L-Arginine depletion by arginase reduces nitric oxide production in endotoxic shock: an electron paramagnetic resonance study

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Abstract **Nitric oxide (NO) synthesis was measured in the liver, lung, spleen and kidney of lipopolysaccharide-treated male rats using the nitric oxide spin trap, iron (II)-diethyldithiocarbamate ($FeDETC_2$). Nitric oxide formation in vivo was determined by the increase in intensity of the characteristic triplet hyperfine EPR spectrum of [NO-$FeDETC_2$]. Intravenous bovine liver arginase, at a dose which completely depleted circulating arginine, significantly reduced the formation of nitric oxide in these tissues. The general decrease in NO levels was confirmed by the decrease in plasma nitrite levels. These results directly demonstrate that NO formation in endotoxic shock depends on extracellular arginine; depletion of plasma arginine may be a useful therapeutic strategy.**

Key words: Nitric oxide; Arginase; Lipopolysaccharide; Endotoxic shock; Arginine

1. Introduction

Nitric oxide is a highly reactive free radical which is synthesised from L-arginine by a family of nitric oxide synthases [1,2]. The calcium-dependent, or constitutive NOS, as found in endothelium and neurones, rapidly synthesizes small amounts of nitric oxide in response to increases in intracellular calcium, whereas the calcium independent NOS, also known as inducible NOS (iNOS) is under transcriptional control [3–5]. Synthesis of iNOS is stimulated by cytokines or endotoxin and, once the enzyme is formed, nitric oxide is produced in large amounts for several hours independently of intracellular calcium levels. We have previously demonstrated by immunohistochemistry the widespread distribution of inducible NOS in rats given lipopolysaccharide [6].

NO produced by the inducible enzyme is a major mediator of macrophage cytotoxicity [7–9] and has been implicated as a mediator in septic shock [10–12] and inflammation. The stable end products of NO are NO_2^- and NO_3^- and these have previously been employed as a measure of NO production [13]. Demonstration of the relative importance of constitutive and inducible NOS in pathological conditions has been hampered by the lack of specific inhibitors of the individual isoforms of NOS. Thus L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) inhibit both constitutive and inducible enzymes and may lead to changes in blood pressure or blood flow which could confound interpretation of in vivo studies designed to elucidate the role of inducible NOS. The inducible enzyme is critically dependent on an extracellular supply of substrate L-arginine [14–16], and in vitro depletion of L-arginine in the medium inhibits macrophage cytotoxicity [16]. The apparent Michaelis constant for L-arginine in NO synthesis by iNOS in murine macrophages is 73 μM [14] and plasma levels of L-arginine are around 225 μM. Griffith et al. [17] have provided evidence that it is possible to decrease NO synthesis in the guinea pig by using arginase to deplete plasma arginine. In the present study, we have investigated the effect of arginase on induced NO production by electron paramagnetic resonance (EPR) spectroscopy and the spin-trap iron(II) diethyldithiocarbamate ($FeDETC_2$), recently developed by Vanin et al., and shown to specifically trap NO formed in animal tissues in vivo [18]. NO generated in rats given LPS alone, LPS/L-NMMA or LPS/arginase was directly measured from the NO-$FeDETC_2$ EPR signal observed from tissue homogenates.

2. Materials and methods

2.1. Materials

L-NMMA was a gift from Dr. S. Moncada (Wellcome Research Laboratories, Beckenham, Kent). Rabbit anti-iNOS antiserum [19] was a gift from Dr. V. Riveros-Moreno (Wellcome Research Laboratories) and the streptavidin–biotin peroxidase system from Dakopatts (High Wycombe, Bucks, UK). The anti-macrophage monoclonal ED1 was obtained from Serotec (Kidlington, Oxfordshire).

Pseudomonas oleovorans was from National Collection of Type Cultures (Colindale, London, UK). LPS *Escherichia coli* serotype 0127:B8, bovine liver arginase and all other reagents used were obtained from Sigma Chemical Co. (Poole, Dorset).

2.2. LPS administration

Experiments were carried out on inbred male Lewis rats weighing 170–270 g, with all injections given in normal saline. LPS (4 mg/kg) was administered intraperitoneally ($n = 28$). Control rats were given $FeDETC_2$ only ($n = 2$). Thirty minutes prior to sacrifice all rats were given DETC (500 mg/kg) intraperitoneally and $FeSO_4$ (50 mg/kg), sodium citrate (250 mg/kg) subcutaneously. 4 h after LPS administration, some rats were given arginase i.v., (2,500 U/rat, $n = 11$), or L-NMMA, i.p. (50 mg/kg, $n = 7$) or equivalent amounts of saline.

