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Calcitriol increases nitric oxide production and modulates microbicidal capacity against *Mycobacterium bovis* in bovine macrophages

Ángel García-Barragán, José A. Gutiérrez-Pabello, Edgar Alfonseca-Silva*

Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, 04510, Ciudad de México, Mexico

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ABSTRACT

Bovine tuberculosis, a re-emerging infectious disease caused by *Mycobacterium bovis*, can be transmitted to humans. Global prevalence of *M. bovis* in humans is underestimated and represents a serious public health risk in developing countries. In light of this situation, it is important to note that our understanding of the immunopathogenesis of human tuberculosis can be improved by studying this disease in the bovine model. Stimulation of the bovine innate immune system with calcitriol (1,25(OH)2D3) leads to an increase in bactericidal molecules involved in macrophage antimicrobial activity. It is unknown, however, if calcitriol´s effect on bovine macrophages impacts intracellular bacterial replication. With these considerations in mind, this study sought to investigate the specific role of calcitriol in tuberculosis control in bovine macrophages, in the hopes of uncovering information applicable to human tuberculosis. As such, infection with *M. bovis* was shown to induce expression of *CYP27B1* and *VDR* genes in macrophages. Moreover, addition of 1,25(OH)2D3 to cultures of macrophages previously infected with mycobacteria and/or activated by LPS triggered cellular expression of nitric oxide synthase (*NOS2*) and increased nitrite concentrations, both indicators of nitric oxide (NO) production. By means of a microbicidal assay, addition of 1,25(OH)2D3 was seen to increase macrophage phagocytosis and to decrease mycobacterial intracellular replication. Thus, taken together, our results show that calcitriol can help stimulate the innate immune system of bovines by increasing phagocytosis and decreasing intracellular replication of microorganisms, such as *M. bovis*, in macrophages, through the VDR pathway.

1. Introduction

Tuberculosis is undoubtedly one of the most studied infectious diseases in the world. *Mycobacterium bovis*, which belongs to the *M. tuberculosis* complex (MTBC), primarily affects cattle but is also responsible for many cases of tuberculosis in humans. As such, this pathogen represents a significant public health risk in developing countries. However, coordination between public and veterinary health authorities is inadequate, thus limiting our ability to exchange relevant information and develop strategic and effective control measures for human and bovine tuberculosis [1]. The bovine model of tuberculosis may explain some unknown aspects of immune responses in human tuberculosis, offering new opportunities to improve our understanding of the immunopathogenesis of this important disease [2].

Innate immunity is an evolutionarily conserved system that represents an animal´s first line of defense against disease. It is crucial for the rapid activation of antimicrobial response mechanisms against pathogens, which in turn are detected by pattern recognition receptors (PRRs) such as Toll Like Receptors (TLRs) [3,4]. Some significant functions of innate immunity are regulated by calcitriol ($1,25(OH)_2D_3$), the active form of vitamin D. This fat-soluble hormone derived from cholesterol is also responsible for the regulation of calcium homeostasis during the peripartum period and lactation, an important function which is often studied in cattle [5,6].

Vitamin D precursors are mainly obtained from limited food sources (vitamin D_2) or by photochemical conversion of 7-dehydrocholesterol in skin exposed to solar ultraviolet B irradiation (290–315 nm; vitamin D_3) [7]. As neither of these metabolites possess biological activity, they must undergo two modifications in order to become biologically active [8]. First, both forms of vitamin D are hydroxylated in the liver at position 25 by cytochrome P-450 enzymes (CYP2R1, CYP27A1 and CYP2D25) in order to produce calcidiol ($25(OH)D_3$), the form of

Abbreviations: $1,25OH_2D_3$, calcitriol; MOI, multiplicity of infection; LPS, lipopolysaccharide; NO, nitric oxide; *NOS2*, nitric oxide synthase 2 gene

* Corresponding author at: Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Av. Universidad 3000, Col. UNAM, Ciudad de México, 04510, Mexico.

