Neuroscience 167 (2010) 872–879

CHONDROITIN SULFATE INHIBITS LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN RAT ASTROCYTES BY PREVENTING NUCLEAR FACTOR KAPPA B ACTIVATION

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Abstract—Chondroitin sulfate (CS) is a glucosaminoglycan (GAG) currently used for the treatment of osteoarthritis because of its antiinflammatory and antiapoptotic actions. Recent evidence has revealed that those peripheral effects of CS may also have therapeutic interest in diseases of the CNS. Since neuroinflammation has been implicated in different neuronal pathologies, this study was planned to investigate how CS could modulate the inflammatory response in the CNS by using rat astrocyte cultures stimulated with lipopolysaccharide (LPS). We have evaluated different proteins implicated in the nuclear factor kappa B (NFκB) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways employing RT-PCR, western blot and immunofluorescence techniques. At 10 μM, CS prevented translocation of p65 to the nucleus, reduced tumour necrosis factor alpha (TNF-α) mRNA and mitigated cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) induction by LPS. However, it did not modify LPS-induced IP-10 and SOCS-1 mRNA, proteins that participate in the JAK/STAT pathway. The results of this study indicate that CS can potentially reduce neuroinflammation by inhibition of NFκB. Therefore endogenous GAGs could afford neuroimmunomodulatory actions under neurotoxic conditions.

Key words: chondroitin sulfate, glucosaminoglycan, astrocytes, lipopolysaccharide, NFκB.

Recent evidence has revealed that the peripheral antiapoptotic and antiinflammatory effects of chondroitin sulfate (CS) may also have therapeutic interest in diseases of the CNS. Chondroitin sulfate is a glucosaminoglycan (GAG) that forms part of the extracellular matrix. In the CNS this matrix forms a special net surrounding certain neurons called perineuronal net. It has been shown that certain components of this net have neuroprotective actions against many injuries of the CNS. CS has shown neuroprotective actions in cultured neurons exposed to excitatory amino acids (Okamoto et al., 1994b), or glutamate (Okamoto et al., 1994a). In our group, we have also described the neuroprotective actions of CS against oxidative stress in human neuroblastoma SH-SY5Y cells (Canas et al., 2007).

Glial cells are mediating the main inflammatory response of the CNS, being astrocytes the mayor glial cells in the brain. Although microglia are the main glial cells involved in neuroinflamamtory responses, the responses of astrocytes to proinflammatory triggers might also be relevant because of the impact that astrocytic responses might locally have on adjacent blood vessels. In response to CNS deterioration by aging or other noxious stimuli, astrocytes are activated, undergoing a morphological and structural change accompanied by an overproduction of proinflammatory cytokines (Takuma et al., 2004). There is growing evidence that suggests the importance of brain inflammatory response in many neurodegenerative disorders like Alzheimer disease (AD) or cerebral ischemia (Salminen et al., 2009; Shah et al., 2009). Glial activation may result beneficial since it could promote tissue repair and pathogen elimination, among others. However, excessive glial activation may be harmful and can promote neuronal death (Hanisch, 2002).

Cytokine production is a consequence of glial activation (Zielasek and Hartung, 1996). Cytokines take part in a wide range of cell processes like modulation of immune and inflammatory responses. These molecules are rapidly induced after cellular injury, inflammation or infection (Allan and Rothwell, 2001). Tumour necrosis factor alpha (TNF-alpha) and IL-1 are two major proinflammatory cytokines in the glial cells during CNS inflammation, and they induce the synthesis of adhesion molecules and more cytokines. TNF-alpha can activate intracellular pathways like MAPK pathway and nuclear factor kappa B (NFκB) (Kaushal and Schlichter, 2008).

On the other hand, lipopolysaccharide (LPS) is a component of the cellular membrane of Gram negative bacteria. LPS promotes a very powerful inflammatory response by stimulating the production of cytokines like TNF-alpha. It has been shown that one of the major proinflammatory

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Abbreviations: COX, 2, cyclooxygenase 2; CS, chondroitin sulfate; GAG, glucosaminoglycan; iNOS, inducible nitric oxide synthase; JAK/STAT, Janus kinase/signal transducer and activator of transcription; LPS, lipopolysaccharide; NFκB, nuclear factor kappa B; TLR4, toll-like receptor 4; TNFα, tumour necrosis factor alpha.

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2010.02.069

pathways activated by LPS is NFκB (Zielasek and Hartung, 1996), and that LPS induces overexpression of cyclooxygenase 2 (COX-2) and prostanoids (Wu, 2005; Kohno et al., 2008). Therefore, stimulation of cultured astrocytes by LPS is a good *in vitro* model for astroglial activation.

LPS stimulation also modulates NFκB proteins, that are in the cytoplasm of resting cells in an inactive form bound to inhibition factor IκB. After cell activation by a wide variety of extracellular ligands (LPS, interleukins), IκB is phosphorylated, ubiquitinized and finally degraded by the proteasome (Cheng et al., 1994). Once free of its inhibitor, the NFκB subunit p65 is translocated to the nucleus and transcribes target genes (Kaltschmidt et al., 1999). NFκB is present in neuronal and glial cells; the target genes in those cells related to immune and inflammatory responses are proinflammatory cytokines like IL-1 and TNF-alpha (Sethi et al., 2008), adhesion molecules like VCAM-1 and ICAM-1 (Quinlan et al., 1999) and enzymes like inducible nitric oxide synthase (iNOS) and COX-2 (Sethi et al., 2008).

