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Cellular bioenergetics changes in magnocellular neurons may affect copeptin expression in the late phase of sepsis

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ABSTRACT

We investigated whether inflammatory mediators during cecal ligation and puncture (CLP)-induced sepsis may diminish copeptin expression in magnocellular neurons, thus affecting arginine-vasopressin (AVP) synthesis. The transcript abundance of IL-1β, IL-1R1, iNOS and HIF-1α was continuously elevated. IL-1β, iNOS and cytochrome c protein levels progressively increased until 24 h. Immunostaining for these proteins was higher at 6 and 24 h, as also seen in the annexin-V assay, while copeptin was continuously decreased. This suggests that increased IL-1β and NO levels may cause significant bioenergetics changes in magnocellular neurons, affecting copeptin expression and compromising AVP synthesis and secretion in the late phase of sepsis.

1. Introduction

Clinical studies report that high arginine-vasopressin (AVP) plasma levels can be found in patients in the early phase of sepsis, in an attempt to restore blood pressure, which tends to decrease due to inflammatory mediators. Nonetheless, in the late phase, despite progressive hypotension, the plasma AVP levels are low, contributing to septic shock and death (Landry et al., 1997; Sharshar et al., 2003a). Moreover, infusion of a low dose of exogenous AVP decreases norepinephrine requirement in septic patients, while maintaining or increasing blood pressure, systemic resistance and urine output in vasodilatory shock (Holmes et al., 2001). There are also clinical studies indicating impaired baroreflex sensitivity (Holmes et al., 2001), depletion of neurohypophyseal hormone content (Holmes et al., 2001; Sharshar et al., 2002), overproduction of nitric oxide (NO) and oxidative stress in AVP neurons (Holmes et al., 2001) as reasons for the AVP secretion impairment. Corresponding findings were also seen in experimental sepsis in previous work from our group (Correa et al., 2007; Pancoto et al., 2008; Oliveira-Pelegrin et al., 2009, 2013).

During sepsis, the excessive production and release of inflammatory mediators may affect AVP synthesis. By using cecal ligation and puncture (CLP) to induce sepsis, we in fact saw a decrease in AVP expression in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus (Oliveira-Pelegrin et al., 2010a, 2010b). Septic patients and rats were also reported to show changes in AVP content, as well as neuronal and glial apoptosis in regions related to autonomic control, including SON and PVN hypothalamic nuclei (Sharshar et al., 2003b; Sonneville et al., 2010). We recently reported an increased expression of cleaved caspase-3 in SON magnocellular neurons of CLP-induced septic rats (Oliveira-Pelegrin et al., 2013) suggesting that apoptosis was occurring in these neurons. Increased cytokine levels, particularly interleukin-1β (IL-1β), are thought to trigger the inducible isoform of NO synthase (iNOS) gene expression in the hypothalamus (Wong et al., 1996b, 1997). Once induced, iNOS produces large NO levels, which may act dually on mitochondrial bioenergetics affecting oxygen consumption and enhancing the generation of superoxide anions by decreasing the electron flow through cytochrome c oxidase. These changes may result in a "metabolic hypoxia" and hydrogen peroxide formation (Mander and Brown, 2004; Mander et al., 2005; Erusalimsky and Moncada, 2007), which may further stimulate iNOS expression and, consequently, an increase in NO levels (Guix et al., 2005). This metabolic hypoxia may also induce the expression and stability of the α subunit of hypoxia-induced factor 1 (HIF-1α) (Chavez et al., 2000; Sharp and Bernaudin, 2004; Erusalimsky and Moncada, 2007). Dimerization of HIF-1α with the constitutive HIF-1β subunit generates the functional transcription factor HIF-1, which regulates the expression of various genes involved in cellular energy metabolism and in the apoptosis pathway (Bruick, 2000; Sharp and Bernaudin, 2004).

Apoptosis can be triggered by various stimuli that activate the extrinsic and/or intrinsic pathway upstream of the caspase cascade. The extrinsic apoptosis pathway is induced by the activation of death receptors, which in turn belong to the tumor necrosis factor receptor

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superfamily (TNFRS). On the other hand, the intrinsic apoptosis pathway is mainly associated with mitochondrial and other intracellular stress signals (Sola et al., 2013). Oxidative stress promotes the movement of pro-apoptotic proteins to the mitochondrial surface, which changes the permeability of the mitochondrial membrane leading to transient pore formation and consequent release of proteins related to the activation of the intrinsic apoptosis pathway, such as cytochrome c (Mignotte and Vayssiere, 1998; Erusalimsky and Moncada, 2007). An early signal at this stage of the apoptosis process is the exposure of phosphatidylserine (PS), which can be detected by its affinity for annexin-V (van Engeland et al., 1998).

