators, some of these methods do not solely detect NO, but also other NO_x and chemically unrelated compounds such as volatile thiols, alkenes (chemiluminescence) and hydrogen peroxide (HbO_2 oxidation) [24–27] which may also be present in biological samples. Some investigators have failed to detect NO by chemiluminescence or e.p.r. technique in the superfusates from stimulated endothelial cells [27–29] or have detected little NO in the absence of strong reducing conditions [30]. In another study, the recovery of NO generated by activated macrophages was reported to be poor in comparison with that of L-arginine-derived nitrite and nitrate [2].

As a result of the technical difficulties involved in its detection, the identity of the NO_x generated by the NO synthase reaction remained unknown, as until now the molecular mechanism of this reaction has not been established [31–33]. Recently spin-trapping experiments identified a carbon-centred radical in lipid extracts from acetylcholine-stimulated arteries [34] in addition to an unidentified radical in cytosol from L-arginine-supplemented neuroblastoma cells [35] and platelets [36]. Although paramagnetic dinitrosyl-iron complexes with intracellular iron–sulphur proteins were detected in activated macrophages by e.p.r. spectroscopy [2,6,7], so far no attempt has been made to quantify the NO included in these paramagnetic complexes.

We have now applied a recently developed e.p.r. technique [37] to detect L-arginine-derived NO on a stoichiometric basis in cell-free incubation mixtures. In addition, we have assessed whether other NO_x, such as nitrite, hydroxylamine or *S*-nitrosothiols, could account for e.p.r.-detectable NO generated by NO synthases.

MATERIALS AND METHODS

Materials

Cassella-Höchst, Frankfurt, Germany, supplied 3-morpholinosydnonimine (SIN-1). Serva, Heidelberg, Germany, supplied diethyldithiocarbamate (DETC), Hepes, NADPH, superoxide dismutase (bovine erythrocyte), N^G-nitro-L-arginine. Boehringer, Mannheim, Germany, supplied porcine brain

Abbreviations used: hfs, hyperfine structure; DETC, diethyldithiocarbamate; SIN-1, 3-morpholinosydnonimine; NO_x, oxygenated nitrogen species; $Fe^{II}(DETC)_3$, ferrous diethyldithiocarbamate complex; $[Fe^{II}(DETC)_2(NO)]$, ferrous mononitrosyl dithiocarbamate complex; $[Fe^{II}(S_2O_3^{2-})_2(NO)_2]$, ferrous dinitrosyl thiosulphate complex; EDRF, endothelium-derived relaxing factor.

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calmodulin, dispase II (*Bacillus polymyxa*). Sigma, Deisenhofen, Germany, supplied antipain, leupeptin, pepstatin, chymostatin, 2′,5′-ADP–agarose, lipopolysaccharide (*Escherichia coli* serotype 055:B5), FAD and FMN. NEN–du Pont, Dreieich, Germany, supplied L-[^{3}H]arginine, [α-^{32}P]GTP and L-[^{14}C]citrulline. Dr. Schircks Laboratories, Jona, Switzerland, supplied (6*R*)-5,6,7,8-tetrahydrobiopterin. Wet baker's yeast (commercial mixture of *Saccharomyces cerevisiae* and *S. carlsbergensis*) was bought in a local store. Soluble guanylyl cyclase was purified to apparent homogeneity from bovine lung as described in [38]. Aqueous NO solution (0.2 mM; pH 7.0) was prepared by equilibration of oxygen-free quartz-distilled water with NO gas purified from commercial NO (99.99 %; Messer-Griesheim, Rheinfelden, Germany) by fractional sublimation in a high-vacuum system. [$Fe^{II}(S_2O_3^{2-})_2(NO)_2$] complex and *S*-nitroso-L-cysteine were prepared as described in [39] and [29] respectively. [$^{15}N_2$]N^G-L-Arginine (99 % ^{15}N; MSD Isotopes) was kindly provided by R. J. Reynolds Tobacco Company, Winston–Salem, NC, U.S.A.

