Brain ischemia induces serine phosphorylation of neuronal nitric oxide synthase by Ca^{2+}/calmodulin-dependent protein kinase II in rat hippocampus[1]

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KEY WORDS serine; phosphorylation; nitric oxide synthase; calmodulin kinase; KN 62; brain ischemia; hippocampus

ABSTRACT

AIM: To investigate whether brain ischemia induces serine phosphorylation of neuronal nitric oxide synthase (nNOS) by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and the interaction between CaMKIIα and nNOS in rat hippocampus. **METHODS:** Brain ischemia was induced by bilateral carotid artery occlusion procedure. Phosphorylation and the interaction of proteins were studied by immunoprecipitation and immunoblotting. We investigated during brain ischemia serine phosphorylation and amount of nNOS in crude membranes fraction (P) and cytosolic fraction (S), interaction between CaMKIIα and nNOS, and the effects of 1-[*N,O-bis*-(5-isoquinolinesulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenylpiperazine (KN-62, a selective inhibitor of CaMKII) on phosphorylation and the interaction of proteins in P. **RESULTS:** Serine phosphorylation of nNOS in P increased persistently during brain ischemia, and 15 min ischemia-induced serine phosphorylation of nNOS was attenuated significantly by KN-62. But there was no serine phosphorylation of nNOS in S. The distributions of nNOS were not affected by ischemia and KN-62. However, the binding levels of both CaMKIIα with nNOS and Thr^{286} autophosphorylated CaMKIIα with nNOS increased after ischemia, and were diminished by KN-62. **CONCLUSION:** CaMKII interacted with nNOS and regulated serine phosphorylation of nNOS during brain ischemia.

INTRODUCTION

Constitutively expressed nitric oxide synthase (NOS) has three isoforms, including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), which has been isolated and cloned[1]. NOS catalyzed *L*-arginine to generate *L*-citruline and nitric oxide (NO), a gaseous messenger molecule, the overproduction of which plays a critical role in glutamate-induced neurotoxicity after ischemia[2]. In mutant mice deficient in nNOS and subsequent NO production, infarct size becomes smaller and neuronal injury is less than those in normal mice after brain ischemia[3]. nNOS locates on the membrane of mitochondria, endoplasmic reticulum, in pre- and postsynaptic profiles and in the cytosol[4]. Posttranslational myristoylation presumably is a determinant for the subcellular localization, but may not be the only factor. No significant differ-

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ence was detected between the soluble and particulate enzymes including their specific activity[5]. It has been reported that phosphorylated nNOS at Ser^{847} decreases enzyme activity to approximate 70 % of unphosphorylated enzyme[6]. The Ca^{2+}/CaM-dependent nNOS is coupled to *N*-methyl-*D*-aspartate receptor (NMDAR) on the membranes by PSD95 protein[7]. Ca^{2+} influx through NMDAR stimulates nNOS and other signaling molecules including the Ca^{2+}/calmodulin(CaM)-dependent protein kinaseII (CaMKII)[8] .

CaMKII, a ubiquitously expressed Ca^{2+}/CaM-dependent protein kinase with broad substrates, is enriched in the brain and constitutes approximate 1 %-2 % of the total protein in the hippocampus[9]. Autophosphorylation at Thr^{286} of the α isoform of CaMKII modifies its function, and converts the kinase into a Ca^{2+}/CaM independent state[10]. After decapitation of rats, the translocation to postsynaptic density from cytosol of CaMKII increases to approximate 10 % of the protein content in the postsynaptic density fraction[11]. CaMKII translocates immediately and completely to crude membranes fraction from cytosolic fraction during brain ischemia[12]. Substrates phosphorylated by CaMKII are involved in many aspects of cellular functions including membrane current, neurotransmitter synthesis and release[10]. Mutant mice knocked out CaMKIIα are deficient in long-term potentiation (LTP), which supports that CaMKIIα is necessary for LTP[13]. It has been documented that in the brain nNOS is one of the substrates of CaMKII, and the phosphorylation of nNOS at Ser^{847} by CaMKII peaks at 15 min ischemia with 0.5 h reperfusion in the hippocampus[14].

