Widespread basal cytochrome P450 expression in extrahepatic bovine tissues and isolated cells

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ABSTRACT

Periparturient cattle face increased risk of both metabolic and infectious diseases. Factors contributing to this predisposition include oxidized polyunsaturated fatty acids, also known as oxylipids, whose production is altered during the periparturient period and in diseased cattle. Alterations in the production of oxylipids derived from cytochrome P450 (CYP450) enzymes are over-represented during times of increased disease risk and clinical disease, such as mastitis. Many of these same CYP450 enzymes additionally regulate metabolism of fat-soluble vitamins, such as A, D, and E. These vitamins are essential to maintaining immune health, yet circulating concentrations are diminished near calving. Despite this, a relatively small amount of research has focused on the roles of CYP450 enzymes outside of the liver. The aim of this paper is to describe the relative gene expression of 11 CYP450 in bovine tissues and common in vitro bovine cell models. Eight tissue samples were collected from 3 healthy dairy cows after euthanasia. In vitro samples included primary bovine aortic and mammary endothelial cells and immortalized bovine kidney and mammary epithelial cells. Quantitative real-time-PCR was carried out to assess basal transcript expression of CYP450 enzymes. Surprisingly, CYP450 mRNA was widely expressed in all tissue samples, with predominance in the liver. In vitro CYP450 expression was less robust, with several cell types lacking expression of specific CYP450 enzymes altogether. Overall, cell culture models did not reflect expression of tissue CYP450. However, when CYP450 were organized by activity, certain cell types consistently expressed specific functional groups. These data reveal the widespread expression of CYP450 in individual organs of healthy dairy cows. Widespread expression helps to explain previous evidence of significant changes in CYP450-mediated oxylipid production and fat-soluble vitamin metabolism in organ microenvi-

ronments during periods of oxidative stress or disease. As such, these data provide a foundation for targeted functional experiments aimed at understanding the activities of specific CYP450 and associated therapeutic potential during times of increased disease risk.

Key words: cytochrome p450, dairy cow, oxylipid, vitamin D

INTRODUCTION

During the periparturient period, inflammation in dairy cattle becomes dysregulated, causing impaired immune health. Dysregulated inflammation predisposes cattle to disease and excessive inflammatory tissue damage, resulting in reduced productivity (Sordillo and Raphael, 2013). Many factors contribute to the dysregulation of inflammation during the periparturient period, including the altered generation of oxidized PUFA inflammatory mediators, collectively termed oxylipids, and an inadequate amount of fat-soluble vitamins necessary for abrogating oxidative stress (Sordillo, 2016).

Oxylipids are derived non-enzymatically from exposure to reactive oxygen species and enzymatically through cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450)-mediated pathways (Gabbs et al., 2015; Sordillo, 2018). Despite the historical focus placed on the COX and LOX pathways, recent work among cattle has suggested that the CYP450 pathway has a significant role in influencing health and disease. In studies profiling oxylipids in periparturient cows compared with those in other lactation stages, and cows with coliform mastitis compared with healthy controls, changes in the production of CYP450-derived oxylipids outnumbered those from either COX or LOX pathways in plasma and milk (Mavangira et al., 2015; Kuhn et al., 2017). Surprisingly, the differences noted in milk outnumbered those found in plasma, suggesting substantial local regulation of CYP450-derived oxylipids in the mammary gland.

Despite previous work showing significant production of CYP450-derived oxylipids in the mammary gland, little information is available regarding extrahepatic expression of CYP450 enzymes that may offer insight

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Table 1. Proprietary TaqMan primer or probe reference information and gene activity

Gene	NCBI reference sequence ¹	TaqMan assay ID	Activity ²
<i>CYP1A1</i>	XM_005222018	Custom assay ³	Epoxygenase, STωH
<i>CYP2C19</i>	NM_001109792.2	Bt03268476_m1	Epoxygenase, STωH
<i>CYP2E1</i>	NM_174530.2	Bt03237496_m1	Epoxygenase, STωH
<i>CYP2J2</i>	XM_002707809	Custom assay ³	Epoxygenase, STωH, 25-hydroxylase
<i>CYP3A4</i>	NM_001099367.1	Bt03293209_sH	Epoxygenase, STωH, 25-hydroxylase
<i>CYP4A11</i>	NM_001077908.1	Bt03243984_m1	TCωH
<i>CYP4F2</i>	NM_001035042.1	Bt03221777_m1	TCωH, vitamin E and LTB4 metabolism
<i>CYP24A1</i>	NM_001191417.1	Bt04306544_m1	24-Hydroxylase
<i>CYP26A1</i>	NM_001168662.1	Bt04280827_m1	Vitamin A metabolism
<i>CYP27A1</i>	NM_001083413.2	Bt03255966_m1	25-Hydroxylase
<i>CYP27B1</i>	NM_001192284.1	Bt04311113_g1	1α-Hydroxylase
<i>EPHX2</i>	NM_001075534.1	Bt03241449_m1	Epoxide hydrolase
<i>RPS9</i>	NM_001101152.2	Bt03272017_m1	Reference gene

¹National Center for Biotechnology Information reference sequence found in the nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>).

²STωH = subterminal ω-hydroxylase; TCωH = terminal carbon ω-hydroxylase; LTB4 = leukotriene B4.

