Biochem. J. (1990) 270, 833–836 (Printed in Great Britain)

Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat

Richard G. KNOWLES, Martin MERRETT, Mark SALTER and Salvador MONCADA
Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

Nitric oxide (NO) synthase in rat brain was found to be constitutive and Ca^{2+}-dependent. The enzyme in rat lung or liver (predominantly in parenchymal cells) was not constitutive, but was induced by endotoxin treatment and was Ca^{2+}-independent. The NO synthases in rat brain and liver or lung are therefore distinct both in their properties and in their regulation.

INTRODUCTION

The L-arginine:NO pathway was originally identified in vascular endothelial cells [1,2] and has since been shown to occur in cytotoxic macrophages [3,4], neutrophils [5–7], the brain [8–10], adrenal gland [11], EMT-6 adenocarcinoma cells [12,13] and liver cells [14]. The NO synthases studied so far are all soluble, NADPH-dependent, and form citrulline as a co-product of NO [4,8,9,11,15–18]. However, the properties of the enzyme in different cells suggest that there is more than one type of NO synthase [8,11,19].

Experiments *in vitro* with macrophages, liver cells and EMT-6 adenocarcinoma cells in culture have shown that the synthesis of NO does not occur in these cells unless they have been exposed to inducing agents such as endotoxin or cytokines [3,4,12–14]. In contrast, the NO synthase in the brain, adrenal gland and vascular endothelium appears to be constitutive, i.e. it is present in these tissues without exposure to inducing agents [8,9,11,15,16]. In the present study we have investigated whether *in vivo* the NO synthase is constitutive in the brain, liver and lung of the rat, and whether the enzyme can be induced in these tissues by treatment of the animals with endotoxin. Our results show that a Ca^{2+}-independent NO synthase is induced in both the lung and liver of rats treated with endotoxin, whereas brain NO synthase is constitutive, unchanged by endotoxin, and entirely Ca^{2+}-dependent.

MATERIALS AND METHODS

Materials

Trichloroacetic acid-extracted lipopolysaccharide (endotoxin) from *Salmonella typhimurium* was obtained from Sigma. L-[*guanidino*-^{14}C]Arginine was obtained from NEN/DuPont (Stevenage, Herts., U.K.). Other chemicals were obtained from Sigma, Boehringer Mannheim or BDH.

Treatment of rats and preparation of soluble tissue extracts

Male Wistar rats (200–300 g, Charles River), fed *ad libitum*, were injected intraperitoneally with various doses of endotoxin prepared in pyrogen-free 0.9% NaCl. At 3, 6, 12, 19 and 43 h after endotoxin treatment, the liver, lungs and brain were removed under pentobarbitone anaesthesia (60 mg/kg) after 2–3 min perfusion of the liver with 0.9% NaCl, via the hepatic portal vein, to remove most of the blood. The tissues were cooled on ice before homogenization (in an Ultra-Turrax homogenizer) with 5 vol. of a buffer containing 10 mM-Hepes, 0.32 M-sucrose, 0.1 mM-EDTA, 1 mM-dithiothreitol, 10 μg of soybean trypsin inhibitor/ml, 10 μg of leupeptin/ml, 2 μg of aprotinin/ml and 1 mg of phenylmethanesulphonyl fluoride/ml, adjusted to pH 7.4 (at room temperature) with NaOH. The homogenates were then centrifuged at 100000 *g* for 1 h. The liver supernatant was filtered (0.2 μm-pore Acrodisc; Gelman Sciences, Northampton, U.K.). The supernatants were stored on ice until assayed for NO synthase activity within 4 h of preparation. In some experiments brain supernatant was passed through Dowex 50W (Na^+ form) to remove endogenous L-arginine.

In experiments to determine which liver-cell types contained NO synthase, a mixed liver-cell population was obtained by a collagenase perfusion essentially as described in ref. [20], after ligation and removal of a ventral lobe. The cells were then fractionated to yield parenchymal and non-parenchymal fractions as described in ref. [21]. The cells were then centrifuged (200 *g* for 5 min) before being sonicated (3 × 10 s at 10 μm amplitude with a Soniprep 150 instrument) in 5 vol. of the homogenization buffer described above, centrifuged and filtered before assay for NO synthase activity.

