Neurobiology of Disease 33 (2009) 57–71

Contents lists available at ScienceDirect

Neurobiology of Disease

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

The endocannabinoid system is modulated in response to spinal cord injury in rats

Daniel Garcia-Ovejero [a], Angel Arevalo-Martin [a], Stefania Petrosino [b,c], Fabian Docagne [d], Carlos Hagen [a], Tiziana Bisogno [b], Masahiko Watanabe [e], Carmen Guaza [d], Vincenzo Di Marzo [b,*], Eduardo Molina-Holgado [a,*]

[a] Laboratorio de Neuroinflamacion, Unidad de Neurología Experimental (Research Unit associated to the Instituto Cajal, CSIC, Madrid), Hospital Nacional de Paraplejicos, Toledo, Spain
[b] Endocannabinoid Research Group, Institute of Biomolecular Chemistry, C.N.R., Pozzuoli (NA), Italy
[c] Department of Pharmaceutical Sciences, University of Salerno, Fisciano, Italy
[d] Neuroimmunology Group, Instituto Cajal, CSIC, Madrid, Spain
[e] Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan

ARTICLE INFO

Article history:
Received 15 May 2008
Revised 3 September 2008
Accepted 9 September 2008
Available online 30 September 2008

Keywords:
Diacylglycerol lipase
Injury
Cannabinoids
CB1
CB2
Palmitoylethanolamide
Astrocytes

ABSTRACT

Endocannabinoids are lipid mediators with protective effects in many diseases of the nervous system. We have studied the modulation of the endocannabinoid system after a spinal cord contusion in rats. In early stages, lesion induced increases of anandamide and palmitoylethanolamide (PEA) levels, an upregulation of the synthesizing enzyme NAPE-phospholipase D and a downregulation of the degradative enzyme FAAH. In delayed stages, lesion induced increases in 2-arachidonoylglycerol and a strong upregulation of the synthesizing enzyme DAGL-α, that is expressed by neurons, astrocytes and immune infiltrates. The degradative enzyme MAGL was also moderately increased but only 7 days after the lesion. We have studied the cellular targets for the newly formed endocannabinoids using RT-PCR and immunohistochemistry against CB_1 and CB_2 receptors. We observed that CB_1 was constitutively expressed by neurons and oligodendrocytes and induced in reactive astrocytes. CB_2 receptor was strongly upregulated after lesion, and mostly expressed by immune infiltrates and astrocytes. The endocannabinoid system may represent an interesting target for new therapeutical approaches to spinal cord injury.

Introduction

Most human spinal cord injuries (SCIs) result from accidents that fracture or dislocate the spinal column. Some of them produce penetrating wounds but many result in transient compression or contusion of the spine. Spinal lesions are frequently suffered by young healthy people and constitute a very important physical and psychological burden and an elevated and sustained economical cost for the individuals, their families and their communities (Ackery et al., 2004; Ho et al., 2007). There are no current treatments for spinal cord injury. Surgery, physical therapy and rehabilitation are still the most effective strategies to improve the quality of life and autonomy of injured patients (Baptiste and Fehlings, 2007; Schwab et al., 2006; Thuret et al., 2006). This situation contrasts with the variety of approaches employed in basic research to get any improvement in animal motor or sensitive outcomes after experimental SCI, including cell transplantations, pharmacology, electrotherapy, blockage of myelin inhibitors or combined therapies (Baptiste and Fehlings, 2007; Bradbury and McMahon, 2006; Reier, 2004; Schwab et al., 2006; Thuret et al., 2006). This scenery, that frequently shows discrepancies between the treatments in the laboratories and the results obtained in clinical trials, can have many interpretations, like species differences or inadequate evaluation methods (Schwab et al., 2006). It is also probable that we still ignore key players involved in the development of the damage and the tissue response to the insult.

