Modulation of the bovine innate immune response by production of 1 α ,25-dihydroxyvitamin D₃ in bovine monocytes

C. D. Nelson,*† T. A. Reinhardt,* T. C. Thacker,‡ D. C. Beitz,§† and J. D. Lippolis*¹

*Periparturient Diseases of Cattle Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA 50010

†Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames 50011

‡Bacterial Disease of Cattle Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA 50010

§Department of Animal Science, Iowa State University, Ames 50011

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 1 α -hydroxylase (1 α -OHase). Based on human studies, it was 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 nonstimulated monocytes with 1,25(OH)₂D₃ increased expression of the gene for the vitamin D 24-hydroxylase (24-OHase) enzyme by 51 $\pm$ 13 fold, but 1,25(OH)₂D₃ induction of 24-OHase expression was blocked by lipopolysaccharide (LPS) stimulation. In addition, 1,25(OH)₂D₃ increased the gene expression of inducible nitric oxide synthase and the chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) in LPS-stimulated monocytes 69 $\pm$ 13 and 40 $\pm$ 12 fold, respectively. Next, the ability of bovine monocytes to express 1 α -OHase and produce 1,25(OH)₂D₃ was tested. Activation of monocytes with LPS, tripalmitoylated lipopeptide (Pam3CSK4), or peptidoglycan caused 43 $\pm$ 9, 17 $\pm$ 3, and 19 $\pm$ 3 fold increases in 1 α -OHase gene expression, respectively. Addition of 25(OH)D₃ to LPS-stimulated monocytes enhanced expression of inducible nitric oxide synthase and RANTES and nitric oxide production in a dose-dependent manner, giving evidence that activated monocytes convert 25(OH)D₃ to 1,25(OH)₂D₃. In conclusion, bovine monocytes produce 1,25(OH)₂D₃ in response to toll-like receptor signaling, and 1,25(OH)₂D₃ production in monocytes increased the expression of genes involved in the innate immune system. Vitamin D status of cattle might be important for optimal innate immune function because 1,25(OH)₂D₃ production in activated monocytes and subsequent upregulation of

inducible nitric oxide synthase and RANTES expression was dependent on 25(OH)D₃ availability.

Key words: vitamin D, bovine innate immunity, nitric oxide, RANTES

INTRODUCTION

For several decades now, it has been known that there is an endocrine mechanism to regulate renal production of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] as a way to regulate the concentration of 1,25(OH)₂D₃ systemically (Horst and Reinhardt, 1983). The primary function of renal 1,25(OH)₂D₃ production was considered to be maintenance of calcium homeostasis (Horst, 1986). It has become evident that 1,25(OH)₂D₃ modulates the immune response of several species, including cattle (Waters et al., 2001). Furthermore, activated human macrophages produce 1,25(OH)₂D₃ as part of the immune response to regulate 1,25(OH)₂D₃ concentration at the site of inflammation (Liu et al., 2006). Local control of 1,25(OH)₂D₃ concentration regulates genes involved in immune responses locally rather than systemically (Schauber et al., 2007). Existence of a mechanism to control 1,25(OH)₂D₃ production and gene expression locally in humans and mice suggests that there might be a similar mechanism 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 D₃ [25(OH)D₃] in the liver (Horst and Reinhardt, 1983). The major circulating metabolite of vitamin D is 25(OH)D₃, and the concentration of 25(OH)D₃ in blood is relatively stable in cattle (Sommerfeldt et al., 1983). Conversion of 25(OH)D₃ to 1,25(OH)₂D₃ is accomplished by the enzymatic activity of 1 α -hydroxylase (1 α -OHase; Sakaki et al., 2005). The ligand for the vitamin D receptor is 1,25(OH)₂D₃; the vitamin D receptor is activated upon binding 1,25(OH)₂D₃ (Reinhardt et al., 1989). The activated vitamin D receptor regulates expression of genes that contain functional vitamin D response elements in their promoters (Lin and White, 2004). It is estimated that greater than 1,000 genes are regulated

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¹Corresponding author: john.lippolis@ars.usda.gov

by $1,25(\text{OH})_2\text{D}_3$ (Wang et al., 2005), and the vitamin D receptor is present in most tissues and cell types (Lin and White, 2004). Therefore, $1,25(\text{OH})_2\text{D}_3$ concentration is regulated tightly to control its effects on gene expression.

