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CALCIUM-DEPENDENT INHIBITION OF CONSTITUTIVE NITRIC OXIDE SYNTHASE

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Summary: The objective of these investigations was to study the regulatory properties of brain constitutive NO synthase. NOS activity was determined in 18,000 X g supernatant by conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline in the presence of NADPH. The expression of catalytic activity of NOS required the presence of calcium ion and calmodulin. The preincubation of enzyme preparations at 37 °C in standard reaction mixture led to time-dependent inhibition of L-citrulline formation. This inhibition also required the presence of calcium ion during preincubation phase, and the enzyme remained calmodulin-dependent as exhibited by sensitivity to calmodulin antagonists trifluoperazine (TFP) and calcineurin. The modified enzyme showed significant decrease in the Vmax with NADPH and L-arginine without any change in apparent Km. Inclusion of protease inhibitors, leupeptin, pepstatin A, PMSF and soyabean trypsin inhibitor to the preparations did not alter preincubation-dependent inhibition of NO synthase. Thus, the calcium-dependent inhibitory phenomenon was not due to either the denaturation or proteolysis or the loss of calmodulin sensitivity of NO synthase. These observations indicate that cytosolic isoform of constitutive NO synthase undergoes dual regulation by physiological concentrations of calcium ion.

It has been well established that nitric oxide-nitric oxide synthase system constitutes an integral cellular component of the signal transduction mechanisms involved in hormonal regulation of various physiological processes (1). Studies with cytosolic NO synthase indicate that the enzyme is a flavoprotein which contains heme, FMN/FAD moieties , and requires NADPH and molecular oxygen as cofactors (2,6). The enzyme requires calcium ions for catalytic activity (12) and is modulated by nitroblue tetrazolium and cytochrome C (3,6,16), arginine derivatives (4) and calmodulin antagonists (5).

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We have recently demonstrated that in the presence of calcium ion, the cytosolic isoform of constitutive NO synthase from rat cerebral generates superoxide ions as well as hydrogen peroxide from NADPH, which participate in the catalytic conversion of L-arginine to citrulline and nitric oxide (6). Studies by other investigators using purified brain NO synthase have shown that oxidation of NADPH and generation of hydrogen peroxide requires the presence of calcium ion (3,7). The present investigations were undertaken to develop further understanding into the characteristics of calcium interaction in the regulation of constitutive NO synthase.

MATERIALS AND METHODS

Preparation of NO Synthase: Sprague-Dawley rats (150-200g) were killed by decapitation. Whole brains were quickly removed, cleaned and immersed in ice-cold 0.25 M sucrose solution. Cerebral hemispheres were excised and homogenized in 6 volume (w/v) of ice-cold 0.25M sucrose containing 20 mM Tris-HCl (pH 8.0), 1 mM ethylenediamine-tetraacetate (EDTA), 1 mM dithiothreitol (DTT), phenylmethylsulfonyl fluoride (100 mg/liter), leupeptin (10 mg/liter), pepstatin A (10 mg/liter) and soyabean trypsin inhibitor (10 mg/liter) using glass homogenizer with Teflon pestle. The homogenate was centrifuged at 18,000 x g for 15 minutes. The supernatant was collected and stored at -80 °C, and employed as source for nitric oxide synthase.

Determination of Nitric Oxide Synthase Activity: The NO synthase activity was determined by conversion of ^{3}H L-arginine to ^{3}H L-citrulline. Unless otherwise indicated, the standard reaction mixture contained 50 mM Tris-HCl (pH 7.6), 2.0 mM calcium chloride, 100 μM NADPH, 100 μM L-Arginine, approximately 190,000 to 380,000 dpm of L-[2,3,4,5-^{3}H] arginine-HCl (64.2 Ci/m mole), and 80-120 μg enzyme protein in a final incubation volume of 100 μl. Enzyme reactions were carried out at 37 °C for 8 minutes and terminated with the addition of 400 μl Stop Buffer (20 mM sodium acetate, pH 5.5, 2 mM EDTA, 2 mM EGTA and 1 mM L-citrulline) Prior to use, ^{3}H L-arginine was purified by anion-exchange chromatography using Dowex AG 1-X8, OH^- form, to remove traces of ^{3}H-citrulline (8). For each observation, duplicate determinations were made on 3-4 separate NO synthase preparations. The data reported here represents results from representative experiments.

