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Determination of nitric oxide synthase activity in rat, pig and rabbit prostate glands

Juliana L. Di Iulio *, Chun Guang Li, Michael J. Rand

Pharmacology Research Unit, Department of Medical Laboratory Science, Royal Melbourne Institute of Technology, Melbourne, Vic. 3001, Australia

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Abstract

The conversion of L-arginine to L-citrulline by nitric oxide synthase and other enzymes was studied in rat, pig and rabbit prostate glands by incubating preparations of the glands with [^{3}H]L-arginine and measuring [^{3}H]L-citrulline formation. The nitric oxide synthase inhibitor N^G-nitro-L-arginine, (100 μM) reduced [^{3}H]L-citrulline production in preparations from all three species. The arginase inhibitor L-valine (60 mM) inhibited [^{3}H]L-citrulline production in rat and pig but not in rabbit prostate preparations. Omission of calcium or NADPH significantly reduced nitric oxide synthase-like activity in preparations from all three species but arginase-like activity was not significantly affected. The results indicate that the rabbit prostate contains the greatest amount of calcium-dependent nitric oxide synthase activity, the rat and pig prostates also have arginase-like enzymatic activity and the rat prostate contains an additional unidentified enzyme that converts L-arginine to L-citrulline. © 1997 Elsevier Science B.V.

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1. Introduction

Activity attributed to nitric oxide synthase has been demonstrated by biochemical assay involving the production of citrulline from arginine in the prostate gland of the rat (Burnett et al., 1992) and human (Ehrén et al., 1994, 1996; Burnett et al., 1995), raising the possibility that nitric oxide may play a role in prostatic function. In addition, there is immunohistochemical evidence that a calcium-dependent neuronal nitric oxide synthase is present in the rat prostate gland (Burnett et al., 1992). Addicks et al. (1996) showed there was an extensive network of nitrergic nerves associated with blood vessels, smooth muscle cells and secretory cells of the human prostate, possibly implicating nitric oxide in the regulation of blood flow, smooth muscle tone, neurotransmitter release and secretory functions. The aim of this study was to determine nitric oxide synthase activity in rat, pig and rabbit prostate glands. Preliminary accounts have been communicated previously (Di Iulio et al., 1996a,b).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (250–350 g) were killed by a blow to the head and New Zealand white rabbits were killed with an overdose of pentobarbitone. These animals were bled out and the prostate gland removed. Pig prostate glands were obtained from a slaughter house.

2.2. Isolated prostate tissue

Prostate glands were rinsed in chilled physiological salt solution of the following composition (mM) NaCl, 118; KCl, 4.7; $NaHCO_3$, 2.5; $MgSO_4$, 0.45; KH_2PO_4, 1.03; $CaCl_2$, 2.5; D-(+)-glucose, 11.1; disodium edetate, 0.067 and ascorbic acid, 0.014, to remove traces of blood. The ventral lobe of the pig prostate gland was cut into six pieces (approximately 2 g each) and frozen in liquid nitrogen. Rabbit prostate glands were frozen whole. The specimens were thawed and assayed within 2 to 4 weeks after collection. It has been previously shown in other tissues that nitric oxide synthase activity was not diminished by freezing and short term storage (Di Iulio et al., 1995). Rat prostate glands weighed approximately 350 mg

* Corresponding author. Fax: (61-3) 9660-3015.

and glands from 2–3 rats were pooled to obtain sufficient material for each enzyme preparation and were assayed fresh.

2.3. Preparation of homogenates

The tissues were homogenised using a Ystral tissue homogeniser (West Germany) for 20 s in ice-cold buffer (pH 7.8; 1 g of wet tissue in 5 ml of buffer) containing 20 mM HEPES, 0.32 M sucrose, 1 mM ethylenediaminetetra-acetic acid (disodium salt) and 1 mM dithiothreitol. The homogenate was centrifuged at $1000 \times g$ for 15 min at 4°C and the supernatant was collected.

In most experiments, the supernatant was passed through a column containing 2 ml of Dowex (50ZS-200, Na^+ form) to remove endogenous arginine and methylarginines.

