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Expression of *1alpha-HYD* and *24-HYD* in bovine kidney mediated by vitamin D_3 supplementation

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ABSTRACT. In order to better understand vitamin D_3 in cattle metabolism, we quantified *1alpha-HYD* and *24-HYD* gene expression. In the kidneys of 35 male Nellore cattle, these were divided into a control group and two treatment groups (2 x 10^6 international units of vitamin D_3 administered for 2 or 8 consecutive days pre-slaughter). Vitamin D_3 supplementation resulted in a significant increase in *1alpha-HYD* gene expression; however, significantly increased *24-HYD* gene expression was only detected in cattle that had 8 days of supplementation. The finding of upregulation of *24-HYD* due to vitamin D supplementation is in line with the expected rise in 24,25-di-hydroxy-vitamin D_3 synthesis observed when plasma vitamin D_3 concentrations are high, stimulating excretion by the organism. On the other hand, upregulation of *1alpha-HYD* was unexpected, since

vitamin D_3 supplementation has been reported to impact these two genes in opposite directions. We conclude that vitamin D_3 metabolism in these animals is more complex than previously reported.

Key words: Gene expression; Kidney; Bovine; Calcium

INTRODUCTION

Vitamin D occurs in two forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). In animal tissue, sunlight exposure converts 7-dihydro-cholesterol present under the skin into vitamin D_3 (Gaman and Sherrington, 1981; Bondi, 1988; Robert et al., 2002). A major function of vitamin D in the body is the regulation and maintenance of serum calcium and phosphorus homeostasis, by increasing intestinal calcium uptake and minimizing renal loss of these minerals, with the aim of normal bone mineralization (Gaman and Sherrington, 1981).

Synthesis of the major circulating form of vitamin D, 25-hydroxyvitamin D_3 [25(OH) D_3] is catalyzed by the hepatic enzyme vitamin D-25-hydroxylase (*25-HYD*). The subsequent fate of 25(OH)D_3 is determined mainly by two renal enzymes, vitamin D-24-hydroxylase (*24-HYD*) and 25-hydroxyvitamin D-1α-hydroxylase (*1alpha-HYD*). [25(OH)D_3 bound to its serum transport protein (transcalciferin) is carried to the kidneys where hydroxylation by the action of the mitochondrial enzyme *1alpha-HYD* occurs, forming 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the physiologically active form of vitamin D (Robert et al., 2002). *1alpha-HYD* is an enzyme of renal origin, also found in bone and placenta, activated directly by parathyroid hormone (PTH) due to a decrease in serum phosphate, or indirectly due to decreased concentration of calcium ions in the plasma (Jones et al., 1998). Although *1alpha-HYD* activity has been demonstrated at several ectopic sites, circulating levels of 1,25$(OH)_2D_3$ appear to reflect the expression of this enzyme in the kidney.

24-HYD expression appears to occur in all vitamin D responsive tissues and the function of this enzyme remains somewhat unclear (Omdahl and May, 1997). On the one hand, under higher levels of 1,25$(OH)_2D_3$, *24-HYD* acts as a negative regulatory enzyme which, through side chain hydroxylation reactions, inactivates 1,25$(OH)_2D_3$. The *1alpha-HYD* and *24-HYD* enzyme pathways act synergistically to maintain optimal levels of physiologically active vitamin D (Henry, 1997).

Supplementation with vitamin D has been used in cattle to increase meat tenderness. However, results are not clear and could be dependent on the animal's ability to inactivate excess vitamin D. The details of the mechanisms regulating the level of 1,25$(OH)_2D_3$, especially in response to dietary factors, are not clear in cattle. In this study, quantitative real-time PCR was used to check whether there is renal regulation of mRNA levels of the *1alpha-HYD* and *24-HYD* genes under different conditions of vitamin D_3 supplementation. This work aimed to determine whether an interaction between vitamin D_3 supplementation and sunlight exposure conditions could promote differential expression of *1alpha-HYD* and *24-HYD* genes.

