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BIOSYNTHESIS OF NITRIC OXIDE AND CITRULLINE FROM L-ARGININE BY CONSTITUTIVE NITRIC OXIDE SYNTHASE PRESENT IN RABBIT CORPUS CAVERNOSUM

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SUMMARY: The objective of this study was to determine whether a constitutive isoform of nitric oxide (NO) synthase is present in rabbit corpus cavernosum that could account for the involvement of the L-arginine-NO pathway in neurogenically-elicited relaxation of the corpus cavernosum and, therefore, penile erection. Citrulline was determined by monitoring the formation of ^{3}H-citrulline from ^{3}H-L-arginine. NO was determined by monitoring the formation of total NO_x (NO + nitrite [NO_2^-] + nitrate [NO_3^-]) by chemiluminescence after reduction of NO_x to NO by acidic vanadium (III). Equimolar quantities of NO plus citrulline were generated from L-arginine and the formation of both products was time-dependent at 37°C. NO synthase activity was distributed almost entirely to the cytosolic fraction. Enzymatic activity was completely dependent on NADPH, calmodulin, and calcium. Addition of tetrahydrobiopterin increased NO synthase activity by about 30 percent. The NO synthase inhibitor N^G-nitro-L-arginine, abolished enzymatic activity. The Km for L-arginine was 17 μM and the Vmax of the reaction was 18 pmol/min/ mg protein. These observations indicate that a cytosolic, constitutive isoform of NO synthase, like that found in brain neuronal tissue, is present in rabbit corpus cavernosum.

Studies from this (1-3) laboratory and other laboratories (4-7) have shown that electrical stimulation of isolated preparations of corpus cavernosum from the penis of rabbits and humans elicits smooth muscle relaxation that is attributed to activation of the L-arginine-NO-cyclic GMP pathway. The corpus cavernosum is an extensive trabecular network of arterioles consisting of smooth muscle and a single layer of endothelial cells lining the intimal surface. Vascular smooth muscle relaxation results in filling of the corpus cavernosum with blood and, therefore, penile erection. Electrical stimulation results in excitation of the nonadrenergic-noncholinergic (NANC) neurons innervating the corpus cavernosum, and the consequence is inhibition of vascular smooth muscle tone and development of a relaxation response (8,9). NANC neurotransmission triggers the

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biosynthesis of NO from L-arginine and NO activates guanylate cyclase, thereby stimulating cyclic GMP formation in the vascular smooth muscle and initiating relaxation (1). Although the identity of the neurotransmitter of the NANC neuronal pathway in the corpus cavernosum is unknown (10), NO is a good candidate as NO synthase has been shown to be localized in the NANC neurons innervating the small intestine and bovine retractor penis (11,12).

The objective of this study was to determine whether a constitutive, calcium-calmodulin activated, isoform of NO synthase is present in the cytosolic fraction from corpus cavernosum. The presence of such a cytosolic enzyme system would account for the involvement of the L-arginine-NO pathway in NANC-mediated relaxation of the corpus cavernosum. Neuronal NO synthase in the cerebellum is localized to the cytosolic fraction (13,14), whereas NO synthase in vascular endothelial cells is localized to the membrane fraction (15). Since electrically-elicited relaxation of the corpus cavernosum is endothelium-independent (1,4), and NO synthase is essentially absent from smooth muscle cells unless enzyme synthesis is induced by the presence of cytokines, a constitutive isoform of NO synthase should be present in the cytosolic fraction from NANC neuronal tissue. In the present study the entire corpus cavernosum, consisting primarily of smooth muscle, endothelial cells, connective tissue, and neuronal tissue, was examined for the presence of NO synthase activity.

MATERIALS AND METHODS

Chemicals and solutions: L-Arginine, L-citrulline, NADPH, calmodulin, calmidazolium, trifluoperazine, dithiothreitol, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, N^G-nitro-L-arginine, EDTA, and EGTA were purchased from Sigma Chemical Co. Tetrahydrobiopterin was purchased from Schircks laboratories (Jona, Switzerland). Dowex AG50W-X8 (H^+ form) 100-200 mesh, Dowex AG 1-X8, acetate form, 100-200 mesh, and Tris base (electrophoresis grade) were purchased from Bio-Rad Laboratories. Vanadium (III) chloride was obtained from Aldrich Chemical Co. Sodium nitrite and sodium nitrate were obtained from Fisher Chemical Co. Aquasol-2 was purchased from Du Pont Company/NEN Research Products.

