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INVESTIGATION OF THE MECHANISMS OF NEUROPROTECTION MEDIATED BY Ro5-4864 IN BRAIN INJURY

EILAM PALZUR,[a] AVIRAM SHARON,[b†] MONA SHEHADEH[a] AND JEAN FRANCOIS SOUSTIEL[a,b*]

[a] *Eliachar Research Laboratory, Medical Center of the Galilee, Faculty of Medicine in the Galilee, University of Bar Ilan, Naharia 22100, Israel*

[b] *Department of Neurosurgery, Medical Center of the Galilee, Faculty of Medicine in the Galilee, University of Bar Ilan, Naharia 22100, Israel*

Abstract—Increasing evidence has established the involvement of the 18-kDa translocator protein (TSPO) in the process of mitochondrial membrane permeabilization and subsequent apoptosis through modulation of the mitochondrial permeability transition pore. Recent studies have shown that treatment with Ro5-4864, a TSPO ligand, resulted in a neuroprotective effect in traumatic brain injury. Yet, the nature of this effect remained uncertain as mature neurons are considered to be lacking the TSPO protein. In order to investigate the mechanism of Ro5-4864-mediated neuroprotection, the neuro-inflammatory and neurosteroid response to cortical injury was tested in sham-operated, vehicle, cyclosporine A (CsA) and Ro5-4864-treated rats. As anticipated, the levels of interleukin 1β and tumor necrosis factor α, as well as the astrocyte and microglia cellular density in the injured area were all decreased by CsA in comparison with the vehicle group. By contrast, no visible effect could be observed in Ro5-4864-treated animals. None of the groups showed any significant difference with any other in respect with the expression of brain-derived neurotrophic factor. Double immunofluorescence staining with NeuN and TSPO confirmed the absence of TSPO in native neurons though showed clear evidence of co-localization of TSPO in the cytoplasm of NeuN-stained injured neurons. Altogether, this study shows that the neuronal protection mediated by Ro5-4864 in brain injury cannot be solely attributed to an indirect effect of the ligand on glial TSPO but may also represent the consequence of the modulation of upregulated TSPO in injured neurons. This observation may be of importance for future pharmacological research in neurotrauma.

Key words: 18-kDa translocator protein, mitochondrial permeability transition pore, Ro5-4864, cyclosporine A, traumatic brain injury, neuroinflammation.

INTRODUCTION

Since its first description by Braestrup and Squires (1977), the 18-kDa translocator protein (TSPO), formerly known as peripheral benzodiazepine receptor, has gained increasing attention, recently leading to the recent completion of its three dimensional structure by Guo et al. (2015). Characterized by a widespread distribution across tissues and species, the TSPO has been shown to participate in a vast array of important biological functions such as steroids synthesis and regulation, mitochondrial respiration and mitochondrial membrane permeabilization. This latter functional aspect of TSPO was first suggested by its co-precipitation with the voltage-dependent anion channel and the adenine nucleotide translocator, both formerly presumed to be components of the mitochondrial permeability transition pore (mPTP) (McEnery et al., 1992). This observation led to the hypothesis that TSPO may represent a constituent of the mitochondrial permeability transition pore (mPTP) with a potential therapeutic interest in oncological research through modulation of the apoptotic process in cancer cells (Banker et al., 2002; Bono et al., 1999; Decaudin et al., 2002; Gonzalez-Polo et al., 2005; Hirsch et al., 1998; Maaser et al., 2001). More recently, the same concept of mPTP modulation by TSPO ligands was oppositely explored for mitochondrial protection. Addition of a specific anti-TSPO antibody in isolated intact rat brain mitochondria resulted in delayed calcium-induced dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and diminished cyclosporine A (CsA)-sensitive calcium efflux, which are both indicative of mPTP inhibition (Azarashvili et al., 2007). In a recent experimental animal studies, we provided evidence supporting the potential neuroprotective effect of the TSPO ligand Ro5-4864 in traumatic brain injury and showed that cell protection was mediated by preservation of $\Delta\Psi_m$ and mitochondrial respiration (Soustiel et al., 2008, 2011). Yet, in a critical review of the potential therapeutic implications of TSPO ligands in neurotrauma, Papadopoulous and Lecanu, while emphasizing the very low levels of TSPO in neurons, suggested that the neuroprotective effect provided by Ro5-4864 may rather be mediated by steroid synthesis in activated reactive glial cells or reflect the impact of TSPO on tissue repair and regeneration.

*Correspondence to: J. F. Soustiel, Department of Neurosurgery, Galilee Medical Center, P.O.B. 21, Naharia 22100, Israel. Tel: +972-4-9107651; fax: +972-4-9107205.

E-mail address: JeanS@gmc.gov.il (J. F. Soustiel).

† Equal contribution.

Abbreviations: CsA, cyclosporine A; GFAP, anti-glial fibrillary acidic protein; IL-1β, interleukin-1β; mPTP, mitochondrial permeability transition pore; ROI, regions of interests; TSPO, translocator protein.

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Accordingly, the purpose of the present study was to elucidate the possible routes of neuroprotection mediated by Ro5-4864 in a rat model of traumatic brain injury.

