



Effects of neonatal inflammation on the inflammatory and oxidative profile during experimental sepsis in adult life

Adroaldo Lunardelli^a, Carolina Luft^b, Leonardo Pedrazza^a, Bianca Andrade Martha^a, Jarbas Rodrigues de Oliveira^a, Márcio Vinícius Fagundes Donadio^{a,b,*}

^a Laboratório de Pesquisa em Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Brazil

^b Centro Infant, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Brazil

HIGHLIGHTS

- Neonatal inflammation programs fear and anxiety behavior in mice.
- Neonatal LPS alters mitochondrial respiratory chain and Ang II receptors.
- The effects of neonatal inflammation are sex-dependent.
- Sepsis-induced inflammatory cytokines response is not altered by neonatal LPS.

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ABSTRACT

The present study aimed to evaluate the long-term effects of neonatal inflammation on the inflammatory and oxidative profile during experimental sepsis in adult life. Neonatal Balb/c mice received different treatments on day 10: LPS i.p. injection (100 g/kg) (nLPS) or saline i.p. injection (nSal). As adults, fear/anxiety behavior was evaluated in the elevated plus maze. The following week, saline solution or LPS was administered and, after 12 h, serum (inflammatory cytokines), liver (mitochondrial complexes and oxidative stress) and adrenal gland samples (angiotensin II type 1 and 2 receptors) were collected. There was an increase in the fear/anxiety behavior in the nLPS group. Neonatal administration of LPS increased the mRNA expression of the AT₁ receptor and decreased the mRNA expression of the AT₂ receptor in the adrenal glands of males. The complexes II and III increased in the nLPS saline male group when compared to control. The LPS administration in adult females, regardless of the neonatal treatment, induced a decrease of the glutathione enzyme activity. There were no differences in the inflammatory cytokines. The results showed that neonatal inflammation influenced mitochondrial respiratory chain metabolism and angiotensin II receptors in a sex-dependent manner. Balb/c mice fear and anxiety behaviors in adulthood were programmed by early life inflammatory stress.

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1. Introduction

The neonatal period is a very important stage in the development of different systems [1] and is also characterized by great plasticity and reorganization [2]. It seems that interventions during this important period may cause a programming effect, that is, a “mark” on the fetal and neonatal development period that will remain in later life. The concept of programming has been used to explain the process by which an organism adapts to environmental events, resulting in stable changes in phenotype. This usually occurs in early periods of development, when

exposure to adverse environments can disrupt the process of cell proliferation and differentiation leading to long-term effects [3].

The administration of lipopolysaccharide (LPS) in the neonatal period generates immunologic stressful stimuli [4] and is able to stimulate the expression of cytokines in the central nervous system and cause brain changes in certain regions of the hippocampus of adult rats [5]. It is suggested that a first stimulus (genetic or environmental) during critical periods of development hinders the ontogeny of neural systems, which produces an enhanced propensity toward a second stimulus in adult life [6]. This immunologic “double challenge” may induce hypothalamic-pituitary-adrenal axis (HPA) modifications, which provides significantly reduced circulating proinflammatory cytokines (IL-1 beta, TNF-alpha, and IL-6) [7], hyperalgesia [8], increased sensitivity to stress and increased anxiety and depression-related behavior [9]. Moreover, in response to stressful situations, the renin-angiotensin

* Corresponding author at: Centro Infant, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6690, 2° andar, Porto Alegre, Rio Grande do Sul CEP 90610-000, Brazil.

E-mail address: mdonadio@pucrs.br (M.V.F. Donadio).

system (RAS) synthesizes angiotensin II (Ang II), promoting the modulation of neuroendocrine systems and stimulating the expression of angiotensin II type 1 (AT₁) or type 2 (AT₂) receptors at peripheral levels [10]. This peptide is involved in the regulation of the release of corticotrophin, adrenocorticotrophic hormone (ACTH) and in the synthesis and secretion of corticosterone, which potentially contributes to the modulation of HPA axis activity [11].

Conversely, mitochondria are the key source of cellular ATP and their structure and function are markedly affected by pathophysiologic processes associated with the host's response to invading pathogens. Organic dysfunction occurs in the course of sepsis as a result of reduced oxygen availability due to hemodynamic disturbances of the macro and microcirculation. It is widely accepted that the impairment of mitochondrial function appears to correlate with organ failure in septic patients [12]. In addition, mitochondrial production of reactive oxygen species (ROS) increases during the sepsis inflammatory response. It has been suggested that mitochondrial ROS may regulate the NF- κ B levels in immune cells, inducing the inflammatory response [13].

Sexual differences in physiology and function of peripheral immune system of vertebrates have been documented for decades. Females of many species typically have increased immune response and resistance to infection compared to males. These differences between genders have been mostly attributed to the immunomodulatory effects of sex steroid hormones. Generally, exogenous estradiol has stimulant effects on humoral immunity. However, it can enhance or suppress cell-mediated immunity, depending on the dose. Exogenous testosterone usually depresses both humoral immunity as mediated by cells, increasing susceptibility to bacterial and viral infections. Certainly, the difference between the number of glial cells and levels of immune molecules is likely to have profound sex-specific effects on the function of the neuroimmune system during an early-life immune challenge, so that males and females may have different responses to the neonatal immune system activation [14].

