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Elevated expression of CAPON and neuronal nitric oxide synthase in the sciatic nerve of rats following constriction injury

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ABSTRACT

Neuronal nitric oxide synthase (nNOS) has been implicated in peripheral nerve lesions and regeneration. The CAPON adaptor protein interacts with the PDZ domain of nNOS, helping to regulate nNOS activity at post-synaptic sites in neurones, but it is not known whether its expression is altered in sciatic nerves after chronic nerve constriction injury. In the present study, the spatiotemporal expression of CAPON was determined in chronically constricted rat sciatic nerves. Similar to the level of protein expression, CAPON mRNA was significantly up-regulated for almost 5 weeks following sciatic nerve injury. Immunohistochemistry demonstrated that increased CAPON was found mainly in S-100-positive Schwann cells. In addition, co-immunoprecipitation demonstrated an interaction between CAPON and nNOS in Schwann cells and the interaction was enhanced in injured sciatic nerves. CAPON may be involved in peripheral nerve regeneration through regulation of nNOS activity.

Introduction

Injuries to peripheral nerves are a consequence of trauma, disease or surgical procedures that require section of peripheral nerves (Belkas et al., 2004). Unlike elements of the central nervous system (CNS), peripheral nerves can regenerate when damaged. After a short (1–2 days) latency to cross the injury site, axones regenerate at a steady rate along the distal nerve, facilitated by reactive Schwann cells and intact endoneurial tubules, which enhance axonal elongation and target re-innervation (Valero-Cabre et al., 2004). Therefore, constriction injuries can be used to investigate the cellular and molecular mechanisms of peripheral nerve regeneration and to assess the role of different factors in the regeneration process (Udina et al., 2003).

Nitric oxide (NO), a short-lived free radical, is synthesised from L-arginine by NO synthases (NOS), including endothelial NOS (eNOS), inducible or macrophage NOS (iNOS) and neuronal NOS (nNOS) (Griffith and Stuehr, 1995). Protein–protein interactions represent an important mechanism in the control of NOS spatial distribution and activity (Alderton et al., 2001; Dedio et al., 2001; Zimmermann et al., 2002). Targeting nNOS to appropriate sites in a cell is mediated by interactions with its post-synaptic density protein-95/discs-large/zona occlusens-1 (PDZ) domain, which consists of a consensus sequence of about 100 amino acids that organise and recruit proteins to the plasma membrane (Kornau et al., 1995; Fanning and Anderson, 1996).

nNOS is larger than eNOS and iNOS because of an amino terminal extension that contains a PDZ domain (Cho et al., 1992; Ponting and Phillips, 1995); post-synaptic density protein-95 (PSD-95) has two PDZ domains, PDZ1 and 2, which connect the carboxy terminus of the NR2B subunit of N-methyl-D-aspartate receptor (NMDAR) and nNOS (Brenman et al., 1996). In the pre-synaptic PDZ domain of synapsin1, Dexras1 interacts with nNOS (Fang et al., 2000). nNOS also has a carboxy terminal PDZ ligand, which connects with the carboxy terminal PDZ ligand of nNOS (CAPON) (Jaffrey et al., 1998). These interactions are thought to facilitate the targeting of nNOS to distinct intracellular sites.

As an adaptor protein for nNOS, CAPON forms a ternary complex with synapsin and nNOS, or with Dexras1 and nNOS, which direct and enhance the delivery and specificity of the NO signal (Jaffrey et al., 1998, 2002; Fang et al., 2000). CAPON, via the PDZ domain of nNOS, regulates nNOS activity at post-synaptic sites in neurones (Jaffrey et al., 1998). Neuronal CAPON expression in the facial motor nucleus decreases after axotomy and recovers with re-innervation (Che et al., 2000). These changes suggest that neuronal CAPON regulates nNOS stability, localisation and possibly expression during synapse formation and muscle re-innervation (Che et al., 2000; Segalat et al., 2005; Chang et al., 2008).

Previous data have been shown that nNOS and CAPON interact both in vitro and in vivo in the mouse cerebellum (Jaffrey et al., 1998), whereas nothing was known about their physiological interaction in the rat sciatic nerve. Work from our laboratory

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Table 1
Numbers of animals used at each time point in the experiment.

