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Inhibition of nitric oxide-induced nuclear localization of CAPON by NMDA receptor antagonist in cultured rat primary astrocytes

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ABSTRACT

Astrocytes play a key role in regulating aspects of inflammation in the central nervous system. It was observed that nNOS had located in the nucleus of cultured cerebral cortical astrocytes of 7 days. In the present study, we found that carboxy-terminal PDZ ligand of nNOS (CAPON) mainly located in the nucleus of astrocytes stimulated with NO donor sodium nitroprusside (SNP) or GSNO or N-methyl-D-aspartate (NMDA) receptor agonist-NMDA. However, originally, it was localized mostly in the cytoplasm of normal astrocytes. Immunocytochemistry showed that nNOS was co-localized with CAPON in the nucleus of astrocytes stimulated with SNP. In addition to the nuclear localization, treatment with SNP increased the mRNA and protein expression of CAPON. When SNP was removed from media, CAPON accumulated in nucleus transported back to cytoplasm. MK801, an inhibitor of NMDA receptor, was able to reverse the nuclear localization of CAPON resulted from SNP, suggesting that there is a functional relationship of NO with NMDA receptor in the regulation of the nuclear localization of CAPON. These findings provide a new insight in the understanding of the physical and pathological significances of CAPON/nNOS/NMDA receptor.

1. Introduction

Astrocytes are the major glial cell population in the central nervous system (CNS). They play various roles for neurons, for example, as structural support, metabolic delivery of nutrients, maintaining the ionic environment inside the CNS and preserving the blood–brain barrier, etc. In addition, more active roles of astrocytes have been suggested, such as immunocompetent cells in CNS. Data from in vivo experiments have shown that in response to immune insults in CNS, glial cells are activated firstly and secret various cytokines to induce some indirect responses in leptomeningeal cells in response to immune insults in CNS (Perry, 2004; Wang et al., 2008). Activated astrocytes overexpressed inflammatory mediators such as proinflammatory cytokines, cyclooxygenase-2, MMP-9 and nitric oxide synthase (NOS) (Lee et al., 2008; Wang et al., 2008).

Nitric oxide (NO) is a diffusible free radical implicated in a wide variety of physiological and pathological processes ranging from vasodilatation and neurotransmission to antimicrobial activity, excitotoxicity, and neurodegeneration (Dawson and Snyder, 1994; Vincent, 1994; Vincent et al., 1999). NO is biphasic, low concentration of NO physiologically promotes cell survival and proliferation (Jeremy et al., 1999); but higher amounts caused cell growth arrest and cell death. It is synthesized through the conversion of L-arginine to citrulline by the NOS enzymes (Hirsch et al., 1993). There are three NOS isoforms, neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). Usually, iNOS is most often associated with inflammatory conditions in which it is produced in large amounts by monocyte/macrophage lineage cell types. Under some circumstances, nNOS and eNOS are also inducible, but show a longer temporal expression profile than iNOS (Hirsch et al., 1993; Murphy et al., 1993; Ma et al., 1994).

Carboxy-terminal PDZ ligand of nNOS (CAPON) was first identified in the rat as a nNOS binding protein, capable of disrupting the association of nNOS with the postsynaptic density scaffolding proteins PSD93 and PSD95 through the binding of the carboxyl terminus of CAPON to nNOS (Jaffrey et al., 1998). With the amino terminus binding either to a direct target of NO-mediated activation by S-nitrosylation (Fang et al., 2000) or to Synapsin (Jaffrey et al., 2002), CAPON resulted in the localization of nNOS to the presynaptic terminals (Xu et al., 2005). CAPON also has an N-terminal phosphotyrosine-binding (PTB) domain whose

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ligands include dexamethasone-induced Ras protein 1 (Dexras1), which is a member of the Ras family (Fang et al., 2000). CAPON helps regulate nNOS stability, localization, and possibly expression during synapse formation and muscle reinnervation (Jaffrey et al., 1998).

