Diet-induced alterations in hepatic progesterone (P₄) catabolic enzyme activity and P₄ clearance rate in lactating dairy cows

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Abstract

Elevated rates of steroid clearance may lead to lower reproductive success in several mammalian species. Cytochrome P450 (EC 1.14.14.1) and aldo-keto reductases (AKR; EC 1.1.1.145–151) are involved in the first phase of steroid inactivation, before second phase conjugation and excretion of the steroid metabolite. The current objectives were to determine liver blood flow (LBF), hepatic enzyme activity, and metabolic clearance rate (MCR) of progesterone (P₄) in dairy cows consuming isoenergetic and isonitrogenous diets formulated to cause divergent insulin secretion. Insulin concentrations increased by 22% in cows fed the high cornstarch diet, and both cytochrome P450 2C and cytochrome P450 3A activities were decreased ($P < 0.05$) by ~50%, while AKR1C tended ($P < 0.10$) to be lower in

cows fed the high cornstarch diet. LBF was similar between the two diets (1891 ± 91 l/h). MCR of P₄ tended ($P < 0.10$) to be lower in cows fed the high cornstarch diet (25 ± 5 l/h $\times$ BW^{0.75}) versus the high fiber diet (40 ± 6 l/h $\times$ BW^{0.75}). The half-life of P₄ was increased ($P < 0.05$) in cows fed the high cornstarch diet (73 ± 10 min) versus the high fiber diet (24 ± 10 min). In summary, cows with elevated insulin concentrations and lower enzyme activity showed a decrease in P₄ clearance without any changes in LBF. This dietary relationship with hepatic enzyme activity may explain some of the observed alterations in steroid profiles during the estrous cycle or gestation of the high producing dairy cow.

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Introduction

Progesterone (P₄) is needed to maintain a successful pregnancy, and removal of the corpus luteum will lead to termination of pregnancy unless exogenous P₄ is provided (McDonald *et al.* 1952, Csapo 1956). P₄ can influence uterine secretion of nutrients and growth factors that are essential for early embryonic development, and P₄ supplementation during early gestation, from several independent reports, improves pregnancy rates in dairy cows (reviewed by Mann & Lamming (1999)). Rhinehart *et al.* (2009) indicated a greater involvement in steroid clearance versus steroid production in regulating peripheral concentrations of P₄ in pregnant lactating dairy cows. Selection for milk production over the last six decades has been accompanied with a decrease in reproductive performance, which might be explained by increased metabolic stress and high energy demands. Independent reports have found a positive association between dry matter intake (DMI), liver blood flow (LBF), and the metabolic clearance rate (MCR) of P₄ (Parr *et al.* 1993a, Sangsritavong *et al.* 2002). Elevated steroid concentrations after improving energy balance (EB) or treatment with insulin have been attributed to increased steroid

production, while a paucity of *in vivo* studies have addressed endogenous regulators or diet-induced modifications of the enzymes responsible for P₄ inactivation.

P₄ is inactivated in the liver by the addition of hydroxyl groups to the steroid nucleus producing a 21-hydroxyprogesterone or 6 β -hydroxyprogesterone metabolite via cytochrome P450 2C (CYP2C) or cytochrome P450 3A (CYP3A) respectively (Murray 1991, 1992). In addition, several authors have observed contributions of the aldo-keto reductase 1C (AKR1C) subfamily (3 α -hydroxysteroid dehydrogenase and 20 α -hydroxysteroid dehydrogenase activities) to P₄ inactivation (Penning *et al.* 2000). Saad *et al.* (1994) found a 40% decrease in the formation of 6 β -hydroxytestosterone (primarily CYP3A activity) in rat hepatocytes exposed to 10 nM insulin versus 1 nM insulin. Smith *et al.* (2006) found a dose-dependent decrease in the fractional rate constant of P₄ decay in a murine hepatocyte cell line challenged with increasing physiological concentrations of insulin. Utilizing the same cell line and experimental conditions, Lemley *et al.* (2009) reported a dose-dependent decrease in the activities of both CYP2C and CYP3A after challenging hepatocytes with increasing physiological concentrations of insulin. Similar to the *in vitro*

data, Smith *et al.* (2006) found a decrease in P₄ clearance in anestrus ewes orally gavaged with sodium propionate (gluconeogenic substrate) versus sodium acetate (energy control group). In a follow-up experiment, Lemley *et al.* (2008a) found approximately a 50% decrease in both hepatic CYP2C and CYP3A activities at 1 h post-feeding in ovariectomized ewes supplemented with sodium propionate versus sodium acetate.