Isolation of endothelial and macrophage cytosols

Endothelial cells were isolated from freshly obtained porcine aortae by digestion with dispase [14]. Murine bone-marrow-derived macrophages cultured for 6 days in L929-cell conditioned medium [40] were stimulated for 20 h with lipopolysaccharide (10 μg/ml, *E. coli* serotype 055:B5) and were then harvested from culture dishes with a rubber policeman. After washing thrice with Hepes buffer (15 mM, pH 7.5) supplemented with sucrose (0.32 M) the cells were sonicated (6 × 5 s, 100 W) in ice-cold sucrose-free Hepes buffer containing proteinase inhibitors (antipain, leupeptin, pepstatin, chymostatin; 10 μg/ml each). Cytosols obtained by centrifugation (1 h; 100000 ***g***) were stored at −70 °C under N_2. In some experiments cytosol was passed over a desalting column to remove low-molecular-mass thiols and L-arginine. Protein was determined by dye binding (Bio-Rad, München, Germany), with BSA as standard.

Purification of endothelial NO synthase by affinity chromatography

All operations were performed in a coldroom (5–10 °C). Cytosolic fractions (5 mg of protein) were loaded on an ADP–agarose column (2 ml bed volume), which was subsequently washed with low-, high (0.6 M-NaCl included)- and low-ionic-strength buffer [40 mM-Tris/HCl (pH 7.4)/5 mM-L-arginine/3mM-dithiothreitol/2μM-FAD/2μM-FMN/2μM-tetrahydrobopterin/10 % (v/v) glycerol]. NO synthase was then eluted with 8 mM-NADPH, concentrated and, in some experiments, passed over a desalting column to remove low-molecular-mass constituents such as thiols (see below). By using this procedure, a 20-fold purified enzyme (determined by the increase in specific enzyme activity) was obtained with 20 % recovery.

Preparation of samples for e.p.r. spectroscopy

The procedure described recently was followed [37]. A yeast suspension (200 mg/ml in 15 mM-Hepes, pH 7.5) was boiled (30 min) and was subsequently loaded with DETC (2.5 mg/ml) for 30 min at 37 °C. The yeast was centrifuged and resuspended in the same volume of fresh Hepes solution. The yeast (final concn. 40 mg/0.7 ml) was incubated (37 °C) in a stoppered plastic vial with NO synthase preparations supplemented with 0.3 mM-L-arginine, 0.1 mM-NADPH, 1 μM-calmodulin, 60 μM-$CaCl_2$ (resulting in a 2 μM free Ca^{2+} concn. [14]), 0.1 μM-(6*R*)-tetrahydrobiopterin, 2 mM-GSH (omitted as indicated), 15 mM-Hepes, pH 7.5, 4 mM-$MgCl_2$, 1 μM-superoxide dismutase, bovine γ-globulin (0.1 mg/ml) and 0.1 mM-EGTA. In some experiments aq. 0.1 mM-NO, $NaNO_2$, hydroxylamine, *S*-nitroso-L-cysteine or SIN 1 were added instead of L-arginine. After a defined incubation period, usually 1 h, and addition of sodium dithionite (1 mg/ml) to some samples for 3 min, all samples were immediately frozen in cylindrical form (4.9 mm × 40 mm) by immersion in liquid N_2. The frozen samples fitted exactly into the e.p.r. cuvette (quartz Dewar with 5 mm inner diameter, filled with liquid N_2). For kinetic e.p.r. measurements the incubations were performed in a flat thermostatically controlled (37 °C) quartz cuvette (liquid-filled space 0.1 mm × 10 mm × 100 mm; Brucker, Karlsruhe, Germany) placed into the cavity of the e.p.r. spectrometer as described in [37]. E.p.r. spectra were recorded on Brucker e.p.r. 420 and Varian e.p.r. spectrometers at liquid-N_2 temperature (−196 °C) and at 37 °C at a microwave frequency of 9.330 GHz, a microwave power of 20 mW and modulation amplitude 0.1–1 mT (1–10 G). To calculate the concentration of NO trapped, we compared the intensity of the first-derivative signal (which is usually recorded on e.p.r. spectrometers) of [$Fe^{II}(DETC)_2NO$] with that of a standard, [$Fe^{II}(S_2O_3^{2-})_2(NO)_2$], as indicated in Figs. 1(*b*)–1(*d*) below by double-headed arrows. By double integration the intensities of both signals were calibrated in terms of spin concentration. This approach is correct, because both paramagnetic species possess identical electron spin ($s = 1/2$) [41,42] and exhibit identical saturation behaviour with increasing microwave power (results not shown). Data were corrected for a weak background [$Fe^{II}(DETC)_2(NO)$] signal in yeast due to NO from an unknown source [37].