E-mail address: eas@unam.mx (E. Alfonseca-Silva).

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vitamin D most commonly found in circulation; calcidiol is then converted to calcitriol by the mitochondrial enzyme CYP27B1, also known as 1-α hydroxylase [9,10]. Most of the body's calcitriol is synthesized in the kidney's proximal tubules; however, calcitriol synthesis can also occur in several extra-renal sites in cells that express *CYP27B1* [9].

Activation of human monocytes and macrophages through TLR2/1 leads to $1,25(OH)_2D_3$ synthesis by the CYP27B1 enzyme at the infection site [11,12]. This suggests that calcitriol is critical to the control of intracellular pathogens [13] through the activation of multiple genes, such as those that code for antimicrobial beta-defensin peptides and cathelicidin, which contain specific sites in their promoters known as Vitamin D Response Elements (VDREs) [14]. Antimicrobial activity therefore depends partially on the vitamin D signaling pathway, making this a significant factor in the successful destruction of intracellular pathogens such as *M. tuberculosis* in human monocytes and macrophages [11,13,15–17]. Additionally, several studies evaluating the effect of calcitriol on innate immune response in cattle indicate that this vitamin is essential for the control of *M. bovis,* the causal agent of bovine tuberculosis [18].

Besides beta-defensin production, bovine monocytes also require calcitriol to produce nitric oxide (NO) [19–21]. In macrophages, NO has been associated with increased restriction of intracellular growth, as well as elimination per se, of *M. bovis* [22,23]. Nevertheless, the role of calcitriol in the control of *M. bovis* by bovine macrophages is not fully understood. Therefore, the aim of this study was to evaluate the functional role of calcitriol in the microbicidal ability of macrophages to restrict intracellular growth of *M. bovis*.

2. Material and methods

2.1. Animals

Animal procedures were carried out according to the regulations on the use of research animals established by the School of Veterinary Medicine and Animal Science (FMVZ) of the National Autonomous University of Mexico (UNAM). Animals from a Holstein Friesian tuberculosis-free (non-exposed unvaccinated and/or challenged) herd belonging to an Animal Health and Production Research and Teaching Center (FMVZ-UNAM) were selected as peripheral blood donors. All animals selected were over 5 years of age and maintained under the following conditions: housed in an open-air pen; fed a diet based on corn silage, hay oats, mineral salts and supplemental balanced feed; and supplied with unlimited access to drinking water. The mean serum calcidiol concentration of these test animals was 64.9 ng/mL.

Calcidiol measurement in bovine serum and cell culture medium was performed by chemiluminescence using LIAISON® 25 OH Vitamin D TOTAL Assay (DiaSorin, Saluggia, Italy).

2.2. Reagents

Lipopolysaccharide O26:B6 (LPS) from *Escherichia coli* (Sigma Chemical Company, St. Louis, MO, USA) was prepared at a concentration of 1 mg/mL in 1× PBS and stored at −20 °C. Vitamin D ($1,25(OH)_2D_3$) (Sigma Chemical Company, St. Louis, MO, USA) was diluted in pure ethanol at a concentration of 10 μg/mL and stored in dark conditions at −80 °C. The final concentration was confirmed by UV spectroscopy at 264 nm using an extinction coefficient of 18,200 M^{-1} per cm.

2.3. Bacteria

The international reference strain *M. bovis* AN5 was cultured and collected in logarithmic phase with constant stirring at 37 °C in Middlebrook 7H9 liquid medium enriched with 10% OADC (Becton, Dickinson, Sparks, MD, USA) and 0.5 g/L of Tween 80 (Sigma Chemical Company, St. Louis, MO, USA). Bacteria were washed twice with 1× PBS and resuspended in fresh CRPMI (Sigma Chemical Company, St. Louis, MO, USA) medium supplemented with 15% fetal bovine serum (Gibco® by Life Technologies, Grand Island, NY, USA). Bacteria were then harvested, aliquoted and kept at −80 °C until use. Mycobacteria concentration was determined by counting Colony Forming Units (CFU) on Middlebrook 7H10 agar plates (Becton, Dickinson, Sparks MD, USA).

