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Nitric Oxide Synthase from Bovine Pancreas: Purification and Characterization

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Nitric oxide synthase, NOS (EC.1.14.13.39), was purified from bovine pancreas over 5,500-fold with a 7.6% yield using 30% ammonium sulfate precipitation, and 2',5'-ADP-agarose and calmodulin-agarose affinity chromatography. The purified bovine pancreatic NOS (bpNOS) showed a single band on SDS-PAGE corresponding to an apparent molecular mass of 160 kDa, whereas it was 320 kDa on non-denaturating gel-filtration. This indicated a homodimeric nature of the enzyme. The specific activity of the purified bpNOS was 31.67 nmol L-citrulline fored/mtn/mg protein and an apparent K_m for L-arginine was 15.72 μM. The enzyme activity was dependent on Ca^{2+} and calmodulin, and to a lesser extent on NADPH, FAD and FMN. H_4B was not required as a cofactor for the activity. In an inhibition experiment with L-arginine analogues, N^G-nitro-L-arginine (NNA) had the most potent inhibitory effect on bpNOS, and N^G, $N^{G'}$-dimethyl-L-arginine (symmetric; sDMA) did not have any inhibitory effect. Immunohistochemical analysis of the bovine pancreas using brain type NOS antibody (anti-bNOS antibody) revealed that acinar cells showed strong immunoreactivity against the antibody.

Key words : Nitric oxide, Nitric oxide synthase, Purification, Exocrine secretion, Pancreas

INTRODUCTION

Nitric oxide (NO) has diverse physiological functions such as cell signaling in the cardiovascular, central and peripheral nervous system, and as an antimicrobial and antitumorigenic action in the cell mediated immunity (Moncada *et al.*, 1991; Bredt and Snyder, 1992). The enzyme responsible for the synthesis of NO from L-arginine, nitric oxide synthase, NOS (EC. 1. 14. 13. 39) (Nathan and Xie, 1994), has been demonstrated in the endothelium (Moncada *et al.*, 1988; Furchgott and Vanhoutte, 1989), brain (Ignarro, 1989), macrophage (Marletta *et al.*, 1988), neutrophils (Nathan, 1992), and liver (Evans *et al.*, 1992). NOS has been purified from various tissues and cell types of different species and was found to exist in several isoforms, three of which have been cloned (Stuehr and Griffith, 1992; Förstermann *et al.*, 1991a). The activities of all three isoforms are distributed among the soluble and particulate fractions of cells: Isoform I from brain (bNOS) and isoform II from cytokine-induced macrophage (mNOS) are mostly soluble proteins (Bredt and Snyder, 1990; Schmidt *et al.*, 1991; Stuehr *et al.*, 1991; Hevel *et al.*, 1991), whereas isoform III from endothelial cells (eNOS) is myristoylated and predominantly in the particulate fraction (Förstermann *et al.*, 1991b; Pollock *et al.*, 1992). In addition to its various roles in physiological functions, NO has also been implicated in pancreatic exocrine secretion (Gukovskaya and Pandol, 1994; Xu *et al.*, 1994; Gokovskaya and Pandol, 1995; Wrenn *et al.*, 1994). Recently, Gukovskaya and Pandol (1994) have obtained an evidence to show that NO is produced in response to agonists in pancreatic acini and is responsible for mediating an increase in cGMP caused by the agonist. Several recent reports confirmed the earlier observation of Gukovskaya and Pandol (1994) and suggested NO to be a putative secretory mediator molecule (Gokovskaya and Pandol, 1995; Wrenn *et al.*, 1994). Nevertheless, the NO-producing enzyme in pancreas has never been purified to homogeneity and has never been investigated at molecular level. Furthermore, subcellular localization of the enzyme in pancreas has not yet been studied with immunohistochemically. In order to understand the physiological and pathophysiological roles of NO as a secretory mediator in pancreatic exocrine secretion, we felt of great importance to identify and characterize the NOS ex-

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pressed in the pancreas. Therefore, we purified and characterized NOS from bovine pancreas in the present study, and further localization of NOS in pancreatic cells was examined by immunohistochemical method.