Detection of NO synthase activity by purified soluble guanylyl cyclase

NO synthase activity was measured by activation of a purified soluble guanylyl cyclase (EC 4.6.1.2.) [14]. Either cytosol, or partially purified NO synthase, was incubated (usually 1 h, 37 °C) under conditions similar to those described for e.p.r. measurements, except for the inclusion of soluble guanylyl cyclase (1 μg/ml), 0.4 mM-[α-^{32}P]GTP (0.2 μCi), 0.1 mM-cyclic GMP, 1 mM-3-isobutyl-1-methylxanthine, 3.5 mM-phosphocreatine and 4.8 units of creatine phosphokinase (140 μl incubation vol.). In some experiments, yeast was omitted. The reactions were stopped by precipitation with $ZnCO_3$ and cyclic [^{32}P]GMP was isolated by chromatography on acid alumina. L-Arginine-dependent formation of cyclic [^{32}P]GMP was taken as a semiquantitative measure of NO synthase activity and was expressed as nmol of cyclic GMP formed/min of incubation per mg of pure guanylyl cyclase. Concentrations of cytosol and NO synthase were chosen to avoid maximal activation of guanylyl cyclase.

Measurement of NO synthase activity by formation of L-[^{3}H]citrulline

NO synthase preparations were incubated as described for the guanylyl cyclase assay, except that soluble guanylyl cyclase and [α-^{32}P]GTP were omitted and L-[^{3}H]arginine (0.05 μCi) as well as L-citrulline (0.1 mM, to saturate possible L-citrulline-transforming pathways) were included. Reactions were terminated by addition of sodium acetate (10 mM)/EDTA (1 mM), pH 5.0, and L-[^{3}H]citrulline was isolated by chromatography on a cation-exchange resin (AG 50W-X8; 100–200 mesh; Bio-Rad) as described in [43]. L-[^{3}H]Citrulline was quantified by liquid-scintillation counting. The amount of L-citrulline formed was calculated from values corrected for blanks and recovery. The recovery was 80 %, as assessed with L-[^{14}C]citrulline, and was not affected by the presence of cytosols.

Removal of thiols

For experiments under 'thiol-free' conditions, cytosols, purified NO synthase and soluble guanylyl cyclase were passed over a desalting column. The effectiveness of this procedure to remove low-molecular-mass thiols was ascertained by titration

with Ellman's reagent under non-denaturing conditions [44] which readily detects less than 1 μM-thiol (molar absorption coefficient at 412 nm 14000 litre·mol^{-1}·cm^{-1}). Since guanylyl cyclase, cytosols and purified NO synthase were diluted 300-, 100- and 20-fold in the final incubation mixture respectively, the starting contamination with low-molecular-mass thiols was probably below 50 nM.

Data evaluation

All assays were done in duplicate and were repeated at least three times. Data are presented as means ± S.E.M. Where indicated, data were compared by Student's t test for paired data. $P < 0.05$ was taken as the level of significance.

RESULTS

Detection of L-arginine-derived NO by e.p.r. spectrometry

DETC-loaded heat-killed yeast exhibited a weak background e.p.r. signal which was due to [$Fe^{II}(DETC)_2NO$] and the DETC complex with copper at $g = 2.035$ and 2.0 respectively (Figs 1a and 1e). The yeast bound free NO, from either an aqueous NO solution (Figs. 1b and 1f) or that released from the pharmacological NO donors sydnonimine SIN-1 and S-nitroso-L-cysteine (results not shown). The accumulated paramagnetic [$Fe^{II}(DETC)_2NO$] complex exhibited a typical e.p.r. signal with $g_{\perp} = 2.035$ and $g_{\parallel} = 2.02$ with unresolved triplet hyperfine structure (hfs) at $g_{\perp}$ in frozen state (Fig. 1b), and an isotropic triplet at $g_{av.} = 2.03$ in liquid phase (Fig. 1f). Similar e.p.r. signals were observed, when cytosol from freshly isolated porcine aortic endothelial cells (Figs. 1c and 1g) or lipopolysaccharide-stimulated murine bone-marrow-derived macrophages (Fig. 2b) was incubated for 1 h with all the ingredients required for maximal activation of the respective NO synthases [45,46]. As calculated from the signal intensity of the representative recordings shown in Figs. 1 and 2, 4.1 μM-[$Fe^{II}(DETC)_2NO$] was generated by endothelial (Fig. 1c) and 2.9 μM by macrophage-derived cytosol (Fig. 2b). Since there was a considerable variation between individual cytosol preparations, comparisons of different experimental conditions were made only within the same preparation. The requirement of NO synthase for Mg^{2+} was tested by omission of Mg^{2+} from incubations. NO formation by endothelial cytosol (130 μg of protein/ml) as detected by e.p.r. spectrometry was unaffected (6.5 ± 0.2 μM-NO/h with versus 6.5 ± 0.3 μM-NO/h without Mg^{2+}; $n = 3$).

