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Vitamin D signaling in the bovine immune system

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Vitamin D signaling in the bovine immune system

by

Corwin D. Nelson

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Biochemistry and Immunobiology

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Iowa State University

Ames, Iowa

2010
DEDICATION

I would like to dedicate this dissertation to my wife, Gwen, and my children, Kaelyn, Nolan, and Madilyn. I am very thankful for their love, encouragement and support through my undergraduate and graduate education. I would not have completed this without them.
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LIST OF ABBREVIATIONS

1,25(OH)$_2$D$_3$ = 1,25-Dihydroxyvitamin D$_3$
1α-OHase = 25-Hydroxyvitamin D 1α-hydroxylase; CYP27B1
24-OHase = Vitamin D 24-hydroxylase; CYP24A1
25(OH)D$_3$ = 25-Hydroxyvitamin D$_3$
FBS = Fetal bovine serum
IL = Interleukin
IFN-γ = Interferon gamma
iNOS = inducible nitric oxide synthase
LPS = Lipopolysaccharide
*M. bovis* PPD = Purified protein derivative of *Mycobacteria bovis*
PAM$_3$CSK$_4$ = synthetic tripalmitoylated-lipopeptide analogous to triacyl-lipopeptide from *Mycobacteria*
PBMC = Peripheral blood mononuclear cell
PBS = Phosphate buffered saline
PGN = Peptidoglycan
RANTES = regulated upon activation, normally T-cell expressed and secreted; CCL5
RPS9 = Ribosomal protein S9
S100 A12 = S100 calcium binding protein A12; Calgranulin C
TLR = Toll-like receptor
VDR = Vitamin D receptor
VDRE = Vitamin D response element
**ABSTRACT**

Vitamin D has predominantly been associated with calcium homeostasis, but recently been associated with several infectious diseases. The active vitamin D metabolite, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), is known to regulate the expression of numerous genes throughout the body. 1,25(OH)$_2$D$_3$ is synthesized from 25-hydroxyvitamin D$_3$ by the enzyme 1α-hydroxylase (1α-OHase; CYP27B1). In the endocrine system, 1α-OHase expression, and subsequently 1,25(OH)$_2$D$_3$ synthesis, is tightly regulated in the kidney in response to calcium homeostasis. 1,25(OH)$_2$D$_3$ produced in the kidney acts systemically to regulate the expression of genes related to calcium homeostasis. In contrast, we have found that bovine monocytes (precursors to macrophages) express 1α-OHase upon activation by toll-like receptor recognition of pathogen associated molecular patterns. Furthermore, we found that 1α-OHase is highly expressed in CD14$^+$ cells (monocytes and macrophages) from peripheral blood mononuclear cell cultures stimulated with a purified protein derivative of *Mycobacteria bovis* and from the infected mammary glands of cows with mastitis. Production of 1,25(OH)$_2$D$_3$ by 1α-OHase in bovine monocytes up-regulated the expression of the genes for iNOS and the chemokine RANTES in monocytes. Up-regulation of iNOS resulted in increased production of nitric oxide, a molecule that is considered to have several important roles in immune function. In stimulated peripheral blood mononuclear cell cultures, IFN-γ, IL-17A, and IL-17F, pro-inflammatory cytokines associated with T-cells, were down-regulated by 1,25(OH)$_2$D$_3$ that was presumably produced by monocytes. Finally, regulation of gene expression by 1,25(OH)$_2$D$_3$ produced by 1α-OHase in monocytes depended on the concentration of 25(OH)D$_3$. The circulating concentration of 25(OH)D$_3$ is
controlled by dietary intake of vitamin D$_3$ and sunlight exposure, so dietary intake of vitamin D$_3$ and sunlight exposure may impact immune function in cattle. Current requirements for vitamin D in cattle are based on the regulation of calcium homeostasis, but those requirements may be inadequate for proper immune function. Therefore, further investigation is needed to determine vitamin D requirements for proper immune function in cattle.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Vitamin D historically has been associated with calcium homeostasis (Horst et al., 2003). In recent years, though, vitamin D has gained popularity for the role it has in the immune system (Adams et al., 2007b; DeLuca, 2008). A noteworthy discovery was made by several groups in 2004 and 2005 that 1,25(OH)_{2}D_{3} up-regulated the expression of cathelicidin in human macrophages and enhance the antimicrobial activity of macrophages (Gombart et al., 2005; Wang et al., 2004). Shortly after, Liu et. Al. showed that human macrophages expressed 1α-hydroxylase (1α-OHase) upon activation by toll-like receptor signaling (Liu et al., 2006). Enzymatic activity of 1α-OHase converts 25-hydroxyvitamin D_{3} to 1,25(OH)_{2}D_{3}. In activated human macrophages, production of 1,25(OH)_{2}D_{3} by 1α-OHase enhanced cathelicidin expression and antimicrobial activity (Liu et al., 2006). These discoveries led to the concept that vitamin D signaling in the immune system functions through intracrine and paracrine mechanisms (Hewison, 2010).

Studies with humans and mice suggest that over 1,000 genes may be regulated by 1,25(OH)_{2}D_{3} and that 1,25(OH)_{2}D_{3} regulates numerous immune responses (Wang et al., 2005). Yet, in bovine immune cells, 1,25(OH)_{2}D_{3} is only known to repress interferon-γ production and enhance nitric oxide production. So, one objective of this dissertation project was to identify genes with immunological function that are regulated by 1,25(OH)_{2}D_{3} in cattle. Meanwhile, the kidney has been the only known source for 1,25(OH)_{2}D_{3} in cattle, but evidence from humans and mice suggests that immune cells also synthesize 1,25(OH)_{2}D_{3} (Stoffels et al., 2007; Stoffels et al., 2006). Therefore, another objective of this dissertation
project was to find if extra-renal production of $1,25(OH)_2D_3$ occurs to regulate immune responses through intracrine and paracrine mechanisms.

The information gained from this project could lead to a better understanding of factors that affect host defense mechanisms in cattle. Bacterial and viral diseases of cattle result in major economic losses and threaten food safety and supply. As a result, identifying the mechanisms and effects of vitamin D signaling in the bovine immune system may have substantial implications for livestock production.

**Dissertation Organization**

This dissertation contains five chapters and two appendices. A review of the literature on vitamin D and immunology is given in chapter 1. Chapter 2 has been published in the Journal of Dairy Science (Nelson et al., 2010). That paper consists of several in vitro experiments that show production of $1,25(OH)_2D_3$ by $1\alpha$-OHase in bovine monocytes regulates expression of the genes for iNOS and RANTES in monocytes. Chapter 3 is a manuscript describing research that gives evidence that $1\alpha$-OHase is expressed only by CD14 positive cells (monocytes/macrophages) in peripheral blood mononuclear cell cultures stimulated with a purified protein derivative of *Mycobacteria bovis*. That manuscript also gives evidence that production of $1,25(OH)_2D_3$ by monocytes can regulate gene expression in surrounding cells. Chapter 4 is a manuscript describing research that provides in vivo evidence that $1\alpha$-OHase is expressed by macrophages in the mammary gland during mastitis. Chapter 5 is a general conclusion and contains ideas for future research. References for each chapter can be found after the chapter in which they appear. Appendix A contains unpublished results on the effects of $25(OH)D_3$ and $1,25(OH)_2D_3$ on monocyte anti-bacterial
activity. Appendix B contains preliminary results on 1α-OHase gene expression in bovine mammary epithelial cells and the effects of 1,25(OH)₂D₃ on gene expression in bovine mammary epithelial cells.

**Literature Review**

**History of vitamin D research**

In the 19th century, sunlight exposure was prescribed as a cure for rickets and tuberculosis (McCarthy, 2001; Rajakumar, 2003). Nearly a century ago, vitamin D was discovered as the factor in butterfat and cod liver oil that prevented rickets (McCollum et al., 2002). A few years later, vitamin D was found to be synthesized in skin exposed to sunlight; which explained why sunlight exposure could cure rickets. However, why sunlight exposure cured tuberculosis was not clear until recently (Liu et al., 2006). Here is an overview of the events that led to the discovery of vitamin D and the physiological roles the vitamin has in both diseases.

The study of rickets ultimately led to the discovery of vitamin D. In 1914, E.V. McCollum and E.B. Hart found that a fat-soluble factor in butterfat was necessary for normal growth in rats (Rajakumar, 2003). Soon after, Edward Mellanby discovered that a fat-soluble factor in butterfat or cod liver oil could prevent rickets (Carpenter and Zhao, 1999). In 1922, McCollum described a “fourth vitamin” that, as far as he could tell, “regulated the metabolism of bones” (McCollum et al., 2002). The process of irradiating food with UV light was patented by Harry Steenbock in 1924 because he found that irradiated food could prevent rickets (Steenbock, 1924). In 1925, A. F. Hess suggested that UV light activated...
cholesterol in the skin to make it anti-rachitic (Wolf, 2004). In the early 1930s, Windaus and
Rosenheim discovered that UV irradiation of ergosterol from plants produced vitamin D\textsubscript{2}
(Wolf, 2004). Windaus finally discovered that cholecalciferol (vitamin D\textsubscript{3}) was derived from
7-dehydrocholesterol in the skin by UV irradiation in 1937 (Wolf, 2004). Discovering that
vitamin D\textsubscript{3} was synthesized in skin exposed to UV light explained earlier observations that
exposure to UV light could prevent rickets.

After discovery of vitamin D, research efforts focused on how it regulated calcium
homeostasis. In the late 1960s, Hector Deluca found that vitamin D\textsubscript{3} was converted rapidly
to 25-hydroxyvitamin D\textsubscript{3} (DeLuca, 1969). Soon after, several groups showed that 25(OH)D\textsubscript{3}
was converted to 1\(\alpha\),25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) and that 1,25(OH)\textsubscript{2}D\textsubscript{3} was the
active metabolite (DeLuca, 1976; Norman and Wong, 1972). Around that time, a receptor for
1,25(OH)\textsubscript{2}D\textsubscript{3}, now known as the vitamin D receptor (VDR), was shown to associate with
chromatin (Tsai et al., 1972). Identification of a receptor for 1,25(OH)\textsubscript{2}D\textsubscript{3} that acted as a
transcription factor provided an explanation for how vitamin D regulated calcium
homeostasis. Eventually, 1,25(OH)\textsubscript{2}D\textsubscript{3} was found to be produced in the kidney by the
enzyme 25-hydroxyvitamin D 1\(\alpha\)-hydroxylase (1\(\alpha\)-Ohase) (DeLuca, 1976). Altogether,
these discoveries led to the concept of the vitamin D endocrine system, where 1,25(OH)\textsubscript{2}D\textsubscript{3}
production was regulated in the kidney to control the circulating concentration of
1,25(OH)\textsubscript{2}D\textsubscript{3} and 1,25(OH)\textsubscript{2}D\textsubscript{3} acts as a hormone to regulate gene expression systemically
(DeLuca, 1980; Horst and Reinhardt, 1983).

Evidence slowly emerged that vitamin D contributed to immune function in the early
1980s. The VDR was shown to be expressed in immune cells (Provvedini et al., 1983).
Several studies showed that 1,25(OH)\textsubscript{2}D\textsubscript{3} affected immune cell proliferation and
differentiation (Koeffler et al., 1984; Lemire et al., 1984; Mangelsdorf et al., 1984). In 1983, pulmonary macrophages from sarcoidosis patients were shown to produce 1,25(OH)$_2$D$_3$ (Adams et al., 1983). Activation of macrophages with interferon gamma (IFN-γ) then was shown to induce production of 1,25(OH)$_2$D$_3$ (Koeffler et al., 1985). In the 1990s, it was shown that 1,25(OH)$_2$D$_3$ could prevent disease onset in mouse models of autoimmune disorders (Cantorna et al., 1996, 1998a). Also during that time, vitamin D deficiency was linked to the incidence of multiple sclerosis (Hayes et al., 1997).

In terms of host defense against infectious disease, two separate research groups showed that 1,25(OH)$_2$D$_3$ induced cathelicidin expression in human macrophages and epithelial cells (Gombart et al., 2005; Wang et al., 2004). This discovery became even more prominent a few years later when Liu and co-workers showed that toll-like receptor (TLR) signaling induced 1,25(OH)$_2$D$_3$ production by 1α-OHase in macrophages (Liu et al., 2006). Production of 1,25(OH)$_2$D$_3$ up-regulated the expression of cathelicidin and subsequently killing of intracellular *Mycobacteria tuberculosis* (Liu et al., 2007b). Even though production of 1,25(OH)$_2$D$_3$ in macrophages had been shown two decades earlier, this discovery was the first time a solid link between vitamin D and host defense was made. That discovery provided some explanation for the 19$^{th}$ century observation that sunlight exposure could cure tuberculosis.

In cattle, most of the information on vitamin D in cattle relates to its role in calcium homeostasis. A major metabolic disease in dairy cattle is milk fever, or severe hypocalcemia; so, a great deal of effort from the 1970s until recently was spent understanding the role of vitamin D in this disease (Horst, 1986; Horst et al., 1997; Horst and Reinhardt, 1983). Those efforts have provided information on the vitamin D endocrine
system and vitamin D requirements for proper calcium homeostasis. Several studies have shown \(1,25(\text{OH})_2\text{D}_3\) regulates immune function in cattle (Ametaj et al., 1996; Nonnecke et al., 2003; Reinhardt et al., 1999; Waters et al., 2001), but knowledge about vitamin D signaling in the bovine immune system lags behind what is known about vitamin D signaling in the human immune system.

**Vitamin D metabolism**

Vitamin D\(_2\) and vitamin D\(_3\) metabolites are found in plasma of cattle (Sommerfeldt et al., 1983). Vitamin D\(_2\) is derived from ergosterol in plants and vitamin D\(_3\) is derived from 7-dehydrocholesterol in animals (Figure 1). In cattle, vitamin D\(_2\) and vitamin D\(_3\) metabolism occurs through the same pathway, with exceptions in digestion in the rumen and side chain catabolism (Horst et al., 1990; Horst and Littledike, 1979; Zimmerman et al., 2001). Even though both forms contribute to the overall signaling events of vitamin D, vitamin D\(_3\) is the predominant form in cattle because it is synthesized in the skin and supplemented in the diet (Horst et al., 1981). Therefore, the focus of this review will be on vitamin D\(_3\). The metabolic pathway of vitamin D\(_3\) is shown in Figure 1.

Vitamin D\(_3\) is synthesized in the skin from 7-dehydrocholesterol by irradiation with UVB light (Holick, 2008). Vitamin D\(_3\) also can be acquired in the diet (McDermott et al., 1985). The NRC recommendation of vitamin D\(_3\) intake for lactating dairy cows is about 20,000 international units (IU; 25 ng = 1 IU) per day depending on animal size and milk production (NRC, 2001). The recommendation for calves is 600 IU/day (NRC, 2001). Many dairy operations, however, have supplemented the diets of lactating cattle with about 40,000 IU/day and most calf milk replacers provide about 10,000 IU/day (Weiss, 1998).
Vitamin D₃ is converted to 25(OH)D₃ in the liver by several cytochrome P450 enzymes (Dusso et al., 2005). CYP27A1 is recognized most often as the enzyme that converts vitamin D₃ to 25(OH)D₃, but CYP2C11, CYP2R1, and CYP3A4 also have been shown to catalyze the conversion (Dusso et al., 2005). Conversion of vitamin D₃ to 25(OH)D₃ is not tightly regulated; so, most vitamin D₃ that is acquired in the diet or synthesized in the skin is quickly converted to 25(OH)D₃ (Horst et al., 1994). 25(OH)D₃ is the most abundant vitamin D metabolite in plasma of cattle (Horst et al., 1981).

The concentration of 25(OH)D₃ in plasma is relatively stable over time (Sommerfeldt et al., 1983). It does not change in response to calcium homeostasis or health status, but depends on dietary intake and sunlight exposure (Horst et al., 1997; Waldron et al., 2003). As a result, the concentration of 25(OH)D₃ is used as a measure of vitamin D status (Hollis et al., 2007). In cattle, the concentration of 25(OH)D₃ in plasma typically ranges from 20 to 50 ng/ml (Horst et al., 1994). Heavy supplementation of vitamin D₃ (> 50,000 IU/day for lactating cow or 10,000 IU/day for calf) can result in plasma 25(OH)D₃ concentrations ranging from 50 to 100 ng/ml (McDermott et al., 1985; Nonnecke et al., 2009). Excess supplementation of vitamin D₃ in the diet can cause calcification of soft tissues to occur when the plasma 25(OH)D₃ concentration exceeds 200 ng/ml (Littledeike and Horst, 1982). In the absence of dietary supplementation, the plasma 25(OH)D₃ concentration depends on sun exposure and is subject to seasonal variation (Hymoller et al., 2009).