In this report, we further studied the subcellular distributions and serine phosphorylation of nNOS in the hippocampus during the early part of brain ischemia, and whether CaMKII was involved in nNOS serine phosphorylation after its autophosphorylation and translocation, and the functional interaction between nNOS and CaMKII.

MATERIALS AND METHODS

Antibody Anti-CaMKIIα antibody (Product Number: C6974) and anti-nNOS antibody for immunoblotting (Product Number: N7280) were purchased from Sigma, anti-nNOS antibody for immunoprecipitation (Catalog: sc-648) was from Santa Cruz; anti-p-CaMKIIα (Thr286 phosphorylated, Catalog: V1111) antibody was from Promega (Madison, WI, USA); anti-p-Ser antibody was prepared by our laboratory.

Animal surgical procedures Adult male Sprague-Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 250-300 g were subjected to 3, 5, 15, and 30 min brain ischemia by four-vessel occlusion method as described before[15]. Briefly, under anesthesia with chloral hydrate (0.3 g/kg, ip), vertebral arteries were electroncauterized then common carotid arteries were exposed. Ischemia was induced by occluding common carotid arteries with aneurysm clips after rats recovered for 24 h, except that the arteries of sham control rats were not occluded other operations were the same. Rats lost their righting reflex within 30 s and those whose pupils were dilated and unresponsive to light were selected for the experiments. Rectal temperature was maintained about 37 ºC in the whole process. When necessary, 20 min before 15 min ischemia, a volume of 5 μL KN-62 which was dissolved in Me_2SO at a concentration of 1 g/L or Me_2SO as vehicle control was injected into the left cerebral ventricle (anteroposterior, -0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma) using a stepper-motorized microsyringe (Stoelting, Wood Dale, IL) within 5 min.

Tissue preparation Rats were decapitated immediately after ischemia. Both sides of hippocampus were removed, and frozen quickly in liquid nitrogen. Samples were homogenized in 1:10 (W/V) ice-cold homogenization buffer (HB) containing 50 mmol/L 3-(*N*-morpholino) propanesulfonic acid (pH 7.4), 100 mmol/L KCl, 320 mmol/L sucrose, 0.5 mmol/L $MgCl_2$, 0.2 mmol/L dithiothreitol, inhibitors of phosphatase and protease (20 mmol/L β-glycerophosphate, 20 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L each of edetic acid, egtazic acid, sodium orthovanadate, *p*-nitrophenyl phosphate, phenylmethylsulfonyl fluoride, and benzamidine and 5×10^{-3} g/L each of aprotinin, leupeptin, and pepstatin A). The homogenates were centrifuged at 800×*g* for 15 min at 4 ºC, then supernatants were centrifuged at 100 000×*g* for 30 min at 4 ºC in a TL 100.2 rotor (Backman, Palo Alto, CA), the supernatant cytosolic fraction (S) was collected, the pellet crude membranes fraction (P) was re-suspended in HB containing 0.1 % Triton X-100 for 10 s by sonication. The samples were stored at -80 ºC before use.

Immunoprecipitation and immunoblotting Samples (400 μg) which were diluted four fold in immunoprecipitation (IP) buffer containing 50 mmol/L *N*-

[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES, pH 7.1), 150 mmol/L NaCl, 1 mmol/L $ZnCl_2$, 1.5 mmol/L $MgCl_2$, 10 % glycerol, 1 % Triton X-100, and inhibitors of phosphatase and protease as indicated above. Samples were preincubated with 20 μL protein A sepharose CL-4B (Amersham, Uppsala, Sweden) for 1 h. Nonspecific protein A was removed by centrifuging. The supernatants were incubated with anti-p-Ser or anti-CaMKIIα or anti-nNOS antibody for 4 h or overnight at 4 ºC, and the reaction system continued incubating with protein A for 2 h. Samples were centrifuged at 10 000×*g*, and the pellets were washed three times with IP buffer. Pellets containing bound proteins were eluted by adding 4-fold SDS-PAGE sample buffer (12 μL) and boiled in 100 ºC water for 5 min. Samples were centrifuged, and supernatants were used for immmunoblotting. For immunoblotting, proteins (50 μg) were separated by 10 % SDS-PAGE and were electrotransferred onto nitrocellulose membranes (NC, pore size, 0.45 μm). The membranes were blocked with blocking buffer for 3 h, then probed with anti-nNOS or anti-CaMKIIα or anti-p-CaMKII antibody overnight at 4 ºC. Detection was performed using alkaline phosphatase conjugated goat anti-rabbit IgG (1:10000) and developed using BCIP/NBT color substrate. The bands on the membranes were scanned and analyzed by an image analyzer (Labworks Software, UVP Inc, Upland, CA).