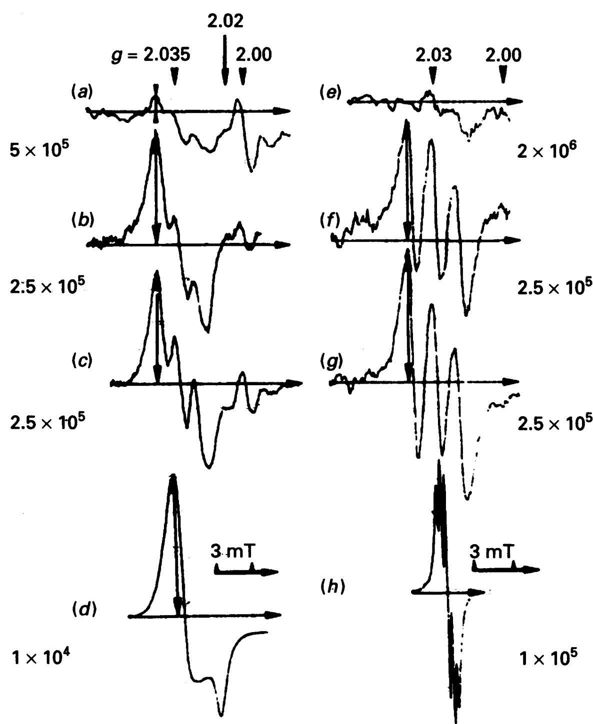

Fig. 1. Representative e.p.r. spectra of heat-killed DETC-loaded yeast (60 mg/ml) incubated (1 h; 37 °C) without (a, e) or with aqueous NO (0.1 mM; b, f), or with endothelial cytosol (0.13 mg/ml; c, g) containing all ingredients required for NO synthase activity as defined in the text

Samples were frozen immediately after 1 h of incubation, without dithionite treatment (see below). The amplitudes of the e.p.r. signal (indicated by double-headed arrows) were calibrated by the signal from $Fe(S_2O_3^{2-})_2(NO)_2$ standards (0.1 mM-NO/ml; d, h). Spectra were recorded in frozen samples (0.7 ml) at −196 °C (a–d) or in liquid aqueous phase at 37 °C (e–h). The horizontal arrow indicates the orientation of the magnetic field. Values beneath the recordings indicate the relative instrument gain. Instrument settings: microwave frequency 9.33 GHz; field sweep 30 mT; sweep time 2 min; microwave power 20 mW; modulation amplitude 1 mT (a–g) or 0.05 mT (h).

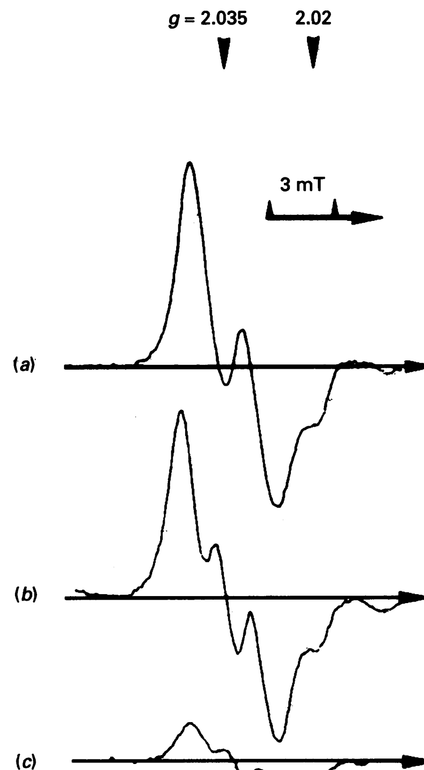

Fig. 2. Representative e.p.r. spectra of heat-killed DETC-loaded yeast (60 mg/ml) incubated (1 h; 37 °C) with cytosol from lipopolysaccharide-activated murine bone-marrow-derived macrophages (0.03 mg of protein/ml) containing [^{15}N]N^G-L-arginine (0.1 mM; a), [^{14}N]N^G-L-arginine (0.1 mM; b) or [^{14}N]N^G-L-arginine (0.1 mM) and N^G-nitro-L-arginine (0.3 mM; c) as well as all ingredients required for NO synthase activity as defined in the text

Spectra were recorded with frozen samples (0.7 ml) at −196 °C, with instrument settings identical with that in Fig. 1 (gain 10^5). Sodium dithionite (1 mg/ml) was added 3 min before freezing the incubation mixtures. Macrophage cytosol generated 7.0 ± 0.1 (a), 7.7 ± 0.5 (b) and 2.0 ± 0.1 (c) μM-NO ($n = 4$).

