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Induction of NADPH-Dependent Diaphorase and Nitric Oxide Synthase Activity in Aortic Smooth Muscle and Cultured Macrophages

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SUMMARY

Lipopolysaccharide (LPS), either alone or in combination with cytokines, induces nitric oxide (NO) synthase activity in cells that normally release little or no NO. In arterial smooth muscle cells and various macrophage cell lines, NO synthase activity is induced after several hours of incubation with LPS. In brain, NADPH-dependent diaphorase activity has been associated with constitutive NO synthase. Here we show that incubation of rat aorta or cultured macrophages with LPS causes a time-dependent induction of NO synthase. The NO synthase activity in both rat aorta and macrophages was calcium independent and inhibited by N^G-monomethyl-L-arginine and N^G-nitro-L-arginine. We also found that LPS caused a time-dependent induction in NADPH-dependent diaphorase activity in both rat aorta and cultured macrophages. The diaphorase activity was mainly NADPH dependent and NADH independent. NO synthase activity and NADPH-diaphorase activity in crude cytosol from LPS-treated macrophages were found to co-purify, using 2′,5′-ADP-Sepharose followed by Superose-6 gel permeation chromatography.

Macrophages are induced in the presence of LPS and/or cytokines to release large quantities of oxides of nitrogen (1). L-Arginine is the substrate for the formation of these nitrogen oxides, and NO is an intermediate (2–4). Vascular smooth muscle cells can also be induced by LPS and/or cytokines to release a factor with properties similar to those of NO (5–7). The enzyme responsible for NO formation, NO synthase, also exists as a constitutive enzyme in endothelial cells and in brain. The constitutive NO synthases known so far are calcium/calmodulin dependent and present in the particulate fraction of endothelial cells (8, 9) and in the soluble fraction of central (10, 11) and peripheral (12) neurons. On the other hand, the inducible NO synthase in vascular smooth muscle (5, 7) and macrophage cells (13) is mainly soluble and calcium independent. Other cofactor requirements for NO synthase activity appear to be the same for the induced (14, 15) and the constitutive (16, 17) isoforms, because all require NADPH and BH_4 for NO production and all isoforms investigated so far contain/require FAD and FMN (see Ref. 18). However, the arginine analogs MeArg and NO_2Arg show selective inhibition of constitutive and inducible isoforms of NO synthase. NO_2Arg more potently inhibits NO release from endothelial cells *in vitro* (19, 20), the vasculature *in vivo* (21), and purified constitutive NO synthase from endothelial cells (17) and brain (10, 11), whereas MeArg more potently inhibits NO formed by cytokine-treated cells (22).

Recently, NADPH-diaphorase has been shown to co-purify with NO synthase from the brain, suggesting that constitutive NO synthase has NADPH-diaphorase activity (23). Because of the differences between the inducible and constitutive NO synthases, we investigated the possibility that NADPH-diaphorase could be induced after exposure of tissue or cells in culture to LPS. Some of these results have been presented in preliminary form (24).

Materials and Methods

The composition of the Krebs' solution was as follows (in mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl \cdot 6H_2O$, 2.5; $NaHCO_3$, 25; and D-glucose, 5.6. NBT, NADPH, LPS from *Escherichia coli*, L-arginine, NO_2Arg, and Krebs' salts were obtained from Sigma Chemical Co. (St. Louis, MO). L-[2,3,4,5-^{3}H]Arginine monohydroch-

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ABBREVIATIONS: LPS, lipopolysaccharide; NO, nitric oxide; NO_2Arg, N^G-nitro-L-arginine; MeArg, N^G-monomethyl-L-arginine; BH_4, tetrahydrobiopterin; NBT, nitro blue tetrazolium; NE, norepinepherine; SOD, superoxide dismutase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

loride (62 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Bradford reagent was obtained from Bio-Rad (Richmond, CA). MeArg was the kind gift of Dr. J. F. Kerwin, Jr. (Abbott Laboratories). BH_4 was obtained from Dr. B. Schirks Laboratories (Jona, Switzerland). All other reagents were of the highest grade available.