NO produced by nNOS is important for N-methyl-D-aspartate (NMDA) receptor-dependent neurotransmitter release, neurotoxicity (Kang et al., 2004). Stimulation of NMDA receptors activates nNOS and the production of NO (Rameau et al., 2003). Previous study showed that activation of glutamate-NMDA receptors stimulates nNOS, resulting in S-nitrosylation and activation of Dexras1 (Cheah et al., 2006). Altered sub-cellular localization of nNOS can reduce activation of nNOS in response to NMDA receptor activation in hyperammonemia (Loesch et al., 1994). Functional NMDA receptors require NMDA receptor 1 (NR1) subunits in combination with NR2A–D or NR3 (Petralia et al., 1994; Watkins and Collingridge, 1994; Brimecombe et al., 1997; Dingledine et al., 1999). The NR2 subunits show distinct spatiotemporal distributions and modulate channel function (Varney et al., 1996; Scherzer et al., 1998; Sprengel et al., 1998; Chen et al., 1999; Hoffmann et al., 2000; Sun et al., 2000; Lynch and Guttmann, 2002). Functional NMDA receptor expression in reactive astrocytes is based on their intracellular calcium response to NMDA (Krebs et al., 2003).

It has been observed the nuclear localization of nNOS in cultured cerebral cortical astrocytes of rats (Yuan et al., 2004). In the present study we found that CAPON transferred from cytoplasm into the nucleus in inflammatory astrocytes resulting from overxepression of NO. Using NO production as an index of astrocytic cell function, we examined the changes of CAPON including space, time and localization in astrocytes resulting from NO overproduction and analyzed the possible mechanism of nuclear localization of CAPON.

2. Materials and methods

2.1. Chemical reagents

Cell culture reagents including Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum (FBS) were obtained from Gibco-BRL. Sodium nitroprusside (SNP); S-nitrosoglutathione (GSNO); NMDA, an agonist of NMDA receptor; MK801, an inhibitor of NMDA receptor and antibody against GFAP or nNOS were purchased from Sigma. Antibody against CAPON was obtained from Santa Cruz.

2.2. Cell cultures

Primary astrocytes were prepared from cerebral cortex of newborn Sprague–Dawley rats (supplied by the University Laboratory Animal Services Centre, the Nantong University). Briefly, after careful removal of menings, the neopallium were dissected out and digested with trypsin for 10 min at 37 °C. Single cell suspension was obtained by trituration, then was filtered with nylon meshes of 400 oculus and cells were seeded onto poly-D-lysine (50 μg/ml) coated plates. The cells cultured were containing DMEM/F12, 15% heat inactivated fetal bovine serum (FBS), 50 U/ml penicillin, 50 mg/ml streptomycin and L-glutamine. Throughout the study, after subculturing twice, 12–13-day-old cells were used. More than 95% of cells were glial fibrillary acidic protein (GFAP) positive astrocytes, as described previously (Iadecola et al., 1995). Cells were washed twice with PBS and then treated with various reagents. After each treatment, the culture supernatants were collected until assayed for NO concentration.

2.3. RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was isolated from astrocytes using Trizol (Invitrogen, Carlslab, CA). RNA concentration was determined by absorption at 260 nm, and the 260/280 nm absorption ratio of the samples were >1.9. cDNA was produced using reverse transcriptase kit (Fermentas), each sample contains approximately 5 μg RNA. Specific DNA bands were amplified and analyzed by real-time PCR. The primer pairs used for amplification of CAPON (GenBank accession number NM-138922) were 5′-GTGGGCAGCCCCTTAGGTA-3′ (sense); 5′-GATGCCTGACTCTCGGAACTT-3′ (antisense) and 5′ (FAM)-CCTTTGGTCTTCTCCTGCCCTTTT-(TAMRA) 3′ (probe). β2-microglobulin (GenBank accession number NM-012512) was used as an internal control and was detected using the following primers: 5′-GTCTTTCTACATCCTGGCTCACA-3′ (sense); 5′-GACGGTTTTGGGCTCCTTCA-3′ (antisense) and 5′(FAM)-CACCCACCGAGACCGATGTATATGCTGC-(TAMRA) 3′ (probe). The reaction mixes included 10× PCR buffer, 20 mM magnesium chloride, 0.2 mM deoxyNTP, and 10 nmol TaqMan probe with a pair of 10 nmol CAPON primers, 10 nmol β2-M primers, and probe of each molecule. Real-time PCR was performed in a Rotor Gene 3000 Detector (Perkin-Elmer/Applied Biosystems, Foster City, CA). The thermal cycling program consisted of 3 min at 94 °C, followed by 40 cycles of 20 s at 94 °C and 1 min at 60 °C. The reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected (threshold cycle, Ct). Each reaction was performed in quadruplicate and the average Ct value was used in all analysis.

