Neuroscience 164 (2009) 520–529

NEUROPROTECTIVE EFFECTS OF A LIGAND OF TRANSLOCATOR PROTEIN-18kDa (Ro5-4864) IN EXPERIMENTAL DIABETIC NEUROPATHY

S. GIATTI,[a] M. PESARESI,[a] G. CAVALETTI,[b] R. BIANCHI,[c] V. CAROZZI,[b] R. LOMBARDI,[d] O. MASCHI,[e] G. LAURIA,[d] L. M. GARCIA-SEGURA,[f] D. CARUSO[e] AND R. C. MELCANGI[a]*

[a]*Department of Endocrinology, Pathophysiology and Applied Biology, Center of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Via Balzaretti 9, Milano, Italy*

[b]*Department of Neurosciences and Biomedical Technologies, University of Milano "Bicocca", Monza, Italy*

[c]*Department of Molecular Biochemistry and Pharmacology, "Mario Negri" Institute for Pharmacological Research, Milano, Italy*

[d]*Neuromuscular Diseases Unit, Fondazione IRCCS National Neurological Institute "Carlo Besta", Milano, Italy*

[e]*Department of Pharmacological Sciences, University of Milano, Milano, Italy*

[f]*Instituto Cajal, C.S.I.C., Avenida Doctor Arce 37, 28002 Madrid, Spain*

Abstract—Peripheral neuropathy represents an important complication of diabetes involving a spectrum of structural, functional and biochemical alterations in peripheral nerves. Recent observations obtained in our laboratory have shown that the levels of neuroactive steroids present in the sciatic nerve of rat raised diabetic by a single injection of streptozotocin (STZ) are reduced and that, in the same experimental model, treatment with neuroactive steroids, such as progesterone, testosterone and their derivatives show neuroprotective effects. On this basis, an interesting therapeutic strategy could be to increase the levels of neuroactive steroids directly in the nervous system. With this perspective, ligands of translocator protein-18 kDa (TSPO) may represent an interesting option. TSPO is mainly present in the mitochondrial outer membrane, where it promotes the translocation of cholesterol to the inner mitochondrial membrane, and, as demonstrated in other cellular systems, it allows the transformation of cholesterol into pregnenolone and the increase of steroid levels. In the diabetic model of STZ rat, we have here assessed whether treatment with Ro5-4864 (i.e., a ligand of TSPO) could increase the low levels of neuroactive steroids in sciatic nerve and consequently to be protective in this experimental model. Data obtained by liquid chromatography–tandem mass spectrometry show that treatment with Ro5-4864 was able to significantly stimulate the low levels of pregnenolone, progesterone and dihydrotestosterone observed in the sciatic nerves of diabetic rats. The treatment with Ro5-4864 also counteracted the impairment of NCV and thermal threshold, restored skin innervation density and P0 mRNA levels, and improved Na^+,K^+-ATPase activity. In conclusion, data here reported show for the first time that a TSPO ligand, such as Ro5-4864, is effective in reducing the severity of diabetic neuropathy through a local increase of neuroactive steroid levels.

Key words: streptozotocin, neuroactive steroids, TSPO, rat, myelin proteins, peripheral nerve.

Peripheral neuropathy is one of the most important complications of diabetes affecting nearly half of diabetic patients. In the nervous system, hyperglycemia is responsible for increased production of free radicals (Vincent et al., 2004; Edwards et al., 2008) and, together with a decreased neurotrophic support, damages axons and myelin sheaths. While the disease progresses, the clinical signs of the nerve degeneration are present as decrease of nerve action potential amplitude and slowing of nerve conduction velocity (NCV), reduction in thermal sensitivity and altered activity of the enzyme Na^+,K^+-ATPase that is responsible for the maintenance of the potential difference throughout the nerves (Dobretsov et al., 2007; Said, 2007; Tomlinson and Gardiner, 2008).

