Journal of Neuroimmunology 258 (2013) 10–16

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

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

Cleaved caspase-3 expression in hypothalamic magnocellular neurons may affect vasopressin secretion during experimental polymicrobial sepsis

Gabriela Ravanelli Oliveira-Pelegrin, Paulo José Basso, Aline Souza Soares, Mariana Rossin Martinez, Karin Dorota Riester [1], Maria José Alves Rocha *

Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 16 August 2012
Received in revised form 14 December 2012
Accepted 14 February 2013

Keywords:
Cecal ligation and puncture
Supraoptic nucleus
Paraventricular nucleus
Cytokines
Nitric oxide

ABSTRACT

We investigated whether the vasopressin (AVP) secretion deficiency observed during cecal ligation and puncture (CLP)-induced sepsis may be caused by apoptosis in hypothalamic magnocellular neurons. Plasma cytokines (TNF-α, IL-1β and IL-6) and nitrate levels were increased during sepsis and plasma AVP levels were higher in the early phase returning to basal levels in the late phase. Concomitantly, expression of the apoptosis effector, cleaved caspase 3, was increased in magnocellular neurons, inferring that this increase in hypothalamic neurons may be caused by cytokines and elevated nitrate levels. This in turn could compromise AVP secretion in the late phase of sepsis.

1. Introduction

Sepsis is a systemic inflammatory response that results from a complex interaction between host and infectious agent (Bone et al., 1992; Vincent and Korkut, 2008) leading to excessive production and release of inflammatory mediators that may directly or indirectly activate the central nervous system causing encephalopathy and affecting certain body functions, including hormone secretion (McCann et al., 2000; Kovacs, 2002; Sharshar et al., 2004; Semmler et al., 2005; Berg et al., 2011).

Among the neuroendocrine changes occurring during sepsis, the biphasic response of vasopressin (AVP) secretion has been extensively studied (Landry et al., 1997; Sharshar et al., 2003a; Correa et al., 2007; Oliveira-Pelegrin et al., 2009, 2010a, 2010b). AVP is a neurohypophyseal hormone synthesized in magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. The increase in AVP plasma levels commonly denoted in the initial phase of sepsis is considered an attempt to revert the drop in blood pressure. Yet in the late phase, AVP secretion is inappropriately low, this contributing to vasodilatation and organ dysfunction.

When investigating possible causes for the deficiency in AVP levels in the late phase of polymicrobial sepsis we observed several factors that apparently contribute to this syndrome, such as a decrease in AVP gene expression (Oliveira-Pelegrin et al., 2010b), a change in hypothalamic hormone content, and also an inability to replenish the neurohypophyseal stocks of AVP (Oliveira-Pelegrin et al., 2009). Additionally, a reduction or blockage of neuronal activation in different brain region related to autonomic and neuroendocrine functions has also been observed in septic animals (Correa et al., 2007; Bruhn et al., 2009). Taken together, these findings suggested that neuronal impairment may occur in the late phase of sepsis, and this hypothesis is in line with observations in patients that died due to sepsis and which evidenced neuronal and glial apoptosis in regions related to autonomic functions were observed (Sharshar et al., 2003b).

Apoptosis, an ATP-dependent mechanism, can be triggered by various stimuli that induce extrinsic and/or intrinsic death signaling pathways (Bantel and Schulze-Osthoff, 2009). These pathways lead to the activation of apoptotic effector caspases, with caspase-3 being one of the most important ones, although neither unique nor ubiquitous. Caspase-3 activation results in cleavage of key protein in the apoptosis process (Mignotte and Vayssiere, 1998; Porter and Janicke, 1999).

During sepsis the brain is exposed to several stimuli that can trigger apoptosis pathways (Semmler et al., 2005; Alexander et al., 2008; Berg et al., 2011). To understand their effects we herein investigated the activation of caspase-3 in hypothalamic magnocellular neurons during experimental polymicrobial sepsis.

* Corresponding author at: Departamento de MEF-Faculdade de Odontologia de Ribeirão Preto, USP., Avenida do Café s/n, CEP 14040-904 Ribeirão Preto-SP, Brazil. Tel.: +55 16 3602 3974; fax: +55 16 3633 0999.
E-mail address: mjrocha@forp.usp.br (M.J.A. Rocha).
[1] Present Address: Fakultät für Biologie, Abteilung für Neuropharmakologie, Eberhard Karls Universität Tübingen, Tübingen, Germany.

0165-5728
http://dx.doi.org/10.1016/j.jneuroim.2013.02.007

2. Material and methods

2.1. Animals

Male Wistar rats (250 ± 30 g) provided by the Animal Facility of the Campus of Ribeirão Preto, University of São Paulo, were housed in controlled temperature (25 ± 1 °C) and photoperiodic (12:12 h night:day cycle) conditions, with food (Nuvilab CR-1, NUVITAL) and tap water available ad libitum. All experimental protocols were approved and performed according to the guidelines of the Ethics Committee of the University of São Paulo - Campus Ribeirão Preto. Humane endpoints in shock research (Nemzek et al., 2004) were used as criterion to euthanize CLP-animals in high suffering, immediately before or soon after the studied time-points defined in this study.

2.2. Cecal ligation and puncture surgery

Animals were randomly assigned to one of two groups, CLP group and sham-operated or non-manipulated animals (control). All experiments were performed at the same time of day (08:00–10:00 AM). Induction of severe sepsis was performed by a cecal ligation and puncture (CLP) procedure. Briefly, rats were anesthetized with a short-acting anesthetic agent (tribromoethanol; 2.5%, 250 mg/kg i.p.; Acros Organics) in order to minimize deleterious effects of anesthesia on cardiovascular functions. Under sterile surgical conditions the animals were subjected to a midline laparotomy. The cecum was carefully isolated to avoid damage to the blood vessels. Subsequently, the cecum was ligated below the ileocecal valve, without causing bowel obstruction, and punctured ten times with a 16-gauge needle allowing fecal contents to spill into the peritoneum. The abdominal cavity was closed in two layers, and all animals received a subcutaneous injection of saline (20 mL/kg body weight). Sham-operated animals were submitted to laparotomy, the cecum was manipulated but neither ligated nor punctured. The animals were allowed to recover in their cages with free access to food and water.

