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EPR evidence for nitric oxide production from guanidino nitrogens of L-arginine in animal tissues in vivo

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Administration of Fe^{2+}-citrate complex (50 mg/kg of $FeSO_4$ or $FeCl_2$ plus 250 mg/kg of sodium citrate) subcutaneously in the thigh or *Escherichia coli* lipopolysaccharide (LPS, 1 mg/kg) intraperitoneally, (i.p.) to mice induced NO formation in the livers in vivo at the rate of 0.2–0.3 μg/g wet tissue per 0.5 h. The NO synthesized was specifically trapped with Fe^{2+}-diethyldithiocarbamate complex ($FeDETC_2$), formed from endogenous iron and diethyldithiocarbamate (DETC) administered i.p. 0.5 h before decapitation of the animals. NO bound with this trap resulted in the formation of a paramagnetic mononitrosyl iron complex with DETC (NO-$FeDETC_2$), characterized by an EPR signal at $g_\perp = 2.035$, $g_\parallel = 2.02$ with triplet hyperfine structure (HFS) at $g_\perp$. This allowed quantification of the amount of NO formed in the livers. An inhibitor of enzymatic NO synthesis from L-arginine, N^G-nitro-L-arginine (NNLA, 50 mg/kg) attenuated the NO synthesis in vivo. L-Arginine (500 mg/kg) reversed this effect. Injection of L-[*guanidineimino*-$^{15}N_2$]arginine combined with Fe^{2+}-citrate or LPS led to the formation of the EPR signal of NO-$FeDETC_2$ characterized by a doublet HFS at $g_\perp$, demonstrating that the NO originates from the guanidino nitrogens of L-arginine in vivo.

Introduction

It is now generally accepted that nitric oxide (NO) is synthesized in biological systems as a result of the NO-synthase-catalyzed guanidino-nitrogen oxidation of L-arginine [1–10]. However, this has not been clearly demonstrated due to equivocal methods used for NO detection. Experiments with L-[*guanidineimino*-$^{15}N_2$]arginine as a nitric oxide source are required for final verification of this proposed pathway for endogenous NO production. Such experiments have been performed [2–6]; however, ^{15}N was detected only in nitrite and nitrate, stable products of the enzymatic oxidation of L-arginine. Direct incorporation of ^{15}N into NO has not been demonstrated in animal tissues in vivo.

Recently, the iron(II) diethyldithiocarbamate complex ($FeDETC_2$) has been used to specifically trap and detect NO formed in animal tissues in vivo from exogenous and endogenous sources [10–15]. Free NO bound with this trap resulted in the formation of a stable paramagnetic mononitrosyl iron complex with diethyldithiocarbamate (NO-$FeDETC_2$), which is characterized by an EPR signal at $g_\perp = 2.035$, $g_\parallel = 2.020$ with triplet hyperfine structure (HFS) at $g_\perp$. The latter originated from the interaction of the unpaired electron with the ^{14}N nucleus of the NO group (^{14}N nuclear spin, $I = 1$). If NO is produced from the guanidino nitrogens of L-arginine, the injection of L-[*guanidineimino*-$^{15}N_2$]arginine into organisms should result in an EPR signal for NO-$FeDETC_2$ with a doublet HFS at $g_\perp$ due to the ^{15}N nuclear spin ($I = 1/2$). This study was undertaken to obtain direct evidence for NO production from L-[*guanidineimino*-$^{15}N_2$]arginine using $FeDETC_2$ as a specific trap for free NO and EPR as a probe for ^{15}N incorporation in NO-$FeDETC_2$.

Materials and Methods

Materials

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0128, B12), $FeSO_4 \cdot 7H_2O$, $FeCl_2 \cdot 4H_2O$, $CaCl_2$, and $NaNO_2$ were obtained from Sigma Chemical Co. L-Arginine, N^G-nitro-arginine (NNLA), N^G-

Abbreviations: DETC, diethyldithiocarbamate; $FeDETC_2$, Fe^{2+} DETC complex; LPS, *E. coli* lipopolysaccharide; NO, nitric oxide; NO-$FeDETC_2$, mononitrosyl iron complex with DETC; NNDA, N^G-nitro-D-arginine; NNLA, N^G-nitro-L-arginine.