2.4. Measurement of arginine metabolism

A 150 μl sample of supernatant was incubated for 60 min at 37°C with 26.5 μl of a buffer (pH 7.8) containing (final concentration) 1 mM NADPH, 10 μCi [^{3}H]L-arginine, 2 μM flavin adenine dinucleotide, 1 mM magnesium acetate, 5 U/ml calmodulin, 1 μM (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (tetrahydrobiopterin) and 50–800 μM calcium chloride. At the end of the incubation period, the reaction was terminated by the addition of 330 μl of a chilled buffer (pH 7.8) containing 20 mM HEPES, 5 mM L-arginine and 4 mM ethylenediaminetetra-acetic acid (disodium salt).

The mixture was applied to 2 ml Dowex (Na^+ form) columns and [^{3}H]L-citrulline was eluted with 2×2 ml of water. The eluate was collected in 20 ml scintillation vials to which 12 ml of scintillation fluid (Ultima Gold, Packard, USA) was added. [^{3}H]L-citrulline levels were then measured in a liquid scintillation counter (Minaxi Tri-Carb 4000 Series; United Technologies Packard, USA).

For all experiments, blanks were prepared using the same protocol, but the tissue homogenate was replaced with 150 μl of tissue free buffer. The radioactivity eluted from the Dowex columns after addition of the tissue-free blank was $0.76 \pm 0.09\%$ ($n = 31$) of the total added to the solution. The blank value was subtracted from the total value obtained with each tissue fraction.

The efficacy of the Dowex column in retaining [^{3}H]L-arginine and allowing the elution of [^{3}H]L-citrulline was determined in control experiments.

The protein content of the supernatant was determined by the Bradford dye binding assay (Bradford, 1976), and metabolism of arginine was expressed as the amount of [^{3}H]L-citrulline produced per mg of protein after 60 min incubation.

In some experiments, arginine metabolism was determined in the presence of either 100 μM N^G-nitro-L-arginine (pilot studies showed that 100 μM N^G-nitro-L-arginine maximally inhibited [^{3}H]L-citrulline production) or 60 mM L-valine (which has been shown in other tissues to inhibit arginase activity, Di Iulio et al., 1995).

2.5. Drugs and chemicals

HEPES, ethylenediaminetetra-acetic acid (disodium salt), calcium chloride and L-valine were obtained from BDH (UK). L-Arginine hydrochloride, dithiothreitol, NADPH, N^G-nitro-L-arginine, bovine albumin, Dowex, flavin adenine dinucleotide and calmodulin were obtained from Sigma (USA). Tetrahydrobiopterin was from ICN (USA). The radiochemicals L-[2,3-^{3}H] arginine (specific activity 40–44.2 Ci/mmol) and L-[ureido^{14}C] citrulline, (specific activity 59.5 mCi/mmol) were purchased from DuPont/NEN (Australia). The Bradford protein assay reagent was from Bio-Rad (USA). Physiological salt solution chemicals and sucrose were obtained from Ajax Chemicals (Australia).

2.6. Statistics

Data are expressed as means ± standard error of the means (S.E.M) and n indicates the number of experiments. Significant differences between means were determined using Student's t-test. Probability levels less than 0.05 were considered significant.

2.7. Ethics

This study was approved by the Animal Experimentation Ethics Committee of the Royal Melbourne Institute of Technology and conformed to the guide lines laid down by the National Health and Medical Research Council of Australia.

3. Results

3.1. [^{14}C]L-citrulline and [^{3}H]L-arginine recovery

In control experiments for validating the methodology, recovery of [^{14}C]L-citrulline and [^{3}H]L-arginine after addition to 2 ml of Dowex ion exchange columns and elution was $90.6 \pm 0.34\%$ ($n = 6$) and $0.44 \pm 0.01\%$ ($n = 6$), respectively. Thus the columns selectively retained arginine and allowed the separation of citrulline from it.