Assay of NO synthase

NO synthesis was measured by a method based on that of Feelisch & Noack [22], in which the oxidation of oxyhaemoglobin to methaemoglobin by NO is monitored spectrophotometrically. The absorption difference between 401 and 411 nm was continuously monitored with a dual-wavelength recording spectrophotometer (Shimadzu UV-3000) by using a band width of 2 nm, at 37 °C. The incubations contained 1.6 μM-oxyhaemoglobin, 1 mM-$MgCl_2$, 40 mM-potassium phosphate, pH 7.2, and up to 20% (v/v) enzyme extract. NO synthesis was initiated by addition of L-arginine (1 mM) and NADPH (1 mM).

The rate of NO production by NO synthase in brain and lung extracts was linear for 10 min. With liver extracts the rate remained linear for only 2–3 min. However, if 50 mM-L-valine was included as an inhibitor of arginase [23], NO synthesis was then linear for 10 min. The initial rate of NO synthesis was found to be linear with the concentration of tissue extract over the range used (0–3 mg of tissue protein/ml; results not shown). The presence of moderate concentrations (up to 50 μM) of haemoglobin in tissue extracts did not affect this assay for NO synthase, unlike that using the stimulation of guanylate cyclase [19]. The rate of NO formation was calculated by using the absorption coefficient of methaemoglobin for the wavelength pair 401 minus 411 nm, which was found to be 38600 $M^{-1} \cdot cm^{-1}$ in the spectrophotometer used.

The validity of this method for measuring NO synthase activity

Abbreviation used: NMMA, N^G-monomethylarginine.

Table 1. Substrate requirements and inhibitor specificity of the brain and liver NO synthases

The data are means ± S.D. from triplicate determinations of NO synthase activity assayed by the haemoglobin-oxidation method: * significantly different from control ($P < 0.01$). The brain enzyme was treated with Dowex to remove endogenous L-arginine before use. Substrates were present at 100 μM and inhibitors at 300 μM.

	NO synthase (% of control)	
	Brain	Liver
L-Arginine + NADPH (Control)	100	100
L-Arginine alone	0 ± 3.5*	0 ± 0.7*
NADPH alone	0 ± 2.7*	0 ± 0.5*
D-Arginine + NADPH	0 ± 3.1*	0 ± 0.9*
L-Homoarginine + NADPH	31 ± 15*	54 ± 1.5*
Control + L-NMMA	3 ± 3.4*	8 ± 1.7*
Control + D-NMMA	89 ± 7.4	88 ± 9.9
Control + L-canavanine	86 ± 2.2*	54 ± 1.5*

Table 2. NO synthase activity in brain, lung and liver in control and endotoxin-treated rats

The data are means ± S.E.M. of NO synthase activities from 3–6 rats, assayed by the oxidation of haemoglobin by NO, or (in parentheses) by [^{14}C]citrulline formation: * significantly different from control ($P < 0.05$). The endotoxin dose was 4 mg/kg body wt., injected 6 h before removal of tissues.

	NO synthase activity (nmol/min per g of tissue)	
	Control	Endotoxin
Brain	4.9 ± 0.63 (9.8 ± 0.65)	4.7 ± 0.95 (9.8 ± 1.06)
Lung	0.0 ± 0.06 (0.0 ± 0.07)	12.2 ± 3.00* (3.1 ± 0.76*)
Liver	0.6 ± 0.12	15.7 ± 3.20*

in tissue extracts of both brain and liver was shown by: (1) the absence of a rate in the absence of NADPH, (2) the absence of a rate in the absence of L-arginine, and the inability of D-arginine to substitute for it, and (3) the stereospecific inhibition by N^G-monomethylarginine (NMMA) (Table 1).

NO synthase activity assayed by this method was compared with the activity as assessed by production of radiolabelled citrulline from L-arginine. Enzyme extracts were incubated with 30 μM-L-[*guanidino*-^{14}C]arginine (sp. radioactivity 0.6 μCi/ml) and 1 mM-NADPH in 40 mM-potassium phosphate buffer (pH 7.2) containing 1 mM-$MgCl_2$. In some incubations 1 mM-L-citrulline was included to minimize the further metabolism of [^{14}C]citrulline formed in the assay. The samples were incubated for 10 min at 37 °C before termination of the reaction by addition of 0.1 vol. of 20% (v/v) $HClO_4$. After centrifugation (10000 *g* for 2 min), [^{14}C]citrulline in the supernatant was separated from [^{14}C]arginine by ion-exchange chromatography, essentially as described in ref. [9], and quantified by liquid-scintillation counting.

