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Effect of FK506 and cyclosporine A on the expression of BDNF, tyrosine kinase B and p75 neurotrophin receptors in astrocytes exposed to simulated ischemia *in vitro*

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Abstract

We investigated whether the immunosuppressive drugs, FK506 and cyclosporine A, increase BDNF protein and/or mRNA expression in ischemic astrocytes and if an increase could be related to changes in the nuclear expression of p-CREB, p-Erk1/2 and p-Akt. The influence of these immunosuppressants on protein and mRNA levels of TrkB and $p75^{NTR}$ receptors was also examined. On day 21, cultures of rat astrocytes were subjected to ischemic conditions simulated *in vitro* (combined oxygen glucose deprivation, OGD) for 8 h and exposed to FK506 (10−1000 nM) and cyclosporine A (0.25−10 μM). FK506 and cyclosporine A (at 1000 nM and 0.25 μM, respectively) stimulated the expression and release of BDNF in cultured rat cerebral cortical astrocytes exposed to OGD. The immunosuppressants at these doses simultaneously increased p-CREB and p-Erk1/2 expression in the nuclear fraction of astrocytes. The results RT-PCR and Western blot analysis provided further evidence of a modulating influence of the drugs on the expression of *trkB* and *$p75^{NTR}$* genes and their protein products in ischemic astrocytes. Published by Elsevier Ltd on behalf of International Federation for Cell Biology.

Keywords: FK506; Cyclosporine A; Astrocytes; Ischemia; BDNF; p-CREB; TrkB; $p75^{NTR}$

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family which includes nerve growth factor (NGF), neurotrophin (NT)-3 and NT-4/5 (Patapoutian and Reichardt, 2001). Numerous observations demonstrate the importance of BDNF for brain development, regulation of synaptic plasticity and protection of neurons against different kinds of insults (Ballarin et al., 1991; Zhou et al., 1996). Decreased BDNF level may also contribute to brain and spinal cord ischemic injury (Tokumine et al., 2003). In the CNS, BDNF is expressed and released by glial cells as well as neurons (Koyama et al., 2005). For this reason, astrocyte-derived BDNF could play a significant role in maintaining brain functions under both physiological and pathological conditions.

BDNF released on cell surface selectively binds to TrkB tyrosine kinase receptor and also interacts with low affinity with $p75^{NTR}$, a member of the tumor necrosis factor receptor family. In cells co-expressing $p75^{NTR}$ with tyrosine kinase receptors (Trks), their interaction either increases neurothrophin binding affinity, reduces Trks responses to non-preferred ligands, or facilitates apoptosis (Benedetti et al., 1993). Astrocytes express both types of receptors (Condorelli et al., 1994). Concomitant upregulation of TrkB and $p75^{NTR}$ follows global transient cerebral ischemia, suggesting that an imbalance of $p75^{NTR}$ and TrkB receptor-mediated signals is involved in ischemic brain injury (Ferrer et al., 1998). Therefore, it seems that restoration of the correct ratio of expression of the two receptors can be a target for neuroprotection.

Stimulation of *bdnf* and *trkB* genes transcription depends on the cyclic AMP-dependent response element binding protein (CREB) transcription factor. Activated CREB after phosphorylation at Ser 133 (p-CREB) binds to cAMP response element (*CRE*) in *bdnf* promoter III or second *CRE* of the two

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CRE sites located within the P2 promoter of *trkB* (Deogracias et al., 2004). Protein kinase A (PKA), protein kinase C (PKC), Akt, Erk1/2 and p38 mitogen activated protein kinases (MAPK), and Ca^{2+}-calmodulin kinase (CaMK) phosphorylate the Ser 133 residue of the CREB (Rao et al., 2007). However, BDNF-induced CREB activation in glial cells is mediated mainly through the activation of Erk1/2 and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways (Kim et al., 2007). On the other hand, some evidence indicates that *CRE*-mediated transcription depends not only on the activity of the protein kinases phosphorylating CREB, but also on the activity of calcineurin (PP2B), i.e. the Ca^{2+}/calmodulin-dependent protein phosphatase. Dephosphorylation of CREB by calcineurin via protein phosphatase-1 (PP1) is crucial for the transcriptional competence of p-CREB (Bito et al., 1996).

