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INCREASED PHOSPHO-ADDUCIN IMMUNOREACTIVITY IN A MURINE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Abstract—Adducins α, β and γ are proteins that link spectrin and actin in the regulation of cytoskeletal architecture and are substrates for protein kinase C and other signaling molecules. Previous studies have shown that expressions of phosphorylated adducin (phospho-adducin) and protein kinase C are increased in spinal cord tissue from patients who died with amyotrophic lateral sclerosis, a neurodegenerative disorder of motoneurons and other cells. However, the distribution of phospho-adducin immunoreactivity has not been described in the mammalian spinal cord.

We have evaluated the distribution of immunoreactivity to serine/threonine-dependent phospho-adducin at a region corresponding to the myristoylated alanine-rich C kinase substrate-related domain of adducin in spinal cords of mice over-expressing mutant human superoxide dismutase, an animal model of amyotrophic lateral sclerosis, and in control littermates. We find phospho-adducin immunoreactivity in control spinal cord in ependymal cells surrounding the central canal, neurons and astrocytes. Phospho-adducin immunoreactivity is localized to the cell bodies, dendrites and axons of some motoneurons, as well as to astrocytes in the gray and white matter.

Spinal cords of mutant human superoxide dismutase mice having motoneuron loss exhibit significantly increased phospho-adducin immunoreactivity in ventral and dorsal horn spinal cord regions, but not in ependyma surrounding the central canal, compared with control animals. Increased phospho-adducin immunoreactivity localizes predominantly to astrocytes and likely increases as a consequence of the astrogliosis that occurs in the mutant human superoxide dismutase mouse with disease progression.

These findings demonstrate increased immunoreactivity against phosphorylated adducin at the myristoylated alanine-rich C kinase substrate domain in a murine model of amyotrophic lateral sclerosis. As adducin is a substrate for protein kinase C at the myristoylated alanine-rich C kinase substrate domain, the increased phospho-adducin immunoreactivity is likely a consequence of protein kinase C activation in neurons and astrocytes of the spinal cord and evidence for aberrant phosphorylation events in mutant human superoxide dismutase mice that may affect neuron survival.

Key words: adducin, motoneuron, motor neuron disease, protein kinase C, spinal cord, superoxide dismutase.

Adducins are heteromeric proteins composed of α, β and γ subunits that cross-link actin with spectrin by bundling and capping actin filaments, and these proteins are widely expressed in many cell types including neurons and glial cells (Bennett and Baines, 2001). Adducin α and γ are ubiquitously expressed, in contrast to the more restricted distribution of β-adducin which is found in CNS tissue and erythrocytes (Bennett and Baines, 2001). Each adducin isoform has an N-terminal globular head domain, a neck domain, and a C-terminal protease-sensitive tail containing a myristoylated alanine-rich C kinase substrate (MARCKS)-related domain (Bennett and Baines, 2001).

The interaction of adducin with spectrin and actin is regulated by calcium and calmodulin, and by several protein kinases (Bennett and Baines, 2001; Kimura et al., 1998; Matsuoka et al., 1998; Shima et al., 2001). Adducin is phosphorylated by a relatively small number of serine/threonine (ser/thr) and tyrosine (tyr) protein kinases including protein kinase C (PKC), cyclic AMP-dependent protein kinase (protein kinase A, PKA), Fyn, and rho-associated kinase (rho-kinase), and is de-phosphorylated by myosin phosphatase (Bennett and Baines, 2001; Kimura et al., 1998; Matsuoka et al., 1998; Shima et al., 2001). The RTPS-serine (ser726 in α-adducin, ser713 in β-adducin and ser662 in γ-adducin) of the MARCKS-related domain is a ser/thr phosphorylation site for PKC (Matsuoka et al., 1998). Phosphorylation of adducin by phorbol esters that activate PKC increases phospho-adducin (p-Add) immunoreactivity in cultured non-neuronal cells, especially in the cytosol, an effect that is attributed to translocation of p-Add to the cytosol (Gilligan et al., 2002; Kaiser et al., 1989). Translocation may inhibit the activity of adducin in promoting spectrin-actin complexes (Matsuoka et al., 1998). Tyr phosphorylation of β-adducin by Fyn leads to translocation of adducin from the cytoplasm to the plasma membrane (Shima et al., 2001).