5 h after LPS, the animals were killed and the relevant tissues frozen immediately in liquid nitrogen. Sections taken were fixed for 48 h in buffered formal saline for histology and immunohistochemistry. Plasma was collected for NO_3^-/NO_2^- and arginine levels. Homogenized tissue samples were analysed for total protein by the modified Lowry method [20], by absorbance at 660 nm.

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Abbreviations: NOS, nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; DETC, diethyldithiocarbamate; EPR, electron paramagnetic resonance.

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2.3. Immunohistochemistry

Paraffin sections were dewaxed, microwaved in citrate buffer and incubated with the iNOS antibody (1:2,000) at 4°C with a biotinylated polyclonal swine anti-rabbit secondary antibody [6]. Macrophages were labelled with the mouse monoclonal antibody ED1 (1:500), with a biotinylated rabbit anti-mouse secondary antibody. Both were detected with streptavidin/biotin peroxidase complex with diaminobenzidine as chromagen and haematoxylin counterstain.

2.4. Plasma nitrite/nitrate and arginine analysis

Blood was anticoagulated with heparin, plasma was separated and frozen for analysis. The plasma was treated with NaOH (0.5 M), and the protein precipitated with $ZnSO_4$ (10%). Nitrate was reduced to nitrite with nitrate reductase from *Pseudomonas oleovorans* and assayed using the Griess Reaction, with the lower limit of detection being 7.5 μM [6]. Arginine levels in plasma were determined by HPLC as described previously [21].

2.5. EPR spectroscopy

Tissue samples removed from the rat immediately after killing were homogenized in an equal weight of 0.2 M HEPES buffer, pH 7.4, placed in quartz EPR tubes (ca. 3.4 mm i.d.), and frozen in liquid nitrogen. X-band EPR spectra were recorded on a Bruker ESP 300 spectrometer fitted with an Oxford Instruments ESR 900 liquid helium flow cryostat. Spectral baselines were corrected by subtraction of a cavity/water spectrum recorded under identical conditions. The concentration of NO-$FeDETC_2$ was estimated by careful measurement of the peak (g = 2.047)-to-baseline height, after normalisation of the data. This provides a measure of the amount of NO available for trapping by $FeDETC_2$.

3. Results

The plasma L-arginine concentration in normal Lewis rats was estimated to be 225 ± 18 μM (n = 12). Preliminary experiments showed that a single dose of 2,500 units of arginase reduced plasma arginine to undetectable levels for 4 h and that at 8 h plasma arginine was 17 ± 1 μM. This dose was chosen for further experiments. LPS treatment alone reduced plasma arginine levels to less than a quarter (50 ± 9.3 μM) and arginase administration after LPS resulted in undetectable levels of arginine one hour later.

NO_3^-/NO_2^- levels were undetectable in control animals but increased to 293 μM ± 36 after LPS (Fig. 1). This rise in NO_3^-/NO_2^- was significantly attenuated by arginase (123.7 ± 15 μM) or by L-NMMA (85 ± 14 μM).

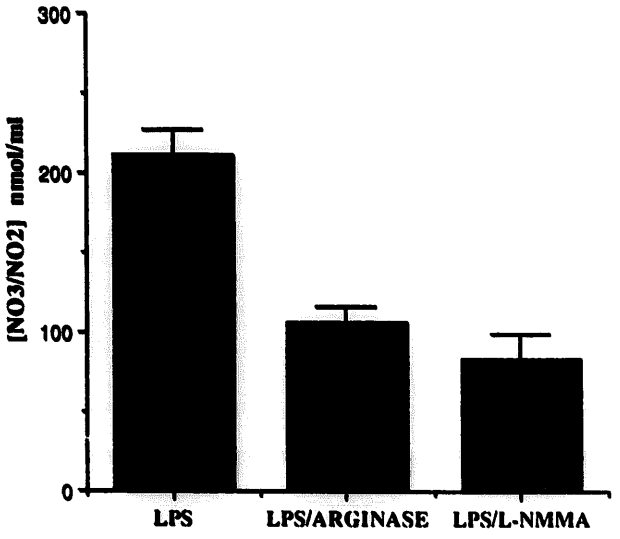

Fig. 1. Plasma NO_3^-/NO_2^- levels in endotoxin-treated rats with or without arginase or L-NMMA. Results expressed as mean ± S.E.M. (n = 6–10 where $P < 0.01$ using an unpaired Student's t-test).