MATERIALS AND METHODS

Materials

L-[2,3,4,5-^{3}H]-L-arginine monohydrochloride (57Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). Pepstatin A, leupeptin, chymostatin, antipain, aprotinin, calmodulin, NADPH, FAD, FMN, 2'5'-ADP agarose resin, calmodulin agarose resin, Tris, N^G-monomethyl-L-arginine (MMA), N^G-nitro-L-arginine (NNA), N^G, N^G-dimethyl-L-arginine (asymmetric; aDMA), N^G,$N^{G'}$-dimethyl-L-arginine (symmetric; sDMA) and N^G-nitro-L-arginine-methylester (NAME) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein Pak 300SW was from Waters (Milford, MA, U.S.A.). Anti-bNOS (brain) antibody was from Transduction Laboratories (Lexington, Kentucky, U.S.A.). Goat anti-rabbit antibody and alkaline phosphatase conjugate substrate kit were from Bio-Rad (Hercules, CA, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Assay of bpNOS activity

Activity of bpNOS was determined by a method based on the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described previously (Bredt and Snyder, 1990) with minor modification. Briefly, the incubation mixture contained 50 μL of an enzyme sample, 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1.5 mM $CaCl_2$, 1 mM NADPH, 2 μg calmodulin, 100 μM H_4B, 10 μM FAD, 10 μM FMN, and 25 μM L-arginine (approximately 200,000 dpm of L-[2,3,4,5-^{3}H]-L-arginine HCl) in a total volume of 200 μL and the mixture was incubated for 8 min at 37°C. The reaction was terminated by the addition of 1 mL of ice-cold Dowex-50W (Na^+ form, 200-400 mesh) which had been preequilibrated with 20 mM sodium acetate buffer (stop reaction buffer; pH 5.5), containing 1 mM L-citrulline, 2 mM EDTA and 0.2 mM EGTA, and the reaction mixtures were left for 5 min followed by centrifugation at 11,000×g for 10 min. The supernatant was collected into a suitable water-miscible scintillate and the radioactivity was counted in a PHARMACIA 1209 Rackbeta liquid scintillation counter. The specific activity was expressed as nmol L-citrulline formed/min/mg protein.

Purification of bpNOS from bovine pancreas

The enzyme was purified from 60 g of fresh bovine pancreas from a local slaughterhouse by the methods of Bredt and Snyder (1990) and Seo *et al.* (1995) with minor modifications. After washing with saline, bovine pancreases were carefully trimmed off lipids, blood vessels and ducts and then kept immediately in -70°C deep freezer. Frozen bovine pancreases were thawed and homogenized in 4 volumes of ice-cold buffer A (50 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, 100 mg/L PMSF, 10 mg/L soybean trypsin inhibitor, 2.5 mg/L pepstatin A, 2.5 mg/L leupeptin, 2.5 mg/L chymostatin, 2.5 mg/L antipain, 2.5 mg/L aprotinin, pH 7.4). All subsequent procedures were carried out at 4°C. The homogenate was centrifuged at 100,000×g for 60 min. Solid ammonium sulfate (176 g/L, corresponding to 30% saturation) was added to the resulting supernatant, followed by centrifugation for 20 min at 10,000×g. The precipitate was resuspended in buffer B (10 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 100 mg/L PMSF, 10 mg/L soybean trypsin inhibitor, 2.5 mg/L pepstatin A, 2.5 mg/L leupeptin, 2.5 mg/L chymostatin, 2.5 mg/L antipain, 2.5 mg/L aprotinin, pH 7.4). After centrifugation for 20 min at 10,000×g, the supernatant was carefully decanted and mixed with 2 mL of 2',5'-ADP-agarose which had been equilibrated with buffer B. The slurry was stirred for 30 min and subsequently transferred to a fritted column. The column was washed with about 20 column volumes of buffer B containing 0.5 M NaCl, and then with buffer B alone. Then bpNOS was eluted with buffer B containing 10 mM NADPH. Protein-containing fractions were pooled and equilibrated with buffer C (50 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 100 mg/L PMSF, 1.5 mM $CaCl_2$, pH 7.4) with ultrafiltration membrane values of 30,000 cut-off. The equilibrated pool from the 2',5'-ADP-agarose was incubated with calmodulin-agarose and non-coupled Sepharose 4B, 1:8 (v/v) which had been equilibrated with buffer C, for 15 min at 4°C and then transferred to fritted column. The column was washed with about 20 column volumes of buffer C and successively 20 column volumes of buffer C containing 0.3 M NaCl. Finally, bpNOS was then eluted with buffer D (50 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 100 mg/L PMSF, 1 M NaCl, 10% glycerol, 5 mM EGTA, pH 7.4).