These previous results in sheep were the first evidence linking elevated insulin concentrations with lowered activity of the enzymes responsible for first phase P₄ biotransformation *in vivo*. We are currently extending these observations to the lactating dairy cow, which has high energy demands that lead to high rates of hepatic P₄ inactivation (Sangsritavong *et al.* 2002); moreover, alterations of steroid clearance *in vivo* have been limited to induction of mixed function mono-oxygenases using phenobarbital (Thomford & Dziuk 1986). We are unaware of any experiments modifying hepatic P₄-metabolizing enzyme activity and P₄ clearance through variations in dietary energy sources. Current understanding of the relative contributions of first phase mixed function monoxygenase (cytochrome P450s) or oxidoreductase (AKRs) activities in modulating peripheral concentrations of P₄ are limited to studies that rely on physiological parameters that are irreproducible *in vivo*. This study describes a role for modifying P₄ clearance by altering the type of dietary energy provided while maintaining adequate nutrition for gestation. The current objectives were to determine LBF, cytochrome P450 and AKR activities, and MCR of P₄ in dairy cows consuming isoenergetic and isonitrogenous diets formulated to cause divergent insulin secretion.

Materials and Methods

Animals and feeding

All experimental procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee #A0913. Eleven Holstein dairy cows (3 primiparous and 8 multiparous) were housed in tie stalls at the North Dakota State University dairy research barn (Fargo, ND, USA) in the Fall of 2008. Cows were randomly assigned to a high cornstarch diet or an isoenergetic and isonitrogenous high fiber diet (Table 1) in a crossover experimental design containing two 14-day periods, with 10 days of diet acclimation and the remaining 4 days were used for sampling (Fig. 1). Diets prior to the start of the experiment were different from both experimental diets, but contained the same forages (corn silage and chopped alfalfa hay) provided in a total mixed ration. Cows were 63 ± 4 days in milk and were milked twice daily at 0400 and 1600 h. Cows were provided each diet as a total mixed ration directly after each milking, ~40% of the feed at 0400 h and 60% of the feed at 1600 h. DMI was kept constant for each cow across treatments in order to avoid confounding effects of DMI with

Table 1 Experimental diet composition and average (± S.E.M.) nutrient composition for high cornstarch (n=4) or high fiber diet (n=4)

	High cornstarch	High fiber
Diet composition		
Ingredient (% of DM)		
Corn silage	31.1	36.7
Chopped alfalfa hay	17.8	15.3
Ground corn	12.5	6.1
Corn gluten meal	1.8	1.5
Corn gluten feed	5.4	5.1
Wheat middlings	7.3	5.2
Soybean meal (47% CP)	8.5	5.0
Soybean hulls	–	23.1
Refined cornstarch	15.1	–
Mega-Lac (Ca soaps of fatty acids)	–	1.9
Dicalcium phosphate	0.29	0.8
Limestone	0.1	–
Magnesium oxide	0.04	0.04
Trace mineral salt	0.13	0.13
Vitamin premix ^a	0.08	0.08
Nutrient composition (% of DM)		
NDF	28.4 ± 1.1	39.9 ± 0.7
ADF	16.1 ± 0.6	26.0 ± 0.4
NFC ^b	50.7	35.3
Starch	33.8 ± 1.5	19.1 ± 0.6
CP	14.7 ± 0.5	14.9 ± 0.4
Ether extract	2.3 ± 0.1	2.9 ± 0.2
Energy content, NEI Mcal/kg DM ^b	1.58	1.58

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrates; NEI, net energy used for lactation.
^aVitamin ADE premix (North American Nutrition Companies, Inc., Brookville, OH, USA); vitamin A, 9920.9 IU/g; vitamin D₃, 2204.6 IU/g, vitamin E, 4.4 IU/g.
^bBased on tabular values (NRC 2001).

differences due to dietary treatment. The feed allocated to each cow was based on her voluntary intake during the first 5 days of the experiment. On day 6, cows received a CIDR (Pfizer Inc., New York, NY, USA) containing 1.38 g P₄, which was removed at the start of the P₄ infusion experiment. After removal of CIDRs between periods 1 and 2, cows were monitored twice daily for estrous behavior, and all cows used in the present study were determined to be cycling. On day 11, at 0800 h both jugular veins were catheterized with Tygon tubing (Thermo Fisher Scientifics, Waltham, MA, USA; 1.02 mm i.d. and 1.78 mm o.d.) and kept patent for the next 3 days by flushing with 10 U/ml heparin (Columbus Serum Co., Columbus, OH, USA). On day 12, cows were administered 25 mg of lutalyse (Pfizer Inc.; to remove endogenous P₄, corpus luteum, before P₄ infusions) prior to the 1600 h milking, and liver biopsies were taken 3.77 ± 0.09 h post-feeding (1600 h feeding). A portion of the liver tissue was immersed in potassium phosphate buffer and immediately used to determine CYP2C and CYP3A activities. The remaining liver sample was snap-frozen in liquid nitrogen, and used for determining AKR1C activity, and CYP2C mRNA and CYP3A mRNA