A	Naïve	5 d	7 d	10 d	14 d	21 d	28 d	35 d	Total number
RT-PCR	3	3	3	3	3	3	3	3	24
Western blot	4	4	4	4	4	4	4	4	32
Immunofluorescence	4	–	8	–	–	–	–	–	12
Immunoprecipitation	4	–	4	–	–	–	–	–	8
Total number	15	7	19	7	7	7	7	7	76

showed that expression of CAPON protein increased in the sciatic nerve after sciatic nerve transection (Shen et al., 2008), but there are no studies specifically addressing the changes in expression of CAPON after sciatic nerve constriction and nothing is known about the physiological interaction of nNOS and CAPON in the rat sciatic nerve.

This study was undertaken to investigate temporal and spatial patterns of CAPON expression at gene and protein levels in an adult rat sciatic nerve constriction injury model. Co-immunoprecipitation was performed to examine whether changes in CAPON expression correlated with changes in nNOS. In addition, the cellular localisation of CAPON was also assessed in normal and injured sciatic nerves of rats.

Materials and methods

Animals and surgery

All protocols using animals were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals[2] and guidelines for the International Association for the Study of Pain and were conducted according to the Animals Care and Use Committee of Nantong University and approved by the Jiangsu Province Animal Care Ethics Committee (20060205-156).

Male Sprague Dawley rats (Experimental Animal Center, Nantong University, China) weighing 180–220 g were randomly divided into eight groups including one normal group and seven surgery groups (Table 1). These included animals used for reverse transcription-PCR (RT-PCR), Western blot analysis, co-immunoprecipitation and immunofluorescence. The animals were housed individually in standard rodent cages with sawdust bedding and provided with food and water ad libitum on a 12:12 h day/night cycle.

The chronic constriction injury was induced according to the model of Bennett and Xie (1988), with some modifications. The rats were anaesthetised using sodium pentobarbital (40 mg/kg) intraperitoneally and the common sciatic nerve of the left hind leg at the mid-thigh level was exposed and freed of connective tissue by blunt dissection through the biceps femoris. Proximal to the sciatic trifurcation, approximately 7 mM of nerve was freed and four loose ligatures of 4-0 chromic gut were placed around the sciatic nerve. Care was taken to tie the ligatures such that the diameter of the nerve was just barely constricted when viewed with a microscope using a dissecting microscope (40× magnification).

After surgery, all animals received 10 mg/kg naloxone hydrochloride (Sigma Aldrich) intraperitoneally as an anaesthetic antagonist to prevent further cooling of the animals, prevent respiratory depression in the absence of further surgical stimulation and hasten recovery. At 5, 7, 10, 14, 21, 28 and 35 days after surgery, rats were anaesthetised using sodium pentobarbital (50 mg/kg) intraperitoneally and killed by decapitation.

RNA extraction and reverse transcription-PCR

Fresh tissues of both sciatic nerves from three rats at each time point were harvested and frozen rapidly in liquid nitrogen. Total RNA was extracted using Trizol Reagent (Life Technologies). cDNA was synthesised from total RNA using the Revert-Aid First Strand cDNA Synthesis Kit (Fermentas Life Sciences). Specific cDNA fragments were amplified by TaqMan DNA polymerase (Life Technologies) using specific primers for CAPON, nNOS and glyceraldehyde phosphate dehydrogenase (GAPDH) (Table 2). Each reaction (25 μL) contained 2 μL cDNA, 2.5 μL 10× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 10 μM each primer and 2.5 U TaqMan DNA polymerase. Amplification protocols are shown in Table 2. PCR products were detected by electrophoresis in a 2% agarose gel containing 0.5 μg/mL ethidium bromide. Optical densities of bands were analysed with a Molecular Dynamics densitometer (Scion).

[2] http://oacu.od.nih.gov/regs/index.htm.