3.2. Production of citrulline

The total amounts of [^{3}H]L-citrulline produced from [^{3}H]L-arginine by preparations of rat and pig prostate glands were 7.24 ± 0.15 ($n = 4$) and 24.26 ± 7.8 ($n = 4$) fmol [^{3}H]L-citrulline per mg protein, respectively. When determined after passage of the supernatant of the homogenate over 2 ml of Dowex ion exchange resin, the amounts of [^{3}H]L-citrulline were significantly increased,

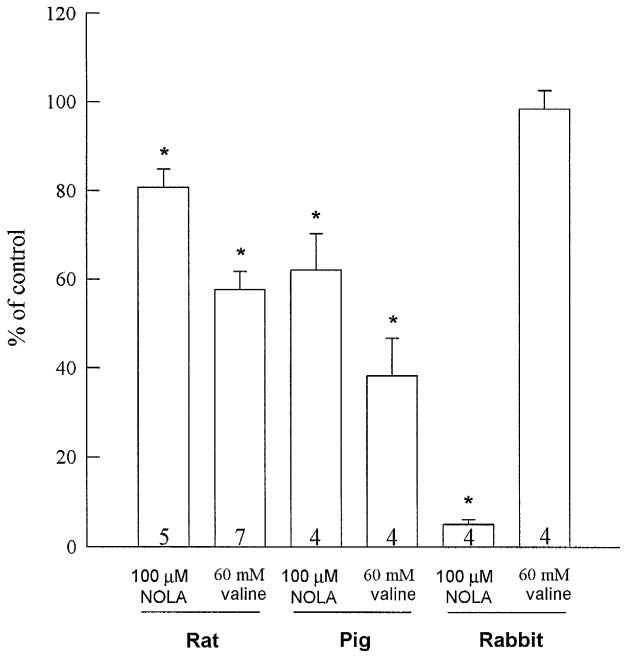

Fig. 1. The effects of N^G-nitro-L-arginine (100 μM) and L-valine (60 mM) on [^{3}H]L-citrulline production in preparations of rat, pig and rabbit prostate gland expressed as percentages (%) of the corresponding control total [^{3}H]L-citrulline production. In absolute terms (fmol [^{3}H]L-citrulline per mg protein in 60 min), the control values were: rat = 14.2 ± 0.3.7; pig = 41.7 ± 11.8; rabbit = 408.8 ± 7.8. Each column represents the means ± SEM; number of experiments (n) is indicated at the base of each column. A significant difference from control is indicated by an asterisk ($^*P < 0.05$).

being 14.22 ± 3.69 ($n = 4$) and 41.69 ± 11.77 ($n = 4$) fmol [^{3}H]L-citrulline per mg protein, respectively. In all subsequent experiments this procedure was performed before assay, including preparations of rabbit prostate.

In preparations of rabbit prostate, [^{3}H]L-citrulline production was considerably greater than in the other two species studied, being 408.78 ± 7.8 ($n = 4$) fmol [^{3}H]L-citrulline per mg protein.

3.3. Inhibition of L-arginine metabolism

The nitric oxide synthase inhibitor N^G-nitro-L-arginine (100 μM) significantly reduced [^{3}H]L-citrulline production in prostate preparations from all three species (Fig. 1). The inhibition was considerably greater in rabbit than in rat or pig preparations.

The arginase inhibitor L-valine (60 mM) significantly reduced [^{3}H]L-citrulline production in rat and pig prostate preparations, but had no significant effect in the rabbit prostate preparation (Fig. 1).

3.4. Effect of cofactors on nitric oxide synthase-like activity

These experiments were carried out in the presence of 60 mM L-valine in the case of rat and pig prostate preparations to eliminate arginase-like activity, but this was not necessary for rabbit prostate preparations. The production of [^{3}H]L-citrulline was significantly reduced by the omission of calcium and NADPH from the reaction mixture in prostate gland preparations from all three species (Fig. 2).

