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Production of anti-peptide antibody of rat brain nitric-oxide synthase[1]

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KEY WORDS nitric-oxide synthase; NADPH dehydrogenase; peptides; epitopes; antibodies; enzyme-linked immunosorbent assay; Western blotting; immunohistochemistry; cerebellum

AIM: To raise antibody of rat brain nitric-oxide synthase (bNOS) through immunizing animal with a peptide of bNOS that can represent the holoprotein. **METHODS:** The amino acid sequence for the bNOS was analyzed by GenePro computer program. According to the hydrophilicity, hydrophobicity, antigenicity, and the potentiality to form protein second structures of α-helix, β-sheet and β-turn, the structure of bNOS was predicted. The peptide 277 - 287 was selected that was predicted to be in the antigen epitope of bNOS. The peptide was chemically synthesized, coupled to keyhole limphet hemocyanin carrier protein and injected into rabbits to raise antibody. The specificity of the antibody was tested by enzyme-linked immunosorbent assay, immunohistochemistry, and Western blotting. **RESULTS:** The antibody bound the protein in rat cerebellum extract. In Western blotting, the antibody bound the protein band of 150 kDa in SDS-PAGE, and the binding was inhibited by peptide conjugated with carrier protein. In immunohistochemistry, the stain was collocated with the stain in NADPH-dehydrogenase histochemistry. **CONCLUSION:** The antibody against the peptide recognized the natural bNOS in rat brain, and the peptide 277 - 287 was located on the surface of bNOS.

Nitric-oxide (NO), a messenger in the central nervous system, is a labile free radical with short half life and can not be stored in cells. Nitric-oxide synthase (NOS) plays a critical role in regulation of NO production. The functions of NO in some physiological and pathological processes can be understood through the study on NOS. NOS has been the subject of intensive study, focusing on the mode of action[1], distribution in different tissues, organs and species[2,3], as well as comparisons between the different isoforms of enzyme at the molecular level[4]. Several ways have been used to detect NOS. The C-terminal portions of all NOS isoforms show high homology to NADPH cytochrome P-450 reductase[4]. So NADPH dehydrogenase histochemical staining has been used to detect NOS in the study of distribution[3]. mRNA analysis is another way to detect the level of NOS mRNA in the tissue. Antibody can provide a direct means to quantitate protein, and the immunological methodologies afford high sensitivity and selectivity. In this study, we raised an antibody of rat brain nitric-oxide synthase (bNOS) by immunizing animal with a peptide of bNOS that can represent the holoprotein.

MATERIALS AND METHODS

Animals White rabbits ($n=2$), ♀, 1.8 and 1.9 kg. Sprague-Dawley rats ($n=20$), ♂, 270 - 330 g.

Chemicals Keyhole limphet hemocyanin (KLH), *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical Co. Bovine serum albumin (BSA), diamino benzidin tetrahydro chloride (DAB), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Shanghai Lizhu Dongfeng Biotechnology Co. Nitrocellulose (NC) membrane was purchased from Serva Co. Horseradish peroxidase sheep-anti-rabbit IgG (HRP-IgG) was purchased from Sino-American Biotechnology Co. Microtiter plate was purchased from Huangyan Xingfeng Plastics Products Co.

Computer predictions The characteristics of amino acid sequence of NOS was analyzed by GenePro software program. The parameters for amino acid type were hydrophilicity and hydrophobility, antigenicity, α-helix potential, β-sheet potential, and β-turn potential. The derived parameter was plotted as a function of residue number. The peptides on the surface of NOS were selected according to all these parameters. The sequence of NOS was compared with

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NADPH cytochrome P-450 reductase and other isoforms of NOS. The peptide with high bNOS specificity was selected as target peptide.

Antigen The peptide was synthesized by solid-phase method. To better approximate its structure in the native protein, peptide was synthesized with a free amino terminus and an amidated carboxyl terminus. A carboxyl-terminal cysteine was added to peptide to allow an ordered chemical bonding to the carrier protein[5]. The synthetic peptide was purified by preparative reversed-phase high performance liquid chromatography (HPLC), and then lyophilized and stored at −20 ℃.