The kidney was the only known source for $1,25(\text{OH})_2\text{D}_3$ in cattle, and regulation of $1\alpha\text{-OHase}$ expression in the kidney is mainly in response to calcium homeostasis (Horst, 1986). In contrast, $1\alpha\text{-OHase}$ 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, $1,25(\text{OH})_2\text{D}_3$ increases the expression of cathelicidin directly via a vitamin D response element in the cathelicidin promoter (Gombart et al., 2005). It was found that $1,25(\text{OH})_2\text{D}_3$ induction of cathelicidin expression in human macrophages was necessary for the killing of intracellular *Mycobacterium tuberculosis* (Liu et al., 2007). In cattle, $1,25(\text{OH})_2\text{D}_3$ modulated the immune response in vitro by increasing nitric oxide production and decreasing $\text{IFN-}\gamma$ production in peripheral blood mononuclear cells (Waters et al., 2001). Production of $1,25(\text{OH})_2\text{D}_3$ in the kidney does not increase as part of the immune response in cattle (Waldron et al., 2003); so, if $1,25(\text{OH})_2\text{D}_3$ modulates the immune response in vivo, there would seem to be a source other than the kidney. It was hypothesized that bovine monocytes express $1\alpha\text{-OHase}$ and produce $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ on bovine monocytes isolated from peripheral blood and test the ability of bovine monocytes to express $1\alpha\text{-OHase}$ and convert $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$.

MATERIALS AND METHODS

Animals

A group of 12 healthy, midlactation 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 legends. 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 described previously (Stabel et al., 1997). Briefly, peripheral blood was collected into $2\times$ 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 3 times 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 $\mu\text{g/mL}$ 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 $10\times$ 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% CO_2 . Lipopolysaccharide from *Serratia marcescens* (Sigma-Aldrich); Pam3CSK4 (InvivoGen, San Diego, CA), a synthetic tripalmitoyl lipopeptide; and peptidoglycan from *Staphylococcus aureus* (InvivoGen) were in endotoxin-free water. Both $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ (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 $18,200\text{ M}^{-1}/\text{cm}$. 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 $25(\text{OH})\text{D}_3$. Ketoconazole (Sigma-Aldrich) was solubilized in PBS at pH 2.5 and diluted to 50 $\mu\text{g/mL}$ in PBS at pH 7.

Measurement of Relative Gene Expression

Ribonucleic acid was isolated from monocytes using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the 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 the manufacturer's instructions. Reactions consisted of 12.5 μL of 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

Table 1. Primer sequences for real-time PCR

Gene ¹	Accession no. ²	Strand ³	Sequence 5'-3'	Product size (bp)	Primer efficiency
1 α -Hydroxylase (1 α -OHase)	XM_588481	F	TGGGACCAGATGTTTGCATTTCGC	80	1.09
		R	TTCTCAGACTGGTTCCTCATGGCT		
24-Hydroxylase (24-OHase)	XM_591370	F	GAAGACTGGCAGAGGGTCAG	97	1.01
		R	CAGCCAAGACCTCGTTGATT		
Cathelicidin 4 (CATH4)	NM_174827	F	AGAGTGTCATCCTACCTGGAA	136	0.97
		R	TTTCACTGTCCAGAAGCCCGAA		
Cathelicidin 5 (CATH5)	NM_174510	F	GTGCTTGGGAAGAAGTATGGCCCAA	63	1.04
		R	GGCCCACAATTACCCCAATTCTGA		
Cathelicidin 6 (CATH6)	NM_174832	F	TCCAGTCATTCCGCTACTCC	91	1.11
		R	CAGAAATCCAAAAGCCTGAGC		
Interleukin-1 β (IL-1 β)	NM_174093	F	GCCTTGGGTATCAAGGACAA	90	1.04
		R	TTTGGGGTCTACTTCCTCCA		
iNOS ⁴	NM_001076799	F	GATCCAGTGGTTCGAACCTGC	128	0.97
		R	CAGTGATGGCCGACCTGATG		
RANTES	NM_175827	F	CACCCACGTCCAGGAGTATT	116	1.01
		R	CTCGCACCCACTTCTTCTCT		
Ribosomal protein S9 (RPS9)	NM_001101152	F	GTGAGGTCTGGAGGGTCAAAA	108	1.02
		R	GGGCATTACCTTCGAACAGA		
S100A12	NM_174651	F	GCTGAAGCAGCTGATCACAA	102	0.93
		R	TCTTTATCGGCATCCAGGTC		

¹iNOS = inducible nitric oxide synthase; RANTES = regulated upon activation, normal T-cell expressed and secreted; S100A12 = S100 calcium binding protein A12.

²<http://www.ncbi.nlm.nih.gov>.

³F = forward; R = reverse.

⁴Primer sequence from (Aalberts et al., 2007).