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nitro-D-arginine (NNDA), sodium citrate and sodium diethyldithiocarbamate (DETC) were obtained from Serva. L-[*guanidineimino*-$^{15}N_2$]Arginine (99 atom% ^{15}N) was purchased from MSD Isotopes.

Sample preparation

The experiments were conducted on mongrel male white mice (20–22 g) in winter (December–January, 1990–1991) or spring (March–April, 1991). All agents were injected into mice in 0.2 ml of physiological salt solution intraperitoneally (i.p.) (except Fe^{2+}-citrate complex and $CaCl_2$, which were administered subcutaneously in the thigh) at the following doses: LPS, 1 mg/kg, 0.5 or 3.5 h before decapitation of the animals; L-[*guanidineimino*-$^{15}N_2$]arginine, 60 mg/kg, 1 h before decapitation; L-arginine, 500 mg/kg; NNLA, 50 mg/kg; NNDA, 50 mg/kg; DETC, 500 mg/kg; $CaCl_2$, 75 mg/kg; and Fe^{2+}-citrate complex, 50 mg/kg of $FeSO_4 \cdot 7H_2O$ or $FeCl_2 \cdot 4H_2O$ + 250 mg/kg of sodium citrate, all administered 0.5 h before decapitation. $FeCl_2 \cdot 4H2O$ was used in the experiments with $CaCl_2$ injection. The control mice were injected with DETC solution only. After death, the liver and other organs (kidney, heart, spleen, lung) were isolated, frozen in liquid nitrogen, and analyzed with an ESR-V (USSR) or an ESR-"Radiopan" (Poland) radiospectrometer.

Solutions of NO-$FeDETC_2$ in dimethylsulphoxide, containing ^{14}NO or ^{15}NO were prepared quantitatively as described elsewhere [14], using $Na^{14}NO_2$ and $Na^{15}NO_2$ (Reachim, USSR) as a sources of NO.

EPR spectrometry

EPR spectra were recorded at 77 K, a microwave frequency of 9.330 GHz, a microwave power of 5 mW and modulation amplitude of 0.3 mT. Quantification of the NO-$FeDETC_2$ concentrations was accomplished using the method of double integration of the EPR signals with the nitroxyl radical, 2,2',6,6'-tetramethylpiperidol-1-oxyl, as a standard paramagnetic sample.

EPR spectra of NO-$FeDETC_2$ with various ratios ^{15}NO and ^{14}NO were generated by computer simulation.

Results

As previously reported [10–15], administration of DETC alone to test animals is sufficient for the formation of $FeDETC_2$ and NO-$FeDETC_2$ in all tissues examined with the exception of blood. Endogenous iron from pool of free iron and from the iron-depositing systems reacts with exogenous DETC to form $FeDETC_2$ which binds NO to produce the mononitrosyl iron complex, NO-$FeDETC_2$.

In the current study, the formation of such complexes in the livers of control mice 30 min after administration of DETC was observed. The amount of NO, trapped as NO-$FeDETC_2$, was dependent on the season when the animals were subjected to the experimental procedures (Table I).

TABLE I

The quantities of nitric oxide trapped by $FeDETC_2$ in the livers of mice in vivo

All agents were injected 30 min before decapitation of the animals. The data shown are expressed as the mean + S.E. mean of n observations from different animals. Probability levels of less than 0.05 were taken to indicate significance in all cases.

Time of experiments	Animals were injected with DETC +	NO (ng/g of liver)	Number of animals (n)
March–April	control mice (only DETC)	100 ± 10	20
	+ Fe-Cit [a]	270 + 70	20
	+ Fe-Cit + NNLA	50 + 5	20
	+ Fe-Cit + NNDA	220 + 30	20
	+ Fe-Cit + NNLA + LA [a]	140 + 80	10
March–April	control mice (only DETC)	100 + 10	10
	+ LPS	100 + 20	16
	+ LPS [b]	320 + 40	19
December–January	control mice (only DETC)	20 ± 1	41
	+ $CaCl_2$	20 + 5	10
	+ Fe-Cit	200 + 20	67
	+ Fe-Cit + $CaCl_2$	300 + 25	48

[a] Abbreviations: Fe-Cit, Fe^{2+}-citrate complex; LA, L-arginine.