EXPERIMENTAL PROCEDURES

Surgical procedures

All animal procedures were approved by the Bar-Ilan University Animal Care Committee and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was based on a model of cortical contusion extensively described in previous studies (Soustiel et al., 2008). Briefly, Sprague–Dawley adult male rats were anesthetized by intraperitoneal injection of Equithesin (4 ml/kg of body weight). Through a sagittal incision in the scalp, the parietal bone was drilled out with a diamond burr creating a craniectomy of 5 mm of diameter. The dura was then widely opened under magnification and the craniectomy connected to a vacuum pump through a specially designed transparent connector. Negative pressure of 400 mbar (0.605 ATA) was applied to the cortical surface for 10 s. During exposure to the intense negative pressure, the torn cortical tissue can be visualized through the transparent connector for lesion control. The skin was then sutured and the animal allowed to resume normal activity. Sham-operated animals received a skin incision followed by drilling of the skull though were not submitted to cortical injury. At timing defined by the study protocol (see below), animals were re-anesthetized and sacrificed.

Study design

Following the injury, animals were allocated into three groups as follows: group 1 — dimethylsulfoxide 1% in saline (DMSO–vehicle); group 2 — Ro5-4864 dissolved in vehicle; group 3 — Cyclosporine A (CsA, Sandimmune, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) dissolved in saline. For dot blot analysis, a fourth group consisted in sham-operated animals as described above. Five animals were included into each group for dot blot analysis whereas eight rats were used for immunostains and immunofluorescence studies. Animals received two intraperitoneal injections at 30 min and 24 h post injury. Dosage for Ro5-4864 was defined as the highest possible dosage compatible with absence of toxicity since dosage higher than 5 mg/kg has been found to cause epileptic seizures (Nakamoto et al., 1996; Shiotani et al., 2000). The dosage elected for CsA, 20 mg/kg, was based on numerous previous studies (Galluzzi et al., 2009; Sullivan et al., 2011).

Cytokines analysis (Dot blot analysis)

Cytokines are important molecules released by TSPO-expressing cells such T lymphocytes, mononuclear and macrophage cells that may significantly affect functions of astrocytic and microglial cells involved in the inflammation process and therefore modify the outcome of the injury. Accordingly, assessment of levels of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) was performed in the injured tissue. Based on several previous studies exploring the temporal profile of cytokines release after brain injury (Dalgard et al., 2012; Ross et al., 1994; Stover et al., 2000), rats were sacrificed at 24 h. The injured area was dissected under magnification and immersed in iced cold lysis buffer (10 mM NaH_2PO_4, 1 mM $MgCl_2$, 30 mM NaCl, 0.02% NaN_3) containing protease inhibitors (Complete Mini, Boehringer, Mannheim, Germany). The tissue was then homogenized with a 3 × 30 inch polytron homogenizer (× 620 CAT, D-7813 Staufen, Germany). Crude homogenates were centrifuged at 16,000*g* for 30 min at 4 °C and the supernatant recovered. The protein concentrations were measured by Bradford assay. Two microliter of the brain lysate sample, containing 100 μg of protein, were applied to a nitrocellulose membrane. In addition, purified rat IL-1β (Sigma–Aldrich, Saint Louis, MO, USA) and TNF-α and brain-derived neurotrophic factor (R&D Systems, Minneapolis, MN, USA) with a known concentration (12.5, 25, 50, 100, 200, 400 and 800 pg/ml) were also applied to the nitrocellulose membrane to be used as extrapolation standards.

Following transfer, the membranes where blocked with TBS w. Tween 20 (Tris Buffer Saline: 23 mM TRIZMA BASE, 136 mM NaCl, 0.5% Tween 20, pH-7.6) containing 5% (wt/vol) dried milk (Carnation, Glendale, CA, USA) overnight at 4 °C, and incubated with first antibodies either anti-IL-1β or anti-TNF-α antibody (R&D Systems, Minneapolis, MN, USA), all diluted at 1:500 in TBS w. Tween 20 containing 3% (wt/vol) dried milk, for 3 h at room temperature. After rinsing with TBS, the membranes where incubated with secondary antibody diluted at 1:5000 in TBS w. Tween for 1 h at room temperature.

After extensive washing, proteins were detected using enhanced chemiluminescence western blotting reagents (Pierce, Rockford, IL, USA) and exposed to X-Omat LS (Kodak scientific imaging film, Kodak USA). To quantify protein immunoreactivity, films were scanned and densitometry was performed. The optical density values for IL-1β and TNF-α for each dot were normalized to that of the related positive controls standard curves.

Brain-derived neurotrophic factor analysis (Dot blot analysis)

Brain-derived neurotrophic factor is an important trophic protein released by activated microglia that can affect the fate of the injured brain cells. Since the expression of BDNF has been shown to be regulated by neurosteroids, the synthesis of which is one of the most clearly established functions of TSPO, otherwise highly expressed in microglia, it may be hypothesized that treatment with TSPO ligands may result in modulation of BDNF release through release of neurosteroids. Accordingly, BDNF levels were quantified in the injured tissue, using the same dot blot technique used for cytokines. As for cytokines, the time elected for assessment of BDNF levels (day 4) was based on previous studies showing high levels of BDNF up to day 6 (Chen et al., 2002; Iwamoto et al., 1996). Purified BDNF and first antibody anti-BDNF (R&D Systems, Minneapolis,