Figure 1 The crossover experimental design consisted of two identical 14-day periods that ran consecutively to one another. On day 11, cows were infused with BSP to estimate liver blood flow at 1, 2, 3, and 4 h post-feeding. On day 12, cows were injected with luteal phase before the 1600 h milking, and a liver biopsy was taken 3.77 ± 0.09 h post-feeding. On day 13, plasma samples were taken to determine insulin (closed triangles) and progesterone (P_4 ; open circles) concentrations. CIDRs were removed after the start of P_4 infusion, i.e. at 0 h post-feeding. MCR of P_4 was determined at 1, 2, 3, and 4 h post-feeding, and P_4 decay was determined at the end of infusion, i.e. at 4, 4.5, 5, 6, 8, and 10 h post-feeding.

expression. Milk production was recorded and milk samples were collected at each milking on day 11, 12, 13, and 14, and samples were analyzed for lactose, protein, and fat percent (Dairy One, Inc., Ithaca, NY, USA). Average body weight (BW; 626 ± 12 kg) was determined by weighing cows before milking, after milking, and halfway between milkings on days 11, 12, and 13 of each period respectively. Feed intake, milk composition, and BW were used to estimate EB over the 4-day sampling period using the equation:

$$EB = NEc - (NEL + NEr),$$

where $NEc = 1.58 \text{ Mcal/kg} \times \text{DMI}$,

$$NEL = MY \times ((0.0929 \times \% \text{ fat}) + (0.0563 \times \% \text{ protein}) + (0.0395 \times \% \text{ lactose})),$$

$$NEr = (BW^{0.75}) \times 0.08$$

(NEc , net energy consumed; NEL , net energy used for lactation; NEr , net energy required; [NRC 2001](#)).

LBF, MCR of P_4 , and insulin concentrations

On day 11, directly after the 1600 h milking, cows were infused with 7.5 mg/ml bromosulphthalein (BSP) at an average infusion rate of 2.77 ± 0.07 ml/min. A stock solution of BSP (20 mg/ml) was diluted to 7.5 mg/ml in 1 l saline bags. Saline bags were attached to an i.v. system containing a

100 ml burette (Columbus Serum Co.), which allowed for calculations of flow rates that were estimated every half an hour. The i.v. systems for each cow were attached to a 12 channel peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL, USA) containing silicone tubing with a 2.79 mm i.d. allowing for a maximal flow rate of 4 ml/min. Catheters and tubing were gas sterilized prior to use with Anprolene gas ampoules (Andersen Products Inc., Haw River, NC, USA), and all catheter lines were flushed with 70% ethanol before and after infusions. Jugular blood samples were collected into pre-chilled EDTA containing tubes at 1, 2, 3, and 4 h post-feeding. BSP concentrations in plasma were determined using the methods from [Sangsritavong et al. \(2002\)](#). Briefly, 50 μ l plasma was mixed with 150 μ l 0.5 M ammonium hydroxide (Sigma Chemical Co.), and absorbance was determined at wavelengths 580 and 426 nm on a Spectra Max Plus plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA). BSP concentrations were corrected for any hemolysis in the plasma using the equation:

$$\text{BSP concentrations} = ((\text{absorbance at } 580 \text{ nm}) - (0.2 \times \text{absorbance at } 426 \text{ nm})) - A.$$

The value A is given by absorbance at 580 nm before BSP infusion $-(0.2 \times \text{absorbance at } 426 \text{ nm})$. The constant 0.2 was determined by hemolyzing blood samples and comparing their absorbance at 580 and 426 nm after the addition of 0.5 M ammonium hydroxide. A standard curve of BSP (0–50 μ g/ml) in cow plasma was used to calculate peripheral concentrations. The MCR of BSP was calculated using the equation: $\text{MCR of BSP (l/h)} = \text{infusion rate } (\mu\text{g/h}) / \text{BSP concentration in plasma } (\mu\text{g/l})$. LBF was calculated using the equation: $\text{LBF (l/h)} = \text{MCR of BSP (l/h)} / 0.8 \times (1 - \text{hematocrit fraction})$.