The peptide was coupled to KLH after cysteine activation by MBS, or to BSA by glutaral.

Coupled to KLH[6,7] KLH 5 mg was reacted with MBS 1.2 mg. The reaction product KLH-MBS was passed through Sephadex G-25 column equilibrated with PBS 50 mmol·L^{-1} (pH 7.4) to remove free MBS. KLH-MBS recovery from peak fractions of the column eluate (monitored by absorbance at 280 nm) was estimated to be 80 %. The collected effluent was lyophilized and the KLH-MBS was redissolved and reacted with 3 mg peptide. The reaction solution was stirred for 3 h at 20 ℃ and then dialyzed against PBS to remove uncoupled peptide and kept frozen at −20 ℃.

Coupled to BSA[8] Peptide 1.5 mg and BSA 3 mg were dissolved separately in 1 mL PBS 50 mmol·L^{-1} (pH 7.4). The 2 solutions were mixed and stirred. 0.2 % glutaral 1.5 mL was added dropwise in 20 min with stirring. The solution was incubated at 4 ℃ for 100 − 120 min. Glycine 875 μL was added from a stock in PBS 1 mol·L^{-1} (pH 7.4) to a final concentration of 200 mmol·L^{-1}. The solution was stirred for 1 h and dialyzed against PBS and kept frozen at −20 ℃.

Antibody On d 1, the rabbit was injected 500 μg peptide-KLH in complete Freund's adjuvant (1:2) intradermally. On d 28, the rabbit was injected 400 μg peptide-KLH in incomplete Freund's adjuvant (1:2) intradermally. In the 9th wk and the 14th wk, the rabbit was injected 200 μg peptide-KLH in incomplete Freund's adjuvant (1:2) intradermally. Small amount of rabbit blood was taken 7 − 10 d after each injection, and the rabbits were exsanguinated 4 wk after the final booster.

Enzyme-linked immunosorbent assay ELISA method A. The microtiter plate was coated with serial diluted antigen solutions, which contained 25 − 300 ng peptide-BSA in 15 mmol·L^{-1} Na_2CO_3/$NaHCO_3$, pH 9.6. The plate was blocked by blocking buffer (2 % BSA, wt/vol, 0.4 % gelatin, wt/vol, in PBS 50 mmol·L^{-1}, pH 7.4. After wash, the rabbit antiserum diluted in blocking buffer was added to the plate and incubated at 37 ℃ for 1 − 1.5 h. After wash, the diluted HRP-IgG was added to each well, followed by incubation at 37 ℃ for 1 h and wash. The chromogenic enzyme reaction was initiated by addition of 50 μL per well of 8 mg o-phenylenediamine and 0.5 μL of 30 % H_2O_2 dissolved in 1 mL of citric phosphate buffer (Na_2HPO_4, 0.2 mol·L^{-1}, citric acid, 0.1 mol·L^{-1}, pH 5.0). Incubated for 15 min. The reaction was stopped by adding of 50 μL of H_2SO_4 1 mol·L^{-1} The microtiter plate was read at 490 nm.

ELISA method B (competitive immunoassay) The microtiter plate was coated with the peptide-BSA solution (300 ng/well) and blocked as above. The diluted antiserum (1 : 2000) was mixed with same volume of serial diluted peptide-BSA or diluted brain extract. The mixture was incubated at 37 ℃ for 1 h. The plate was washed and the reaction was continued with the conjugated and substrate as in method A.

Tissue process and protein content determination Brain was homogenized in 2 volumes of Tris-HCl 50 mmol·L^{-1}, containing sucrose 320 mmol·L^{-1}, edetic acid 1 mmol·L^{-1}, dithiothreitol 1 mmol·L^{-1}, phenylmethyl-sulfonyl fluoride (PMSF) 100 mg·L^{-1}. The homogenate was centrifuged at 100 000 / g at 4 ℃ for 30 min. Protein concentration was estimated by the Coomessie brilliant blue protein assay using BSA as the standard[9].