RESULTS AND DISCUSSION

NO synthase in the brain

The soluble fraction from the whole brain of control rats was found to contain a constitutive NO synthase as determined both by the haemoglobin assay and by measuring the rate of formation from L-[^{14}C]arginine of [^{14}C]citrulline, the co-product of NO (Table 2). Brain NO synthase activity measured by the haemoglobin assay was entirely Ca^{2+}-dependent, since addition of 100 μM-EDTA to incubations completely abolished the formation of NO ($n = 3$). This is consistent with previous reports showing the Ca^{2+}-dependence of NO synthase from rat brain synaptosomes [8] and cerebellum [9,18]. The brain NO synthase required L-arginine and NADPH for activity. L-Homoarginine was a poor substrate for the enzyme. The brain NO synthase was inhibited by L-NMMA, but not D-NMMA, and was only slightly inhibited by L-canavanine.

The activity of the NO synthase measured in the soluble fraction of whole brain was approx. 5 nmol/min per g of tissue (Table 2), equivalent to 200 pmol/min per mg of protein [since there was 25 ± 2.8 ($n = 3$) mg of soluble protein/g of brain]. This is greater than that observed in the soluble fraction from synaptosomes measured either by formation of nitrite (a breakdown product of NO), or by the stimulation of soluble guanylate cyclase, of 40 pmol/min per mg of protein [19]. This suggests that the NO synthase is likely to be widely distributed in the brain, and is not only or predominantly present in nerve terminals.

Treatment of rats with 2 mg of endotoxin/kg had no effect on brain NO synthase activity (Table 2, Fig. 1*a*).

NO synthase in the lung and liver

In contrast with the brain, the lung of control rats contained no detectable NO synthase as assessed by the synthesis of either NO or citrulline from L-arginine (Table 2). There is a report suggesting that a DEAE-Sepharose-purified fraction from bovine lung does contain constitutive NO synthase [17], but, since the assay used was not quantitative, it is not clear whether the activity observed would be below the limit of detection of the

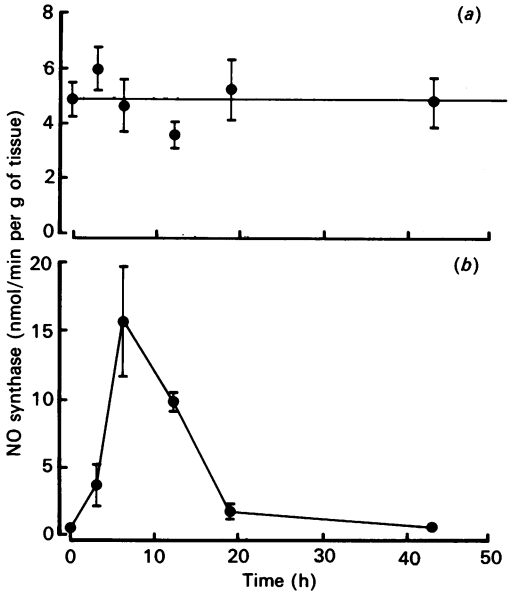

Fig. 1. NO synthase activity in rat brain (*a*) and liver (*b*) after injection of endotoxin

The data shown are the NO synthase activities of brain or liver extracts, assayed by the haemoglobin method at various times after injection of 2 mg of endotoxin/kg, as means ± S.E.M. ($n = 3$).

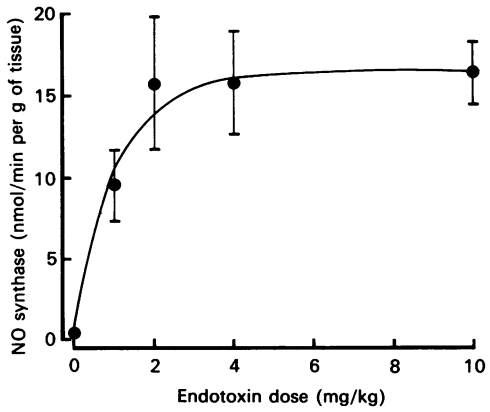

Fig. 2. Dose-dependence of induction by endotoxin of NO synthase activity in the liver

The data shown are the NO synthase activities of liver extracts, assayed by the haemoglobin method at 6 h after injection of various doses of endotoxin, as means ± S.E.M. ($n = 3$).

