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Inhibition of Tetrahydrobiopterin Synthesis Reduces *in Vivo* Nitric Oxide Production in Experimental Endotoxic Shock

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Nitric oxide synthesis requires the cofactor tetrahydrobiopterin. We have examined the effect on nitric oxide synthesis in experimental endotoxic shock of 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of GTP cyclohydrolase I, the first and rate limiting enzyme for tetrahydrobiopterin synthesis. Rats given lipopolysaccharide (LPS, 10 mg/kg) showed a large rise in plasma nitrate at 4 and 8 hours which was significantly reduced by DAHP (1 g/kg) given at the same time as LPS. There was a 40–50% reduction in the haem-NO signal detected in kidney by electron paramagnetic resonance spectroscopy. LPS produced hypotension at 3 hours and 6 hours and this was ameliorated at 6 hours in rats given DAHP. DAHP abolished the rise in kidney tetrahydrobiopterin levels seen 4 hours after LPS but no effect was seen on induction of inducible nitric oxide synthase (iNOS) as assessed by immunohistochemistry and reverse transcriptase PCR, consistent with the effect of DAHP being by reduction of tetrahydrobiopterin levels. The results show that inhibition of tetrahydrobiopterin synthesis is an effective strategy to reduce nitric oxide synthesis by iNOS *in vivo.*

Nitric oxide (NO) is a highly reactive free radical and potent vasodilator which is synthesised from L-arginine by nitric oxide synthase (NOS). NOS exists in three different isoforms (1). Two of these are dependent for their activity on intracellular calcium levels and were first demonstrated to be constitutively expressed in nervous tissue (NOS I, ncNOS) and endothelium (NOS III, ecNOS). The other isoform (NOS II, iNOS) is independent of intracellular calcium and its activity is controlled principally at the levels of transcription and translation. iNOS is induced in a range of cells by pro-inflammatory cytokines and lipopolysaccharide (LPS) (2). NO produced by iNOS has been implicated in inflammation and in the hypotension of endotoxic shock and there is thus considerable interest in strategies for inhibiting NO *in vivo.*

The activity of NOS is dependent on the availability of co-factors including tetrahydrobiopterin (BH_4). The exact role of BH_4 is unknown but it may be important in maintaining NOS in an active configuration, or may have a regulatory redox role (3,4). The need for an adequate supply of BH_4 has been shown for purified macrophage iNOS (5,6) and for intact cells including macrophages, (7,8) fibroblasts (9) and smooth muscle cells (10,11). In human endothelial cells, inflammatory cytokines increase NO synthesis by increasing BH_4 levels (12,13). The rate-limiting enzyme for *de novo* BH_4 synthesis is GTP cyclohydrolase (GTPCH) and it is up-regulated in a variety of cells by stimuli which also induce iNOS (10,11,14). *In vivo,* increased GTPCH mRNA and enzyme activity have been demonstrated in the tissues of LPS-treated rats (15,16). 2,4-Diamino-6-hydroxypyrimidine (DAHP) is an inhibitor of GTPCH and has been used extensively *in vitro* to elucidate the role of BH_4 in NO synthesis (9,10). We have now studied the effect of administration of DAHP *in vivo* on NO synthesis, tissue BH_4 levels and blood pressure in rats given LPS.

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; BH_4, tetrahydrobiopterin; GTPCH, GTP cyclohydrolase; DAHP, 2,4-diamino-6-hydroxypyrimidine; EPR, electron paramagnetic resonance.

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MATERIALS AND METHODS

Lipopolysaccharide administration. Inbred male Lewis rats (200–300g) were given an intraperitoneal injection of lipopolysaccharide (LPS) *Escherichia coli* serotype 0127:B8 (10 mg/kg, Sigma Chemical Co., Poole Dorset).

Four or eight hours after administration, animals were sacrificed and the kidneys were frozen immediately in liquid nitrogen. Blood was taken into heparinised syringes. Samples of kidney, liver, spleen and lung were placed in formal saline for 24 hours and embedded in paraffin wax for immunohistochemistry.