On day 13, directly after the 1600 h milking, cows were infused with 50 μ g/ml P_4 at an average infusion rate of 2.45 ± 0.09 ml/min. A stock solution of 1 mg/ml P_4 in ethanol was diluted into one liter saline bags to a final concentration of 50 μ g/ml. Infusion procedures were similar to the BSP infusions, except that CIDRs were withdrawn 10 min after the start of infusions (~ 5 min before feeding) to assure tissue saturation with P_4 . Steady-state concentrations of P_4 were reached within 1 h of infusions, which were determined during a preliminary experiment at the West Virginia University Animal Sciences Farm, Morgantown, WV, USA. A jugular plasma sample was collected at 0, 1, 2, 3, and 4 h post-feeding. The MCR of P_4 was calculated using the equation: $\text{MCR of } P_4 \text{ (l/h)} = \text{infusion rate } (\mu\text{g/h}) / P_4 \text{ concentration in plasma } (\mu\text{g/l}) / 0.6$ ([Sangsritavong et al. 2002](#)). After completion of the 4 h P_4 infusion, blood samples were collected at 4.5, 5, 6, 8, and 10 h post-feeding. These samples were used to determine the fractional rate constants of P_4 decay (k). Briefly, k was calculated for each individual cow with SigmaPlot 8.0 (Aspire Software International, Ashburn, VA, USA) using the equation: $P_t = P_0 e^{-kt}$ (where P_t is the P_4 concentrations at any given time; P_0 is the initial

P₄ concentrations; *t* is the time) as described by Lemley *et al.* (2008a). The fractional rate constants of P₄ decay (*k*) were then used to calculate the half-life of P₄ using the equation: $t_{1/2} = \ln(2)/k$. P₄ concentrations were determined using RIA (Sheffel *et al.* 1982) with a sensitivity of 100 pg/ml and intra- and inter-assay coefficient of variation (CV) of 4.3 and 6.7% respectively. Briefly, the P₄ standard curve ranged from 0.1 to 4 ng/ml. One hundred microliters of plasma were extracted with 3 ml of petroleum ether and separated from the aqueous phase by freezing with dry ice in 2-propanol. Dextran-coated charcoal was used to separate free and bound ligand. The antibody for the P₄ assay was previously characterized by Butcher (1977). The only cross reactions of the 18 steroids tested were: pregnenolone, 15%; 5 α -pregnane-3,20-dione, 15%; 17 α -hydroxy-4-pregnene-3,20-dione, 3%; testosterone, 2%; corticosterone, 1%; 20 α -hydroxy-4-pregnen-3-one, 0.8%; 17 α -hydroxy-pregnenolone, 0.6% (Butcher 1977). Insulin concentrations over the 10-h sampling period were determined using RIA (Lemley *et al.* 2008a) with a sensitivity of 0.05 nM and intra- and inter-assay CV of 4.2 and 6.4% respectively. The assay was validated to measure bovine insulin in our laboratory. Briefly, plasma dilutions from two cows were assayed separately for insulin and found to be parallel with the standard curve. In addition, a plasma sample was spiked with bovine insulin (Sigma Chemical Co.) to determine percent recovery, which was >90%.

Cellular fractionation and enzymatic activity

On day 12, the 1600 h feeding was staggered for each cow by 5 min to assure similar liver biopsy times post-feeding (3.77 ± 0.09 h post-feeding). Briefly, the hair was removed from the animals' right side, and the skin was scrubbed twice with betadine (Columbus Serum Co). After determining the location of the tenth intercostal space, 2% lidocaine hydrochloride (Columbus Serum Co.) was administered as a local anesthetic. The skin was punctured using a scalpel, and the liver samples (1.1 ± 0.1 g/biopsy) were collected using a biopsy needle machined at the West Virginia University Physics Department (Morgantown, WV, USA) following the specifications of Swanson *et al.* (2000). CYP2C and CYP3A activities were assessed on ~200 mg of fresh liver sample following our previously published protocol (Lemley *et al.* 2008a). Briefly, liver samples were submerged in phosphate buffer and homogenized using a Dounce homogenizer. Microsomes were collected and concentrated using differential centrifugation techniques (modified from Nelson *et al.* (2001)). Homogenized tissue was centrifuged at 10 000 *g* for 10 min. Pellets were discarded and the supernatants were centrifuged at 100 000 *g* for 60 min. The microsomal pellets were resuspended in phosphate buffer, and the activity of cytochrome *c* reductase (product number CY0100; Sigma Chemical Co.) was used to standardize CYP2C and CYP3A activities. CYP2C activity was measured as the non-ketoconazole-inhibitable, omeprazole-dependent oxidation of NADPH. Microsomes were pre-incubated for 15 min

