Journal of Neuroimmunology 183 (2007) 133 – 145

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Lymphocytes upregulate signal sequence-encoding proopiomelanocortin mRNA and beta-endorphin during painful inflammation *in vivo*

Nicolle Sitte [a,1], Melanie Busch [a,*,1], Shaaban A. Mousa [a], Dominika Labuz [a], Heike Rittner [a], Carmen Gore [a], Hans Krause [b], Christoph Stein [a], Michael Schäfer [a]

[a] *Department of Anesthesiology and Critical Care Medicine, Charité University Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany*

[b] *Department of Urology, Charité University Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany*

Received 5 October 2006; received in revised form 15 November 2006; accepted 29 November 2006

Abstract

Proopiomelanocortin (POMC)-derived beta-endorphin$_{1-31}$ (END) released from immune cells inhibits inflammatory pain. We examined the expression of END and POMC mRNA encoding the signal sequence required for entry of the nascent polypeptide into the regulated secretory pathway in lymphocytes of rats with inflamed hindpaws. Within 12 h of inflammation, END increased in popliteal lymph nodes and at 96 h the intraplantar neutralization of END exacerbated pain. Lymphocytes expressed POMC, END, and full-length POMC mRNA. Semi-nested PCR revealed 8-fold increased exon 2–3 spanning POMC mRNA. Thus, painful inflammation enhances signal sequence-encoding lymphocytic POMC mRNA needed for regulated secretion of functionally active END.

Keywords: Opioid peptides; Pain; Immune system; Complete Freund's Adjuvant; Neuro-immune interaction

1. Introduction

Immune cell-derived beta-endorphin$_{1-31}$ (END) can elicit pain control by activating opioid receptors on sensory nerve terminals within peripheral inflamed tissue (Stein et al., 1990b, 2003). The secretion of END can be triggered by stress, cytokines, catecholamines, corticotropin-releasing factor (CRF) or chemokines (Binder et al., 2004; Cabot et al., 1997; Mousa et al., 2004; Rittner et al., 2006). With prolonged inflammation the number of opioid containing immune cells, the tissue END content, and the efficacy of pain control increase (Machelska et al., 2003; Mousa et al., 2001; Rittner et al., 2001). Immunosuppression reduces stress- or CRF-induced analgesia (Przewlocki et al., 1992; Schäfer et al., 1994; Stein et al., 1990b) and the reconstitution of immunosuppressed rats with lymphocytes reverses this effect (Hermanussen et al., 2004).

END is derived from proopiomelanocortin (POMC) and is processed within the pituitary and various types of non-neuronal cells (Heijnen et al., 1991; Raffin-Sanson et al., 1999; Sharp and Linner, 1993; Slominski et al., 2000; Westly et al., 1986). The first exon of the POMC gene includes promotor binding sites and the mRNA cap region, which are typically untranslated. The second exon encodes the signal peptide sequence necessary for directing the nascent polypeptide to the regulated secretory pathway (Kalies and Hartmann, 1998). The sequences of functionally active

Abbreviations: CFA, Complete Freund's Adjuvant; CP, crossing point; CRF, corticotropin releasing factor; END, beta-endorphin; END$^{-/-}$, beta-endorphin knock out; i.pl., intraplantar; ir, immunoreactive; LN, lymph node(s); M, DNA standard marker; NC, negative control; POMC, proopiomelanocortin; PPT, paw pressure threshold; PT, pituitary; RIA, radioimmunoassay; rpL19, ribosomal protein L19; RT, reverse transcriptase; qRT-PCR, quantitative RT-PCR; SIG, signal sequence; WT, wild type.

* Corresponding author. Forschungseinrichtung für Experimentelle Medizin, Klinik für Anästhesiologie und operative Intensivmedizin, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Krahmerstr. 6-10, 12207 Berlin, Germany. Tel.: +49 30 8445 2131; fax: +49 30 8339 389.

E-mail address: melanie.busch@charite.de (M. Busch).

[1] Both authors have equally contributed to the present study and share first-authorship.

0165-5728/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2006.11.033

peptides such as adrenocorticotrophic hormone, melanocyte-stimulating hormones, and END are contained within the third exon (Drouin et al., 1985).

Previous studies indicate that translation products of POMC transcripts lacking the signal sequence are neither processed to authentic peptides nor secreted (Clark et al., 1990; Rees et al., 2002). Several studies detected truncated POMC transcripts in naïve lymphocytes (Buzzetti et al., 1989; Cabot et al., 1997; DeBold et al., 1988; Lacaze-Masmonteil et al., 1987; Oates et al., 1988; Przewlocki et al., 1992), while findings of full-length POMC mRNA are limited to a single study (Stephanou et al., 1991). However, full-length POMC mRNA may be present under pathological conditions, as demonstrated in a T lymphoma cell line (Buzzetti et al., 1989) and after mitogen treatment of lymphocytes *in vitro* (Lyons and Blalock, 1997). In the present study we set out to examine the expression of exon 1–3 and exon 2–3 spanning POMC mRNA in lymphocytes from rats with Complete Freund's adjuvant (CFA)-induced paw inflammation. In addition, we quantified exon 2–3 spanning POMC transcripts and END in relation to the development of inflammatory signs and hyperalgesia, we confirmed the functional relevance of END in producing intrinsic pain inhibition *in vivo* and we verified the specificity of the END antibody by use of $END^{-/-}$ mice.