NO determination: NO was determined as total NO_x generated in the enzymatic reaction, which includes NO + NO_2^- + NO_3^-. Almost all of the NO_x was present as the oxidized species, NO_2^- + NO_3^-. NO in oxygen-containing solutions is chemically unstable and undergoes rapid oxidation to NO_2^-. The presence of biological tissue catalyzes this oxidation and promotes further oxidation of NO and NO_2^- to NO_3^- (16). Measurement of all three species is necessary in order to determine NO accurately. NO_2^- + NO_3^- were measured by chemiluminescence after sample reduction in boiling acidic vanadium (III) by a modification of a method described previously (17). Acidic vanadium (III) at 98°C quantitatively reduces both NO_2^- and NO_3^- to NO, which is quantified by a chemiluminescence detector (Dasibi Chemiluminescence NO_x Analyzer, model 2108; Glendale, CA) after reaction with ozone. Samples (100 μl) were injected with a gas-tight syringe into 100 ml of 0.1 M vanadium (III)

chloride in 2 N HCl maintained at 98°C under an atmosphere of oxygen-free nitrogen. Signals from the detector were analyzed with a Hewlett Packard HP 3396 Series II Integrator and recorded as areas under the curve. Extensive experimentation was conducted to validate and standardize this procedure for the quantification of varying proportions of NO_2^- and NO_3^- in a common solution. Standard curves for NO_2^-, NO_3^-, and combinations of both anions were linear over a broad concentration range of 100 pmol to 4 nmol of NO_x^- (NO_2^- and/or NO_3^-). All sample concentrations of NO_x^- fell within this range.

Citrulline determination: Citrulline was determined by monitoring the formation of ^{3}H-citrulline from ^{3}H-L-arginine by a modification of a procedure described previously (13). Samples (2 ml) prepared as described below were applied to columns (1 cm diameter) containing 1 ml of Dowex AG50W-X8, Na^+ form, 100-200 mesh (prepared from the H^+ form), that had been pre-equilibrated with 20 mM sodium acetate, pH 5.5, containing 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA (Stop Buffer). The eluate (2 ml) was collected into a liquid scintillation vial. Columns were eluted with 2 ml of water and collected into another vial. Aquasol-2 (10 ml) was added to each vial and samples were counted in a Beckman LS 3801 liquid scintillation spectrometer. Citrulline was recovered in the first 4 ml of Dowex column eluate to the extent of 96%, and data were corrected to account for such recovery.

Protein determination: Protein concentrations in tissue fractions were determined by the Bradford, Coomassie brilliant blue method as described by Bio-Rad. Bovine serum albumin was used as the standard.

NO synthase assay: Rabbit corpus cavernosum was used as the source of NO synthase. Rabbits (New Zealand White, males, 3 kg) were sacrificed by decapitation and the corpora cavernosa were excised, cleaned of the fibrous tunica albuginea, rinsed and stored frozen at -75°C. Homogenates (25% w/v) of corpus cavernosum were prepared in 50 mM Tris HCl, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 2 μM leupeptin at 0-4°C with the aid of a tissue grinder fitted with a ground glass pestle. In most of the experiments, homogenates were centrifuged at 20,000 x g for 60 min at 4°C and the supernatant was used as the source of NO synthase. In the subcellular distribution study, various tissue fractions were tested as indicated in the text. Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine, 100 μM NADPH, 10 μM tetrahydrobiopterin, 2 mM $CaCl_2$, 1 μg calmodulin, 0.10 - 0.40 mg tissue fraction protein, and other test agents as indicated, in a final incubation volume of 100 μl. Samples that were analyzed for citrulline also contained from 200,000 dpm to 400,000 dpm of L-[2,3,4,5-^{3}H]arginine HCl (77 Ci/mmol; Amersham) that was previously purified by anionic exchange chromatography on columns of Dowex AG 1-X8, OH^- form (prepared from the acetate form, 100-200 mesh in order to remove traces of ^{3}H-citrulline (15). Enzymatic reactions for the determination of citrulline were terminated by addition of 2 ml of ice-cold Stop Buffer, and samples were chromatographed as described above. Enzymatic reactions for the determination of NO did not contain ^{3}H-L-arginine and were terminated by addition of 200 μl of ice-cold 50 mM Tris HCl, pH 7.4, containing 10 mM EDTA. Aliquots of 100 μl were assayed for NO_x by chemiluminescence after chemical reduction to NO as described above.