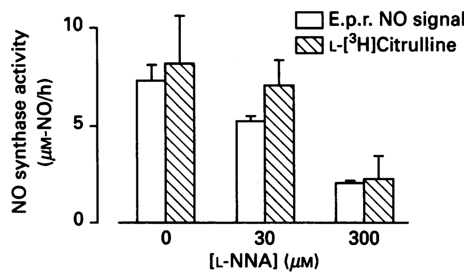

Fig. 3. Inhibition of macrophage NO synthase by N^G-nitro-L-arginine

DETC-loaded yeast was incubated (1 h; 37 °C) with macrophage cytosol (0.03 mg of protein/ml) as described in Fig. 1, in the absence or presence of N^G-nitro-L-arginine (L-NNA, 0.03 and 0.3 mM). NO synthase activity was measured in parallel incubations by e.p.r. spectroscopy of frozen samples after addition of sodium dithionite (open columns) and by formation of L-[^{3}H]citrulline from L-[^{3}H]arginine (hatched columns).

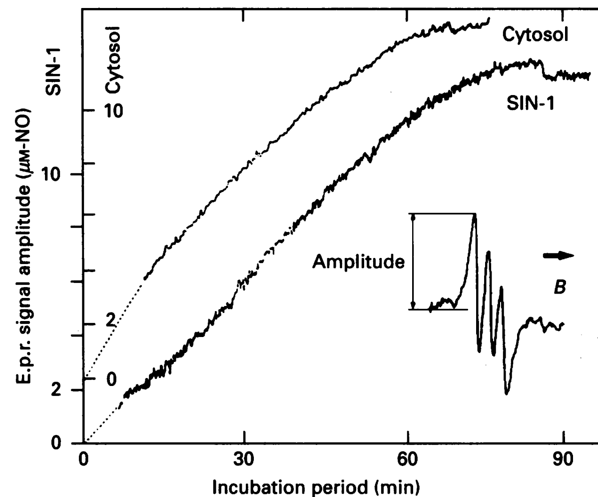

Fig. 4. On-line e.p.r. registration of NO formation by endothelial cytosol and by SIN-1

DETC-loaded yeast was incubated at 37 °C with endothelial cytosol (0.26 mg/ml; upper tracing) and NO synthase ingredients (see the text) or with sydnonimine SIN-1 (lower tracing) in a flat quartz cuvette placed inside the cavity of the e.p.r. spectrometer. After adjustment of instrument settings (indicated by broken lines) the magnetic field (B) was fixed to the first hyperfine line of the isotropic triplet e.p.r. signal to record the increase in the signal amplitude during the incubation. After 90 min the complete e.p.r. spectrum was recorded (inset) and the total amplitude was measured as indicated. The amplitude was calibrated in terms of NO concentration (μM) as described. Note the different zero points of both recordings. The results represent one of three experiments.

Source of nitrogen incorporated into [$Fe^{II}(DETC)_2(NO)$]

Formation of the paramagnetic mononitrosyl complex in cytosolic samples depended on the presence of L-arginine and NADPH (results not shown). In the presence of the stereospecific NO synthase inhibitor, N^G-nitro-L-arginine, e.p.r.-detectable NO formation by endothelial (results not shown) and macrophage cytosol (Figs. 2c and Fig. 3) was diminished. To demonstrate the source of the NO nitrogen atom, L-arginine-depleted macrophage and endothelial cytosol was incubated with the isotopically labelled [$^{15}N_2$]N^G-L-arginine instead of the naturally abundant [$^{14}N_2$]N^G-L-arginine. The typical doublet e.p.r. signal of [$Fe^{II}(DETC)_2(^{15}NO)$] was observed with macrophage (Fig. 2a) and endothelial cytosol (results not shown), owing to the hyperfine interaction of the ^{15}N nucleus with the unpaired electron in the paramagnetic centres. Since the e.p.r. signal derived from ^{15}NO accounted for all the paramagnetic centres, the nitrogen atom included in NO can be said to be derived exclusively from one guanidino nitrogen of L-arginine.