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 $2^{-\Delta\Delta C_t}$ 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 μ L of culture supernatant or culture media with 0 to 100 μ M sodium nitrite to 100 μ L 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*^G-monomethyl-L-arginine (Sigma-Aldrich), a nitric oxide synthase inhibitor, was added as a control treatment. There was no accumulation of nitrite in the culture supernatant when *N*^G-monomethyl-L-arginine 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, $\Delta\Delta C_t$ values were used as the response variables in the analyses. Mean $\Delta\Delta C_t$ values $\pm$ SE were transformed ($2^{-\Delta\Delta C_t}$) 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 $P < 0.05$.

Figure 1. Effects of 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 of LPS and 4 ng/mL of 1,25(OH)₂D₃ as indicated for 24 h. Relative expression of (A) 24-hydroxylase (24-OHase) and cathelicidin (CATH) 4, 5, and 6 and (B) inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), RANTES (regulated upon activation, normal T-cell expressed and secreted), and S100 calcium binding protein A12 (S100A12) was determined using real-time PCR and the $2^{-\Delta\Delta C_t}$ method. The mean fold increase shown for each gene is relative to the nontreated control. Error bars represent SE, n = 4. Means with different letters are different, $P < 0.05$.

RESULTS

Effects of 1,25(OH)₂D₃ on Monocytes

Initially the effect of 1,25(OH)₂D₃ on 24-hydroxylase (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). The effects of 1,25(OH)₂D₃ on cathelicidin gene expression were tested. The expression of CATH4, CATH5, or CATH6 was not increased by 1,25(OH)₂D₃ treatment in nonstimulated or LPS-stimulated monocytes (Figure 1A).

Of the other genes tested, inducible nitric oxide synthase (iNOS), regulated upon activation, normal T-cell expressed and secreted (RANTES), and S100 calcium binding protein A12 gene expression were upregulated by treatment with 4 ng/mL of 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 S100 calcium binding protein A12 gene expression (Figure 1B). Interleukin-1β gene expression increased slightly ($P > 0.05$) in nonactivated and LPS-activated monocytes treated with 4 ng/mL of 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

Figure 2. Effects of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] dose on LPS-stimulated monocytes. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL of LPS and 0 to 4 ng/mL of 1,25(OH)₂D₃ as indicated for 24 h. Relative expression of RANTES (regulated upon activation, normal T-cell expressed and secreted; A) and inducible nitric oxide synthase (iNOS; B) was determined using real-time PCR and the $2^{-\Delta\Delta C_t}$ method. The mean fold increase shown for RANTES and iNOS expression is relative to the nontreated 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, $P < 0.05$.

1,25(OH)₂D₃ and peaked with addition of 0.4 ng/mL of 1,25(OH)₂D₃ (Figure 2A). Both iNOS expression and nitric oxide production increased with 1,25(OH)₂D₃ dose in LPS-activated monocytes ($P < 0.05$; Figures 2B and 2C).

Expression 1 α -OHase in Monocytes

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

Activity of 1 α -OHase in Monocytes

Increasing the concentration of 25(OH)D₃ 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 α -OHase activity is necessary for the effects of 25(OH)D₃ on activated monocytes, ketoconazole, a competitive inhibitor of 1 α -OHase, was used to block conversion of 25(OH)D₃ to 1,25(OH)₂D₃. Treatment with ketoconazole decreased the effects of

Figure 3. Toll-like receptor signaling induces 1 α -hydroxylase (1 α -OHase) expression in bovine monocytes. Monocytes were isolated from peripheral blood of 6 cows and treated with 100 ng/mL of LPS, 5 μ g/mL of Pam3CSK4 (Pam3), or 5 μ g/mL of peptidoglycan (PGN) for 24 h. Relative expression of 1 α -OHase was determined using real-time PCR and the $2^{-\Delta\Delta C_t}$ method. The mean fold increase shown for 1 α -OHase expression is relative to the nonstimulated control. Error bars represent SE, n = 6. ***Mean is different from control, $P < 0.001$.

Figure 4. Gene expression of RANTES (regulated upon activation, normal T-cell expressed and secreted) and inducible nitric oxide synthase (iNOS) and nitric oxide production increase with 25-hydroxyvitamin D₃ [25(OH)D₃] dose. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL of LPS and 0 to 100 ng/mL of 25(OH)D₃ as indicated for 24 h. Relative expression of RANTES (A) and iNOS (B) was determined using real-time PCR and the $2^{-\Delta\Delta C_t}$ method. The mean fold increase shown for RANTES and iNOS expression is relative to the nontreated 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.

25(OH)D₃ on RANTES and iNOS gene expression and nitric oxide production but not 1 α -OHase gene expression in LPS-stimulated monocytes (*P* < 0.05; Figure 5). Furthermore, the effects of ketoconazole were reversed when exogenous 1,25(OH)₂D₃ was added to the culture media (*P* < 0.05). Addition of 25(OH)D₃ to monocytes that were not activated with LPS increased RANTES gene expression (*P* < 0.05) even though 1 α -OHase gene expression was not elevated.