25(OH)D₃ is the precursor for the biologically active metabolite, 1,25(OH)₂D₃ (Horst et al., 1994). 25(OH)D₃ is converted to 1,25(OH)₂D₃ by addition of a hydroxyl group in the 1α position by cytochrome P450 enzyme CYP27B1 (Sakaki et al., 2005). CYP27B1 also is
known as the 25-hydroxyvitamin D 1α-hydroxylase (1α-Ohase) and is the only known enzyme that converts 25(OH)D₃ to 1,25(OH)₂D₃ (Panda et al., 2001).

The circulating concentration of 1,25(OH)₂D₃ is regulated by the kidney in response to calcium homeostasis (Horst et al., 2003). The normal concentration of 1,25(OH)₂D₃ in plasma ranges from 5-20 pg/ml in cattle, but during severe hypocalcemia it may elevate to >300 pg/ml (Horst et al., 1994). When the calcium concentration drops, parathyroid hormone (PTH) is released, which elevates 1α-OHase expression in kidney causing an increase in the circulating concentration of 1,25(OH)₂D₃ (Engstrom et al., 1987; Hove et al., 1984). 1,25(OH)₂D₃ stimulates calcium absorption in the intestine and calcium reabsorption in bones and the kidney (Horst et al., 2003). Extra-renal 1α-OHase expression does occur but is regulated in response to other stimuli and will be discussed later.

Both 25(OH)D₃ and 1,25(OH)₂D₃ are substrates for CYP24A1 (Engstrom et al., 1984). CYP24A1, or 24-hydroxylase (24-OHase), is another cytochrome P450 enzyme that adds a hydroxyl group at the 24 positions of both 25(OH)D₃ and 1,25(OH)₂D₃ (Sakaki et al., 2005). The expression of 24-OHase increases with the concentration of 1,25(OH)₂D₃ (Beckman et al., 1994; Reinhardt et al., 1989). 24-Hydroxyvitamin D₃ metabolites are essentially inactive; so, the function of 24-OHase serves as a feedback regulator of 1,25(OH)₂D₃ synthesis (Reinhardt and Horst, 1989). 24-hydroxyvitamin D₃ metabolites undergo further side chain oxidation in the kidney to eventually form more polar metabolites, which are excreted in the bile (Horst et al., 1994).
Transport of vitamin D metabolites

Specialized mechanisms exist to transport vitamin D metabolites because they are hydrophobic compounds (Dusso et al., 2005). Nearly all vitamin D metabolites in serum are bound by the vitamin D binding protein (DBP) (Brown et al., 1999). DBP is a member of the albumin family of serum proteins and is produced in the liver (Haddad, 1995). It has multiple functions besides vitamin D binding, including actin binding, macrophage activation, and fatty acid transport (Speeckaert et al., 2006). DBP has the highest affinity for 25(OH)D₃, the major vitamin D metabolite in serum (Haddad et al., 1992). The concentration of DBP in human serum is about 5 µM, which is over 100X greater than the concentration of 25(OH)D₃ in serum (Brown et al., 1999). As a result, over 99.9% of 25(OH)D₃ and 99% of 1,25(OH)₂D₃ in serum are bound by DBP (White and Cooke, 2000). Even still, only 5% of DBP in serum is occupied by vitamin D metabolites (Haddad, 1995).

Transport of vitamin D metabolites in serum by DBP is an important process in vitamin D metabolism and signaling (Brown et al., 1999). DBP extends the lifetime of 25(OH)D₃ in serum and allows for storage of 25(OH)D₃ in plasma that is readily accessible (Cooke and Haddad, 1989). The maintenance of free versus bound 1,25(OH)₂D₃ by DBP is another important role because free 1,25(OH)₂D₃ has greater access to target cells (Bikle and Gee, 1989). In fact, vitamin D toxicity is thought to occur when DBP becomes saturated with 25(OH)D₃ and 1,25(OH)₂D₃ is freed (Vieth, 2007).

Vitamin D metabolites not bound by the DBP will enter the cell by diffusion; however, facilitated uptake of 25(OH)D₃ bound by DBP also occurs (Dusso et al., 2005). Megalin and cubulin, members of the low density lipoprotein receptor family, have been shown to facilitate endocytosis of DBP in kidney proximal tubule cells and mammary
epithelial cells (Nykjaer et al., 1999; Rowling et al., 2006). Subsequently, synthesis of 1,25(OH)$_2$D$_3$ in those cells depended on megalin-mediated endocytosis of DBP-bound 25(OH)D$_3$. Upon endocytosis, DBP is degraded in the lysosome and the bound vitamin D metabolites are released (Adams et al., 2007a). There are several intracellular DBPs (IDBP) that are known to transport vitamin D metabolites in the cell (Adams et al., 2007a). The IDBPs are members of the inducible heat shock protein family (hsp-70) that seem to promote synthesis and action of 1,25(OH)$_2$D$_3$ by facilitating transport of 25(OH)D$_3$ to the mitochondria and 1,25(OH)$_2$D$_3$ to the vitamin D receptor (VDR) (Adams et al., 2004). Therefore, intracellular transport of vitamin D metabolites also seems to be an important process in vitamin D metabolism and signaling.

**Genomic actions of 1,25(OH)$_2$D$_3$**

The biological function of 1,25(OH)$_2$D$_3$ is to regulate gene expression (Horst et al., 2005). 1,25(OH)$_2$D$_3$ regulates gene expression by activating the vitamin D receptor (VDR) (Koszewski et al., 1996; Pike et al., 2007). The VDR is a nuclear hormone receptor that has a ligand binding domain, a domain that forms a heterodimer with the retinoid X receptor (RXR), and a DNA binding domain (Dusso et al., 2005). The ligand binding domain contains a ligand binding site with an affinity for 1,25(OH)$_2$D$_3$ ($K_d = 100$ pM) that is about 100 times greater than the affinity for 25(OH)D$_3$ (Reinhardt et al., 1989). Binding of 1,25(OH)$_2$D$_3$ to the VDR promotes heterodimerization of the VDR with the RXR and nuclear localization (Barsony and Prufer, 2002). The DNA binding domains of the VDR/RXR heterodimer recognize DNA sequences, known as vitamin D response elements (VDREs), in the promoter regions of vitamin D responsive genes (Lin and White, 2004). There are a few
different types of VDREs that are recognized by the VDR/RXR heterodimer, and the type of VDRE recognized by the VDR/RXR heterodimer depends on the orientation of the heterodimer (Lin and White, 2004). The most common VDRE is known as a DR3, which is a direct repeat of PuG(G/T)TCA separated by 3 bases (Lin and White, 2004). An inverted repeat of that sequence separated by nine bases is another VDRE that is recognized by the VDR/RXR heterodimer but is not as common (Lin and White, 2004).

As mentioned earlier, 24-OHase expression increases with 1,25(OH)\textsubscript{2}D\textsubscript{3} concentration. Analysis of the 24-OHase promoter in several species has revealed the presence on multiple VDREs, which explains why it is so responsive to 1,25(OH)\textsubscript{2}D\textsubscript{3} in many different tissues (Vaisanen et al., 2005). Besides 24-OHase, in silico analysis of the human and murine genomes has revealed that nearly 1,000 genes have promoters with potential VDREs (Wang et al., 2005). Regulation of each gene would depend on the presence of the VDR and accessibility of the promoter, but the wide distribution of VDREs does suggest that 1,25(OH)\textsubscript{2}D\textsubscript{3} has a multitude of effects throughout the body (Carlberg and Dunlop, 2006).

**Regulation of immune responses by 1,25(OH)\textsubscript{2}D\textsubscript{3}**

Initial evidence that 1,25(OH)\textsubscript{2}D\textsubscript{3} acts in immune cells came from observations that 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibited proliferation of lymphocytes and promoted differentiation of monocytes to macrophages (Koeffler et al., 1984; Lemire et al., 1984; Mangelsdorf et al., 1984). Recently, 1,25(OH)\textsubscript{2}D\textsubscript{3} has been shown to enhance antimicrobial activity of human macrophages by directly up-regulating expression of the genes for cathelicidin antimicrobial peptide and defensin β4 (DEFB4) (Liu et al., 2009; Wang et al., 2004). Subsequently,
cathelicidin-dependent killing of *Mycobacterium tuberculosis* in human macrophages has been shown (Liu et al., 2007b). The promoter of the cathelicidin gene in humans contains a VDRE (Wang et al., 2004). Cathelicidin gene expression is not regulated by 1,25(OH)$_2$D$_3$ in mice, however, and the presence of a VDRE in the cathelicidin promoter is primate specific (Gombart et al., 2009). In regards to cattle, there are 11 cathelicidin genes and none of them are known to be regulated by 1,25(OH)$_2$D$_3$ (Zanetti, 2004).

The effects of 1,25(OH)$_2$D$_3$ on innate immunity go beyond enhancement of antimicrobial peptide gene expression. 1,25(OH)$_2$D$_3$ up-regulates CD14 and NOD2 gene expression in human monocytes (Liu et al., 2006; Wang et al., 2010). Both of those genes are involved in recognition of pathogens. In dendritic cells, 1,25(OH)$_2$D$_3$ enhanced CCL22, a chemokine that attracts regulatory T-cells, and CD300LF, an inhibitory cell surface receptor (Szeles et al., 2009). Migration of myeloid dendritic cells to the lymph nodes increased in mice treated with 1,25(OH)$_2$D$_3$ (Enioutina et al., 2009). In that study, migration of myeloid dendritic cells to lymph nodes was less in VDR knock out mice than in wild type mice.

In cattle, 1,25(OH)$_2$D$_3$ increases nitric oxide production by stimulated peripheral blood mononuclear cells (PBMCs) (Waters et al., 2001). Nitric oxide is considered to have an antimicrobial and signaling role in the immune system (Bogdan, 2001). Two studies have shown that 1,25(OH)$_2$D$_3$ increases inducible nitric oxide synthase (iNOS) expression in human monocytes (Martineau et al., 2007; Rockett et al., 1998). In mouse monocytes, there is not evidence that nitric oxide production is increased by 1,25(OH)$_2$D$_3$, but *M. tuberculosis* killing depended on nitric oxide production (Thoma-Uszynski et al., 2001). Furthermore, iNOS knockout mice developed more severe lesions than did wild type mice when infected
with *M. tuberculosis* (MacMicking et al., 1997; Waters et al., 2004). Killing of *M. tuberculosis* by human monocytes, however, was not dependent on induction of iNOS expression (Thoma-Uszynski et al., 2001). Instead, it seems to depend on induction of cathelicidin expression by 1,25(OH)$_2$D$_3$ (Liu et al., 2007b).

In addition to the effects 1,25(OH)$_2$D$_3$ has on innate immune functions, it also regulates adaptive immunity (Cantorna et al., 2004). The general effects that 1,25(OH)$_2$D$_3$ has on adaptive immunity are suppression of pro-inflammatory responses and enhancement of regulatory responses (Cantorna et al., 2008). 1,25(OH)$_2$D$_3$ inhibits onset of models of multiple sclerosis and inflammatory bowel disease in mice (Cantorna et al., 1996; Cantorna et al., 2000). The exact mechanism 1,25(OH)$_2$D$_3$ has in preventing disease onset in each case is not entirely clear. Activated T-cells, however, have a functional VDR, and studies with VDR knockout mice show the VDR is necessary for 1,25(OH)$_2$D$_3$-mediated inhibition of autoimmune disease (Froicu et al., 2003; Veldman et al., 2000).

In activated human PBMCs, IFN-γ production is down-regulated by 1,25(OH)$_2$D$_3$ (Reichel et al., 1987). IFN-γ is expressed by antigen-specific T$_{H1}$-cells and functions as a macrophage activator (Hu and Ivashkiv, 2009). 1,25(OH)$_2$D$_3$ down regulated IFN-γ production in T-cell cultures that were activated with anti-CD3 (Baeke et al., 2010). In a human T-cell line that over-expressed the VDR and a reporter gene under control of the IFN-γ promoter, the VDR bound the IFN-γ promoter and repressed expression of the reporter gene (Cippitelli and Santoni, 1998). Several studies in cattle have shown that 1,25(OH)$_2$D$_3$ represses antigen-specific IFN-γ responses to *M. bovis* in vitro (Ametaj et al., 1996; Nonnecke et al., 2003; Waters et al., 2001). IFN-γ production is considered to be particularly
important in pathogenesis of *Mycobacteria* infections (de Almeida et al., 2008; Thacker et al., 2007), so the effects of 1,25(OH)$_2$D$_3$ on IFN-γ may have significant implications in those diseases.

Besides IFN-γ, 1,25(OH)$_2$D$_3$ down-regulated IL-2 production and enhanced IL-4 production by T-cells (Bemiss et al., 2002; Cantorna et al., 1998b). Down-regulation of IL-2 may explain why 1,25(OH)$_2$D$_3$ decreases T-cell proliferation (Cantorna et al., 2004). In contrast, enhancement of IL-4 may explain why 1,25(OH)$_2$D$_3$ enhances antibody production (Reinhardt et al., 1999). Direct regulation of IL-2 and IL-4 production by 1,25(OH)$_2$D$_3$, however, has not been shown.

Several studies in mice and humans have provided evidence that 1,25(OH)$_2$D$_3$ promotes development of regulatory T-cells (Ghoreishi et al., 2009; Jeffery et al., 2009; Unger et al., 2009). Regulatory T-cells are characterized by FoxP3, and CTLA4 and 1,25(OH)$_2$D$_3$ increased the number of FoxP3$^+$CTLA4$^+$ cells in human CD4$^+$ T-cell cultures stimulated with anti-CD3 (Jeffery et al., 2009). IL-10 production also is associated with a regulatory T-cell response (de Almeida et al., 2008). IL-10 is a suppressor of macrophage activation and has been implicated in 1,25(OH)$_2$D$_3$-mediated inhibition mouse models of multiple sclerosis and inflammatory bowel disease (Froicu et al., 2006; Spach et al., 2006). Yet, direct regulation of IL-10 production by 1,25(OH)$_2$D$_3$ has not been shown and 1,25(OH)$_2$D$_3$ has not been shown to act directly in regulatory T-cells.

Recently, IL-17A production by T-cells was shown to be down-regulated by 1,25(OH)$_2$D$_3$ (Jeffery et al., 2009). IL-17A is a member of the IL-17 family of cytokines, most of which are known to be produced by a specific lineage of CD4$^+$ T-cells called T$_{H17}$-cells (Dong, 2008). Although γδT-cells also express IL-17 cytokines (O'Brien et al., 2009).
IL-17A has been the most widely studied of the IL-17 cytokines and its primary functions seem to be activation of cytokine and chemokine genes in fibroblasts and epithelial cells and recruitment of neutrophils (Dong, 2008). IL-17A has been implicated in the progression of multiple sclerosis (Dong, 2008). In cattle, in vitro IL-17A production positively correlated with protection against tuberculosis (Vordermeier et al., 2009).

**Synthesis of 1,25(OH)₂D₃ in the immune system**

In contrast to regulation of calcium homeostasis, which is regulated by the concentration of 1,25(OH)₂D₃ in plasma, 1,25(OH)₂D₃-immune responses do not seem to be regulated by the circulating concentration of 1,25(OH)₂D₃ (Hewison, 2010). The concentration 1,25(OH)₂D₃ needed to regulate responses of bovine PBMCs in vitro was at least 400 pg/ml (Waters et al., 2001). The normal circulating concentration of 1,25(OH)₂D₃ in cattle is 5-20 pg/ml (Horst et al., 1994). The circulating concentration of 1,25(OH)₂D₃ was shown to rise above 100 pg/ml in a case of bovine tuberculosis (Rhodes et al., 2003) but did not change in cattle infused with lipopolysaccharide (LPS; Waldron et al., 2003). Therefore, the circulating concentration of 1,25(OH)₂D₃ does not reach the concentration of 1,25(OH)₂D₃ that was required to regulate immune responses in PBMCs; so, regulation of immune responses by 1,25(OH)₂D₃ would likely be regulated synthesis of 1,25(OH)₂D₃ in immune cells at the site of infection.

Activated macrophages can synthesize 1,25(OH)₂D₃ from 25(OH)D₃ in the human and murine immune systems (Hewison, 2010). The first evidence of extra-renal synthesis of 1,25(OH)₂D₃ by macrophages was shown over two decades ago when pulmonary macrophages from sarcoidosis patients were shown to synthesize 1,25(OH)₂D₃ (Adams et al.,
1983). In human and mouse monocytes and macrophages, 1α-OHase gene expression is
induced by IFN-γ or toll-like receptor (TLR) recognition of pathogen-associated molecules
(Liu et al., 2006; Stoffels et al., 2007; Stoffels et al., 2006). Expression of 1α-OHase in
monocytes and macrophages enabled conversion of 25(OH)D₃ to 1,25(OH)₂D₃ (Hewison et
al., 2007). In human monocytes, synthesis of 1,25(OH)₂D₃ by 1α-OHase up-regulated
cathelicidin gene expression and subsequently antimicrobial activity (Liu et al., 2006).
Furthermore, it was shown that synthesis of 1,25(OH)₂D₃ and subsequent up-regulation of
cathelicidin expression in activated monocytes correlated with the concentration of
25(OH)D₃ in serum from humans (Adams et al., 2009).

