J. Dairy Sci. 101:1–15
https://doi.org/10.3168/jds.2017-14143

Intramammary 25-hydroxyvitamin D_3 treatment modulates innate immune responses to endotoxin-induced mastitis

Kathryn E. Merriman,* Jessi L. Powell,† Jose E. P. Santos,† and Corwin D. Nelson†[1]
*Animal Molecular and Cellular Biology Graduate Program and
†Department of Animal Sciences, University of Florida, Gainesville 32611

ABSTRACT

Vitamin D signaling in response to pathogen-associated molecules contributes to activation of innate immune responses of bovine monocytes. We hypothesized that lipopolysaccharide (LPS) of bacteria associated with mastitis in dairy cows activates the vitamin D pathway in innate immune cells of the udder and that increasing availability of 25-hydroxyvitamin D_3 [25(OH)D_3] would augment expression of vitamin D-associated genes. The objective of this experiment was to determine the effects of intramammary LPS and 25(OH)D_3 treatments on activation of the vitamin D pathway and innate immune responses of mammary immune cells. Individual mammary quarters of 5 lactating cows were treated with placebo control, 100 μg of 25(OH)D_3, 5 μg of LPS, or a combination of 100 μg of 25(OH)D_3 and 5 μg of LPS. Somatic cells from milk were evaluated for percentage of neutrophil and macrophage populations and expression of genes associated with vitamin D metabolism and innate immunity. Data from samples collected from 4 to 12 h after challenge were analyzed for main effects of LPS and 25(OH)D_3 treatments, treatment interactions, and simple effects of 25(OH)D_3 treatment. Data from samples collected at the time of challenge were used as covariates. The percentages of neutrophils in milk at 8 h postchallenge were 58 ± 10, 82 ± 11, 89 ± 10, and 63 ± 10% of total cells in milk from control, 25(OH)D_3, LPS, and LPS plus 25(OH)D_3 glands, respectively, such that the interaction of LPS and 25(OH)D_3 was significant. Expression of the vitamin D 1α-hydroxylase (*CYP27B1*) and vitamin D receptor genes was upregulated by LPS treatment in total cells, macrophages, and neutrophils in milk. In addition, expression of the vitamin D 24-hydroxylase (*CYP24A1*) gene in milk somatic cells was upregulated by 25(OH)D_3 and LPS treatments. The inducible nitric oxide synthase (*iNOS*), chemokine (C-C-motif) ligand 5 (*CCL5*), β-defensin 3 (*DEFB3*), *DEFB7*, and *DEFB10* genes were upregulated by LPS treatment in total cells and neutrophils from milk. Expression of *iNOS* in milk somatic cells tended to be affected by the interaction between LPS and 25(OH)D_3, such that 25(OH)D_3 tended to increase *iNOS* in the absence of LPS but not in the presence of LPS. Furthermore, expression of *CCL5* in macrophages was downregulated by 25(OH)D_3. In conclusion, intramammary endotoxin challenge activates the vitamin D pathway in mammary macrophages and neutrophils, and intramammary 25(OH)D_3 treatment alters the percentage of neutrophils and expression of immune genes in milk somatic cells.
Key words: vitamin D, innate immunity, mastitis, dairy cow

INTRODUCTION

Mastitis in dairy cattle is a major concern in regards to animal welfare, milk quality, and profitability for dairy producers, and it eventually affects consumers (Ruegg, 2012). Mastitis is almost always caused by infection of the mammary gland, and environmental bacterial pathogens such as *Escherichia coli* and *Streptococcus uberis* are the most common agents in farms that have adopted control measures for mastitis. The innate response of the mammary gland to a bacterial pathogen triggers an influx of immune cells, primarily neutrophils, and production of inflammatory cytokines and bactericidal molecules (Rainard and Riollet, 2006). Although the innate immune response to bacterial infections of the mammary gland has been well-characterized, a better understanding of the mechanisms influencing innate defenses of the mammary gland is needed to improve treatment and prevention efforts for mastitis.

Several reports using models of cattle, human, and rodent innate immune responses have documented a role for vitamin D in the innate immune response to pathogens. Specifically in cattle, pathogen-associated

Received November 16, 2017.
Accepted March 13, 2018.
[1] Corresponding author: cdnelson@ufl.edu

molecules such as LPS and peptidoglycan stimulated expression of the 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1) in cultures of peripheral blood monocytes (Nelson et al., 2010b, 2011). The CYP27B1 enzyme catalyzes conversion of 25-hydroxyvitamin D_3 [**25(OH) D_3**] to the active vitamin D hormone, 1,25-dihydroxyvitamin D_3 [**1,25(OH)$_2$$D_3$**]. The 1,25(OH)$_2$$D_3$ primarily acts to regulate gene expression upon its association with intracellular vitamin D receptors (**VDR**). Synthesis of 1,25(OH)$_2$$D_3$ from 25(OH)D_3 in LPS-stimulated cultures of bovine monocytes upregulated expression of β-defensin (*DEFB*) 3, *DEFB4*, *DEFB6*, *DEFB7*, *DEFB10*, inducible nitric oxide synthase (*iNOS*), and chemokine (C-C motif) ligand 5 (*CCL5*) genes (Nelson et al., 2010b; Merriman et al., 2015). Altogether, these data indicate a potential for targeted enhancement of defense against bacterial infections via the vitamin D pathway.