Western blotting Proteins in brain extract were separated by SDS-PAGE and transferred to an NC membrane[10,11]. The transferred NC membranes were processed in PBS and blocked with 2 % BSA, wt/vol, 0.4 % gelatin, wt/vol, in PBS 0.05 mol·L^{-1}, pH 7.4. The blots were incubated with antiserum diluted 1:100 in blocking buffer. Blots were washed and incubated with diluted HRP-IgG. The blots were washed. The substrate solution of chromogenic reaction containing DAB 0.5 g·L^{-1}, 0.03 % H_2O_2 in Tris-HCl 0.05 mol·L^{-1}, pH 7.6. The substrate solution was prepared just before use. The reaction was developed at room temperature (25 ± 3 ℃) with agitation and stopped by removing the substrate and rinsing with PBS.

Histochemistry Rat was anesthetized with sodium pentobarbital 40 mg·kg^{-1} and transcardially perfused with 50 mL vascular rinse (NaCl 8 g·L^{-1}, KCl 0.25 g·L^{-1}, $NaHCO_3$ 0.5 g·L^{-1}, phosphate buffer 10 mmol·L^{-1}, pH 7.4) followed by fixative 500 mL (4 % paraformaldehyde in phosphate buffer 10 mmol·L^{-1}, pH 7.4) for about 30 min. The brain was transferred to phosphate buffer 0.1 mol·L^{-1} containing 15 % sucrose. Coronal sections (80 μm thick) were cut on the Vibratome (Lancer Brunswick Co) and mounted onto gelatin-coated slides.

After being blocked in 4 % calf serum, 0.4 % Triton X-100 in PBS, pH 7.4, sections were incubated with antiserum at a 1 : 200 dilution at 4 ℃ overnight. The sections were washed and incubated with anti-rabbit IgG conjugated to horseradish peroxidase at 20 ℃ for 3 h. After wash and the substrate solution containing DAB 0.5 g·L^{-1},

0.03 % H_2O_2 in Tris-HCl 50 mmol · L^{-1}, pH 7.6 was added. The reaction was stopped by removing the substrate and rinsing with PBS. After immunohistochemical staining the sections were slipped with cover. Negative control sections received identical treatment except for the primary antibody.

To ascertain the relationship between NOS-immunocreactivity and NADPH-dehydrogenase reactivity, adjacent sections of brain tissue to those obtained for measurement of immunoreactivity were processed for NADPH-dehydrogenase histochemistry[12]. The sections were treated at 37 ℃ with Tris-HCl, 50 mmol · L^{-1}, pH 8.0, 0.2 % Triton X-100, nitroblue tetrazolium (NBT) 0.5 mmol · L^{-1}, NADPH 1 mmol · L^{-1} for 30 min for demonstration of NADPH-dehydrogenase activity.

RESULTS

Computer predictions The first 2 parameters were hydrophilicity and antigenicity. The height of each spike was proportional to the relative strength of each amino acid. The opposite characteristic were hydrophobility. The secondary structure predictions are displayed for each of the respective baselines proportional to the potentiation. The height of each spike was proportional to the relative value of respective properties of each amino acid in sequence.

Several regions in the amino acid sequence of rat bNOS were predicted to be on the surface of the native protein (Tab 1).

Tab 1. Peptides of rat brain NO synthase that were predicted to be on surface of native protein.

