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PROGESTERONE ATTENUATES DEMYELINATION AND MICROGLIAL REACTION IN THE LYSOLECITHIN-INJURED SPINAL CORD

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Abstract—Progesterone treatment of mice with experimental autoimmune encephalomyelitis has shown beneficial effects in the spinal cord according to enhanced clinical, myelin and neuronal-related parameters. In the present work, we report progesterone effects in a model of primary demyelination induced by the intraspinal injection of lysophospatidylcholine (LPC). C57Bl6 adult male mice remained steroid-untreated or received a single 100 mg progesterone implant, which increased circulating steroid levels to those of mouse pregnancy. Seven days afterwards mice received a single injection of 1% LPC into the dorsal funiculus of the spinal cord. A week after, anesthetized mice were perfused and paraffin embedded sections of the spinal cord stained for total myelin using Luxol Fast Blue (LFB) histochemistry, for myelin basic protein (MBP) immunohistochemistry and for determination of OX-42+ microglia/macrophages. Cryostat sections were also prepared and stained for oligodendrocyte precursors (NG2+ cells) and mature oligodendrocytes (CC1+ cells). A third batch of spinal cords was prepared for analysis of the microglial marker CD11b mRNA using qPCR. Results showed that progesterone pretreatment of LPC-injected mice decreased by 50% the area of demyelination, evaluated by either LFB staining or MBP immunostaining, increased the density of NG2+ cells and of mature, CC1+ oligodendrocytes and decreased the number of OX-42+ cells, respect of steroid-untreated LPC mice. CD11b mRNA was hyperexpressed in LPC-treated mice, but significantly reduced in LPC-mice receiving progesterone. These results indicated that progesterone antagonized LPC injury, an effect involving (a) increased myelination; (b) stimulation of oligodendrocyte precursors and mature oligodendrocytes, and (c) attenuation of the microglial/macrophage response. Thus, use of a focal demyelination model suggests that progesterone exerts promyelinating and anti-inflammatory effects at the spinal cord level.

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; LFB, Luxol Fast Blue; LPC, lysophospatidylcholine; MBP, myelin basic protein; MS, multiple sclerosis; OPC, oligodendrocyte precursor cells; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

Key words: demyelination, lysolecithin, microglial reaction, progesterone, remyelination.

Multiple sclerosis (MS) is a major neurodegenerative disease that causes neurological disability in an estimated 2.5 million people worldwide. MS presents a gender preference, with a woman to man ratio of 2.6, although the incidence in women increases after menopause (Confavreux et al., 1998; Hughes, 2004). Interestingly, hormonal factors have been considered to play a pathogenic role in MS. Early studies report an association between lesion size of MS patients, low circulating progesterone levels and high estradiol levels during the sex cycle, suggesting a beneficial role of progesterone (Bansil et al., 1999; Pozzilli et al., 1999). The PRIMS (Pregnancy in Multiple Sclerosis) study concluded that relapses of MS are less frequent during the last trimester of pregnancy but reappear following delivery, supporting a protective role of sex steroids (El-Etr et al., 2005). This evidence led to the suggestion that progesterone-induced immunosuppression during human pregnancy may prevent relapses of MS (Confavreux et al., 1998; Hughes, 2004; Druckmann and Druckmann, 2005).

Conclusive demonstrations regarding the neuroprotective and promyelinating role of progesterone in the central and peripheral nervous system (Melcangi et al., 2000; Azcoitia et al., 2003; Brinton et al., 2008; Schumacher et al., 2008; De Nicola et al., 2009), stimulated trials on its therapeutic value for MS and models of the disease such as experimental autoimmune encephalomyelitis (EAE) and cuprizone-induced demyelination. Earlier reports have shown variable effects of progesterone in EAE, ranging from inactivity, increased vulnerability of neurons to disease improvement if estradiol is combined with progesterone (Kim et al., 1999; Bebo et al., 2001; Hoffman et al., 2001). In particular, the synthetic progestin medroxyprogesterone acetate has shown a protective effect in EAE (Elliot et al., 1973).

More recent evidence supports that progesterone provides beneficial effects to rodents with EAE. For example, progesterone treatment prior to EAE induction with myelin oligodendrocyte glycoprotein (MOG) attenuates the clinical scores of the disease, slightly delays disease onset and decreases demyelination foci, according to Luxol Fast Blue staining (LFB), myelin basic protein (MBP) and proteolipid protein (PLP) protein and mRNA expression. Key genes of motoneuron function and axonal parameters are also enhanced in EAE mice receiving progesterone (Garay et al., 2007, 2008, 2009). Another group has shown that progesterone given at the time of EAE induction reduces peak score and the cumulative disease index, decreases proinflammatory and increases antiflammatory chemokine secretion

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(Yates et al., 2010). A recent study employing steroid treatment at the time EAE symptoms started has shown a progesterone-induced nuclear sublocalization of the Olig1 transcription factor involved in remyelination in addition to providing clinical benefit (Yu et al., 2010). In cotherapy with estrogens, progesterone is also effective to counteract not only EAE-induced spinal cord demyelination but also cuprizone-induced demyelination of the corpus callosum (Acs et al., 2009; Garay et al., 2008). Thus, evidence gathered in rodents with demyelination caused by EAE induction and cuprizone treatment support a potential therapeutic value for steroids in experimental models of MS.