DAHP administration. DAHP was dissolved in phosphate buffered saline (PBS, 10mM pH 6.5) immediately prior to use. DAHP (1 g/kg) was administered intraperitoneally with LPS or 4 hours after LPS. Animals receiving LPS only were given an equivalent amount of PBS. The animals were sacrificed 8 hours after LPS.

Immunohistochemistry. iNOS antibody was a gift from Dr V. Riveros-Moreno (Wellcome Research Laboratories, Beckenham, UK) (17). Paraffin sections were dewaxed in xylene, rehydrated and microwaved in citrate buffer (10mM, pH 6.0) for 15 minutes. Primary antibody was incubated overnight at 4°C. A biotinylated secondary antibody was enhanced with streptavidin-biotin-peroxidase (Dako, High Wycombe, Bucks, UK) and developed with diaminobenzidine. Macrophages were detected with the monoclonal antibody ED-1 (1:500, Serotec, Oxford, UK.) using the same developing system.

Plasma nitrate/nitrite levels. Blood was anticoagulated with heparin and plasma removed. Nitrate was reduced to nitrite by bacterial nitrate reductase using *Pseudomonas oleovorans* (National Collection of Type Cultures, Colindale, London, UK) (18) and the supernatant assayed via the Griess reaction as described previously (19). The lower limit of detection was 7.5 μM.

Tetrahydrobiopterin analysis. Kidneys were thawed and homogenised (20% w/v) in perchloric acid (0.1 M) containing dithioerythreitol (6.5 mM) and diethylenetriaminepentaacetic acid (2.5 mM). Following centrifugation (15 000g for 3 minutes) supernatants were diluted 1:2 with the perchloric acid (+ additives) and analysed for tetrahydrobiopterin by HPLC and electrochemical detection (20).

Blood pressure measurements. Systolic blood pressure was measured in conscious rats using a tail cuff blood pressure monitor (Harvard Apparatus, Edenbridge, Kent, UK.). Animals were accustomed for 3 days prior to LPS administration. Measurements were taken prior to LPS/DAHP administration and 3 and 6 hours subsequently.

Electron paramagnetic resonance (EPR) spectroscopy. Kidneys were thawed and homogenised in an equal weight of HEPES buffer (0.2 M, pH 7.4) containing EDTA (10 mM). Samples were placed in quartz EPR tubes (3 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 at 15 K. Spectra were an average of 5 scans and normalised for direct comparison.

Protein determination. Kidney homogenates, taken at the same time as EPR sample preparation, were assayed by the modified Lowry method for total protein, using bovine serum albumin as a standard (21).

Reverse transcription polymerase chain reaction. RNA was isolated from frozen kidney by homogenisation in RNAzol B (Biogenesis, Bournemouth, UK) following manufacturers instructions. RNA (5 μg) from each sample was reverse transcribed with Moloney murine leukaemia virus enzyme (GIBCO BRL, Paisley, UK) using random hexamers. The resultant cDNA was amplified by the polymerase chain reaction (PCR) using primers for iNOS as described previously (22). PCR was carried out for 35 cycles with the annealing temperature of 61°C. PCR products were analysed on 1.5% agarose gel and visualised with ethidium bromide staining and ultraviolet transillumination.

Statistics. Data are presented as mean ± SEM. Comparisons between groups were by Mann-Whitney U test.

RESULTS

Administration of LPS (10 mg/kg) caused a large rise in plasma nitrate after 4 hours, increasing further by 8 hours (Figure 1). DAHP (1 g/kg) administered with LPS significantly reduced plasma nitrate production at both 4 and 8 hours. Normal rats or rats given DAHP alone showed no detectable nitrate. There was no reduction in plasma nitrate at 8 hours when DAHP was given 4 hours after LPS.