Gel filtration chromatography

To estimate the native molecular weight of bpNOS, 10 mM NADPH eluate from the 2',5'-ADP-agarose column was concentrated using a Ultrafree-15 Centrifugal Filter Device (Millipore, U.S.A.). The concentrated sample was passed through a Protein Pak 300SW (Waters, U.S.A.) gel filtration column, which had been equilibrated with buffer E (50 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 100 mg/L PMSF, 0.15

M NaCl, pH 7.4) or buffer F (50 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 100 mg/L PMSF, 0.35 M NaCl, pH 7.4). This procedure was performed with a FPLC system (Pharmacia LKB Biotechnology, Sweden) at 4°C. Fractions (0.4 mL) were collected at a flow rate of 0.2 mL/min and protein were determined at 280 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native gel electrophoresis

Mini-protein II electrophoresis system (Bio-Rad, U.S.A.) was employed according to the manufacturer's instruction.

Western blot analysis

Western blot analysis was performed by the method described (Towbin *et al.*, 1979) with a minor modification. Samples were electrophoresed on 7.5% or 6% polyacrylamide slab gels at pH 8.3. Proteins were subsequently transferred to PVDF (Millipore, U.S.A.) membrane for 2 h at 60 mA with Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, U.S.A.). Each membrane was blocked by incubation with 5% skim milk in Tris-buffered saline (TBS), pH 7.4, for 1 h at room temperature by gentle shaking. After washing with 0.1% Tween-20 and 0.2% sodium azide in TBS (TTBS), the membrane was reacted overnight at room temperature with a commercially obtained anti-bNOS antibody (first antibody) diluted to 1:500 in TTBS and was then incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit antibody (second antibody) diluted to 1:1000 in TTBS. After washing with TTBS, the membrane was processed in 10 mL of a solution containing 5-bromo-4-chloroindolyl phosphate (BCIP), nitrobluetetrazolium (NBT) and color development reagent. Depending on the coloring of the membrane, development was terminated with TTBS solution.

Immunohistochemical study of NOS in bovine pancreas

The bovine pancreatic tissue was immunostained by the methods of Stein *et al.* (1985) with minor modification. The fresh bovine pancreas was fixed with neutral buffered formalin (NBF). The fixed tissue was dehydrated with serial gradient concentration of ethanol and then with xylene. After paraffin infiltration and embedding, fixed tissue was cut in 7~8 μm sections and a section was placed on cleaned glass slides. After deparafinization with xylene, the section was dehydrated, washed with well running water and then transferred to TBS solution. Dehydrated sections was incubated with primary antibody (anti-bNOS antibody) at the appropriate dilution for 45 min and then the excess antiserum was washed off with TBS. The sample was further incubated in alkaline phosphatase-conjugated secondary antibody at the appropriate dilution for 45 min. The sample was then washed in TBS and subsequently incubated with developing buffer containing NBT and BCIP. Finally, the sample was counterstained with hematoxylin and mounted as appropriately.

Protein determination

Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of NOS from bovine pancreas

The purification of bpNOS was accomplished by four step procedures as summarized in Table I; namely 100,000×g supernatant, 30% ammonium sulfate precipitate, 2',5'-ADP-agarose and calmodulin-agarose affinity chromatography. The data presented in Table I are one of the several typical experiments under the same conditions and were highly reproducible. The bpNOS was unstable in the crude supernatant, and its instability was not improved by various commercially available protease inhibitors. The purified NOS preparation had a specific activity of 31.67 nmol/min/mg, representing 5,500-fold enrichment from the crude 100,000×g supernatant fraction with an overall recovery of 7.6%. This relatively poor yield during purification was most likely due to the instability of the

Table I. Purification of NOS from bovine pancreas

Fraction	Protein (mg)	Total activity (nmoles of L-citrulline/min)	Specific activity (nmoles of L-citrulline/min/mg protein)	Yield (%)	Purification folds
100,000×g Supernatant	1,863	10.54	0.00566	100	1
30% Ammonium-sulfate fraction	205	5.76	0.0281	55	5
2',5'-ADP agarose	1.1	3.23	2.919	30.6	515
Calmodulin agarose	0.0254	0.805	31.67	7.6	5,595

The enzyme activity was determined by conversion of L-arginine to L-citrulline as described. The data are one of three independent procedures and were highly reproducible.