	Peptide	Sequence
1	RGGPAKAEMKD	220 – 230
2	KEQSPTSGKQS	277 – 287
3	QPDGSTLGDPA	487 – 497
4	GNGDPPENGEK	810 – 820
5	SREPDRPKKYV	1 311 – 1 321
6	SRLRDDNRYHE	1 382 – 1 392

These regions were hydrophilic, antigenic, and of high β-turn potential, low α-helix potential, and low β-sheet potential. Peptides 4, 5, and 6 were in the domain reputed to be similar to cytochrome P-450 reductase[6]. Peptide 3 was in the highly conserved region 380 – 700 that was homologous with the other 2 isoforms of NOS[13]. Peptide 2 contained 3 serines. They might be the substrate of PKC and/or PKA. Peptide 2 might be located in the region of great importance in the enzyme regulation. Peptide 2 was selected as target peptide and chemically synthesized.

Antipeptide antibody The titer of the rabbit sera against the peptide was assessed in an ELISA test. The wells were coated with peptide linked to BSA to get rid of the antibodies against the carrier protein KLH and to measure specifically the anti-peptide response. The peptide induced good antibody response. After the 3rd booster, the titer of one rabbit was 1:4000 (Fig 1).

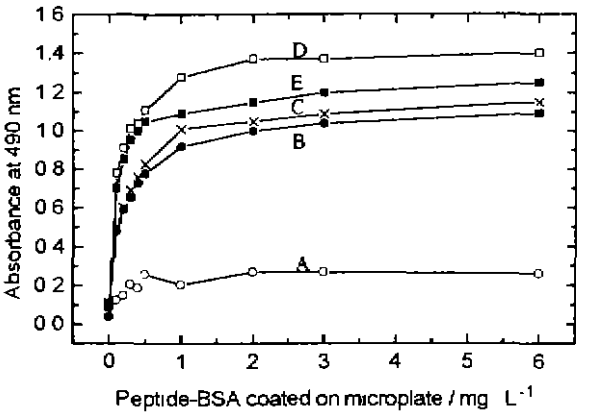

Fig 1. ELISA of rabbit antiserum reacting with peptide-BSA. A) pre-immuno-serum (1:1000); B) antiserum of rabbit 1 (1:1500); C) antiserum of rabbit 1 (1:1000); D) antiserum of rabbit 2 (1:2000); E) antiserum of rabbit 2 (1:4000).

ELISA The binding of the antibody to the native rat brain enzyme was measured by competitive ELISA. Each point in the ELISA corresponded to the mean of at least 3 determinations. As the concentration of cerebellar extracts increased, the bond decreased. The declination of the curve was similar to the curve of synthetic antigen. Hence, the antibody bound the protein in cerebellar extract. The content of bNOS in cerebellar extract was higher than that in cerebral cortex extract of the same protein concentration (Fig 2).

Western blotting The rabbit antibody bound the protein of 150 kDa in cerebellar extract. This was consistent with the reported molecular weight of rat bNOS[14]. There were some bands of low

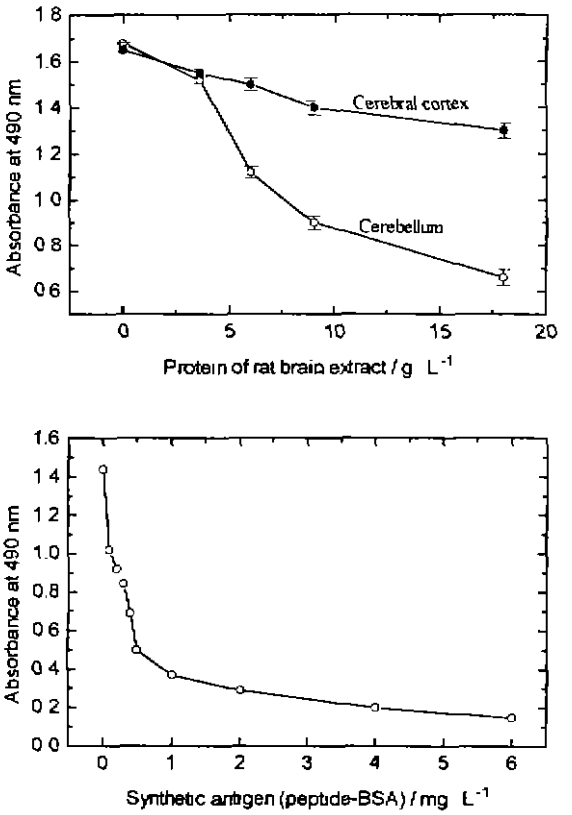

Fig 2. Competitive ELISA of brain extract or synthetic antigen. $n = 3$, $\bar{x} \pm s$.

molecular weights, about 100, 80, 70, and 47 kDa (Fig 3).