RESULTS AND DISCUSSION

Preliminary experiments were conducted with the supernatant fraction derived from centrifugation of homogenates at 20,000 x g for 60 min at 4°C. These initial experiments

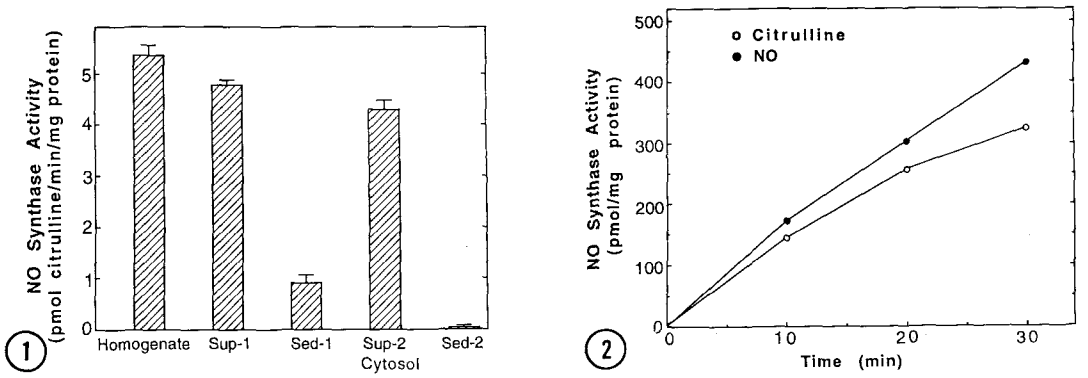

Fig. 1. Subcellular distribution of NO synthase activity in rabbit corpus cavernosum. Tissue homogenates were prepared and aliquots were removed for NO synthase and protein assays. The homogenate was then centrifuged at 10,000 x g for 20 min to yield the supernatant and sediment fractions. The sediment was resuspended in homogenizing buffer to a volume equivalent to that of the starting homogenate and assayed (Sed-1). An aliquot of the supernatant was assayed (Sup-1) and the remainder was centrifuged at 100,000 x g for 60 min to yield the supernatant and sediment fractions. The sediment was resuspended in homogenizing buffer to a volume equivalent to that of the starting volume of Sup-1 and assayed (Sed-2). The supernatant was also assayed (Sup-2; Cytosol). Enzymatic reactions were conducted at 37°C for 30 min in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine, 100 μM NADPH, 10 μM tetrahydrobiopterin, 2 mM $CaCl_2$, 1 μg calmodulin, and appropriate tissue fractions in a final volume of 100 μl. Values are expressed as pmol citrulline formed per min per mg of original starting homogenate protein. Data represent the mean ± S.E.M. of 4 - 6 determinations from 3 separate experiments.

Fig. 2. Time course of NO and citrulline formation from L-arginine by NO synthase from rabbit corpus cavernosum. Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 100 μM L-arginine, 100 μM NADPH, 10 μM tetrahydrobiopterin, 2 mM $CaCl_2$, 1 μg calmodulin, and supernatant (20,000 x g x 60 min) containing 0.36 - 0.39 mg protein. Reaction mixtures that were assayed for ^{3}H-citrulline contained approximately 400,000 dpm of L-[2,3,4,5-^{3}H]arginine. Data points represent mean values of 4 determinations from 2 separate experiments.

revealed that NADPH, tetrahydrobiopterin, and calmodulin had to be added to enzyme reaction mixtures in order to obtain maximal rates of conversion of L-arginine to citrulline. A subcellular distribution study was conducted to determine the localization of NO synthase in rabbit corpus cavernosum. Fig. 1 illustrates that most of the enzymatic activity of the starting homogenate was present in the cytosolic fraction. The low activity in Sed-1 likely represents contaminating cytosol. Despite the fact that the entire corpus cavernosum, a heterogeneous mixture of cells including endothelial cells, was analyzed, little or no NO synthase activity was found in the membrane or particulate fractions. Endothelium-derived NO synthase is primarily membrane-bound, whereas neuronally-derived NO synthase is primarily cytosolic or soluble (13-15). The constitutive isoform of NO synthase is essentially absent from smooth muscle cells. Therefore, the principal localization of the constitutive

isoform of NO synthase in corpus cavernosum to the cytosolic fraction represents indirect evidence that NO synthase is distributed primarily to neuronal tissue, perhaps the NANC neurons that innervate the corpus cavernosum smooth muscle cells. Immunocytochemical studies are in progress with polyclonal antibody raised to cytosolic NO synthase purified from rat cerebellum to determine whether NO synthase in the corpus cavernosum is localized to neuronal structures.

