Journal of Steroid Biochemistry & Molecular Biology 154 (2015) 274–284

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

A functional progesterone receptor is required for immunomodulation, reduction of reactive gliosis and survival of oligodendrocyte precursors in the injured spinal cord

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 26 August 2015
Accepted 8 September 2015
Available online 11 September 2015

Keywords:
Astrocytes
Knockout mice
Microglia
Neuroinflammation
Progesterone receptor
Spinal cord injury

ABSTRACT

The anti-inflammatory effects of progesterone have been increasingly recognized in several neuropathological models, including spinal cord inflammation. In the present investigation, we explored the regulation of proinflammatory factors and enzymes by progesterone at several time points after spinal cord injury (SCI) in male rats. We also demonstrated the role of the progesterone receptor (PR) in inhibiting inflammation and reactive gliosis, and in enhancing the survival of oligodendrocyte progenitors cells (OPC) in injured PR knockout (PRKO) mice receiving progesterone. First, after SCI in rats, progesterone greatly attenuated the injury-induced hyperexpression of the mRNAs of interleukin 1β (IL1β), IL6, tumor necrosis factor alpha (TNFα), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), all involved in oligodendrocyte damage. Second, the role of the PR was investigated in PRKO mice after SCI, in which progesterone failed to reduce the high expression of IL1β, IL6, TNFα and IκB-α mRNAs, the latter being considered an index of reduced NF-κB transactivation. These effects occurred in a time framework coincident with a reduction in the astrocyte and microglial responses. In contrast to wild-type mice, progesterone did not increase the density of OPC and did not prevent apoptotic death of these cells in PRKO mice. Our results support a role of PR in: (a) the anti-inflammatory effects of progesterone; (b) the modulation of astrocyte and microglial responses and (c) the prevention of OPC apoptosis, a mechanism that would enhance the commitment of progenitors to the remyelination pathway in the injured spinal cord.

1. Introduction

Cells in the spinal cord respond differently to spinal cord injury (SCI), with some becoming unfortunate targets while others playing an effector role. The first category includes neurons and oligodendrocytes. Neurons suffer necrosis, apoptosis, oxidative damage, and chromatolysis [1,2] concomitant with the activation of several molecules associated with neuropathology [3,4] SCI also causes oligodendrocyte death by apoptosis and the release of proinflammatory mediators, mechanisms leading to axonal demyelination and functional impairment [1,5]. Oligodendrocyte loss is followed by a wave of oligodendrocyte precursor cell (OPC) proliferation, although in the absence of adequate support their survival and differentiation is compromised [6,7]. In contrast to neurons and oligodendrocytes, astrocytes and microglia become activated after SCI and produce proinflammatory mediators, oxygen free radicals and neurotoxic levels of nitric oxide as part of a process known as reactive gliosis [8,9]. Astrocytes, change their phenotype with strong expression of glial fibrillary acidic protein (GFAP), vimentin and S100β, and they show early hypertrophy and late proliferation depending on the severity of the lesion [9]. Marked changes also occur in microglia, which present a more reactive, proinflammatory phenotype [10]. Reactive astrocytes and microglia release proinflammatory mediators which reciprocally regulate each other, producing a feed-forward mechanism that propagates secondary injury and inflammation after spinal cord trauma [8,11].

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http://dx.doi.org/10.1016/j.jsbmb.2015.09.011
0960-0760/

In this context, therapies aimed at blocking the innate immune response and holding back the glial reaction may be relevant for preserving functions of the damaged spinal cord. For example, the glucocorticoid methylprednisolone (MP) has been used as a standard therapy for humans with SCI. Clinical trials (NASCIS II and III) have demonstrated therapeutic benefits for these patients [12]. However, the true efficacy of MP in addition to adverse effects has raised questions regarding the value of this steroid for SCI [13,14]. Progesterone emerges as much more promising candidate for SCI considering its potent neuroprotective, promyelinating, anti-inflammatory and anti-nociceptive effects [15–21].

Improved functional recovery resulting from progesterone treatment of rats with SCI has been demonstrated by the improved motor outcome in the Basso–Bresnahan–Beattie scale for locomotion and CatWalk gait analysis [22]. At the neurochemical level, a strong myelinating drive follows progesterone treatment of injured rats. As in models of experimental demyelination, the steroid enhances the density of OPC, promotes their differentiation into myelinating oligodendrocytes, and increases the expression of central myelin proteins after SCI [23–25]. In this regard, restraining oligodendrocyte death improves recovery after SCI [26]. Besides exerting direct effects on neurons and oligodendrocyte lineage cells [27,28], progesterone's neuroprotective and remyelinating actions are associated with inhibition of the activation and proliferation of astrocytes and microglia [29,30].

The effects of progesterone and its metabolites in the nervous system are pleiotropic and mediated by the classical progesterone receptor (PR), membrane progesterone receptor component 1 (PGRMC1), membrane progesterone receptors (mPR) and neurotransmitter receptors [15,31–34]. Recently, the roles of progesterone binding to the classical PR or after conversion to allopregnanolone have been reconsidered for neurological disorders [35,36]. Therefore, it was important to determine whether PR is required for the effects of progesterone on inflammatory and glial responses after SCI.