³Information regarding the creation of custom TaqMan primer and probe sets can be found in Table 2.

into the mechanisms behind local regulation of these lipid mediators (Mavangira et al., 2015; Kuhn et al., 2017). Given the diverse activities of CYP450 enzymes (Table 1), functional studies are necessary to understand the specific CYP450 enzymes contributing to oxylipid production. Establishing a transcript expression profile of CYP450 enzymes builds a foundation for such studies, given that extrahepatic expression for many CYP450 has not yet been shown.

The multiple activities of individual CYP450 and redundant activities between them create a complex network of oxylipid and vitamin regulation with multiple instances of substrate and function crossover. Understanding the unique activities of individual CYP450 and overlapping activities between them is key to understanding their roles in health and disease. For example, several CYP450 may metabolize arachidonic acid into oxylipids, 7 of which were included in this study (Figure 1A). Currently, no study in cattle has established the relative contribution of differing CYP450 to the overall oxylipid pool. Due to this, CYP450 that metabolize PUFA chosen for this study were selected based on their significant contribution to the oxylipid pool in other species, such as CYP2C19, CYP2E1, CYP3A4, CYP4A11, and CYP4F2, or previous research in cattle that has explored the activity of a specific CYP450, such as CYP1A1 or CYP2J2 (Lasker et al., 2000; Yang et al., 2001; Fer et al., 2008; Zhang et al., 2018).

A subset of these PUFA-metabolizing CYP450, the CYP450 family 4 (**CYP4**) class, including CYP4A11 and CYP4F2, have predominantly terminal carbon ω-hydroxylase activity (**TCωH**), producing the highly pro-inflammatory 20-hydroxyeicosatetraenoic acid (**20-HETE**) as their primary product (Powell et al., 1998). In contrast, CYP1A1, CYP2C19, CYP2E1, CYP2J2,

and CYP3A4 generally act as epoxygenases, creating 4 isomers of epoxyeicosatrienoic acids (**EET**) as a primary product. These EET are further metabolized by the non-CYP450 enzyme soluble epoxide hydrolase (**EPHX2**) to secondary and presumably less anti-inflammatory oxylipid metabolites. As such, EPHX2 is an enzymatic step that has been targeted in other veterinary species for therapeutic intervention for the reduction of pain due to inflammation (Bylund et al., 1998; Guedes et al., 2017). Secondarily, and to a lesser extent, these latter 5 epoxygenases metabolize arachidonic acid by hydroxylating carbons 16–19, thus acting as subterminal ω-hydroxylases (**STωH**), a class of oxylipids whose activities are only beginning to be understood in veterinary research.

Whereas oxylipids are direct mediators of inflammation, fat-soluble vitamins A, D, and E act upon inflammation both directly, through regulation of inflammatory signaling, and indirectly, as antioxidants curtailing oxidative stress (Sordillo, 2016). Although the mechanisms by which fat-soluble vitamins can influence inflammation differ from oxylipids, their regulation shares many of the same CYP450 enzymes that regulate oxylipid production. For example, epoxygenases CYP2J2 and CYP3A4 also act as a 25-hydroxylase of vitamin D, along with CYP27A1, to form 25-hydroxyvitamin D₃ [**25-(OH)D₃**; Figure 1B]. Although several CYP450 have been identified as 25-hydroxylases in other species, no studies have conclusively identified the relative contribution of each of these enzymes to the overall production of 25-(OH)D₃ in cattle. It has been shown, however, that polymorphisms in *CYP2J2* and *CYP27A1* lead to reduced serum 25-(OH)D₃ and ultimately are associated with incidence of milk fever (Casas et al., 2013; Pacheco et al., 2018). Although these studies do not provide direct evidence of activ-

Figure 1. Cytochrome P450 enzymes are placed upon their organ of highest mRNA expression as follows: (A) from left to right, spleen, liver, kidney; (B) clockwise from upper left, liver, spleen, kidney; (C) kidney; (D) liver. (A) Several cytochrome P450 enzymes are capable of metabolizing PUFA; shown here are pathways of arachidonic acid metabolism. Cytochrome P450 enzymes (backed by blue and on the left) act as epoxigenases producing epoxyeicosatrienoic acids (EET) that can be further metabolized by the enzyme EPHX2 to dihydroxyeicosatrienoic acids (DHET). Cytochrome P450 enzymes (backed in red, on the right) have predominantly terminal carbon ω -hydroxylase activity, producing 20-hydroxyeicosatetraenoic acid (20-HETE). (B) Cytochrome P450 enzymes involved in the activation and inactivation of vitamin D. Vitamin D is obtained from either exposure of the skin to UV light or directly from the diet, as either vitamin D₂ or vitamin D₃. Shown is the cascade for vitamin D₃ as the initial compound is metabolized to 25-hydroxyvitamin D₃ [25-(OH)D₃] by at least 2 cytochrome p450 enzymes in cattle, CYP27A1 and CYP2J2. Then, 25-(OH)D₃ is metabolized by the 1 α -hydroxylase CYP27B1 to produce active vitamin D, 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃]. Ultimately, both 25-(OH)D₃ and 1,25-(OH)₂D₃ are inactivated by CYP24A1 to molecules of low bioactivity such as 24,25-dihydroxycholecalciferol or calcitroic acid. (C) All 8 analogs of vitamin E are hydroxylated by CYP4F2 to form analog-specific forms of 13'-carboxychromanol. 13'-carboxychromanol is readily metabolized by other means to water-soluble metabolites, some of which have significant bioactivity. (D) Vitamin A is a group of compounds, including carotenoids and retinyl esters, ultimately metabolized to forms of retinoic acids, including all-*trans* retinoic acid (RA), 9-*cis* RA, and 13-*cis* RA. Such RA are metabolized by cytochrome P450 family 26 enzymes, specifically CYP26A1 evaluated in this study, to metabolites with little bioactivity. Figure created with BioRender.com.

ity or discount the potential activity of other CYP450 25-hydroxylases, they nonetheless provide evidence of specific CYP450 that have clinical effects on the health of cattle. Metabolism of vitamin D continues with the activation of 25-(OH)D₃ to 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], the active form of vitamin D, solely by CYP27B1. Last in this cascade, CYP24A1 alone is responsible for the ultimate degradation of 25-(OH)D₃ and 1,25-(OH)₂D₃ to inactive compounds (Christakos et al., 2016).