Macrophages are not the only immune cells that express 1α-OHase and synthesize
1,25(OH)₂D₃. Monocyte-derived dendritic cells were shown to express constitutively low
amounts of 1α-OHase, but 1α-OHase expression was increased by stimulation with LPS
(Fritsche et al., 2003). Dendritic cells from 1α-OHase knock out mice did not migrate to the
lymph nodes as well as did dendritic cells from wild type mice (Enioutina et al., 2009).
Synthesis of 1,25(OH)₂D₃ by dendritic cells also enhanced CCR10, a chemokine receptor,
expression by T-cells (Sigmundsdottir et al., 2007). Overall, synthesis of 1,25(OH)₂D₃ by
1α-OHase in dendritic cells may have multiple effects on adaptive immunity through
intracrine and paracrine mechanisms (Hewison, 2010). Several studies have provided
evidence for 1α-OHase expression in B-cells and T-cells, so 1,25(OH)₂D₃ synthesis also is
possible in those cells (Chen et al., 2007; Sigmundsdottir et al., 2007).

Human keratinocytes express 1α-OHase in response to transforming growth factor-β
(TGF-β) (Schauber et al., 2007). Treatment of keratinocytes with 25(OH)D₃ and TGF-β
enhanced cathelicidin and CD14 gene expression (Schauber et al., 2007). Increased
expression of those genes in keratinocytes depended on synthesis of 1,25(OH)₂D₃ by 1α-OHase in keratinocytes.

Mammary epithelial cells also express 1α-OHase and synthesize 1,25(OH)₂D₃ (Kemmis et al., 2006). 1α-OHase expression in mammary epithelial cells was regulated during development and involution of the mammary gland (Zinser and Welsh, 2004). Mammary epithelial cells have immune functions that can be activated by TLR signaling (Boulanger et al., 2001; Wellnitz and Kerr, 2004). Therefore, bovine mammary epithelial cells may have the ability to synthesize 1,25(OH)₂D₃ in response to TLR signaling, but regulation of 1α-OHase expression in mammary epithelial cells in response to immune stimuli has not been shown.

**Activation of innate immunity**

Toll-like receptor (TLR) signaling activated intracrine vitamin D signaling in human monocytes and macrophages (Liu et al., 2006; Stoffels et al., 2007). TLRs are a group of innate receptors that recognize pathogen associated molecular patterns such as LPS from Gram negative bacteria, peptidoglycan from Gram positive bacteria, or double stranded RNA from viruses (Uematsu and Akira, 2006). When these receptors bind their respective ligands they activate the immune system (Brightbill et al., 1999). In cattle there are 10 different TLRs, each recognizing a different molecular pattern, and they seem to be homologous to the human TLR family (McGuire et al., 2006).

Of the TLR family, TLR4 has been studied the most in cattle. TLR4 recognizes LPS from Gram negative bacteria (Ibeagha-Awemu et al., 2008). Recognition of LPS by TLR4 is aided by CD14 and the LPS binding protein (De Schepper et al., 2008). CD14 is mainly
found on the cell surface on monocytes and macrophages and, to a lesser extent, neutrophils (Rainard and Riollet, 2006). The TLR2/1 and TLR2/10 heterodimers can recognize triacyl-lipopeptides from *Mycobacteria* species such as *M. tuberculosis* (Guan et al., 2010; Takeda et al., 2002). Antimicrobial activity of macrophages against *M. tuberculosis* is triggered by the TLR2/1 signaling pathway (Thoma-Uszynski et al., 2001). The signaling mechanism of the TLR2/10 heterodimer, however, is not clear and is distinct from the signal transduced by the TLR2/1 heterodimer (Guan et al., 2010). In contrast, the TLR2/6 heterodimer can recognize diacyl-lipopeptides from *Mycobacteria* species (Takeda et al., 2002). Pam$_2$CSK$_4$ and Pam$_3$CSK$_4$ (dipalmitoyl- and tripalmitoyl-cystiene,serine, lysine$_4$) are synthetic lipopeptides that have been developed and are recognized by the TLR2/6 and TLR2/1 heterodimers, respectively (Aliprantis et al., 1999). TLR3, TLR7, and TLR8 are involved in recognition of viral pathogens (Kumar et al., 2009). TLR3 recognizes double-stranded viral RNA and TLR7 and TLR8 will recognize single-stranded viral RNA (Kawai and Akira, 2007). TLR9 can recognize bacteria CpG DNA sequences, whereas TLR5 recognizes flagellin from bacteria (Kumar et al., 2009).

In general, activation of TLRs by their respective ligands triggers nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling cascades (Doyle and O'Neill, 2006; Weiss et al., 2008). Activation of these pathways by TLRs typically occurs through myeloid differentiating factor 88 (MyD88) and toll-interleukin 1 receptor adaptor protein (TIRAP) (O'Neill, 2006). MyD88-independent activation of signaling cascades by TLRs, however, is known to occur (Ibeagha-Awemu et al., 2008). Activation of TLR signaling pathways induces the transcription of genes for cytokines and chemokines in cattle. Interleukin-1 (IL-1), IL-6, IL-8/ CXCL8, IL-12, RANTES/CCL5 (regulated upon activation,
normally T-cell expressed and secreted), and tumor necrosis factor-α (TNF-α) are examples of cytokines and chemokines that are induced by TLR signaling in bovine macrophages and mammary epithelial cells (De Schepper et al., 2008; Ibeagha-Awemu et al., 2008; Lahouassa et al., 2007; McClenahan et al., 2006; Pareek et al., 2005; Weiss et al., 2008).

**Comparison of bovine, human, and murine immune systems**

Most of what is known about vitamin D signaling in the immune system comes from studies in humans and mice. However, there are some significant different differences among the immune systems of cattle, humans, and mice. Recent publication of the bovine genome revealed that cattle have a high number of genes encoding for antimicrobial peptides such as 11 cathelicidin genes and over 100 defensin genes (Elsik et al., 2009). In contrast, there is only one cathelicidin gene and less than 60 defensin genes in the human and mouse genomes. Many of the antimicrobial peptides in cattle are constitutively expressed on the skin and epithelial lining of the digestive and respiratory tracts (Tetens et al., 2010; Zanetti, 2004).

The prevalence of γδT-cells in cattle is another distinct difference between the bovine, human, and murine immune systems (Davis et al., 1996). Unlike αβT-cells, γδT-cells have innate abilities to recognize pathogen-associated molecular patterns (Kerns et al., 2009; Martin et al., 2009). γδT-cells activated through innate receptors have been shown to produce IFN-γ and IL-17A (Martin et al., 2009; Pietschmann et al., 2009), which are cytokines that are associated with T\textsubscript{H}1 and T\textsubscript{H}17 cells of the αβT-cell lineage. 1,25(OH)\textsubscript{2}D\textsubscript{3} decreases IFN-γ and IL-17A production and activation of both γδT-cells and αβT-cells.
(Baeke et al., 2010; Jeffery et al., 2009; Waters et al., 2003). Therefore, the prevalence and innate characteristics of γδT-cells in cattle requires consideration in regards to the effects that 1,25(OH)\(_2\)D\(_3\) has on immune function.

**Conclusion**

From the time vitamin D was discovered it has been known to regulate calcium homeostasis. Regulation of calcium homeostasis by vitamin D occurs by tight regulation of 1,25(OH)\(_2\)D\(_3\) synthesis in the kidney. Numerous studies have shown a definite role for 1,25(OH)\(_2\)D\(_3\) in regulating immune responses (Adams and Hewison, 2010). The predominant source of 1,25(OH)\(_2\)D\(_3\) in the immune system seems to be monocytes and macrophages that are activated by TLR signaling (Adams et al., 2007b; Liu et al., 2007a). In cattle, 1,25(OH)\(_2\)D\(_3\) has been shown to regulate a few immune responses, most notably down-regulation of IFN-γ and up-regulation of nitric oxide production (Waters et al., 2001). The kidneys, however, have been the only known site of 1,25(OH)\(_2\)D\(_3\) synthesis in cattle.

Evidence from studies with mouse and human monocytes and macrophages suggests that bovine monocytes and macrophages will produce 1,25(OH)\(_2\)D\(_3\) in response to TLR signaling.

**References**


Adams, J.S., Chen, H., Chun, R., Ren, S., Wu, S., Gacad, M., Nguyen, L., Ride, J., Liu, P., Modlin, R., Hewison, M., 2007a, Substrate and enzyme trafficking as a means of


Froicu, M., Zhu, Y., Cantorna, M.T., 2006, Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice. Immunology 117, 310-318.


Weiss, D.J., Souza, C.D., Evanson, O.A., Sanders, M., Rutherford, M., 2008, Bovine monocyte TLR2 receptors differentially regulate the intracellular fate of


Figure 1. A) Structures of vitamin D$_2$ (plant derived) and vitamin D$_3$ (animal derived). B) Metabolic pathway of vitamin D$_3$ showing major metabolites.
CHAPTER 3. PRODUCTION OF 1,25-DIHYDROXYVITAMIN D₃ BY 1α-HYDROXYLASE IN CD14+ CELLS DIRECTS PERIPHERAL BLOOD MONONUCLEAR CELL IMMUNE RESPONSES

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Abstract

In cattle, the kidney has been the only known site for production of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) from 25-hydroxyvitamin D₃ (25(OH)D₃) by 1α-hydroxylase (1α-Ohase). Based on human studies, we hypothesized that bovine monocytes could produce 1,25(OH)₂D₃ upon activation and 1,25(OH)₂D₃ would regulate expression of vitamin D responsive genes in monocytes. First, the effects of 1,25(OH)₂D₃ on bovine monocytes isolated from peripheral blood were tested. Treatment of non-stimulated monocytes with 1,25(OH)₂D₃ increased expression of the gene for the vitamin D 24-hydroxylase (24-OHase) enzyme by 51 ± 13 fold, but 1,25(OH)₂D₃ induction of 24-OHase expression was blocked by lipopolysaccharide (LPS) stimulation. Also, 1,25(OH)₂D₃ increased the gene expression of inducible nitric oxide synthase (iNOS) and the chemokine

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RANTES in LPS-stimulated monocytes 69 ± 13 and 40 ± 12 fold respectively. Next, the ability of bovine monocytes to express 1α-OHase and produce 1,25(OH)$_2$D$_3$ was tested. Activation of monocytes with LPS, tripalmitoylated lipopeptide (Pam3CSK4), or peptidoglycan (PGN) caused 43 ± 9, 17 ± 3, and 19 ± 3 fold increases in 1α-OHase gene expression respectively. Addition of 25(OH)D$_3$ to LPS-stimulated monocytes enhanced expression of iNOS and RANTES and nitric oxide production in a dose dependent manner, giving evidence that activated monocytes convert 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$. In conclusion, bovine monocytes produce 1,25(OH)$_2$D$_3$ in response to toll like receptor signaling and 1,25(OH)$_2$D$_3$ production in monocytes increased the expression of genes involved in the innate immune system. Evidence suggests that vitamin D status of cattle may be important for optimal innate immune function because 1,25(OH)$_2$D$_3$ production in activated monocytes and subsequent up-regulation of iNOS and RANTES expression was dependent on 25(OH)D$_3$ availability.

**Introduction**

For several decades now, it was known that an endocrine mechanism exists to regulate renal production of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) as a way to regulate the concentration of 1,25(OH)$_2$D$_3$ systemically (Horst and Reinhardt, 1983). The primary function of renal 1,25(OH)$_2$D$_3$ production was considered maintenance of calcium homeostasis (Horst, 1986). It has become evident that 1,25(OH)$_2$D$_3$ modulates the immune response of several species, including cattle (Waters et al., 2001). Furthermore, activated human macrophages produce 1,25(OH)$_2$D$_3$ as part of the immune response to regulate
Local control of 1,25(OH)_2D_3 concentration regulates genes involved in immune responses locally rather than systemically (Schauber et al., 2007). Existence of a mechanism to control 1,25(OH)_2D_3 production and gene expression locally in humans and mice suggests that a similar mechanism may exist in cattle.

Vitamin D, acquired in the diet or by radiation of 7-dehydrocholesterol with UVB light in the skin, is converted to 25-hydroxyvitamin D_3 (25(OH)D_3) in the liver (Horst and Reinhardt, 1983). The major circulating metabolite of vitamin D is 25(OH)D_3 and the concentration of 25(OH)D_3 in blood is relatively stable in cattle (Sommerfeldt et al., 1983). Conversion of 25(OH)D_3 to 1,25(OH)_2D_3 is accomplished by the enzymatic activity of 1α-hydroxylase (1α-Ohase; Sakaki et al., 2005). 1,25(OH)_2D_3 is the ligand for the vitamin D receptor (VDR), which is activated upon binding 1,25(OH)_2D_3 (Reinhardt et al., 1989). The activated VDR regulates expression of genes that contain functional vitamin D response elements (VDRE) in their promoters (Lin and White, 2004). It is estimated that over 1,000 genes are regulated by 1,25(OH)_2D_3 (Wang et al., 2005), and the VDR is present in most tissues and cell types (Lin and White, 2004). Therefore, 1,25(OH)_2D_3 concentration is regulated tightly in order to control its effects on gene expression.

The kidney was the only known source for 1,25(OH)_2D_3 in cattle and regulation of 1α-OHase expression in the kidney is mainly in response to calcium homeostasis (Horst, 1986). In contrast, 1α-OHase was expressed in human monocytes and macrophages in response to activation by toll like receptor (TLR) recognition of pathogen-associated molecules (Liu et al., 2006). In human macrophages, 1,25(OH)_2D_3 increases the expression of cathelicidin directly via a VDRE in the cathelicidin promoter (Gombart et al., 2005). It
was found that 1,25(OH)\(_2\)D\(_3\) induction of cathelicidin expression in human macrophages was necessary for killing of intracellular *Mycobacteria tuberculosis* (Liu et al., 2007). In cattle, 1,25(OH)\(_2\)D\(_3\) modulated the immune response in vitro by increasing nitric oxide production and decreasing IFN-\(\gamma\) production in peripheral blood mononuclear cells (Waters et al., 2001). Production of 1,25(OH)\(_2\)D\(_3\) in the kidney does not increase as part of the immune response in cattle (Waldron et al., 2003); so, if 1,25(OH)\(_2\)D\(_3\) modulates the immune response in vivo, there would seem to be a source other than the kidney. It was hypothesized that bovine monocytes express 1\(\alpha\)-OHase and produce 1,25(OH)\(_2\)D\(_3\) at the site of infection in response to TLR signaling to direct local regulation of vitamin D responsive genes. The objectives were to assess the effects of 1,25(OH)\(_2\)D\(_3\) on bovine monocytes isolated from peripheral blood and test the ability of bovine monocytes to express 1\(\alpha\)-OHase and convert 25(OH)D\(_3\) to 1,25(OH)\(_2\)D\(_3\).

**Materials and Methods**

**Animals**

A group of 12 healthy, mid-lactation Holstein cows at the National Animal Disease Center were used. The number of cows used for each experiment ranged from 4 to 6 and is specified in the figure legend. The care and treatment of the cows used were approved by the National Animal Disease Center animal care and use committee.
Monocyte isolation and culture conditions

Monocytes were isolated by adherence to tissue culture flasks as previously described (Stabel et al., 1997). Briefly, peripheral blood was collected into 2X acid citric dextrose and the mononuclear cell fraction was separated by density gradient centrifugation. Cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) plus 10% fetal bovine serum (FBS; Hyclone, Waltham, MA) and incubated at 37°C for 1 h in tissue culture flasks. Lymphocytes were then removed by washing 3X with warm PBS. Monocytes were dislodged from the tissue culture flasks using cold PBS plus 20 mM EDTA. Monocytes were pelleted and resuspended to a concentration of $10^7$ cells/mL in RPMI 1640 containing 50 µg/mL gentamicin (Invitrogen, Carlsbad, CA) and placed in 24-well or 96-well non-tissue culture treated polystyrene plates (Becton Dickinson, Franklin Lakes, NJ).