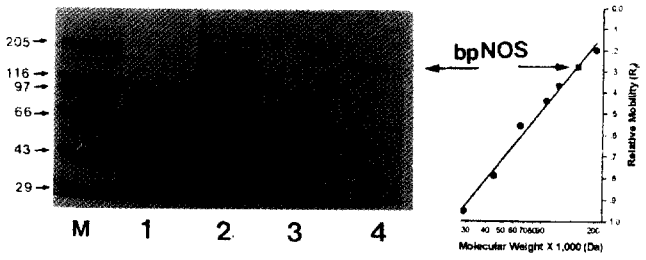

Fig. 1. SDS-PAGE analysis of purified bovine pancreatic NOS. A: 7.5% polyacrylamide gel was stained with Coomassie blue. Lanes: M (molecular weight standard), myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa); lane 1, 100,000×g supernatant; lane 2, 30% ammonium sulfate precipitates; lane 3, 10 mM NADPH eluates on 2',5',-ADP-agarose; lane 4, 5 mM EGTA eluates on calmodulin agarose. Apparent molecular mass of the single protein band in the purified bpNOS preparation is designated by the closed square (■) and is about 160 kDa.

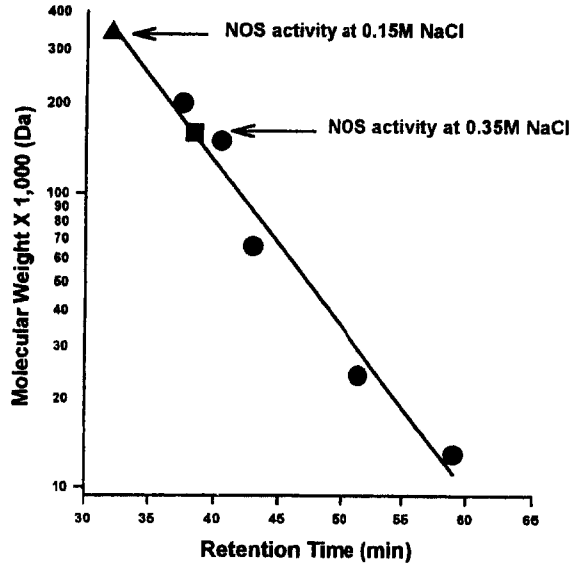

Fig. 2. Estimation of native molecular mass of bovine pancreatic NOS with gel filtration chromatography. Gel filtration with purified bpNOS was conducted with different salt concentrations on Protein Pak 300SW column (Waters, U.S.A.). When eluted with buffer containing 0.15 M NaCl, bpNOS activity was eluted at the region corresponding to a molecular mass of 320 kDa. When eluted with 0.35 M NaCl containing buffer, bpNOS activity was eluted at the region corresponding to a molecular mass of 160 kDa. The apparent molecular mass of the native bpNOS is indicated by arrows at each salt concentration. Molecular weight standards were used β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

enzyme caused by loss of cofactors or stabilizing factors. The enzyme was apparently homogeneous, since on SDS-PAGE, the active fraction showed a single band corresponding to a molecular mass of 160 kDa (Fig. 1). It should be noted that an isoform of NOS called I (bNOS) which is constitutively expressed in brain was first purified from rat and porcine cerebellum (Bredt and Snyder, 1990; Schmidt *et al.*, 1991; Mayer *et al.*, 1990). This isoform is a soluble enzyme with a molecular mass of 150 to 160 kDa on SDS-PAGE (Bredt and Snyder, 1990; Schmidt *et al.*, 1991). Thus, bpNOS is very similar to bNOS in that they are both soluble and their molecular mass on SDS-PAGE is about 150~160 kDa.