2.3. Experimental protocol

Following sham-operation or CLP surgery, the animals were decapitated at 4, 6, 24 or 48 h for blood collection and removal of the brain. Plasma was used for measurement of cytokines, nitric oxide (NO) and AVP levels. Brains were snap-frozen on dry ice and stored at −80 °C until SON and PVN dissection. These hypothalamic nuclei were carefully microdissected and processed for reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) for caspase-3 transcript levels, or Western blotting for detection of cleaved caspase-3 (CC3) protein. Another set of animals was deeply anesthetized and perfused with 4% paraformaldehyde (4% PFA) in 0.1 M phosphate buffered saline (0.1 M PBS) at 6 and 24 h after surgery. Brains were removed, post-fixed for 4 h and immersed in 30% sucrose in PBS for cryoprotection. Cryostat sections of 30 μm thickness containing the hypothalamus were processed for CC3 immunohistochemistry.

2.4. Measurement of plasma cytokine levels

Plasma was used for measuring TNF-α, IL-1β and IL-6 levels by using kits for specific enzyme-linked immunosorbent assay (DuoSet ELISA Development system) (R&D Systems, Minneapolis, MN, USA). The assays were performed following the manufacturer's instructions and the detection limits for TNF-α, IL-1β and IL-6 were 5, 5 and 21 pg/mL, respectively. According to the manufacturer's datasheet, the TNF-α, IL-1β and IL-6 assays do not exhibit any cross-reactivity or interference.

2.5. Plasma nitrate quantification

Total nitrate was determined by using the purge system of a Sievers Instruments Nitric Oxide Analyzer (NOA model 280i, Boulder, CO, USA). Plasma samples were deproteinized using cold absolute ethanol and then injected into a reaction vessel containing vanadium trichloride (VCl_3), which converts nitrate to NO. The NO produced was detected as ozone induced by chemiluminescence. Peak NO values of samples were determined using a standard curve established with sodium nitrate solutions of various concentrations (5, 10, 25, 50 and 100 μM).

2.6. Radioimmunoassay for vasopressin

The AVP radioimmunoassay was performed as previously described (Correa et al., 2007). Briefly, 1 mL plasma samples were extracted using acetone/petroleum ether, lyophilized and stored at −80 °C until analysis. Standard reagents and incubation protocols were used for the peptide assays. For peptide labeling, ^{125}I was purchased from a commercial supplier (Amersham) and an AVP antiserum (Peninsula Lab.) was used at a final dilution of 1:40,000 in a phosphate buffer (pH 7.5) supplemented with BSA (0.5%). A non-equilibrium assay was used with an incubation volume of 500 μL and an incubation time of 4 days at 4 °C. Bound hormone was separated from unbound by a secondary antibody produced in the laboratory of J. Antunes-Rodrigues and Lucila L.K. Elias (Univ. São Paulo, Ribeirão Preto), where the RIA was performed. The AVP antiserum is specific and shows essentially no cross-reactivity with oxytocin. The minimum detection limit was 0.9 pg/mL and the coefficients of intra- and inter-assay variation were 7% and 11%, respectively.

2.7. Microdissection of the SON and PVN nuclei

In a cryostat, the brain was placed in a brain matrix (Insight Equipment LTDA, Ribeirão Preto, Brazil) and cut based on rat brain atlas coordinates (Swanson, 1998) with optic chiasm as anatomical landmark for reproducibility among the dissections. A single section of approximately 1-mm thickness was taken and the PVN and SON regions were carefully dissected by using punch needles of 1400 μm and 1200 μm diameter, respectively (Palkovits, 1973).

2.8. Primer design, RNA extraction and reverse transcription

Gene-specific primers for rat caspase-3 (NM_012922.2), GAPDH (NM_017008) and 18S (M11188) were designed based on GenBank sequences. The sequences were as follows: caspase-3 (+) cag tgg tgg aca tga cga c, (−) agt tgg tat tat ggt ctg tcc; GAPDH: (+) tca cca cca tgg aga agg c, (−) gct aag cag ttg gtg gtg ca; 18S: (+) acg gaa ggg cac cac cag ga and (−) cac cac cac cca cgg aat cg. The reference gene (GAPDH and 18S) primer combinations had already been validated in previous studies (Oliveira-Pelegrin et al., 2010b).

The tissue punches were homogenized in 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for total RNA extraction. All samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) to remove any contaminating genomic DNA. RNA purity and quantity were assessed by spectrophotometry using a Synergy H1 Take 3 system (BioTek) and Gen5 software. First-strand cDNA synthesis was carried out using the following protocol. Two μg of total RNA, 1 μL of oligo$(dT)_{12-18}$ primer (0.5 μg/μL, Invitrogen, Carlsbad, CA, USA) and 1 μL of dNTP mix (10 mM) (Invitrogen, Carlsbad, CA, USA) were incubated at 69 °C for 5 min and chilled on ice. Subsequently, 4 μL of 5× First Strand Buffer, 2 μL of DTT (0.1 M) and 1 μL of RNaseOUT Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA, USA) were added and the samples incubated for 2 min at 42 °C. Next, SuperScript™ II Reverse Transcriptase (200 U, Invitrogen, Carlsbad, CA, USA) was added and the reaction incubated at 42 °C for 50 min