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NEUROPROTECTIVE EFFECTS OF DIHYDROPROGESTERONE AND PROGESTERONE IN AN EXPERIMENTAL MODEL OF NERVE CRUSH INJURY

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Abstract—A satisfactory management to ensure a full restoration of peripheral nerve after trauma is not yet available. Using an experimental protocol, in which crush injury was applied 1 cm above the bifurcation of the rat sciatic nerve for 20 s, we here demonstrate that the levels of neuroactive steroids, such as pregnenolone and progesterone (P) metabolites (i.e. dihydroprogesterone, DHP, and tetrahydroprogesterone, THP) present in injured sciatic nerve were significantly decreased. On this basis, we have focused our attention on DHP and its direct precursor, P, analyzing whether these two neuroactive steroids may have neuroprotective effects on biochemical, functional and morphological alterations occurring during crush-induced degeneration-regeneration. We demonstrate that DHP and/or P counteract biochemical alterations (i.e. myelin proteins and Na^+,K^+-ATPase pump) and stimulate reelin gene expression. These two neuroactive steroids also counteract nociception impairment, and DHP treatment significantly decreases the up-regulation of myelinated fibers' density occurring in crushed animals. Altogether, these observations suggest that DHP and P (i.e. two neuroactive steroids interacting with progesterone receptor) may be considered protective agents in case of nerve crush injury.

Key words: rat sciatic nerve, neuroactive steroids, myelin proteins, reelin, morphology, nociception.

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Abbreviations: ANOVA, analysis of variance; Ct, cycle threshold; Cx32, connexin 32; DHP, dihydroprogesterone; MAG, myelin-associated glycoprotein; MAL, myelin and lymphocyte protein; MBP, myelin basic protein; MS/MS, tandem mass spectrometry; P, progesterone; PBS, phosphate buffer solution; PCR, polymerase chain reaction; PMP22, peripheral myelin protein 22; PREG, pregnenolone; P0, glycoprotein zero; SFI, sciatic functional index; THP, tetrahydroprogesterone; 5α-R, 5α-reductase.

Peripheral nerves are frequently exposed to physical injury and this can result in a severe functional impairment and decreased quality of life because of sensory and motor loss of function.

In the last decade, several therapeutic approaches have been developed to stimulate the regeneration of the nerve, such as the administration of neurotrophins (Terenghi, 1999; Boyd and Gordon, 2003; Gordon et al., 2003), of extracellular matrix molecules (Woolley et al., 1990; Labrador et al., 1998), or the application of electrical stimulation (Nix and Hopf, 1983; Al-Majed et al., 2000). Unfortunately, all these methods are limited in scope because they do not ensure a full restoration of function. Therefore, new strategies that simultaneously potentiate axonal regeneration, promote remyelination and the recovery of nerve functions are needed.

Recent observations obtained in experimental models of peripheral neuropathy, such as diabetes, aging, hereditary neuropathy, etc., indicate that neuroactive steroids exert protective effects (Azcoitia et al., 2003; Sereda et al., 2003; Melcangi and Garcia-Segura, 2006; Leonelli et al., 2007; Roglio et al., 2007, 2008). Interestingly, in some of these experimental models, we demonstrated that neurodegenerative process itself affects the levels of neuroactive steroids present in peripheral nerves (Roglio et al., 2007; Caruso et al., 2008a,b).

In addition, studies in experimental models of peripheral neuropathy due to physical injury demonstrated promising protective effects of neuroactive steroids. For instance, in the experimental model of rat nerve transection, progesterone (P) and its metabolite, dihydroprogesterone (DHP), significantly increase the gene expression of a myelin protein, glycoprotein zero (P0) (Melcangi et al., 2000). After cryolesion of sciatic nerve in male mice, administration of P or its precursor, pregnenolone (PREG), to the lesion site increases the extent of myelin sheath formation (Koenig et al., 1995). Moreover, P-loaded chitosan prostheses produce the best-guided nerve regeneration response in rabbit facial nerve after axotomy (Chavez-Delgado et al., 2005). Finally, in peripheral nerve injury models, such as hamster facial motoneuron, rat sciatic motoneuron, rat pudendal motoneuron, etc., testosterone and its derivative, dihydrotestosterone, accelerate regeneration and functional recovery of nerves (Yu, 1982; Vita et al., 1983; Tanzer and Jones, 1997, 2004; Jones et al., 2001; Huppenbauer et al., 2005).

Nerve crush injury in rat is a commonly used axonotmetic model (i.e. an experimental model in which injury has the potential to recover and recover completely) to study

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regeneration after nerve damage, characterized by a complete interruption of nerve axon and myelin. After a nerve is wounded, the distal part of axons undergo a process called Wallerian degeneration. In these conditions, several studies documented biochemical and functional alterations such as changes in the expression levels of myelin proteins (Gupta et al., 1990, 1993; LeBlanc et al., 1990; Snipes et al., 1992; Gillen et al., 1995; Venezie et al., 1995; Chandross et al., 1996) as well as of extracellular matrix proteins (Panteri et al., 2006; Lorenzetto et al., 2008), increase in the Na^+,K^+-ATPase activity and changes in the expression of its subunits (Kawai et al., 1997), decrease in nerve conduction velocity (Mert et al., 2004) and loss of sensorimotor function (Varejão et al., 2004). Different methods of inducing nerve injury have been reported in the literature, including various surgical instruments (Navarro and Kennedy, 1989; Bridge et al., 1994; Kingery et al., 1994) and compression devices (Radevik and Lundborg, 1977; Chen et al., 1992; Oliveira et al., 2001) with different position on the nerve and crush durations. The ability of the axon to regenerate and recover sensory and motor functions is deeply dependent on the type of lesion.