The omission of flavin adenine dinucleotide or tetrahydrobiopterin slightly but significantly reduced [^{3}H]L-citrulline production in pig prostate preparations but had no

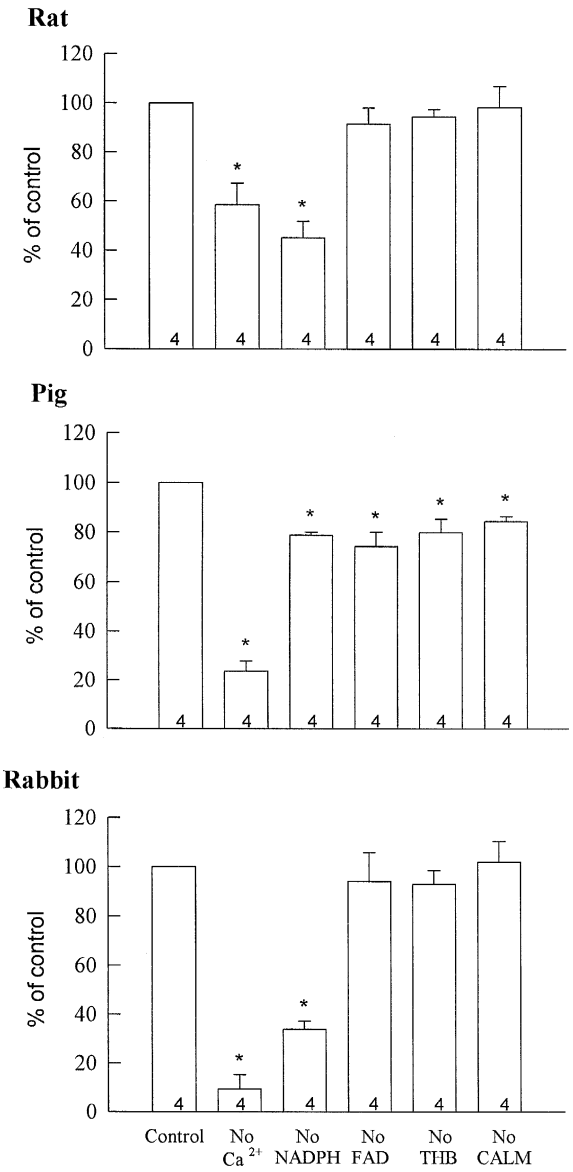

Fig. 2. The formation of [^{3}H]L-citrulline in the presence of L-valine (60 mM) in preparations of rat, pig and rabbit prostate glands in the absence of either 50 μM calcium (+4 mM ethylenediaminetetra-acetic acid), 1 mM NADPH, 2 μM flavin adenine dinucleotide (FAD), 1 μM tetrahydrobiopterin (THB) or 5 U/ml calmodulin (CALM). The production of [^{3}H]L-citrulline was expressed as a percentage of the corresponding control. Control values for each species in fmol citrulline per mg protein were: rat = 12.2 ± 0.6; pig = 37.3 ± 9.5; rabbit = 312.2 ± 56.2. Each column represents the means ± SEM; number of experiments (n) is indicated at the base of each column. A significant difference from control is indicated by an asterisk ($^*P < 0.05$).

effect in rat or rabbit prostate preparations. The omission of calmodulin had no effect in rat or rabbit prostate preparations and slightly but significantly reduced it in pig prostate preparations (Fig. 2).

3.5. Effect of cofactors on arginase-like activity

A preliminary characterisation of the arginase-like activity was carried out in preparations of rat and pig prostate glands. The addition, of L-valine (60 mM) significantly reduced [^{3}H]L-citrulline produced in the absence of calcium (4.80 ± 0.5, $n = 4$ and 4.50 ± 0.84, $n = 4$ fmol [^{3}H]L-citrulline per mg protein, respectively) or NADPH (4.98 ± 086, $n = 4$ and 18.11 ± 5.2, $n = 4$ fmol [^{3}H]L-citrulline per mg protein, respectively) when compared to control samples containing calcium (50 μM), NADPH (1 mM) and L-valine (60 mM) (9.58 ± 0.5, $n = 4$ and 24.74 ± 6.5, $n = 4$ fmol [^{3}H]L-citrulline mg protein).