Characterization of bpNOS

Estimation of native molecular weight of bpNOS: Although denatured forms of NOS, whether constitutive or inducible, have been reported to have molecular weights in the range of 130 to 160 kDa, the native proteins showed a molecular weight ranging about 200 to 300 kDa (Bredt and Snyder, 1990; Schmidt *et al.*, 1991; Stuehr *et al.*, 1991). Thus, to estimate the molecular mass of presently purified bpNOS in its native state, gel filtration chromatography on Protein-Pak 300SW column (Waters, U.S.A.) eluted with different salt concentrations was conducted. In the protein profiles of buffer E containing 0.15 M NaCl elution, bpNOS activity was eluted in the region of 320 kDa while the enzyme activity was detected in the region of 160 kDa with high salt concentration (0.35 M NaCl) of buffer F (Fig. 2). Since, the non-denaturing gel electrophoresis analysis did not show a band of 160 kDa but showed a band corresponding to 320 kDa, the native bpNOS seems to be a homodimer of 160 kDa subunit that easily associates into a dimer. Although Western blot analysis with an anti-brain type NOS antibody (anti-bNOS antibody) did not manifest a single immunoreactive band on active fraction of gel filtration eluate, a strong immunoreactive band corresponding to an apparent molecular mass of 160 kDa was detected (Fig. 3C). This Western blot analysis of bpNOS with a major immunoreactive band at the region of 160 kDa corresponded well with that of rat cerebellum NOS. In the case of non-denaturing polyacrylamide gel, bpNOS was also cross-reacted with an anti-bNOS antibody and showed a immunoreactive band corresponding to an apparent molecular mass of 320 kDa (Fig. 3D). It is concluded that bpNOS exists as a homodimer (about 320 kDa) of two identical subunit (about 160 kDa), as determined by gel filtration chromatography and Western blot analysis.

Cofactor dependency of bpNOS: Table II presents the cofactor dependency of the purified bpNOS. The purified enzyme was found to be dependent on Ca^{2+}, NADPH and calmodulin for its activity and also dependent on FAD and FMN to a lesser extent, however, independent of H_4B. Schmidt *et al.* (1992) reported that the cofactor requirement varied depend-

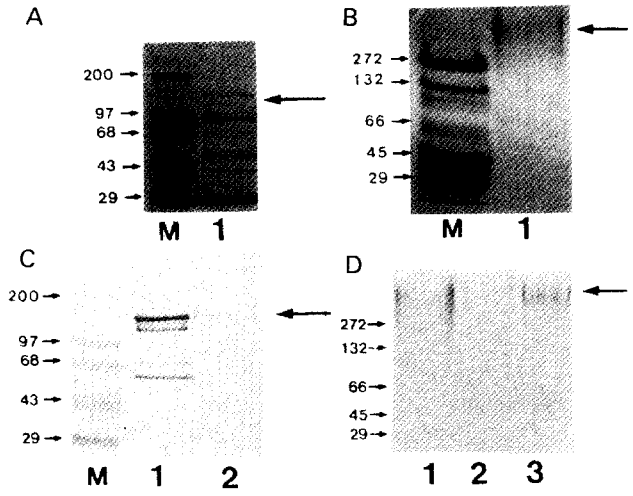

Fig. 3. Comparisons of SDS-PAGE, native gel electrophoresis and Western blot analysis of bovine pancreatic NOS activity fraction on gel filtration chromatography. SDS-PAGE (A) and native gel electrophoresis (B) were carried out with purified bpNOS, and Western blot analysis was also conducted with the same gels (C,D). Purified bpNOS indicated by arrows on SDS-PAGE and native gel electrophoresis exhibited at 160 kDa and 320 kDa region, respectively. Western blot analysis with the same gels showed identical position as with SDS-PAGE or native gel electrophoresis, and bpNOS band was shown in the identical position with NOS of 100,000×g supernatant of rat cerebellum. A: A 7.5% polyacrylamide gel was stained with Coomassie blue. Lane M (molecular weight standard): myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), Lane 1: NOS activity fraction on gel filtration. B: A 6% polyacrylamide gel for native gel electrophoresis was stained with Coomassie blue. Lane M (molecular weight standard): urease trimer (272 kDa), bovine serum albumin trimer (132 kDa), bovine serum albumin dimer (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa); 1, NOS activity fraction on gel filtration. C: Western blot analysis of partially purified bpNOS with SDS-polyacrylamide gel. Lane M (molecular weight standard): myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), Lane 1, bpNOS after gel filtration; Lane 2, 100,000×g supernatant of rat cerebellum. D: Western blot analysis of partially purified bpNOS with native gel. Lane 1, bpNOS after gel filtration; Lane 2, 30% ammonium sulfate fraction; Lane 3, 100,000×g supernatant of rat cerebellum.

ing on the purification procedures employed and suggested that cofactors are lost during isolation with some procedures. As shown in Table II, as well as by others, all the constitutive forms of NOS have been shown to require Ca^{2+} and calmodulin for their activities and intracellular Ca^{2+} levels therefore can stringently regulate constitutive NOS activity in the cells. The result in the Table II suggests that bpNOS is Ca^{2+}/calmodulin dependent isoform of NOS like as bNOS or eNOS.