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Abbreviations: ALS, amyotrophic lateral sclerosis; DAB, diaminobenzidine tetrahydrochloride; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; MARCKS, myristoylated alanine-rich C kinase substrate; mSOD, mutant superoxide dismutase; NGS, normal goat serum; NMDA, *N*-methyl-D-aspartate; p-Add, phosphoadducin; PBS, phosphate-buffered saline; PBST, Triton X-100 in 0.1 M phosphate-buffered saline; PFA, paraformaldehyde; PKC, protein kinase C; ROI, region of interest; ser/thr, serine/threonine; SOD, superoxide dismutase; TPLSM, two photon laser scanning confocal microscopy; tyr, tyrosine, wt, wildtype.

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Only a few studies have examined the distribution of adducin immunoreactivity in the nervous system. In rat brain, α-adducin is localized to regions with high densities of synapses, such as the CA1 and CA3 regions of the hippocampus, cerebral cortex and synaptic terminals of parallel fibers in the cerebellum (Seidel et al., 1995). The distribution of adducin isoforms in mammalian spinal cord has not been determined. Seidel et al. (1995) also found significant immuno-labeling of α-adducin in the processes of glia, both in the hippocampus and cerebellum. High levels of ser/thr p-Add have been detected in the dendritic spines of cultured rat hippocampal neurons and in the hippocampi of young rats (Matsuoka et al., 1998).

Recent studies of protein kinase and phosphoprotein expression using multi-immunoblotting techniques have shown that p-Add is expressed in both murine and human spinal cord (Hu et al., 2003a,b). Interestingly, the expression of p-Add is significantly increased in spinal cord tissue from patients who died with amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder of motoneurons and descending motor pathways, compared with spinal cord tissue from patients who died without neurological disease (Hu et al., 2003b). Significant elevations in the activities and expressions of PKC in spinal cord tissue from ALS patients have been found compared with control tissue (Lanius et al., 1995; Hu et al., 2003b). The explanation for the increased expression of p-Add in spinal cord tissue from patients with ALS is unknown, but could result from increased phosphorylation of Add by PKC.

In this study we have examined the distribution of p-Add in spinal cords of control mice and mice over-expressing mutant human superoxide dismutase (SOD). Mice over-expressing mutant superoxide dismutase (G93A; mSOD) develop a neurodegenerative disorder characterized by progressive motoneuron loss where the progression of neurodegeneration is dependent on the extent of over-expression of mSOD as well as on the specific mSOD mutation.

EXPERIMENTAL PROCEDURES

Animals

Transgenic mice over-expressing human mutant SOD1 protein (G93A mSOD mice) were bred from progenitor stock obtained from the Jackson Laboratory (Bar Harbor, ME, USA; strain B6SJL-TgN (SOD1-G93A)1Gur; Gurney et al., 1994). The mSOD expression was confirmed by a PCR-based assay described on the Jackson Laboratory website. Animals were treated in accordance with the requirements of the SFU Animal Care Committee and the Canadian Council for Animal Care and institutional approval certificates are available on request. These requirements are in accordance with guidelines produced by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were monitored closely following the onset of clinical symptoms and killed at a pre-determined end-point based on the appearance of a set of behavioral markers (including hind limb ataxia, and an inability to forage due to paralysis of the hindlimbs, so-called 'end-stage disease'). Age-matched wildtype (wt) littermates that were phenotypically normal and PCR assay negative served as controls. All efforts were made to minimize the number of animals used and to minimize animal suffering. Mean survival was 121.8±2.0 days (mean±S.E.M., $n=18$) until the end-point. Quantitative estimates of motoneuron loss in mSOD mice at the 'end stage' indicate an ~60% loss in motoneuron pools of the lumbosacral spinal cord (Hamson et al., 2002; Mohajeri et al., 1998).