Vitamins A and E also can be degraded by CYP450. In addition to its TCωH activity, CYP4F2 is the single CYP450 responsible for the degradation of analogs of vitamin E to their inactive forms (Figure 1C; Sontag and Parker, 2002). Further, the cytochrome P450 family 26 (CYP26) class of enzymes degrades active forms of vitamin A to less bioactive metabolites (Figure 1D; Ross and Zolfaghari, 2011). As with CYP450-derived oxylipids, the regulation of these vitamins is disturbed during times of greatest need (LeBlanc et al., 2004; Holcombe et al., 2018). Regulation of vitamin metabolism is another essential aspect of understanding how CYP450 enzymes affect cow health and disease.

Modeling the function of CYP450 enzymes will require an understanding of their transcriptional expression in cell culture systems that are relevant to bovine health and production. Bovine mammary alveolar epithelial cells (MAC-T) and Madin-Darby bovine kidney cells (MDBK) are commonly sought for their immortalized nature, providing reproducible results through many passages (Madin and Darby, 1958; Huynh et al., 1991). On the other hand, primary cells, such as bovine aortic endothelial cells (BAEC) and bovine mammary endothelial cells (BMEC), are valued for their phenotypic similarities to in vivo counterparts (Yang et al., 2001; Ryman et al., 2016).

Although CYP450 have the capacity to significantly affect inflammation by regulating vitamin metabolism and contributing to the oxylipid pool, their relative expression in cattle and potential for study using in vitro models is largely unknown. The aim of this study was to describe the relative basal transcript expression of CYP450 enzymes relevant to the regulation of inflammation in healthy cattle. Additionally, with the need for in vitro models for functional studies of CYP450 enzymes, expression of these CYP450 was evaluated in both primary and immortalized cell lines of endothelial and epithelial cells, respectively. Many studies have evaluated a single CYP450 or a small subset of such enzymes; however, none have explored such a wide range of enzymes among the many tissues of cattle nor presented data in a manner that allows for comparison between CYP450, as described herein. Furthermore, no studies as of yet have evaluated relative expression

of CYP450 between commonly used in vitro bovine models. This report expands our current understanding of CYP450 expression in cattle, showing novel tissue expression of several enzymes and offering a foundation for future functional assays to build from.

MATERIALS AND METHODS

Chemicals and Reagents

QIAzol lysis reagent and RLT buffer were purchased from Qiagen Sciences (Germantown, MD). Chloroform was purchased from Thermo Fisher Scientific (Fair Lawn, NJ) and Dulbecco's modified Eagle's medium was obtained from Gibco (Thermo Fisher Scientific). Collagenase and Hanks' balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO). Ham's F-12K medium was purchased from Irvine Scientific (Santa Ana, CA).

Animals and Tissue Sampling

Three multiparous Holstein cows were culled from the Michigan State University Dairy Teaching and Research Center (East Lansing) for non-disease-related reasons. Parity ranged from lactation 3 to 7, with all cows between 74 and 163 DIM. Production in the previous lactation ranged from 8,095 to 13,641 kg of milk on a 305-d milking average. Two cows were open, and 1 was pregnant at the time of culling. The cows were milked twice daily, kept in a freestall barn, offered water ad libitum, and fed a TMR twice daily.

Cows were euthanized by captive bolt and exsanguination, and tissues were collected within 15 min of euthanasia. Samples of liver, kidney, heart, skeletal muscle, spleen, mammary gland, lung, and uterus were taken aseptically by removing roughly 0.5-cm pieces of organ tissue from areas devoid of serosal surfaces or large vasculature. Samples were placed in 2-mL Eppendorf tubes, flash frozen in liquid nitrogen, and stored at -80°C for less than 3 mo before RNA extraction.

Primary Cell Isolation

Bovine mammary endothelial cells and BAEC were collected as described previously (Aherne et al., 1995). Briefly, for BAEC isolation, a 10-cm full-circumference section of descending aorta, just distal to the branch of the subclavian artery, was collected at a commercial abattoir and immediately submerged in HBSS with 0.05 mg/mL gentamicin on ice. Pudendal artery for BMEC isolation was collected by taking a 4-cm full-circumference sample of pudendal artery near the cut edge of the mammary gland, submerged immediately

in HBSS with 0.05 mg/mL gentamicin, and placed on ice. Once transported to the laboratory for processing, aorta samples were cut lengthwise and laid flat in a solution of 2 mg/mL collagenase in Krebs-Ringer bicarbonate with 4% BSA and allowed to incubate at 37°C for 10 min. Pudendal artery samples were rinsed with HBSS and clamped at one end, filled with a solution of 2 mg/mL collagenase in Krebs-Ringer bicarbonate with 4% BSA, clamped at the remaining open end, and incubated for 10 min at 37°C. After each incubation, the collagenase solution was collected and the luminal sides of tissues were rinsed with HBSS, collecting the rinse solution. The rinse solution was added to collected collagenase and samples were centrifuged at $160 \times g$ for 10 min at 15°C. Resulting cellular pellets were plated in T25 cell culture flasks until confluent in Ham's F12K medium containing 10% fetal bovine serum, 10 mM HEPES buffer, 0.25% sodium bicarbonate, 2 mM L-glutamine, 1% 1:1 antibiotic:antimycotic, 100 µg/mL heparin, 10 µg/mL insulin, 5 µg/mL transferrin, and 40 ng/mL sodium selenite.

