Neuroscience 137 (2006) 231–239

NEUROPROTECTIVE EFFECTS OF TACROLIMUS (FK506) IN A MODEL OF ISCHEMIC CORTICAL CELL CULTURES: ROLE OF GLUTAMATE UPTAKE AND FK506 BINDING PROTEIN 12 kDa

C. LABRANDE,[a] L. VELLY,[b] B. CANOLLE,[a] B. GUILLET,[a] F. MASMEJEAN,[c] A. NIEOULLON[c] AND PASCALE PISANO[a]*

[a]*Laboratoire de Pharmacodynamie, Faculté de Pharmacie, Université de la Méditerranée, Marseille, France*

[b]*Département d'Anesthésie-Réanimation, CHU Timone, Université de la Méditerranée, Marseille, France*

[c]*IC2N (UMR 6186), Centre National de la Recherche Scientifique (CNRS), Université de la Méditerranée, Marseille, France*

Abstract—Background: The mechanisms underlying the neuroprotective effects of the immunosuppressant tacrolimus, observed *in vivo*, remain unclear. Here we quantify these effects *in vitro*, and evaluate the potential involvement of the glutamate and/or immunophilin FK506 binding protein 12 kDa in tacrolimus-induced neuroprotection.

Methods: Primary cultures of neurons and astrocytes from rat cerebral cortex were subjected to transient oxygen-glucose deprivation. Neuronal injury was evaluated by cell counting after immunostaining experiments, lactate dehydrogenase release and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction. The involvement of the immunophilin FK506 binding protein 12 kDa was explored using an anti-FK506 binding protein 12 kDa antibody, (3-3-pyridyl)-1-propyl(2 s)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidine carboxylate and rapamycin. Extracellular glutamate and glutamate uptake were respectively measured by high performance liquid chromatography and L-[^{3}H]glutamate incorporation.

Results: When added during either oxygen-glucose deprivation or reoxygenation, FK506 (50–500 pM) offered significant neuroprotection. During oxygen-glucose deprivation, it was able to reverse the oxygen-glucose deprivation-induced increase in extracellular glutamate and decrease in glutamate uptake and this effect was reversed in the presence of threo-3-methyl glutamate, a specific inhibitor of glutamate transporter-1. Blocking FK506 binding protein 12 kDa inhibited the neuroprotection induced by tacrolimus added during either oxygen-glucose deprivation or reoxygenation. Tacrolimus-induced neuroprotection was also reversed in the presence of rapamycin, an immunosuppressant FK506 binding protein 12 kDa ligand devoid of neuroprotective properties and (3-3-pyridyl)-1-propyl(2 s)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidine carboxylate, a non-immunosuppressant ligand of FK506 binding protein 12 kDa, exerteing neuroprotective effects.

Conclusion: The beneficial effects of tacrolimus during *in vitro* ischemia/reperfusion seem to indicate the restoration of a glutamate transporter-1-mediated activity and could be mediated by a FK506 binding protein 12 kDa pathway. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: immunosuppressive drugs, neuroprotection, oxygen-glucose deprivation, immunophilins.

The immunosuppressive compound tacrolimus (FK506) has been reported to be a powerful neuroprotective agent in animal models of cerebral ischemia *in vivo* (Sharkey and Butcher, 1994; Sharkey et al., 1996 and Ide et al., 1996) when injected before or after injury (for review, see Macleod et al., 2005). As an immunosuppressant, tacrolimus is known to inhibit calcineurin-mediated T-cell activation by forming a complex with the immunophilin FK506 binding protein 12 kDa (FKBP12) (Wiederrecht et al., 1992). During ischemia–reperfusion injuries, the involvement of FKBP12 in tacrolimus-induced neuroprotection is still under debate (Tanaka et al., 2002; Gold et al., 1999 and Brecht et al., 2003) and other mechanisms have been explored. Either tacrolimus-mediated protection from free radical-induced deterioration of mitochondrial function after middle cerebral artery occlusion (Nakai et al., 1997), or an *in vitro* (Kikuchi et al., 1998; Dawson et al., 1993; Toung et al., 1999; Butcher et al., 1997) antiexcitotoxic effect mediated by the attenuation of *N*-methyl-D-aspartate (NMDA)-elicited/NO-related free radical production via inhibition of NO synthase, has been suggested.

