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Evaluation of leptin receptor expression on buffalo leukocytes

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ABSTRACT

Experimental evidences support a direct role for leptin in immunity. Besides controlling food intake and energy expenditure, leptin was reported to be involved in the regulation of the immune system in ruminants. The aim of this work was to highlight the expression of leptin receptor (LEPR) on *Bubalus bubalis* immune cells using a multi-approach assessment: flow cytometry, confocal microscopy and gene expression analysis. Flow cytometric analysis of LEPR expression showed that peripheral blood monocytes were the predominant cells expressing LEPR. This result was corroborated by confocal microscopy and RT-PCR analysis. Moreover, among lymphocytes, LEPR was mainly expressed by B lymphocytes and Natural Killer cells. Evidence of LEPR expression on buffalo blood leukocytes showed to be a good indicator of the responsivity of these cells to leptin, so confirming the involvement of leptin in buffalo immune response.

1. Introduction

Leptin is one of the most important hormones secreted by adipocytes and performs a variety of biological actions on feeding, metabolism and the neuroendocrine axis. Leptin mediates its effects by binding with the leptin receptor (LEPR), a transmembrane protein member of class I cytokine receptor family (Tartaglia et al., 1995).

Peelman et al. (2014) provided an extensive overview on the structure of leptin receptor. The protein can be divided into three parts: extracellular, trasmembrane and cytoplasmic. The extracellular part is composed of a N-terminal domain (NTD), two cytokine receptor homology domains (CRH1 and CRH2), an immunoglobulin-like domain (IGD), and two membrane-proximal fibronectin type III (FN III) domains. In human at least six different LEPR isoforms are found, all generated from a single gene by alternative splicing. All isoforms, namely LEPRa to LEPRf, share identical extracellular and transmembrane domains, but are characterized by intracellular domains of variable length. The longest isoform (LEPRb) contains domains implicated in signal transduction pathways involving Janus kinase (JAK) and Signal Transducers and Activators of Transcription (STAT) (Tartaglia, 1997). The soluble isoform (LEPRe) can regulate serum leptin concentration, and serves as a carrier protein delivering the hormone to its membrane receptors activating signal transduction into the cell (Gorska et al., 2010).

Bovine *LEPR* gene, located on chromosome 3q33 (Pfister-Genskow et al., 1997), is composed of eighteen exons and was fully characterized by sequencing the whole coding region and part of the 5′flanking region (De Matteis et al., 2012). Since the buffalo genome is not yet fully assembled or annotated and considering the high sequence homology between cattle and buffalo (almost 97% for the coding and regulatory regions of genes), previous studies (De Matteis et al., 2015) investigated *LEPR* gene in buffalo using the database of the bovine genome. Results highlighted that cDNA sequence of *LEPR* gene is highly conserved in mammals where similarity of buffalo with other mammals was: 99% with bovine, 96% with ovine, 91% with porcine, 87% with human and 81% with mouse.

In human and mouse, leptin receptor isoforms are expressed in a variety of peripheral tissues including brain, liver, kidney, pancreas, placenta, lung, skeletal muscle, heart, hematopoietic cells, and peripheral blood mononuclear cells (PBMC) (Tartaglia et al., 1995; Tsiotra et al., 2000; Margetic et al., 2002) but their levels of expression may differ between them (Fei et al., 1997; Kielar et al., 1998). Experimental evidences support a direct role for leptin in the regulation of immune response (Matarese et al., 2005)

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and in support of this, cells connected with immune and inflammatory response express LEPR (Sanchez-Margalet et al., 2003; Papathanassoglou et al., 2006; Gorska and Wasik, 2012). Leptin-deficient mice (*ob/ob*) and leptin receptor-deficient mice (*db/db*) display an array of immune abnormalities (Fantuzzi and Faggioni, 2000). It has been confirmed that human circulating leukocytes express leptin receptor and the predominant expressing cell type are monocytes (Zarkesh-Esfahani et al., 2001; Papathanassoglou et al., 2006).

In ruminants, Bartha et al. (2005) hypothesized that besides controlling food intake and energy expenditure, leptin may be involved in the regulation of the immune system. Some studies were carried out in bovine to clarify the role of leptin as a regulator of immune function (Ahmed et al., 2007; Ahmed et al., 2008; Lacetera et al., 2009). The stimulatory effects of leptin on mitogenic response of bovine PBMC was demonstrated and it was reported that leptin activates monocytes/macrophages to produce some cytokines that stimulate proliferation of T lymphocytes (Ahmed et al., 2007). Other authors reported that macrophages phagocytosis was affected by leptin (Florez-Diaz and Kegley, 2005). Finally, Ahmed et al. (2008) demonstrated the suppressive effect of leptin on the mitogenic response of bovine purified T lymphocytes. Altogether, these evidences indicated that leptin might be a target molecule to modulate the immune function in ruminants.

Moreover, several polymorphisms in bovine *leptin* and *leptin receptor* genes play a role in the variability of hematological parameters during peripartum (Napolitano et al., 2010; Orrù et al., 2012). To date, in *Bubalus bubalis* the expression of *LEPR* mRNA was demonstrated only in the alveolar epithelial cells of the mammary gland (Sayed-Ahmed et al., 2003). Mammary epithelial cells are involved in the initial combat against microorganisms through the expression of cytokines and acute phase proteins, which regulate the immune response (Wellnitz and Kerr, 2004). However, to our knowledge, no evidence has yet been reported in water buffalo on the distribution of the leptin receptor molecules on the leukocytes cell surface.