Cells were detached via incubation with 0.05% trypsin, diluted serially, and plated in 96-well plates. To exclude fibroblasts and other cell types, wells containing only colonies derived from a single cell were selected for propagation. Wells were selected by typical endothelial cobblestone morphology for further propagation and confirmed by von Willebrand factor staining. Cells were frozen at passage 3 and grown to passage 6 for RNA isolation, representing the earliest passage at which cells could realistically be used for assays.

Cell Line Culture

Madin-Darby bovine kidney cells were obtained from the American Type Culture Collection (Manassas, VA), and MAC-T cells were obtained from the USDA Agricultural Research Service as a line of primary mammary alveolar cells transfected with SV-40 large T-antigen for immortalization (Huynh et al., 1991). Both MDBK and MAC-T cells were cultured in Dulbecco's modified Eagle's medium (+ 4.5 g/L D-glucose) devoid of phenol red, sodium pyruvate, or L-glutamine. Added to this medium were the following: 10% fetal bovine serum, 10 mM HEPES buffer, 0.25% sodium bicarbonate, 2 mM L-glutamine, 1% 1:1 antibiotic:antimycotic, 100 µg/mL heparin, 10 µg/mL insulin, 5 µg/mL transferrin, and 40 ng/mL sodium selenite. Cells were incubated at 5% CO₂ and 37°C.

RNA Extraction

Tissues were cut into 30- to 40-mg pieces with a sterile scalpel while still frozen and added to 450 µL of QIAzol

lysis reagent (Qiagen). Samples of muscle tissue were cut into 80-mg pieces for homogenization. A TissueRuptor II tissue homogenizer (Qiagen) was run at maximum allowable speed for 1 min for tissue homogenization. An additional 450 µL of QIAzol was added to samples, which were then incubated at room temperature for 5 min. After incubation, 180 µL of chloroform was added to each tube, and tubes were vigorously shaken for 15 s and incubated at room temperature for 3 min. Samples were centrifuged at $12,000 \times g$, 4°C, for 15 min, and the upper aqueous layer was removed and added to a new 2-mL tube for RNA extraction.

Cultured cells were grown on 100-mm cell culture treated dishes. Once confluent, dishes were washed twice with HBSS and 600 µL of buffer RLT (Qiagen) was added for cell lysis. Buffer RLT was collected, added to QIAshredders nested in 2-mL Eppendorf tubes, and spun at $16,000 \times g$ for 2 min. Flow-through was stored at -20°C.

Once all tissue and cell culture samples were obtained, RNA was extracted according to the Qiagen RNeasy protocol with DNase digest, using a QIAcube (Qiagen). RNA quantity and quality were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher).

Reverse Transcription

For cDNA generation, RNA was diluted with nuclease-free water to standardize all samples. A master mix of 10× reverse-transcription buffer, 25× dNTP, 10× random primers, Multiscribe reverse transcriptase, RNase inhibitor, and RNase nuclease-free water from a high-capacity cDNA reverse-transcription kit with RNase inhibitor (all kit components from Applied Biosystems, Vilnius, Lithuania), was added at an equal volume to diluted RNA. Samples were placed in a PTC-200 Peltier Thermo Cycler (MJ Research, Waltham, MA), which ran as follows: stage 1, 25°C for 10 min; stage 2, 37°C for 2 h; stage 3, 85°C for 5 min; stage 4, hold at 4°C.

Real-Time PCR

Real-time PCR was carried out with predesigned TaqMan primers and FAM-MGB probes (Applied Biosystems, Pleasanton, CA) for those genes listed in Table 1. Two genes were created as custom TaqMan primers, listed in Table 2. All primers and probes were used in a 7500 Fast Real-time PCR system (Applied Biosystems). Genes were evaluated in triplicate with 2× TaqMan Gene Expression Master Mix (Applied Biosystems), 20× Custom TaqMan Gene Expression Assay Mix (Applied Biosystems), sample cDNA (50

Table 2. Primer sequences for custom TaqMan primers

Gene	Direction	Primer sequence (5'–3')	bp	Accession no. ¹
<i>CYP1A1</i>	Forward	TTGTGAACCACTGGCAGATCAA	64	XM_005222018.4
	Reverse	GGCCGGAAGTCAGATGGAT		
<i>CYP2J2</i>	Forward	TCCTACCTCATCCAGACGATAAGAG	78	XM_002707809.6
	Reverse	GGAAACGGCATTGTTGATTGTCA		

¹Accession numbers from National Center for Biotechnology Information gene bank database (<https://www.ncbi.nlm.nih.gov/gene>).

ng/well for tissues, 100 ng/well for cells), and nuclease-free water for a total of 10 µL per reaction well. A 20× pre-designed TaqMan Gene Expression Assay for *RPS9* was used as endogenous control (Applied Biosystems). Thermal cycling conditions for the Fast 2-step PCR system were as follows: stage 1, 95°C for 20 s; stage 2, 95°C for 3 s; stage 3, 60°C for 30 s, with 40 cycles of stages 2 and 3. Data were recorded and compiled using ExpressionSuite Software version 1.0.4 (Applied Biosystems) and analyzed using DataAssist Software version 3.01 (Applied Biosystems, Foster City, CA). Data from tissues and in vitro cell samples, having no standard cell type for comparison, are expressed as $2^{-\Delta CT}$.