NO formation induced by LPS administration can be directly measured by its binding to haem proteins by EPR spectroscopy. NO binding to these proteins gives rise to a unique EPR spectrum with a characteristic triplet splitting due to the hyperfine coupling of the nitrogen nucleus of bound NO. At 40 K, the EPR spectrum of kidney homogenates from rats administered LPS alone (Figure 2a) displayed a prominent resonance centred at $g \sim 2.0$, with a peak at $g \sim 2.07$ and a triplet splitting centred at $g \sim 2.01$, typical of haem-NO formation. The broad feature at $g \sim 1.94$ is due to reduced iron-sulphur proteins. DAHP given simultaneously with LPS significantly decreased (by 40–50%) the haem-NO signal intensity (Figure 2b). However, only a small decrease in intensity of the haem-NO EPR signal was observed when DAHP was given four hours after LPS (data not shown). The haem-NO signal observed in the difference spectrum shown in Figure 2 (lower spectrum) shows a splitting of $A(^{14}N) \sim 1.7$ mT. Similar signals have been observed for the

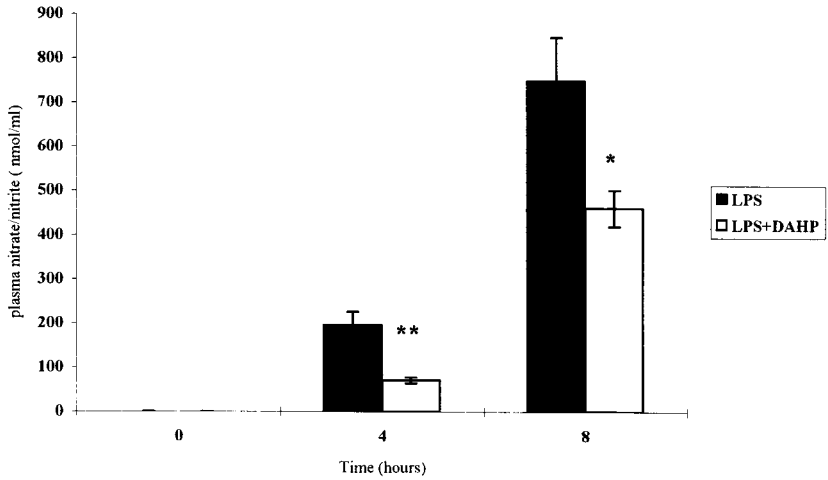

FIG. 1. Plasma nitrate/nitrite levels (nmol/ml) at varying times following LPS (10 mg/kg) and LPS with DAHP (1 g/kg) administration. Normal and DAHP treated rats had no detectable nitrate/nitrite and represent time 0 above. Plasma was incubated with *P. oleovorans,* plasma protein was precipitated and the resultant nitrite assayed via the Griess reaction. * $p < 0.05$, ** $p < 0.01$ compared with LPS nitrate/nitrite levels ($n = 6$).

NO-adducts of Type II haem proteins such as cytochrome *c* oxidase and haemoglobin (23) DAHP administration caused a significant reduction in kidney BH_4 levels compared with normal rats (Figure 3). BH_4 increased dramatically with LPS at 4 and 8 hours and this rise was completely inhibited at 4 hours by DAHP given at the same time as LPS and markedly attenuated at 8 hours.