Western blot analysis

Rats were killed with an overdose of isoflurane by inhalation. Sciatic nerves (equivalent to that used for total RNA isolation) were collected, frozen in dry ice and stored at −70 °C until assayed. Each sample was weighed, homogenised in a lysate buffer containing 1 M Tris–HCl, pH 7.5, 1% Triton X-100, 1% Nonidet P-40 (NP-40), 10% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5 M ethylene diamine tetraacetic acid (EDTA), 10 μg/mL leupeptin, 10 μg/mL aprotinin and 1 mM phenyl methylsulfonyl fluoride (PMSF) (Sigma Aldrich), then centrifuged at 12,000 *g* for 30 min. The supernatant was collected and protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad Laboratories).

Protein (50 μg) was mixed with an equal volume of loading buffer (0.1 M Tris–HCl buffer containing 0.2 M dithiothreitol, 4% SDS, 20% glycerol and 0.1% bromophenol blue), separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidine difluoride filter (PVDF) membranes (Millipore). The membranes were blocked with 5% non-fat powdered skim milk in 20 mM Tris, 150 mM NaCl and 0.05% Tween-20 (TBST).

After 2 h at room temperature, the membranes were washed with TBST three times, then incubated overnight with primary rabbit polyclonal antibodies against CAPON (1:800; Santa Cruz), mouse monoclonal antibody against nNOS (1:2000; clone NOS-B1, catalogue number N2280, Sigma Aldrich) and primary rabbit polyclonal antibodies against GAPDH (1:1000, clone FL-335, catalogue number Sc-25778, Santa Cruz) at 4 °C. After being washed with TBST three times, a goat–anti-rabbit IgG or a goat–anti-mouse IgG conjugated to horseradish peroxidase (1:2000; Southern-Biotech) was added for an additional 2 h at room temperature and the blots were visualised using an enhanced chemiluminescence detection system (ECL kit, Amersham Biosciences). After exposing the membranes to film plates (Eastman Kodak), the plates were developed and then scanned using a Molecular Dynamics densitometer (Scion). Relative amounts of CAPON were quantified by optical density analysis and normalised to GAPDH levels, a domestic loading control.

Co-immunoprecipitation

Six animals received chronic constriction injuries and, 1 week later, the sciatic nerves were homogenised in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.1% SDS; 0.5% sodium deoxycholate; 5 mM EDTA; 10 mg/mL leupeptin; 10 mg/mL aprotinin and 1 mM PMSF). The homogenates were centrifuged at 12,000 *g* for 20 min at 4 °C and the supernatants were collected.

Aliquots of supernatant (0.2 mL) were incubated with 25 μL of protein G Sepharose beads (Santa Cruz) at 4 °C for 3 h, followed by centrifugation at 12,000 *g* for 30 min. The pre-cleared lysates were diluted to approximately 1 mg/mL total protein and reacted with the appropriate primary antibody overnight at 4 °C: rabbit polyclonal antibody against CAPON (1:800; clone R-300, catalogue number Sc-9138, Santa Cruz), mouse monoclonal antibody against nNOS (1:2000; clone NOS-B1, catalogue number N2280, Sigma Aldrich) and mouse monoclonal antibody against β-actin (1:3000; clone AC-15, catalogue number A1978, Sigma Aldrich) overnight at 4 °C, after which 30 μL of protein G Sepharose beads were added and the incubation continued with gentle rotation for 2 h. The antibody against β-actin served as an uncorrelated antibody in both co-immunoprecipitations, for which no cross-reaction was observed with either CAPON or nNOS.

The Sepharose beads were washed three times with phosphate buffered saline (PBS), then resuspended in 30 μL SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1%, bromophenol blue), heated to 95 °C for 5 min and then briefly centrifuged. The supernatants were analysed by SDS–PAGE, followed by immunoblotting for the appropriate second IgG, as described above. For control purposes, the primary antibody was omitted and replaced with beads.

Double or single immunofluorescent staining

Normal rats or rats with sciatic nerve injuries were deeply anaesthetised with pentobarbital (50 mg/kg) and perfused through the left ventricle with 500 mL of 0.9% saline, followed by 500 mL of 4% paraformaldehyde in 0.1 M PBS. Segments of sciatic nerve (1 cm long) centred at the constricted area were carefully dissected free and post-fixed for an additional 4–6 h in the same fixative, followed by immersion in increasing concentrations of phosphate buffered sucrose solution (for cryoprotection): 10% for 1 day, 20% for 1 day, then 30% for 3 days at 4 °C. The specimens