Characterization of nitric oxide synthases in non-adrenergic non-cholinergic nerve containing tissue from the rat anococcygeus muscle

¹Jane A. Mitchell, Hong Sheng, Ulrich Förstermann & Ferid Murad

Department 47S, AP10/2, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A. and Department of Pharmacology Northwestern University Medical School, Chicago, IL 60611, U.S.A.

Tissue homogenates prepared from rat anococcygeus muscle converted L-arginine to L-citrulline indicating the presence of nitric oxide (NO) synthase. NO synthase activity was also found in crude and partially-purified soluble and particulate fractions prepared from the homogenates. Both soluble and particulate NO synthase were dependent on NADPH, 5,6,7,8-tetrahydrobiopterin and calcium, and inhibited by N^G-nitro-L-arginine. Tissue homogenates or crude cytosolic and membrane fractions from rat vas deferens, which does not contain NO releasing non-adrenergic non-cholinergic neurones, had no NO synthase activity.

Keywords: Nitric oxide; arginine; cyclic GMP; N^G-nitro-L-arginine; endothelium-derived relaxing factor; guanylyl cyclase; 5,6,7,8-tetrahydrobiopterin

Introduction Electrical stimulation of autonomic nerves to various tissues produces relaxations which are not mediated by adrenergic or cholinergic transmitters. These non-adrenergic non-cholinergic nerves (NANC; Gillespie, 1972) release an unidentified transmitter which in some tissues, such as the rat anococcygeus, resembles NO or a related molecule (Gillespie & Sheng, 1990). In brain tissue, the NO-like material is synthesized by a cytosolic Ca²⁺-dependent isoform of NO synthase that has recently been purified (Bredt & Snyder, 1990; Schmidt *et al.*, 1991). Vascular endothelium also releases a relaxing factor (EDRF), with characteristics similar to NO, which is formed from L-arginine (Palmer *et al.*, 1988) by a particulate isoform of NO synthase (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991). Here we show that NANC innervated rat anococcygeus muscle contains both soluble and particulate NO synthase activity.

Methods Male Wistar rats were anaesthetized with CO₂ gas and killed by decapitation. The anococcygeus muscle or vas deferens was removed and immediately placed in liquid nitrogen. For each enzyme preparation between 30 and 50 frozen muscles were homogenized in ice-cold buffer 1 (Tris/HCl 50 mM; EDTA 0.1 mM; EGTA 0.1 mM; 2-mercaptoethanol 12 mM; leupeptin 2 µM; pepstatin A 1 µM and phenylmethylsulphonyl fluoride 1 mM) with a tissue homogenizer (Polytron; Switzerland). The homogenate was filtered through muslin before being homogenized a second time on ice with a glass tissue grinder and teflon pestle (25 strokes). The homogenate was centrifuged at 100,000 *g* for 1 h at 5°C. The supernatant fraction (crude cytosol) was then either assayed directly or applied to a DEAE anion exchange column (DEAE-Sephacel; Pharmacia; 0.5 ml). The column was washed with 10 volumes of buffer 1 and eluted with buffer 1 containing 0.3 M NaCl. This procedure removes contaminating haemoglobin and concentrates protein. The 100,000 *g* pellet was either resuspended by homogenization and assayed directly or washed with buffer 1 containing 1 M KCl (5 min exposure at 5°C) before being centrifuged at 100,000 *g* for 30 min at 5°C. The supernatant was discarded and the KCl washed membrane fraction resuspended in buffer 1. This procedure removes any loosely associated soluble proteins from the particulate fraction.

Protein concentrations were measured by use of the Bradford reagent (Bio-Rad, Richmond, CA, U.S.A.) with bovine serum albumin as standard. Bovine aortic endothelial cells were cultured and homogenized as described previously (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991).

Enzymatic activity was measured by the ability of cytosolic or particulate fractions of anococcygeus muscle to form [³H]-L-citrulline from [³H]-L-arginine (DuPont/NEN) (Bredt & Snyder, 1990; Schmidt *et al.*, 1991) or by guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation in rat foetal lung fibroblast (RFL-6) cells as previously described (Förstermann *et al.*, 1991; Schmidt *et al.*, 1991). All tissue culture products were purchased from Gibco Laboratories (Grand Island, NY). Tetrahydrobiopterin (BH₄) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistics Results are presented as the mean ± s.e.mean, where *n* is the number of values. The statistical difference between groups was evaluated by Student's unpaired *t* test. *P* < 0.05 was taken to be statistically significant.