Cytokine receptor signalling mechanisms depend mainly on the activation of two protein families, the Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). JAKs are proteins with tyrosine kinase activity and are constitutively associated to the receptor; when activated, JAKs are transphosphorylated and in this conformation they can phosphorylate STATs, which are in an inactivated conformation in the cytosol. Once phosphorylated, STATs traslocate to the nucleus and transcribe its target genes (Rawlings et al., 2004).

Because of its peripheral antiinflammatory actions, CS is currently used in the clinic for the treatment of osteoarthritis (Campo et al., 2008b). Since CS has proven to have neuroprotective properties (Okamoto et al., 1994a,b; Canas et al., 2007; Sato et al., 2008), the aim of this study was to evaluate if CS could also display antiiflammatory actions in the CNS by using rat astrocytes stimulated by LPS as a model of neuroinflammation. We have found that CS can reduce the inflammatory response caused by LPS stimulation of astrocytes by inhibiting the translocation of p65 to the nucleus and reducing the production of TNF-alpha and inducible proteins COX-2 and iNOS. This new mechanism of action could complement CS neuroprotective properties and could therefore be of interest in different pathologies of the CNS where neuronal cell death and neuroinflammation take place, as happens to be the case in certain neurodegenerative diseases or brain ischemia.

EXPERIMENTAL PROCEDURES

Rat astrocyte cell culture

Two to three days old Sprague Dawley rat pups were used. The experiments were performed after approval of the protocol by the institutional Ethics Committee, in accordance with the law in force (European Directive 86/609/EEC and Real Decreto 1201/2005), following the Research Council's Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. The animals were decapitated, immediately craneotomized and both cortices removed. Cortices were placed in a Petri dish with cold PBS and, under the microscope, meninges of each cortex were discarded. Once cleaned, all cortices were submerged in DMEM and a mechanical dissection was made. Then, the tissue was passed to a 50 ml Falcon tube where mechanical disgregation was made, passing each time through lower diameter size pipettes (10, 5 ml, and finally a Pasteur pipette), 15–20 times each. Thereafter, the mixture was centrifuged 5 min at 1000 rpm and the supernatant was discarded. The pellet was resuspended in DMEM containing 20% fetal bovine serum (FBS) with streptomycin and penicillin. Finally, the homogenate was passed through a 70 μm filter and seeded in 75 cm^2 flasks.

Subculture for purified astrocytes

Three days after seeding, the culture medium was changed to DMEM with 10% FBS. A week later, 4 μM cytosine arabinoside was added to each flask. After 3 days incubation, the flasks were placed on a horizontal shaker at 250 rpm for 3 h. at 37 °C. Then, the medium was replaced by PBS:trypsin (0.25% EDTA) 5:1 until the morphology of the astrocytes changed and they adopted a rounded form and detached; at this point, FBS:PBS 1:5 was added to block the enzymatic action of trypsin. Thereafter, the medium was collected and centrifuged 5 min at 1000 rpm and the pellet was resuspended in DMEM with 10% FBS with penicillin and streptomycin and seeded in Petri dishes.

Incubation of drugs

The inflammatory stimulus used was 10 μg/ml LPS. At 10 μM, CS was co-incubated with LPS for 2 or 4 h, depending on the experimental protocol. CS used in this study was a highly purified mixture of chondroitin 4 and 6 sulfate of bovine origin in a concentration not less than 98%, with an average molecular weight of ~15–16 kDa, and a 4-sulfated/6-sulfated ratio (4s/6s ratio–the ratio between the sulfated groups located in position 4 and 6 on N-acetyl-D-galactosamine) of 2.

p65 immunocytochemistry

Twelve well plates containing polylisine treated glass coverslips were seeded with 2 ml medium containing 6×10^4 cells/ml. Twenty four hours after plating, 10 μg/ml LPS alone or in combination with 10 μM CS were added to the cells for 2 or 4 h. Thereafter, the cells were washed three times with cold PBS and fixed with 4% paraformaldehyde during 30 min at room temperature. Then, another three washes with cold PBS were made and cells were permeabilized with 0.2% triton X 100 in PBS.

After permeabilization, cells were washed three times with PBS and blocked by adding 3% goat serum during 60 min at room temperature. After overnight incubation at 4 °C with anti-p65 (1:250) (Santa Cruz) with 1% goat serum, cells were washed with PBS and the secondary anti-rabbit antibody with green fluorescence was added for 1 h. Finally, the medium was replaced by cold PBS, and photographs were taken in a fluorescent microscope with an objective 20×.

Isolation of nuclear extracts

Cultured medium from plates was removed and astrocytes were detached with cold PBS and centrifuged at 1000 rpm for 5 min at 4 °C. Pellets were resuspended in 1 ml of hypotonic lysis buffer (Cellytic Nuclear Extraction Kit, SIGMA, Madrid, Spain), pipetting 10–20 times and maintained on ice for 15 min. Then, they were centrifuged at 2300 rpm for 5 min at 4 °C. Pellets were resuspended in 400 μl lysis buffer by passing five times through a 25G needle in a 1 ml syringe. Samples were then centrifuged at 11,000 rpm for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 140 μl extraction buffer from the kit,