4. Discussion

The total production of [^{3}H]L-citrulline from [^{3}H]L-arginine was considerably greater in rabbit prostate preparations than in those of pig or rat (the least). The nitric oxide synthase inhibitor N^G-nitro-L-arginine reduced [^{3}H]L-citrulline in preparations of rabbit, pig and rat prostates by 95, 38 and 19%, respectively, indicating the presence of nitric oxide synthase, but in greatly varying quantities between species. These findings are reflected in functional studies in which a nitrergic relaxation was readily demonstrable in the rabbit prostate, but not in the pig or rat prostates (unpublished observations). Taken together, the findings indicate the presence of neuronal nitric oxide synthase in the rabbit prostate, whereas the nitric oxide synthase in pig and rat prostates may be endothelial.

Passage of the supernatants from rat and pig prostate homogenates over 2 ml of Dowex ion exchange resin significantly increased [^{3}H]L-citrulline production. It has previously been shown that this procedure removes endogenous L-arginine and the endogenous inhibitor of nitric oxide synthase dimethylarginine (King et al., 1995). Therefore, the increased [^{3}H]L-citrulline production may be due either to the removal of dimethylarginine from the homogenate thus removing a restraint on nitric oxide synthase activity, or to the removal of endogenous L-arginine allowing a greater proportion of the [^{3}H]L-arginine to be converted to [^{3}H]L-citrulline rather than cold L-arginine to L-citrulline which would not be detected by the assay system, or to both factors.

The arginase inhibitor L-valine (Fuentes et al., 1994) significantly inhibited [^{3}H]L-citrulline production in preparations of rat and pig prostate indicating that some of the [^{3}H]L-arginine was utilised in the urea cycle to produce [^{3}H]L-citrulline and possibly other radiolabelled metabolites. The proportions of the total [^{3}H]L-citrulline formed by arginase were about 40% in rat and 60% in pig prostate preparations. In contrast, L-valine did not alter [^{3}H]L-citrulline production in rabbit prostate preparations.

Since arginase activity was detected in rat and pig prostate preparations, further characterisation of nitric oxide synthase activity in these was performed in the presence of L-valine. Nitric oxide synthase activity was markedly reduced in the absence of calcium (and in the presence of 4 mM disodium ethylenediaminetetra-acetic acid) in prostate preparations of all three species, indicating that the nitric oxide synthase was of the constitutive calcium-dependent type.

The omission of NADPH reduced nitric oxide synthase activity in preparations from all three species. Nitric oxide synthase activity in the rat and rabbit prostate gland preparations was not affected by the omission of exogenous flavin adenine dinucleotide, tetrahydrobiopterin or calmodulin, suggesting that the endogenous levels of these compounds in the preparations were sufficient for the requirements of the enzyme. In pig prostate preparations, nitric oxide synthase activity was slightly but significantly reduced by the omission of flavin adenine dinucleotide and tetrahydrobiopterin and calmodulin, suggesting that the endogenous levels were not adequate for optimal activity.

The arginase-like activity of rat and pig prostate preparations was not inhibited by the omission of calcium or NADPH from the reaction mixture.

In rabbit prostate preparations almost all (95%) of the [^{3}H]L-citrulline production was due to Ca^{2+}-dependent nitric oxide synthase. In pig prostate preparations, [^{3}H]L-citrulline production was almost entirely accounted for by the activities of a calcium-dependent nitric oxide synthase (40%) and an arginase-like enzyme (60%). However, in rat prostate preparations, nitric oxide synthase (20%) and an arginase-like enzyme (40%) contributed to [^{3}H]L-citrulline production, but 40% of the total [^{3}H]L-citrulline production was not due to either. The residual [^{3}H]L-citrulline production seen particularly in rat prostate preparations appears to be due to a so far unidentified L-arginine-utilising enzyme. Failure to recognise the presence of arginase or other enzymes that metabolise L-arginine may lead to misleading findings in biochemical assays of nitric oxide synthase activity.

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