Preparation of rat aortae. Male Sprague-Dawley rats were anesthetized by using CO_2 gas and were sacrificed by decapitation. The thoracic aortae were removed and placed into ice-cold Krebs' solution. Aortae were cleaned of connective tissue and opened longitudinally, and the endothelial layer was removed by gentle rubbing with tissue paper. Each aorta was cut into four pieces of approximately 1 cm^2. When vessels were removed from beagle dogs or ferrets, the animals were first given a lethal dose of sodium pentabarbital (Abbott Laboratories). The vessels were then incubated at 37° in Krebs' solution (gassed with 95% CO_2/5% O_2), in the presence or absence of LPS (300 ng/ml). At different time points after the beginning of the incubation, pieces of vessel were removed from the incubation medium, frozen in liquid nitrogen, and stored at −70°. The frozen pieces of tissue were homogenized on ice in the following buffer: Tris·HCl, 50 mM; EDTA, 0.1 mM; EGTA, 0.1 mM; 2-mercaptoethanol, 12 mM; leupeptin, 2 μM; pepstatin, 1 μM; and phenylmethylsulfonyl fluoride, 1 mM. The vessel homogenates corresponding to the designated incubation time were then assayed for NO synthase and NADPH-diaphorase activity. Protein concentration was measured by the method of Bradford (25), with bovine serum albumin as the standard.

Measurement of vascular responses. Rat aortic strips were prepared as described above and mounted in organ baths containing Krebs' solution, at 37°, gassed with 95% CO_2/5% O_2. The strips were placed under a resting tension of 2 g and equilibrated for 30 min, with washing every 15 min. Dose-response curves with NE (1–1000 nM) were then performed. Subsequently, the tissues were washed and allowed to return to base-line tension before being incubated with LPS (100 or 300 ng/ml) for 24 hr. Then dose-response curves with NE were repeated. Tension was recorded using isometric Harvard transducers and was displayed on a MI² chart recorder.

Tissue culture. The macrophage cell line J774 was purchased from the American Type Culture Collection (Rockville, MD). The cells were grown to confluence (80–90%), in culture flasks (75 cm^2), in F-12 Ham's nutrient mixture containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, before being removed with trypsin and seeded once more onto culture dishes (176.6 cm^2). Cells were maintained at 37° in 95% air/5% CO_2. Once the cells were confluent, agents were added to the medium at the designated time points. Rat fetal lung fibroblast RFL-6 cells were obtained from Stanford University and cultured as described above, in 12-well tissue culture plates.

Measurement of NO synthase activity. NO synthase activity was measured in tissue homogenates as the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine, as previously described (10, 11). Briefly, 50 μl of tissue homogenate (approximately 100 μg of protein) were incubated in the presence of L-arginine/l-[^{3}H]arginine (10 μM; 5000 Bq/tube), NADPH (1 mM), and calcium (2 mM). The reaction volume was made up to 100 μl by the addition of HEPES (10 mM; pH 7.5), and the reaction mixture was incubated for 20 min. The reaction was stopped by the addition of 1 ml of 20 mM HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). The reaction mixture was then applied to Dowex-50W (Na^+ form; Sigma) columns (1 ml), and the eluted L-[^{3}H]citrulline was measured using a Beckman scintillation counter. In addition, NO synthase activity was measured as cGMP accumulation in RFL-6 cells, as previously described (8, 9, 11, 17). NO synthase was purified, using methods already described, on 2′,5′-ADP-Sepharose (Pharmacia, Piscataway, NJ) (10, 11, 17), followed by Superose-6 gel permeation (Pharmacia) (17).

Measurement of NADPH-diaphorase activity. Tissue homogenates were incubated with NBT (1 mM) and NADPH (1 mM) for 10 min at 25°, in a volume of 100 μl. The reaction was terminated by the addition of 100 μl of H_2SO_4 (0.1 M) and 200 μl of dimethylsulfoxide. Samples were then centrifuged at 10,000 × *g* for 5 min, and the absorbance, at 595 nm, of the supernatant fraction was measured using a plate reader (Bio-Tek Instruments EL3) (E = 12,600/M/cm). Although the pellet retained a portion of the formazan stain, the absorbance of the supernatant fraction was clearly representative of the dye in the whole homogenate.

Statistics. All values represent mean ± standard error from n experiments. Statistical difference between groups was assessed by Students t test for unpaired data, and a p value of <0.05 was taken as significant.

Results

LPS-induced loss in vascular response to NE. After incubation with LPS (100 or 300 ng/ml), the efficacy of NE in contracting aortic strips was significantly reduced (Fig. 1). In separate experiments, LPS-treated aortae were incubated with MeArg (100 μM) for 5–10 min after administration of a submaximal concentration of NE (10 nM). This treatment partially restored the response of NE, from 38.7 ± 2.7% of control in the absence of MeArg to 65.7 ± 3.2% of control in the presence of MeArg (n = 3).

