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Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases

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The distribution of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthase (NOS) was studied in rabbits and in control and endotoxin-treated rats and guinea-pigs. There was a widespread localization of NOS which differed for the two forms of the enzyme and which showed marked differences between species. Endotoxin induced the activity of the Ca^{2+}-independent NOS in many tissues and also increased the activity of Ca^{2+}-dependent NOS in the rat ileum and caecum. These results demonstrate the differential distribution of NOSs in control and endotoxin-treated animals and emphasize the widespread biological role of nitric oxide (NO).

Nitric oxide; Nitric oxide synthase; Endotoxin; Rat; Guinea-pig; Rabbit

1. INTRODUCTION

The L-arginine:NO pathway was originally identified in vascular endothelial cells [1,2] and has since been shown to occur in many other cell-types [2–9] although no systematic study has been carried out to identify its functional location in different tissues in the body. There are at least two distinct types of nictric oxide synthase (NOS; provisionally EC 1.14.13.39); a Ca^{2+}-dependent enzyme that has been shown to be present in the brain [10,11], vascular endothelium [12,13], platelet [9] and adrenal gland [14] and a Ca^{2+}-independent enzyme expressed in macrophages [4,15], parenchymal liver cells [7,8], neutrophils [6], vascular endothelial cells [16,17] and the vascular smooth muscle layer [18–20] after challenge with endotoxin or cytokines. The Ca^{2+}-dependent enzyme synthesises NO as part of a transduction mechanism for the regulation of soluble guanylate cyclase. Nitric oxide formed from the Ca^{2+}-independent enzyme acts as a cytotoxic agent for tumour cells, bacteria, fungi and protozoa (reviewed in [21]) and may play a role in pathological vasodilatation and host tissue damage in endotoxin shock and other conditions of altered immunological status [16,18,19,22,23].

Recently, the Ca^{2+}-dependent NOS has been purified from rat cerebellum and antibody raised from the enzyme has been used to determine the antigenic localization of this NOS in the rat [24]. In the present study we have investigated the functional distribution of both the Ca^{2+}-dependent and -independent NOS in a number of species in control animals and in those treated with endotoxin. Our results show a widespread pattern of NOS localization that would not have been predicted from the antigenic localization.

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase

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2. MATERIALS AND METHODS

2.1. *Materials*

Trichloroacetic acid-extracted lipopolysaccharide (endotoxin) from *Salmonella typhimurium* was obtained from Sigma. L-[U-^{14}C]Arginine was obtained from Amersham, UK. RU-486 was a gift from Roussel-UCLAF. Other chemicals were obtained from Sigma, Boehringer Mannheim or BDH.

2.2. *Treatment of rats and preparation of soluble tissue extracts*

Male Wistar rats (200–300 g, Charles River) and Duncan-Hartley guinea-pigs (400–500 g, Halls) were starved for 24 h and were injected intraperitoneally with various doses of endotoxin prepared in pyrogen-free 0.9% NaCl. Endotoxin preparations were briefly (5 s) sonicated in a F.S.1006 sonicating water bath (DECON) to ensure homogeneity of preparation. At various times after injection, tissues were removed under pentobarbitone anaesthesia (60 mg/kg) after 2–3 min perfusion via the hepatic portal vein (towards the liver) with 0.9% NaCl (outflow via the inferior vena cava and hepatic portal vein behind the point of cannulation). New Zealand white rabbits (2.4–3.0 kg) were fed ad libitum and tissues were removed without saline perfusion after an overdose of pentobarbitone. Endotoxin was not administered to the rabbits. Tissues were freeze-clamped and stored at −70°C until use.

Tissues were extracted by 0–4°C by homogenization (with a Ystral homogenizer) in 5 vols. of a buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL-dithiothreitol, 100 μg/ml phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 2 μg/ml aprotinin brought to pH 7.0 at 20°C with HCl. The homogenates were then centrifuged at 100 000 × *g* for 30 min. The supernatants were stored on ice for up to 2 h before use.

2.3. *Assay of NOS*

Nitric oxide synthesis was measured either spectrophotometrically,

by determining the N^G-monomethyl-L-arginine-inhibitable oxidation of oxyhaemoglobin (401–421 nm, ε = 77 200 M^{-1}) by NO as described previously [18], or by the conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline as described below. The limit of detection in both these assays was approx. 0.05 nmol/min/g. The radiochemical assay was used in instances when tissue extracts were highly coloured (e.g. spleen) or in instances where activity was not linear with tissue concentration in the haemoglobin NOS assay (e.g. ileum) but was linear in the radiochemical assay. The radiochemical assay was also used in some experiments in which higher precision was required.