Efficiency (E) of PCR was calculated based on the slope of a standard curve of tissue samples, serially diluted. The slope of the standard curve was used to calculate E, using the equation $E = (10^{-1/\text{slope}} - 1) \times 100$. Calculated efficiency was 100.04% for *CYP1A1* and 99.99% for *CYP2J2*, verifying acceptable efficiency for these TaqMan custom primer and probe sets.

RESULTS

Tissue Expression

Gene expression of CYP450 enzymes in tissues of major organs of dairy cattle was surprisingly widespread, with every CYP450 evaluated found to be expressed in at least 1 sample of every tissue type (Figure 2). As expected, the liver, in general, expressed all CYP450 to the highest degree, with the only exceptions being *CYP2J2*, *CYP2A1*, and *CYP27B1*.

Oxylipid Metabolism. The CYP450 that metabolize oxylipids from PUFA substrate in this study include *CYP1A1*, *CYP2C19*, *CYP2E1*, *CYP2J2*, *CYP3A4*, *CYP4A11*, and *CYP4F2*. Of the CYP450 with significant epoxygenase and STωH activity, including *CYP1A1*, *CYP2C19*, *CYP2J2*, *CYP2E1*, and *CYP3A4*, all were found to have greatest expression in the liver except for *CYP2J2*. Instead, *CYP2J2* was expressed in splenic and lung tissue 6.06-fold and 1.42-fold above liver expression, respectively ($2^{-\Delta\Delta CT}$, reference gene *RPS9*). Additionally, *CYP1A1* and *CYP3A4* each had their second-greatest expression in lung tissue, whereas *CYP2E1* and *CYP2C19* had the lowest tissue expres-

sion in the lung. Although there are inherent differences between the isolation of specific CYP450 and reverse transcriptase efficiency, samples were processed as a single batch, ensuring appropriate RNA quality, and used a single lot of reverse transcriptase to reduce these effects. Comparing CYP450, we found *CYP2C19* to be expressed to a considerably greater amount in the liver than the next closest epoxygenase (*CYP2C19* $2^{-\Delta CT}$ of 87.69, compared with 28.09 of *CYP2E1* or 5.19 of *CYP1A1*; reference gene *RPS9*) and was the predominate epoxygenase in the mammary gland, heart, and uterus, in each case followed in expression by *CYP1A1* and *CYP2E1* sequentially. In the lung, *CYP2C19* dominated epoxygenase expression with little comparable expression by any other epoxygenase. Interestingly, the spleen was comparatively devoid of epoxygenase expression apart from *CYP2J2*.

Of the 2 TCωH enzymes, *CYP4A11* was most highly expressed in the liver and secondarily in the kidney, whereas *CYP4F2* had a significantly greater expression in the kidney than any other tissue. Additionally, *CYP4F2* was also the only CYP450 not reliably expressed in each tissue type, with samples of skeletal muscle, spleen, and uterus devoid of expression of this enzyme. Despite increased expression of *CYP4F2* in the kidney compared with the liver, overall transcription of *CYP4F2* paled in comparison to *CYP4A11* in all tissues.

Although not a CYP450 enzyme, the EPHX2 enzyme is instrumental in secondary metabolism of CYP450-derived epoxides, such as metabolizing EET to DHET. We found that *EPHX2* was expressed to the greatest amount in the liver compared with reference gene *RPS9* ($2^{-\Delta CT}$ of 1.3), and to a lesser amount in other tissues, having its second highest expression in the kidney ($2^{-\Delta CT}$ of 0.31), followed by other organs, which were expressed at similar amounts ($2^{-\Delta CT}$ ranging from 0.012 to 0.12).

Vitamin D Metabolism. Of the 3 25-hydroxylases evaluated in this study, *CYP27A1* and *CYP3A4* were both expressed to the highest extent in liver tissue, whereas *CYP2J2* was expressed most highly in the spleen. Between CYP450, *CYP27A1* was found to be the most highly expressed 25-hydroxylase in every tissue type and had the most consistent expression profile between tissues.

We found *CYP27B1* expressed to a considerably greater amount in the kidney than in any other tissue, with the next highest expression in heart and uterine tissues. The 24-hydroxylase *CYP24A1* was also relatively highly expressed in the kidney compared with

all other tissues, apart from the uterus. Interestingly, 1 sample of uterine tissue was found to have expression of *CYP24A1* comparable to those found in the kidney, whereas the remaining 2 uterine tissue samples had levels near that of liver.