Measurement of ^{3}H- Citrulline Formed: Citrulline formed in the NO synthase reaction was measured by the procedure described earlier (5). 0.5 ml of reaction mixture generated above, was applied over 1 ml of Dowex AG 50 W-X8, Na^+ form, 100-200 mesh which was pre-equilibrated with Stop Buffer. The eluate (0.5 ml) was collected in scintillation vials. Columns were eluted with additional 2 ml of water (4 fractions of 0.5 ml each) To each vial, 5 ml of scintillation cocktail (ScintiVerse) was added prior to counting in LKB Liquid Scintillation Spectrometer

Preincubation Treatment: Cytosolic NO synthase preparations were preincubated at 37°C, for the indicated periods with each experient, in the standard mixture containing 50 mM Tris-HCl (pH 7.6), 2.0 mM Calcium chloride with and without 100μM NADPH prior to the initiation of incubation as decribed above. In omission experiment, some of the ingradients (as shown in Table I) were omitted during the preincubation phase. These components were, however, replenished during the incubations with ^{3}H-L-arginine.

Protein Determination: Protein contents of the enzyme preparations were measured by Bio-Rad Assay (Bradford reagent containing Coomassie blue) with bovine serum albumin as standard.

Reagents and Isotopes: Dowex AG 50 (100-200 mesh), Dowex AG 1 were procured from Bio-Rad, Richmond, CA. L-[2,3,4,5 ^{3}H] Arginine (64.2 Ci/mmole) was obtained from Amersham, Arlington Height, IL. NADPH, L-arginine, L-citrulline, bovine brain calmodulin, calcineurin and other reagents were purchased from Sigma Chem.Co. St. Louis, MO.

RESULTS

Initial characterization of 18,000 X g cytosol from rat cerebral cortex revealed significant NO synthase activity (approximately 90-100 p mole L-citrulline formed/mg protein/min), and required the presence of calcium and NADPH. The basal activity of NOS obtained in the present investigations was consistent with other reports in the literature (4,5). The preincubation of crude enzyme preparations in complete reaction mixture at 37°C, prior to addition of L-arginine, led to significant loss in citrulline formation. The data expressed in Figure 1 shows that preincubation caused 66 and 90% loss of activity in 2 and 5 minutes, respectively.

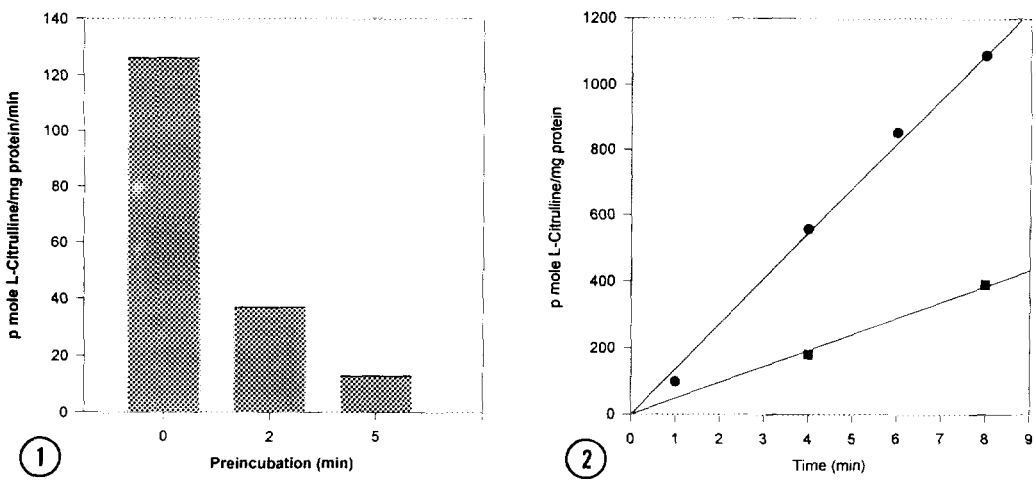

FIG.1. Effect of preincubation on nitric oxide synthase activity. Rat brain cytosolic fractions (0.12 mg protein) were preincubated at 37°C in complete reaction mixture, as described in the Materials and Methods section, for varying lengths of time. Incubations were initiated with the addition of 100 µM ^{3}H-L-arginine and continued for 8 minutes. The ^{3}Hcitrulline formed was determined as described.