Time-dependent induction of NO synthase by LPS. LPS (300 ng/ml) produced a time-dependent induction of NO synthase in aortic segments, which was maximal at 24 hr (n = 3) (Fig. 2A). To a lesser extent, NO synthase activity increased in a time-dependent manner in aortae incubated in Krebs' buffer without LPS (n = 3) (Fig. 2A). NO synthase was also induced in J774 macrophages after incubation with LPS (100 ng/ml), reaching a maximum at 30 hr (n = 3) (Fig. 3A). In addition to citrulline production, homogenates prepared from LPS-treated aortae and J774 macrophages increased cGMP levels in RFL-6 cells in the presence but not the absence of SOD (20 units/ml) (n = 3) (Fig. 4). Homogenates from freshly excised aortae or untreated J774 macrophages had no effect on cGMP levels in RFL-6 cells (n = 3) (Fig. 4). NO synthase activity in both rat aorta and J774 cells was significantly inhibited by submaximal concentrations of MeArg and NO_2Arg at all time points (n = 3) (data not shown), with IC_{50} values of 15 and 25 μM for rat aorta and 15 and 15 μM for J774 cells, respectively. However, a portion of the NO synthase (as measured by citrulline formation) activity in rat aorta but not J774 macrophages was resistant to inhibition by MeArg or NO_2Arg (100 μM). This activity increased proportionally with incubation time with LPS, reaching a maximum, at 24 hr, of 67% of

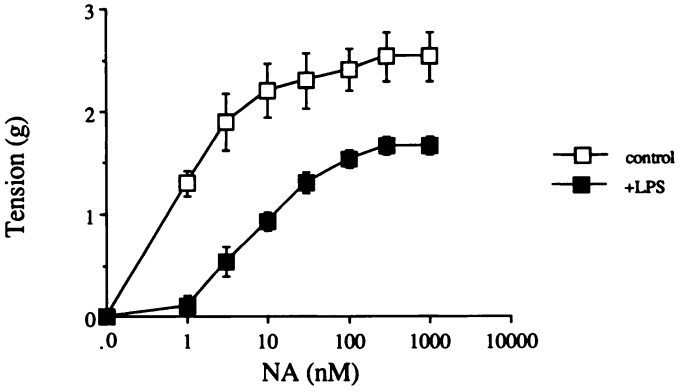

Fig. 1. Reduction in contractile response of rat aorta to NE after incubation with LPS (300 ng/ml). □, Control strips of rat aorta; ■, LPS-treated strips (24 hr). Each *point* shows the mean ± standard error of three to six experiments.

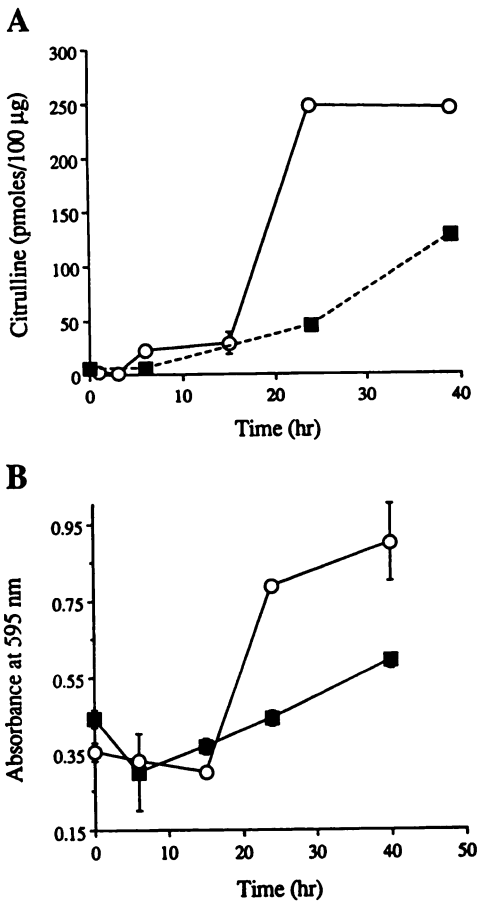

Fig. 2. Time-dependent induction of NO synthase and NADPH-diaphorase in rat aortic strips. A, NO synthase activity, measured as L-[^{3}H] citrulline production from L-[^{3}H]arginine in the presence of NADPH (1 mM), BH_4 (3 µM), calmodulin (30 units/ml), and calcium (2 mM). B, Amount of NADPH-diaphorase activity, measured by absorbance at 595 nm in the presence of NADPH (1 mM) and NBT (1 mM). O, Strips incubated with LPS (300 ng/ml); ■, strips incubated without LPS (control). Each *point* shows the mean ± standard error of three experiments. Similar results were obtained in three other preparations.