Their proportion depended on the length of storage of the preparation. When the extract was stored overnight at room temperature, the low molecular weight band was obvious, while the 150-kDa band was very weak. So the low molecular weight protein might correspond to the degraded 150-kDa bNOS and could also be recognized by the antibody.

Histochemistry NADPH-dehydrogenase reactivity and bNOS immunoreactivity were compared on adjacent sections. The cerebellum was densely stained in both immunohistochemistry and NADPH-dehydrogenase histochemistry. The stain formed a colored network in gray matter of cerebellum (Fig 4, Plate 1).

The striatum was sparsely stained in immunofhistochemistry and NADPH-dehydrogenase histochemistry. These data indicate close associations between NADPH-dehydrogenase neurons and bNOS immunoreactivity neurons.

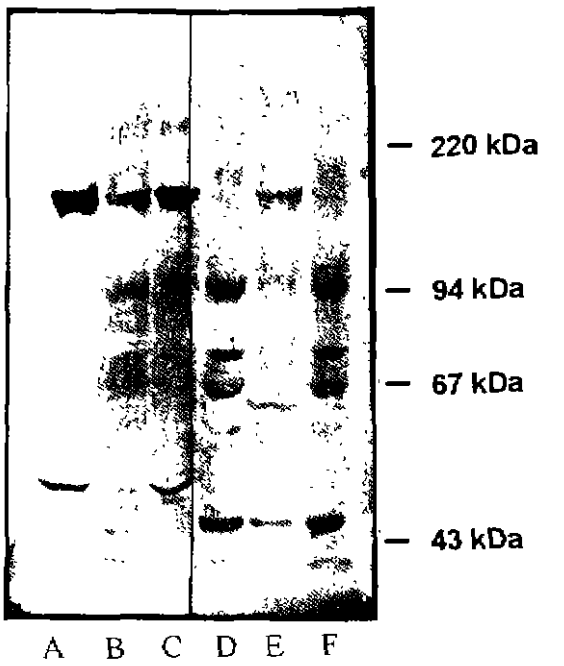

Fig 3. Western blots. Rabbit antibody against peptide-KLH reacting with: rat cerebellar extract which was freshly made (A and C); rat brain stem extract freshly made (B); rat cerebellar extract which had been stored overnight at room temperature (D and F); rat brain stem extract stored overnight (E).

DISCUSSION

In the present studies, an antibody of rat brain nitric-oxide synthase (bNOS) was raised by immunizing animal with a peptide of bNOS that can represent the holoprotein. Based on the amino acid sequence of bNOS, the peptide with strong antigenicity was selected. In order to get high specificity, the reductase domain that is homologous with NADPH cytochrome P-450 reductase, and/or the highly conserved region, from 380 – 700, which is homologous with other 2 NOS isoforms were avoided. The peptide 277 – 287 which is highly hydrophilic, antigenic and has the substrate sequence of PKC was used to prepare antibodies. The capacity of the antibody to recognize specifically a protein corresponding to bNOS is supported by the following arguments: 1) The antibody recognized the protein with a 150-kDa molecular mass in Western blotting. Weak bands of low molecular mass were also seen, and their proportion depended on the length of storage of the preparation suggest-

ing that the bands corresponded to partly degraded NOS in tissue process. 2) The results of competitive immunoassay showed that antibody could bind the protein molecule in cerebellar extract. 3) In immunohistochemical staining, cerebellum was densely stained. Striatum and hippocampus were sparsely stained. The staining was collocated with NADPH-dehydrogenase histochemical staining. And these results were also consistent with the reported distribution of rat bNOS in brain[3]. The results suggest that the antibody could recognize not only the denatured protein after SDS-PAGE separation, but also the natural protein in cerebellar extract and in fixed tissue. We concluded that the peptide 277 – 287 was in the antigenic epitope and the antibody raised against this peptide can recognize the original bNOS.