Considering the evidence that inflammatory processes in the neonatal period can generate stable alterations, the high prevalence of adverse situations early in life and severe states in adulthood, such as sepsis, it is justified to further investigate how early-life stress may influence the adult inflammatory response, which could contribute to a better understanding and management of these situations. Thus, the objective of this study was to evaluate the long-term effects of neonatal inflammation on the inflammatory and oxidative profile in experimental sepsis in adulthood. The activity of mitochondrial complexes, oxidative stress, inflammatory cytokines, expression of angiotensin II receptors, as well as the behavior and possible sexual differences in Balb/c mice were evaluated.

2. Materials and methods

Experiments were performed with laboratory Balb/c mice, which were housed under controlled conditions ($24 \pm 2^\circ\text{C}$), 12-h light–dark cycle, 55 to 65% humidity, and provided with food and water ad libitum. The animals were handled according to the Guiding Principles for the Care and Use of Animals in Research and Teaching adopted by the American Physiological Society.

The research was approved by the Research Ethics Committee for Animal Use of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), under n° 11/00,233.

2.1. Experimental procedures

For all experiments, the adult animals were initially divided into two groups, according to the treatment during the neonatal period. On day 10 after birth, the litter was standardized between 6 and 8 animals (balanced by sex) and separated from their mothers for five minutes. At this time, a similar number of pups received different treatments (intraperitoneally) and were divided into two groups: nLPS, received

100 μL of 100 $\mu\text{g}/\text{kg}$ of LPS (serotype *Escherichia coli* O26:B6; Sigma-Aldrich, USA) dissolved in saline solution - (0.9% NaCl-sterile); and nSal (control) received equivalent volume of sterile saline vehicle. After the procedure, the litter returned to their mothers until day 21 of life when the weaning occurred. Following, the animals were separated by sex in boxes containing approximately 3 to 5 animals, with no other interventions until adulthood.

On day 49 after birth, the animals were weighed and submitted to the experiments. Initially, fear and anxiety behavior of each animal were measured using an elevated plus maze (EPM). The following week, the animals underwent intraperitoneal administration of saline or LPS (septic induction using 20 mg/kg [15]) in the same final volume, and were divided into four experimental groups: (i) nSal–Saline, animals that received saline on day ten after birth and saline solution when adults; (ii) nSal–LPS, animals that received saline on day ten after birth and LPS when adults; (iii) nLPS–Sal, animals that received LPS on day ten after birth and saline when adults; and (iv) nLPS–LPS, animals that received LPS on day ten after birth and LPS when adults.

After 12 h, the animals were euthanized by decapitation for blood collection in order to obtain serum samples for the measurement of interferon-gamma (INF- γ), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF- α), transforming growth factor-beta 1 (TGF- β 1) and interleukin 6 (IL-6). The liver of mice was removed by laparotomy and quickly placed in ice. The organ was homogenized (1:20 p/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 IU/mL heparin) pH 7.4. The homogenate was centrifuged at $750 \times g$ for 10 min. The supernatant was kept at -70°C until being used for the determination of oxidative stress parameters through the measurement of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD) and nitric oxide (NO). The activity of mitochondrial complex II, II–III and IV were also measured. To this end, the tissue was subjected to three freezing cycles in order to expose the mitochondrial enzymes catalytic site. The adrenal gland was also removed, weighed and frozen for subsequent quantification of mRNA expression of angiotensin AT₁ and AT₂ receptors, using the reverse transcription polymerase chain reaction (RT-PCR).

2.2. Elevated plus-maze (EPM)

The apparatus consisted of two open arms ($30 \times 5 \text{ cm}$) and two closed arms ($30 \times 5 \times 15 \text{ cm}$) extending from a common central platform ($5 \times 5 \text{ cm}$). The apparatus was constructed from non-abrasive black material, and elevated 66 cm above the floor. Mice were placed individually on the center square facing a closed arm and were kept in the apparatus for 10 min. The distance covered, time spent in each arm or central square and latency between the beginning of the procedure and the animal entry into the open arm were recorded and analyzed using behavioral scoring software Any-Maze (Stoelting).

2.3. TGF- β 1 quantification

TGF- β 1 concentration was measured in serum samples using a commercial kit supplied by R&D systems (R&D systems, Inc., Minneapolis, USA). The kit contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound TGF- β 1. The color reaction was detected in 540/570 nm in a plate reader. Results were calculated according to a standard curve concentration. TGF- β 1 levels were expressed as $\mu\text{g}/\text{mL}$.

2.4. Cytokine quantification

Multiple circulating serum cytokines (INF- γ , TNF- α , IL-6 and MCP-1) were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, USA). Acquisition was performed with a FACSCanto II flow cytometer

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