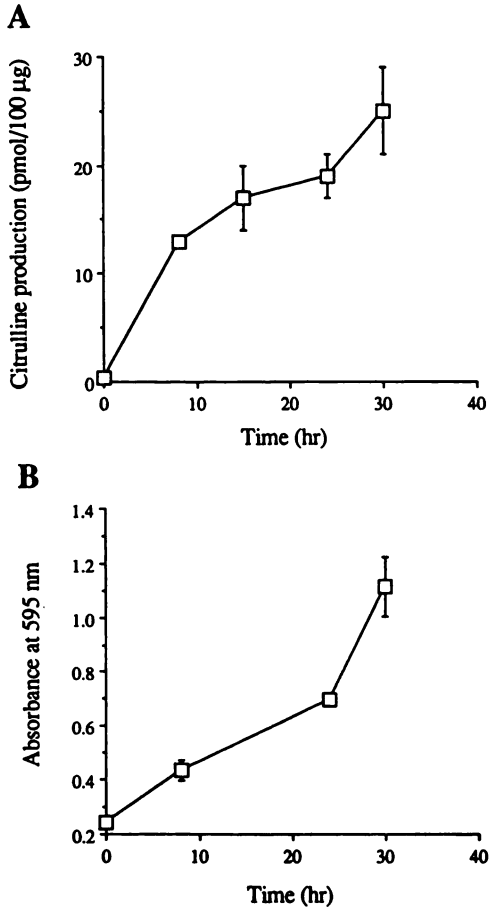

Fig. 3. Time-dependent induction of NO synthase and NADPH-diaphorase in J774 macrophages. Experimental details are the same as in Fig. 2. A, NO synthase activity; B, NADPH-diaphorase activity. Each *point* shows the mean ± standard error of three experiments.

control activity in the presence of MeArg and 79% of control activity in the presence of NO_2Arg ($n = 3$).

Coincubation of rat aorta with LPS and dexamethasone (300 nM) (24 hr) greatly attenuated NO synthase induction, by >80% (Fig. 5), but had less effect on induction in J774 cells (34 ± 6% inhibition; $n = 5$). LPS-induced NO synthase in both rat aorta and J774 cells was unaffected by removal of calcium and addition of EGTA (1 mM) ($n = 3$) (data not shown).

Time course for induction of NADPH-diaphorase activity. NADPH-diaphorase activity was induced after incubation of rat aorta or J774 cells with LPS (300 or 100 ng/ml, respectively). In rat aorta, induced NADPH-diaphorase reached a maximum at 24 hr. Similarly to NO synthase activity, NADPH-diaphorase activity was induced in rat aorta incubated without LPS, but to a significantly lesser extent ($n = 3$) (Fig. 2B). In J774 cells, NADPH-diaphorase activity reached a maximum at 30 hr ($n = 3$) (Fig. 3B). In both tissues and cells, all NADPH-diaphorase activity was virtually abolished in the absence of NADPH and significantly ($p < 0.05$) reduced when NADPH was replaced with NADH (1 mM), by 69 ± 5% in rat aorta and 58 ± 3% in J774 homogenates ($n = 3$). Coincubation of rat aorta with dexamethasone (300 nM) (24 hr) significantly inhibited the amount of NADPH-diaphorase activity (Fig. 5), but to a lesser extent than seen with NO synthase.

NO synthase co-purified with NADPH-diaphorase present in the crude cytosol of LPS-treated (1 µg/ml, for 16 hr) macrophages. Based on formazan formation in crude cytosol (5.53 nmol/mg/min), the specific activity increased approximately 10-fold, to 55.6 nmol/mg/min, after partial purification by chromatography on 2′,5′-ADP-Sepharose (10, 11, 17). Both NO synthase and NADPH-diaphorase could be further co-purified using Superose-6 gel permeation chromatography (17), resulting in a specific activity of 476.7 nmol of formazan/mg/min.

Induction of NO synthase and NADPH-diaphorase in other tissues. In separate experiments, it was found that NO synthase and NADPH-diphorase could also be induced in other vascular tissues, such as rat vena cava, canine carotid artery, canine jugular vein, and ferret aorta (Table 1).