MATERIAL AND METHODS

Experimental conditions

We used a randomized complete block design (three different categories of initial

body weight after a period of adaptation to high concentrate diet) under a 3 x 2 factorial arrangement, with 3 regimens of vitamin D_3 supplementation and 2 systems of sunlight exposure, resulting in 6 treatments with 7 replications. For the data of expression of specific genes from bovine kidney, 3 treatments lost 2 parcels each, leaving 5 replicates for each treatment and 1 lost one parcel, leaving 6 replicates (35 animals). A linear mixed model was used to perform this combined analysis, in which the effect of vitamin D_3 supplementation, sunlight exposure conditions, and their interactions were considered to be fixed, while the effect of blocks nested within the effect of the sunlight exposure conditions was considered to be random.

Animals

Nellore cattle (*Bos indicus*) from a commercial herd were used. They had an average weight of 435 kg and average age of 30 months. After adaptation to a high-concentrate diet, the animals were divided into three weight groups (light = 376 ± 29 kg, intermediate = 403 ± 16 kg, and heavy = 457 ± 17 kg), which represented the different growth potentials before the beginning of the experimental feedlot period. The animals within each weight group were randomly assigned to the six treatments (three levels of vitamin D_3 supplementation x two sunlight exposure conditions).

Treatments

After a 56-day period of adaptation to a high-concentrate diet, the animals were confined for 45 days with different sunlight exposure conditions. On the 25th day of exposure to different sunlight exposure conditions, all animals were conditioned to receive part of their diet in a small trough to simulate the vitamin D_3 supplementation procedure, before receiving the remaining portion of the diet. On the 37th or 43rd day at the feedlot, the animals began supplementation with vitamin D_3 for 8 or 2 days respectively.

The animals were housed in individual pens to permit control of consumption, especially of vitamin D_3, and received the following treatments: 1) no vitamin D_3 supplementation (Vit. 0) and no shade (N = 6); 2) Vit. 0 with shade (50% UV filtration ratio) (N = 7); 3) 2 x 10^6 IU vitamin D_3 for two consecutive days prior to slaughter (Vit. 2) and no shade (N = 5); 4) Vit. 2 with shade (N = 5); 5) with 2 x 10^6 IU vitamin D_3 for eight consecutive days prior to slaughter (Vit. 8) and no shade (N = 7); and 6) Vit. 8 with shade (N = 5). Vitamin D_3 was given orally, mixed with the concentrate. After slaughter, tissue samples of kidneys (cortex) were collected, frozen in liquid nitrogen and stored at -80°C until extraction of total RNA.

RNA extraction and cDNA synthesis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) following manufacturer instructions. Total RNA concentration was determined by spectrophotometry (HITACHI, U-200) and integrity was verified by 1% agarose electrophoresis. Reverse transcription was performed using 1 µg total RNA and the ImProm-II Reverse Transcription System kit (Promega, Madison, WI, USA) as described by the kit manufacturer. After completion of reactions, each cDNA was diluted 1:4 using DNase- and RNase-free water.

Gene expression analysis

Primers were designed using Primer3 software (Table 1). To avoid possible amplification of contaminating genomic DNA, primer sets were designed over intron-exon boundaries. The expression of *1alpha-HYD*, *24-HYD* and reference genes *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *EEF1A2* (eukaryotic translation elongation factor-1-alpha-2), *RPL-19* (ribosomal protein L-19) were analyzed by quantitative RT-PCR using the LightCycler® 480 instrument and SYBR Green I (Roche Diagnostics, Mannheim, Germany), according to manufacturer instructions. Each sample was analyzed in a total reaction volume of 20 μL, consisting of 2 μL 10X diluted cDNA, 10 μL SYBR Green I Master 2X and 2 mM each primer and water. The reaction setup for all primers was: 95°C for 2 min; 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 6 s; and a final extension at 72.0°C for 3 min. Melting curve analysis was performed immediately after PCR reaction. Every RT-qPCR experiment included a negative control (non-template control) and a calibrator, which is a pool from all samples for a known gene (*GAPDH*). The expression was recorded as cycle threshold (Ct).

Table 1. Genes and primers used in Real Time PCR.