18 μl of tissue extract was added to 10 ml plastic tubes prewarmed to 37°C containing 100 μl of a buffer consisting of 50 mM potassium phosphate, pH 7.2, 60 mM L-valine, 120 μM NADPH, 1.2 mM L-citrulline, 24 μM L-arginine and L-[U-^{14}C]arginine (150 000 dpm), 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$. Samples were incubated for 10 min at 37°C before termination of the reaction by removal of substrate and dilution by addition of 1.5 ml of 1:1 (v/v) H_2O/Dowex-50W (200–400, 8% cross-linked, Na^+-form). The Na^+-form of Dowex-50 was prepared by washing the H^+-form of the resin with 1 M NaOH 4 times and then washing with H_2O until the pH was less than 7.5. 5 ml of H_2O was added to the resin incubate mix, left to settle for 10 min and 4 ml of supernatant was removed and examined for the presence of [^{14}C]citrulline by liquid-scintillation counting. The activity of the Ca^{2+}-dependent NOS was determined from the difference between the [^{14}C]citrulline produced from control samples and samples containing 1 mM EGTA; the activity of the Ca^{2+}-independent enzyme was determined from the difference between samples containing 1 mM EGTA and samples containing 1 mM EGTA and 1 mM N^G-monomethyl-L-arginine (an inhibitor of NOS). L-[U-^{14}C]Arginine was purified by adding the radiolabel in 20 mM HEPES (2 ml), pH 5.5, to a 1 ml column of Dowex resin (prepared as above), washing the column with 8 ml of H_2O and eluting with 4 ml of 0.5 M ammonia. The eluant was freeze-dried and resuspended in 2% ethanol.

3. RESULTS AND DISCUSSION

Table I shows the distribution of Ca^{2+}-dependent and Ca^{2+}-independent NOS in control and endotoxin-treated rats. It is clear that there is a wide distribution of the Ca^{2+}-dependent enzyme in control animals but that the Ca^{2+}-independent enzyme is absent. This finding is consistent with previous data demonstrating the requirement for endotoxin or cytokines for the expression of the Ca^{2+}-independent form of the enzyme [4,6–8,16–19]. The distribution of the Ca^{2+}-dependent NOS activity demonstrates a far greater tissue specificity than might be predicted from results obtained using antibody-staining with antibodies raised to the cerebellar enzyme [24]. Substantial Ca^{2+}-dependent NOS activity is seen in parts of the gastrointestinal tract, the thymus, skin and skeletal muscle. The activity in these areas is unlikely to be a consequence of the level of general nerve innervation or vascular endothelial content, as little or no activity is seen in other nerve-rich areas (e.g. duodenum) or in tissues rich in vascular endothelium (e.g. lung, liver, see Table II). Treatment of rats with 4 mg/kg endotoxin induces NOS in a variety of tissues; this induction does not correlate with the presence or absence of the Ca^{2+}-dependent enzyme in the tissue. The expression of large amounts of NOS in the gastrointestinal tract may account for the production of NO which may have a protective role in the gut [25,26]. The distribution of NOS is quite different in skeletal muscle from diaphragm muscle; both have the Ca^{2+}-dependent enzyme but the Ca^{2+}- independent one is only induced in the diaphragm. The function of the Ca^{2+}-dependent enzyme in muscle is unknown; however, it is possible that activation of Ca^{2+}-dependent NOS during muscle contraction may play a role in exercise-induced hyperaemia in the rat although this may not be the case in all other species (see below).