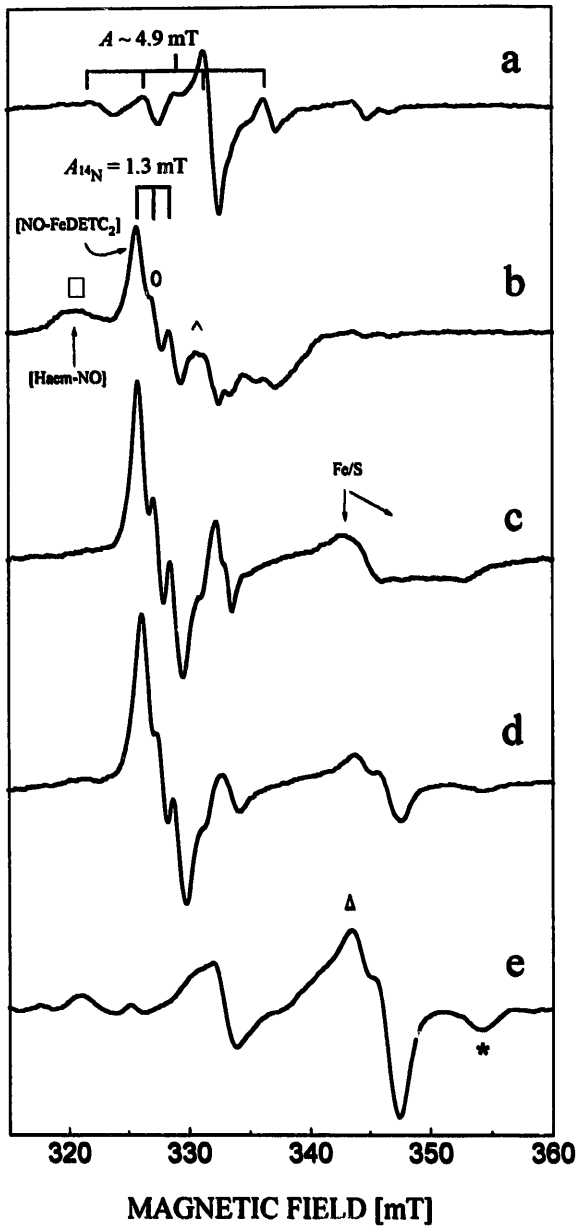

Fig. 2. X-band EPR spectra from endotoxin-treated rats. Spleen homogenate from rats treated with DETC and Fe-citrate alone 30 min before sacrifice (a), and representative spectra of (b) spleen, (c) kidney and (d) liver homogenates from rats administered LPS (5 h before death) and DETC and Fe-citrate (30 min prior to death); (e) liver homogenate from an untreated rat. Measurement conditions were: temperature, 15 K; microwave power, 20 mW; microwave frequency, 9.34 GHz; modulation amplitude, (a–d) 0.49, (e) 0.98 mT; time constant, 0.08 s; sweep rate (a–d) 1.19, (e) 2.38 mT/s. The spectra are an average of 2 scans, and have been normalised to allow direct comparison. The g-factors are indicated in the figure as: g = 2.08, □; g = 2.035, ○; g = 2.02, ^; g = 1.93, △; g = 1.88, *).

Nitric oxide was detected by EPR using the spin trap $FeDETC_2$ [18]. Fig. 2a shows the g = 2 region of the EPR spectrum of spleen removed from a rat treated with $FeDETC_2$ only. At 15 K, the tissue exhibited an intense quartet signal at

