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Expression of autocrine prolactin and the short isoform of prolactin receptor are associated with inflammatory response and apoptosis in monocytes stimulated with *Mycobacterium bovis* proteins

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ABSTRACT

Increased levels of prolactin (PRL) have recently been associated with carcinogenesis and the exacerbation of autoimmune diseases, and might be involved in the progression of tuberculosis (TB). To investigate the relationship between PRL and prolactin receptor (PRLr) expression with inflammatory response and apoptosis in monocytes, we used THP-1 cells stimulated with antigens of the *Mycobacterium bovis* AN5 strain culture filtrate protein (CFP-*M. bovis*). Western blot (WB), real-time Polymerase chain reaction (PCR), and immunocytochemistry were performed to identify both PRL and PRLr molecules. PRL bioactivity and proinflammatory cytokine detection were assessed. The results showed that PRL and PRLr messenger RNA (mRNA) were synthesized in THP-1 monocytes induced with CFP-*M. bovis* at peaks of 176- and 404-fold, respectively. PRL forms of 60 and 80 kDa and PRLr isoforms of 40, 50, and 65 kDa were also identified as time-dependent, while 60-kDa PRL, as well as 40-, and 50-kDa PRLr, were found as soluble forms in culture media and later in the nucleus of THP-1 monocytes. PRL of 60 kDa released by monocytes exhibited bioactivity in Nb2 cells, and both synthesized PRL and synthesized PRLr were related with nitrite and proinflammatory cytokine levels proapoptotic activity in CFP-*M. bovis*-induced monocytes. Our results suggest the overexpression of a full-autocrine loop of PRL and PRLr in monocytes that enhances the inflammatory response and apoptosis after priming with *M. bovis* antigens.

1. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by excessive and/or prolonged cytokine production that might affect immunoendocrine communication and favor important alterations in essential biological functions (Bellavance and Rivest, 2012). Studies in blood from patients with TB showed increased levels of interferon-gamma (IFN-γ), interleukin 10 (IL-10), and IL-6, accompanied by a modest increase in cortisol, prolactin (PRL), and thyroid hormone levels and markedly augmented concentrations of growth hormone (Rey et al., 2007). Culture supernatants from peripheral blood mononuclear cells (PBMC) of patients with TB stimulated with *Mycobacterium tuberculosis* antigens inhibit Dehydroepiandrosterone secretion by a human adrenal cell line, indicating that immune cells from these patients can directly affect the synthesis of this hormone. These immunoendocrine interactions may play an adverse role during TB under conditions of the development of protective immune responses, tissue damage control, metabolic disorders, and disease aggravation (Bottasso et al., 2013).

Pro- and anti-apoptotic responses are a result of *Mtb* infection in the host cell. The pro-apoptotic response is beneficial to the host because it impedes the survival and replication of *Mtb* in macrophages, while necrotic cell death is an advantage for intracellular bacteria in inducing escape and allowing it to infect new cells (Lee et al., 2006; O'Sullivan et al., 2007). On the other hand, anti-apoptosis gene expression is one evasive strategy of *Mtb* virulent strains, in contrast with non-pathogenic *Mtb* strains in macrophages; thus, the capacity of *Mtb* to inhibit apoptosis is a virulence factor (Keane et al., 2000).

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PRL is a hormone/cytokine produced in the anterior pituitary, but also can be synthesized in an extra-pituitary manner, where it can exert an influence on diverse cellular processes. PRL has been involved in autoimmunity due to its immunoregulatory and lymphoproliferative effects (Adan et al., 2013; Legorreta-Haquet et al., 2012). The PRL receptor (PRLr) shares structure and signal transduction pathways with the hematopoietic/cytokine receptor superfamily and is expressed in a variety of immune cells, in which this hormone can be pro- or anti-inflammatory by regulating proliferation, survival, and the release of inflammatory mediators.

The expression of PRLr and PRL messenger RNA (mRNA) in T lymphocytes has suggested possible autocrine or paracrine PRL immune effects. Likewise, expression of an autocrine loop of PRL in lymphocytes (Xu et al., 2010) implies that PRL and its receptor must be synthesized and that the ligand is also secreted for the same cell, in addition to that released PRL possesses bioactivity in the synthesizing cell (e.g., proliferative responses). The effects of PRL are dependent on the expressed isoforms of PRLr; long forms (LFs) and intermediate forms (IFs) have been associated with increased cell proliferation or anti-apoptotic effects, while short forms (SFs) and soluble short forms (SSFs) have been described as dominant-negative (Meng et al., 2004).

An acute-phase response mouse model showed differential expression of PRLr in various lymphoid and non-lymphoid organs (Corbacho et al., 2004). In fibroblasts treated with proinflammatory cytokines, expression of the LF of PRLr (100 kDa) has previously correlated with JAK–STAT-5b activation and the suppression of interferon regulatory factor 1 (IRF-1) (Corbacho et al., 2003), but also with inhibition of IRF-1 and inducible nitric oxide synthase (iNOS) expression (Corbacho et al., 2003). In mononuclear phagocytes, reprogramming is a regulatory process useful during inflammatory response, driven by several cytokines (Ma et al., 2003) and some hormones (Flierl et al., 2007). PRL may help to maintain homeostasis during inflammatory responses throughout differential PRLr isoform expression (Adan et al., 2013). Expression of PRLr isoforms has been identified in several tissues throughout the body, suggesting transcriptional and post-translational regulation of PRLr (Trott et al., 2003). Expression of several isoforms also suggests the activation of alternative signal transduction pathways (Martinez-Neri et al., 2015; Qazi et al., 2006). Peritoneal macrophages respond to PRL, secreting nitric oxide (NO) (Tripathi and Sodhi, 2007), IL-1β, tumor necrosis factor-alpha (TNF-α), and IFN-γ through the activation of the JAK2–STAT1 pathway (Tripathi and Sodhi, 2008). Although expression of PRL in PBMC has been noted (Dogusan et al., 2001; Matera et al., 2000), the precise role and mechanism of the action of PRL in mononuclear phagocytes continue to remain elusive. Recently, activation of the human PRL extra-pituitary promoter in lipopolysaccharide (LPS)-activated monocytes was noted as being highly regulated and involved with the resolution phase of inflammation (Semprini et al., 2012).