Results The specific NO synthase activity in homogenates prepared from rat anococcygeus was approximately half of that found in homogenates of bovine aortic endothelial cells (Table 1). The specific activity of the particulate fraction from rat anococcygeus increased following washing with KCl (presumably this step removes some inhibitory material) and was then unaffected by a second KCl wash (data not shown). The specific NO synthase activity of cytosolic fractions increased following DEAE anion exchange chromatography (Table 1). The cytosolic fraction (1 µg protein ml⁻¹), treated with phosphocellulose (Schmidt *et al.*, 1991) to remove haemoglobin, stimulated cyclic GMP levels in RFL-6 cells more than 3 fold (from 0.7 ± 0.2 to 3.0 ± 0.2 pmol cyclic GMP/10⁶ RFL-6 cells 3 min⁻¹) in the presence of L-arginine (100 µM), NADPH (100 µM) and superoxide dismutase (SOD; 20 U ml⁻¹). This increase was reduced by more than 50% to 1.3 ± 0.1 pmol cyclic GMP/10⁶ RFL-6 cells 3 min⁻¹ (*n* = 3) in the absence of SOD or in the presence of 3 µM haemoglobin (1.2 ± 0.5 pmol cyclic GMP/10⁶ RFL-6 cells 3 min⁻¹; *n* = 3). No activity was found in homogenate, cytosol or membrane

¹ Author for correspondence at: 47S, AP10/2, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.

Table 1 Nitric oxide (NO) synthase activity in preparations from various tissues

Tissue	Preparation	Citrulline	
		(pmol mg ⁻¹ protein 30 min ⁻¹)	n
BAE	Homogenate	40.7 ± 3.7	3
Rat AC	Homogenate	19.6 ± 1	9
Rat AC	Cytosol	17.6 ± 2	14
Rat AC	Pellet	33.1 ± 10	6
Rat AC	KCl-washed pellet	78.5 ± 26	17
Rat AC	DEAE-eluted cytosol	171.0 ± 16.5	3
Rat vas	Homogenate	0	3
Rat vas	Cytosol	0	3
Rat vas	KCl-washed pellet	0	3

The NO synthase activity found in preparations from cultured bovine aortic endothelial cells (BAE), rat anococcygeus (Rat AC), or rat vas deferens (Rat vas) was measured with the formation of [³H]-L-citrulline from [³H]-L-arginine (pmol mg⁻¹ protein 30 min⁻¹). In each case the following co-factors were present: NADPH (1 mM), L-arginine (10 μM), [³H]-L-arginine (35 nM), tetrahydrobiopterin (3 μM) and calcium (2 mM). Citrulline formation was measured over a 30 min time course at 25°C (n = 3–17).

fractions (n = 3) prepared from rat vas deferens. Although this is a similar uro-genital non-vascular smooth muscle preparation it does not contain NO releasing NANC nerves (Sheng, H. unpublished observations). The cytosolic and particulate forms of NO synthase from rat anococcygeus appeared similar in their regulation and requirement of co-factors. Both enzymes were dependent on Ca²⁺ and NADPH and, to differing extents, partially dependent on BH₄ (Figure 1). In addition both enzymes were strongly inhibited by the specific NO synthase inhibitor N^G-nitro-L-arginine (10 μM; Hobbs & Gibson, 1990), (Figure 1).

Discussion Our results show that the rat anococcygeus muscle contains both cytosolic and particulate NO synthase. The particulate activity did not decline following repeated washes with 1 M KCl indicating that it represents an integral membrane protein or is anchored in the membrane. As described for NO synthase from brain (Mayer *et al.*, 1990; Schmidt *et al.*, 1991) both soluble and particulate enzymes from rat anococcygeus required NADPH and BH₄. The finding that the particulate enzyme had a greater requirement for BH₄ than the soluble could be explained by higher levels of endogenous BH₄ in the soluble fraction.

It is now well-established that brain tissue contains soluble but not particulate NO synthase (Bredt & Snyder, 1991; Schmidt *et al.*, 1991). NO synthase from NANC tissue appears to be different in that substantial enzymatic activity was found to be associated with the particulate fraction.

The data presented here add further to the evidence that the NANC transmitter is NO (or a related molecule) formed from L-arginine. An alternative explanation could be that an unknown NANC transmitter activates NO synthase in smooth muscle cells. This seems unlikely as preparations from rat vas deferens had little or no enzyme activity. In addition the NO synthase in smooth muscle cells is known to be Ca²⁺-independent and only appears following induction with endotoxin and/or cytokines (Rees *et al.*, 1990). As NANC-derived NO synthase is activated by Ca²⁺ we propose that field stimulation results in Ca²⁺ influx into the respective neurones which activates NO synthase, thereby controlling the amount of neurotransmitter released.

Purification of NANC derived NO synthase will allow for the generation of antibodies to the protein which can then be used for histochemical localization of the enzyme within the tissue.

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Figure 1 Effect of removal of various co-factors on the activity of crude cytosolic (a) and KCl-washed particulate (b) nitric oxide (NO) synthase isolated from rat anococcygeus. Control activity was measured by [³H]-L-citrulline formation from [³H]-L-arginine in the presence of NADPH (1 mM), L-arginine (10 μM) tetrahydrobiopterin (BH₄; 3 μM) and calcium (2 mM). In the absence of BH₄ or NADPH enzyme activity was reduced. Significant reduction, *P* < 0.05. When either calcium was removed and EGTA (0.1 mM) added (-Ca²⁺), or N^G-nitro-L-arginine added (10 μM; +NNA) to the preparation NO synthase activity was virtually abolished. The columns represent means ± s.e.mean from 3–6 experiments; number of experiments shown in parentheses.

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