Statistical analysis Values were expressed as the mean±SD. Statistical analysis of the results was carried out by one way analysis of variance (ANOVA) followed by the Duncan's new multiple range method. $P<0.05$ was considered significant.

RESULTS

Effect of brain ischemia on nNOS serine phosphorylation and the distributions of nNOS nNOS serine phosphorylation in P increased persistently and significantly after 3 min ischemia ($P<0.05$, Fig 1A). Interestingly, there was no serine phosphorylation of nNOS in S (Fig 1B). nNOS was found in both P (Fig 1C) and S (Fig 1D), and the distributions of nNOS did not change during brain ischemia ($P>0.05$).

Effect of KN-62 on the increased nNOS serine phosphorylation KN-62, a selective inhibitor of CaMKII, was administered 20 min before 15 min ischemia through left cerebral ventricle. The elevation of nNOS serine phosphorylation induced by 15 min ischemia was inhibited significantly by KN-62 in P ($P<0.05$), but not by Me_2SO ($P>0.05$, Fig 2A). The distributions and amounts of nNOS in P were not affected by KN-62 ($P>0.05$, Fig 2B).

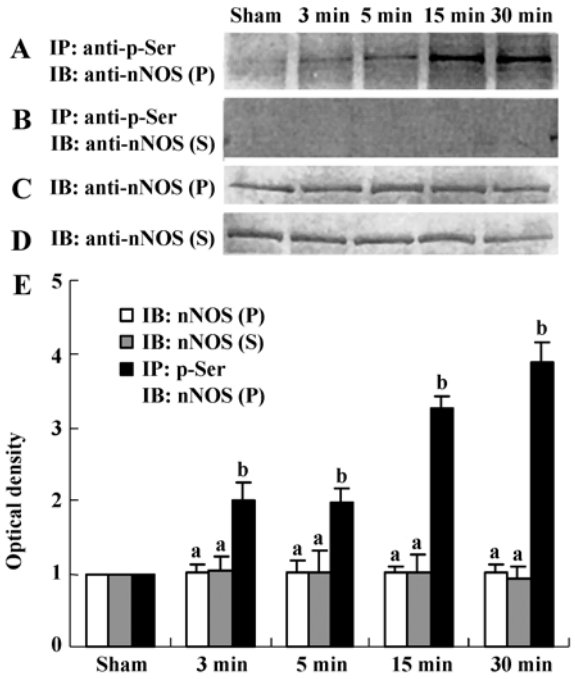

Fig 1. Immunoblotting analysis of serine phosphorylation and the distributions of nNOS in P and S during brain ischemia. (A, B) IP with anti-p-Ser antibody and (A, B, C, D) IB with anti-nNOS antibody. (E) Quantitative representation of nNOS in P and S, and nNOS serine phosphorylation in P. n=3. Mean±SD. $^aP>0.05$, $^bP<0.05$ *vs* sham. P, crude membranes fraction; S, cytosolic fraction; IP, Immunoprecipitation; IB, immunoblotting.

Effects of brain ischemia on the interaction of nNOS with CaMKIIα or p-CaMKIIα The associations of nNOS with CaMKIIα and p-CaMKIIα were examined by co-immunoprecipitation. nNOS and CaMKIIα can coimmunoprecipitate with each other (Fig 3A). Moreover, the nNOS and CaMKIIα complex level was up regulated remarkably after a 3-min ischemia ($P<0.05$). CaMKIIα level increased significantly during ischemia ($P<0.05$, Fig 3B). The phosphorylated CaMKIIα and that collocating with nNOS increased markedly ($P<0.05$, Fig 3D, 3E).