2.4. Purification of bovine macrophages

Macrophages were obtained from peripheral blood mononuclear cells (PBMCs) as previously described [24], but with some modifications. Blood was collected by jugular venipuncture, using 60 mL syringes with citric acid dextrose in aseptic conditions. PBMCs were isolated using a Histopaque gradient with a specific density of 1.077 g/mL (Sigma Chemical Company, St. Louis, MO, USA). Cells were washed and resuspended in fresh CRPMI 1640 medium with 2 mM of L-glutamine, 0.1 mM of non-essential amino acids, 1 mM of sodium pyruvate and 20 mM of sodium bicarbonate (Sigma Chemical Company, St. Louis, MO, USA), and were then incubated in 6-well, ultra-low adhesion plates (Costar®, Corning Incorporate, NY, USA) for 2 h at 37 °C with 5% CO_2. Non-adherent cells were removed, whereas adherent cells were cultured in CRPMI enriched with 12% autologous serum (CRPMI has a mean calcidiol concentration of 16 ng/mL.) for 12 days until occurrence of macrophage differentiation. Cells were harvested with a cold solution of 1x PBS with 2 mM of EDTA and plated in 24-well, cell-culture plates (Costar®, Corning Incorporate, NY, USA). To determine gene expression of *CYP24 A1* and *VDR*, 5×10^5 macrophages were plated, stimulated with 4, 20 and 40 ng/mL of $1,25(OH)_2D_3$, and incubated at 37 °C for 24 h with 5% CO_2.

2.5. RNA extraction and synthesis of cDNA

A total of 5×10^5 macrophages were infected with *M. bovis* AN5 at a multiplicity of infection of 2 bacteria per macrophage (MOI) 2:1. They were treated with or without 4 ng/mL of $1,25(OH)_2D_3$, and with or without 100 ng/mL of *E. coli* O26: B6 LPS for 24 h at 37 °C with 5% CO_2. Culture supernatants were collected and stored at −80 °C until use. Total RNA from macrophages was isolated using TRIzol Reagent and purified with DNase I RNase-free, according to the manufacturer's recommendations (Thermo Fisher Scientific, Carlsbad, CA, USA). RNA integrity and quality were evaluated using 1% agarose gel electrophoresis stained with SYBR® Safe gel (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), and by spectrophotometry (NanoDrop ND-1000, Wilmington, DE, USA) in nuclease-free water within a reading range of 260–280 nm.

Complementary DNA (cDNA) was synthesized using the RevertAid H Minus First Strand cDNA Synthesis kit, following the manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA, USA). The cDNA was diluted 1 in 10 in nuclease-free water and stored at −80 °C until use.

2.6. Real time PCR

Specific primers were used to detect previously published bovine *CYP24A1*, *NOS2*, *VDR*, and *CYP27B1* genes [21]. *RPL32* was used as an internal control. Primers were designed with QuestSM IDT's Primer software, using the following sequence: 5′-AAG GGC CAG ATC TTG ATG CC-3′ forward and 5′ -CTT GAC GTT GTG GAC CAG GA-3′ reverse. Real Time PCR was performed using a Light Cycler 480 thermocycler and an SYBR Green I Master Kit (Roche Diagnostics GmbH, Mannheim, Germany) as a detection system, adhering to the manufacturer's instructions. The specificity of each primer was verified using the melting curve analysis and agarose gel for the detection of non-specific PCR products. The threshold cycle (Ct) of each sample was employed to determine gene expression in relative expression units (UREs), using the $2^{\wedge\Delta\Delta Ct}$ comparative method.