FIG.2. Effect of preincubation on time course of L-citrulline generation by NO synthase. Brain cytosol was preincubated for 0 (closed circles) and 3 minutes (closed squares) at 37°C prior to the addition of 100 µM ^{3}H-L-arginine and 100 µM NADPH. L-citrulline formed at different times of incubation was determined as described above.

TABLE I

Effect of omission of different reaction ingredients on preincubation-dependent NOS inhibition

Ingredient(s) Omitted	NO Synthase Activity (p mole L-Citrulline/mg protein/min)
None	33.5
NADPH*	35.4
L-Arginine*	39.6
Ca^{2+}	103.5

*These substances were, however, present during the incubation period.

While the preincubation of NOS for 3 minutes decreased the enzyme activity significantly, the rate of L-citrulline formation remained linear upto 8 minutes of incubation with L-arginine as shown in Figure 2. The reasons for the dimunition of catalytic activity were further investigated by the omission of individual ingradients of the reaction mixture during the preincubation phase. It was observed that decreased enzyme activity was not related to the depletion of either NADPH or L-arginine concentrations during the preincubation (Table I). Also the presence of either of substrates in preincubation did not affect the NOS inhibition (data not shown). However, the omission of calcium ions from the preincubation media completely restored the NOS activity to original basal level. The effects of calcium ion on preincubation were further evaluated by adding increasing concentrations of the cation. The results shown in Table II indicate that both, the preincubation-dependent effect as well as the catalytic conversion of L-arginine to L-citrulline required the presence of calcium ions. However, the concentration requirements of calcium ion varied for the two events. Whereas, the basal catalytic activity of NOS reached optimal level at 100 μM, the maximum preincubation effect on NOS was observed at 1.0 mM calcium.

Because of the known requirement of calmodulin for the catalytic activity of NO synthase, the possibility of altered sensitivity to calmodulin in the preincubated preparation was tested. The exogenous addition of bovine brain calmodulin during the preincubation did not alter NOS activity. However, the addition of 100 μM trifluoperazine (TFP) or calcineurin

TABLE II

Effects of Ca^{2+} ion concentration on NO synthase activity

Additions	NO Synthase Activity (p mole L-Citrulline/mg protein/min)	
	Control	Preincubation
None	0.0	0.0
$CaCl_2$ (10μM)	0.0	0.0
$CaCl_2$ (100μM)	104.3	72.0
$CaCl_2$ (1000μM)	107.1	41.1
$CaCl_2$ (2000μM)	109.0	43.5

(9.0 Units) did lead to the decrease of NOS activity both in the control as well as preincubated preparations, and the degree of NOS inhibition by calmodulin-antagonists was quite comparable between the control and treated enzyme (Table III).

TABLE III

Effect of calmodulin antagonists on NO synthase activity

Additions	NO Synthase Activity (p mole L-Citrulline/mg protein/min)	
	Control	Preincubation
None	103.5	56.8
Calmodulin, 1.0μM	102.0	50.5
Trifluoperazine, 100μM	8.5	5.7
Calcineurin, 9.0 Units	63.8	35.5