Enzymatic characterization of bovine pancreatic NOS: Analogues of L-arginine represent the largest and potentially most useful class of NOS inhibitors and a variety of L-arginine analogues have been found to be inhibitors of NOS activity. Most widely utilized and studied of these analogues is N-methyl-arginine (Hibbs *et al.*, 1994). An another widely used arginine analogue whose mechanism of inhibition has been studied is NNA. NOS isoforms display modest differences in their sensitivity to various L-arginine analogues (Bredt and Snyder, 1994). For inhibition studies on bpNOS activity, two L-arginine analogues were divided into two groups, one of which are synthetic group such as NNA, NAME and the other native inhibitor group such as MMA, aDMA and sDMA.

In an inhibition experiment using L-arginine analogues on bpNOS, NNA was found to be most potent inhibitor on bpNOS, being times more potent than MMA (Table III). Interestingly, aDMA was found to be more potent than MMA on bpNOS. The L-arginine analogues were also examined for their kinetic properties on bpNOS (Fig. 4). Both native and synthetic inhibitor groups showed competitive inhibition pattern evidenced by Lineweaver-Burk plot, but sDMA did not have inhibitory effect on bpNOS. Finally, the

Table II. Cofactor dependency of NOS from bovine pancreas

Cofactor omitted dependency	L-citrulline formation in the absence of cofactor (nmol/min/mg protein)	Enzyme activity (%)	Relative (%)
None	1.80	100	
Ca^{2+}	N.D[a]	N.D	100
NADPH	0.42	23	77
Calmodulin	0.20	11	89
FAD, FMN	1.16	64	36
H_4B	1.82	100	-

Each cofactor was replaced with H_2O in the reaction mixture, and the activity was assayed with 10 mM NADPH eluates on 2',5'-ADP agarose affinity chromatography.
[a]N.D., Not detected.

Table III. Characterization of bovine pancreatic NOS

Parameter	Value
Denatured molecular mass (kDa)	160
V_{max}, nmol of citrulline per mg protein	7.82
K_m (L-arginine), μM	15.72
K_i (NNA), μM	1.05[†]
K_i (NAME), μM	15.46[†]
K_i (MMA), μM	5.42[¶]
K_i (aDMA), μM	3.38[¶]
K_i (sDMA), μM	N.I.[a¶]
Optimum temperature (°C)	37
Optimum pH	7.4

The enzyme activity was determined by conversion of L-arginine to L-citrulline with the purified bpNOS. Data were representive of three independent determinations.
[a]N.I., No inhibitory effect
[†]: Synthetic L-arginine analogue
[¶]: Native L-arginine analogue