Antipeptide antibody of bNOS provided a direct means to quantitate the level of NOS in intact cells. One limitation of this procedure is the potential for reacting with degraded bNOS. This is not usually a problem for immunoblotting assays, as the degraded bNOS can be separated from bNOS by SDS-PAGE prior to assay.

REFERENCES

1 Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites. J Biol Chem 1992; **267**: 10976 – 81.

2 Springall DR, Riveros-Moreno V, Buttery L, Suburo A, Bishop AE, Merrett M, *et al*. Immunological detection of nitric oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoforms.
Histochemistry 1992; **98**: 259 – 66

3 Vincent SR, Kimura H. Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 1992; **46**: 755 – 84.

4 Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase.
Nature 1991; **351**: 714 – 8.

5 Czernik AJ, Girault J-A, Nairn AC, Chen J, Snyder G, Kebabian J, *et al*. Production of phosphorylation state-specific antibodies. Methods Enzymol 1991; **201**: 264 – 83.

6 Liu FT, Zinnecker M, Hamaoka T, Katz DH.
New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates
Biochemistry 1979; **18**: 690 – 7.

7 Green N, Alexander H, Olson A, Alexander S, Shinnick TM, Sutcliffe JG, *et al*. Immunogenic structure of the influenza virus hemagglutinin. Cell 1982; **28**: 477 – 87.

8 Reichlin M. Use of glutaraldehyde as a coupling agent for proteins and peptides. Methods Enzymol 1980; **70**: 159 – 65.

9 Bradford MM A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; **72**: 248 – 54.

10 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature 1970; **227**: 680 – 5.

11 Towbin H, Staehelin T, Gordon J.
Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.
Proc Natl Acad Sci USA 1979; **76**: 4350 – 4.

12 Zhang ZG, Chopp M, Gautam S, Zaloga C, Zhang RL, Schmidt HHHW, *et al*. Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat.
Brain Res 1994; **654**: 85 – 95.

13 Förstermann U, Nakane M, Tracey WR, Pollock JS. Isoforms of nitric oxide synthase: functions in the cardiovascular system.
Eur Heart J 1993; **14** (1 Suppl): 10 – 5.

14 Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme.
Proc Natl Acad Sci USA 1990; **87**: 682 – 5.

15 Benjamin DC, Berzofsky JA, East IJ, Gurd FRN, Hannum C, Leach SJ, *et al*. The antigenic structure of proteins: a reappraisal. Annu Rev Immunol 1984; **2**: 67 – 101.

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大鼠脑一氧化氮合酶抗肽抗体的制备[1]

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关键词 一氧化氮合酶; NADPH 脱氢酶类; 肽; 抗原决定簇; 抗体; 酶联免疫吸附法; 蛋白质印迹; 免疫组织化学; 小脑 R392.33

目的: 用大鼠脑一氧化氮合酶(bNOS)的一个片段免疫家兔, 产生可识别该酶本身的抗体. **方法**: 根据亲水性、抗原性及产生 α-螺旋、β-折叠、和 β-转角的可能性, 预测肽 277 – 287 位于该酶的抗原决定簇中. 肽 277 – 287 经化学合成, 与血蓝蛋白交联, 免疫家兔, 获得抗体. 特异性用 ELISA、免疫组织化学和蛋白印迹分析法鉴定. **结果**: 抗体与大鼠小脑提取液可特异性结合; 免疫组织化学染色结果与 NADPH-黄递酶组织化学染色结果一致. 它可结合分子量约 150 kDa 的蛋白. **结论**: 该抗肽抗体可专一性识别大鼠脑组织中的 bNOS.