All treatments were added to heat-inactivated FBS at 10X the desired final concentration, and FBS was added to wells containing monocytes to a final concentration of 10%. Monocytes were incubated with the treatments for 24 h at 37°C in 5% CO₂. LPS from Serratia marsescens (Sigma Aldrich), Pam3CSK4 (InvivoGen, San Diego, CA), a synthetic tripalmitoyl lipopeptide, and peptidoglycan (PGN) from Staphylococcus aureus (InvivoGen) were in endotoxin-free water. Both 25(OH)D₃ and 1,25(OH)₂D₃ (Sigma Aldrich) were diluted to a concentration of 100 ng/µL in 100% ethanol. Concentrations were confirmed by UV spectroscopy using an extinction coefficient of 18,200 $M^{-1}$ per cm. The final concentration of ethanol did not exceed 0.04% for any of the treatments, and a treatment of ethanol alone at 0.04% was used as a control for the effects of ethanol. The lot of FBS used contained no more than 20 ng/mL of 25(OH)D₃. Ketoconazole (Sigma Aldrich) was solubilized in PBS at pH 2.5 and diluted to 50 µg/mL in PBS at pH 7.
Measurement of relative gene expression

RNA was isolated from monocytes using an Rneasy Mini kit (Qiagen, Valencia, CA) according to manufacturer’s instructions and eluted with 50 µL of Rnase-free water. The RNA was reverse transcribed to cDNA in a 20 µL reaction using a High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) with 10 µL of RNA sample and 20 units of Rnase Inhibitor (Applied Biosystems). Reactions were incubated at 37°C for 2 h and heated to 85°C for 5 s. The cDNA samples were diluted 1:10 in sterile water and stored at -20°C. Quantitative PCR was performed with the 7300 Real-Time PCR System (Applied Biosystems) according to manufacturer’s instructions. Reactions consisted of 12.5 µL SYBR® Green PCR Master Mix (Applied Biosystems), 2.5 µL each of 10 µM forward and reverse primers and 7.5 µL of diluted cDNA. Primers pairs were designed with primer3 (http://frodo.wi.mit.edu/primer3) (Rozen and Skaletsky, 2000) to span intron-exon boundaries. Primer sequences along with the efficiency of replication for each primer pair are in Table 1. The efficiency of each primer pair was calculated using the equation

\[
\text{Efficiency} = -1 + 10^{(-1/\text{slope})}
\]

where slope equals the slope of a standard curve generated with known dilutions of cDNA in the PCR reactions. Primer specificity was determined by gel electrophoresis and melting curve analysis. Relative quantification of mRNA transcripts was accomplished using the \(2^{-\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001). The gene for ribosomal protein S9 (RPS9) was used as the reference gene (Janovick-Guretzky et al., 2007), and stability of RPS9 expression was checked by comparison with β-actin expression. For each experiment, the control sample was used as the calibrator, and expression of each gene is reported as fold increase relative to the control.
Measurement of nitric oxide production

The concentration of nitrite in the culture supernatant at the end of the incubation period was used as an indicator of nitric oxide produced by monocytes. Nitrite concentration was measured by adding 100 µL of culture supernatant or culture media with 0 to 100 µM sodium nitrite to 100 µL of Griess reagent (0.5% sulfanilamide, 2.5% phosphoric acid, and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride) (Sigma Aldrich) in a 96-well clear bottom plate. The reactions were incubated for 10 min at room temperature. Absorbance at 550 nm in each well was measured using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were converted to micromoles per liter using a standard curve. To ensure that nitrite accumulation in the culture supernatant was a result of nitric oxide synthase activity, 1 mM of \(N^\text{G}-\text{monomethyl-L-arginine} \) (L-NMMA) (Sigma Aldrich), a nitric oxide synthase inhibitor, was added as a control treatment. There was no accumulation of nitrite in the culture supernatant when L-NMMA was added as a treatment.

Statistical analysis

Response variables were analyzed as a completely randomized block design with PROC GLM (SAS Institute, Inc., Cary, NC). The model accounted for effects of treatment and cow. Experimental units were blocked according to cow to account for variation of monocyte responses between cows. For gene expression, \(\Delta \Delta CT\) values were used as the response variables in the analyses. Mean \(\Delta \Delta CT\) values ± SE were transformed (\(2^{-\Delta \Delta CT}\)) and presented as the mean fold increase relative to control. Multiple-comparison tests of the means were made with the Tukey adjustment. Differences were considered significant at \(P < 0.05\).
Results

Effects of 1,25(OH)₂D₃ on monocytes

Initially the effect of 1,25(OH)₂D₃ on 24-OHase expression in monocytes was tested because 24-OHase is known to be a vitamin D-responsive gene. The 24-OHase expression in monocytes increased with 1,25(OH)₂D₃ treatment ($P < 0.05$; Figure 1A). Surprisingly though, the effects of 1,25(OH)₂D₃ on 24-OHase expression were greatly reduced when monocytes were activated with LPS ($P < 0.05$; Figure 1A). Potential VDREs were found in the promoters of the bovine Cathelicidin 4 (CATH4), CATH5 and CATH6 genes (results not shown) using the NHR SCAN website (http://asp.ii.uib.no:8090/cgi-bin/NHR-scan/nhr_scan.cgi; Sandelin and Wasserman, 2005). However, the expression of CATH4, CATH5, or CATH6 was not increased by 1,25(OH)₂D₃ treatment in non-stimulated or LPS-stimulated monocytes (Figure 1A).

Of the other genes tested, iNOS, RANTES, and S100A12 gene expression were upregulated by treatment with 4 ng/mL 1,25(OH)₂D₃ alone ($P < 0.05$; Figure 1B). The combination of LPS and 1,25(OH)₂D₃ treatments, resulted in increases of both iNOS and RANTES gene expression relative to either treatment alone ($P < 0.05$; Figure 1B). There was no synergistic effect of LPS and 1,25(OH)₂D₃ on S100A12 gene expression (Figure 1B). IL-1β gene expression increased slightly ($P > 0.05$) in non-activated and LPS-activated monocytes treated with 4 ng/mL 1,25(OH)₂D₃ (Figure 1B).

In LPS-activated monocytes, RANTES expression was increased by addition of 0.04 ng/mL ($P < 0.05$) of 1,25(OH)₂D₃ and peaked with addition of 0.4 ng/mL 1,25(OH)₂D₃
Both iNOS expression and nitric oxide production increased with 1,25(OH)\textsubscript{2}D\textsubscript{3} dose in LPS-activated monocytes ($P < 0.05$) (Figures 2B and 2C).

**Expression 1\(\alpha\)-OHase in monocytes**

The ability of bovine monocytes to express 1\(\alpha\)-OHase upon activation with TLR ligands was tested. Activation of bovine monocytes with LPS, Pam3CSK4, or PGN triggered a large increase in 1\(\alpha\)-OHase gene expression relative to non-activated monocytes ($P < 0.001$; Figure 3).

**Activity of 1\(\alpha\)-OHase in monocytes**

Increasing the concentration of 25(OH)D\textsubscript{3} in the culture media to physiological concentrations increased RANTES and iNOS gene expression and nitric oxide production in LPS-stimulated monocytes in a dose-dependent manner (linear effect, $P < 0.001$; Figure 4).

To determine if 1\(\alpha\)-OHase activity is necessary for the effects of 25(OH)D\textsubscript{3} on activated monocytes, ketoconazole was used to block conversion of 25(OH)D\textsubscript{3} to 1,25(OH)\textsubscript{2}D\textsubscript{3}. Ketoconazole is a non-specific competitive inhibitor of 1\(\alpha\)-OHase enzyme activity (Schuster et al., 2001). Treatment with ketoconazole decreased the effects of 25(OH)D\textsubscript{3} on RANTES and iNOS gene expression and nitric oxide production, but not 1\(\alpha\)-OHase gene expression in LPS-stimulated monocytes ($P < 0.05$; Figure 5). Furthermore, the effects of ketoconazole were reversed when exogenous 1,25(OH)\textsubscript{2}D\textsubscript{3} was added to the culture media ($P < 0.05$). Addition of 25(OH)D\textsubscript{3} to monocytes that were not activated with LPS increased RANTES gene expression ($P < 0.05$) even though 1\(\alpha\)-OHase gene expression was not elevated.
Discussion

It has been known that 1,25(OH)$_2$D$_3$ modulates bovine immune responses by increasing nitric oxide production by PBMC in vitro (Waters et al., 2001). This study more specifically revealed that 1,25(OH)$_2$D$_3$ enhanced iNOS gene expression in activated monocytes. It showed for the first time that RANTES expression was increased by 1,25(OH)$_2$D$_3$. But, the concentration of 1,25(OH)$_2$D$_3$ needed to increase iNOS and RANTES was much higher than the normal concentration of 1,25(OH)$_2$D$_3$ in serum which is less than 50 pg/mL (Horst and Reinhardt, 1983). Also, the concentration of 1,25(OH)$_2$D$_3$ in serum did not increase during infection in cattle (Waldron et al., 2003). It was shown in this study that bovine monocytes converted 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ in response to TLR signaling; providing 1,25(OH)$_2$D$_3$ at the site of infection. Furthermore, physiological concentrations of 25(OH)D$_3$, which typically range from 20 to 50 ng/mL, were sufficient to increase iNOS and RANTES gene expression through the actions of 1α-OHase in monocytes.

An interesting observation in terms of regulating 1,25(OH)$_2$D$_3$ concentration at the site of infection was the regulation of 24-OHase expression. Inactivation of 1,25(OH)$_2$D$_3$ occurred by hydroxylation at the 24 position by 24-OHase (Reinhardt and Horst, 1989). Expression of 24-OHase normally is up-regulated by 1,25(OH)$_2$D$_3$ as a means to limit the concentration of 1,25(OH)$_2$D$_3$ (Goff et al., 1992). The same regulation of 24-OHase expression occurs in non-activated bovine monocytes. Activation of monocytes with LPS, blocks induction of 24-OHase expression by 1,25(OH)$_2$D$_3$. Without 24-OHase, 1,25(OH)$_2$D$_3$ produced in monocytes will not be degraded and continue to regulate gene expression. Inhibition of 24-OHase expression in LPS-activated monocytes seems to amplify the effects
of 1,25(OH)₂D₃ on iNOS and RANTES gene expression. Physiologically, this may be another means to increase the concentration of 1,25(OH)₂D₃ at the site of infection and increase the expression of iNOS and RANTES and possibly other genes as well. The specifics of this observation will have to be studied further to better understand the physiological impact.

A major difference exists between humans and cattle in regards to the effects of 1,25(OH)₂D₃ on the innate immune response. In human monocytes, production of 1,25(OH)₂D₃ increased the expression of cathelicidin, which enhanced killing of intracellular Mycobacteria tuberculosis (Liu et al., 2007). In contrast, the bovine cathelicidin genes with potential VDREs in their promoters were not affected by 1,25(OH)₂D₃. Regulation of cathelicidin expression by 1,25(OH)₂D₃ does not occur in mice either (Gombart et al., 2005) and is thought to be primate specific (Gombart et al., 2009).

In cattle, production of 1,25(OH)₂D₃ by monocytes enhanced production of nitric oxide by increasing the expression of iNOS. Nitric oxide is known to have several effects physiologically and was considered a fundamental component of the antimicrobial response (Bogdan, 2001). Studies with iNOS-deficient mice revealed that production of nitric oxide aided in the resolution of M. tuberculosis and M. bovis infections (MacMicking et al., 1997, Waters et al., 2004). Nitric oxide is not necessary for killing of intracellular M. tuberculosis in human macrophages though (Thoma-Uszynski et al., 2001), and no studies have definitively shown that nitric oxide is necessary for bovine macrophages to kill bacteria. The primary function of nitric oxide in the bovine immune response, therefore, may be regulation of blood flow (Lacasse et al., 1996) and signaling (Bansal et al., 2009) rather than antimicrobial. Regardless, nitric oxide production occurred during the course of several
major diseases of cattle such as Johne’s disease (Waters et al., 2003), mastitis (Blum et al., 2000, Bouchard et al., 1999), and tuberculosis (Palmer et al., 2007). The concentration of 25(OH)D₃ in cattle then may have considerable implications in such diseases because nitric oxide production in monocytes is dependent on 25(OH)D₃ concentration in vitro.

It was shown that 1,25(OH)₂D₃ production in bovine monocytes increased RANTES gene expression. Also known as chemokine (C-C motif) ligand 5 (CCL5), RANTES is a chemo-attractant for T-helper cells and monocytes to the site of inflammation (Schall, 1991). RANTES was implicated in the clearance of viral infections in humans, likely by the recruitment of other immune cells to the site of infection (Levy, 2009). Little information exists on the importance of RANTES in the bovine immune response and most of what is known about the chemokine is drawn from studies in other species. RANTES expression was induced by TLR signaling in cattle (Pareek et al., 2005, Widdison et al., 2008). Based on evidence from this study, induction of RANTES expression in bovine monocytes was mediated by production of 1,25(OH)₂D₃ in monocytes in response to TLR signaling.

Finally, this study provides evidence that vitamin D status of cattle is important for proper immune function. It is clear that RANTES and iNOS expression and nitric oxide production in activated monocytes increased with 25(OH)D₃ concentration up to 100 ng/mL in vitro. The concentration of serum 25(OH)D₃ in cattle supplemented with the recommended amount of dietary vitamin D typically ranges from 20 to 50 ng/mL (McDermott et al., 1985). Serum 25(OH)D₃ concentrations above 50 ng/mL can be reached by additional supplementation, so it may be possible to boost RANTES expression and nitric oxide production during an immune response in cattle. When the 25(OH)D₃ concentration exceeds 200 ng/mL in serum, calcification of soft tissue occurred (Horst et al., 1994).
Current dietary recommendations for vitamin D supplementation of cattle are largely based on the amount of vitamin D needed to maintain proper mineral homeostasis in dairy cattle, not for proper immune function. Therefore, future studies are needed to determine what concentration of 25(OH)D₃ in cattle is needed for proper immune function.

Acknowledgments

The authors thank Randy Atchison, Duane Zimmerman, and Derrel Hoy for their technical assistance.

References


## Tables

### Table 1. Primer sequences for real-time PCR

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\(^1\) Primer sequence from (Aalberts et al., 2007).
Figure 1. Effects of lipopolysaccharide (LPS) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) treatment on gene expression in bovine monocytes. Monocytes were isolated from peripheral blood of 4 cows and treated with 100 ng/mL LPS and 4 ng/mL 1,25(OH)₂D₃ as indicated for 24 h. Relative expression of (A) 24-hydroxylase (24-OHase), cathelicidin (CATH) 4, 5 and 6, and (B) inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), RANTES, and S100A12 was determined using real-time PCR and the 2⁴∆∆Ct method. The mean fold increase shown for each gene is relative to the non-treated control. Error bars represent SE, n = 4. Means with different letters are different, P < 0.05.
Figure 2. Effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) dose on lipopolysaccharide-stimulated monocytes. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL lipopolysaccharide and 0 to 4 ng/mL of 1,25(OH)₂D₃ as indicated for 24 h. Relative expression of RANTES (A) and inducible nitric oxide synthase (iNOS; B) was determined using real-time PCR and the 2^{ΔΔCT} method. The mean fold increase shown for RANTES and iNOS expression is relative to the non-treated control. (C) Nitric oxide production was determined by measuring the amount of nitrite in the culture supernatant with the Griess assay. Error bars represent SE, n = 6. Means with different letters are different, $P < 0.05$. 

(A) RANTES mRNA

(B) iNOS mRNA

(C) Nitrite
**Figure 3.** Toll-like receptor signaling induces $1\alpha$-hydroxylase ($1\alpha$-Ohase) expression in bovine monocytes. Monocytes were isolated from peripheral blood of 6 cows and treated with 100 ng/mL lipopolysaccharide (LPS), 5 µg/mL Pam3CSK4 (Pam3), or 5 µg/mL peptidoglycan (PGN) for 24 h. Relative expression of $1\alpha$-OHase was determined using real-time PCR and the $2^{\Delta\Delta Ct}$ method. The mean fold increase shown for $1\alpha$-OHase expression is relative to the non-stimulated control. Error bars represent SE, n = 6. ***Mean is different from control, $P < 0.001$. 
Figure 4. RANTES, inducible nitric oxide synthase (iNOS) and nitric oxide increase with 25-hydroxyvitamin D₃ (25(OH)D₃) dose. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL lipopolysaccharide (LPS) and 0 to 100 ng/mL 25(OH)D₃ as indicated for 24 h. Relative expression of RANTES (A) and iNOS (B) was determined using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. The mean fold increase shown RANTES and iNOS expression is relative to the non-treated control. (C) Nitric oxide production was determined by measuring the amount of nitrite in the culture supernatant with the Griess assay. Error bars represent SE, n = 6. Means with different letters are different, $P < 0.05$. *Linear effect, $P < 0.001$. 
Figure 5. RANTES, inducible nitric oxide synthase (iNOS) and nitric oxide are dependent on 1α-hydroxylase (1α-Ohase) activity. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL lipopolysaccharide (LPS), 75 ng/mL 25-hydroxyvitamin D₃ (25(OH)D₃), 5 µg/mL ketoconazole, and 0.4 ng/mL of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) as indicated for 24 h. Relative expression of 1α-OHase (A), RANTES (B) and iNOS (C) was determined using real-time PCR and the 2⁻ΔΔCt method. The mean fold increase shown for each gene is relative to the non-treated control. (D) Nitric oxide production was determined by measuring the amount of nitrite in the culture supernatant with the Griess assay. Error bars represent SE, n = 6. Means with different letters are different, P < 0.05.
CHAPTER 3. PRODUCTION OF 1,25-DIHYDROXYVITAMIN D₃ BY 1α-HYDROXYLASE IN CD14+ CELLS DIRECTS PERIPHERAL BLOOD MONONUCLEAR CELL IMMUNE RESPONSES

A paper to be submitted to the journal of Veterinary Immunology and Immunopathology

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Abstract

Studies in several species have shown that vitamin D contributes to immune function besides the well-known role it has in regulation of calcium homeostasis. We have previously shown that activated bovine monocytes express 1α-hydroxylase (1α-Ohase), the enzyme that converts 25-hydroxyvitamin D₃ (25(OH)D₃) to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). Production of 1,25(OH)₂D₃ in monocytes up-regulated iNOS and RANTES gene expression. In other studies, IFN-γ and IL-17A production in stimulated PBMC cultures was down-regulated by treatment with exogenous 1,25(OH)₂D₃. In this study we wanted to determine if the monocytes in stimulated PBMC cultures expressed 1α-OHase and if addition of 25(OH)D₃ to stimulated PBMC cultures could regulate IFN-γ, IL-17A, and IL-17F.