The aim of the present study was therefore to assess the expression of LEPR in *Bubalus bubalis* peripheral blood leukocytes with a multi-approach assessment: flow cytometry, confocal microscopy and RT-PCR.

2. Materials and methods

2.1. Animals and blood samples collection

The trial was performed on ten lactating water buffaloes. All animals were maintained under the same management and feeding conditions at the CREA-PCM Research Centre. The CREA-PCM is authorized to use farm animals for experimental design (as stated in DM 26/96-4 of Italian Welfare Ministry). Peripheral blood samples were taken by from the external jugular vein into evacuated tubes containing EDTA as anticoagulant. The management and care of the animals were in compliance with the 86/609EEC European Union directive.

2.2. Flow cytometry analysis and detection of LEPR on peripheral blood leukocytes

Since no buffalo specific antibody to detect LEPR is commercially available, we tested two commercial anti-human leptin receptor antibodies: a PE (R-Phycoeritrin)-conjugated monoclonal mouse IgG2b (Clone 52263-R&D Systems Inc.) and a PE-conjugated rabbit polyclonal antibody (bs-0961R-Bioss Inc.). The monoclonal antibody does not cross-react with either buffalo or bovine cells (data not shown). Hereby, we used the rabbit polyclonal antibody testing a series of different antibody concentrations searching for the saturating concentration (20 μg/mL for 5×10^5 cells dissolved in 50 μL).

Flow cytometry analysis of leptin receptor protein on the surface of leukocytes was performed after saturation of Fc receptors. To prevent unspecific binding, 500 μL of EDTA whole blood was lysed with 1:10 *v/v* of 1× Ammonium Chloride lysing solution (10× stock solution: NH_4Cl 80 g, $KHCO_3$ 10 g, Na_4EDTA 3.7 g, final volume 1 L), washed twice with Phosphate Buffer Solution (PBS) supplemented with 1% Bovine Serum Albumin (BSA) (Sigma Aldrich) and then 5×10^5 cells were saturated with 20 μg/mL of polyclonal rabbit IgG (Bioss Inc.) in PBS with BSA 1% (Sigma Aldrich) for 30 min at room temperature. Live/Dead Fixable Dead Cell Stain kit (Invitrogen, Molecular Probes) was performed to test the viability of cells. The viability was always ≥97%.

Two panels of antibodies (Panel A and Panel B) were designed and optimized for the identification of the subsets of buffalo leukocytes that express LEPR. The Panel A of antibodies was designed for the identification of monocytes, B lymphocyte. Panel A included the PE-conjugated anti-LEPR rabbit polyclonal antibody as well as the following monoclonal antibodies (mAbs): the mouse anti-human CD14 (clone TÜK4) conjugated with Allophycocynin (APC) (Miltenyi, Biotech, Germany) and the anti-bovine CD21 (Clone CC21) conjugated with Fluorescein Isothiocyanate (FITC) (AbD Serotec). The mouse anti-human CD14 antibody, clone TÜK4, recognizes the human CD14 cell surface antigen and is strongly expressed on the surface of monocytes and macrophages (Simmons et al., 1989; Jungi et al., 1997; Sopp and Howard, 1997); the mouse anti-bovine CD21 monoclonal antibody, clone CC21, recognizes the bovine CD21 cell surface antigen, a ~145 kDa single pass type I membrane glycoprotein and in cattle its expression is restricted to B lymphocytes (Naessens et al., 1990; Tenaya et al., 2012). The Panel B of antibodies was designed for the identification of T lymphocytes and Natural Killer cells. Panel B included the PE-conjugated anti-LEPR rabbit polyclonal antibody and the following primary not conjugated mAbs: mouse anti-bovine CD4 (clone ILA11A, IgG2a, Washington State University, Pullman, WA) validated on buffalo by Davis et al. (2001), mouse anti-bovine CD8 (clone CC63, IgG2a, AbD-Serotec), used for the identification of T lymphocytes (Gutierrez et al., 1999) and mouse anti-bovine-CD335 (clone AKS1, IgG1, AbD Serotec) for the identification of Natural Killer cells (Storset et al., 2004). All mAbs have been already used in cattle. On the other hand, mAbs have already been tested in buffalo in our laboratory by flow cytometric analysis (data not shown). These antibodies showed the same flow cytometric pattern of reactivity observed on bovine leukocytes and for this reason, they were considered for flow cytometry in buffalo.

The staining of cells from ten buffaloes was performed in 96-well V bottom plates with 5×10^5 cells/well/50 μL of PBS and 1% BSA. For Panel A three-color staining, cells were incubated for 15 min on ice and in the dark with 20 μg/mL of anti-LEPR, 20 μg/mL of anti-CD14 and 20 μg/mL of anti-CD21. For Panel B three-color staining, cells were incubated with the primary antibodies anti-CD4, anti-CD8, and anti-CD335. These mAbs were titrated and then used in saturating conditions at 7.5 μg/mL in 50 μL of PBS and 1% of BSA. After 15 min of incubation on ice, cells were washed 3 times with PBS/BSA 1%. Then, the anti-LEPR PE-conjugated antibody was added to cells and incubated with the subtype specific secondary antibodies for 15 min on ice and in the dark. A subset of specific secondary antibodies conjugated with Alexa 647 (anti-mouse IgG1 mAb, Abcam) and Pe-Cy7 (anti-mouse IgG2a, Thermo Fisher Scientific) was used. To evaluate the background fluorescence, a control staining with isotype control was performed.

Flow cytometry analysis was performed on Cytomics FC500 Flow Cytometry Analyzer (Beckman Coulter Inc.) and each treatment was performed in duplicate. For the analysis of triple-stained