Neuroscience 155 (2008) 104–113

ANALYSIS OF FK506-MEDIATED PROTECTION IN AN ORGANOTYPIC MODEL OF SPINAL CORD DAMAGE: HEAT SHOCK PROTEIN 70 LEVELS ARE MODULATED IN MICROGLIAL CELLS

M.-S. GUZMÁN-LENIS, C. VALLEJO, X. NAVARRO AND C. CASAS*

Group of Neuroplasticity and Regeneration, Institute of Neurosciences and Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Abstract—Functional loss after spinal cord injuries is originated by primary and secondary injury phases whose underlying mechanisms include massive release of excitatory amino acids to cytotoxic levels that contribute to neural death. Attenuation of this excitotoxicity is a key point for improving the functional outcome after injury. One of the drugs with potential neuroprotective actions is FK506, a molecule widely used as an immunosuppressant. FK506 may exert neuroprotection via inhibition of calcineurin by binding the FKBP12, or by binding other immunophilins such as FKBP52, leading to modulation of heat shock proteins (Hsp) 90 and 70. In the present study, we used an *in vitro* model of organotypic culture of rat spinal cord slices to assess whether FK506 is able to protect them against glutamate excitotoxicity. The results showed that FK506 promoted a significant protective effect on the spinal cord tissue at concentrations of 50 and 100 nM. Hsp70 induction was restricted to microglial cells in spinal cord slices treated with either glutamate or FK506. In contrast, the combination of both agents led to a transient reduction in Hsp70 levels in parallel to a marked reduction in IL-1β precursor production by glial cells. The use of geldanamycin, which promotes persistent induction of Hsp70 in these cells as well as in motoneurons, did not produce tissue neuroprotection. These observations suggest that FK506 might protect spinal cord tissue by targeting on microglial cells and that transient downregulation of Hsp70 on these cells after excitotoxicity is a relevant mechanism of action of FK506.

Key words: FK506, spinal cord injury, organotypic culture, motoneuron, microglia, neuroprotection.

Spinal cord injuries (SCI) disrupt ascending and descending spinal pathways resulting in a devastating loss of sensory and motor functions, generally leading to long-lasting disability of affected patients. The initial impact causes immediate mechanical damage of the spinal cord resulting in massive cell death. A cascade of pathological events, known as secondary injury, develops hours to days after the lesion, resulting in further exacerbation of tissue loss and functional deficits (Tator and Fehlings, 1991; Lu et al., 2000). During this period secondary changes take place distant from the initial injury site and may severely compromise the ability of spared systems that contribute to recovery. For example, atrophy or dysfunction of motor neurons below the injury can extend over periods of weeks or months after SCI (Chang, 1998; García-Alias et al., 2006). Secondary injury changes include a rise of extracellular excitatory amino acid and free radicals concentrations to cytotoxic levels within minutes following trauma (Liu et al., 1991a, 1999; Farooque et al., 1996; Xu et al., 1998; McAdoo et al., 1999; Panter et al., 1990; Park et al., 2004), contributing to neural cell death (Choi, 1988; Lipton and Rosenberg, 1994). Prevention of excitotoxic-mediated cell death has thus been considered critical for improving the functional outcome after SCI. One of the drugs with potential neuroprotective actions for the treatment of SCI is FK506 or tacrolimus, a molecule widely used as an immunosuppressant to prevent rejection in organ transplantation. Several experimental studies have demonstrated that treatment with FK506 increases axonal regeneration after peripheral nerve injuries (Gold et al., 1995; Steiner et al., 1997; Udina et al., 2002, 2004), and prevents axonal damage in toxic neuropathies (Gold et al., 2004b), More recently, it has been shown that FK506 enhances functional recovery, neural protection and axonal sprouting in animal models of SCI (López-Vales et al., 2005, 2006; Madsen et al., 1998; Bavetta et al., 1999) suggesting a novel application for this drug. The mechanisms underlying this neuroprotective action remain unclear, so their elucidation may be useful for the design of selective drugs that mimic the protective and regenerative effects of FK506 but obviate immunosuppression and related side effects (Gold and Villafranca, 2003). The immunosuppressive action of FK506 involves its binding to the 12-kDa immunophilin FK506-binding protein (FKBP12) and consequent inhibition of the calcium-dependent phosphatase calcineurin (Liu et al., 1991b). However, FK506 may exert other actions by binding to different immunophilins, such as FKBP52, leading to modulation of heat shock protein (Hsp) 90 activity (McLaughlin et al., 2002; Davies and Sanchez, 2005) and consequent downstream up-regulation of Hsp70 (Klettner and Herdegen, 2003; Gold et al., 2004b). The aim of the present work was to assess whether application of FK506 is able to protect the spinal cord from an excitotoxic insult, and further investigate its possible mechanisms of action. For this purpose, we have used an *in vitro* model of excitotoxic lesion based on organotypic culture of spinal cord slices.