On-line registration of NO accumulation by e.p.r. spectroscopy

When frozen incubation mixtures containing [$Fe^{II}(DETC)_2$-(NO)] were thawed and re-recorded at 37 °C, the e.p.r. signal remained constant over 1 h, indicating that the mononitrosyl complex was highly stable. Therefore NO accumulation in aqueous solution could be monitored continuously over prolonged time by e.p.r. spectroscopy. Incubations with endothelial cytosol or with SIN-1 were performed in a quartz flat cell continuously exposed to the microwave radiation and the magnetic field inside the e.p.r. cavity, with the magnetic field strength fixed to the first (low-field) component of the triplet e.p.r. signal (indicated by 'Amplitude' in the inset to Fig. 4) of the [$Fe^{II}(DETC)_2(NO)$] complex. As Fig. 4 shows, the kinetic tracings start after adjustment of instrument settings, which took about 10 min after introducing the incubation mixture into the prewarmed flat cell. The exact starting point was extrapolated

Table 1. Effect of reducing agents on NO synthase activity as measured by e.p.r. spectroscopy, [^{3}H]-L-citrulline formation and guanylyl cyclase activation

The activity of ADP–agarose-purified endothelial (EC-NOS) and cytosolic macrophage (MQ-NOS) NO synthase was measured by e.p.r. spectrometry {[$Fe^{II}(DETC)_2NO$]} by formation of L-[^{3}H]citrulline (L-Citrulline) and by activation of soluble guanylyl cyclase. Similarly, NO release and guanylyl cyclase activation was measured with S-nitroso-L-cysteine (Cys-NO). Incubations (1 h; 37 °C) were performed either thiol-free or in the presence of GSH (2 mM; +GSH). E.p.r. measurements were conducted with sodium dithionite (1 mg/ml)-treated (+Dithio) and untreated samples (−Dithio). Guanylyl cyclase activity was determined in the presence (+Yeast) and absence of DETC-loaded yeast (−Yeast; 57 mg/ml) respectively. The first line lists background levels of e.p.r.-detected NO, L-[^{3}H]citrulline and guanylyl cyclase activity, which were subtracted from the values listed below. *Indicates a significant effect of GSH ($P < 0.05$). Abbreviation: n.d., not determined.

Addition	[$Fe^{II}(DETC)_2NO$] (μM/h)		L-Citrulline (μM/h)	Guanylyl cyclase activity (nmol/min per mg)	
	−Dithio	+Dithio	+Yeast	+Yeast	−Yeast
+GSH	0.5±0.2	1.3±0.2	0.0±0.3	150±33	145±37
EC-NOS (2 μg/ml)	0.5±0.2	0.9±0.2	1.0±0.3	70±12	345±17
+GSH	1.3±0.2*	1.5±0.1*	1.3±0.3	75±11	354±25
MQ-NOS (30 μg/ml)	2.2±0.3	4.6±1.6	8.9±2.9	346±22	1293±175
+GSH	2.9±0.4*	7.1±0.9*	8.2±2.0	360±15	1240±245
Cys-NO (10 μM)	5.5±1.1	9.9±1.5	n.d.	333±32	787±19
+GSH	8.6±3.3*	n.d.	n.d.	358±17	760±10

(broken lines) from the amplitude of the e.p.r. spectrum recorded at the end of each experiment (Fig. 4, inset). The rate of NO formation, as represented by the increase in the e.p.r. amplitude with time, was constant for about 1 h. Owing to the high concentration of cytosolic protein (0.26 mg/ml) and SIN 1 (0.1 mM), accumulation of NO ceased after 1 h because of saturation of traps. Indeed, when the quantity of NO traps available in the samples was measured by a short exposure to an excess of NO {anaerobic equilibration with NO gas [40 kPa (300 mmHg)] for 10 min}, a maximum of 10–15 μM-NO was trapped by 40 mg of yeast. Similar values were obtained after 90 min in the experiment shown in Fig. 4. When lower concentrations of cytosol or SIN-1 were used, the linear phase of NO accumulation was extended above 1 h (results not shown).