Gene	NCBI (Gene ID)	Local	Primer sequence	Product (bp)
1alpha-HYD	539630	Exon 6	5'-TCTCCTGGGCTCTGTATGAA-3'	221
		Exon 7	5'-TTCACCCACACAAATGTCTC-3'	
24-HYD	540080	Exon 3	5'-TGGACGACAAAATCAACGAG-3'	140
		Exon 5	5'-CTCATACAGCACAAGGCAGA-3'	
GAPDH	281181	Exon 1	5'-GGCGTGAACCACGAGAAGTATAA-3'	194
		Exon 2	5'-CCCTCCACGATGCCAAAGT-3'	
EEF1	282220	Exon 6	5'-GCAGCCATTGTGGAGATG-3'	196
		Exon 7	5'-ACTTGCCCGCCTTCTGTG-3'	
RPL-19	510615	Exon 1	5'-GAAATCGCCAATGCCAAC-3'	
		Exon 2	5'-GAGCCTTGTCTGCCTTCA-3'	410

The efficiency for each gene and sample was determined by linear regression analysis of the fluorescence data obtained with quantitative real-time PCR through the LinRegPCR software (Ramakers et al., 2003). The amount of mRNA (R_0) and the amplification efficiency was calculated with equations 1, 2 and 3.

$$log\ R = log\ (E + 1) \times n + log\ R_0;\ intercept = log\ R_0,\ slope = log\ [E + 1] \quad \text{(Equation 1)}$$

$$R_0 = 10^{Intercept} \quad \text{(Equation 2)}$$

$$E = 10^{Slope} - 1 \quad \text{(Equation 3)}$$

From the efficiency of individual samples, the observed Cts were adjusted for a general efficiency, defined as 2.0 (Equation 4). Thus, all the following analyses had the same base efficiency of 2.0.

$$Ct_{2ij} = log_2 (E\_gene_{ij}^{Ct\_gene(ij)}) \quad \text{(Equation 4)}$$

where, Ct_{2ij} is Ct sample ij corrected for efficiency 2.0; $E\_gene_{ij}^{Ct\_gene(ij)}$ is the actual efficiency calculated by the LingRegPCR, high Ct sample for gene ij.

A mathematical model (Equation 5) that includes the fixed effect of the reference genes (*GAPDH*, *EEF1A2* and *RPL-19*) and a random effect associated with the natural variability of the individual sampled was used to obtain the best linear unbiased predictor (BLUP) values of random sample effect. These values were used to obtain an adjusted quantification cycle (aCt) of the target genes (*1alpha-HYD* and *24-HYD*).

$$Y_{ij}=\mu+g_i+a_j+e_{ij} \quad \text{(Equation 5)}$$

where, Y_{ij} is the aCt for reference gene i in animal j; μ is the overall average aCt; g_i is the fixed effect of ith gene reference (i = 1, ..., 5); a_j is the random effect associated with the animal considering $a_j \sim NID(0, \sigma_a^2)$; and e_{ij} is the residual random effect, with $e_{ij} \sim NID(0, \sigma_e^2)$.

After Ct adjustment, a general linear model that includes the effects of vitamin D_3, sunlight exposure, block and interaction between vitamin D_3 and sunlight exposure (Equation 6) was used to study gene expression with PROC GLM of SAS (2000). A Tukey test was applied to test the difference between treatments, with a significance level at $P < 0.05$.

$$Y_{ijkl} = \mu + v_i + s_j + vs_{ij} + b_k + e_{ijkl} \quad \text{(Equation 6)}$$

where, Y_{ijkl} is the aCt of *1alpha-HYD* or *24-HYD*; μ is the constant associated with the model; v_i is the effect of vitamin D_3, where i = 0, 2 and 8; s_j is the effect of sunlight exposure, where j = 0 and 1; vs_{ij} is the interaction between vitamin D_3 and sunlight exposure; b_k is the effect of block, where k = 1, 2 and 3; and e_{ijkl} is the error associated with each observation.

RESULTS

Optimal amplification conditions for each gene were established and reaction specificity for each set of primers was verified by melting temperature analysis and agarose gel electrophoresis, in which a single band corresponding to the expected product length was observed for each gene. Melting temperature analysis revealed the absence of nonspecific product amplification.