Figure 2. Relative abundance (mean $\pm$ SEM) of gene transcripts for *CYP1A1*, *CYP2C19*, *CYP2E1*, *CYP2J2*, *CYP3A4*, *CYP4A11*, *CYP4F2*, *CYP24A1*, *CYP26A1*, *CYP27A1*, *CYP27B1*, and *EPHX2* found in liver, kidney, lung, mammary gland, heart, skeletal muscle, spleen, and uterine tissues from healthy, lactating dairy cows ($n = 3$). §Presented value excludes sample not found during PCR cycling. #Presented value contains samples with cycle to threshold value >35 cycles. §§Presented value excludes 2 samples not found during PCR cycling. Values are presented as transcript abundance ($2^{-\Delta CT}$) relative to endogenous control gene *RPS9*.

Vitamin A Metabolism. We found *CYP26A1* to be transcribed in all tissue types, with the highest expression in the liver, followed closely by uterine tissue. Moderate expression was noted in heart, kidney, and mammary tissue, with low expression in lung, skeletal muscle, and spleen.

Cell Culture Expression

Oxylipid Metabolism. Gene expression of oxylipid-producing CYP450 in cultured bovine cells was far less robust than noted in tissues. We found that *CYP2C19* and *CYP4F2* were not reliably expressed by any cell type evaluated, *CYP4A11* only consistently expressed in BAEC, and *CYP2E1* only consistently expressed in BAEC and MAC-T (Table 3). Additionally, *CYP2J2* was not expressed by a clone of the MDBK cell line. The MAC-T cell line expressed both *CYP1A1* and *CYP2J2* to considerably greater amounts, over 10-fold, compared with the next highest-expressing cell type (Figure 3). Expression of *CYP3A4* varied between cell types but was nonetheless reliably expressed in each sample. We found that *EPHX2* was similarly expressed among cell types but considerably lower in MDBK cells.

Vitamin D Metabolism. Of all 25-hydroxylases evaluated, *CYP27A1* was expressed to the highest amount and was consistently expressed among cell types, aside from MAC-T cells, for which *CYP27A1* had measurable yet low expression (Figure 3). The final 2 steps of vitamin D metabolism, *CYP27B1* and *CYP24A1*, were both reliably expressed in all cell types. Although both *CYP27B1* and *CYP24A1* had greater expression in MAC-T cells compared with either endothelial cell type, MDBK expression was converse, representing the lowest expressing cell type of *CYP27B1* yet highest expressing cell type of *CYP24A1*.

Vitamin A Metabolism. In cell culture models, *CYP26A1* was consistently expressed among all cell types, with greatest expression in MAC-T and lowest in MDBK cells. Compared with other CYP450, however, *CYP26A1* was expressed to a comparatively lesser amount (Figure 3).

DISCUSSION

Circulating concentrations of oxylipids and fat-soluble vitamins A, D, and E fluctuate during the periparturient period in cattle, contributing to a state of oxidative stress and dysfunctional inflammation at this time (Sordillo, 2016, 2018). Despite influencing inflammation through differing mechanisms, these metabolites share regulation by the CYP450 enzymatic pathway. Nevertheless, before this study, little was known about

Table 3. Cell samples with cycles to threshold (CT) values >35 cycles or undetected (n = 3)

Gene	Cell type ¹			
	BAEC	BMEC	MAC-T	MDBK
<i>CYP2C19</i>	30.05	37.33	35.54 ²	27.03 ²
<i>CYP2E1</i>	28.86	35.94 ²	34.27	35.56 ²
<i>CYP4A11</i>	33.62	29.07 ²	37.09 ²	36.99 ²
<i>CYP4F2</i>	— ³	— ³	33.31 ²	36.95 ²

¹BAEC = bovine aortic endothelial cell; BMEC = bovine mammary endothelial cell; MAC-T = bovine immortalized mammary epithelial cells; MDBK = Madin-Darby bovine kidney epithelial cell.

²Excludes samples with no expression.

³No expression found in any sample (100 ng of RNA).

the extrahepatic expression of specific CYP450 in dairy cattle. Surprisingly, every CYP450 evaluated herein, apart from *CYP4F2*, was transcriptionally expressed in every tissue sampled. Although extrahepatic expression was relatively low for many CYP450, local effects of CYP450 should not be overlooked. For example, infusion of LPS into the mammary gland results in isolated activation of CYP450 involved in the regulation of vitamin D without changes in activation of CYP450 noted outside of the mammary gland (Merriman et al., 2018).

Conclusions drawn from these data are limited by the understanding that gene expression does not directly relate to activity of an enzyme. Post-transcriptional and translational modification through phosphorylation, glycosylation, and protein degradation are all known to modify the activity of CYP450 (Aguiar et al., 2005). In general, however, CYP450 activity is influenced most by substrate availability, such as the induction of *CYP24A1* by 1,25-(OH)₂D₃ or *CYP26A1* by vitamin A (Krüger et al., 2005; Merriman et al., 2017). As such, detailing mRNA expression to understand the breadth of CYP450 distribution in various organs is needed as a first step for functionality research.

Samples with cycles to threshold (CT) values of greater than 35 cycles were not included in Figure 3, as quantitative accuracy diminishes beyond this point (Lorenz, 2012). Such samples, included in Table 3, cannot be compared directly to those with reliable expression. Tissue samples included bovine liver, kidney, mammary gland parenchyma, lung, spleen, heart, skeletal muscle, and full-thickness uterus from healthy, multiparous, lactating cows.