Recovery of NO by e.p.r. spectrometry

To assess whether NO as detected by e.p.r. spectrometry fully accounts (stoichiometrically) for L-arginine oxygenation by NO synthases, formation of L-[^{3}H]citrulline from L-[^{3}H]arginine was measured in parallel with e.p.r. incubations. Although reproducible results were obtained with the assay of citrulline in crude macrophage cytosol, for unknown reasons reliable data could not be obtained with crude endothelial cytosol. Therefore endothelial NO synthase was partially purified by ADP–agarose affinity chromatography, yielding a 20 fold-enriched enzyme preparation (specific activity 750 ± 200 and 40 ± 10 nmol of $NO \cdot h^{-1} \cdot mg^{-1}$ for purified enzyme and cytosol respectively), as estimated by e.p.r. spectrometry. If an excess of traps was provided, the NO and L-citrulline generated were detected in a stoichiometric ratio (1:1) with purified endothelial NO synthase (Table 1), but in a substoichiometric ratio (0.4:1) with macrophage cytosol (Table 1). A similarly low recovery (40 ± 10 %) was observed with NO released from sydnonimine SIN 1 (results not shown) and from *S*-nitroso-L-cysteine (10 μM; Table 1). However, when we treated the specimen with the strong reductant sodium dithionite at the end of the incubations with NO donors, we observed 100 % recovery of NO detected as $[Fe^{II}(DETC)_2NO]$ (Table 1).

Exclusion of nitrite, hydroxylamine and *S*-nitroso-L-cysteine as immediate products from L-arginine oxygenation

Since the e.p.r. method like all other NO detection methods cannot, in principle, discriminate between NO and labile NO donors in aqueous solution, we tested whether other NO_x besides NO could account for $[Fe^{II}(DETC)_2NO]$ complex formation by NO synthases. Incubation of yeast with a sodium nitrite (1–100 μM) or a hydroxylamine hydrochloride solution (10 μM), either in the absence or presence of L-arginine-depleted (desalted) endothelial cytosol, did not significantly increase the background NO signal (0.4 ± 0.2 μM-NO/h). Furthermore, the significantly increased background after addition of dithionite (1.1 ± 0.5 μM) was not affected by nitrite or hydroxylamine. Therefore these compounds are probably not intermediates in the endothelial and macrophage NO synthase reactions.

S-nitrosothiols are also difficult to differentiate from NO in aqueous solution and were assumed to be identical with EDRF [11,29]. Since NO and thiols cannot spontaneously form *S*-nitrosothiols under physiological conditions [47], one should assume that they are formed as a result of the NO synthase reaction, if at all. Such a reaction requires that a thiol (such as the GSH routinely included in the incubations described above) is accepted as a substrate by NO synthase, resulting in the formation of an *S*-nitrosothiol. To measure NO_x generated by NO synthase in the absence of low-molecular-mass thiols, purified thiol-depleted (desalted) soluble guanylyl cyclase, a biological target of NO [1,3,4], was used as a detector of NO_x. Incubations were performed with thiol-depleted macrophage cytosol and purified endothelial NO synthase in the absence of GSH, and L-citrulline formation under these conditions was compared. The activation of guanylyl cyclase and formation of L-citrulline were not significantly different in thiol-free and thiol-supplemented (GSH) preparations (Table 1). However, the recovery of e.p.r.-detectable NO was significantly decreased (Table 1; −GSH) in the absence compared with in the presence of GSH (Table 1; +GSH). The inclusion of GSH also increased the recovery of e.p.r.-detectable NO released from SIN-1 and *S*-nitroso-L-cysteine in a manner similar to dithionite (Table 1), whereas activation of guanylyl cyclase by both NO donors was not affected, in accordance with recent findings [48]. Guanylyl cyclase incubations performed for 10 min, 30 min and 1 h in the absence and presence of thiols yielded similar results, suggesting a linear rate of cyclic GMP formation for that period of time.

DISCUSSION

Though NO has previously been detected in substoichiometric amounts with respect to nitrite and nitrate in extracellular medium [2], it is still not clear whether NO diffuses freely from the generator to the target cell or whether it is bound to some carrier. Potential post-synthetic reactions could be traced and some of the proposals made concerning the identity of EDRF could be excluded, if one could be sure that NO is the immediate product generated by NO synthases. Unfortunately, this is questioned [34–36,49], and the molecular mechanism of the NO synthase reaction(s) still needs to be clarified [31–33]. We now demonstrate for the first time that NO derived from the guanidino nitrogen of L-arginine accumulates *stoichiometrically* with respect to L-citrulline in cell-free incubation mixtures of endothelial and macrophage NO synthases (experiments with [^{15}N]N^{G}-L-arginine). A similar conclusion was drawn in previous reports, where formation of ^{15}N-labelled nitrite and nitrate as well as formation of ^{15}NO complexes with haem and non-haem-iron proteins was observed with activated macrophages supplemented with [^{15}N]N^{G}-L-arginine [2,6,7,50]. However, in these and another study where ^{15}NO released from endothelial cells was detected by mass spectrometry [22], the stoichiometric conversion of the guanidino nitrogen of L-arginine into ^{15}NO was not demonstrated.