A therapeutic strategy able to counteract diabetic neuropathy is not currently available and the clinical treatments are limited to hyperglycemia control. Our recent data obtained in the streptozotocin (STZ)-induced experimental model of diabetic neuropathy suggested that neuroactive steroids have potential neuroprotective activity in peripheral nerves (Roglio et al., 2008). We observed that progesterone (PROG) and its metabolite dihydroprogesterone (DHP) are able to counteract the increase in the number of fibers with myelin infoldings induced by STZ treatment in the sciatic nerve (Veiga et al., 2006). Neuroactive steroids are also able to influence biochemical and functional parameters of peripheral nerves. For instance, treatment with PROG, or with its derivatives DHP or tetrahydroprogesterone (THP), counteracted the impairment of NCV and thermal threshold, restoring skin innervation density and myelin protein mRNA levels, such as glycoprotein zero (P0) and peripheral myelin protein 22 (PMP22), and improving of Na^+,K^+-ATPase activity (Leonelli et al., 2007). Similar effects have been also ascertained in case of other neuroactive steroids, such as testosterone (T) and its derivatives, dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol) (Roglio et al., 2007).

*Corresponding author. Tel: +39-02-50318238; fax: +39-02-50318204.
E-mail address: roberto.melcangi@unimi.it (R. C. Melcangi).

Abbreviations: DHP, dihydroprogesterone; DHT, dihydrotestosterone; IENF, intra-epidermal nerve fiber; LC–MS/MS, liquid chromatography–tandem mass spectrometry; NCV, nerve conduction velocity; P0, glycoprotein zero; PMP22, peripheral myelin protein 22; PREG, pregnenolone; PROG, progesterone; STZ, streptozotocin; T, testosterone; THP, tetrahydroprogesterone; TSPO, translocator protein-18 kDa; 3α-diol, 5α-androstane-3α,17β-diol.

0306-4522/09 $ - see front matter
doi:10.1016/j.neuroscience.2009.08.005

In agreement with these findings we observed that neuroactive steroid levels in plasma and in peripheral nerves are affected by diabetes with a general decrease of their levels (Caruso et al., 2008; Pesaresi et al., 2009). In particular, a statistically significant decrease of PROG and T was observed in plasma while the levels of the precursor of PROG, pregnenolone (PREG), of T and its derivatives (DHT and 3α-diol) were significantly decreased in the sciatic nerve of STZ rats (Pesaresi et al., 2009).

On this basis, an interesting therapeutic strategy could be to increase the levels of neuroactive steroids directly in the nervous system. With this perspective, ligands of translocator protein-18 kDa (TSPO) may represent an interesting option. TSPO is a mitochondrial protein, particularly enriched in the contact site between the outer and inner mitochondrial membrane. In rats, it is expressed in various peripheral tissues such as kidney (Braestrup and Squires, 1977), blood cells—like macrophages, platelets and lymphocytes (Benavides et al., 1984; Zavala et al., 1984; Cahard et al., 1994), steroidogenic glands (Gavish et al., 1999; Brown and Papadopoulos, 2001; Papadopoulos et al., 2006), in central (Chen et al., 2004; Papadopoulos et al., 2006; Veneti et al., 2006; Veiga et al., 2007; Cosenza-Nashat et al., 2009) and peripheral nervous system (Lacor et al., 1996, 1999; Schumacher et al., 2007). Since its first characterization in the rat kidney, TSPO has been extensively studied due its implications in a number of important cell functions. It is involved in the regulation of cellular proliferation (Alho et al., 1994), immunomodulation (Zavala, 1997), porphyrin transport and heme biosynthesis (Taketani et al., 1995), anion transport (Basile et al., 1988), apoptosis (Hirsch et al., 1998) and steroidogenesis (Besman et al., 1989). This latter feature is the best characterized. The tertiary structure of TSPO conforms as a 5α helices transmembrane pore able to directly bind cholesterol and to transfer it into the inner mitochondrial membrane (Besman et al., 1989; Papadopoulos et al., 1997a,b). Here resides the enzyme cytochrome P450 side chain cleavage (P450scc) that converts cholesterol in PREG, which represents the limiting step of steroidogenesis (Melcangi et al., 2008). Indeed, reports from different laboratories indicate that TSPO ligands stimulate steroid synthesis in adrenal, placental, testicular, ovarian and glial cells (Brown and Papadopoulos, 2001; Papadopoulos et al., 2001; Lacapere and Papadopoulos, 2003; Giatzakis and Papadopoulos, 2004). Interestingly, Schwann cells also express TSPO (Schumacher et al., 2007) and a previous study has shown that its agonist Ro5-4864 increases the concentrations of PREG in the rat sciatic nerve (Lacor et al., 1999).