*Correspondence to: C. Casas, Unitat de Fisiologia Mèdica, Edif. M, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Tel: +34-935811348; fax: +34-935812986.
E-mail address: caty.casas@uab.cat (C. Casas).

Abbreviations: EthD, ethidium homodimer-1; FKBP12, 12-kDa immunophilin FK506-binding protein; Hsp, heat shock protein; LPS, lipopolysaccharide; NO, nitric oxide; P, postnatal day; PBS, phosphate-buffered saline; SCI, spinal cord injury; TUJ, βIII-tubulin.

0306-4522/08$32.00+0.00
doi:10.1016/j.neuroscience.2008.04.078

EXPERIMENTAL PROCEDURES

Organotypic spinal cord and glial cell cultures

Organotypic slice cultures were prepared from Sprague–Dawley rats of postnatal day 7 (P7), using a modification of previously described methods (Stoppini et al., 1991; Rothstein et al., 1993). Briefly, pups were killed by over-exposure to CO_2 and decapitated, and the thoracic segment of their spinal cord was removed and placed in ice-cold high glucose (6 mg/ml) Hanks' balanced salt solution (HBSS) (Sigma, St. Louis, MO, USA). Under sterile conditions, meninges and roots were removed and the spinal cord was sectioned into 350 μm slices with a McIlwain Tissue Chopper (Gomshall, Surrey, UK). Up to five sections were carefully transferred onto Millicell-CM (Millipore, Bedford, MA, USA) culture plate inserts and placed into a six-well plate (Iwaki, Asahi Techno Glass, Chiba, Japan) containing 1.5 ml of Neurobasal medium (Gibco, Grand Island, NY, USA) with 1× B27 (Gibco), 2 mM glutamine, 6 mg/ml glucose and antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin), and incubated at 37 °C in 5% CO_2 for 24 h. The following day, the medium was changed to remove antibiotics and incubated for the next 6 days to stabilize the culture. The medium was changed twice per week until 15 days *in vitro* (DIV). All procedures involving animals were approved by the Ethics Committee of our institution, and followed the European Communities Council Directive 86/609/EEC. We have exercised extreme care to minimize pain. All surgical procedures were carried out under deep general anesthesia. We have proceeded according to the requirements of the procedures themselves, always validated and approved by the local veterinary and animal welfare authorities. We have used the minimum number of animals required to carry out the experiments.

Astrocytes/microglia mixed primary cultures were prepared as previously described (Agullo et al., 1990). Briefly, the cerebellum from P7 rats was dissociated by successive passages through nylon cloths (211 μm-Ø, 135 μm-Ø mesh) and cells were plated (1.2×10^6 cells/well) in Dubecco's modified Eagle's medium (Sigma) with 10% fetal-calf serum (FCS), 100 UI/ml penicillin and 10 μg/ml streptomycin, and incubated at 37 °C in 5% CO_2. After 15 days in culture cells were harvested after the addition of 100 ng/ml lipopolysaccharide (LPS, Sigma) for 20 h to promote glial reactivity.

Excitotoxicity

Two-week-old spinal cord slices cultured in serum-free medium were placed in modified Locke's buffer (137 mM NaCl; 2.5 mM $CaCl_2$; 5 mM KCl; 5.6 mM D-glucose; 0.3 mM KH_2PO_4; 4 mM $NaHCO_3$; 0.3 mM Na_2HPO_4; 0.01 mM glycine; 10 mM Hepes) with 50 μM glutamate, and incubated for 30 min. We have previously found that this concentration of glutamate was able to induce around 50% cell death in the spinal cord slices 2 days after the insult compared with those submitted to long-term cold stress (4 °C for 24 h) (Guzmán-Lenis and Casas unpublished observations). After washing out glutamate traces with medium, the inserts were transferred to warm-fresh medium with or without FK506 (Fujisawa Pharmaceuticals, Osaka, Japan) and incubated for 24 or 48 h. The concentrations of FK506 used (0.5–500 nM) were within the range of those previously described in *in vitro* assays (Dawson et al., 1993; Gold et al., 1999; Klettner and Herdegen, 2003; Muramoto et al., 2003). In other cultures, either cyclosporine A (Tocris, Bristol, UK) or geldanamycin (Sigma) was added instead of FK506. Sham-control samples were manipulated in the same way without the addition of glutamate.