Our approach is more direct than other methods for NO detection (formation of methaemoglobin [16], determination of nitrite [18], bleaching of ferredoxin [8]) employed with oxygen-containing aqueous media, since NO is part of the paramagnetic species detected by e.p.r. spectroscopy, whereas, by reaction with oxyhaemoglobin and ferredoxin, NO is destroyed. The e.s.r. signal recorded for $[Fe^{II}(DETC)_2NO]$ is a 'fingerprint' of this complex in fact [39,42]. The failure of previous investigators to detect NO in cell-free incubation mixtures by e.p.r. spectroscopy [34–36] may be ascribed to insufficient trapping techniques. We took advantage of the high stability of $[Fe^{II}(DETC)_2NO]$ in oxygenated media to trap NO quantitatively. Though data are not available in the literature, our findings suggest a high rate and stability constant of $Fe(DETC)_2NO$ formation, since traps in about 10-fold lower concentration than oxygen competed successfully with inactivation of NO by oxygen, a reaction that proceeds in a few seconds. Also, an efficient competition of traps for NO with a haem moiety of soluble guanylyl cyclase was evident from the finding that NO-induced activation of purified guanylyl cyclase was inhibited by DETC-loaded (see Table 1), but not the DETC-free yeasts (results not shown).

Concerning the identity of a putative labile NO-precursor (NO_x) generated by NO synthases, we were able to rule out nitrite and hydroxylamine, since these compounds did not yield

a nitrosyl e.p.r. signal under our cell-free incubation conditions. It was more difficult to exclude *S*-nitrosothiols, since *S*-nitroso-L-cysteine generated a similar e.p.r. signal in our test system. There is also evidence that a thiol is released concomitantly with NO_x from endothelial cells [29], resulting in the proposal that EDRF may be an *S*-nitrosothiol [29]. If NO synthases generated *S*-nitrosothiols as intermediates, they must use a thiol as a substrate. Thus it follows that omission of thiols should impair NO synthesis. A significant decrease in e.p.r.-detectable NO was in fact observed when incubations were performed with thiol-depleted cytosols or partially purified NO synthase preparations in the absence of low-molecular-mass thiols. However, this effect was not related to impaired NO synthase activity, since formation of the biologically active NO_x measured by activation of purified soluble guanylyl cyclase and of L-citrulline was unaffected by thiol-free conditions. It is rather unlikely that traces of low-molecular-mass thiols (below 50 nM) present in the 'thiol free' incubation mixtures were sufficient to catalyse formation of *S*-nitrosothiols for more than 1 h at a steady rate in the presence of oxygen. Our findings are in accordance with the observation that the macrophage NO synthase remains active for at least 30 min in the absence of thiols at 37 °C [45]. It is known that $[Fe^{II}(DETC)_2NO]$ oxidizes to 'e.p.r.-silent' $[Fe^{II}NO(DETC)_2X]$, where X = halogen anions or NO_2. This complex is transformed into the parent paramagnetic complex under reductive conditions [51,52]. We probably induced such transformation in our system by addition of dithionite and/or GSH. Therefore the effect of thiol depletion on the recovery of NO generated by NO synthases (and also pharmacological NO donors) seems to be related to the formation of e.p.r.-silent nitrosyl complexes, and not to direct effects on NO synthase activity. For this reason, it is unlikely that thiols participate as intermediate NO_x in L-arginine-dependent NO synthesis.

Our present findings strongly suggest that NO is in fact the NO_x generated by NO synthases. One question still remains unanswered, that is, in which form is NO released from the generator cell? Since NO cannot form *S*-nitrosothiols by direct reaction with thiols under physiological conditions (neutral pH) [47], a fact which is frequently overlooked, we must search for other potential post-synthetic pathways to explain the divergent findings on the nature of EDRF. The hypothesis that EDRF is a nitrosyl iron complex with thiol ligands [53] is under study [54].

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