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expression in addition to iNOS and RANTES expression. PBMCs from steers that were vaccinated with *Mycobacterium bovis* (*M. bovis*) BCG were stimulated with a purified protein derivative of *M. bovis* (*M. bovis* PPD) and gene expression was measured by real-time PCR. Stimulation with *M. bovis* PPD induced 1α-OHase gene expression in PBMC cultures. 1α-OHase expression was induced in CD14+ cells, but not in CD14- cells. Treatment of stimulated PBMC cultures with 1,25(OH)\(_2\)D\(_3\) or 25(OH)D\(_3\) up-regulated iNOS and RANTES expression. In contrast, both 1,25(OH)\(_2\)D\(_3\) and 25(OH)D\(_3\) down-regulated IFN-\(\gamma\), IL-17A and IL-17F gene expression in stimulated PBMC cultures. In conclusion, it appears that conversion of 25(OH)D\(_3\) to 1,25(OH)\(_2\)D\(_3\) by 1α-OHase in monocytes can regulate IFN-\(\gamma\), IL-17A, and IL-17F responses to stimulation in PBMCs.

**Introduction**

Vitamin D is best known for the role it has in calcium homeostasis through the endocrine system. However, in recent years it has become evident that vitamin D also has an important role in the immune system. Vitamin D status has been linked to the autoimmune disorders multiple sclerosis, inflammatory bowel disease, and type 1 diabetes (Cantorna and Mahon 2004, Hayes 2000). Serum 25-hydroxyvitamin D\(_3\) (25(OH)D\(_3\)) concentrations, the primary measure of vitamin D status, also were inversely correlated with the number of upper respiratory track infections in people (Ginde, Mansbach and Camargo 2009). In other studies, the risk of tuberculosis and influenza A infection were reduced by vitamin D supplementation (Martineau et al. 2007, Urashima et al. 2010). The link between vitamin D status and microbial infections in people is explained by a direct effect of vitamin D
signaling has on immune function (Adams and Hewison 2010). Therefore, understanding the mechanisms of vitamin D signaling in the bovine immune system has implications for viral and bacterial diseases in cattle.

The vitamin D metabolite that regulates gene expression is 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) (Hewison 1992, Reinhardt and Hustmyer 1987). 1,25(OH)$_2$D$_3$ is the primary ligand for the vitamin D receptor (Reinhardt, Ramberg and Horst 1989). The VDR controls the expression of genes that have promoters with accessible vitamin D response elements when activated by 1,25(OH)$_2$D$_3$ (Koszewski, Reinhardt and Horst 1996, Pike et al. 2007). The VDR is expressed in many tissues and cells throughout the body, including cells of the immune system (Provvedini et al. 1983, Veldman, Cantorna and DeLuca 2000). Examination of the human and murine genomes has revealed that nearly one thousand genes may be controlled through the actions of 1,25(OH)$_2$D$_3$ (Wang et al. 2005).

A number of genes with known immune function are regulated by 1,25(OH)$_2$D$_3$. In human monocytes and macrophages, 1,25(OH)$_2$D$_3$ enhances antimicrobial activity by inducing cathelicidin and defensin beta 4 gene expression (Gombart, Borregaard and Koeffler 2005, Liu et al. 2009, Wang et al. 2004). In activated bovine monocytes, 1,25(OH)$_2$D$_3$ enhanced inducible nitric oxide synthase (iNOS) and regulated upon activation, normally T-cell expressed, and secreted (RANTES/CCL5) gene expression (Nelson et al. 2010). It has been shown on multiple occasions that 1,25(OH)$_2$D$_3$ suppresses antigen induced IFN-γ production in peripheral blood mononuclear cell (PBMC) cultures from cattle (Ametaj et al. 1996, Nonnecke et al. 2003, Waters et al. 2001). Recently, 1,25(OH)$_2$D$_3$ was shown to suppress IL-17A production by human CD4 positive T-cells (Jeffery et al. 2009, Tang et al. 2009). IFN-γ and IL-17A have been associated with the adaptive immune...
response to *Mycobacteria bovis* (*M. bovis*) in cattle (Thacker, Palmer and Waters 2007, Vordermeier et al. 2009), so regulation of those genes by 1,25(OH)$_2$D$_3$ has implications in bovine tuberculosis.

Regulation of vitamin D responsive genes is dependent on the concentration of 1,25(OH)$_2$D$_3$. In the vitamin D endocrine system, 1,25(OH)$_2$D$_3$ is produced from circulating 25-hydroxyvitamin D$_3$ (25(OH)D$_3$) by 1α-hydroxylase (1α-Ohase) in the kidney (Horst, Goff and Reinhardt 2005). Control of the circulating 1,25(OH)$_2$D$_3$ concentration occurs by tight regulation of 1α-OHase expression in the kidney mainly in response to calcium homeostasis via the parathyroid hormone (Engstrom et al. 1987). However, several studies from over two decades ago showed that macrophages produce 1,25(OH)$_2$D$_3$ (Adams et al. 1983, Koeffler et al. 1985). Now, evidence shows that regulation of immune responses by 1,25(OH)$_2$D$_3$ occurs through intracrine and paracrine mechanisms of vitamin D signaling rather than through the vitamin D endocrine system (Hewison 2010). In the case of bovine tuberculosis 1,25(OH)$_2$D$_3$ was found to accumulate in granulomas (Rhodes et al. 2003). We have found that bovine monocytes express 1α-OHase in response to toll-like receptor (TLR) signaling (Nelson et al. 2010). Production of 1,25(OH)$_2$D$_3$ from 25(OH)D$_3$ in monocytes up-regulated monocyte iNOS and RANTES expression. In human monocytes, an intracrine mechanism of vitamin D signaling was shown to control cathelicidin and defensin beta 4 expression (Liu et al. 2009, Liu et al. 2006). In addition to monocytes, activated B-cells and T-cells also have been shown to express 1α-OHase (Chen et al. 2007, Sigmundsdottir et al. 2007), so conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ is possible in several types of immune cells.
Evidence suggests that regulation of innate and adaptive immune responses by 1,25(OH)$_2$D$_3$ occurs through production of 1,25(OH)$_2$D$_3$ by 1α-OHase in activated immune cells (Hewison 2010). However, in cattle it is not known if immune cells, other than monocytes, express 1α-OHase upon activation. Conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ by 1α-OHase in monocytes regulated iNOS and RANTES gene expression, but regulation of gene expression by conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ in PBMC cultures is not known. Therefore, the objectives of this study were to 1) evaluate 1α-OHase gene expression in PBMC cultures and 2) determine the effects of 25(OH)D$_3$ on iNOS, RANTES, IFN-γ, IL-17A, and IL-17F in PBMC cultures.

**Materials and Methods**

**Animals**

A group of 10 adult Holstein steers at the USDA National Animal Disease Center (NADC; Ames, IA) were used for this study. The NADC animal care and use committee approved the care and treatment of animals for this study. The steers were vaccinated with *Mycobacterium bovis* bacilli Calmette-Guerin (BCG) by subcutaneous injection 5 months prior to the experiments performed in this study.

**Peripheral blood mononuclear cell cultures**

Blood from the jugular vein was collected in 2X acid citrate dextrose. Blood was centrifuged and buffy coats were collected. Contaminating RBCs were removed by hypotonic lysis. PBMCs were isolated by density gradient centrifugation. PBMC were
resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 50 µg/ml gentamicin (Invitrogen, Carlsbad, CA). PBMCs were cultured at a concentration of $10^7$ cells/ml in 96-well tissue culture plates or T-25 tissue culture flasks for 24 h at 37°C in 5% CO$_2$.

Purified protein derivative from *M. bovis* (*M. bovis* PPD) was added at 10 µg/ml to PBMC cultures. The vitamin D metabolites 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ (Sigma-Aldrich) were diluted to 100 ng/ml in 100% ethanol and added to fetal bovine serum (FBS; Hyclone, Waltham, MA) at 10X the final desired concentration. The concentrations of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ in ethanol were confirmed by UV spectroscopy. FBS with and without the vitamin D metabolites was added to PBMC cultures to a final concentration of 10% FBS.

**Cell sorting**

PBMCs from six steers were stimulated with *M. bovis* PPD for 16 h in T-25 flasks. Cells were removed from the flasks with cold PBS. Cells were washed and labeled with mouse anti-bovine CD14 IgG$_1$ antibody (CAM36A; VMRD, Pullman, WA) and a PE-labeled antibody against mouse IgG$_1$ (Southern Biotech, Birmingham, AL). Labeled cells were separated based on fluorescence intensity using the BD FACSAria Cell Sorting System (BD Biosciences, San Jose, CA). Approximately $10^6$ CD14- and CD14+ cells were collected from each PBMC culture.

**Relative gene expression**

RNA was isolated from PBMC using the Rneasy Mini Kit (Qiagen, Valencia, CA). RNA samples were reverse transcribed to cDNA in 20 µl reactions using the High Capacity
Reverse Transcription Kit with Rnase inhibitor and random primers (Applied Biosystems, Foster City, CA). The reverse transcription reactions were incubated for 2 h at 37°C followed by 5 s at 85°C and finally cooled to 4°C. The cDNA samples were diluted 1:10 in water and stored at -20°C.

The amount of specific cDNA transcripts in each sample was determined using the 7300 Real-Time PCR System (Applied Biosystems). Each reaction contained 12.5 µl SYBR Green Master Mix (Applied Biosystems), 7.5 µl of cDNA sample, and 5 µl of 10 µM forward and 10 µM reverse primers. Reactions were incubated as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Primer sets were designed with Primer3 (http://frodo.wi.mit.edu/primer3) (Rozen and Skaletsky 2000) to span intron-exon boundaries and are listed in Table 1. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The efficiency of each primer set was determined as previously described (Nelson et al. 2010) and fit the criteria required for quantification by real-time PCR (Livak and Schmittgen 2001). The specificity of each primer set was verified by melting curve analysis and gel electrophoresis. The amounts of cDNA transcripts were normalized to ribosomal protein S9 (RPS9) cDNA. The relative expression of each gene was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expression of each gene is relative to the normalized amount of each cDNA transcript in the non-stimulated controls for each experiment.
**Statistical Analysis**

Analysis of variance was performed using PROC GLM of SAS (SAS Institute INC., Cary, NC). The model accounted for effects of treatment and steer. For analysis of gene expression, ΔΔCt values were used in the analyses. The average ΔΔCt values ± SE was transformed using the equation $2^{\Delta\Delta Ct}$. The expression of each gene is presented as the mean fold increase ± SE relative to non-stimulated controls. Multiple comparison tests of the means were made using the Tukey adjustment.

**Results**

*1α-OHase expression*

PBMC cultures were treated with a purified protein derivative of *M. bovis* (*M. bovis* PPD) for 24 hrs and 1α-OHase gene expression was measured by qPCR. 1α-OHase gene expression in the stimulated PBMC cultures was 8 fold higher than in the non-stimulated cultures (Fig. 1). *M. bovis* PPD stimulated PBMCs also were sorted according to surface expression of CD14 by FACS to find which population of cells were expressing 1α-OHase (Fig. 2B-D). 1α-OHase gene expression was 80 fold higher in CD14+ cells compared to CD14- cells from the stimulated PBMC cultures (Fig 2A). Furthermore, 1α-OHase expression in the CD14- fraction was not different from 1α-OHase expression in non-stimulated PBMCs.
**Regulation of PBMC responses by vitamin D signaling**

The biologically active metabolite of vitamin D₃ is 1,25(OH)₂D₃ and the enzymatic activity of 1α-OHase synthesizes 1,25(OH)₂D₃ from 25(OH)D₃. Circulating concentrations of 25(OH)D₃ and 1,25(OH)₂D₃ in cattle under normal conditions can range from 20 to 100 ng/ml and 5 to 50 pg/ml, respectively (Horst, Goff and Reinhardt 1994). PBMC cultures were treated with M. bovis PPD and 100 ng/ml 25(OH)D₃, 400 pg/ml of 1,25(OH)₂D₃, or 0.04% ethanol (vehicle) for 24 hrs and iNOS, RANTES, IFN-γ, IL-17A, and IL-17F gene expression was measured. iNOS and RANTES gene expression was significantly higher in PBMC cultures that were treated with M. bovis PPD and 25(OH)D₃ or 1,25(OH)₂D₃ than in cultures that were treated with M. bovis PPD alone. (Fig. 3A and B). Whereas, IFN-γ, IL-17A, and IL-17F gene expression was significantly lower in PBMC cultures that were treated with M. bovis PPD and 25(OH)D₃ or 1,25(OH)₂D₃ than in cultures that were treated with M. bovis PPD alone (Fig. 3C-E).

**iNOS, RANTES, IFN-γ, IL-17A, and IL-17F expression in CD14+and CD14- cells**

The CD14+ and CD14- cells from M. bovis-stimulated PBMC cultures that were evaluated for 1α-OHase expression (Fig. 2) also were evaluated for iNOS, RANTES, IFN-γ, IL-17A, and IL-17F expression (Fig. 4). Like 1α-OHase, iNOS gene expression was much greater in the CD14+ cells than in the CD14- cells (Fig. 4A). In contrast, IFN-γ gene expression was much greater in the CD14- cells than in the CD14+ cells (Fig. 4C). RANTES, IL-17A and IL-17F expression was greater in the CD14- than in the CD14+ cells, but the difference was not significant (Fig. 4B, D and E).
Discussion

Several studies have shown that 1,25(OH)$_2$D$_3$ regulates genes that are important for innate and adaptive immunity in cattle (Nelson et al. 2010, Waters et al. 2001). Expression of 1α-OHase in immune cells allows for intracrine and paracrine control of 1,25(OH)$_2$D$_3$-mediated immune responses rather than endocrine control. In this study, we showed that 1α-OHase expression was induced in the CD14+ cells (monocytes) in PBMC cultures that were stimulated with *M. bovis* PPD. Subsequently, addition of 25(OH)D$_3$ regulated expression of several genes that have an important role in immune function. In a previous study we showed that regulation of gene expression in monocytes by treatment with 25(OH)D$_3$ depended on conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ by 1α-OHase in monocytes (Nelson et al. 2010). Therefore, regulation of gene expression by addition of 25(OH)D$_3$ in this study evidently occurred by synthesis of 1,25(OH)$_2$D$_3$ by 1α-OHase in monocytes.

Data from this study indicates that synthesis of 1,25(OH)$_2$D$_3$ by 1α-OHase in monocytes up-regulated iNOS and RANTES expression in stimulated PBMC cultures. Previously we showed that synthesis of 1,25(OH)$_2$D$_3$ in monocytes up-regulated iNOS and RANTES expression in monocytes (Nelson et al. 2010). However, RANTES was originally discovered as a T-cell cytokine (Schall et al. 1988), so in PBMC cultures 1,25(OH)$_2$D$_3$ produced in monocytes also may act on T-cells to up-regulate RANTES expression. Up-regulation of RANTES may enhance attraction of other immune cells to the site of an infection, whereas, up-regulation of iNOS may enhance nitric oxide-mediated antimicrobial activity and signaling of macrophages (Bogdan 2001, Levy 2009).
In contrast to iNOS and RANTES, 25(OH)D₃ down-regulated IFN-γ expression in stimulated PBMC cultures. Several previous studies have shown that exogenous 1,25(OH)₂D₃ down-regulated antigen-specific IFN-γ expression in PBMCs (Ametaj et al. 1996, Nonnecke et al. 2003, Waters et al. 2001). However, this is the first study to give evidence that endogenous synthesis of 1,25(OH)₂D₃ down-regulates IFN-γ expression in PBMCs. IFN-γ is considered a T-cell cytokine and is associated with disease severity in bovine tuberculosis (Thacker et al. 2007, Vordermeier et al. 2002). Therefore, synthesis of 1,25(OH)₂D₃ in monocytes may have an important role in the pathogenesis of tuberculosis by regulating IFN-γ expression in T-cells.