Effects of KN-62 on the interaction of nNOS with CaMKIIα or p-CaMKIIα Following administration with KN-62, the up-regulation of nNOS and CaMKIIα complex level induced by 15 min ischemia was blocked ($P<0.05$, Fig 4A). Importantly, the increase of p-CaMKIIα collocating with nNOS was diminished accordingly ($P<0.05$, Fig 4B). Both were not

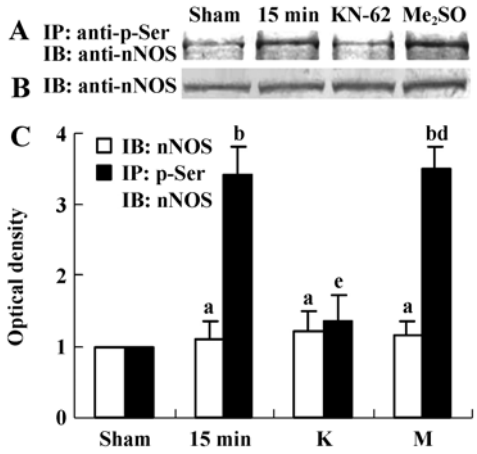

Fig 2. Effect of KN-62 on the elevation of nNOS serine phosphorylation induced by 15 min-ischemia in P. (A) Sample proteins were immunoprecipitated with anti-p-Ser antibody and (A, B) immunoblotted with anti-nNOS antibody. (C) Quantitative representation of nNOS and nNOS serine phosphorylation in P. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* sham. [d]*P*>0.05, [e]*P*<0.05 *vs* 15 min-ischemia. P, crude membranes fraction; IP, immunoprecipitation; IB, immunoblotting; K, KN-62; M, Me_2SO.

affected by Me_2SO (P>0.05, Fig 4A, 4B).

DISCUSSION

nNOS, a soluble and membrane-bound enzyme, appears to localize in specific populations of neurons in the brain and in the nonadrenergic, noncholinergic, autonomic nervous system[16]. In the present study, nNOS was found in both P and S. nNOS serine phosphorylation in P increased markedly and there was no serine phosphorylation of nNOS in cytosolic fraction during brain ischemia, suggesting that nNOS in P was phosphorylated by the protein kinase of the membranes. Although little is known about the factors determining its subcellular distribution, the unchanging levels of nNOS in P and S indicated that brain ischemia was not a factor affecting the distributions of nNOS.

CaMKII constitutes approximately 1 %-2 % of the total protein in the hippocampus[9]. It translocates immediately and completely to crude membranes fraction from cytosolic fraction during brain ischemia[12]. The CaMKIIα level in P increased rapidly and remained high after 3 min and the CaMKIIα level in S decreased rapidly and thoroughly[12]. In this study, nNOS serine phosphorylation in P increased significantly and that in S was not detected. KN-62 (a selective CaMKII inhibitor) diminished the increase of nNOS serine phosphorylation, and did not affect the amount of nNOS in P. These