Epoxygenases

The epoxygenases examined in this study were *CYP1A1*, *CYP2C19*, *CYP2E1*, *CYP2J2*, and *CYP3A4* (Fer et al., 2008). Unlike previous reports, we found that *CYP1A1* was not distributed evenly among tis-

sues but had a preponderance in the liver similar to other CYP450 (Darwish et al., 2010). Prominent lung expression, compared with other CYP450, however, is

in line with a previous report in human tissue profiling. Such lung expression may underscore the importance of ST ω H activity also noted from epoxigenases such as

Figure 3. Relative abundance (mean $\pm$ SEM) of gene transcripts for *CYP1A1*, *CYP2J2*, *CYP3A4*, *CYP24A1*, *CYP26A1*, *CYP27A1*, *CYP27B1*, and *EPHX2* in bovine primary aortic endothelial cells (BAEC), bovine primary mammary endothelial cells (BMEC), bovine immortalized mammary epithelial cells (MAC-T), and Madin-Darby bovine kidney cells (MDBK; $n = 3$). #Presented value contains sample with cycle to threshold value >35 cycles. Values are presented as transcript abundance ($2^{-\Delta CT}$) relative to endogenous control gene *RPS9*.

CYP1A1. Relatively high lung expression of *CYP1A1* may support the necessity of 19(R)-HETE production in the local pulmonary environment, where 20-HETE is potentially vasoactive (Bièche et al., 2007; Fan et al., 2016). Robust hepatic *CYP2C19* expression is unsurprising, given its role in xenobiotic metabolism; however, the greater hepatic expression relative to other xenobiotic-metabolizing CYP450 is not conserved in other species (Bièche et al., 2007; Hirota et al., 2013). Additionally, the extrahepatic expression of *CYP2E1* and *CYP3A4* was unexpected and novel, as previously published findings in cattle found little to no extrahepatic expression (Krüger et al., 2005; Darwish et al., 2010).

Interestingly, we found robust splenic expression of *CYP2J2* compared with its expression in other tissues, as opposed to the relatively pronounced cardiac expression seen in other ruminants (Messina et al., 2010). In this regard, among CYP450 evaluated, *CYP2J2* was the highest-expressing CYP450 in splenic tissue as well. This relatively greater splenic expression may be due to the preference of CYP2J2 for producing EET rather than linoleic acid-derived oxylipids or subterminal HETE. Because EET are a vasodilatory class of oxylipids, such increased expression relative to other tissues may facilitate at least partial control over splenic and hepatic blood flow (Fer et al., 2008; Di Pascoli et al., 2016).

When measuring oxylipids derived from the CYP450 enzymatic pathway, in addition to absolute values, it has proven beneficial to put outcomes in a context comparing the primary produced oxylipid to the metabolite formed from EPHX2. For example, in dairy cattle, the ratio of primary to secondary metabolites of the ω -6 PUFA linoleic acid (epoxyoctadecenoic acid to dihydroxyoctadecenoic acid), is increased significantly in the milk and plasma of cows with coliform mastitis despite not all oxylipid concentrations significantly differing between sick and healthy animals (Mavangira et al., 2015). Differences in oxylipid abundance such as this have led others to focus on EPHX2 as a therapeutic target with clinically beneficial results, such as the amelioration of inflammation and pain from laminitis in horses (Guedes et al., 2017). Interestingly, despite its close-knit activity to CYP450 epoxigenases, *EPHX2* expression was not distributed similarly to any epoxigenase enzyme. Nevertheless, *EPHX2* had widespread expression throughout all tissues. As such, EPHX2 may indeed significantly contribute to oxylipid metabolism not only systemically but at the local level in tissues throughout the body.

Expression of epoxigenases in differing bovine in vitro cell types was overall less robust than expression

in tissues, likely due to the cellular heterogeneity of tissues and known reduction of CYP450 expression soon after removal from parent tissues (Xiang et al., 2019). In general, no in vitro cell type expressed a profile of epoxigenases similar to the profile of any tissue. The low expression of *CYP3A4* and *CYP2E1* in cultured bovine cells, compared with the organ types from which they were originally derived, has indeed been noted previously (Talbot et al., 2016).

ω -Hydroxylases

In addition to epoxide formation of mid-chain double bonds, CYP450 hydroxylate PUFA at the terminal carbon. Terminal carbon ω -hydroxylases CYP4A11 and CYP4F2 produce the highly pro-inflammatory oxylipid 20-HETE from arachidonic acid (Johnson et al., 2015). Although not the only 2 enzymes to produce 20-HETE, CYP4F2 and CYP4A11 are the major contributors to 20-HETE production in other species (Lasker et al., 2000). Although the relative input of each CYP4 to 20-HETE production is unknown in cattle, these data suggest that on a transcription basis, CYP4A11 is the greater of the 2, with production predominantly in the liver and kidney.

Aside from arachidonic acid, CYP4F2 has several substrates, including the oxylipid leukotriene B4 (LTB4) and vitamin E (Zhang et al., 2000; McDonald et al., 2009; Schmölz et al., 2016). Interestingly, despite reported widespread expression of *CYP4F2* in cattle, this was the only CYP450 enzyme not found consistently in all tissues (Haga et al., 2015). Nonetheless, bovine CYP4 enzymes have proven to participate in mediating immune functions in organ microenvironments. Indeed, 20-HETE production in the mammary gland appears to be locally regulated (Mavangira et al., 2015; Kuhn et al., 2017). Cattle with lower expression of cutaneous *CYP4F3*, another CYP450 which metabolizes LTB4, also show resistance to ectoparasites by maintaining greater amounts of LTB4 (Tabor et al., 2017). Differential expression of *CYP4F2* may also alter the rate of degradation of vitamin E. Certainly in other species, reduced expression of *CYP4F2* slows the breakdown of vitamin E; however, the contribution of CYP4F2 to circulating vitamin E concentrations in cattle is unknown (Bartolini et al., 2017). The lack of consistent expression of TC ω H *CYP4F2* and *CYP4A11* cell culture models underscores why such research is so difficult to undertake. Although the CYP4 family of enzymes shows indications of important functions in organ microenvironments, a lack of cell culture models, as supported by these data, has prevented the study of such functions without transfection.