It is well known that a massive increase of extracellular glutamate levels occurs during ischemia, which induces excitotoxic neuron death mainly due to overstimulation of NMDA receptors (Benveniste et al., 1984). Under normal conditions, the extracellular concentrations of glutamate depend on degree of release into the synaptic cleft and of uptake into cells by high affinity glutamate transporters located both on neurons (excitatory amino acid carrier 1, EAAC1) and glial cells (glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT1)). The role of glutamate uptake in the regulation of the extracellular glutamate concentrations during ischemia has recently been re-assessed as an inhibition (Jabaudon et al., 2000) and even an inversion (Rossi et al., 2000) of the transport process, thus allowing the neurotransmitter to be released from the nervous cells and astrocytes into the extracellular

*Correspondence to: P. Pisano, Labouratoire de Pharmacodynamie, Faculté de Pharmacie, 27, bd Jean Moulin-13005 Marseille, France. Tel: +33-0-4-91-83-56-24; fax: +33-0-4-91-80-29-24.
E-mail address: pisano@pharmacie.univ-mrs.fr (P. Pisano).

Abbreviations: BSS_0, glucose-free balanced salt solution; BSS_{20}, glucose balanced salt solution; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DIV, days *in vitro*; EAAC1, excitatory amino acid carrier 1; FKBP12, FK506 binding protein 12 kDa; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; GLT1, glutamate transporter-1; GPI1046, (3-3-pyridyl)-1-propyl(2 s)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidine carboxylate; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; MEM, Minimum Essential Medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; OD, optical density; OGD, oxygen-glucose deprivation; 3MG, threo-3-methyl glutamate.

0306-4522/06$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.08.080

space. Extracellular glutamate level has not previously been considered as a major determinant of the neuroprotective effect of tacrolimus on *in vivo* models of ischemia (Drake et al., 1996). However, a possible anti-excitotoxic effect of FK506 based on the preservation of glutamate uptake during ischemia has never been explored.

The aim of this study was first to quantify, on primary mixed cultures of neurons and astrocytes from rat cerebral cortex subjected to transient oxygen-glucose deprivation (OGD), the neuroprotection afforded by tacrolimus, added either before injury or during reoxygenation. Secondly, the potential involvement of glutamate and FKBP12 in tacrolimus-induced neuroprotection was analyzed.

EXPERIMENTAL PROCEDURES

The experimental protocols, involving animals and their care, strictly conformed to the guidelines of the French Agriculture and Forestry Ministry (decree 87–848). In all experiments attention was paid to the regulations of local authorities for handling laboratory animals, the European Communities Council Directive (86/609/EEC). Particular efforts were made to minimize animal suffering and to reduce the number of animals used.

Preparation of cell cultures

Mixed cortical neuronal–glial cell cultures were prepared from the cortex of 18-days-old Wistar rat fetuses. Briefly, after removal of meningeal, striatal, hippocampal tissues and olfactory bulbs, cerebral cortices were pooled and maintained in ice-cold phosphate buffered saline solution (PBS; Bio-Whittaker, Emerainville, France) supplemented with glucose (33 mM). Cells were mechanically dissociated from forebrains in dissociation medium by trituration through a fire polished 9-inch Pasteur pipette. After centrifugation (400 r.p.m. for 10 min) the pellets were resuspended in Minimum Essential Medium with Earle's salts L-glutamine free (MEM; Life Technologie, Invitrogen, Cergy Pontoise, France), supplemented with glucose 15 mM (final concentration 20 mM), calf serum 5%, horse serum 5% (Sigma, Lyon, France), penicillin 50 UI/ml and streptomycin 50 μg/ml. Cells were plated to achieve a confluent monolayer (10^5 cells/cm^2) on plastic 12 or 24-well culture plates (Costar, Vitaris, Baar, France) previously coated with poly-ornithine (10 μg/ml). Cells were incubated at 37 °C in a humidified 6% CO_2 and 94% air atmosphere. Experiments were performed after synaptogenesis, on mature cultures, in a serum free-medium, at 13 days *in vitro* (DIV). Cell types at DIV 13 were analyzed by immunochemical staining and determined to be 51±8% neurons, 35±6% astrocytes and 14±8% undetermined cells.