~321.6, 326.0, 331.0, 336.1 mT ($a \approx 4.9$ mT), characteristic of DETC complexed with endogenous copper [18]; this signal was detected at temperatures up to 100 K. A weaker signal to higher field at $g \approx 1.94$ was also present in the spectrum and assigned to reduced iron-sulphur proteins. Endotoxin administration to the rat resulted in the detection of a prominent new signal in the $g = 2$ region, from spleen, liver, kidney and lung (Fig. 2b–d). The EPR spectrum now displayed the characteristic three-line spectrum of NO-$FeDETC_2$ similar to that demonstrated previously by other workers [18,22], at $g_z = 2.02$, $g_{yx} = 2.035$, with a triplet hyperfine structure ($a = 1.3$ mT) at g_{yx}, due to the interaction of the unpaired electron on the ^{14}N nucleus of NO complexing with free $FeDETC_2$ (see Fig. 2). The NO-$FeDETC_2$ signal is readily distinguished from background signals due to endogenously-reduced iron-sulphur clusters. The EPR signal of free $FeDETC_2$ is no longer visible. This could be due to overlap by the more intense signal from the mononitrosyl complex, or a more likely explanation may be that all of the spin-trap has converted to NO-$FeDETC_2$, due to excess amounts of NO being formed by iNOS. The latter suggestion is supported by the observation of a broad feature at $g \sim 2.08$ which is indicative of haem-nitrosyl complex formation [23]. Kidney and liver from rats treated with endotoxin displayed similar spectra (Fig. 2c–d), though with more intense signals at $g \approx 1.93$ and $g \approx 1.88$ from iron-sulphur clusters. Both the g-value and lineshape of these signals are diagnostic of the mitochondrial iron-sulphur proteins, such as NADH dehydrogenase and succinate dehydrogenase (Complexes I and II of the respiratory chain, respectively). Furthermore, there was no evidence for NO formation in the homogenate of a control animal, although particularly prominent signals were observed from these iron-sulphur proteins (Fig. 2e).

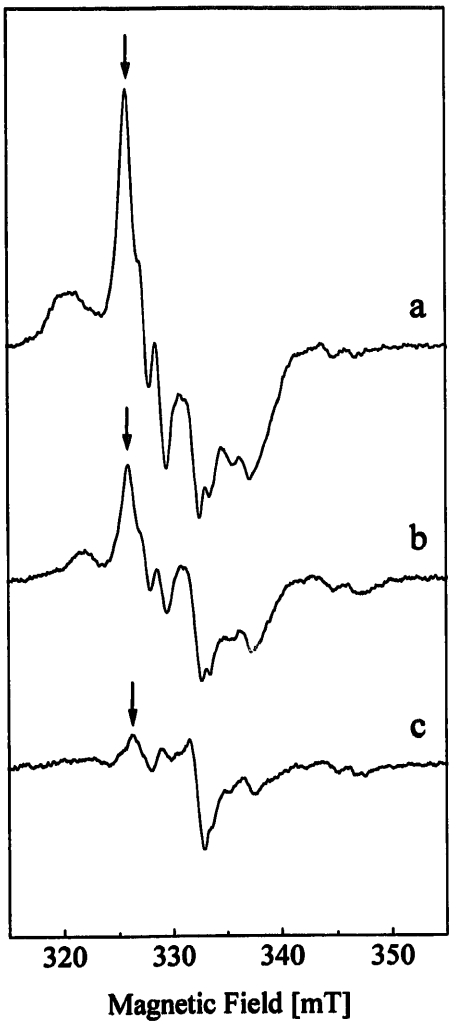

Fig. 3. Effects of arginase and L-NMMA on the endotoxin-stimulated NO-$FeDETC_2$ EPR signal (indicated by the arrows) from the spleen of LPS-treated rats. Animals were injected with LPS five hours before sacrifice, and either (a) saline, (b) arginase or (c) L-NMMA 4 h after LPS. Protein concentration, (a) 114, (b) 171 and (c) 215 mg/g wet tissue. Measurement conditions were as for Fig. 2b.

Upon administration of arginase 4 h after LPS a marked decrease was observed in the endotoxin-stimulated NO-$FeDETC_2$ signal, as indicated by the arrows in Fig. 3 (cf. spectra a and b). This decrease was even more noticeable in the spleen from rats administered with the inhibitory analogue, L-NMMA (Fig. 3c). In the presence of L-NMMA the amplitude of the NO-$FeDETC_2$ signal was so attenuated that the EPR signal from free $FeDETC_2$ could now be resolved (cf. spectrum 2a with 3c). Similar results were obtained with rat lung, liver and kidney (data not shown). The effects of arginase and L-NMMA on the intensity of the mononitrosyl complex signal are summarised in Fig. 4.

Endotoxin treatment caused an influx of macrophages into all tissues examined and macrophage numbers were increased 2-fold over normal tissues. No difference in macrophage influx was apparent between LPS, LPS/arginase, and LPS/L-NMMA treated tissues.