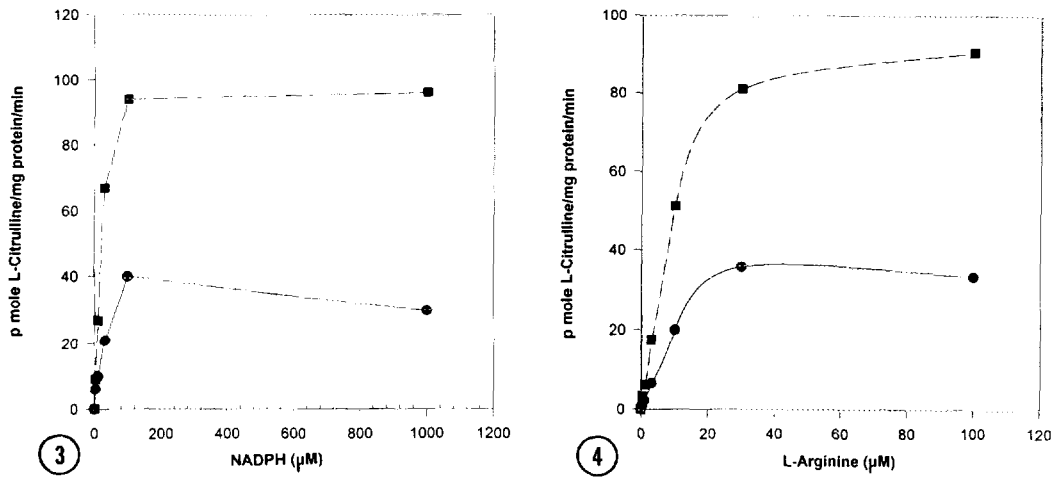

FIG.3. Effect of preincubation on NO synthase activity as function of NADPH concentrations. Brain cytosolic fractions (0.126 mg protein) were preincubated for 0 (closed squares) and 3 minutes (closed circles) at 37 °C in the presence of 2 mM calcium chloride. Reactions were initiated with the addition of 100 μM ^{3}H-L-arginine and different concentrations of NADPH. The ^{3}H-L-citrulline formed was measured as described earlier.

FIG.4. Effect of preincubation on NO synthase activity as function of L-arginine concentrations. NO synthase preparation (0.12 mg protein) was preincubated with 2 mM calcium for 0 (closed squares) and 3 minutes (closed circles). Reactions were initiated with the simultaneous exposure of preincubated NO synthase preparations to different concentrations of ^{3}H-L-arginine and 100 μM NADPH and the ^{3}H-L-citrulline formed was determined.

The effect of preincubation was also studied on the kinetic properties of NOS. When preincubated enzyme preparations were simultaneously exposed to both substrates, significant decrease in the V_{max} was observed with NADPH (Fig.3) as well as L-arginine (Fig.4). The NOS activity decreased from 98.5 to 40.0 pmole L-citrulline/mg protein/min with no changes in the apparent Km values. The apparent Km values for the two substrates were observed to be approximately 10-20 μM.

DISCUSSION

The brain cytosol exhibited significant NO synthase activity as determined by conversion of L-arginine to L-citrulline. The reaction rates were linear with protein concentrations and time upto 8 minutes at 37°C. However, preincubation of cytosol in complete reaction mixture led to significant loss of NO synthase activity. The rate of L-citrulline formation were not altered upto 8 minutes, whereas, the preincubation-dependent inhibition was apparent within 2 minutes (Figure 1). Thus, the inhibition of NO synthase did not appear

to be due to the denaturation. The possibility of proteolysis of the enzyme preparation, as the cause of lower activity, was also ruled out because of the combined presence of various protease inhibitors in the homogenizing buffer.

The complete reversal of the preincubation effect by omission of calcium ion (Table I) and and dependence on calcium (Table II) revealed the importance of cation in the inhibitory phenomenon . However, the concentrations of calcium required for optimal preincubation effect were significantly higher (1.0 mM) than those required for catalytic activity (100 μM) of NO synthase (Table II).

The observed inhibition of NO synthase activity by calmodulin antagonists TFP and calcineurin indicated the characteristic involvement of calmodulin in the formation of L-citrulline (Table III), as noted by investigators (15). These data also suggest that during preincubation the calmodulin sensitivity of NO synthase system was not compromised since the degree of inhibition by calmodulin-antagonists was of the same magnitude in the control and preincubated preparations. The addition of exogenous calmodulin, however, was without effect, presumably, due to endogenous saturating concentrations of calmodulin in the crude enzyme preparations.

The precise mechanism(s) underlying the calcium-dependent down-regulation of NO synthase is not understood at this time. Calcium ion and calmodulin are required for the catalytic conversion of L-arginine to nitric oxide and L-citrulline (6,12). Earlier studies from this and other laboratories indicate that catalytic activity of constitutive NO synthase involves generation of superoxide anion and hydrogen peroxide (6,10), and calmodulin participates in the transfer of electrons to the heme moiety of the enzyme (11). We have previously reported the preincubation-dependent dimunition of oxygen-radical stimulation of guanylate cyclase (also a heme protein) through some unknown mechanism (13,14). In the present studies, we have not measured the level of hydrogen peroxide generation after preincubation. It is, however, conceivable that during preincubation either calcium or some "calcium-derived intermediate" interacts with a presumed regulatory (noncatalytic) site of NO synthase, whose alteration leads to the diminished generation of oxidizing equivalents at catalytic site resulting in the lower production of nitric oxide and L-citrulline. Additional studies are currently underway in our laboratory to precisely understand the mechanism(s) of calcium-dependent down-regulation of NO formation.

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