[b] In these experiments LPS was injected 3.5 h before decapitation of the animals.

When the animals were treated with DETC and Fe^{2+}-citrate simultaneously, the formation of NO-$FeDETC_2$ in liver was sharply augmented (Table I). In the tissues of other organs of these animals, the content of NO-$FeDETC_2$ was 5–10-times lower. There was no difference between the effects of $FeSO_4$ or $FeCl_2$. When DETC and Fe^{2+}-citrate complex were administered together with NNLA, an attenuation of the level of NO-$FeDETC_2$ was observed (Table I). This decrease was more pronounced (up to total inhibition) with increasing NNLA doses (up to 350 mg/kg, data not shown). Similar effects of NNLA were also observed in the control animals (data not shown). NNDA (D-isomer of NNLA) did not affect the production of NO-$FeDETC_2$ in the animal tissues examined (Table I). Combined administration of NNLA and L-arginine restored the level of NO-$FeDETC_2$ induced by Fe^{2+}-citrate (Table I), but this effect was achieved only with a high dose of L-arginine (500 mg/kg) and a dose of NNLA producing incomplete suppression of NO-$FeDETC_2$ formation (50 mg/kg). These results are consistent with Fe^{2+}-citrate induced enzymatic formation of NO from L-arginine in the tissues examined. The competitive inhibitor of NO-synthase, NNLA, reversible inhibits this process. When all chemicals used (DETC, $CaCl_2$, Fe^{2+}-citrate, and LPS) were mixed in aqueous solution, no NO-$FeDETC_2$ signals were observed in the EPR spectrum of the solutions.

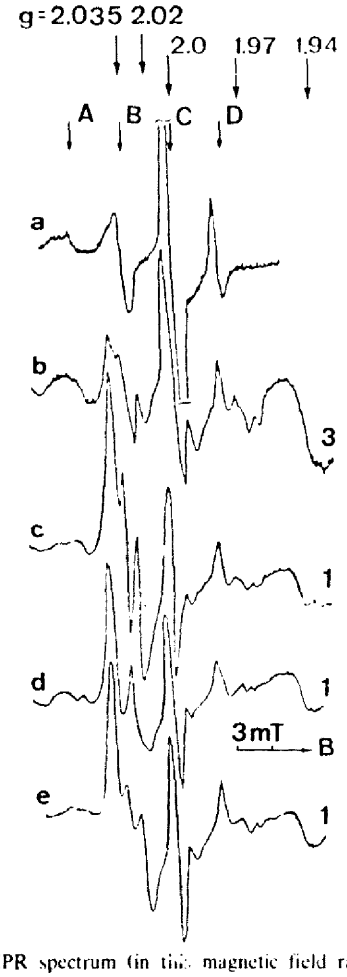

Fig. 1. The EPR spectrum (in the magnetic field range) of the Cu^{2+}-DETC complex (a). 'A, B, C, D' (top) indicate the position of the four components of the HFS at $g_{\parallel}$ of the Cu^{2+}-DETC complex. Typical EPR spectra of mouse liver preparations of control animals, injected with DETC only (b), animals injected with DETC + Fe^{2+}-citrate (c), animals injected with DETC + Fe^{2+}-citrate + L-[*guanidineimino*-$^{15}N_2$]arginine (d), animals injected with DETC + LPS + L-[*guanidineimino*-$^{15}N_2$]arginine (e). Spectrometer gain is shown on the right (arbitrary units). Recordings were made at 77 K, $p = 5$ mW, $f = 9.330$ GHz, modulation amplitude = 0.3 mT. The spectra were redrawn by hand.

To confirm that the NO trapped by $FeDETC_2$ originated directly from L-arginine and not nitrite which accumulated during the reaction, the animals were injected with $NaNO_2$ at doses up to 15 mg/kg. Even at this concentration, which was 50-times greater than the NO concentration detected in the liver (0.3 μg/kg of wet tissue), NO-$FeDETC_2$ was not detected in these animals.