with 250 μ M ketoconazole. CYP2C enzymatic reactions contained CYP3A-inhibited microsomes, 2.5 mM omeprazole, and 250 μ M NADPH. CYP3A activity was measured as the nifedipine-dependent oxidation of NADPH. CYP3A enzymatic reactions contained fresh microsomes, 200 μ M nifedipine, and 250 μ M NADPH. AKR1C activity was determined in cytosolic cellular fractions using the specific substrate 1-acenaphthenol following the methods of Palackal *et al.* (2002) and Savlik *et al.* (2007). Briefly, AKR1C enzymatic reactions contained 150–650 μ g of cytosolic protein, 250 μ M 1-acenaphthenol and 500 μ M NADP. The 1-acenaphthenol-dependent reduction of NADP was standardized using cytosolic protein. All solutions were added to u.v. star 96-well plates (PGC Scientifics, Frederick, MD, USA), and the oxidation of NADPH or reduction of NADP was determined by measuring the amount of light absorbed at 340 nm for 5 min at 37 °C. The rate of oxidized NADPH or reduced NADP was determined to be linear over the 5-min period. The extinction coefficient for NADPH (6220 l/mol $\times$ cm) was used to calculate oxidized NADPH or reduced NADP per unit time.

CYP2C and CYP3A mRNAs expression

Liver samples were stored at –80 °C until total cellular RNA was extracted using TRIzol reagent (Invitrogen) and precipitated with 2-propanol following the manufacturer's protocol. Concentrations of RNA in each sample were determined using a Nanodrop ND-1000 spectrophotometer. One microgram of RNA was electrophoresed through a 1.5% agarose gel to determine sample purity and for visualization of 28S and 18S rRNA bands. Real-time RT-PCR was performed as previously described (Costine *et al.* 2007). Briefly, samples were diluted to 1 μ g RNA/ μ l and reverse transcribed using moloney murine leukemia virus reverse transcriptase (Promega) following the manufacturer's protocol. β -actin was used as a reference gene for measurements of bovine CYP2C and CYP3A genes (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>). Primers for β -actin (accession no. NM_001009784; forward: 5'-ATGAGCTGCCCGATGGTC-3'; reverse: 5'-GGATGTCCACGTCACACTTC-3'), CYP2C (accession no. XM_587518; forward: 5'-TATGGACTCCTGCTCCTGCT-3'; reverse: 5'-CATCTGTGTAGGCATGCAG-3'), and CYP3A (accession no. BT030557; forward: 5'-GTGCCAATCTCTGTGCTTCA-3'; reverse: 5'-CCAGTTCCAAAGGCAGGTA-3') were synthesized (Integrated DNA Technologies, Inc., Coralville, IA, USA). Amplification was optimal at an annealing temperature of 63.1 °C, and efficiencies for β -actin, CYP2C, and CYP3A were 1.89, 1.95, and 1.85 (theoretical yields of 94.5, 97.5, and 92.5% respectively). The relative abundance of mRNAs for CYP2C and CYP3A was corrected for PCR efficiency, standardized using β -actin, and expressed relative to a pooled sample, as described by Costine *et al.* (2007).

Feed analysis

Dry matter of diets was determined by oven drying at 60 °C for 48 h. Ether extraction of diets was performed according to AOAC (1990) using a Soxtec Foss Tecator (Foss Analytical, Hillerød, Denmark). Ash content and organic matter were determined using the procedure described by AOAC (1990). Diets were ashed at 500 °C for 16 h. Neutral detergent fiber and ADF content were determined using an Ankom 200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA). Total nitrogen and crude protein were analyzed according to AOAC (1990) using an automated Tecator digestion system (Tecator, Inc., Herndon, VA, USA). Sugars in the diet were determined by the extraction procedure adapted from Deriaz (1961). Reducing sugars were determined spectrophotometrically with potassium ferricyanide. Starch content of the diets was determined by the procedure of Smith (1969).

Statistical analysis

The effects of diet on the dependent variables were tested with the MIXED procedure of SAS (SAS software version 9.1, SAS Institute Inc., Cary, NC, USA). The model statement included period (period that dietary treatment was applied), order (order of dietary treatments), parity, and diet, while days in milk were used as a covariant. Cow within order was considered random, and means were separated using the PDIF option of the LSMEANS statement. MCR of P₄ and insulin concentrations were tested using repeated-measures ANOVA of the MIXED procedure of SAS with an autoregressive covariance structure, and means were separated using the DIFF option of the LSMEANS statement. Pearson correlation coefficients were determined using the CORR procedure of SAS. Least-square means and S.E.M. are reported. Trends were declared at $P < 0.10$ and $P > 0.05$. Statistical significance was declared at $P \leq 0.05$.