Both the heart and the aorta express Ca^{2+}-independent NOS after treatment of the animal with endotoxin;

Table I

The distribution of NOS in control and endotoxin-treated rats

Tissue	Endotoxin	NOS (nmol/min per g of tissue)	
		Ca^{2+}-dependent	Ca^{2+}-independent
Oesophagus	−	1.04 ± 0.18	0
	+	0.99 ± 0.25	0.60 ± 0.16*
Stomach	−	2.20 ± 0.48	0
	+	2.31 ± 0.05	0
Duodenum	−	0	0
	+	0	2.56 ± 0.09*
Ileum	−	0.41 ± 0.04	0
	+	0.65 ± 0.01*	0.62 ± 0.01*
Caecum	−	0.56 ± 0.03	0
	+	1.44 ± 0.08*	1.00 ± 0.04*
Colon/Rectum	−	1.34 ± 0.32	0
	+	1.39 ± 0.13	0
Diaphragm	−	0.10 ± 0.01	0
	+	0.15 ± 0.04	1.25 ± 0.23*
Skeletal Muscle	−	0.94 ± 0.16	0
	+	0.76 ± 0.18	0
Heart	−	0.05 ± 0.01	0
	+	0.10 ± 0.02	0.19 ± 0.03*
Spleen	−	0.10 ± 0.01	0
	+	0.09 ± 0.03	3.64 ± 0.01*
Thymus	−	0.45 ± 0.03	0
	+	0.47 ± 0.04	0.33 ± 0.04*
Kidney	−	0	0
	+	0	0.14 ± 0.03*
Skin	−	0.52 ± 0.24	0
	+	0.34 ± 0.11	0
Aorta	−	0.47 ± 0.07	0
	+	0.28 ± 0.06	0.71 ± 0.16*
Pancreas	−	0	0
	+	0	0
Testes	−	0	0
	+	0	0
Epididymis	−	0	0
	+	0	0

The data are means ± SEM from three rats except for the ileum where n=6. Rats were treated with vehicle (0.9% pyrogen-free NaCl, 2 ml/kg) or 4 mg/kg endotoxin 6 h before sacrifice. NOS was measured by spectrophotometrically determining the oxidation of oxyhaemoglobin. NOS in the ileum and spleen was measured by the conversion of L-[U-^{14}C]arginine to [U-^{14}C]-citrulline. Data for the aorta are taken from [18]. *$P < 0.01$; significantly different from vehicle-treated rats (Student's t-test).

the effects of this increased potential for NO production on heart function are unclear at present (R. Schulz, E. Nava and S. Moncada, unpublished work). The substantial Ca^{2+}-independent NOS activity in the spleen may well reflect the presence of macrophages in the tissue.

A comparison of NOS activity in the rat, guinea-pig and rabbit brain, lung, liver and skeletal musle is shown in Table II. The brain and liver show a similar pattern of activity between species; however, the enzyme activities in the lung and skeletal muscle of the three species are quite different. No measurable activity could be found in rabbit skeletal muscle and this may relate to the lack of a role for NO in exercise-induced hyperaemia in the rabbit [27] in contrast to the rat. The difference in the NOS distribution pattern between lungs from the various species was striking. The rat, as has been shown previously [8], only expressed activity after treatment of the animal with endotoxin whereas the guinea-pig lung had the Ca^{2+}-dependent but not the Ca^{2+}-independent enzyme in both control and treated animals. In the lung from control rabbits both forms of the enzyme were expressed; this is the only evidence we have found that expression of the Ca^{2+}-independent NOS can occur without substantial pharmacological manipulation of the animal and may reflect mild immunological activation of the animals.

Previous work has shown that the Ca^{2+}-dependent enzyme is unchanged in tissues after endotoxin and/or cytokine challenge [8,18,19]. This observation is borne out by most of the tissues in Table I; however, in the ileum and caecum there was a clear increase in the level of activity of the Ca^{2+}-dependent NOS as well as an increase in the Ca^{2+}-independent form of the enzyme. This effect of endotoxin on the enzymes in the ileum was examined further. Fig. 1 shows the time-dependence of

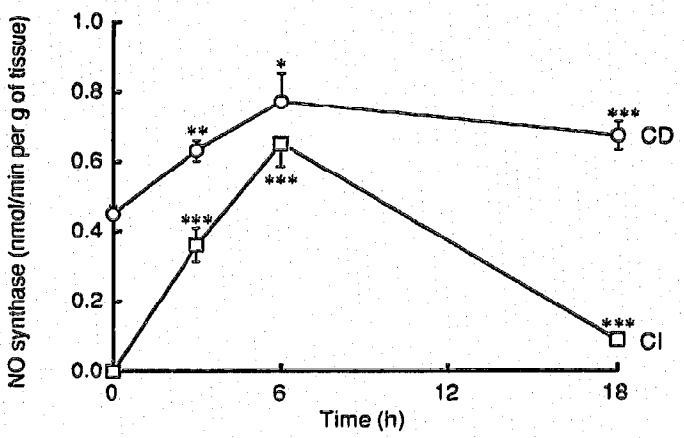

Fig. 1. Time-course of induction by endotoxin of NOS activity in the rat ileum. The data shown are means ± SEM from three rats. Ca^{2+}-dependent (○) and Ca^{2+}-independent (□) NOS was measured from ileum, removed at the time indicted after 4 mg/kg endotoxin administration, by conversion of L-[U^{14}C]arginine to [U-^{14}C]citrulline. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ significantly different from activity at 0 h (Student's *t*-test).