Discussion

Here we show that induction of NO synthase coincides with the induction of NADPH-diaphorase in several tissues. NO synthase activity induced by LPS was characterized by (i) reduction in the contractile response of rat aorta to NE, (ii) production of L-citrulline (the co-product of NO formation from L-arginine), (iii) SOD-dependent activation of soluble guanylyl

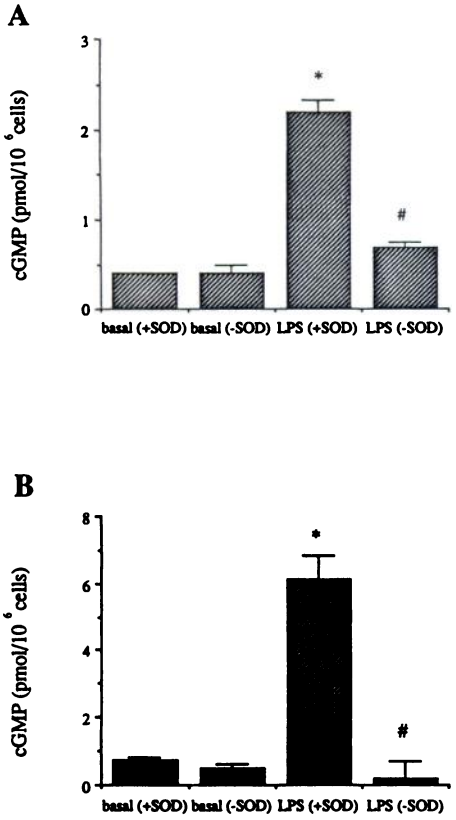

Fig. 4. Characterization of NO synthase activity in aortic smooth muscle and J774 cell homogenates. A, NO synthase in homogenates from aortic smooth muscle; B, NO synthase in homogenates from J774 macrophages. NO synthase activity was measured as cGMP accumulation in RFL-6 cells in the presence of L-arginine (100 μM), calmodulin (30 units/ml), BH_4 (3 μM), NADPH (100 μM), and calcium (2 mM), either in the presence of SOD (20 units/ml) (*+SOD*) or in the absence of SOD (*−SOD*). *basal*, NO synthase activity present in tissue or cells before LPS exposure; *LPS*, activity after 24-hr exposure to LPS (300 ng/ml in the case of aortae and 100 ng/ml in the case of J774 macrophages). Each *column* represents the mean ± standard error of three experiments. *, Significant ($p < 0.05$) increase in NO synthase activity in homogenates from tissue treated with LPS, measured in the presence of SOD, compared with basal; #, significant decrease in the absence of SOD.

cyclase in RFL-6 cells (indicative of NO-like material), and (iv) inhibition by the L-arginine analogs NO_2Arg and MeArg. NADPH-diaphorase was characterized by the NADPH-dependent reduction of NBT to a formazan dye. It was interesting to note that the IC_{50} values for MeArg and NO_2Arg differed between aortic smooth muscle and J774 cells. In J774 macrophages, LPS-induced NO synthase was equally inhibited by the two analogs, whereas the induced enzyme in rat aorta was more potently inhibited by MeArg. This finding suggests that distinct isoforms of NO synthase may be induced by LPS in different cells. However, there was a substantial component of LPS-induced NO synthase activity in rat aorta that was insensitive to inhibition by either MeArg or NO_2Arg. This portion of NO synthase activity increased with incubation time with LPS and could represent either *de novo* synthesis of several isoforms of NO synthase, modification with time of a cytokine-induced isoform, or activities of other citrulline-forming enzymes.

NO synthase induced in both J774 cells and aortic strips was clearly different from the constitutive isoforms reported in neuronal tissue (10–12) and endothelial cells (8, 9, 17), because both induced isoforms were calcium independent and relatively