On this basis, we hypothesized that activation of TSPO might increase neuroactive steroid levels and protect peripheral nerves from degeneration induced by diabetic neuropathy. To this aim, after treatment with Ro5-4864 in STZ-rat model we have evaluated by liquid chromatography–tandem mass spectrometry (LC–MS/MS) the levels of neuroactive steroids, such as PREG, PROG and its derivatives (i.e. DHP, THP and isopregnanolone), T and its derivatives (i.e. DHT and 3α-diol). The effect of this TSPO ligand has been also investigated at neurophysiological, biochemical and neuropathological levels, analyzing antidromic tail NCV, thermal nociceptive threshold, Na^+,K^+-ATPase activity, intra-epidermal nerve fiber (IENF) density and expression of myelin proteins, such as P0 and PMP22.

EXPERIMENTAL PROCEDURES

Materials

5-pregnen-3β-ol-20-one (pregnenolone; PREG), PROG, 5α-pregnane-3, 20-dione (dihydroprogesterone; DHP), 3α-hydroxy-5α-pregnen-20-one (THP), 3β-hydroxy-5α-pregnen-20-one (isopregnanolone), T, 5α-androstane-17β-ol-3-one (dihydrotestosterone; DHT), 3α-diol, were purchased from Sigma Aldrich. 17,21,21,21-D_4-PREG (D4-PREG) was kindly synthesized by Dr. P. Ferraboschi (Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milano, Milano, Italy); 2,2,4,6,6–17α,21,21,21-D_9-PROG (D_9-PROG) was obtained from Medical Isotopes, (Pelham, NH, USA); 2,4,16,16-D_4-17β-estradiol (D_4-17β-E) was obtained from CDN isotope (Pointe-Claire, Quebec-Canada). SPE cartridges (Discovery DS-C18 500 mg) were from Supelco, Milano, Italy. All solvents and reagents were HPLC grade (Sigma Aldrich, Milano, Italy).

Animals

Two-month-old male Sprague–Dawley rats, Crl:CD BR (Charles River, Italy) were utilized. The animals were maintained in the department animal quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 06.30 h). The animals were handled following the European Union Normative (Council Directive 86/609/EEC), with the approval of our Institutional Animal Use and Care Committees. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

Induction of diabetes and experimental treatments

Diabetes was induced by a single i.p. injection of freshly prepared STZ (65 mg/kg; Sigma, Italy) in 0.09 M citrate buffer pH 4.8. Control animals were injected with 0.09 M citrate buffer at pH 4.8. Hyperglycemia was confirmed 48 h after STZ injection by measuring tail vein blood glucose levels using a Glucomen tester (Menarini, Italy). Only animals with mean plasma glucose levels above 300 mg/100 ml were classified as diabetic. Glycemia was also assessed before the treatment with steroids (2 months after STZ injection, see below) and tested at scheduled death, 3 months after STZ. At 2 months after STZ injection two different protocols of TSPO ligand administration were assessed. In the protocol 1, animals were administered every 2 days with 3 mg/kg Ro5-4864 (Sigma, Italy) dissolved in 200 μl of sesame oil (i.e., they received 16 s.c. injections); in the protocol 2, animals were injected once a week with the ligand (i.e., they received four s.c. injections). Control rats received 200 μl of vehicle (sesame oil). Rats were killed 24 h after the last treatment.

Assessment of neuroactive steroids by LC–MS/MS

Neuroactive steroids in sciatic nerves and plasma were extracted according to Pesaresi et al. (2009). Briefly, the internal standards deuterated were added to the samples. The plasma and the sciatic nerves of each animal were independently analyzed. Acetic acid (1%) in methanol was added to the samples and the sciatic nerves were homogenized by sonication. The samples were loaded to C18 cartridges, the steroid fraction was eluted with methanol (5 ml), and the organic phase was reconstituted with