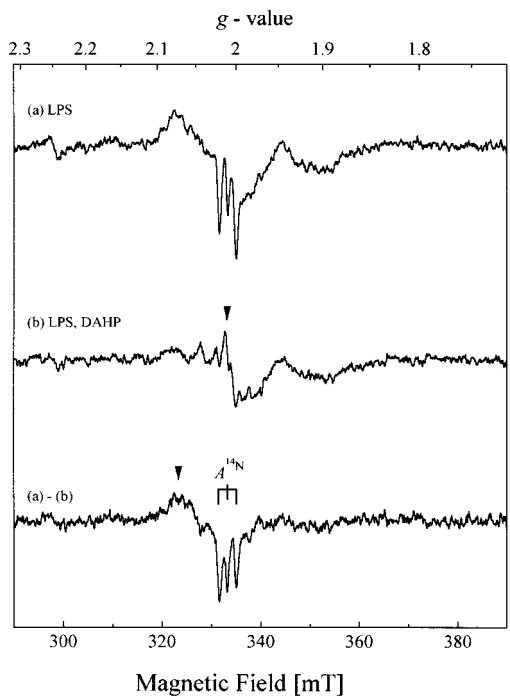

FIG. 2. X-Band EPR spectra from LPS-treated rats, demonstrating the effect of DAHP administration on endotoxin-induced NO formation. Kidney homogenate from rats treated with either LPS alone (a), or LPS and DAHP (b). Measurement conditions were: temperature, 40 K; microwave power, 20 mW; microwave frequency, 9.375 GHz; modulation amplitude, 0.49 mT; time constant, 0.082 s; sweep rate, 2.38 mT/s. The spectra are an average of two scans.

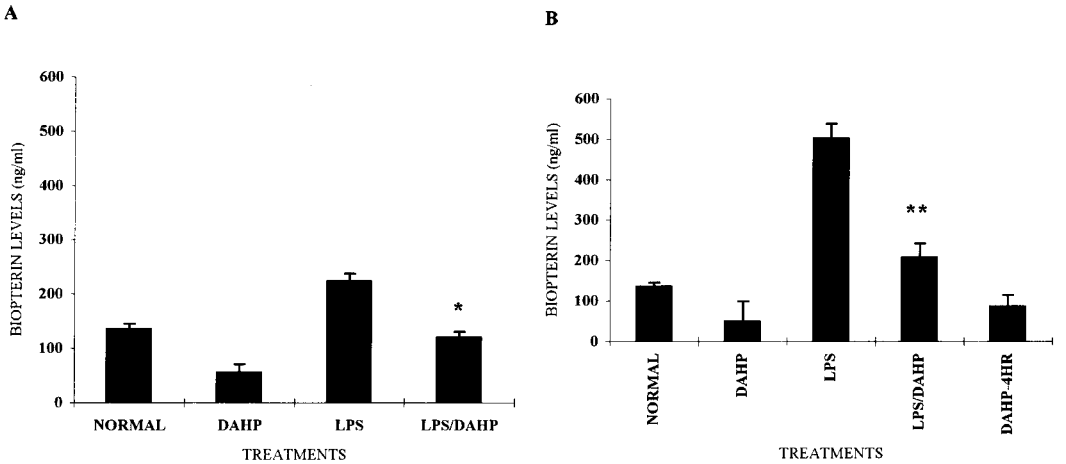

FIG. 3. Tetrahydrobiopterin levels in the kidney of LPS and/or DAHP treated rats. Kidney tetrahydrobiopterin levels (ng/ml) were measured in rats receiving LPS (10 mg/kg), DAHP (1 g/kg) or LPS and DAHP (administered simultaneously) for 4 hours (A) or 8 hours (B). DAHP-4 (B) refers to DAHP administered 4 hours after LPS. Normal rats received no treatment. * $p < 0.05$, ** $p < 0.01$ compared with LPS alone ($n = 6$).

LPS caused hypotension after 3 hours which was not significantly altered 6 hours later (Figure 4). DAHP administration with LPS had no effect at 3 hours but a significant increase in blood pressure towards normal was apparent at 6 hours following administration. DAHP given alone had no effect on blood pressure (data not shown).

Immunohistochemical staining for iNOS was negative in tissues from normal rats and rats treated with DAHP alone. Animals given LPS showed iNOS expression, increasing from 4 to 8 hours in all tissues examined. DAHP had no effect on the staining for iNOS. Macrophage numbers increased approximately 2 fold from normal in all tissues with 4 hours LPS and 3 fold at 8 hours. DAHP did not alter this increase in macrophage influx.

RT-PCR showed increased iNOS mRNA in kidneys from rats given LPS or LPS and DAHP (Figure 5).