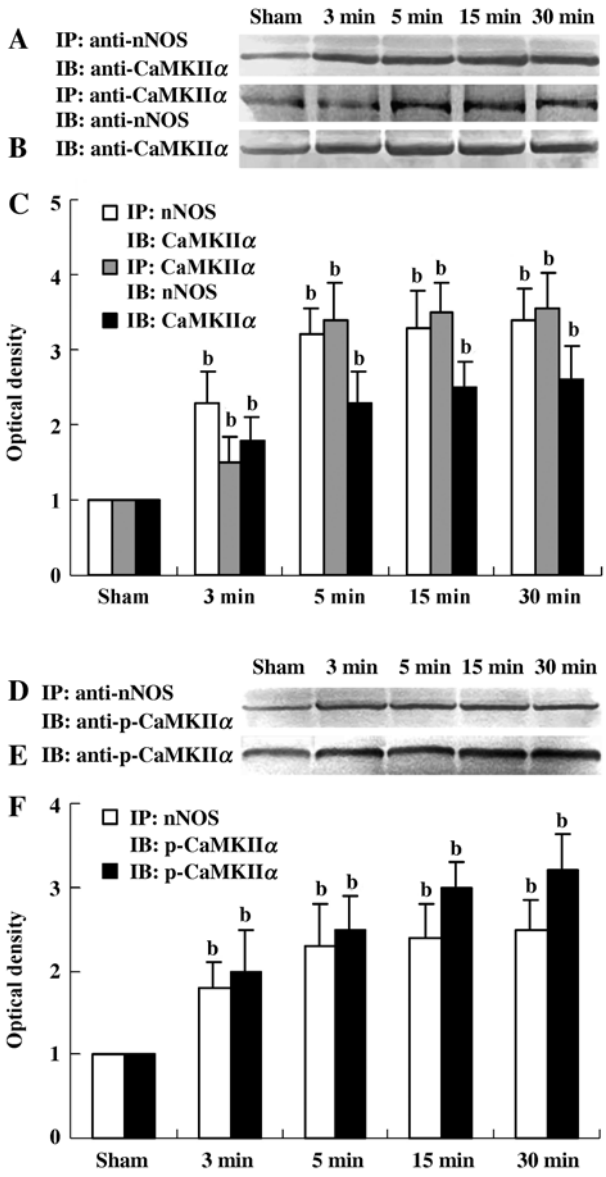

Fig 3. Interactions of nNOS with CaMKIIα or p-CaMKIIα in P during brain ischemia. (A, D) IP with anti-nNOS or anti-CaMKIIα antibody, and IB with anti-CaMKIIα or anti-nNOS or anti-p-CaMKIIα antibody. (B, E) IB with anti-CaMKIIα antibody or anti-p-CaMKIIα antibody. (C, F) Quantitative representation of interaction of nNOS with CaMKIIα or p-CaMKIIα, amounts of CaMKIIα and p-CaMKIIα in P. *n*=3. Mean±SD. [b]*P* < 0.05 *vs* sham. P, crude membranes fraction; IP, immunoprecipitation; IB, immunoblotting.

results supported that CaMKIIα was predominantly implicated in nNOS serine phosphorylation in hippocampus during brain ischemia. The inhibition of the Ser/Thr protein kinase CaMKIIα did not influence the distributions of nNOS.

In the present work, the levels of nNOS and

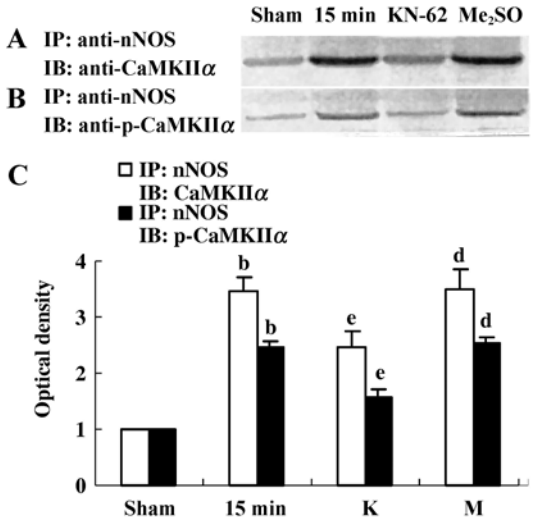

Fig 4. Effect of KN-62 on the interaction of nNOS with CaMKIIα or p-CaMKIIα in P. (A, B) IP with anti-nNOS antibody and IB with anti-CaMKIIα or anti-p-CaMKIIα antibody. (C) Quantitative representation of the interaction of nNOS with CaMKIIα or p-CaMKIIα. n=3. Mean±SD. $^{b}P < 0.05$ *vs* sham. $^{d}P > 0.05$, $^{e}P < 0.05$ *vs* 15 min-ischemia. P, crude membranes fraction; IP, immunoprecipitation; IB, immunoblotting; K, KN-62; M, Me_2SO.