In the present study, using an experimental protocol in which crush injury was applied 1 cm above the bifurcation of rat sciatic nerve for 20 s, we have evaluated whether levels of neuroactive steroids were affected and whether DHP and P had neuroprotective effects on biochemical, morphological and functional alterations occurring during crush-induced degeneration.

EXPERIMENTAL PROCEDURES

Animals and treatments

Animals. Adult male Sprague–Dawley rats, Crl:CD BR (Charles River, Calco, Italy) weighing 300 g 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), and the experimental procedure was approved by the Animal Use and Care Committees of the University of Milano. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

Crush injury. All surgical procedures were performed under deep anesthesia with a premixed solution containing ketamine (80 mg/kg) plus xylazine (5 mg/kg). The sciatic nerve was exposed in the right thigh and crushed just 1 cm above the bifurcation using a Martin clamp 20-307-14 for 20 s. An indelible felt-tip pen was used to mark the site of the lesion. Thereafter, the transected muscles were sutured (Vicryl 3–0 2 EP, Ethicon, Johnson & Johnson Medical, Roma, Italy), the skin wound was closed with suture clips (Martin 18/8, 7.5 mm), and the animals were left to recover under a lamp heating till the awakening. In the control group (i.e. sham-operated animals), the rats were anesthetized, the right sciatic nerves were exposed and the wounds were closed as above.

Treatments. Crushed rats received s.c. injections (every other day since the day after surgery) of 1 mg P or DHP (Sigma-Aldrich, Milano, Italy, Italy) dissolved in 200 μl sesame oil. Control rats received 200 μl of vehicle (sesame oil). Rats were killed 24 h after the last treatment. Biochemical analyses were performed on day 12 after nerve injury (i.e. rats received 6 treatments). Functional assessment was done before and 12, 18, 24 and 36 days after crush; according to treatment schedule, rats received 6, 9, 12 and 18 treatments, respectively. Morphological analysis was conducted on day 36 after crush (i.e. rats received 18 treatments).

Neuroactive steroid levels in sciatic nerves

Steroids in sciatic nerves were extracted according to Caruso et al. (2008a) with minor modification. Briefly, the internal standards deuterium-labeled 17,21,21,21-D_4 PREG (5 ng/sample; D_4-PREG; prepared by Dr. P. Ferraboschi, Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milano, Italy) and 2,2,4,6,6,17α,21,21,21-D_9-P (0.2 ng/sample; D_9-P; Medical Isotopes, Inc., Pelham, NH, USA) were added to the samples. The distal portion of right sciatic nerve of each animal was independently analyzed. Acetic acid (1%) in methanol was added to the samples before homogenization by sonication and loading to C18 cartridges (Discovery DSC-18, 500 mg, Supelco, Milano, Italy). The steroid fraction was eluted with methanol (5 ml) and dried with a gentle stream of nitrogen in a 40 °C waterbath. The organic residue was reconstituted with methanol:water (1:1) before the injection in a RP-C18 analytical column (Hypersil GOLD, Thermo Fisher Scientific Inc., Rodano, Italy, 3 μm, 100 mm$\times$3 mm inner diameter). The high-performance liquid chromatograph (Surveyor LC Pump Plus, Thermo Electron Co., Waltham, MA, USA) was coupled to a linear ion trap mass spectrometer (LTQ, Thermo Electron Co.; LC/MS) equipped with an atmospheric pressure chemical ionization source operating in the positive ion mode. Each steroid was identified on the basis of both the retention time and the tandem mass (MS/MS) spectrum of reference compounds. The quantitative analyses were done monitoring specific ions (selected ion chromatogram mode) in the MS/MS spectrum obtained by collision of precursor ion in MS spectrum as previously reported (Caruso et al., 2008a). Samples were quantified by means of calibration curves using the deuterium labeled internal standards.

Real-time polymerase chain reaction (PCR)

Following total RNA extraction from the distal portion of right sciatic nerves, the samples were processed for real-time PCR to assess the influence of nerve crush injury on 5α-reductase (5α-R) gene expression in sciatic nerve. A 1-μg aliquot of each sample was treated with DNase, to avoid DNA contamination, then reverse transcribed using a High-Capacity cDNA Archive Commercial Kit (Applera, Monza, Italy). The real-time PCR reaction was performed using the TaqMan Universal PCR Master Mix (Applera) with the specific TaqMan MGB probe purchased from Applera. Thermal cycling conditions included a pre-run of 2 min at 50 °C and 10 min at 95 °C. Cycle conditions were 40 cycles at 95 °C for 15 s and 60 °C for 1 min according to the TaqMan Universal PCR Protocol. We utilized the ABI Prism 7000 Sequence Detection System (Applera). Each sample was run in triplicate for the quantification of the 5α-R gene expression as compared with the internal 18s rRNA. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression. To use this method, we performed a validation experiment to demonstrate that the efficiencies of target gene and 18s rRNA amplifications were approximately equal and close to 100%, as suggested by the manufacturer (user bulletin, No. 2; Applera). The Ct, which is the PCR cycle at which the fluorescent signal is first detectable, was then determined for all PCR reactions. For analysis, the Ct value for the internal standard (i.e. 18s rRNA) was first subtracted from the Ct value for the cDNA of interest (i.e. 5α-R). This value is denoted as the ΔCt. Next, the ΔCt value generated from control samples was subtracted from the ΔCt for experimental samples. This equation sets the control sample to a reference value of 0 and generates a $\Delta\Delta$Ct for experimental samples. These values were