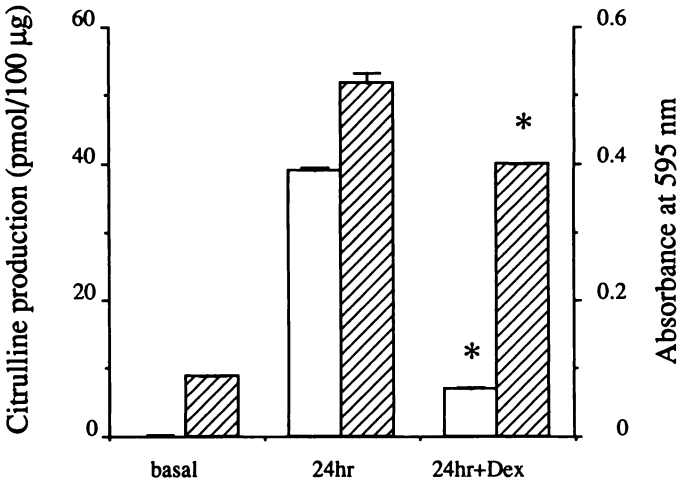

Fig. 5. Effect of coincubation of rat aortic strips with LPS and dexamethasone (*Dex*) (300 nM) for 24 hr. Experimental details are the same as in Fig. 2. □, NO synthase activity; ▨, NADPH-diaphorase activity. Each *point* represents the mean ± standard error of three experiments. *, Significant difference in activity with dexamethasone, compared with incubation with LPS alone (24 hr).

TABLE 1

Induction by LPS (300 ng/ml) of NO synthase and NADPH-dependent diaphorase activity

The enzyme activities are given as percentage of basal activity at the time point required for maximum induction of NO synthase and NADPH-dependent diaphorase. Experimental details are the same as in Fig. 2. The numbers were calculated as the means of three experiments.

Tissue	NO synthase activity	Diaphorase activity	Time
	% of basal	*% of basal*	*hr*
Rat vena cava	400	430	40
Canine carotid artery	1850	1000	40
Canine jugular vein	300	270	40
Ferret aorta	760	375	24

insensitive to NO_2Arg. The finding that NO synthase could be induced in a variety of vascular preparations supports the hypothesis that excessive NO production by vascular smooth muscle cells may be responsible for the hypotension associated with septic shock (26, 27). The novel finding that NO synthase can also be induced in venous tissue *in vitro* suggests that venodilation may occur *in vivo* during sepsis. Indeed, Seaman and Greenway (28) showed that the venous responsiveness to vasoconstrictors was greatly reduced after endotoxin treatment of anesthetized cats. Induction of NO synthase in venous tissue could explain the reduced cardiac output and blood pooling produced by endotoxemia (29, 30).

NO production by LPS/cytokines plays a role in the cytotoxic effects of macrophages. The cytosol of untreated macrophages has no or very little NO synthase activity, whereas cytosol prepared from macrophages incubated with LPS for several hours contains significant NO synthase (2). Although protein synthesis inhibitors and steroids, such as dexamethasone, block the ability of LPS/cytokine-treated vascular smooth muscle cells to release NO (5, 7), it is unclear whether incubation with LPS/cytokines causes induction of NO synthase *per se* or the synthesis of an activating regulatory protein. In the rat aorta, the ability of dexamethasone to inhibit the induction of NO synthase was not fully reproduced in the case of NADPH-diaphorase activity. Although there was a significant reduction in diphorase activity with dexamethasone, the effect was not

as marked as that for NO synthase activity. These findings suggest that other NADPH-diaphorases may be induced along with that activity associated with NO synthase. In our hands, dexamethasone had little effect on NO synthase induction in J774 cells. However, this could be due to the progressive loss of steroid receptors during culture and repeated passage.

Treatment of cells with LPS/cytokines can induce the synthesis of BH_4 via GTP cyclohydrolase (31). BH_4 is a cofactor for the induced NO synthase (14, 15) and constitutive NO synthase from endothelial cells (17) and central (16, 32) and peripheral (12) neuronal tissue. In addition, BH_4 itself can stimulate the reduction of NBT to formazan, in a NADPH-independent manner.[1] Therefore, an alternative explanation for the LPS-induced diaphorase activity could be increased biopterin synthesis. However, because LPS-induced diaphorase activity is NADPH dependent (unlike the effect of BH_4), it would seem unlikely that increased cellular BH_4 has any role in the observed diaphorase activity. On the other hand, it has been suggested that NO synthase may have quinoid-dihydropteridin reductase-like activity (16),[1] and this activity in other systems is NADPH dependent. It is conceivable, therefore, that NADPH-dependent diaphorase results from the NADPH-dependent cycling of BH_4 by NO synthase. Whether, in the absence of L-arginine but in the presence of NADPH, NO synthase could generate BH_4, which could be responsible for part of the observed diaphorase activity, remains the subject of investigation.

The development of specific antibodies that recognize the constitutive neuronal and endothelial NO synthase have led to studies showing co-localization of immunoreactivity with NADPH-diaphorase staining (23). Our results show that induction of NO synthase is associated with increased NADPH-diaphorase activity and that the two activities co-purify. However, we show that other diaphorase activities, which are independent of NO synthase, can also be induced by LPS.

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