To correct for possible variation in RNA extraction, quantification and cDNA synthesis efficiency, *GAPDH*, *EEF1A2* and *RPL-19* were used as reference genes. All reference genes showed low variation between treatments and exhibited overall average Ct values of 20.05 (±0.66), 24.20 (±0.87), and 31.04 (±0.86) cycles, respectively (Table 2).

Table 2. mRNA expression (Ct) of reference genes *GAPDH*, *EEF1A2*, and *RPL-19*.

Supplem.	Ct (means ± SE)					
	GAPDH		EEF1A2		RPL-19	
	No shade	With shade	No shade	With shade	No shade	With shade
Vit. 0[1]	20.14 (0.53)	20.20 (0.61)	24.22 (0.95)	24.36 (0.73)	32.59 (1.70)	31.18 (0.37)
Vit. 2[2]	20.18 (0.69)	20.22 (0.72)	24.60 (0.87)	24.18 (1.05)	31.11 (0.45)	30.71 (0.85)
Vit. 8[3]	19.74 (0.64)	19.86 (0.76)	24.27 (0.76)	23.59 (0.88)	30.34 (0.67)	30.33 (1.10)

[1]without vitamin D_3 feeding; [2]vitamin D_3 supplementation at a dose of 2 x 10^6 IU for 2 consecutive days pre-slaughter; [3]with vitamin D_3 supplementation at a dose of 2 x 10^6 IU for 8 consecutive days pre-slaughter.

Different amounts of sunlight exposure did not alter the expression of *1alpha-HYD* and *24-HYD* genes (P > 0.05). There was no interaction between sunlight exposure conditions and vitamin D_3 supplementation. The expression of the *1alpha-HYD* gene was significantly higher (lower Ct) when the animals were supplemented with 2 x 10^6 IU vitamin D_3 for two and eight consecutive days pre-slaughter, regardless of UV exposure (Table 3). *1alpha-HYD* expression was 7.6-fold greater in animals that received supplementation with 2 x 10^6 IU vitamin D_3 for eight consecutive days prior to slaughter compared to animals without supplementation. Comparison between animals fed for two consecutive days prior to slaughter and those without supplementation showed that the *1alpha-HYD* gene was 3.6-fold more expressed in animals fed for two days.

Table 3. mRNA expression (aCt) of *1alpha-HYD* and *24-HYD* genes.

Supplem.	*1alpha-HYD* gene	*24-HYD* gene
	P > |t|	
Vit. 0[1]	29.90 (0.56)[a]	30.43 (0.46)[a]
Vit. 2[2]	28.06 (0.48)[b]	29.74 (0.53)[ab]
Vit. 8[3]	26.97 (0.62)[b]	28.60 (0.50)[b]

*Least square means of Ct (standard error); [1]without vitamin D_3 feeding; [2]vitamin D_3 supplementation at a dose of 2 x 10^6 IU for 2 consecutive days pre-slaughter; [3]with vitamin D_3 supplementation at a dose of 2 x 10^6 IU for 8 consecutive days pre-slaughter. [ab]Different lowercase letters in the column within each gene significantly differ by Tukey test (P < 0.05).

Regarding the *24-HYD* gene, expression in animals that received supplementation with 2 x 10^6 IU vitamin D_3 for eight consecutive days prior to slaughter was 3.55-fold higher when compared to animals without supplementation and did not differ when compared those fed for only 2 days. When the comparison was made between animals fed for two consecutive days prior to slaughter and those without supplementation, there was no effect of supplementation although mean Ct values were decreased. Importantly, lower Ct values indicate a higher gene expression.

DISCUSSION

Supplementation of vitamin D_3 to increase meat tenderness in beef cattle shows conflicting results. The rationale for the supplementation is based on the assumption that higher levels of vitamin D_3 may contribute to higher calcium levels in plasma and muscle, which would lead to a more active proteolysis by the calpain system.

Previous results from our group, with the same animals used in this study, showed that supplementation with vitamin D_3 did not change plasma or muscle calcium levels (Lobo-Jr et al., 2012). This result could be a consequence of the opposing actions of the kidney enzymes (*1alpha-HYD* and *24-HYD*) that control the physiological levels of the active $1,25(OH)_2D_3$.