4. Discussion

The purpose of these experiments was to study the effect of depletion of circulating L-arginine on the synthesis of NO in vivo. We used a model of endotoxin administration in rats which has previously been shown to lead to synthesis of the inducible isoform of NOS and a marked increase in NO synthesis in multiple organs [6,10,12]. We have used two separate measures of NO synthesis. First, to obtain an indirect measure of whole body NO synthesis, we measured the accumulation of nitrite and nitrate, the stable end-products of NO, in the plasma. Secondly, in order to determine the extent of NO synthesis in individual organs we used EPR spectroscopy to measure the NO adduct formed with the spin trap $FeDETC_2$, a method which has previously been employed to measure organ-specific NO synthesis in mice [18] and rats [22,24]. The insoluble complex remains in the tissues, and the size of the EPR signal obtained in the presence of $FeDETC_2$ is related to the amount of nitric oxide synthesized during the 30-min period before the animals were killed. Using these methods we have demonstrated that LPS led to a marked increase in circulating nitrite and nitrate with NO synthesized in liver, kidney, lung and spleen from 4.5 to 5 h after LPS administration. As expected, the arginine analogue L-NMMA given at 4 h reduced circulating nitrite and nitrate and markedly reduced the synthesis of NO in all four organs.

In order to deplete L-arginine in the plasma we gave rats an intravenous bolus injection of bovine liver arginase which catalyses the conversion of L-arginine to L-ornithine and urea. Arginase is well tolerated and indeed has previously been given for

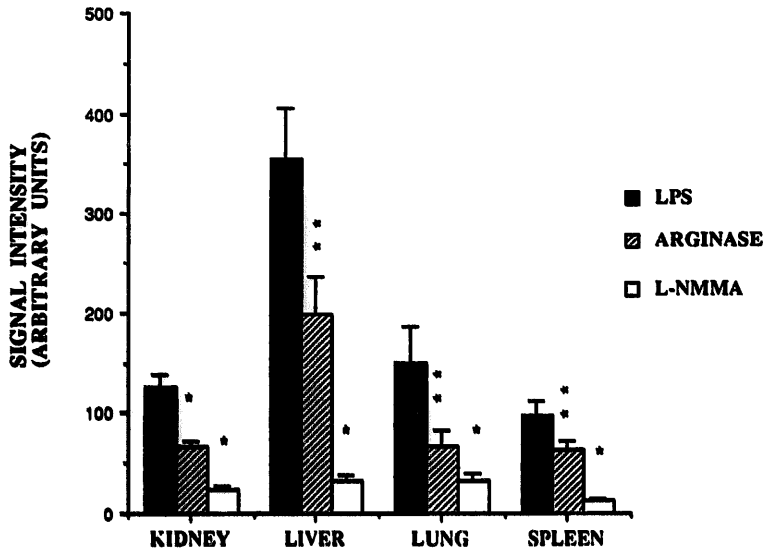

Fig. 4. Effect of arginase/L-NMMA on the EPR signal intensities of the [NO-FeDETC$_2$] complex in LPS treated tissues. Results expressed as mean ± S.E.M., where $^*P < 0.01$, $^{**}P < 0.05$, using unpaired Student's t-test.

days or weeks in experiments examining its possible effect on tumours [25,26]. Importantly, there was no effect on systemic blood pressure (results not shown) implying that synthesis of NO by the vascular calcium-dependent nitric oxide synthase was not inhibited. We therefore gave arginase 4 h after LPS and studied NO synthesis by using FeDETC$_2$. Arginase treatment after LPS reduced plasma arginine to undetectable levels, although interestingly LPS itself led to a marked reduction in L-arginine at 5 h, possibly by consumption to generate NO. In kidney, liver, lung and spleen, arginase significantly reduced the synthesis of NO as detected by EPR although the effect was not as large as that observed with L-NMMA.

It has been suggested that NO may act to down-regulate expression of vascular adhesion molecules and thereby prevent leukocyte infiltration into tissues. However, we found no effect of arginase or L-NMMA on the LPS-induced increase in tissue macrophage numbers.

Our results demonstrate that intracellular stores of L-arginine are insufficient to sustain the massive rise in NO synthesis which occurs after endotoxin administration and that this synthesis depends partly on the supply of extracellular L-arginine. This suggests that measures to lower the availability of L-arginine may be useful for exploring the role of NO synthesis by iNOS at sites of localized inflammation, without the drawbacks associated with inhibition of systemic NO synthesis by the calcium-dependent isoforms of NOS.

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