Results

DMI, milk yield, and milk lactose yield were similar ($P > 0.50$; Table 2) between the two experimental diets. Milk fat percentage was decreased by 15% ($P < 0.001$), while milk fat yield was decreased by 14% ($P < 0.005$) in cows consuming the high cornstarch diet versus the high fiber diet. Milk protein percentage was increased by 6% ($P < 0.01$), while milk protein yield showed a trend ($P = 0.06$) for a 6% increase in cows consuming the high cornstarch diet versus the high fiber diet (Table 2). EB was improved ($P < 0.05$) in cows consuming the high cornstarch diet compared to the high fiber diet (Table 2). Effect of order was not significant for all variables measured.

Insulin response and hepatic enzyme activities are depicted in Table 3. Average insulin concentrations, over the 10-h blood sampling period, were increased by 22% in cows

Table 2 Effect of a high cornstarch diet ($n=11$) versus a high fiber diet ($n=11$) on intake and milk production in lactating dairy cows

	High cornstarch	High fiber	S.E.M.	P value
Dependent variable				
DMI (kg/day)	24.77	24.44	0.82	0.516
Milk yield (kg/day)	37.19	36.82	2.31	0.677
Fat percent (%)	3.54	4.14	0.17	<0.001
Fat yield (kg/day)	1.31	1.53	0.13	0.001
Protein percent (%)	2.91	2.73	0.05	0.005
Protein yield (kg/day)	1.08	1.01	0.06	0.056
Lactose percent (%)	4.83	4.92	0.05	0.070
Lactose yield (kg/day)	1.80	1.81	0.12	0.825
Energy balance (Mcal/day)	4.09	1.94	1.07	0.028

consuming the high cornstarch diet versus the high fiber diet. Plasma insulin concentrations at the time of liver biopsy (3.77 ± 0.09 h post-feeding) were not different between the two diets. CYP2C activity was decreased by 56% for cows fed the high cornstarch compared to the high fiber diet. Similarly, CYP3A activity was decreased by 54% for cows consuming the high cornstarch diet compared to the high fiber diet. AKR1C activity tended to be 19% lower in cows consuming the high cornstarch diet versus the high fiber diet. Average LBF was similar between the two diets (1891 ± 91 l/h; $P > 0.40$). We observed a trend for a decrease ($P = 0.06$) in the average MCR of P₄ (Fig. 2) in cows fed the high cornstarch diet versus the high fiber diet. At 1 h post-feeding, the MCR of P₄ was similar between the two diets; however, hours 2, 3, and 4 tended ($P < 0.10$) to be lower in cows consuming the high cornstarch diet compared to the high fiber diet. The fractional rate constant of P₄ decay was not significantly different ($P = 0.115$) between the two diets; however, the half-life of P₄ was lengthened ($P < 0.01$) in cows fed the high cornstarch diet (73 ± 10 min) versus the high fiber diet (24 ± 10 min; Fig. 3). Expression of CYP2C and CYP3A

Table 3 Average insulin concentrations over the 10-h blood sampling period, insulin concentrations at the time of liver biopsy (3.77 ± 0.09 h post-feeding), and hepatic cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), and aldo-keto reductase 1C (AKR1C) activities in cows consuming a high cornstarch diet ($n=11$) or a high fiber diet ($n=11$)

	High cornstarch	High fiber	S.E.M.	P value
Dependent variable				
Average insulin (nM)	0.18	0.14	0.01	0.048
Biopsy insulin (nM)	0.22	0.17	0.03	0.198
CYP2C (pmol/min × mU reductase)	14.8	33.8	4.2	0.008
CYP3A (pmol/min × mU reductase)	4.24	9.31	1.41	0.027
AKR1C (pmol/min × mg protein)	178.9	221.2	15.2	0.069

Figure 2 Metabolic clearance rate of progesterone, standardized by body weight, for cows consuming the high cornstarch diet (open circles, $n=10$) or the high fiber diet (closed circles, $n=10$). Effect of diet, $P=0.06$; double daggers (‡) represent $P<0.10$.

mRNAs was not different between the two experimental diets (data not shown).

Correlation analysis revealed a positive association between EB and insulin concentrations averaged across the 10-h blood sampling period (Table 4). EB was positively correlated with the half-life of P_4 and negatively correlated with the MCR of P_4 on a per BW basis. Average insulin was positively correlated with P_4 half-life and negatively correlated with CYP2C activity. P_4 half-life was negatively correlated with CYP2C activity, while CYP3A activity and AKR1C activity were positively correlated with the average MCR of P_4 .