Among the mechanisms involved, it is known that progesterone exerts immunomodulatory effects to control the onset or progression of MS and EAE. Thus, increased progesterone levels during pregnancy modulates the immune system, changing a Th_1 pro-inflammatory response into a Th_2 anti-inflammatory response (Confavreux et al., 1998; Hughes, 2004; Druckmann and Druckmann, 2005). Furthermore, progesterone activates lymphocytes to secrete the non-inflammatory cytokines IL_3, IL_4, IL_{10} and to reduce the inflammatory cytokines IFN-γ, TNFα and IL_2 (Szekeres-Bartho and Wegmann, 1986; Blois et al., 2007). The immunomodulatory effects of progesterone applies not only to autoimmune diseases but has also been postulated to alleviate the effects of traumatic central nervous system (CNS) injury (Stein and Fulop, 1998).

In the present work, we induced spinal cord demyelination employing lysolecithin (LPC), to further assess progesterone effects in a focal demyelination model caused by a gliotoxin. A seminal study of Hall (1972) has shown that intraspinal injection of LPC induces changes typical of wallerian degeneration with disruption of the myelin sheath. Later on, Jeffery and Blakemore (1995) demonstrate that LPC demyelination extends well beyond the lesion site, with rapidity of repair. Remyelination that follows loss of myelin requires the recruitment of oligodendrocyte precursor cells (NG2+ cells), that differentiate and mature into myelin-producing oligodendrocytes if properly stimulated (Bruce et al., 2010). However, in contrast to the existence of reports showing the effects of different steroids in the EAE model, only glucocorticoids have been tested in LPC-induced demyelination (Triarhou and Herndon, 1986; Pavelko et al., 1998). In the present work, mice received progesterone prior to induction of a focal demyelination by the intraspinal injection of LPC. Our data showed that progesterone-treated mice showed less spinal cord demyelination, increased the density of NG2+ cells and of mature oligodendrocytes and decreased the expression of a maker of reactive microglia/macrophages, suggesting the possibility for direct steroid effects on the spinal cord.

EXPERIMENTAL PROCEDURES

Experimental animals

Male C57BL/6 mice (9–11 weeks old) remained untreated or received s.c. a single 100 mg progesterone pellet (Sigma-Aldrich, St. Louis, MO, USA) under xylazine (6 mg/kg) and ketamine (75 mg/kg) anesthesia. A week afterwards, both groups of mice were anesthetized as before, secured on a flat surface and a dorsal laminectomy was carried out in the upper thoracic region of the spinal cord closed to the midline. The dura mater was incised and the dorsal funiculus of the spinal cord exposed after opening the pia mater. One microlitre of a 1% solution of LPC (Sigma Aldrich, St. Louis, MO, USA) in sterile phosphate-buffered saline (PBS, pH 7.4) was slowly delivered with a Hamilton syringe into the dorsal funiculus. The injection site was macroscopically localized by the addition of Evan's Blue dye to the LPC solution. The blue spot detected the injection site at T1-T3. The whole procedure was performed under a binocular dissecting microscope. The wound was closed in two layers and mice were left undisturbed for 1 week before killing for the different experiments. In this way, two groups of LPC-injected mice were prepared, that is, with or without progesterone pre-treatment. In addition, we prepared a third. group of steroid-untreated mice that received intraspinal injections of PBS only (control mice). Clinical signs in LPC-injected mice consisted of loss of tail tonicity, body shaking and unsteady gait. Clinical signs were absent from PBS-injected controls. Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Institute's Assurance Certificate # A5072-01) and were approved by the Institute's Animal Care and Use Committee.

LFB histochemistry and MBP immunostaining

Anesthetized mice were perfused transcardially with a solution containing 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.4. Thoracic spinal cords were removed, post-fixed in the same fixative for 2.5 h and embedded in paraffin. All tissues were treated in an identical manner to evaluate toxin-induced-demyelination. Each thoracic spinal cord was embedded in paraffin and subdivided into five blocks (A–E) 200 μm in thickness. From block A, 5 μm coronal slices were cut with a microtome starting on the top edge of the lesion site marked by Evan's Blue staining. A total of eight slices were laid sequentially on a microscope slide, comprising a 40 μm section of the thoracic spinal cord. This procedure was repeated five times, in order to analyse the entire 200 μm section for demyelination. The next block B was discarded. For blocks C and E the procedure was identical to block A, whereas tissue contained in block D was discarded. The first slide from blocks A, C and E was deparaffinized and stained with LFB to mark the area of demyelination. LFB staining was carried out according to Kim et al. (2006). Sections were treated with 95% ethanol and left in LFB solution (0.1 mg% Luxol Fast Blue in 95% ethanol with 10% acetic acid) at 60 °C for 18 h. After several washes, sections were immersed in lithium carbonate, and then 70% ethanol, rinsed in distilled water, dried and mounted with Permount. The area of white matter demyelination, lacking LFB staining, was determined by computerized image analysis and expressed as described below.

MBP immunocytochemistry (Labombarda et al., 2002) was performed in blocks A, C and E previously selected for LFB staining. Five-micrometre sections of paraffin-embedded spinal cords were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Immunocytochemistry was carried out using a 1:500 dilution of rabbit anti-MBP primary antibody (Code AO623, Dako Cytomation, Carpinteria, CA, USA) diluted in PBS containing 1% goat serum. After the overnight incubation at 4 °C and several washes with PBS, sections were incubated with a goat anti-rabbit IgG secondary antibody, diluted 1/200 (Vector laboratories, CA, USA) for 1 h at 22 °C, then with avidin-biotin-peroxidase (ABC) complex for 30 min (ABC kit Vector laboratories, CA, USA) and finally revealed with 0.5 mg/ml diaminobenzidine tetrachloride (Sigma Aldrich, St. Louis, MO, USA) in the presence of 0.01% H_2O_2 for 7 min in the dark. The sections were given a final rinse in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount.

For quantitative evaluation, areas showing negative LFB histochemical staining or MBP immunostaining were delimited at