Table 3. NO synthase in parenchymal and non-parenchymal liver cells

The data are means ± S.E.M. from three fractionation experiments. The assays were carried out in the presence of 1 mM-L-citrulline and 50 mM-L-valine, with 100 μM-L-arginine or 20 μM-L-[^{14}C]arginine as substrate for the haemoglobin-oxidation and [^{14}C]citrulline-formation assays respectively. The results were calculated on the basis of the presence of 72 ± 0.53 ($n = 6$) mg of 100000 *g*-supernatant protein/g of liver, and the reported distribution of cytosol between parenchymal and non-parenchymal cells in the liver: 93.2% parenchymal, 6.8% non-parenchymal [21]. The livers were from rats treated with 4 mg of endotoxin/kg 19 h before removal of the liver.

	NO synthase (nmol/min per g of liver)	
	Haemoglobin assay	Citrulline assay
Whole liver	10.4 ± 0.85	2.5 ± 0.12
Parenchymal-cell fraction	6.0 ± 0.74	3.0 ± 0.04
Non-parenchymal-cell fraction	0.3 ± 0.18	0.2 ± 0.02

assay used in the present study with unpurified enzyme extracts (approx. 0.2 nmol/min per g of tissue). However, it is likely that in the lung there is some constitutive NO synthase in vascular endothelium, since this organ has a most extensive vascular network. The liver contained a low constitutive activity of NO synthase (Table 1).

Treatment of rats with endotoxin (4 mg/kg) caused a substantial induction of NO synthase in the lung and liver. In lung extracts this activity could be measured by formation of either NO or [^{14}C]citrulline (Table 2). In liver extracts [^{14}C]citrulline was not observed in the absence of added L-citrulline and L-valine, presumably because of rapid metabolism of both arginine and citrulline by enzymes of the urea cycle. When liver extracts were assayed in the presence of 1 mM-L-citrulline (to inhibit the metabolism of [^{14}C]citrulline formed) and 50 mM-L-valine (to inhibit the arginase-catalysed degradation of L-[^{14}C]arginine to urea and L-ornithine), then formation of [^{14}C]citrulline was observed (Table 3).

The induction by endotoxin of NO synthase in the liver was maximal at 6 h after injection and declined rapidly after 12 h (Fig. 1*b*). The induction was dose-dependent, with the liver NO synthase at 6 h being induced to half-maximal activity at approx. 0.5–1.0 mg of endotoxin/kg, and with a maximum activity of approx. 17 nmol/min per g of tissue at doses above 2 mg/kg (Fig. 2). This induction of NO synthase is likely to be the cause of the increase in the excretion of urinary nitrate, a breakdown product of NO, which is observed when rats are treated with endotoxin [24,25].

The liver NO synthase was mainly present in the parenchymal-cell fraction (Table 3). The non-parenchymal fraction (which consists of liver macrophages and endothelial cells with some contaminating parenchymal material [21]) had a lower and more variable activity. The induced NO synthase in the liver is therefore predominantly present in parenchymal cells, and less activity is associated with the reticuloendothelial cells. It is not clear which cell type in the lung is induced to synthesize NO synthase, but much of the activity may reside in macrophages in this tissue. Further studies are necessary to clarify this point.

The liver NO synthase was able to utilize L-homoarginine as substrate and was inhibited by L-canavanine (Table 1); both were more effective on the liver enzyme than on that from the brain. Furthermore, the NO synthase activity induced in the lung and liver by endotoxin was largely independent of Ca^{2+}. Addition of EGTA to incubations resulted in only a small inhibition of activity (lung 11 ± 3.0%, liver 7 ± 3.2% of control; means ± S.E.M., $n = 3$). These results suggest that, once induced, the liver and lung NO synthases will catalyse a continuous synthesis of NO from L-arginine over long periods. In contrast, tissues such as the brain, the vascular endothelium and the adrenal gland contain the constitutive Ca^{2+}-dependent NO synthase, which at basal cytosolic Ca^{2+} concentrations is inactive [8,9,11,16]. In these tissues NO synthesis will only occur in short bursts after stimulation by neurotransmitters, hormones or mechanical stimuli which will transiently increase the cytosolic Ca^{2+} concentration.