Endocannabinoids are lipid mediators formed from plasma membrane precursors that bind the cannabinoid receptors CB_1 or CB_2. During the last years, the endocannabinoid system has attracted the attention of researchers working in neural damage and repair and is being considered a promising target for the development of new therapies (Di Marzo, 2008; Pacher et al., 2006). This increasing interest is supported by evidence showing that the endocannabinoids 2-arachidonoylglycerol (2-AG) and arachidonoyl ethanolamide or anandamide (AEA) are produced "on demand" following cerebral ischemia (Amantea et al., 2007; Muthian et al., 2004), excitotoxic damage (Hansen et al., 2001; Marsicano et al., 2003) and traumatic brain injury (Panikashvili et al., 2001) and have been shown to act as neuroprotective and immunomodulatory mediators after lesions of the nervous system (Arevalo-Martin et al., 2008; Di Marzo, 2008; Klein, 2005; Mechoulam, 2002; Pacher et al., 2006). Moreover, some

* Corresponding authors. E. Molina-Holgado is to be contacted at Laboratorio de Neuroinflamación, Unidad de Neurología Experimental, Hospital Nacional de Parapléjicos, SESCAM, 45071 Toledo, Spain. Fax: +34 925247745. V. Di Marzo, Endocannabinoid Research Group, Institute of Biomolecular Chemistry, C.N.R., Via Campi Flegrei 34, Comprensorio Olivetti, 80078 Pozzuoli (NA) Italy.
E-mail addresses: vdimarzo@icmib.na.cnr.it (V. Di Marzo), eduardom@sescam.jccm.es (E. Molina-Holgado).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2008.09.015

components of the endocannabinoid system, that are constitutively expressed in the normal spinal cord such as diacylglycerol lipases, fatty acid amide hydrolase and CB_1 receptor (Bisogno et al., 2003; Cravatt et al., 2004; Farquhar-Smith et al., 2000; Glass et al., 1997; Herkenham et al., 1991; Ong and Mackie, 1999; Romero et al., 2002; Tsou et al., 1998) are modulated after neurodegenerative diseases or after peripheral nerve lesions (Baker et al., 2001; Bilsland et al., 2006; Farquhar-Smith et al., 2000; Hohmann et al., 1999; Lim et al., 2003; Petrosino et al., 2007; Shoemaker et al., 2007; Witting et al., 2004; Wotherspoon et al., 2005; Zhang et al., 2003). Furthermore, malfunction of the endocannabinoid system has been related to the progression of several pathologies, like epilepsy (Ludanyi et al., 2008) or multiple sclerosis (Witting et al., 2006).

We report here that the endocannabinoid system is activated in a clinically relevant model of traumatic spinal cord injury in rats. We also determine the time course of the changes in endocannabinoid levels, the modulation of mRNA expression for endocannabinoid synthesizing and degradative enzymes and changes in cannabinoid receptor expression after this lesion. Our results unveil two different stages of endocannabinoid activation: an acute overproduction of anandamide and the related anti-inflammatory compound palmitoylethanolamide (PEA) and a delayed elevation in 2-AG levels, that are accompanied by modulation of specific synthesizing and degradative enzymes. We also suggest a prominent role of astrocytes as producers and targets for endocannabinoids in SCI.

Material and methods

Animals

Young adult male Wistar rats (295–315 g; 12 weeks of age) were obtained from Harlan-Interfauna Ibérica (Barcelona, Spain) and maintained in our animal facilities on a 12:12-hour light:dark cycle, receiving food and water *ad libitum*. Animals were handled in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals, the principles laid out in the Guidelines for the Use of Animals in Neuroscience Research published by the Society for Neuroscience, and European Union guidelines (Council Directive 86/609/EEC). Experimental procedures were approved by our institutional animal use and care committee, the Ethical Committee for Animal Welfare at the National Paraplegics Hospital (CEBA). Special care was taken to use the minimum number of animals required for statistical accuracy.