In regards to mastitis, a role for vitamin D signaling in defense of the mammary gland has been indicated in dairy cows. Mammary tissue and somatic cells isolated from milk of quarters experimentally infected with *S. uberis* had increased expression of *CYP27B1*, *VDR*, and the gene for the 25-hydroxtvitamin D 24-hydroxylase (*CYP24A1*) compared with healthy control quarters (Nelson et al., 2010a). Furthermore, Lippolis et al. (2011) tested the effects of intramammary 25(OH)D_3 treatment on the outcomes of experimental *S. uberis* mastitis on the basis that vitamin D signaling is activated during mastitis and observed reduced signs of mastitis in cows treated with intramammary 25(OH)D_3. The authors proposed that intramammary 25(OH)D_3 treatments enhanced defense against *S. uberis* infection by increasing immune cell 1,25(OH)$_2$$D_3$ synthesis in the infected quarters. Indeed, the 1,25(OH)$_2$$D_3$ metabolite does influence expression of vitamin D-associated genes in the mammary gland (Merriman et al., 2017). However, the influence of 25(OH)D_3 on mammary immune responses to bacterial pathogens has not been determined.

Intramammary LPS challenge induces acute mastitis in dairy cows and has been used as a model to characterize the innate immune response to bacterial pathogens (Bannerman et al., 2003; Schmitz et al., 2004). We hypothesized that intramammary LPS would stimulate CYP27B1 activity in the mammary gland and that simultaneous intramammary 25(OH)D_3 would enhance immune responses associated with vitamin D signaling in cattle. Therefore, the objective of this experiment was to determine the effects of intramammary LPS and 25(OH)D_3 treatments on activation of the vitamin D pathway and innate immune responses of the mammary gland.

MATERIALS AND METHODS

Animals

Five healthy lactating Holstein cows at the University of Florida's Dairy Unit (Hague, FL) were used for this experiment. Cows were between 300 and 680 DIM, not pregnant, free of mastitis (milk SCC ≤200,000 cells/mL), and otherwise healthy by appearance. Cows were milked twice per day and fed a diet formulated to meet the needs of lactating dairy cows (NRC, 2001). The diet was fed as a TMR and was estimated to provide approximately 40,000 IU of vitamin D_3/d, which is normal for dairy cows in the United States and typically achieves between 50 and 80 ng/mL of 25(OH)D_3 in serum (Nelson et al., 2016a). The University of Florida's Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures for the care and treatment of the animals.

Intramammary LPS and 25(OH)D_3 Treatment

The 25(OH)D_3 was purchased in crystalline form from Cayman Chemical (Ann Arbor, MI) and dissolved to a concentration of 10 mg/mL in ethanol. The concentration and purity of 25(OH)D_3 was confirmed by UV absorption spectroscopy at 228- and 264-nm wavelengths using a molar extinction coefficient of 18,200 $M^{-1}cm^{-1}$. For intramammary 25(OH)D_3 treatments, 100 μg of 25(OH)D_3 was diluted in 10 mL of sterile PBS (Dulbecco's PBS without calcium or magnesium; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum as a carrier for 25(OH)D_3 (characterized serum; Hyclone). The LPS from *E. coli* 026:B6 was purchased in crystalline form from Sigma-Aldrich (St. Louis, MO) and dissolved to a concentration of 5 mg/mL in sterile H_2O. Ten-milliliter solutions of PBS containing 5 μg of LPS (**LIPO**), 100 μg of 25(OH)D_3 (**25D**), 5 μg of LPS and 100 μg of 25(OH)D_3 (**LIPO+25D**), or no LPS or 25(OH)D_3 (control) were prepared for intramammary treatment. The amount of 25(OH)D_3 used was selected on the basis of previous research that showed it did not alter concentrations of 25(OH)D_3 in serum and had inhibitory effects on *S. uberis* mastitis (Lippolis et al., 2011).

Experimental Design

The experiment was a randomized complete block design with a factorial arrangement of treatments. Individual mammary quarters of each cow were considered the experimental units, and cow was the blocking factor. The model used gland-specific responses as previ-