Discussion

In the current study, a high cornstarch diet improved EB and increased peripheral insulin concentrations. Hepatic P_4 catabolic enzyme activities were decreased after feeding a high cornstarch diet, and the MCR of P_4 tended to be lower while the half-life of P_4 was significantly lengthened in cows consuming a high cornstarch diet. Milk yield was not affected by dietary treatments; however, milk fat yield and milk protein yield were altered after feeding a high cornstarch diet versus a high fiber diet. Several researchers have found that replacing corn with soybean hulls increased the ratio of ruminal acetate to propionate with propionate concentration either decreasing (Sarwar *et al.* 1992, Cunningham *et al.* 1993) or remaining constant (Sievert & Shaver 1993, Mansfield & Stern 1994). Providing 15.1% refined cornstarch, in the current experiment, caused a 22% increase in average concentrations of insulin sampled over a 10-h period, which would be expected after increasing propionate availability for hepatic gluconeogenesis.

Butler *et al.* (2004) infused insulin at a constant rate of $0.3 \mu\text{g/kg BW} \times \text{h}$ in dairy cows during 10–14 days of

post-calving, which increased plasma insulin by 2.6-fold and increased peripheral concentrations of both estradiol and testosterone compared with control cows. Although steroid production versus steroid clearance was not measured in the Butler *et al.* (2004) study, it is worthwhile to note that similar catabolic pathways exist between steroids and elevated insulin in this study could have affected peripheral clearance rather than production of estradiol. In early *post partum* dairy cows fed a glucogenic (26.6% starch), mixed (17.9% starch), or lipogenic diet (10.4% starch), Van Knegsel *et al.* (2007) found no difference in DMI or energy intake between dietary treatments; however, EB was improved in multiparous cows fed the glucogenic diet compared with the mixed diet. In the current study, we found improvements in EB, which were presumably due to a decrease in milk fat yield and a shift in energy partitioning due to elevated insulin. Of the reproductive parameters tested in the Van Knegsel *et al.* (2007) study, multiparous cows fed the glucogenic diet tended to have shorter days until first P_4 rise. Mean P_4 concentrations were not different between the three diets, which may be due to the addition of fat and/or the addition of citrus pulp in the lipogenic diet. Hawkins *et al.* (1995) found an increase in P_4 half-life in beef heifers fed a lipogenic diet (170 min) compared with an energy control (113 min). Citrus products contain furanocoumarins (i.e. bergamottin), strong inhibitors of cytochrome P450 enzymes (Sahi *et al.* 2002), which may lead to decreased P_4 clearance after supplementing animals with citrus pulp. This is in agreement with our current findings showing a decrease in cytochrome P450 activity accompanied by a decrease in P_4 clearance.

Researchers have found no change or increased first service conception rates after supplementing cows with P_4 in the form of a CIDR, which can increase peripheral concentrations of P_4 by $\sim 1 \text{ ng/ml}$ (Larson *et al.* 2007, Stevenson *et al.* 2007). However, supplementation with P_4 partially masks the high rates of P_4 clearance in dairy cows and fails to address the issue of excessive steroid clearance due to elevated feed intake during early lactation. Rhinehart *et al.* (2009)

Figure 3 Fractional rate constant of progesterone decay (k ; left two bars) and average half-life of progesterone ($T_{1/2}$; right two bars) in cows consuming a high cornstarch diet (open bars, $n=10$) or a high fiber diet (closed bars, $n=11$). The equation $T_{1/2} = \ln(2)/k$ was used to calculate the half-life of progesterone. Asterisk (*) represents $P<0.01$.

Table 4 Correlation coefficients (*r*) for energy balance (EB, *n*=22), average insulin concentrations over the 10-h sampling period (insulin, *n*=22), average liver blood flow (LBF, *n*=21), progesterone (P₄) half-life (half-life, *n*=21), average metabolic clearance rate of P₄ on a body weight basis (MCR, *n*=20), and cytochrome P450 2C (CYP2C) (2C Act, *n*=22), cytochrome P450 3A (CYP3A) (3A Act, *n*=22), and aldo-keto reductase 1C (AKR1C) activities (AKR Act, *n*=22)

	Insulin	LBF	Half-life	MCR	2C Act	3A Act	AKR Act
EB	0.43 (0.04)	0.20 (0.38)	0.55 (0.009)	-0.45 (0.04)	-0.31 (0.16)	-0.03 (0.91)	-0.16 (0.46)
Insulin		-0.17 (0.45)	0.47 (0.03)	-0.28 (0.24)	-0.59 (0.004)	0.03 (0.88)	0.10 (0.66)
LBF			-0.21 (0.37)	-0.28 (0.24)	0.13 (0.58)	-0.26 (0.26)	-0.04 (0.85)
Half-life				-0.23 (0.35)	-0.51 (0.02)	0.02 (0.94)	-0.04 (0.85)
MCR					0.22 (0.35)	0.45 (0.05)	0.39 (0.08)
2C Act						-0.05 (0.82)	0.06 (0.77)
3A Act							0.38 (0.08)