Conclusions

Our results demonstrate that in addition to the constitutive NO synthase present in brain, vascular endothelium and adrenal glands, which plays a role as a transduction mechanism for the stimulation of the soluble guanylate cyclase [8], there is an NO synthase induced *in vivo* in the liver and lung by endotoxin. This is different from the constitutive enzyme in its lack of Ca^{2+}-dependence, its sensitivity to L-canavanine as an inhibitor, and its ability to utilize L-homoarginine as a substrate. These properties suggest that the liver and lung NO synthase enzymes are similar to that inducible in macrophages *in vitro* [26–28]. This NO synthase is likely to play an important role in various pathophysiological states such as infection and in the host defence against cancer.

We thank Gill Henderson for preparation of the typescript.

REFERENCES

1. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) Nature (London) **327**, 524–526
2. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) Nature (London) **333**, 664–666
3. Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) Science **235**, 473–476
4. Marletta, M. A., Yoon, P. S., Lyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) Biochemistry **27**, 8706–8711
5. McCall, T. B., Boughton-Smith, N. K., Palmer, R. M. J., Whittle, B. J. R. & Moncada, S. (1989) Biochem. J. **262**, 293–296
6. Salvemini, D., De Nucci, G., Gryglewski, R. J. & Vane, J. R. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6328–6332
7. Wright, C. D., Mulsch, A., Busse, R. & Osswald, H. (1989) Biochem. Biophys. Res. Commun. **160**, 813–819

8. Knowles, R. G., Palacios, M., Palmer, R. M. J. & Moncada, S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5159–5162
9. Bredt, D. S. & Snyder, S. H. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9030–9033
10. Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) Nature (London) **336**, 385–388
11. Palacios, M., Knowles, R. G., Palmer, R. M. J. & Moncada, S. (1989) Biochem. Biophys. Res. Commun. **165**, 802–809
12. Amber, I. J., Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1988) J. Leukocyte Biol. **44**, 58–65
13. Lepoivre, M., Boudbid, H. & Petit, J.-F. (1989) Cancer Res. **49**, 1970–1976
14. Curran, R. D., Billiar, T. D., Stuehr, D. J., Hofmann, K. & Simmons, R. L. (1990) in Nitric Oxide from L-Arginine: A Bioregulatory Mechanism (Moncada, S. & Higgs, E. A., eds.), pp. 373–379, Elsevier, Amsterdam
15. Palmer, R. M. J. & Moncada, S. (1989) Biochem. Biophys. Res. Commun. **158**, 348–352
16. Mayer, B., Schmidt, K., Humbert, P. & Bohme, E. (1989) Biochem. Biophys. Res. Commun. **164**, 678–685
17. Mayer, B. & Bohme, E. (1989) FEBS Lett. **256**, 211–214
18. Bredt, D. S. & Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 682–685
19. Knowles, R. G., Palacios, M., Palmer, R. M. J. & Moncada, S. (1990) Biochem. J. **269**, 207–210
20. Seglen, P. O. (1976) Methods Cell Biol. **13**, 29–83
21. Van Berkel, T. J. C. (1982) in Metabolic Compartmentation (Sies, H., ed.), pp. 437–482, Academic Press, New York
22. Feelisch, M. & Noack, E. A. (1987) Eur. J. Pharmacol. **139**, 19–30
23. Kaysen, G. A. & Strecker, H. J. (1973) Biochem. J. **133**, 779–788
24. Wagner, D. A., Young, V. R. & Tannenbaum, S. R. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 4518–4521
25. Leaf, C. D., Wishnok, J. S. & Tannenbaum, S. R. (1990) in Nitric Oxide from L-Arginine: A Bioregulatory Mechanism (Moncada, S. & Higgs, E. A., eds.), pp. 283–290, Elsevier, Amsterdam
26. Kwon, N. S. K., Nathan, C. F. & Stuehr, D. J. (1989) J. Biol. Chem. **264**, 20496–20501
27. Tayeh, M. A. & Marletta, M. A. (1990) in Nitric Oxide from L-Arginine: A Bioregulatory Mechanism (Moncada, S. & Higgs, E. A., eds.), pp. 177–182, Elsevier, Amsterdam
28. Lyengar, R., Stuehr, D. J. & Marletta, M. A. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6369–6373

Received 26 March 1990/5 July 1990; accepted 23 July 1990