In order to elucidate the immunomodulatory role of progesterone after SCI and the involvement of PR in the modulation of inflammatory responses and reactive gliosis and in promoting the survival of OPC, we performed several experiments. We first studied the time-course of the effects of progesterone on the expression of inflammatory enzymes, proinflammatory mediators and their regulatory molecules in rats with SCI. We also used wild-type and PRKO mice with SCI to elucidate: (a) the role of PR for the regulation of proinflammatory mediators and for astrocyte and microglial responses, and (b) the role of PR for OPC viability.

2. Materials and methods

2.1. Progesterone treatment and spinal cord transection

Male Sprague-Dawley rats (250–300 g) were anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and their spinal cords transected at the thoracic level (T10) as described by Labombarda et al. [29] [24]. PRKO mice (PR^{lacz} mice on a C57BL6/129SvEv background) [37] and wild- type mice originally obtained from Baylor College of Medicine (Houston, TX, USA) were bred and maintained at the animal facility of the Instituto de Biologia y Medicina Experimental. In these mice, both PRA and PRB isoforms, transcribed from a single gene, are inactivated. PRKO mice were identified following an established genotyping procedure [37]. Three-month-old male mice were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.), and their spinal cords were transected as described above for the rat experiments. After SCI, animals were housed singly. Urinary bladders were manually expressed twice a day, and infections were prevented by administration of cefalexine (20 mg/kg daily) starting immediately before surgery. In sham-operated animals (CTL), skin and muscle surgery was performed but the spinal cord was not cut. Animals with SCI received daily sc injections of vegetable oil (vehicle) or 16 mg/kg progesterone (Proluton, Schering, Argentina) and were killed 6 h, 24 h, 48 h, 3 or 21 days following surgery. The first progesterone injection was given immediately after injury. Thus, 3 groups of animals were prepared per time period: CTL, SCI and SCI+ progesterone treatment. A CTL+ progesterone group was not included because morphological, neurochemical and molecular evidence have confirmed the absence of progesterone effects in the intact spinal cord [38].

The dose of progesterone chosen prevents oedema, neuronal loss, and improves cognitive responses following brain contusion [21]. In the damaged spinal cord, this progesterone dose reduces secondary damage, preserves white matter, improves locomotor outcome, promotes remyelination, and modulates glial cells involved in the inflammatory response [22,29]. The periods of time (6 h, 24 h, 3 and 21 days) were chosen to compare rapid or long-term effects of SCI and steroid treatment on proinflammatory mediators. The animal procedures described for rats and mice followed the NIH Guide for the Care and Use of Laboratory Animals (Assurance Certificate N A5072-01 to Instituto de Biología y Medicina Experimental) and received approval of the Institute's Animal Care and Use Committee and the CICUAL of the Faculty of Medicine, University of Buenos Aires. Efforts were made to keep the number of lesioned animals to a minimum.

2.2. Real time PCR for semi-quantitative determination of mRNA expression of proinflammatory mediators after spinal cord injury

Six and 24 h, 3 and 21 days after SCI, animals receiving progesterone or vehicle as well as CTL animals ($n = 8$ animal per group), were deeply anesthetized with chloral hydrate (800 mg/kg ip) and killed by decapitation. Spinal cord tissue localized immediately rostral to the lesion site, and equivalent regions form CTL animals were removed and homogenized with a Polytron homogenizer. RNA was extracted and subjected to reverse transcription as previously described [24]. Relative gene expression was determined using the ABI PRISM 7500 sequence Detection System (Applied Biosystems, Foster City, CA). Sequence of primers for mice was designed using the web site http://www.ncbi.nlm.nih.gov/tools/primer-blast Primer sequences for rat and mice are listed in Tables 1 and 2, respectively. Cyclophilin B (Cyc B) was chosen as the housekeeping gene based on the similarity of mRNA expression across all samples templates. Linearity and efficiency of PCR amplification were validated before

Table 1
Rat foward and reverse primers sequences.

Gene	Primer sequence	Reference
IL 1 β	F: 5′CACCTCTCAAGCAGAGCACAG 3′ R: 5′GGGTTCCATGGTGAAGTCAAC 3′	NM_031512.2
IL 1β R1	F: 5′GTTTTTGGAACACCCTTCAGCC 3′ R: 5′ACGAAGCAGATGAACGGATAGC 3′	XM_006244754.2
IL 1 β R2	F: 5′CATTCAGACACCTCCAGCAGTTC 3′ R: 5′ACCCAGAGCGTATCATCCTTCAC 3′	XM_008766984.1
IL1 ra	F: 5′AAGACCTTCTACCTGAGGAACAACC 3′ R: 5′GCCCAAGAACACATTCCGAAAGTC 3′	XM_006233638.2
TNF α	F: 5′TCGTAGCAAACCACCAAGCA 3′ R: 5′CCCTTGAAGAGAACCTGGGAGTA 3′	X66539.1
IL 6	F: 5′AAGTCGGAGGCTTAATTACATATGTTC 3′ R: 5′TGCCATTGCACAACTCTTTTCT 3′	NM_012589.2
Cox-2	F: 5′TTTGTTGAGTCATTCACCAGACAGAT 3′ R: 5′ACGATGTGTAAGGTTTCAGGGAGAAG 3′	S67722.1
iNOS	F: 5′CCAGAGCAGTACAAGCTCAC 3′ R: 5′CCACAACTCGCTCCAAGATC 3′	AY211532.1