We hypothesized that the expression of a PRL autocrine loop may play an important role during the inflammatory response in monocytes stimulated with culture filtrate proteins (CFP)-*Mycobacterium bovis*. The aim of this study was to investigate the relationship between PRL synthesis and PRLr isoforms with the inflammatory response and apoptosis in cells stimulated by CFP-*M. bovis* in monocytes.

2. Material and methods

2.1. Reagents and antibodies

Human recombinant PRL (hrPRL) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA): rabbit anti-PRLr (H-300) against residues from 323–622 in the exon 10 sequence of human PRLr, which recognize LF, IF, delta short isoform 1 (ΔS1), and short isoform 1a (S1a); mouse immunoglobulin G (IgG-1) anti-PRL (E-9) against 96–200 residues, corresponding to exons 3, 4, and 5 of human PRL; goat anti-mouse IgG-horseradish peroxidase (HRP), and goat anti-rabbit IgG-HRP. Mouse IgG1 anti-PRLr (MAB1167) against human PRLr extracellular domain (R&D Systems, Minneapolis, MN, USA) was previously employed to neutralize PRLr function (Kanda and Watanabe, 2007). A mouse IgG1 anti-PRL (6F11) that recognizes an epitope restricted to the carboxyl-terminal disulfide loop conserved among different types of PRL from several species was utilized (QED Bioscience, Inc., San Diego, CA, USA). The anti-human actin mAb was obtained from Chemicon (Temecula, CA, USA). Green-fluorescent Alexa Fluor® 488 goat anti-mouse IgG isotype-specific, orange-Red fluorescent Alexa Fluor® 568 goat anti-rabbit IgG isotype-specific, and 4′-6-diamidino-2-phenylindole (DAPI) were obtained from Gibco (Invitrogen Corp., Carlsbad, CA, USA).

2.2. Experimental procedures with different cell lines and isolated monocytes

THP-1 cells (American Type Culture Collection [ATCC]®) were maintained in RPMI 1640 medium containing 10% (v/v) Fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic at 2×10^5 cell/mL as described. THP-1 cells in 6-well (Nunc) or 96-well plates (Corning) cultured for 0.5, 1, 2, 4, and 8 h were stimulated with CFP-*M. bovis* (50 μg/mL). Nb2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% HS, 10% FBS, and 10% antibiotic–antimycotic as described. The MCF-7 breast cancer cell line (ATCC®) was cultured in RPMI 1640 as reported (Schroeder et al., 2002). Monocytes from PBMC were isolated from the heparinized (5 U/mL) blood of 10 healthy male donors (29.8 ± 7.4 years of age) by means of standard density gradient centrifugation at 400 *g* using lymphocyte separation medium (Sigma Chemical) for 15 min at room temperature as described (Nimura et al., 2006). Cells at the interface were collected and washed three times in cold phosphate-buffered solution (PBS) containing 0.1% bovine serum albumin (BSA). PBMC were maintained for 24 h in RPMI 1640 medium containing 10% (v/v) FBS and 1% (v/v) antibiotic–antimycotic at 5×10^6 cells/mL. Non-adherent cells were removed by washing in BSA–PBS; then, remaining adherent cells (>95% CD14 + cells) were cultured and stimulated for 48 h with CFP-*M. bovis* (50 μg/mL). Healthy donors volunteered to participate and signed informed consent prior to their inclusion in the study. The investigation was performed according to the ethical guidelines of the 2008 Declaration of Helsinki and was approved by the Ethical Investigation and Biosafety Committee of the University Center of Health Sciences at the University of Guadalajara, Mexico. To determine the dose of CFP-*M. bovis* to be employed in this study, we performed dose–response assays using CFP-*M. bovis* (5 and 50 μg/mL). After that, we chose the highest dose of CFP-*M. bovis* for priming cells and did not subsequently detect differences in phenotype.

2.3. Nb2 cell bioassay of THP-1-treated supernatants

Supernatants were obtained by incubating non-confluent THP-1 (7×10^5 cells/mL) for 1, 2, 4, and 8 h with CFP-*M. bovis* (50 μg/mL). The supernatants were concentrated 24-fold using Centricon 10 (Millipore, Billerica, MA, USA). Nb2 cells (4×10^4 cells/mL) were cultured for 60 h with serial dilutions of treated or control concentrated supernatants (5, 10, 20, and 45 μL). Nb2 cell proliferation and viability were measured with reduction of MTT as described. Bioactivity was extrapolated from a standard dose–response curve with hrPRL (1, 10, 100, 500, and 1000 pg/mL). Bioactivity was inhibited with 4 μg of α-human PRL (E-9) for each dilution assayed.

2.4. Real-time RT-PCR

Total RNA was extracted from THP-1 (Trizol, Invitrogen) and complementary DNA (cDNA) was synthesized (Superscript III, Invitrogen).