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Aberrant δPKC activation in the spinal cord of Wobbler mouse: a model of motor neuron disease

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Protein kinase C (PKC) was suggested to play a role in the pathology of amyotrophic lateral sclerosis (ALS) patients. Activation of PKC delta (δPKC) modulates mitochondrially induced apoptosis. The goal of the present study was to define whether δPKC activation occurs in Wobbler mouse spinal cord (a model of motor neuron disease). The level of δPKC in the soluble fraction was significantly decreased in the spinal cord of Wobbler mice, which was associated with a significant increase in δPKC cleavage. Since caspase-3 is known to cleave δPKC, we determined caspase-3 activation in the Wobbler mice spinal cord, immunohistochemically. The results demonstrated intense immunoreactivity for activated caspase-3 in corticospinal tract motor neurons of Wobbler mice spinal cord. We hypothesize from these results that caspase-3 activation cleaves δPKC, which in turn promotes an aberrant signal transduction pathway in the Wobbler spinal cord.

Keywords: Amyotrophic lateral sclerosis; Neuronal death; Apoptosis; Cytochrome *c*; Neurological disorders; Immunohistochemistry; SOD1 mice

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease that results in degeneration of both upper and lower motor neurons of the brain, brain stem, and spinal cord (Brownell et al., 1970). The cause of most types of ALS remains uncertain, and the disease is incurable.

There is a wide range of theories of the etiology of ALS (Bradley, 1995; Bradley and Krasin, 1982; Lange et al., 1983). The two most currently accepted theories are glutamate excitotoxicity (Bristol and Rothstein, 1996; Rothstein, 1995; Rothstein et al., 1992) and oxidative damage (Browne and Beal, 1994). It is possible that oxidative damage occurs via mitochondrial dysfunction. In fact, the involvement of mitochondrial dysfunction in ALS was suggested by histopathological and biochemical mitochondrial abnormalities reported in both sporadic and familial ALS patients (Ferrante et al., 1997). We recently reported mitochondrial respiration dysfunction in two animal models of motor neuron disease: in complex I, III, or IV in the motor cortex and or spinal cord of Wobbler mice (Dave et al., 2003a; Xu et al., 2001) and in complex IV in the brain and spinal cord of the SOD1 transgenic mouse (Kirkinezos et al., 2001). We further demonstrated that mitochondrial dysfunction develops at an early stage of the disease in spinal cord and is more pronounced in the motor cortex later in the disease (Dave et al., 2003a). This type of mitochondrial dysfunction will generate reactive oxygen species, which can promote oxidative stress.

Oxidative stress can affect many cellular systems. One such system is the neuronal signal transduction pathway (Droge, 2002; Finkel, 2001, 2003; Martindale and Holbrook, 2002; Owuor and Kong, 2002). In fact, an abnormal activation of a key signal transduction molecule, protein kinase C (PKC), was suggested as one of the factors involved in the etiology of ALS (Krieger et al., 1996). PKC is a protein kinase that in normal conditions phosphorylates serine and threonine residues, thus changing the kinetics of specific proteins. However, defining how PKC is involved in ALS is complicated, as there are many PKC isozymes that play multiple and sometimes opposite cellular roles. Among the PKC isozymes, PKC delta (δPKC) is a good candidate to play a role in the pathology of ALS, since it is known to modulate mitochondrial-induced apoptosis (Anantharam et al., 2002; Li et al., 1999; Majumder et al., 2000).

Thus, the goal of the present study was to define whether δPKC activation occurs at the onset of disease in Wobbler mouse spinal cord, a model of motor neuron degeneration (Mitsumoto and Bradley, 1982).

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Materials and methods

Animals

Wobbler mice were bred in our facility. The wr/wr mice used for this study were derived from the original colony (Falconer, 1956) and crossbred with a high-fertility NFR/N inbred strain by Dr. C. Hanson at National Institutes of Health, USA (Mitsumoto and Bradley, 1982). Mice transgenic for the mutated human SOD1 (TgN[SOD1-G93A]1Gur) (mhSOD1) and wild-type human SOD1 (TgN[SOD1]2Gur) (whSOD1) were originally obtained from Jackson Laboratories (Bar Harbor, ME) and were bred in our facility. Both lines were originally made and characterized by Gurney et al. (1994). All animals received food and drinking water ad libitum. Mice showing the Wobbler phenotype (genotype wr/wr, the phenotype appears at about 35–45 days of age) were used in these experiments. Littermates having a normal phenotype (genotypes Wr/Wr and Wr/wr) were used as control. SOD1 mice were sacrificed under halothane anesthesia at 65 days of age. At this time point the symptoms of paralysis have not become evident yet and the mice are considered asymptomatic (Kong and Xu, 1998). The experimental protocol was approved by the Animal Care and Use Committee, University of Miami School of Medicine.