Typical EPR spectra from mouse liver are shown in Fig. 1. In addition to the EPR signals which are due to free radicals, Mo^{5+}-complexes, and reduced iron-sulphur proteins with g-factors 2.0, 1.97, and 1.94 respectively, the livers of control animals (treated with DETC only) showed the characteristic EPR signal of NO-$FeDETC_2$ ($g_{\parallel} = 2.035$, $g_{\perp} = 2.020$) and the EPR signal of DETC complexes with endogenous copper (Cu^{2+}) (Fig. 1b). In this magnetic field range the latter is characterized by quartet HFS (A, B, C, D at $g_{\parallel}$ in Fig. 1a) originating from the interaction of the unpaired electron with the copper nucleus ($I = 3/2$) (Fig. 1a). The $g_{\parallel}$ and hyperfine splitting (A) values of this signal are 2.02 and $4 \cdot 10^{-3}$ cm^{-1}, respectively. The second component of this HFS superimposes and partially masks the EPR signal of NO-$FeDETC_2$ in the livers of control animals. However, its intensity can be estimated by using the third (high-field) HFS component which is not overlapped by the components of the EPR signal from Cu^{2+}-DETC complex. This is not the case for the EPR signal from the livers of mice treated with DETC and Fe^{2+}-citrate because of the high intensity of this signal (Fig. 1c). It is characterized by triplet HFS due to the interaction of the unpaired electron with the ^{14}N nucleus of the NO group of the mononitrosyl iron complex.

When DETC with Fe^{2+}-citrate were administered 30 min after L-[*guanidineimino*-$^{15}N_2$]arginine (1 h before decapitation) the EPR signal of NO-$FeDETC_2$ was characterized by doublet HFS at $g_{\perp}$ (Fig. 1d). Comparison of this signal with the computer-simulated EPR spectra of NO-$FeDETC_2$ with various ratios of ^{15}NO and ^{14}NO shown in Fig. 2 (spectra a and b are the experimental spectra, spectra c–f are the computer-generated sums of spectra a and b in varying ratios), reveals approx. 80% enrichment of ^{15}NO in NO-$FeDETC_2$ in livers of animals treated with L-[*guanidineimino*-$^{15}N_2$]arginine. This result is consistent with NO production from the guanidino nitrogens of L-arginine.

The injection of $CaCl_2$ alone had no direct effect on NO synthesis in mouse liver, but did enhance the effect of exogenous Fe^{2+}-citrate (Table I). These experiments were conducted during the winter season (December–January) and this might explain the attenuation of NO synthesis in the livers of control animals due to reduced metabolic activity as compared to animals in the spring (Table I). Despite this apparent seasonal attenuation, Fe^{2+}-citrate stimulated NO formation in the livers of these mice to the same extent as was observed for 'spring' mice.

Bacterial LPS did not stimulate NO-$FeDETC_2$ formation in mouse liver by 30 min after administration; however, NO-$FeDETC_2$ formation was observed at 3–4 h after this treatment. This result is in line with recent data obtained in Moncada's laboratory [16] and indicates the inducible type of NO synthase responsible for NO and mononitrosyl iron complex formation in the livers of mice treated with LPS. As shown earlier with the EPR method [15], L-arginine participated in this process and its inhibitory analog, NNLA, reversibly blocked NO synthesis stimulated by LPS in mouse liver in vivo.

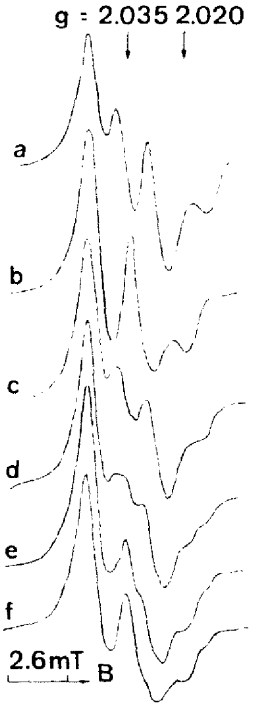

Fig. 2. The shapes of EPR signals of NO-FeDETC$_2$ including ^{14}NO (a) and ^{15}NO (b) only, or at the various ratios of these ligands: ^{15}NO: ^{14}NO = 3:7 (c), 1:1 (d), 2:1 (e), 4:1 (f). The EPR signals a and b were recorded at 77 K, $p = 5$ mW, $f = 9.330$ GHz, modulation amplitude = 0.3 mT. The concentrations of NO-FeDETC$_2$ responsible for the EPR signals a and b were equal. The EPR signals c–f were computer simulated by summing the EPR signals a and b in various ratios.