the change in activity of both forms of the enzyme caused by 4 mg/kg endotoxin. The pattern of induction of the Ca^{2+}-independent form of the enzyme is similar to that seen for the liver Ca^{2+}-independent enzyme [8]. The increase in the Ca^{2+}-dependent enzyme shows a similar time-dependence but with a slower return to control values. The endotoxin dose-dependence of the changes in NOS is shown in Fig. 2. Increases in the activity of the Ca^{2+}-dependent enzyme show a similar dose-dependence to the liver Ca^{2+}-independent enzyme [8]; the ileum Ca^{2+}-independent enzyme is induced more at 10 mg/kg than at 4 mg/kg, unlike the response of the liver Ca^{2+}-independent enzyme which is maximal at these doses [8]. It is not clear whether the increase in activity

Table II

Activity of NOS in the brain, lung, liver and skeletal muscle of the rat, guinea-pig and rabbit

Tissue	Endotoxin	NOS (nmol/min per g of tissue)					
		Rat		Guinea-pig		Rabbit	
		CD	CI	CD	CI	CD	CI
Brain	−	5.43 ± 0.47	0	13.5 ± 1.56	0	4.51 ± 0.38	0
	+	6.16 ± 0.66	0	13.1 ± 1.50	0	ND	ND
Lung	−	0	0	5.87 ± 0.38	0	0.36 ± 0.05	0.26 ± 0.04
	+	0	4.87 ± 0.10*	5.43 ± 0.41	0	ND	ND
Liver	−	0	0	0	0	0	0
	+	0	2.92 ± 0.20*	0	1.65 ± 0.35*	ND	ND
Skeletal muscle	−	0.94 ± 0.16	0	0.65 ± 0.05	0	0	0
	+	0.76 ± 0.18	0	0.71 ± 0.06	0	ND	ND

The data are means ± SEM from three animals except for rat lung where $n=4$. Animals were treated with vehicle (0.9%, NaCl, 2 ml/kg) or 4 mg/kg endotoxin 6 h before sacrifice. NOS was measured by conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline except for skeletal muscle where NOS was measured by spectrophotometric determination of the oxidation of oxyhaemoglobin. ND = not determined. CD = Ca^{2+}-dependent; CI = Ca^{2+}-independent. $*P < 0.005$; significantly different from vehicle-treated animals (Student's *t*-test).

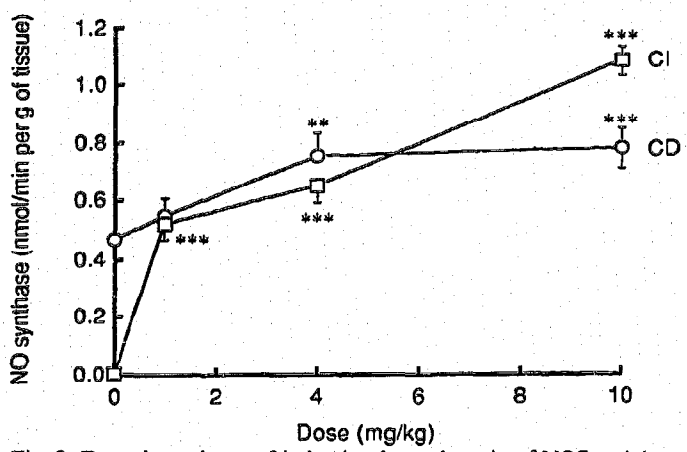

Fig. 2. Dose-dependence of induction by endotoxin of NOS activity in the rat ileum. The data shown are means ±SEM from five rats. Ca^{2+}-dependent (○) and Ca^{2+}-independent (□) NOS was measured from ileum, removed 6 h after endotoxin administration, by conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline. $^{**}P < 0.01$; $^{***}P < 0.005$ significantly different from activity of controls (Student's t-test).

of Ca^{2+}-dependent NOS represents induction, or activation of the existing enzyme.