DISCUSSION

It has been known that 1,25(OH)₂D₃ modulates bovine immune responses by increasing nitric oxide production by peripheral blood mononuclear cells in vitro (Waters et al., 2001). This study more specifically revealed that 1,25(OH)₂D₃ enhanced iNOS gene expression in activated monocytes. It showed for the first time that RANTES expression was increased by 1,25(OH)₂D₃. However, the concentration of 1,25(OH)₂D₃ needed to increase iNOS and RANTES was much greater than the normal concentration of 1,25(OH)₂D₃ in serum, which is less than 50 pg/mL (Horst and Reinhardt, 1983). Also, the concentration of 1,25(OH)₂D₃ 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)D₃ to 1,25(OH)₂D₃ in response to TLR signaling, providing 1,25(OH)₂D₃ at the site of infection. Furthermore, physiological concentrations of 25(OH)D₃, which typically range from 20 to 50 ng/mL, were sufficient to increase iNOS and RANTES gene expression through the actions of 1 α -OHase in monocytes.

An interesting observation in terms of regulating 1,25(OH)₂D₃ concentration at the site of infection was the regulation of 24-OHase expression. Inactivation of 1,25(OH)₂D₃ occurred by hydroxylation at the 24 position by 24-OHase (Reinhardt and Horst, 1989). Expression of 24-OHase normally is upregulated by 1,25(OH)₂D₃ as a means to limit the concentration of 1,25(OH)₂D₃ (Goff et al., 1992). The same regulation of 24-OHase expression occurs in nonactivated bovine monocytes. Activation of monocytes with LPS blocks induction of 24-OHase expression by 1,25(OH)₂D₃. Without 24-OHase, 1,25(OH)₂D₃ produced in monocytes will not be degraded and will 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 might 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 specif-

ics of this observation will have to be studied further to better understand the physiological effect.

There is a major difference between humans and cattle in regard to the effects of $1,25(\text{OH})_2\text{D}_3$ on the innate immune response. In human monocytes, production of $1,25(\text{OH})_2\text{D}_3$ increased the expression of cathelicidin, which enhanced killing of intracellular *M. tuberculosis* (Liu et al., 2007). In contrast, the bovine cathelicidin genes with potential vitamin D response elements in their promoters were not affected by $1,25(\text{OH})_2\text{D}_3$.

In cattle, production of $1,25(\text{OH})_2\text{D}_3$ 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 *Mycobacterium bovis* infections (MacMicking et al., 1997, Waters et al., 2004). No studies have definitively shown that nitric oxide is necessary for bovine macrophages to kill bacteria. 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 $25(\text{OH})\text{D}_3$ in cattle might have considerable implications in such diseases because nitric oxide production in monocytes is dependent on $25(\text{OH})\text{D}_3$ concentration in vitro.

It was shown that $1,25(\text{OH})_2\text{D}_3$ production in bovine monocytes increased RANTES gene expression. Also known as chemokine (C-C motif) ligand 5, RANTES is a chemo-attractant for T-helper cells and monocytes to the site of inflammation (Schall, 1991). In humans, RANTES was implicated in the clearance of viral infections, likely by the recruitment of other immune cells to the site of infection (Levy, 2009). There is little information 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. The expression of RANTES 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 $1,25(\text{OH})_2\text{D}_3$ 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 $25(\text{OH})\text{D}_3$ concentration up to 100 ng/mL in vitro. The concentration of serum $25(\text{OH})\text{D}_3$ in cattle supplemented with the recommended amount of dietary vitamin D typically ranges from 20 to 50

Figure 5. RANTES (regulated upon activation, normal T-cell expressed and secreted), 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 of LPS, 75 ng/mL of 25-hydroxyvitamin D₃ [$25(\text{OH})\text{D}_3$], 5 $\mu\text{g/mL}$ of ketoconazole, and 0.4 ng/mL of 1,25-dihydroxyvitamin D₃ [$1,25(\text{OH})_2\text{D}_3$] as indicated for 24 h. Relative expression of 1α -OHase (A), RANTES (B), and iNOS (C) was determined using real-time PCR and the $2^{-\Delta\Delta\text{Ct}}$ method. The mean fold increase shown for each gene is relative to the nontreated 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, $P < 0.05$.

ng/mL (McDermott et al., 1985). Serum 25(OH)D₃ concentrations above 50 ng/mL can be reached by additional supplementation; so, it might be possible to boost RANTES expression and nitric oxide production during an immune response in cattle. When the 25(OH)D₃ concentration exceeded 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 on the amount 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.

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