Like IFN-γ, IL-17A and IL-17F were down-regulated by 25(OH)D₃. Down-regulation of IL-17A by 1,25(OH)₂D₃ has been observed in human T-cells (Jeffery et al. 2009), but this is the first study to show that either 25(OH)D₃ or exogenous 1,25(OH)₂D₃ down-regulate IL-17A and IL-17F responses in cattle. IL-17 cytokines are predominantly expressed by T_{H17}-cells but also are known to be expressed by γδT-cells (Dong 2008). IL-17A and IL-17F induce antimicrobial and chemokine responses in fibroblasts and epithelial cells and are involved in the recruitment and maturation of neutrophils in mice (Kao et al. 2004, Laan et al. 1999, Chang and Dong 2009). In cattle, IL-17A expression in vitro positively correlated with protection against bovine tuberculosis in vaccinated animals (Vordermeier et al. 2009). Synthesis of 1,25(OH)₂D₃ in monocytes, then, also may have a critical role in the pathogenesis of tuberculosis by regulating IL-17A and IL17F expression.

An important environmental factor that could affect regulation of immune responses by 1,25(OH)₂D₃ is acquisition of vitamin D₃. Vitamin D₃ is acquired in the diet or by
conversion from 7-dehydrocholesterol in skin exposed to ultra-violet B (Horst and Reinhardt 1983). Acquisition of vitamin D₃ affects the circulating concentration of 25(OH)D₃. In this study we showed that expression of several genes that have a role in immune function were affected by treatment with a physiological concentration of 25(OH)D₃. Current recommendations for vitamin D in cattle target a range of 20 to 50 ng/ml of 25(OH)D₃ in plasma (NRC 2001). Vitamin D requirements in cattle are based on vitamin D signaling in the endocrine system and control of calcium homeostasis. Plasma 25(OH)D₃ concentrations below 20 ng/ml are considered deficient for maintenance of the vitamin D endocrine system. However, the minimal requirement for vitamin D may be higher for the immune system. In fact, plasma 25(OH)D₃ concentrations below 30 ng/ml in humans are now considered insufficient for proper vitamin D signaling in the immune system (Adams and Hewison 2010, Holick 2008, Hollis 2005). Therefore, the requirement for vitamin D in cattle needs to be re-evaluated to account for the effects of vitamin D status on the immune system.

**Conclusion**

Evidence from this study suggests that vitamin D signaling in the immune system controls several key aspects of innate and adaptive immunity in cattle. Therefore, vitamin D signaling in the immune system and vitamin D status may be important factors in the defense against viral and bacterial diseases in cattle. However, more investigation is needed to define vitamin D requirements for proper immune function and to find other genes that are regulated by 1,25(OH)₂D₃ in the immune system.
Acknowledgements

We thank Bruce Pesch, Derrel Hoy, Duane Zimmerman, and Randy Atchison (National Animal Disease Center, USDA, Ames, IA) for their technical assistance.

References


### Table 1. Primer sequences for real-time PCR.

<table>
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<th>Gene</th>
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<td>Reverse</td>
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<td>Reverse</td>
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1 Accession numbers for mRNA sequences from NCBI database.
2 Primer sequences have been published previously (Nelson et al. 2010).
3 Primer sequences have been published previously (Raffatellu et al. 2007).
Figure 1. 1α-hydroxylase (1α-Ohase) gene expression in PBMC cultures. PBMC cultures from 8 steers were treated with 0 or 10 µg/ml of *M. bovis* purified protein derivative (PPD) as indicated for 24 h. The amount of 1α-OHase mRNA was determined by quantitative real-time RT-PCR and was normalized to the amount of RPS9 mRNA in each sample. Expression of 1α-OHase is relative to non-stimulated cultures. Error bars represent SE. *** Mean is different from non-stimulated PBMC; *P* < 0.001.
Figure 2. Evaluation of 1α-hydroxylase (1α-Ohase) gene expression in PBMC sub-populations. PBMC cultures from 6 steers were treated with 10 µg/ml of M. bovis PPD for 24 h and sorted by FACS according to CD14 expression on the cell surface. (A) The amount of 1α-OHase mRNA was determined by quantitative real-time RT-PCR and was normalized to the amount of RPS9 mRNA in each sample. Expression of 1α-OHase is relative to 1α-OHase expression in non-stimulated cultures. Error bars represent SE. *** Mean is different from CD14- cells; * * * Mean is different from CD14- cells: P < 0.001. (B-D) Representative histogram and scatter plots of stimulated PBMCs labeled with the CD14 antibody (CAM36A) and the PE conjugated secondary antibody.
Figure 3. Effects of 1,25(OH)_2D_3 and 25(OH)D_3 on gene expression in PBMC cultures. PBMC cultures from 8 steers were treated with 0 or 10 µg/ml of *M. bovis* purified protein derivative (PPD), 400 pg/ml 1,25(OH)_2D_3 and 100 ng/ml of 25(OH)D_3 as indicated for 24 h. The amount iNOS, RANTES, IFN-γ, IL17A, IL-17F mRNA was determined by quantitative real-time RT-PCR. Each gene was normalized to the amount of RPS9 mRNA in each sample. Expression of each gene is relative to non-stimulated cultures. Error bars represent SE, n = 8. * Mean is different from PBMC cultures treated with *M. bovis* PPD alone.
Figure 4. PBMC cultures from 6 steers were treated with 10 µg/ml of *M. bovis* purified protein derivative (PPD) and sorted by FACS according to CD14 expression on the cell surface as shown in figure 2. The amount of iNOS, RANTES, IFN-γ, IL-17A, and IL-17F was determined by quantitative real-time RT-PCR. The amount of mRNA for each gene was normalized to the amount of RPS9 mRNA in each sample. Expression of each gene is relative to non-stimulated cultures. Error bars represent SE. * Means are different; $P < 0.001$. 
CHAPTER 4. CD14+ CELLS IN THE BOVINE MAMMARY GLAND EXPRESS 25-HYDROXYVITAMIN D 1α-HYDROXYLASE DURING BACTERIAL MASTITIS

A paper to be submitted for publication

Corwin D. Nelson\textsuperscript{1,2}, Timothy A. Reinhardt\textsuperscript{1}, Donald C. Beitz\textsuperscript{2,3}, John D. Lippolis\textsuperscript{1}

Abstract

The active vitamin D metabolite, 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}), is an important regulator of immune function. The enzyme that synthesizes 1,25(OH)\textsubscript{2}D\textsubscript{3} from 25-hydroxyvitamin D\textsubscript{3} is 1α-hydroxylase (1α-Ohase; CYP27B1). Several in vitro studies have shown that TLR signaling induces expression of 1α-OHase in monocytes and macrophages. Synthesis of 1,25(OH)\textsubscript{2}D\textsubscript{3} in monocytes and macrophages presumably allows for local control of 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated gene expression. However, induction of 1α-OHase expression at the site of an infection has not been shown. Using \textit{Streptococcus uberis} mastitis in cattle as a model of bacterial infection, we measured 1α-OHase gene expression in mammary tissue and cells isolated from the milk during mastitis. In tissue and secreted cells from the infected mammary glands, 1α-OHase gene expression was much higher compared to expression in tissue and cells from the healthy glands. Separation of the cells by fluorescence activated cell sorting (FACS), revealed that 1α-OHase was predominantly expressed in the CD14\textsuperscript{+} cells from the infected gland. Finally, 24-hydroxylase, inducible

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nitric oxide synthase (iNOS), and RANTES (regulated upon activation, normally T-cell expressed and secreted; CCL5), genes that are upregulated by 1,25(OH)\(_2\)D\(_3\) in cattle, were expressed significantly more in tissue and cells from the infected glands than from the healthy glands. In conclusion, 1\(\alpha\)-OHase is upregulated in CD14\(^+\) cells in an infected mammary gland during mastitis and seems to provide 1,25(OH)\(_2\)D\(_3\) for local regulation of 1,25(OH)\(_2\)D\(_3\)-mediated immune responses. To our knowledge, this study provides the first in vivo evidence that 1\(\alpha\)-OHase expression is upregulated in macrophages in response bacterial infection.

**Introduction**

Vitamin D has been shown to have a role in regulating immune function in addition to the well-known role it has in regulating calcium homeostasis. 1,25-dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)), the active vitamin D\(_3\) metabolite, regulates the expression of several genes involved in host defense and immune function (Adams and Hewison, 2010). Therefore, synthesis of 1,25(OH)\(_2\)D\(_3\) to control vitamin D responsive genes in immune cells is a critical factor in regulating immune function.

The enzyme that synthesizes 1,25(OH)\(_2\)D\(_3\) from 25-hydroxyvitamin D\(_3\) (25(OH)D\(_3\)), the major vitamin D\(_3\) metabolite in plasma, is 1\(\alpha\)-hydroxylase (1\(\alpha\)-Ohase; CYP27B1) (Horst et al., 2003). In the kidney, 1\(\alpha\)-OHase expression is induced by parathyroid hormone in response to calcium homeostasis (DeLuca, 1975; Engstrom et al., 1987). Synthesis of 1,25(OH)\(_2\)D\(_3\) in the kidney regulates the circulating concentration of 1,25(OH)\(_2\)D\(_3\) and the endocrine actions of vitamin D. In monocytes and macrophages, 1\(\alpha\)-OHase is expressed in
response to activation by IFN-γ or TLR signaling (Krutzik et al., 2008; Liu et al., 2006; Nelson et al., 2010; Stoffels et al., 2007). Conversion of 25(OH)D₃ to 1,25(OH)₂D₃ by 1α-OHase in monocytes regulates the expression of vitamin D responsive genes in an intracrine and paracrine manner (Hewison, 2010). In human monocytes, production of 1,25(OH)₂D₃ by 1α-OHase drives cathelicidin gene expression (Liu et al., 2006). In the same way, 1α-OHase activity in bovine monocytes enhances iNOS and RANTES gene expression (Nelson et al., 2010). From in vitro studies, expression of 1α-OHase by macrophages at the site of an infection seems to be an important part of innate immunity. However, no studies have shown that 1α-OHase is expressed at the site of a bacterial infection.

Mastitis in cattle offers a model of bacterial infection to determine if 1α-OHase is expressed in response to bacterial infection in vivo. Mastitis is defined as inflammation of the mammary gland and is most often caused by bacterial infection (Bradley, 2002). Common pathogens that cause mastitis include *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus uberis* (Hogan and Larry Smith, 2003; Olde Riekerink et al., 2008; Petrovski et al., 2009; Sampimon et al., 2009). During acute mastitis the number of somatic cells secreted in milk will often exceed 10⁶ cells/mL. Approximately 80 to 90 percent of somatic cells in milk from an infected gland are neutrophils and the remainder of the cells are macrophages and lymphocytes (Riollet et al., 2000). The advantage of using mastitis is that the infiltrating cells during mastitis can easily isolated from milk using non-invasive procedures; allowing us to study the in vivo immune responses of immune cells to bacterial infection.

TLRs are present in the bovine mammary gland (Reinhardt and Lippolis, 2006) and invasion of the mammary gland by bacteria triggers an innate immune response by TLR
signaling (De Schepper et al., 2008). Based on in vitro evidence that 1α-OHase expression in macrophages is induced by TLR recognition of bacteria (Liu et al., 2006; Nelson et al., 2010; Stoffels et al., 2006), we hypothesized that 1α-OHase expression would be upregulated in macrophages during mastitis. Using mastitis in cattle as a model of bacterial infection, we give the first in vivo evidence that 1α-OHase expression is upregulated in CD14+ cells that are at the site of a bacterial infection.

Materials and Methods

Animals

Eight, mid-lactation Holstein cows at the USDA National Animal Disease Center were used for this study. The National Animal Disease Center Animal Care and Use Committee approved all procedures used in this study. Prior to the study, all cows were healthy and bacteria were not detected in their milk. Mastitis was induced by infusion of 500 cfu of Streptococcus uberis strain 0140 (S. uberis; a gift from Dr. Max Paape, USDA, Beltsville, MD) suspended in 3 mL of PBS into one mammary gland. The contra-lateral gland was infused with an equal volume of PBS and served as the control. The amount of S. uberis in the milk from the control and infected glands was determined by culturing log dilutions of milk samples on blood agar plates for 24 hours at 37°C.
Collection of tissue and cells

Mammary tissue was collected from various locations in the control and infected glands of three cows that were euthanized at the onset of clinical mastitis. Clinical mastitis was defined by rectal temperature, noticeable inflammation, and presence of bacteria in the milk. Tissue was placed in RNAlater (Qiagen, Valencia, CA), snap frozen in liquid nitrogen and stored at -80°C.

Cells were isolated from milk from the control and infected glands and peripheral blood of 5 cows before infection with *S. uberis* and at the onset of clinical mastitis. Cells were isolated from milk by centrifuging the milk at 1000Xg for 20 min. Peripheral blood leukocytes were isolated by lysing the erythrocytes with a hypotonic buffer and centrifuging at 650Xg for 10 min. The cell pellets from milk and blood were washed 3X by resuspending in cold PBS and centrifuging at 650Xg for 10 min. Cells were lysed with RLT buffer (Qiagen) and stored at -80°C or separated by fluorescence activated cell sorting (FACS).

For separation of cells from blood and milk by FACS, cells were labeled with monoclonal anti-bovine CD14 IgG\(_1\) (CAM36A; VMRD, Inc., Pullman, WA) and a PE-conjugated anti-mouse IgG antibody (Southern Biotech, Birmingham, AL). Labeled cells were separated based on fluorescence intensity using the BD FACS\(\text{\textcopyright}\)Aria Cell Sorting System (BD Biosciences, San Jose, CA). Approximately 10\(^6\) CD14\(^+\) and CD14\(^-\) cells with greater than 95% purity were isolated from the infected gland of each animal. The sorted cells were lysed with RLT buffer (Qiagen) and stored at -80°C.
**Real-time PCR**

RNA was isolated from mammary tissue and cells by using an Rneasy Mini Kit (Qiagen). RNA samples were eluted in 50 μL of RNase-free water. Immediately after elution, RNA was reverse transcribed to cDNA by using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 10 μL RNA sample and 20 units of Rnase inhibitor (RNaseOUT, Invitrogen, Carlsbad, CA) in a 20-μL reaction. Reactions were incubated at 37°C for 2 h and heated to 85°C for 5 s. The cDNA samples were diluted 1:10 in water and stored at -20°C. Real-time PCR was performed by using a 7300 Real-Time PCR System (Applied Biosystems). The reactions were incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Each reaction contained 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 2.5μL each of 10 μM forward and reverse primers, and 7.5 μL of diluted cDNA. Sequences for primer pairs in 5’ to 3’ order were: 1α-hydroxylase (1α-Ohase/CYP27B1) forward TGGGACCAGATGTTCATTCGC, reverse TTCTCAGACTGGTTCCTCATGGCT; 24-hydroxylase (24-OHase/CYP24A1) forward GAAGACTGGCAGAGGGTCAG, reverse CAGCCAAGACCTCGTTGATT; β-actin forward GGCATCCTGACCCTCAAGTA, reverse CACACGGAGCTCGTTTGTAGA; iNOS forward GATCCAGTGGTCGAACCTGC, reverse CACCCACGTCCAGGAGTATT; RANTES forward CAGTGATGGCGACCTGATG; RPS9 forward GTGAGGTCTGGAGGGTCAAA, reverse GGGCATTACCTTCGGAACAGA; vitamin D receptor (VDR) forward AGCCACCGGCTTCCATTTCA, reverse AACAGCGCCTCCGCTTCAT. Primers were
purchased from Integrated DNA Technologies (Corralville, IA). The specificity of each primer pair was determined by gel electrophoresis of cDNA products and efficiency was determined by using known dilutions of cDNA. All primer pairs, except for the VDR, have been used previously (Nelson et al., 2010). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). RPS9 was used as the reference gene. RPS9 gene expression also was compared to β-actin gene expression and the relative expression of RPS9 did not differ significantly among treatments.