The protective potential of immunosuppressants such as FK506 and cyclosporine A has been reported in many experimental models of ischemia both *in vivo* and *in vitro* (Phillis et al., 2002; Sinigaglia-Coimbra et al., 2002; Domanska-Janik et al., 2004; Yamaguchi et al., 2006). Most of the experimental data prove the main role of specific calcineurin inhibition in their neuroprotective effects. However, current studies on the neuroprotective properties of immunosuppressants suggest not only inhibition of calcineurin, but also stimulation of BDNF expression. Miyata et al. (2001) have described upregulation of BDNF and TrkB protein expression in the CA1 region of hippocampus after forebrain ischemia in cyclosporine A-treated rats, suggesting the mechanism that the protection of the cells in forebrain in ischemia involves interactions between p-CREB, BDNF and TrkB. However, precise determination of which type of cell showed increased of BDNF synthesis was lacking. Results obtained by Zawadzka and Kaminska (2003) indicate that astrocytes treated with FK506 might be a source of BDNF, but the cells in normoxia were exposed to toxic drug concentrations (30–50 μM), which dramatically reduced viability and induced apoptosis. To our knowledge, there are no data on the influence of these immunosuppressants on the profile of changes in TrkB and $p75^{NTR}$ protein or gene expression in ischemic astrocytes.

On the basis and the results of our previous study (Gabryel et al., 2006a,b), we assume that low concentrations of immunosuppressants affect BDNF protein and/or mRNA expression in ischemic astrocytes and that this is related to changes in expression of p-CREB. We also suppose that these drugs have a normalizing effect on the changes induced by ischemia in the expression of TrkB and $p75^{NTR}$ receptors in astrocytes. To test this hypothesis, primary cultures of cortical rat astrocytes were subjected to ischemia-simulating conditions (combined oxygen glucose deprivation, OGD).

2. Methods

2.1. Cell culture

Astrocytes were isolated from one-day old Wistar rat pups and cultured essentially according to Hertz et al. (1985). The study was approved by the Local Ethics Commission for the Animal Experimentation. Briefly, hemispheres of newborn Wistar rats were removed aseptically from the skulls, freed of the meninges, minced and mechanically disrupted by vortexing in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension was filtered through sterile nylon screening cloth with pore sizes 70 μm (first sieving) and 10 μm (second sieving). The cells were counted in a Coulter Z1 counter (Coulter Counter, Buckinghamshire, UK). The concentration of cells in suspension was adjusted to 1×10^6 cells/ml. The cells were sieved onto plastic dishes of 100 mm in diameter at 1×10^6 cells/dish. Subsequently, cultures were incubated at 37 °C in 95% air and 5% CO_2 with 95% relative humidity (CO_2-Incubator, Kebo-Assab, Stockholm, Sweden). The culture medium initially contained 20% of fetal bovine serum (FBS) and after 4 days was replaced with medium containing 10% FBS. The total volume of culture medium was changed twice a week. The cells were cultured for 2 weeks until confluent. On 14th day *in vitro* (DIV) contaminated microglia and oligodendrocytes cells were removed by shaking at 200 rpm on an orbital shaker for 5 h and incubating with 5 mM L-leucine methyl ester (Simmons and Murphy, 1992). To identify astrocytes, cultures were stained immunocytochemically for glial fibrillary acidic protein (GFAP) (Sigma–Aldrich, St. Louis, MO, USA), a specific marker for astrocytes. Analysis of the cultures has shown that 90–95% of cells were GFAP-positive. About 1–2% of cells in cultures reacted with Ricinus Communis Agglutinin-1, a lectin that binds to surface glycoproteins on microglia (Vector, Burlingame, CA, USA). No neurons, as confirmed by an immunocytochemical staining method using monoclonal antibodies against MAP-2 (Promega, Madison, WI, USA), were detected. All experiments were performed on 21-day old cultures.

2.2. Treatment of astrocyte cultures

Prior to the experiment, the cells were incubated overnight with fresh medium. At the 21st DIV, cultures of astrocytes were placed in the medium deprived of glucose and serum. Osmolarity of the medium was measured and adjusted to 319 mOsm with 20% mannitol (0.9 ml/100 ml) and cell cultures were incubated for 8 h in the ischemia-simulating conditions (oxygen glucose deprivation, OGD): 92% N_2, 5% CO_2 and 3% O_2 at 37 °C (CO_2-incubator, Heraeus, Hanau, Germany). Cells were treated with FK506 (10, 100, 1000 nM) and cyclosporine A (0.25, 1 and 10 μM) during 8 h simulated ischemia. Cyclosporine A was purchased from Sigma–Aldrich, and FK506 from Calbiochem (Darmstadt, Germany). Drugs were dissolved in ethanol at an initial concentration of 1 mM. Further dilutions were performed in the appropriate medium (final concentration in the medium did not exceed 0.1%). Corresponding amounts of ethanol were added to the control cultures.

2.3. Cell viability

Cell viability of astrocytes treated with FK506 and CsA was assayed with the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum

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