DISCUSSION

NO synthesis is important in many pathological conditions and there is considerable interest in ways of inhibiting its synthesis *in vivo*. We now show that inhibition of BH_4 synthesis with the GTPCH inhibitor DAHP *in vivo* suppresses NO synthesis induced by a single dose of LPS as assessed by plasma nitrate levels and the formation of EPR-detectable haem-NO complexes. DAHP was most effective when given at the same time as LPS. A single dose of LPS leads to a rapid increase in iNOS (24) and GTPCH (15) mRNA levels and by 3 hours iNOS protein is detectable in many tissues by immunohistochemistry (17) but by 12 hours iNOS protein is only detectable in the spleen. Thus a single dose of LPS causes a massive reversible increase in iNOS and it is likely that, once synthesised, the enzyme rapidly binds BH_4. When DAHP was given 4 hours after LPS there was no effect on nitrate levels suggesting that BH_4 remains bound to iNOS and continuing synthesis of BH_4 is no longer necessary for NO synthesis. In pathological conditions there may be longer-lasting stimuli to iNOS synthesis with continuous turnover, and in those circumstances DAHP may remain effective when given later.

NO is a potent vasodilator and a likely mediator of the hypotension that follows LPS administration. After LPS administration there is a rapid fall in blood pressure (25) which is thought to be due to enhanced formation of NO by eNOS followed by a delayed phase in which iNOS is implicated. Hypotension in endotoxic shock can be reversed by competitive inhibitors of NOS