Onset of the disease

The onset of disease in Wobbler mice was identified as described earlier (Krieger et al., 1992). Presently, we used two clinical abnormalities, paw condition and walking pattern, to confirm the onset of disease (Dave et al., 2003a,b).

Cell fractionation and Western blot analysis

In brief, Wobbler and control mice were sacrificed under halothane anesthesia at the first signs of the disease (35–45 days of age), while SOD1 mice were sacrificed similarly at 65 days of age. Mice were decapitated, and motor cortex and spinal cord were removed, frozen immediately in liquid nitrogen, and stored at −80°C until the analysis. The motor cortex was identified with the help of a mouse brain stereotaxic atlas (Franklin and Paxinos, 1996). In the case of SOD1 mice, only the spinal cord was removed. At the time of Western blot analysis, the spinal cord and motor cortex were washed once with cold phosphate-buffered saline (PBS, pH 7.4). Ten percent of homogenate was prepared in homogenizing buffer (4 mM ATP, 100 mM KCl, 10 mM imidazole, 2 mM EGTA, 1 mM $MgCl_2$, 20% glycerol, 0.05% Triton X-100, 17 μg/ml PMSF, 20 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, 25 μg/ml aprotinin) using an all-glass homogenizer. The homogenate was then centrifuged at 4°C at 1000 × *g* for 10 min. The supernatant was carefully taken off and recentrifuged at 16,000 × *g* for 15 min to remove any contaminating pellet material. The resulting supernatant is the soluble/cytosolic fraction. The soluble/cytosolic fraction was analyzed for protein content using the Bio-Rad protein assay kit, based on the method of Bradford (1976).

The proteins were separated by 12% SDS-PAGE. Equal amounts of protein from both groups were run on the same gel and analyzed at the same time. Protein was transferred to Immobilon-P (Millipore, MA, USA) membrane and incubated with the primary antibodies anti-δPKC (1:800) (Calbiochem, La Jolla, CA, USA) and anti-cytochrome *c* (1:800) (PharMingen, CA, USA) for the detection of δPKC and cytochrome *c*, respectively. Immunoreactivity was detected using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Pharmacia Biotech, UK). Western images were digitized at 8-bit precision by means of a charge-coupled-device-based (CCD) camera (8–12 bit, Xillix Technologies Corp., Vancouver, Canada) equipped with a 55 mm Micro-Nikkor lens (Nikon, Japan). The camera was interfaced to an advanced image-analysis system (MCID Model M2, Imaging Research, Inc., St. Catherines, Ont., Canada). The digitized immunoblots were subjected to densitometric analysis using MCID software.

Immunohistochemistry

An immunohistochemical procedure was performed as described previously (Olive and Hodge, 2000; Olive et al., 1997; Tsang et al., 2000). Briefly, mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4), coronal spinal cord sections from the cervical region and coronal brain section at 1.34 mm bregma level were cut at 30 μm on a cryostat, and staining was performed on free-floating sections in PBS/0.4% Triton X-100 (PBST). Following preblocking with 10% goat serum, sections were incubated for 24 h at 4°C with a rabbit polyclonal antisera to cleaved caspase-3 (1:100) (Cell Signaling technology, MA, USA) in combination with mouse monoclonal antibody SMI-32 (1:10,000) (Sternberger Monoclonals Inc, Lutheville, MD, USA), against nonphosphorylated epitope in neurofilament H, which specifically stains motor neurons with considerably higher intensity (Tsang et al., 2000). Following overnight washing with PBST, the sections were then incubated with fluorescent secondary antibodies, fluorescein-labeled anti-rabbit (Santa Cruz Biotechnology, CA, USA) and rhodamine-labeled anti-mouse (Santa Cruz Biotechnology), for 24 h at 4°C temperature. To demonstrate translocation of δPKC to the mitochondria, we incubated spinal cord sections with the mouse monoclonal antibody for cytochrome oxidase subunit four (Cox-IV) (1:250) (mitochondrial marker) and rabbit polyclonal antisera to δPKC (1:500). Following overnight washing with PBST, the sections were then incubated with the fluorescent secondary antibodies, Alexa-680 anti-rabbit (Molecular Probes, OR, USA) and Alexa-488 anti-mouse (Molecular Probes), for 24 h at 4°C temperature. The sections were rinsed in PBST, mounted onto microscope slides, and cover slipped using Prolong Antifade kit (Molecular Probes).

Confocal microscopy and image analysis

Sections were viewed on a Carl Zeiss Laser Scanning Microscope 510. The images of the sections were analyzed using LSM 5 image browser. To analyze colocalization of signals emitted from mitochondrial marker Cox-IV and δPKC, we used LSM software (Carl Zeiss). The degree of colocalization is expressed in colocalization coefficient.

Statistical analysis

All data are expressed as mean ± SEM. Statistical comparison between control and Wobbler groups was performed using Student's *t* test. A *P* value of <0.05 was considered significant.