Vitamin D Metabolism

Initial vitamin D from the diet or UV light exposure of the skin is metabolized to 25-(OH)D₃ by several CYP450, including CYP2J2, CYP3A4, and CYP27A1, evaluated in this study. Of bovine 25-hydroxylases, thus far only *CYP2J2* and *CYP27A1* have been associated with measurable contributions to calcium homeostasis. Such findings suggest that although other 25-hydroxylases may significantly produce 25-(OH)D₃, at a minimum CYP2J2 and CYP27A1 play a role in vitamin D metabolism important enough to influence clinical disease (Casas et al., 2013; Pacheco et al., 2018). In other species, CYP2R1 appears to be the primary 25-hydroxylase, and in cattle, gene expression of *CYP2R1* in mammary epithelial cells stimulated with cholecalciferol certainly increases in a dose-dependent manner, as would be expected with substrate supplementation (Télez-Pérez et al., 2012; Christakos et al., 2016). Additionally, *CYP2R1* has been associated with incidence of mastitis; however, it is unclear whether this is due to 25-hydroxylase activity or to a separate mechanism (Naderi et al., 2018). Despite these understandings, no direct evidence of the relative contribution of CYP2R1 to 25-(OH)D₃ has been shown in cattle.

The importance of 25-hydroxylases in health is beginning to draw greater attention, as our understanding of how clinical and subclinical hypocalcemia influence the predisposition to other diseases improves. Given that hypocalcemia is implicated as a contributing factor to several periparturient cow disorders, the importance of 25-hydroxylases goes beyond milk fever alone (Rodríguez et al., 2017). The relatively high expression of *CYP27A1* compared with other 25-hydroxylases bolsters the proposition by Pacheco et al. (2018) that it is indeed one of the primary drivers of 25-(OH)D₃ production in cattle.

The active form of vitamin D₃, 1,25-(OH)₂D₃, is formed solely by CYP27B1 (Christakos et al., 2016). Previously thought to occur primarily in the kidney, these data and recent research in cattle show that expression is found widely throughout the body in many tissues and circulating leukocytes (Nelson et al., 2010a; Nelson et al., 2011). The activity of CYP27B1 outside of the kidney may be critical for the immune-modulating activities of 1,25-(OH)₂D₃. Specifically in the mammary gland, several studies have noted the antibacterial and anti-inflammatory properties of vitamin D, as well as the importance of *CYP27B1* upregulation in response to pathogen insult (Merriman et al., 2017; Merriman et al., 2018). With relatively high expression in lung and uterine tissues, which commonly face pathogen challenge, *CYP27B1* may contribute to the antibacterial defenses of these organs as well.

The final step in the vitamin D pathway is carried out by CYP24A1, which is generally induced by the metabolites it ultimately degrades to maintain appropriate 1,25-(OH)₂D₃ amounts (Christakos et al., 2016). Its induction is inhibited by LPS, however, potentially acting as a defense mechanism against pathogen insult to prolong the activity of 1,25-(OH)₂D₃ (Nelson et al., 2010b).

Vitamin A Degradation

Three CYP450 of the CYP26 family of enzymes (CYP26A1, CYP26B1, CYP26C1) are primarily responsible for the degradation of the active forms of vitamin A, namely all-*trans* retinoic acid (**AT-RA**), 9-*cis* retinoic acid, and 13-*cis* retinoic acid. Of the 3 CYP26 enzymes, CYP26A1 was included in this profile due to its widespread tissue expression in humans, which was recapitulated in the current study despite previous research in cattle showing little extrahepatic expression (Krüger et al., 2005; Ross and Zolfaghari, 2011). Prominent expression in liver samples is conserved with other mammals, whereas robust expression in uterine tissue is not yet may represent a means for the tight regulation of vitamin A necessary during pregnancy in cattle (Topletz et al., 2012).

Similarly, CYP26A1 is also likely a key regulator in the role played by active forms of vitamin A in contributing to the inflammatory response to bacterial insult. Like *CYP24A1*, the induction of *CYP26A1* is significantly reduced by LPS (Zolfaghari et al., 2007). Given the ability of AT-RA to protect bovine cells from oxidative stress and to potentiate the LPS-induced inflammatory cascade, preventing the induction of *CYP26A1* may serve as a protective mechanism by prolonging the activity of AT-RA during infection (Shi et al., 2018; Xu et al., 2019).

CONCLUSIONS

This study provides evidence that CYP450 enzymes are widely transcriptionally expressed throughout all tissues of dairy cattle, although to varying amounts. Such widespread expression in distinct cell types aids in explaining how CYP450-derived oxylipids are differentially produced in localized compartments such as the mammary gland. Furthermore, profiling of commonly utilized in vitro cells revealed significantly lower overall CYP450 expression in cultured cells compared with isolated organ tissues. Nonetheless, these data provide a valuable foundation for future studies aiming to understand the metabolism of vitamins A, D, and E in cattle as well as the production of CYP450-derived oxylipids.

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