Subsequent experiments were conducted with the supernatant fraction derived from centrifugation of homogenates at 20,000 x g for 60 min. This fraction was studied in order to avoid repeated use of an ultra-centrifuge, and is justified on the basis that the microsomal fraction, which is a contaminant in this 20,000 x g supernatant fraction, was essentially devoid of NO synthase activity (Fig. 1). The constitutive isoform of NO synthase catalyzed the conversion of L-arginine to equimolar quantities of NO plus citrulline (Fig. 2). Since the chemical half-life of NO is only several seconds under assay conditions in the required presence of oxygen, it is not possible to monitor the formation of NO itself. NO undergoes spontaneous oxidation to NO_2^- in the presence of oxygen and catalyzed oxidation to NO_3^- in the presence of superoxide anion, contaminating oxyhemoglobin and other oxyhemoproteins, and other tissue-derived oxidants (16). Both NO_2^- and NO_3^- are quantitatively reduced back to NO by refluxing in acidic vanadium (III), as discussed in Materials and Methods. Thus, the determination of both NO_2^- and NO_3^- accumulation in a sample represents a direct measure of any NO that had been formed in that sample.

Experiments were conducted in which completely supplemented enzyme reaction mixtures were compared with reaction mixtures deficient in NADPH or tetrahydrobiopterin, containing calmodulin antagonists, or containing an NO synthase inhibitor (Fig. 3). The constitutive isoform of NO synthase from corpus cavernosum required the addition of NADPH for expression of enzymatic activity. Addition of tetrahydrobiopterin to enzyme reaction mixtures increased NO synthase activity by about 30 percent. Calmidazolium and trifluoperazine, both of which are calmodulin antagonists, nearly abolished NO synthase activity. N^G-substituted analogs of L-arginine act as competitive inhibitors of NO synthase activity (18,19). Fig. 3 illustrates that N^G-nitro-L-arginine nearly abolished NO synthase activity.

Experiments were conducted to determine the Km for L-arginine and the Vmax of the enzymatic reaction. Kinetic experiments were conducted under initial velocity conditions, where the substrate concentration was not limiting. A double reciprocal plot of velocity versus substrate concentration was constructed and a straight line was obtained. The Km for L-arginine was 17 μM and the Vmax of the enzymatic reaction was 18 pmol/min/mg protein. The relatively low Km for L-arginine is consistent with the low Km

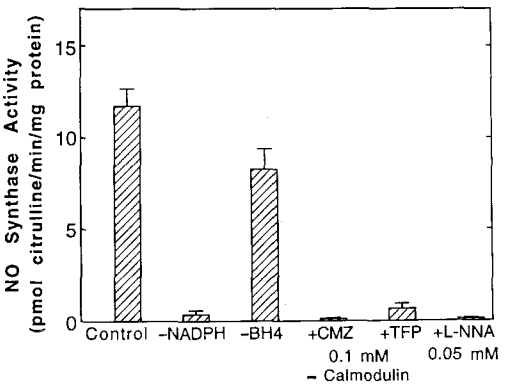

Fig. 3. Dependence of citrulline formation from L-arginine by NO synthase from rabbit corpus cavernosum on NADPH, tetrahydrobiopterin and calmodulin, and inhibition by N^G-nitro-L-arginine. Enzymatic reactions were conducted at 37°C for 30 min in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine, 100 μM NADPH (except -NADPH), 10 μM tetrahydrobiopterin (BH4) (except -BH4), 2 mM $CaCl_2$, 1 μg calmodulin (except - calmodulin), and supernatant (20,000 x g x 60 min) containing 0.21 - 0.39 mg protein. Certain reaction mixtures contained calmidazolium (CMZ), trifluoperazine (TFP), or N^G-nitro-L-arginine (L-NNA) as indicated. Reaction mixtures that were assayed for ^{3}H-citrulline contained from 200,000 dpm to 400,000 dpm of L-[2,3,4,5-^{3}H]arginine. Data represent the mean ± S.E.M. of 4 - 16 determinations from 2 - 7 separate experiments.

reported with NO synthase from other cell sources (20). The Vmax of NO synthase from corpus cavernosum is similar to the Vmax of NO synthase from another NANC-innervated smooth muscle preparation, the anococcygeus (21).

This study reveals that rabbit corpus cavernosum contains a constitutive isoform of NO synthase that is distributed primarily to the cytosolic fraction of cells. Like other constitutive isoforms found in brain and vascular endothelium, NO synthase from corpus cavernosum is dependent on NADPH, calcium, and calmodulin, and enzymatic activity is greater in the presence of tetrahydrobiopterin. NO synthase catalyzes the conversion of L-arginine to equimolar quantities of NO plus citrulline, and enzymatic activity is markedly inhibited or abolished by NO synthase inhibitors and calmodulin antagonists. The cytosolic NO synthase present in corpus cavernosum accounts for the observations from this (1-3,10) and other (4-7) laboratories that NANC neuronal stimulation of corpus cavernosum causes smooth muscle relaxation by mechanisms attributed to the L-arginine-NO-cyclic GMP pathway.

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