**Statistical analysis**

Statistical analysis was performed by using PROC GLM of SAS (SAS Institute Inc., Cary, NC). The model used in the analysis accounted for effects of treatment and cow. ΔΔCt values were used to analyze relative gene expression. Mean ΔΔCt values ± SE were transformed ($2^{-\Delta\Delta Ct}$) and shown as the expression relative to the control. The control treatment is designated in the figure legends. Multiple comparison tests of the means were made with the Tukey-Kramer adjustment.

**Results**

*Streptococcus uberis mastitis*

On separate occasions, 8 cows were infused with 500 CFUs of *S. uberis* strain 0140 in one mammary gland and sterile PBS in the contra-lateral gland. Bacteria were not detected in milk from any of the glands prior to infection and were not detected in the milk from the control glands during mastitis (Fig. 1A). The average amount of *S. uberis* in the
milk from the infected glands during mastitis was $10^8$ CFU/mL (Fig. 1A). Prior to infection cows had normal body temperatures, but during mastitis body temperatures were elevated (Fig. 1B). The number of somatic cells in the milk from the infected glands rose from an average of $10^5$ cells/mL prior to infection to over $10^7$ cells/mL during mastitis (Fig. 1C). The number of somatic cells in the control glands remained near $10^5$ cells/mL on average during mastitis (Fig. 1C). Examination of mammary tissue by microscopy revealed inflammation and the presence of infiltrating cells in the alveoli of the infected mammary gland (Fig. 1D). The onset of mastitis was typically 3 days after infection with S. uberis; which was consistent with previous studies with S. uberis (Bannerman et al., 2004). Therefore, mastitis was effectively induced in these cows by infusion of S. uberis into the mammary gland.

**1α-Hydroxylase gene expression during mastitis**

At the onset of clinical mastitis, secretory mammary tissue was collected from the control and infected glands of 3 cows. In the tissue from the infected mammary glands, 1α-OHase expression was nearly 50 fold greater than 1α-OHase expression in tissue from the healthy glands ($P < 0.001$; Fig. 2A). The alveoli in the infected mammary tissue were packed with infiltrating cells (Fig. 1D); so, we isolated cells from the milk of control and infected glands to determine the contribution of infiltrating cells on 1α-OHase gene expression during mastitis. In cells isolated from milk from the inflamed glands, 1α-OHase gene expression was 40 fold greater than expression in cells from the healthy gland ($P < 0.001$) and over 300 fold greater than peripheral blood leukocytes ($P < 0.001$) Fig. 2B). Finally, separating the cells according to CD14 expression revealed that 1α-OHase was predominantly expressed in the CD14$^+$ cells from the infected glands ($P < 0.001$ compared to
CD14- cells from the infected gland), but not in CD14+ cells from peripheral blood (Fig. 2C). CD14 is a marker for monocytes and macrophages (Brodersen et al., 1998) and typically 10% of the cells isolated from the milk of the infected gland were CD14+ cells (Fig. 2D – F).

Expression of the VDR and vitamin D responsive genes during mastitis

The effects of 1,25(OH)2D3 on gene expression depend on the presence of the VDR. We measured VDR gene expression to find if it was more abundant in the infected mammary gland. VDR expression was slightly higher in mammary tissue from the infected gland than in tissue from the control gland (Fig. 3A). However, VDR expression in the cells isolated from milk of the infected gland was 8 fold higher than VDR expression in cells from the control gland ($P < 0.05$) and 75 fold higher than VDR expression in peripheral blood leukocytes ($P < 0.001$; Fig. 3B). In the cells from the inflamed gland, there was an insignificant difference of VDR expression between the CD14+ and CD14- populations (Fig. 3C). In both populations though, VDR expression was at least 50 fold greater in the cells from the inflamed gland than in peripheral blood leukocytes ($P < 0.05$).

Expression of the gene for 24-hydroxylase (24-OHase), the enzyme that deactivates 1,25(OH)2D3, is very responsive to 1,25(OH)2D3 (Nelson et al., 2010). In mammary tissue, 24-OHase expression was 50 fold higher in the inflamed glands relative 24-OHase expression in the control glands ($P < 0.001$; Fig. 4A). The difference in 24-OHase expression in cells isolated from milk was not as great, but was still 4 fold higher in cells from the inflamed gland than in cells from the control gland or peripheral blood ($P < 0.05$; Fig. 4B). In our previous study, we observed that 24-OHase expression was not upregulated by 1,25(OH)2D3 in LPS-activated monocytes (Nelson et al., 2010). During mastitis, 24-
OHase expression was not significantly higher in CD14+ cells from the infected glands compared to the CD14+ cells from blood, but was higher in CD14− cells from the infected glands compared to CD14− cells from blood ($P < 0.05$; Fig. 4C).

In activated bovine monocytes, 1,25(OH)$_2$D$_3$ increases expression of iNOS and RANTES (Nelson et al., 2010); so, we measured the expression of both genes in mammary tissue and cells from the control and infected glands (Fig. 4D-I). iNOS and RANTES expression was greater ($P < 0.001$ for iNOS and $P < 0.05$ for RANTES) in tissue and cells from the inflamed glands than in tissue or cells from the control glands. Like 1α-OHase, iNOS was predominantly expressed in the CD14+ cells from the infected gland ($P < 0.001$ compared to CD14− cells from the infected glands). RANTES, however, was expressed more in the CD14− population from the infected glands compared with CD14+ cells from the infected glands ($P < 0.05$).

**Discussion**

Several studies have shown that 1α-OHase expression in monocytes and macrophages is induced by TLR signaling (Liu et al., 2006; Nelson et al., 2010; Stoffels et al., 2006), however, this is the first study to show that 1α-OHase expression is up-regulated at the site of a bacterial infection. Evidence from this study also indicates that CD14+ cells are the predominant cell type that express 1α-Ohase, and subsequently produce 1,25(OH)$_2$D$_3$, in response to bacterial infection. In addition, VDR gene expression was elevated in the cells isolated from the milk from the infected mammary gland; which would seemingly enhance their sensitivity to the 1,25(OH)$_2$D$_3$ produced in the infected mammary gland. Upregulation
of 1α-OHase and VDR expression in the infected mammary glands indicates that regulation of gene expression by 1,25(OH)₂D₃ can be controlled locally by the innate immune system. Regulation of vitamin D signaling in the innate immune system, in that case, is distinct from the endocrine system where 1,25(OH)₂D₃ is produced in the kidney and acts systemically to regulate gene expression.

In the infected mammary glands 24-OHase, iNOS, and RANTES gene expression was upregulated; which gives evidence that 1,25(OH)₂D₃ was produced in the infected mammary gland. Upregulation of 24-OHase, in particular, provides evidence that 1,25(OH)₂D₃ was produced in the mammary gland during mastitis because 24-OHase expression is known to be highly upregulated by 1,25(OH)₂D₃ (Reinhardt et al., 1999; Vaisanen et al., 2005). Similarly, iNOS and RANTES expression are enhanced by 1,25(OH)₂D₃ in activated bovine monocytes (Nelson et al., 2010); so, they are presumably upregulated by 1,25(OH)₂D₃ in the mammary gland. Altogether, 1α-OHase expression in the infected mammary gland seems to enable site-specific regulation of genes that are involved in host defense.

The substrate for 1α-OHase is 25(OH)D₃, so production of 1,25(OH)₂D₃, and subsequent regulation of gene expression by 1,25(OH)₂D₃, depends on the availability of 25(OH)D₃. The circulating concentration of 25(OH)D₃ depends on dietary intake of vitamin D and exposure to sunlight (Horst and Reinhardt, 1983; Wagner et al., 2008). In cattle supplemented with the recommended amount of vitamin D, the circulating concentration of 25(OH)D₃ is similar to that in humans and typically ranges from 20 to 50 ng/mL (McDermott et al., 1985). In the absence of dietary intake, however, the circulating concentration of 25(OH)D₃ changes greatly with season in northern latitudes and will be consistently low in
cattle that do not receive sun exposure (Hymoller et al., 2009). Similar observations also have been made in regards to vitamin D status in people (Basile et al., 2007; Holick, 2008; Sherman et al., 1990). Meanwhile, it is evident that 1,25(OH)\(_2\)D\(_3\)-mediated immune responses are dependent on the availability of 25(OH)D\(_3\) (Adams et al., 2009; Nelson et al., 2010). Without adequate supplementation of vitamin D, regulation of immune responses by 1,25(OH)\(_2\)D\(_3\) depends on sun exposure and is subject to seasonal variation.

Finally, additional work is needed to determine how much vitamin D is necessary for optimal immune function. In cattle, 20 ng/mL of 25(OH)D\(_3\) in plasma has been considered adequate for calcium homeostasis (NRC, 2001). For proper immune function, however, 20 ng/mL may not be sufficient. In fact, circulating 25(OH)D\(_3\) concentrations below 30 ng/mL are now considered insufficient for humans (Adams and Hewison, 2010; Holick and Chen, 2008; Hollis, 2005). Currently, there is not information to indicate what circulating concentration of 25(OH)D\(_3\) is necessary for proper immune function in cattle. In regards to immune function in humans, a target range for 25(OH)D\(_3\) also still remains elusive because of the inability to perform tightly controlled experiments. Therefore, efforts to find the optimal range of 25(OH)D\(_3\) concentration for proper immune function in cattle are necessary and may have implications for human health.
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References


Sherman, S.S., Hollis, B.W., Tobin, J.D., 1990, Vitamin D status and related parameters in a healthy population: the effects of age, sex, and season. J Clin Endocrinol Metab 71, 405-413.


Figures

**Figure 1.** Induction of mastitis with *Streptococcus uberis*. Five hundred CFU of *S. uberis* strain 0140 were infused into the mammary glands of 8 healthy cows. The contra-lateral glands were infused with 3 mL of PBS and served as the controls. Prior to infection, all glands were free of bacteria. The average CFU of *S. uberis* in milk (A), rectal temperatures (B), and number of somatic cells in milk (C) prior to infection and during mastitis are shown. Error bars represent the SE. (D) Representative tissue sections at 20X magnification from the control and infected mammary glands of one of the cows with mastitis.
Figure 2. 1α-OHase gene expression during mastitis. The relative amount of 1α-OHase mRNA was determined by quantitative real-time RT-PCR and the 2−ΔΔCt method. 

(A) Seventy-two hours after infusion 3 cows were euthanized and tissue was collected from various locations in the control and infected glands. 1α-OHase expression is relative to 1α-OHase expression in control tissue. 

(B) Cells were isolated from peripheral blood and milk of the control and infected glands of 5 cows prior to infection and during mastitis. 1α-OHase expression is relative to 1α-OHase expression in peripheral blood leukocytes during mastitis. 

(C) Cells from blood and milk of the infected glands of 5 cows were separated according to CD14 expression on the cell surface using FACS. 1α-OHase expression is relative to 1α-OHase expression in peripheral blood leukocytes during mastitis. ANOVA performed by SAS using the general linear model and multiple comparison tests were made using the Tukey adjustment. Error bars represent the SE, ***Mean is different from other means, P < 0.001. 

(D) Representative histogram of CD14 expression on cells isolated from the milk from the infected gland. 

(E and F) Scatter plots of all cells and CD14+ cells isolated from milk from an infected gland.
**Figure 3.** VDR gene expression during mastitis. The relative amount of VDR mRNA was determined by quantitative real-time RT-PCR and the $2^{-\Delta\Delta Ct}$ method. Tissue and cells were collected as in figure 2. (A) VDR expression in tissue from control and infected glands. VDR expression is relative to the amount of VDR in control tissue. (B) VDR gene expression in cells from milk and peripheral blood prior to infection and during mastitis. VDR expression is relative to the amount of VDR in peripheral blood leukocytes during mastitis. (C) VDR gene expression in cells from blood and milk from infected glands separated according to CD14 protein expression. VDR expression is relative to the amount of VDR in peripheral blood leukocytes during mastitis. ANOVA performed by SAS using the general linear model and multiple comparison tests were made using the Tukey adjustment. Error bars represent the SE, *Mean is different from other means, $P < 0.05$. 
Figure 4. Relative gene expression of 24-OHase (A-C), iNOS (D-F), and RANTES (G-I) in mammary tissue and cells from peripheral blood and milk during mastitis. Tissue and cells were collected as in figure 2. The relative amount of mRNA for each gene was determined by quantitative real-time RT-PCR and the $2^{-\Delta\Delta C_t}$ method. Gene expression in tissue is relative to the amount of mRNA of each gene in control tissue. Gene expression in total cells and sorted cells from blood and milk is relative to the amount of mRNA of each gene in peripheral blood leukocytes during mastitis. ANOVA performed by SAS using the general linear model and multiple comparison tests were made using the Tukey adjustment. Error bars represent the SE. *, **Mean is different from other means, $P < 0.05$, $P < 0.001$, respectively.
CHAPTER 5. GENERAL DISCUSSION

Discussion

The studies in this dissertation provide the first evidence that 1α-OHase, the enzyme that converts 25(OH)D₃ to 1,25(OH)₂D₃, is expressed by activated monocytes in cattle. In monocytes, expression of 1α-OHase was induced by TLR ligands, indicating that it is induced upon innate recognition of pathogen-associated molecular patterns. Further investigation with peripheral blood mononuclear cell (PBMC) cultures and mastitis indicated that in the bovine immune system 1α-OHase was predominantly expressed by monocytes and macrophages. For example, 1α-OHase was only expressed by CD14⁺ cells (monocytes and macrophages) in M. bovis PPD-stimulated PBMCs, even though data indicated that lymphocytes also were activated. Most important though, 1α-OHase expression was elevated the infected mammary glands during mastitis and 1α-OHase was predominantly expressed in CD14⁺ cells from the infected mammary glands. Expression of 1α-OHase in CD14⁺ cells from the infected mammary gland gave the first evidence for any species that 1α-OHase is expressed at the site of a microbial infection.

Production of 1,25(OH)₂D₃ by 1α-OHase in monocytes subsequently regulated the expression of several genes that have an important role in the immune system. In monocytes, iNOS and RANTES expression increased with the concentration of 25(OH)D₃. Regulation of iNOS and RANTES by the concentration of 25(OH)D₃ depended on synthesis of 1,25(OH)₂D₃ from 25(OH)D₃ by 1α-OHase in monocytes. In M. bovis PPD-stimulated PBMC cultures, 25(OH)D₃ up-regulated iNOS and RANTES expression and down-regulated
IFN-γ, IL-17A, and IL-17F expression. There is no evidence that these genes are regulated by synthesis of 1,25(OH)₂D₃ in vivo, but 24-OHase, a gene that is only known to be regulated by 1,25(OH)₂D₃, was expressed much higher in infected mammary tissue than in healthy tissue.

Expression of iNOS and RANTES in monocytes was up-regulated by 1,25(OH)₂D₃ that was produced in monocytes, which is an example of intracrine regulation (Hewison, 2010). In contrast, IFN-γ, IL-17A, and IL-17F are generally known as T-cells cytokines (Dong, 2008; Hu and Ivashkiv, 2009); so, in M. bovis PPD-stimulated PBMCs, their expression was down-regulated in a paracrine fashion by 1,25(OH)₂D₃ that was presumably produced in monocytes. RANTES also was originally discovered in T-cells (Schall et al., 1988); so, 1,25(OH)₂D₃ from monocytes also may act in T-cells to up-regulate RANTES expression. Altogether, 1,25(OH)₂D₃ produced in monocytes seems to 1) act in monocytes to up-regulate iNOS and RANTES expression and 2) act in T-cells to down-regulate IFN-γ, IL-17A, and IL-17F expression and up-regulate RANTES expression (Fig. 1). However, this dissertation does not give conclusive evidence that 1,25(OH)₂D₃ is directly down-regulating IFN-γ, IL-17A, and IL-17F expression. In fact, macrophage-produced nitric oxide suppressed proliferation of mitogen-activated lymphocytes from rats (Fecho, 1994). Therefore, upregulation of nitric oxide production by synthesis of 1,25(OH)₂D₃ in monocytes may be causing apoptosis of surrounding T-cells, resulting in down-regulation of IFN-γ, IL-17A, and IL-17F expression. In light of that possibility, further experiments are needed to determine how vitamin D signaling is regulating T-cell associated immune responses.

The genes that are regulated by 1,25(OH)₂D₃ in monocytes and PBMCs play an important role in the bovine immune system. iNOS synthesizes nitric oxide which is
suggested to have several roles in the immune system (Bogdan, 2001). In mice, killing of \textit{M. tuberculosis} by macrophages depended on production of nitric oxide and iNOS knockout mice with tuberculosis develop more severe lesions (Thoma-Uszynski et al., 2001; Waters et al., 2004). However, killing of \textit{M. tuberculosis} in human macrophages does not depend on nitric oxide (Thoma-Uszynski et al., 2001) and 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ did not enhance anti-bacterial activity of monocytes even though nitric oxide production was increased (Appendix A). Besides bactericidal activity, nitric oxide is known to regulate blood flow and induce apoptosis in selected cells (Bogdan, 2001; Lacasse et al., 1996). Production of nitric oxide in the mammary gland has a potential negative effect by inducing apoptosis in mammary epithelial cells (Zhao and Lacasse, 2008). In any case, nitric oxide does have a significant role in host defense, so regulation of it by 1,25(OH)$_2$D$_3$ will likely impact immune function.