Our results indicated that shortly after vitamin D_3 supplementation there was an increase in *1alpha-HYD* before a rise in *24-HYD* gene expression. This is a novel result, since studies in mouse showed decreased *1alpha-HYD* gene expression concomitantly with increased *24-HYD* expression under vitamin D_3 supplementation (Henry, 1997; Murayama et

al., 1999; Healy et al., 2003). This discrepancy could be a consequence of the time of exposure to high levels of vitamin D_3. Our study was short term, while most of the literature deals with long-term supplementation.

The increased expression of *1alpha-HYD* observed after 2 days of supplementation with vitamin D_3 could have resulted in higher circulating levels of the active form of $1,25(OH)_2D_3$; however, this did not result in higher plasma or muscle calcium levels. A possible explanation for this result may involve changes in the vitamin D receptor (VDR) level or function. There is a number of reports indicating that PTH and calcitonin positively regulate, while $1,25(OH)_2D_3$ negatively controls, the expression of *1alpha-HYD* via VDR (Chen et al. 1993; Murayama et al., 1999). The latter showed that in VDR-deficient mice, there was no negative control of *1alpha-HYD* expression by the active vitamin D form. Desensitization of the signaling pathway of active vitamin D or defect in the response of VDR has also been reported as a possible cause for lack of downregulation of *1alpha-HYD* expression under conditions of ectopic administration of the metabolite $1,25(OH)_2D_3$ in homozygous *Klotho* mutant mice (Yoshida et al., 2002). There is also the report of higher and lower expression for *1alpha-HYD* and *24-HYD*, respectively, in VDR null mice (Panda et al., 2004).

Moreover, an apparent insensitivity to vitamin D_3 negative regulatory effect on the expression of *1alpha-HYD* was reported in cells derived from monocytes (Hewison et al., 2003). Those responses appeared to be dependent on variations in the levels of vitamin receptor or receptor ligand ability to recruit coactivators, and the presence of routes that reflect the intracellular response mediated by the receptor. Although those conditions could contribute to the results reported in the present study, all the reports on *1alpha-HYD* regulation by vitamin D_3 are related to gene mutation or knockout in renal cells or extra-renal responses. Those special physiological conditions make it difficult to point out the probable cause of *1alpha-HYD* expression observed. Furthermore, there has been a well-characterized difference between renal and extra-renal regulation of *1alpha-HYD* (Zineb et al., 1998).

Vitamin D_3 supplementation caused increased expression of the *24-HYD* gene. These results corroborate those of another study describing increased expression of *24-HYD* as physiological regulation of $1,25(OH)_2D_3$, thereby preventing the accumulation of toxic levels of the hormone, even when the expression of *1alpha-HYD* is enhanced by PTH and calcitonin (Bartella et al., 2004).

Studies using the *24-HYD*-null mutant mouse provided the first *in vivo* evidence that the C-24 pathway, initiated by the *24HYD* enzyme, is the major catabolic process that functions to regulate the physiological levels of $1,25(OH)_2D_3$ (St-Arnaud et al., 2000). However, increased expression of *24-HYD* detected only after 8 days of supplementation points towards a delayed response. These results suggest that the duration of the experiment or the quantity of vitamin D_3 supplied may have not been sufficient to dictate the whole response to supplementation to promote an inverse regulation of the kidney enzymes.

Exposure of monocyte-derived dendritic cells (moDCs) to $1,25(OH)_2D_3$ increased *24-HYD* gene expression dramatically (up to 3000-fold) at early time points (day 3 of culture), but then decreased the expression on days 7 and 9 (Hewison et al., 2003). On the other hand, the cited authors reported that $25(OH)D_3$ had little effect on *24-HYD* gene expression on day 3, but consistent with its conversion to active ligand, induced *24-HYD* expression at later time points to a similar degree as with exogenous $1,25(OH)_2D_3$. These observations could help explain the delayed response observed for *24-HYD* expression in our study.

The results of this study indicate that short term supplementation of vitamin D_3 could result in higher levels of $1,25(OH)_2D_3$, since the induction of *1alpha-HYD* gene expression occurs before any change in *24-HYD* expression. However, the lack of increased calcium levels in plasma or muscle indicates that further studies are necessary to investigate the role of the receptor in the regulation of vitamin D_3 activity.

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