2.4. NO determination

NO production from cells was determined by measuring nitrite, a stable oxidation product of NO. NO_3^- was reducted into NO_2^-, NO_2^- interacted with specific chromogenic agent and then the chromogenic degree, which represents NO level, was detected by spectrophotometric measurements at 530 nm.

2.5. Immunocytochemistry

The subcultured glial cells, grown on poly-L-lysine coated glass coverslips, were washed in warm phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde for 30 min. Cells were blocked with 10% normal donkey serum in PBS for 2 h, and incubated with primary antibodies (GFAP, CAPON and nNOS) in 2% BSA overnight at 4 °C. After extensive washing three times in 0.01 M PBS at room temperature, the cells were incubated with TRITC-labeled antibody, FITC-labeled antibody and Hoechst 33342 (Sigma–Aldrich Corp.) at 37 °C for 2 h. Finally, cells were washed three times and mounted on slides and analyzed by Leica light microscope (Germany). Three independent experiments were performed.

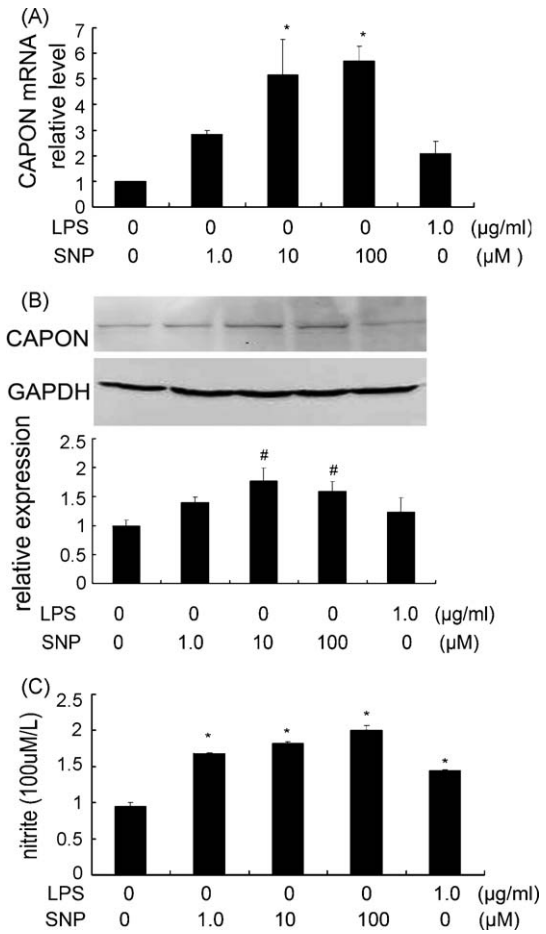

Fig. 1. Expressions of CAPON in rat primary astrocytes stimulated with LPS or SNP. (A) The level of CAPON mRNA was analyzed by using real-time PCR. Cells were treated with different concentrations of SNP (1.0, 10 and 100 μM) or 1.0 μg/ml LPS for 6 h. (B) Western blots of different concentrations of SNP treated for 24 h or LPS stimulated for 24 h, were fractionated by 10% SDS-PAGE, with antibody against CAPON. GAPDH acted as standardization. (C) The concentration of nitrite was measured in supernatants. Astrocytes were stimulated with SNP of different concentrations or 1.0 μg/ml LPS for 6 h. Results from three independent experiments are presented as mean ± S.E.M ($^*p < 0.001$; $^\#p < 0.05$).