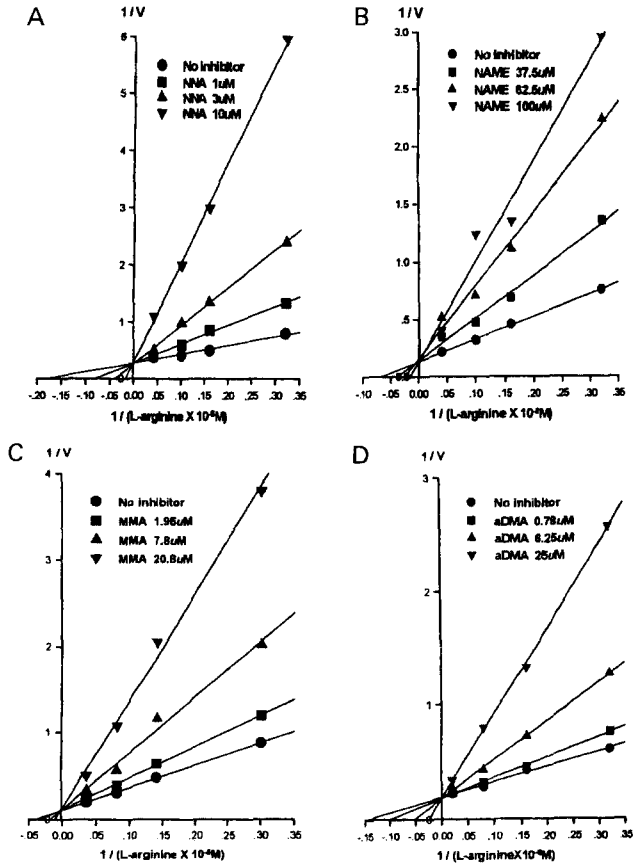

Fig. 4. Double-reciprocal plot of the inhibition of bpNOS by various L-arginine analogues. bpNOS was assayed at 3. 125, 6.25, 10, or 25 μM L-arginine under standard conditions described in "Materials and Methods". Analysis of data revealed that all L-arginine analogues (A; NNA, B; NAME C; MMA D; aDMA) acted as a competitive inhibitor for L-arginine. Maximum velocity (V) was expressed as units of enzyme per milligram of protein by L-citrulline formation method.

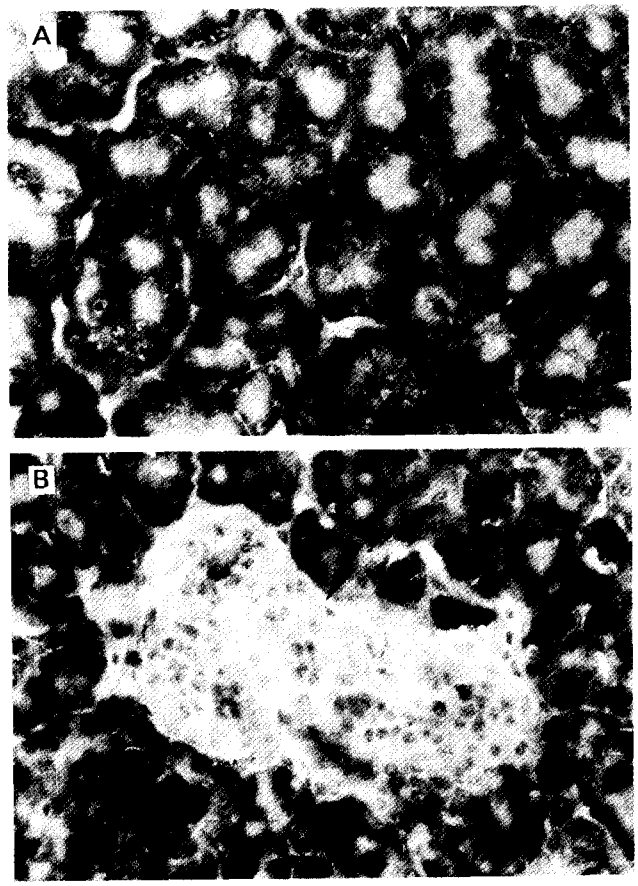

Fig. 5. Immunoreactivity with an antibody against brain type NOS and NOS histochemistry in bovine pancreas. A: immunoreactivity against bpNOS was found in acinar cells in bovine pancreas, and intact nuclei were observed in each acinar cell. B: pancreatic islet cells (indicated by arrow) showed no immunoreactivity, however, acinar cells surrounding pancreatic islet cells showed immunoreactivity against bpNOS.

apparent K_m and V_{max} values for L-arginine at 37°C and pH 7.4 were estimated to be 5.72 μM and 7.82 nmole/min/mg , respectively (Table III).

Immunohistochemical and histochemical analysis

Since the most of the pancreas (>80%) is composed of acinar cell, it is expected the bpNOS to be localized in the acinar cell. Nevertheless, it was necessary to confirm it. Our preliminary study by Western blot analysis using anti-bNOS antibody proved that bpNOS cross-reacted with anti-bNOS antibody. Thus, anti-bNOS antibody was used as primary antibody for immunohistochemical analysis. As shown in Fig. 5A, most of acinar cells in pancreas exhibited strong immunoreactivity against the anti-bNOS antibody, although other cells including pancreatic islet cell did not cross-react with anti-bNOS antibody (Fig. 5B). The above result that the anti-bNOS antibody cross-reacted with NOS only in the acinar cell of pancreas demonstrated that the bpNOS is expressed and mainly located in acinar cells. Taken together, the results suggest that NO can be a messenger molecule for regulation of pancreatic exocrine secretion.

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