2. Materials and methods

2.1. Experimental animals and induction of inflammation

All experiments were approved by the animal care committee of the Senate of Berlin and strictly followed the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Male Wistar rats (225–300 g, Charles River Breeding Laboratories) received an intraplantar (i.pl.) injection of 0.15 ml Complete Freund's Adjuvant (CFA; Calbiochem, La Jolla, CA, USA) or 0.15 ml NaCl (controls) into the right hindpaw under brief isoflurane (Rhodia Organic Fine Ldt., Bristol, UK) anesthesia. The inflammation remained confined to the right paw throughout the observation period.

2.2. Measurement of paw volume and nociceptive thresholds

Handled rats were tested at 2, 6, 24, and 96 h after i.pl. CFA or NaCl injections. The paw volume was determined using a plethysmometer (Ugo Basile, Comerio, Italy) as described earlier (Stein et al., 1990a). Nociception was assessed by measuring the paw pressure threshold (PPT) using an algesiometer (modified Randall–Selitto test; Ugo Basile) as previously described (Stein et al., 1990b). Three consecutive trials, separated by 10 s intervals, were conducted and the average was calculated.

At 96 h of inflammation one group of rats received an i.pl. injection (0.1 ml) of either naloxone-methiodide (100 μg, Sigma-Aldrich, Taufkirchen, Germany) or 0.9% NaCl (control) into the CFA-inflamed paw. Another group of rats received an i.pl. injection (0.1 ml) of a polyclonal rabbit antibody against END (anti-END; 1 μg, Peninsula Laboratories Inc., Belmont, CA, USA) into the CFA-inflamed paw, while control animals were injected with nonimmune IgG (1 μg; Sigma). PPT was assessed before and 5 min after these treatments as described above.

2.3. Immunofluorescence

The expression of POMC and END in lymph nodes (LN) from untreated and CFA-treated rats (24 h after inoculation) were analyzed. Under deep isoflurane anesthesia, rats were perfused transcardially with 0.1 M PBS, followed by fixative solution (PBS containing 4% paraformaldehyde, pH 7.4). The popliteal LN were removed, postfixed for 30 min at 4 °C in the fixative solution, and cryoprotected overnight at 4 °C in PBS containing 10% sucrose. The tissue was embedded in Tissue Tek compound (OCT, Miles, Elkhart, IN, USA), frozen, cut into 8 μm sections, mounted onto gelatin-coated slides, and processed for immunofluorescence (Mousa et al., 2000). To prevent non-specific binding, sections were incubated for 60 min in PBS containing 0.3% Triton X-100, 1% BSA, 5% goat serum, and 5% donkey serum (blocking solution). Sections were then incubated overnight with mouse monoclonal anti-POMC (10 μg/ml; Biogenesis Ltd, Poole, UK; recognizing the N-terminal amino acids 1–50 of POMC, crossreactivities: 100% for N-POMC, <6% for gamma-melanocyte-stimulating hormone and beta-lipotropin) in combination with polyclonal rabbit anti-END_{1-31} (1:1000; Peninsula Laboratories, Belmont, CA, USA; crossreactivities: 100% for alpha-END; 60% for gamma-END; none for adrenocorticotrophic hormone or alpha-melanocyte-stimulating hormone, according to the manufacturer). Then the tissue sections were washed with PBS and incubated with texas red-conjugated goat anti-rabbit antibody in combination with FITC-conjugated donkey anti-mouse antibody (both Vector Laboratories, Burlingame, CA, USA). Unbound antibodies were removed by washing with PBS. Nuclei were stained by incubation with 4′-6′-Diamidino-2-phenylindole (DAPI) for 5 min (10 μg/ml; Sigma). Sections were mounted in vectashield (Vector Laboratories) and viewed under a fluorescence microscope (Zeiss, Jena, Germany) with appropriate filters. The following control experiments were included: preabsorption of anti-END with END (Peninsula Laboratories) and omission of either the primary or the secondary antibodies (Mousa et al., 2000).

2.4. Radioimmunoassay (RIA) and specificity of the END antibody

Rats were sacrificed at different time points after CFA-inoculation by isoflurane overdose. Popliteal LN were dissected, homogenized, and transferred to a 70 μm cell strainer to obtain cell suspensions. Cells were counted and viability was examined by the trypan blue exclusion method.