RANTES is the other gene that was up-regulated by 1,25(OH)$_2$D$_3$ in monocytes and PBMCs. RANTES is a chemokine that was originally discovered in T-cells (Levy, 2009), but has been shown to be expressed by macrophages and epithelial cells in cattle (Pareek et al., 2005; Widdison et al., 2008). The physiological impact of RANTES on immune function in cattle is not clear, but up-regulation RANTES by 1,25(OH)$_2$D$_3$ would likely facilitate attraction of other immune cells to the site of an infection.

In PBMCs, 1,25(OH)$_2$D$_3$ down-regulated IFN-\(\gamma\), IL-17A, and IL-17F. All three of these cytokines are considered to be pro-inflammatory (Dong, 2008; Hu and Ivashkiv, 2009). IFN-\(\gamma\) primarily functions to activate macrophages and dendritic cells (Hu and Ivashkiv, 2009), so down-regulation of IFN-\(\gamma\) expression by 1,25(OH)$_2$D$_3$ produced in activated macrophages may be a regulatory mechanism to limit over-activation of macrophages. IL-
17A and IL-17F act on fibroblasts and epithelial cells to induce cytokine production and neutrophil recruitment (Dong, 2008). Recent studies in mouse models of autoimmune disorders have provided evidence that IL-17A is involved in development of the disease (Joshi et al., 2009; Tang et al., 2009). In cattle, IFN-γ and IL-17A have been associated with progression of tuberculosis and their expression is likely an important component of the immune response to mycobacterial infections (Vordermeier et al., 2002; Vordermeier et al., 2009). In general, 1,25(OH)_{2}D_{3} is known to promote development of T-regulatory cells and immune tolerance (Cantorna et al., 2004); so, inhibition of these pro-inflammatory cytokines by 1,25(OH)_{2}D_{3} may be involved in the promotion of a regulatory phenotype.

There is clear evidence that immune responses in monocytes and PBMCs in vitro depend on the concentration of 25(OH)D_{3}. The current requirements for vitamin D in cattle target a circulating concentration of 20 to 50 ng/mL of 25(OH)D_{3} (NRC, 2001). That range of 25(OH)D_{3} concentration is adequate for maintenance of calcium homeostasis in cattle (Horst et al., 2003). However, discovery of intracrine and paracrine regulation of gene expression by 1,25(OH)_{2}D_{3} indicates that vitamin D requirements for cattle need to be re-examined. Emerging evidence indicates that circulating 25(OH)D_{3} concentrations below 30 ng/mL may be insufficient for proper immune function in people and an increasing number of studies have shown there is a correlation between disease prevalence and vitamin D status in people (Adams and Hewison, 2010). Based on evidence from human studies, then, the current requirements for vitamin D in cattle may be insufficient for proper immune function.
Future Studies

So far, the expression of several genes in monocytes and PBMCs have been shown to be regulated by 1,25(OH)\(_2\)D\(_3\). The physiological impact of vitamin D-mediated regulation of those genes, however, can only be presumed based on evidence from other species. The unique nature of the bovine immune system, compared with the human and murine immune systems, indicates that additional investigation is needed to determine the physiological role of the 1,25(OH)\(_2\)D\(_3\)-regulated genes in the bovine immune system. A better understanding of the function of the 1,25(OH)\(_2\)D\(_3\)-regulated genes will result in a better understanding of how vitamin D status impacts immune function in cattle.

In addition to vitamin D signaling in monocytes and PBMCs, preliminary studies have indicated that mammary epithelial cells express 1\(\alpha\)-OHase and produce 1,25(OH)\(_2\)D\(_3\) in response to unknown stimuli from monocytes (Appendix B). In lactating cows, mammary epithelial cells have an important role in host defense in addition to their role in milk production (Rainard and Riollet, 2006). 1,25(OH)\(_2\)D\(_3\) up-regulated iNOS and S100 A12 gene expression in mammary epithelial cells that were activated with LPS (Appendix B). In human and mouse mammary epithelial cell cultures, 1,25(OH)\(_2\)D\(_3\) inhibits proliferation and has received much attention for the role it may have in prevention of breast cancer (Welsh, 2007). Therefore, investigating how 1\(\alpha\)-OHase expression is triggered in bovine mammary epithelial cells has implications in bovine mastitis and milk production and in human health.

Finally, there is substantial evidence to indicate that vitamin D is beneficial for immune function in cattle, but the optimal circulating concentration of 25(OH)D\(_3\) for proper immune function is not known. Therefore, studies need to be completed to find if prevention
of infectious diseases or severity of disease is affected by vitamin D status. In addition, there is not current information on the use of vitamin D supplements in cattle or the vitamin D status of herds in the United States or worldwide. Efforts to keep track of vitamin D supplementation and vitamin D status in cattle are beneficial because of the potential impact it has on animal health.

**References**


Figure 1. Proposed mechanism for regulation of monocyte and T-cell responses by vitamin D signaling. 1,25D₃, 1,25-dihydroxyvitamin D₃; 25D₃, 25-hydroxyvitamin D₃; 1α-OHase, 1α-hydroxylase; IL-17, interleukin-17; iNOS, inducible nitric oxide synthase; IFN-γ, interferon gamma; MHC, major histocompatibility complex; RANTES, regulated upon activation normally T-cell expressed and secreted; TCR, T-cell receptor; TLR, toll-like receptor; VDR, vitamin D receptor.
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APPENDIX A: EFFECTS OF 25-HYDROXYVITAMIN D\textsubscript{3} AND 1,25-DIHYDROXYVITAMIN D\textsubscript{3} ON MONOCYTE ANTI-BACTERIAL ACTIVITY

Summary

Nitric oxide production by activated monocytes was increased by 1,25(OH\textsubscript{2})D\textsubscript{3} or 25(OH)D\textsubscript{3} (Chapter 2, Figs. 2 and 4). Nitric oxide is considered an antimicrobial molecule; so, we hypothesized that treatment of monocytes with 25(OH)D\textsubscript{3} or 1,25(OH\textsubscript{2})D\textsubscript{3} would enhance their anti-bacterial activity in vitro. For killing of \textit{Streptococcus uberis}, monocytes were pretreated with peptidoglycan with or without 1,25(OH\textsubscript{2})D\textsubscript{3} for 24 h then infected with \textit{S. uberis} and allowed to kill for 1 h and 2 h. 1,25(OH\textsubscript{2})D\textsubscript{3} did not have an effect on the ability of monocytes to kill \textit{S. uberis} (Fig. 1). For \textit{Mycobacteria bovis} BCG killing, monocytes were infected with \textit{M. bovis} BCG and then cultured with or without 25(OH)D\textsubscript{3} for 24, 48, and 72 h. 25(OH)D\textsubscript{3} did not affect killing of \textit{M. bovis} BCG by monocytes (Fig. 2). Based on this evidence, 25(OH)D\textsubscript{3} or 1,25(OH\textsubscript{2})D\textsubscript{3} do not enhance the anti-bacterial activity of monocytes.
Figure 1. Monocytes from two cows were isolated by adherence to tissue culture flasks. The monocytes were transferred to 96-well plates and seeded at 5 X 10⁵ cells/well. Monocytes were treated with 5 µg/mL peptidoglycan (PGN) and 0 (dashed line) or 4 (solid line) ng/mL 1,25(OH)₂D₃ in RPMI 1640 plus 10% FBS and 50 µg/mL gentamicin for 24 h. After 24 h, monocytes were washed and S. uberis was added at to the monocyte cultures at 2:1 and 5:1 CFU S. uberis/monocyte in RPMI 1640 plus 10% FBS. Monocyte were incubated with S. uberis for 30 min then washed 3X to remove extra-cellular S. uberis. Monocytes were cultured then lysed (10% PBS, 0.1% SDS) and log dilutions of lysate plated on blood agar plates or cultured for 1 or 2 more h in RPMI 1640 plus 10% FBS and 100 µg/mL gentamicin (gentamicin was added to kill any remaining extra-cellular bacteria). At 1 h and 2h, monocytes were lysed and log dilutions of lysate was plated on blood agar plates. Each treatment was repeated 6X for each cow giving a total of 12 replicates. Data are shown as the average number of colony forming units (CFU)X1000 of S. uberis in monocytes at 0, 1, and 2 h after infection.
Figure 2. Effects of 25(OH)D₃ on monocyte *M. bovis* BCG killing. Monocytes were isolated from peripheral blood by adherence to tissue culture flasks. Monocytes were removed from the flasks and resuspended to 2.5X10⁶ cells/mL in RPMI 1640 plus 10% FBS. *M. bovis* BCG was added to the monocyte suspension at a concentration of 10⁶ *M. bovis* BCG/mL. The monocyte/BCG suspension was placed in a 96-well plate. After 3 h of incubation, monocytes were washed 2X to remove extra-cellular *M. bovis* BCG. Fresh media (RPMI 1640 plus 10% FBS and 50 µg/mL gentamicin) was added along with 0 or 75 ng/mL 25(OH)D₃. *M. bovis* BCG-infected monocytes were cultured for 0, 24, 48, or 72 h and then lysed with a 10% PBS, 0.1% SDS solution. Log dilutions of the lysates were plated on 7H11 agar plates and cultured for 3 weeks at 37°C. Data are shown as the average number of colony forming units (CFU) X 1000 of *M. bovis* BCG in monocytes of 3 replicates at 0, 24, 48, and 72 h after infection.
APPENDIX B. 1α-HYDROXYLASE EXPRESSION AND EFFECTS OF 1,25-DIHYDROXYVITAMIN D\textsubscript{3} ON GENE EXPRESSION IN BOVINE MAMMARY EPITHELIAL CELLS

A portion of these data appeared in the 2010 Animal Industry Report (Leaflet R2488) published by the Animal Science Department at Iowa State University

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Introduction

The vitamin D\textsubscript{3} hormone is 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}). The hormone can bind and activate the vitamin D receptor (VDR), a nuclear hormone receptor that regulates the expression of genes that contain a vitamin D response element in their promoter. 1,25(OH)\textsubscript{2}D\textsubscript{3} is produced from 25-hydroxyvitamin D\textsubscript{3} (25(OH)D\textsubscript{3}), the predominant circulating form of vitamin D\textsubscript{3}, by the enzyme 1α-hydroxylase (1α-OHase). We have recently shown that bovine monocytes express 1α-OHase in response to toll-like receptor (TLR) recognition of bacteria. Production of 1,25(OH)\textsubscript{2}D\textsubscript{3} by 1α-OHase in activated bovine monocytes increased production of nitric oxide and expression of the chemokine RANTES in monocytes. We also have observed that 1α-OHase is expressed in inflamed mammary tissue during mastitis. Human and mouse ammary epithelial cells have been shown to express 1α-OHase and produce 1,25(OH)\textsubscript{2}D\textsubscript{3}. Production of 1,25(OH)\textsubscript{2}D\textsubscript{3} up-regulated 24-OHase gene expression in human mammary epithelial cells. The objectives of this experiment were to 1) determine the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and lipopolysaccharide (LPS) on iNOS, RANTES, and S100 calcium binding protein A12 (S100 A12) gene
expression in bovine mammary epithelial cells (MEC), and 2) determine if bovine MEC could express 1α-OHase.

**Materials and Methods**

MEC were derived from mammary tissue biopsies of three Holstein cows in mid-lactation. The cells were cultured in RPMI 1640 plus 10% fetal bovine serum and mammary epithelial growth supplement (Invitrogen). Prior to treatment, MEC were transferred to 96-well tissue culture treated plates and cultured until they were confluent.

To test the effects of 1,25(OH)$_2$D$_3$ on gene expression in MEC, MEC cultures derived from 3 cows were treated with 0 or 1 µg/mL LPS along and 0, 0.1, 1 or 10 nM 1,25(OH)$_2$D$_3$ for 16 h in duplicate.

To determine if 1α-OHase expression could be induced in MEC, MEC cultures derived from one cow were treated with 1 µg/mL LPS, 100ng/mL 25(OH)D$_3$, supernatant from resting monocytes, and supernatant from LPS-activated monocytes in triplicate. Monocyte supernatants were prepared by isolating monocytes from peripheral blood and treating them with 0 or 100 ng/mL LPS for 24 h in the absence of 25(OH)D$_3$. Supernatants were collected, spun and filtered to remove monocytes.

After treatment, total RNA was isolated from MEC and reverse transcribed to cDNA. Quantitative real-time PCR using the $2^{-\Delta\Delta C_T}$ method was used to measure relative abundance of 1α-OHase, 24-OHase, iNOS, RANTES and S100 A12. Ribosomal protein S9 (RPS9) was used as the reference gene.
Results and Discussion

Treatment of MEC with 1,25(OH)$_2$D$_3$ caused an increase in 24-OHase gene expression in a dose dependent manner in both the presence and absence of LPS (Figure 1A). The 24-OHase enzyme adds a hydroxyl group to the 24 position of vitamin D metabolites, which inactivates 1,25(OH)$_2$D$_3$. In bovine monocytes, induction of 24-OHase expression by 1,25(OH)$_2$D$_3$ was repressed by LPS treatment.

In MEC treated with LPS, both iNOS and S100 A12 gene expression increased with 1,25(OH)$_2$D$_3$ dose (Figure 1B and C). In the absence of LPS, however, their was only a minimal response to 1,25(OH)$_2$D$_3$ for both genes. Increased expression of iNOS resulted in increased nitric oxide production by MEC (not shown). Nitric oxide may increase antimicrobial activity and blood flow in the mammary gland during mastitis, but it also may lead to MEC apoptosis. The function of S100 A12 is not completely understood, but it has been shown to have chemotactic and antimicrobial properties.

RANTES expression in MEC did not increase with 1,25(OH)$_2$D$_3$ treatment (Figure 1D). Treatment with LPS, however, caused an increase in MEC RANTES expression. In contrast, RANTES expression was up-regulated by 1,25(OH)$_2$D$_3$ treatment in LPS-stimulated bovine monocytes, but was not affected by LPS treatment alone.

Expression of 1α-OHase in MEC was up-regulated by treatment with supernatant from monocyte cultures activated with LPS (Fig. 2A). Direct treatment of MEC cultures with LPS did not increase 1α-OHase gene expression. Addition of 100 ng/mL of 25(OH)D$_3$ to MEC cultures up-regulated 24-OHase gene expression when 1α-OHase was expressed (Fig. 2b).
In conclusion, 1,25(OH)_{2}D_{3} acts in bovine MEC to enhance expression of iNOS and S100 A12 that is induced by TLR recognition of LPS. Therefore, production of 1,25(OH)_{2}D_{3} by 1α-OHase in the mammary gland during mastitis may affect the host defense capabilities of MEC. Expression of 1α-OHase is induced in MEC by a factor secreted by activated monocytes. Further investigation is necessary to find what the factor form monocytes is that activates 1α-OHase gene expression in MEC.
Figures

Figure 1. Mammary epithelial cell cultures were treated with 0 or 1 µg/mL LPS and 0 to 10 nM 1,25-dihydroxyvitamin D₃ for 16 hours. The amount of 24-hydroxylase (24-OHase; A), inducible nitric oxide synthase (iNOS; B), S100 calcium binding protein A12 (S100 A12; C), and RANTES (D) mRNA in MEC was measured by quantitative PCR. The expression of each gene was normalized to ribosomal protein S9 expression. The fold increase is relative to the control treatment. Results are the average gene expression in duplicate cultures derived from three cows. Error bars represent standard error. Means with different letters are different ($P < 0.05$).
Figure 2. Mammary epithelial cells were cultured in 96-well plates and treated with 1 µg/mL LPS, 100 ng/mL 25(OH)D$_3$, supernatant from resting monocytes (M. sup.), and supernatant from LPS-activated monocytes (LPS-stim. M. sup.) as indicated for 24 h. Supernatants from monocytes were prepared by culturing monocytes at $10^7$ monocytes/mL with 0 or 100 ng/mL LPS for 24 h in the absence of 25(OH)D$_3$, then supernatants were collected, spun, and filtered with a 0.2 micron filter to remove monocytes. 1α-Hydroxylase (1α-OHase) and 24-hydroxylase (24-OHase) mRNA in MEC was measured by quantitative PCR. The expression of each gene was normalized to ribosomal protein S9 expression. The fold increase is relative to the control treatment. Results are the average gene expression of 3 replicates. Error bars represent standard error. * $P < 0.05$. 