The EPR signal of NO-FeDETC$_2$ recorded in mouse liver after LPS injection (not shown) was identical to the spectrum of NO-FeDETC$_2$ observed for mice treated with Fe^{2+}-citrate, shown in Fig. 1c. The administration of L-[*guanidineimino*-$^{15}N_2$]arginine (1 h before decapitation) after LPS injection led to an EPR signal in the liver which was a superposition of EPR signals with triplet and doublet HFS (Fig. 1e). A comparison of this signal with the simulated EPR signals presented in Fig. 2 revealed approx. 30% enrichment of ^{15}N in NO-FeDETC$_2$ in livers of animals treated with LPS and L-[*guanidineimino*-$^{15}N_2$]arginine.

Discussion

To date, two types of NO synthase have been described [16–21]. The type 1 enzyme is induced by a set of cytokines and bacterial products and is functional in the macrophages, neutrophils, hepatocytes, tumor and endothelial cells. The type 2 NO synthase acts in response to ligand-receptor-coupling reactions and is localized in the endothelial and cerebellar cells, platelets, and adrenal gland tissue. In contrast with the inducible type of enzyme, the second constitutive type of NO synthase displays Ca^{2+} dependence. Apparently, both types of NO synthase were dealt with in our experiments on mice. The first type was induced with bacterial LPS, the other type was activated in response to Fe^{2+}-citrate. In the latter experiments this activation seemed to be connected with prooxidant action of iron on the cell membranes. This resulted in a membrane lesion with subsequent uptake of extracellular Ca^{2+} by cells. These Ca^{2+} ions activated NO synthase. The observed increase in NO-FeDETC$_2$ formation under the combined action of Fe^{2+}-citrate and $CaCl_2$ is consistent with this hypothesis. It is noteworthy that the inhibitory effect of phenozan, an antioxidant agent, on Fe^{2+}-citrate activation of NO synthase in mouse liver in vivo has been described elsewhere [13]. This type of NO synthase is probably functional in vascular endothelial cells of liver. Another type of NO synthase induced by LPS seems to be localized in hepatocytes or Kuppfer cells [16].

In a previous study [13], it was demonstrated that the administration of DETC alone to control animals resulted in the formation of sufficient FeDETC$_2$ in the liver to trap up to 2 μg NO per g of wet tissue. Consequently, the amount of FeDETC$_2$ formed endogenously from the pool of free iron and from the iron-depositing systems in the test animals did not limit the production of NO-FeDETC$_2$ in this study. The enhanced formation of NO-FeDETC$_2$ in mouse liver induced by the administration of Fe^{2+}-citrate was due only to enhanced formation of NO, not to an increase in the amount of FeDETC$_2$ available to trap it.

The data reported herein demonstrate that L-arginine functions as the substrate of NO synthase in animal tissues in vivo and NO originates from one of two equivalent guanidino nitrogens of this amino acid. However, we can not yet say that L-arginine is the only source of NO in vivo since we failed to observe an EPR signal of NO-FeDETC$_2$ with "pure" doublet HFS. It is likely that incomplete absorption of L-arginine by the test animals made it difficult to obtain such results. Weak absorption of L-arginine might also explain the incomplete restoration of NO-FeDETC$_2$ after combined injection of NNLA and L-arginine (Table I).

Iyenger et al. [2] reported the formation of ^{15}NO-morpholine by activated macrophages treated with morpholine and L-[*guanidineimino*-$^{15}N_2$]arginine. This observation may not be considered as sufficient evidence for the appearance of NO in the cells in a free form. Nitrosomorpholine might have been formed as a result of reactions not involving the participation of free NO. Since FeDETC$_2$ is known to trap free NO [10–15], the formation of ^{15}NO-FeDETC$_2$ observed in this study may be taken as sufficient evidence for the appearance of free NO in our animal model.

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