OGD followed by reoxygenation

Mixed cultures were exposed to a transient OGD as described by Goldberg and Choi (1993) with little modifications (Velly et al., 2003). Cells were placed in a hypoxic chamber (pO_2 <2 mm Hg by constant insertion of 94% N_2 and 6% CO_2). Culture medium was exchanged two times with a glucose-free balanced salt solution (BSS_0–O_2 in mM: NaCl 116, KCl 5.4, $MgSO_4$ 0.8, NaH_2PO_4 1.0, $CaCl_2$ 1.8, $NaHCO_3$ 26.2, Phenol Red 0.025, sucrose 20) bubbled with an anaerobic gas mix (95% N_2, 5% CO_2) for 30 min to remove residual oxygen. Cells were incubated at 37 °C in this solution for 80 min to produce OGD. This OGD duration produced injury selectively limited to neurons with no injury to the glial layer (Velly et al., 2003). OGD was terminated by removing cultures from the chamber, replacing the exposure solution with oxygenated MEM (20 min bubbling with a 95% air, 5% CO_2 aerobic gas mix) supplemented with 20 mM glucose and returning the multi-wells to the incubator under normoxic conditions for 24 h. Cells submitted to OGD and not treated with drugs were called control cells. Sham wash cell cultures, not submitted to OGD, were placed in glucose balanced salt solution (BSS_{20})+O_2 (containing 20 mM glucose and aerated for 20 min with aerobic gas mix) for 80 min. Then BSS_{20}+O_2 was replaced by MEM.

Lactate dehydrogenase (LDH) release

Neuronal injury was quantitatively assessed by measurement of LDH release into bathing medium, immediately after OGD and 24 h after reoxygenation, with Cytotoxicity Detection Kit (LDH) from Roche Diagnostic, Meylan, France. This cytosolic enzyme is released from damaged or destroyed cells and it has been established previously that LDH release correlates linearly with the number of damaged or dying neurons after both excitotoxic and apoptotic injuries. Background LDH levels were determined in sham wash sister cultures and subtracted from experimental values to yield the LDH activity specific to the experimental injury. Results obtained immediately after OGD and 24 h after reoxygenation were pooled and expressed as percent of the maximal LDH level corresponding to a near complete neuronal death without glial cell death (=100%), determined by assaying sister cell cultures exposed to NMDA (Tocris) 300 μM and glycine 1 μM (Tocris, Zürich, Switzerland) for a full 24 h.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction test

Neuronal viability was quantitatively evaluated by the reduction of MTT (Sigma) by the mitochondrial succinate dehydrogenase in living cells. Twenty-four hours after OGD, cultures were incubated with MTT 5 mg/ml (v/v: 1/10) for 3 h at 37 °C. The formazan product formed by the reduction of MTT was solubilized in HCl 0.08 N/isopropanol. Cell viability corresponded to the value of the optical density (OD) read at 570 nm with background subtraction at 630 nm. Values obtained from cultures exposed to NMDA and glycine during the 24 h following the OGD (100% neuronal death) were subtracted to those measured in all experimental conditions. Results were expressed as percent of the OD measured in sham wash cells.

In order to determine the involvement of FKBP12 in the neuroprotection of tacrolimus, we also studied the effect of a mouse monoclonal anti-FKBP12 antibody (Interchim, Montluçon, France) on the tacrolimus-induced inhibition of OGD-elicited MTT reduction. This antibody is directed toward the first 13 amino end sequence of the human FKBP12 protein and does not cross-react with the other members of the immunophilin family. For these experiments, prior to adding tacrolimus (50 pM) and anti-FKBP12 antibody (50 nM) during OGD, cells were permeabilized by saponin (15 μg/ml for 10 min). Negative controls were performed using a purified mouse irrelevant IgG1 (Immunotech, Marseille, France; 50 nM).

Immunocytochemistry

Assessment of the OGD-induced neuronal damage was also performed 24 h after the insult, by astrocytes and neurons immunochemical numeration, as described by Velly et al. (2003). Neurons were labeled with mouse monoclonal anti-microtubule associated protein (MAP2a; 1:500 dilution; Roche Diagnostics) and astrocytes with rabbit monoclonal anti-glial fibrillary acidic protein (GFAP; 1:500 dilution; Dako, Glostrup, Denmark). Secondary antibodies were from Molecular Probes, Invitrogen: Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 546 goat anti-mouse IgG (H+L). Nuclear counterstaining was performed with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma). The numeration was performed on three independent cultures and four distinct fields. Results were expressed as percent of the total