Histology

Experimental animals ($n=8$) and littermate controls ($n=8$) were killed using progressively increasing CO_2 and O_2, to minimize animal distress and rapidly perfused transcardially with phosphate-buffered saline (0.1 M PBS, pH 7.4) followed by buffered 4% paraformaldehyde (PFA; pH 7.4). Spinal cords were dissected out, post-fixed in 4% PFA for 24 h, transferred to a 20% sucrose/PBS solution overnight for cryoprotection, frozen in Tissue-Tek O.C.T compound (Sakura, Zoeterwoude, Netherlands) and sectioned in the transverse plane at 50 μm on a cryostat. Generally, lumbosacral regions of spinal cord were used for study, as these regions are prominently affected in mSOD mice (Gurney et al., 1994). Unless otherwise indicated all figures are obtained from sections of spinal cord from 'end-stage' animals and similarly aged control littermates.

For immunolabeling of p-Add we used a polyclonal primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and a rabbit anti-p-Add antibody (UBI, Lake Placid, NY, USA). For immunolabeling and Western blotting of α and β adducin in spinal cord, polyclonal primary antibodies to anti-α and anti-β adducin were obtained from Santa Cruz Biotechnology. The anti-α and anti-β adducin antibodies react with peptides mapping near the C terminus of each adducin isoform. The anti-p-Add antibodies selectively recognize ser662 phosphorylated adducin γ, ser724 phosphorylated adducin α and ser713 phosphorylated adducin β (UBI). Spinal cord sections were washed three times using 0.3% Triton X-100 in 0.1 M PBS (PBST, pH 7.4). For immunoperoxidase labeling the free-floating sections were first incubated with 0.3% hydrogen peroxide in 10% methanol for 30 min. After blocking with 10% normal goat serum (NGS) in PBST for 1 h, sections were incubated with primary antibodies to p-Add (1:000 for Santa Cruz; 1:200 for UBI) in PBST containing 10% NGS for 48 h at 4 °C. Sections were then incubated with 1:500 biotinylated anti-rabbit secondary antibody (Vector, Burlingame, CA, USA) for 1 h at room temperature, followed by incubation with 1:1000 ABC reagent (Vector) in PBST for 90 min. Color development was performed using diaminobenzidine tetrahydrochloride (DAB) as a substrate for peroxidase and the reaction was stopped by flooding with distilled water.

Immunofluorescence labeling was performed on floating sections after washing and blocking. Primary antibodies were incubated for 48 h at 4 °C at the following dilutions: 1:500 for anti-microtubule-associated protein-2 (MAP-2; mouse monoclonal, Chemicon, Temecula, CA, USA); 1:200 for anti-glial fibrillary acidic protein (GFAP, rat polyclonal antibody; Calbiochem, Temicula, CA, USA), and 1:25,000 for anti-SMI31 (mouse monoclonal antibody against phosphorylated neurofilament; Sternberger, Lutherville, MD, USA). After washing, appropriate secondary antibodies conjugated to FITC or Cy3 (Molecular Probes, Eugene, OR, USA) were used at a dilution of 1:100, or 1:600, respectively, in PBST containing 5% NGS for 1 h at room temperature. For double immunostaining, primary antibodies were applied to the sections simultaneously. Sections were mounted onto slides, coversliped with Vectorshield (Vector). Appropriate controls without primary antibody were evaluated in parallel. Sections were examined using the Olympus BX40 or the Zeiss confocal microscopes (Jena, Germany).

Quantitative analysis of p-Add immunoreactivity

To quantify p-Add immunoreactivity in mSOD and wt mice, regions of interest (ROI) were defined morphologically in the central canal, and from both ventral and dorsal horns of transverse sections of spinal cord (Fig. 1A). To insure the uniformity of analysis,