On the background of all this information we hypothesized that the cellular bioenergetics changes seen during sepsis could trigger alterations in synthesis of the AVP precursor, including that of copeptin, a C-terminal glycopeptide in the AVP precursor preprovasopressin. Copeptin plays an important role in the correct structural formation and proteolytic maturation of AVP (Barat et al., 2004; Struck et al., 2005; Morgenthaler et al., 2008). With this in mind, we analyzed the expression of oxidative stress and apoptosis markers in copeptin-AVP neurons of the SON and associated these with changes in AVP synthesis and basal plasma concentrations typically seen in the late phase of sepsis.

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 or control (sham-operated or non-manipulated animals). All experiments were performed at the same time of day (08:00–10:00 AM). Severe sepsis was induced 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) to minimize deleterious effects of anesthesia on cardiovascular functions. The animals were subjected to a midline laparotomy done under sterile surgical conditions. The cecum was carefully isolated to avoid damage to 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 or 6 (early phase of sepsis) or 24 or 48 h (late phase of sepsis) for removal of the brain, which was snap-frozen on dry ice and stored at −80 °C until SON dissection. The hypothalamic nuclei were carefully microdissected and processed for reverse transcription (RT) and quantitative polymerase chain reaction (qPCR), or Western blot detection. 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 IL-1β, IL-1R1, HIF-1α, iNOS, cytochrome c, and copeptin immunohystochemistry and the annexin-V affinity assay.

2.4. Microdissection of supraoptic nucleus (SON)

The frozen brains were placed in a brain matrix (Insight Equipment LTDA, Ribeirão Preto, Brazil) and cut based on the rat brain atlas coordinates (Swanson, 1998) with the optic chiasm as anatomical landmark for reproducibility among the dissections. A single section of approximately 1 mm thickness was taken and the SON region was carefully dissected by using a punch needle of 1.2 mm diameter (Palkovits, 1973).

2.5. Primer design, RNA extraction and reverse transcription

Gene-specific primers for rat IL-1β (NM_031512), IL-1R1 (Peinnequin et al., 2004), iNOS (Peinnequin et al., 2004), HIF-1α (NM_024359), GAPDH (NM_017008) and 18S rRNA (M11188) were used as in the literature or designed based on GenBank sequences. The sequences were as follows: IL-1β: (+) gca atg gtc ggg aca tag tt, (−) aga cct gac ttg gca gag ga; IL-1R1: (+) gtt ttt gga aca ccc ttc agc c, (−) acg aag cag atg aac gga tag c; iNOS: (+) cat tgg aag tga agc gtt tcg, (−) cag ctg ggc tgt aca aac ctt; HIF-1α: (+) tca agt cag caa cgt gga ag, (−) tat cga ggc tgt gtc gac tg; GAPDH: (+) tca cca cca tgg aga agg c, (−) gct aag cag ttg gtg gtg ca; and 18S rRNA: (+) acg gaa ggg cac cac cag ga, (−) cac cac cac cca cgg aat cg. The reference gene (GAPDH and 18S rRNA) 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 contaminant 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 followed by 15 min at 70 °C. cDNAs were stored undiluted at −20 °C until further use. All cDNA samples were diluted 1:3 with DEPC-treated water before being used as templates in quantitative PCR assays.

2.6. Quantitative real-time PCR (qPCR)

Quantitative real-time PCR studies were performed using Fast EvaGreen Master Mix (Biotium, Hayward, USA) in a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). qPCR reactions, performed in 96-well 0.2 mL thin-wall PCR microplates (Axygen) sealed with film, consisted of 10 μL of Fast EvaGreen Master Mix, 0.8 μL of each forward and reverse primer (10 μM) and 2 μL of 1:3-diluted template cDNA in a total volume of 20 μL completed with DEPC-treated water. Cycling was performed using the optimized conditions in a four-step experimental run protocol: (i) denaturation program (2 min at 96 °C); (ii) amplification and quantification program repeated 45 times (5 s at 96 °C, 5 s at 58 °C, 25 s at 72 °C); melting curve program (60–95 °C with a heating rate of 0.5 °C/s and continuous fluorescence measurement); (iv) cooling down to 4 °C. Melting curves obtained after