The in vitro and in vivo induction of the Ca^{2+}-independent NOS by endotoxin and cytokines has previously been shown to be prevented by concomitant glucocorticoid treatment [16,18,19]. Table III shows the effect of dexamethasone on the induction of NOSs in the lung and ileum. As with the liver [18], there was an almost total inhibition (92 ± 2.4%) of induction in the lung, whereas dexamethasone was less effective in preventing (62 ± 5.9%) the induction in the ileum ($P < 0.005$, Student's t-test). This demonstrates that the effect of glucocorticoids on NOS induction differs from tissue to tissue. Interestingly, the increase in the activity of the Ca^{2+}-dependent enzyme in the ileum was not prevented by dexamethasone treatment (Table III).

RU-486 (Mifepristone), a glucocorticoid antagonist [28], had no significant effect on the induction of NOS in the lung, ileum or brain (Table III). This suggests that at the time of day that these experiments were done (10.00–16.00 h) endogenous glucocorticoids were not limiting the induction of NOS by endotoxin. This is consistent with observations [29] that adrenalectomy does not alter the activity of the glucocorticoid-regulated enzyme, tryptophan 2,3-dioxygenase, at the nadir of the glucocorticoid diurnal rhythm (10.00–14.00 h). The administration of RU-486 at a higher point in the diurnal glucocorticoid rhythm, however, may well influence the induction of NOS.

4. CONCLUSIONS

Our results show a widespread and varied response of Ca^{2+}-dependent and -independent NOSs to endotoxin treatment. Our functional assessment of Ca^{2+}-dependent NOS distribution shows that there is a quite different tissue distribution of this form of the enzyme

Table III

The effect of dexamethasone and RU-486 on the induction of NOS by endotoxin

Tissue	Endotoxin	RU-486	Dexamethasone	NOS (nmol/min per g of tissue) CD	CI
Lung	−	−	−	0	0
	+	−	−	0	4.87 ± 0.10*
	+	+	−	0	5.22 ± 0.45*
	+	−	+	0	0.41 ± 0.05***
Ileum	−	−	−	0.44 ± 0.03	0
	+	−	−	0.83 ± 0.05*	0.68 ± 0.03*
	+	+	−	0.76 ± 0.04*	0.82 ± 0.15*
	+	−	+	0.81 ± 0.08*	0.26 ± 0.02**
Brain	−	−	−	5.43 ± 0.47	0
	+	−	−	6.16 ± 0.66	0
	+	+	−	5.85 ± 0.57	0

The data are means ± SEM from 3 or 4 rats. Animals were treated with vehicle (0.9% pyrogen-free NaCl, 2 ml/kg) or 4 mg/kg endotoxin 6 h before sacrifice. Dexamethasone (3 mg/kg in 0.9% pyrogen-free NaCl) and RU-486 (20 mg/kg in 0.9% pyrogen-free NaCl, 0.1% Triton X- 100) were administered intraperitoneally after brief sonication as for endotoxin (see Materials and Methods). NOS was measured by conversion of L-[U-^{14}C]-arginine to [U-^{14}C]-citrulline. CD = calcium-dependent; CI = calcium-independent. Significantly different from vehicle-treated rats ($^{*}P < 0.005$) and from endotoxin-treated rats ($^{**}P < 0.01$, $^{***}P < 0.005$), Student's t-test.

from that seen in antibody-staining experiments in the rat using antibody raised against cerebellar NOS [24]. Furthermore, recent work demonstrates that the mRNA coding for this cerebellar NOS is absent from rat stomach, skeletal muscle and heart [30], although our data clearly show significant Ca^{2+}-dependent NOS activity in these tissues. This would be consistent with the hypothesis that there may be isoenzymes of the Ca^{2+}-dependent NOS. In support of this, Hope et al. [31] have shown that an antibody which reacts with neuronal NOS (which has been found to have NADPH diaphorase activity) produces weak staining in the cerebellum and does not stain endothelial cells, unlike the staining pattern seen by Snyder and colleagues with antibody raised against purified cerebellar NOS [24].

The results shown here demonstrate the wide distribution of NOS in the body both before and after endotoxin treatment, emphasizing the important role of this enzyme in health and disease.

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