Spinal cord injury

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg, Normon Veterinary Division, Madrid, Spain) and Xilagesic (2% Xylacine, 10 mg/kg, Calier, Barcelona, Spain). Once the absence of reflexes had been checked, the rats were injected with a low dose of atropine (50 μg/kg body weight; Brown Medical, Barcelona, Spain) to reduce salivary and bronchial secretions, and to avoid the induction of bradycardia and possible cardiac arrest by the surgery or xylazine. Artificial tears were applied to the eyes to prevent corneal abrasion and infection. The contusion/compression model we used here is adapted from the weight-drop technique and it produces a moderate contusion similar to that described previously (Basso et al., 1996; Collazos-Castro et al., 2005). Briefly, after shaving and cleaning the back of the animals with ethanol and iodine, a dorsal midline incision was made in the skin and the T8 vertebra was exposed after blunt dissection of the muscle. The spiny apophysis and dorsal lamina of the 8th thoracic vertebra were removed, care being taken not to apply pressure on the underlying spinal cord and to maintain the integrity of the dura mater. The animals were then positioned on a SM-15 Narishige stereotaxic frame (Tokyo, Japan), stabilizing the spiny processes of the T7 and T9 vertebrae with clamps. A circular impounder was placed on the dural surface of the spinal cord and an 11 g weight was dropped from 12.5 mm onto the impounder. The impounder was removed after a 10 second compression following the contusion and the site of injury was closed. The animals were then hydrated and placed in heated blankets for 1 hour. The injury produced by this method is equivalent to that described as "moderate" by other groups (Basso et al., 1995; Basso et al., 1996).

Sham operated animals received the same protocol of laminectomy but without suffering contusion. Postoperative care included a subcutaneous injection of Buprex (Buprenorphine, 0.05 mg/kg; Schering Plough, Madrid, Spain) and a prophylactic sub-cutaneous antibiotic injection 1 hour after the lesion and on the following day (Baytril, Enrofloxacine, 1 mg/kg; Bayer, Kiel, Germany). The animals were fed with wet extruded rodent food and manual bladder expression was employed until they were self voiding (within 10 days). The animals were monitored for hydration and eventual infections until the end of the experiment.

Locomotor assessment (Basso–Bresnahan–Beattie locomotor scale)

A representative sample of the injured animals (n = 10) was evaluated in the open field using the Basso–Bresnahan–Beattie (BBB) locomotor scale according to the instructions published in original papers (Basso et al., 1995; Basso et al., 1996) and imparted in the Spinal Cord Injury Research Training Program at the Spinal Trauma and Repair Laboratories (Ohio State University, Columbus, OH, USA). Briefly, animals were gentled to the open field during several sessions. Then, two trained observers scored rats for 4 minutes at the same time of the day on post-lesional days 1, 7 and 28, one person recording all the data on a score sheet and the other one keeping the animal moving in the open field. Movements elicited by the touch of an examiner were not scored.

Endocannabinoid and palmitoylethanolamide measurement

Animals were sacrificed at 1, 7 or 28 days after the lesion and the back of the animal, where the spinal cord lies was placed in a cold chamber within 1 minute (−20 °C), where the spinal cord was removed in less than 8 minutes. A sample of 1 cm around the epicentre of the lesion and a separate 1 cm sample from the adjacent rostral part of the spinal cord were frozen individually in dry ice. For each time point analyzed 4 lesioned and 4 sham-operated animals were used.

The extraction, purification and quantification of endocannabinoids and palmitoylethanolamide (PEA) require a set of different biochemical steps (Di Marzo et al., 2001). First, the tissue was homogenized and extracted with chloroform/methanol/Tris–HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal standards (200 pmol each of $[^2H]_8$ anandamide, $[^2H]_4$PEA and $[^2H]_5$2-AG). The endocannabinoid internal standards were obtained from Cayman Chemicals, Ann Arbor, MI, USA, and were labelled with 8 deuterium atoms on the arachidonate moiety, in the case of $[^2H]_8$ anandamide, and with 5 deuterium atoms on the glycerol moiety, in the case of $[^2H]_5$2-AG. $[^2H]_4$PEA was synthesized by us from deuterated ethanolamine and palmitoylchloride, and contained 4 deuterium atoms on the ethanol moiety. The lipid-containing organic phase was dried and weighed, and the extract was pre-purified by open bed chromatography on silica columns that were eluted with increasing concentrations of methanol in chloroform. The anandamide, PEA and 2-AG fractions were obtained by eluting the column with 9:1 chloroform/methanol (by vol.) and they were directly analyzed by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface.

LC–APCI–MS analyses were carried out in the selected ion monitoring (SIM) mode, as described previously (Di Marzo et al.,