CaMKIIα complex and the phosphorylated CaMKIIα collocating with nNOS were up-regulated during brain ischemia. KN-62 decreased the up-regulations. Our results suggested that nNOS could interact with CaMKIIα, and CaMKIIα collocating with nNOS was autophosphorylated and auto-activated. KN-62 diminished the association of CaMKIIα with nNOS, and decreased the activation of CaMKIIα. Brain ischemia results in rapid increase in Ca^{2+} concentration of cytoplasma in cell that comes from extracellular Ca^{2+} and intracellular store. CaM, an EF-hand family of Ca^{2+}-binding proteins, is a predominant intracellular receptor for Ca^{2+}. Ca^{2+}/CaM binds to CaM binding domain of CaMKIIα that lays C-terminal to the autoregulatory regions, removing autoinhibitory domain, then exposes ATP-binding sites, protein substrate binding sites and Thr^{286} for autophosphorylation[10]. After translocating to postsynaptic density, autophosphorylation of CaMKIIα at Thr^{286} enables substrates such as nNOS to interact with its protein substrate binding sites in the catalytic core of the kinase, thus serine residues of nNOS may be phosphorylated by active p-CaMKIIα[10]. KN-62, a drug belonging to quinolonesulfonamides, functions as a competitive ATP inhibitor[17] . It disabled substrate phosphorylation and autophosphorylation at Thr^{286} by competing ATP-binding sites of CaMKIIα. Moreover, there is a reciprocal facilitation between binding of Ca^{2+}/CaM and ATP[10] , therefore Ca^{2+}/CaM binding was attenuated by KN-62 and the regulatory domain as a pseudosubstrate inhibited nNOS binding. Our results that the levels of CaMKIIα and phosphorylated CaMKIIα increased in P during brain ischemia were in agreement with previous study, supporting that CaMKIIα autophosphorylates and translocates to crude membranes fraction from cytosolic fraction during brain ischemia[12].

nNOS catalytic activity is likely to be decreased by phosphorylation[18], and Ser^{847} phosphorylated nNOS decreases enzyme activity to 70 % of unphosphorylated enzyme[6]. nNOS catalytic activity regulates the production of NO that medicates not only biological actions but also neurotoxicity triggered by NMDAR activation through PSD95[7]. In this current study, during brain ischemia nNOS serine phosphorylation was increased by CaMKIIα. Previous study showed that in the reperfusion, after the peak of the phosphorylation of nNOS at Ser^{847} at 15 min ischemia with 0.5 h reperfusion, the Ser^{847} phosphorylation decreased below sham level at 2 h reperfusion[14]. Therefore, CaMKIIα might play different roles in the early part of brain ischemia and in the transient brain ischemia followed reperfusion. In the early part of brain ischemia, CaMKIIα might play a neuroprotective role by up-regulation of nNOS serine phosphorylation to reduce NO production.

nNOS can be phosphorylated by cylic AMP-dependent protein kinase (PKA), protein kinase C (PKC), and calcium-calmodulin protein kinases (CAM-K)[1]. In the present work, KN-62 attenuated the elevation of nNOS serine phosphorylation induced by 15 min ischemia nearly to sham level in P. There were unchanging levels of nNOS in S during brain ischemia, but there was no serine phosphorylated nNOS although there were other protein kinases such as PKA, PKC. These results supported the idea that during brain ischemia, it was CaMKII, not other protein kinases, that phosphorylated nNOS at serine residues. Little is known concerning why other protein kinases such as PKA, PKC were not involved in serine phosphorylation of nNOS during brain ischemia, and the precise mechanism of the physical interaction between nNOS and CaMKIIα. It is obscure whether nNOS recruits to nNOS-CaMKIIα complex directly through PDZ domain or there is any other adapter proteins such as PSD95 between them like

NMDAR-PSD95-nNOS complex[19] .

Taken together, CaMKIIα may play a neuroprotective role in the early part of brain ischemia by phosphorylating serine residues of nNOS to down-regulate the activity of nNOS and NO production.

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