Viability test

We analyzed overall cell death in the slices by measuring the degree of ethidium homodimer-1 (EthD) uptake (Live/Death viability assay, Molecular Probes, Eugene, OR, USA). Cultured slices were incubated with 1.5 μM EthD for 24 h after the excitotoxic insult or sham manipulations. Some slices were submitted to cold stress by leaving them at 4 °C for 24 h to induce a maximum injury on the slices. Following the period of incubation, images were taken with the aid of a digital camera (Olympus C-5050) attached to a fluorescence microscope (Olympus BX-70). Analyses were done with the ImageJ software (National Institutes of Health, USA) by adjusting the threshold above the background and fixing the upper level at 256. The integrated density was measured and used to calculate cell death expressed as percentage of EthD uptake with the formula: % EthD uptake=(Dtreat/Dmax)×100, where Dtreat is the integrated density of the treated slice and Dmax is the integrated density of the slice incubated at 4 °C for 24 h, which closely represents maximal lesion (Krassioukov et al., 2002; Adamchik et al., 2000; Frantseva et al., 1999).

Western blot

Cultured slices were homogenized in modified RIPA buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate (Sigma), 0.2% SDS, 100 mM NaCl, 1 mM EDTA) containing protease inhibitor cocktail (10 μl/ml, Sigma). The homogenate was then centrifuged at 13,000×*g* and the supernatant obtained for protein concentration determination using the BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein (50 μg) were separated on 14% SDS-PAGE gel and transferred to nitrocellulose membrane (Whatman, Dassel, Germany). The membranes were then incubated with 5% skimmed milk powder in TBS plus 0.05% tween-20 (TBST) for 30 min and incubated with primary antibodies anti-HSP70 (1:1000; Stressgen, Victoria, BC, Canada), anti-β-actin (1:1000; Sigma) or anti-IL-1β (1:100; Santa Cruz Technology, Santa Cruz, CA, USA) at 4 °C overnight. Horseradish peroxidase–coupled secondary antibody incubation was performed for 90 min at room temperature and after washing, blots were developed using SuperSignal West pico chemiluminescent substrate (Pierce). Signals were analyzed by band densitometry with the Gene Snap and Gene Tools software in a Gene Genome apparatus (Syngene, Cambridge, UK).

Immunohistochemistry

Cultured slices were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) and, after blocking with 3% bovine serum and 0.3% Triton X-100 in 0.1 M Tris–HCl, 0.15 M NaCl at pH 7.4 (TBS), incubated with primary antibody overnight at 4 °C. Primary antibodies used were: anti-SMI32 (Stenberger Monoclonals, Baltimore, MD, USA), anti-GFAP (Chemicon, Temecula, CA, USA), anti-IB4 (Vector, Burlingame, CA, USA), anti-HSP70 and anti-FKBP52 (Stressgen), anti-βIII-tubulin (TUJ) (Covance, Berkeley, CA, USA), and anti-choline acetyltransferase (ChAT) (Chemicon). Incubation with secondary antibodies Cy3 anti-mouse and Cy2 anti-rabbit (Jackson, West Grove, PA, USA) was made overnight at 4 °C. After several washes, slices were dehydrated and mounted with DPX (Fluka, Buchs, Switzerland). The slices were observed with an Olympus BX-51 epifluorescence microscope equipped with a digital camera and processed with Photoshop. Confocal microscopic examinations were performed with a Leica TCS SP2 AOBS laser scanning confocal system (Leica, Heidelberg, Germany). Images were collected with a 1.4 numerical aperture oil-immersion 63× objective. Confocal images were obtained using two separate photomultiplier channels, either concurrently or in separate runs, and were separately projected and merged using a pseudocolor display showing green for Cy2, red for Cy3 and yellow for colocalization.

Statistical analysis

All data presented are representative of at least four cultured slices of every experiment, repeated at least three times indepen-