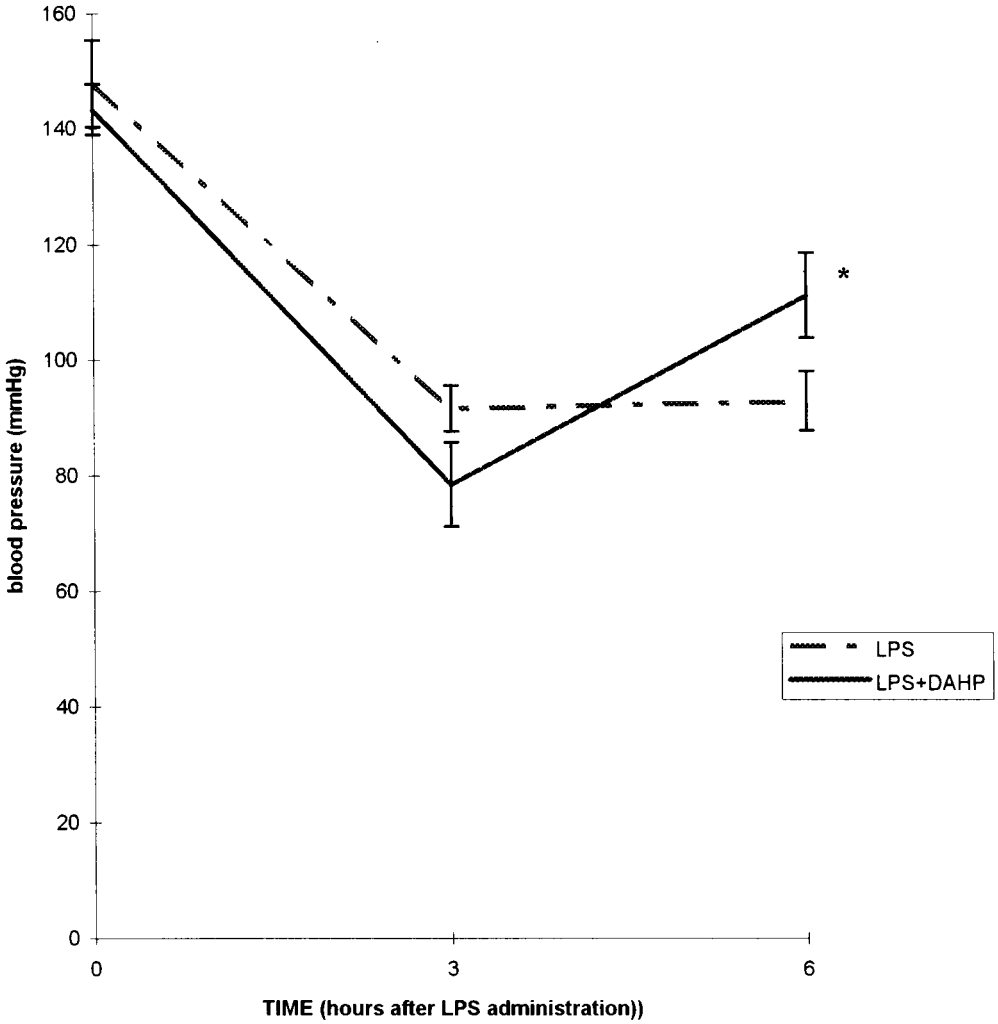

FIG. 4. Effect of DAHP on LPS induced hypotension. The systolic blood pressure of conscious rats was recorded prior to LPS (time 0), 3 and 6 hours following administration of LPS or LPS and DAHP (administered, simultaneously). * $p < 0.05$ compared with the hypotension at 3 hours post-LPS.

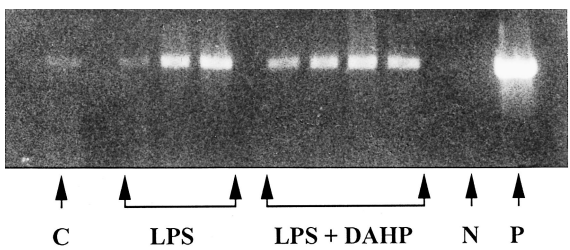

FIG. 5. Inducible NOS mRNA expression in the kidney of rats treated with LPS or LPS and DAHP. LPS (10 mg/kg) was administered for 4 hours either with or without DAHP (1 g/kg). Control (C) rats received saline only. mRNA was isolated, reverse transcribed and PCR was performed with primers for iNOS. Products were separated by gel electrophoresis and visualised with ethidium bromide and UV light. The negative control (N) was saline and the positive control (P) was iNOS cDNA.

(26,27). However, it may be important to block the effect of the induced NO synthesis while maintaining the action of constitutive NO which is required to maintain normal tissue perfusion. It has been suggested that inhibition of BH_4 synthesis might be an effective strategy for treating endotoxic shock since the presumed slow turnover of the endothelial cNOS would limit its sensitivity to inhibition of co-factor synthesis (28). We found that administration of DAHP together with LPS led to a partial amelioration of hypotension at 6 hours although not at three hours. This lack of effect at three hours may indicate that hypotension at this time is still primarily due to activation of endothelial cNOS which is already bound to BH_4.

In order to study the mechanism by which DAHP reduces levels of NO we measured tissue BH_4 levels in the kidney. As expected DAHP reduced BH_4 levels in normal kidney (with no effect on blood pressure). DAHP attenuated the marked increase in BH_4 seen 8 hours after LPS whether given at the same time as LPS or 4 hours later. The reduction even when DAHP was given at 4 hours suggests that there is a continuous turnover of BH_4. We considered the possibility that DAHP might also inhibit the expression of iNOS mRNA or protein as has been shown for macrophages in culture (29). Using immunohistochemistry we found no difference in iNOS expression at 4 or 8 hours and iNOS-mRNA was increased in LPS treated animals whether treated with DAHP or not, although we did not formally quantitate mRNA. We therefore believe that, *in vivo*, the main mechanism of action of DAHP is by reduction of BH_4 levels, rather than direct inhibition of NOS synthesis.

In summary, we have shown that administration of DAHP *in vivo* inhibits the increase in tissue BH_4 levels caused by LPS, reduces NO synthesis and ameliorates hypotension. DAHP may thus be a useful drug for further investigation of the role of NO in inflammation *in vivo.*

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