P values are presented in parentheses.

supplemented exogenous P₄ to lutectomized dairy cows that were previously classified as having high or low endogenous concentrations of P₄. Cows that were previously classified as having high endogenous concentrations of P₄ had a greater P₄ area under the curve compared to the low P₄ group post-lutectomy. Gene expression for components of the endothelin and prostaglandin systems in corpora lutea was not different between the high and low P₄ groups, which may indicate a greater involvement in steroid clearance versus steroid production in regulating peripheral concentrations of P₄ in pregnant lactating dairy cows (Rhinehart *et al.* 2009).

In dairy cows chronically exposed to high insulin via a hyperinsulinemic–euglycemic clamp (96 h exposure), we found a decrease in expression of both *CYP2C* and *CYP3A* mRNAs (Lemley *et al.* 2008b). Both insulin and glucagon have been implicated in increasing or decreasing *CYP3A* expression and activity in rat hepatocytes (Saad *et al.* 1994). In these previous experiments, supraphysiological concentrations of insulin or glucagon modified cytochrome P450 mRNA expression after extended exposure (24, 48, or 96 h). In the current experiment, both *CYP2C* and *CYP3A* activities were altered, while expression of *CYP2C* and *CYP3A* mRNAs was similar between dietary treatments. These results are not surprising due to the evidence associated with specific substrates that regulate cytochrome P450 activity by decreasing or increasing protein turnover, which is independent of mRNA expression (Zangar & Novak 1998). In the current study, AKR1C activity tended to be lower in cows consuming the high cornstarch diet. AKR activity has been previously reported in pigs, cattle, goats, and sheep (Szotakova *et al.* 2004); however, this is the first study linking hepatic AKR1C activity with the MCR of P₄ in lactating dairy cows. Similar to the cytochrome P450 superfamily, the AKR1C enzymes have been implicated in

first phase steroid biotransformation, specifically at the 3 α , 17 β , and 20 α positions of the steroid nucleus (Penning *et al.* 2001, 2004, Shimada *et al.* 2006), and decreased activity would be expected to increase the half-life of P₄ in peripheral circulation.

Parr *et al.* (1993a) found a linear decrease in P₄ concentrations as well as a linear increase in MCR of P₄ as feed intake was increased from half maintenance, maintenance, and twice maintenance requirements. Parr *et al.* (1993b) determined that ~96% of the P₄ entering into the gut and liver was inactivated. This inverse relationship between plasma concentrations of P₄ and feed intake was later associated with a linear increase in LBF (Parr *et al.* 1993b). Sangsritavong *et al.* (2002) found a strong positive association between LBF and MCR of P₄ in lactating dairy cows. Their highest feed intake group (15.2 kg of dry matter consumed within the first 4 h) had a 3500 l/h MCR of P₄ at 4 h post-feeding. In the current study, we found a MCR of P₄ at 4 h post-feeding in cows consuming 25 kg dry matter to be ~3300 and 5100 l/h in the high cornstarch versus the high fiber diet respectively. This depression in MCR of P₄ was not accompanied by a decrease in LBF, which averaged 1891±91 l/h, strengthening the notion that decreasing P₄ catabolic enzyme activity in the liver by modifying the type of energy provided can lower the rate of P₄ inactivation. Miller *et al.* (1963) estimated a half-life of 33.8 min for P₄ in dairy cows that were infused with radiolabeled P₄. These half-life measurements in dairy cows are similar to our own estimates, which are much shorter compared with estimates in beef heifers of 113–170 min (Hawkins *et al.* 1995).

In summary, feeding a high cornstarch diet compared with a high fiber diet stimulated insulin secretion and lowered hepatic cytochrome P450 activity. LBF was not different after

altering energy source, while the MCR of P₄ tended to be lower in cows consuming the high cornstarch diet compared to the high fiber diet. The half-life of P₄, measured at a similar time compared with the time of liver biopsy, was increased threefold in cows consuming the high cornstarch diet versus the high fiber diet. The type of energy provided can modulate the rate of P₄ inactivation in the liver. Feeding a diet that stimulates insulin secretion during early pregnancy may improve peripheral concentrations of P₄. Moreover, elevated P₄ has been associated with increased pregnancy retention and advancement in embryonic development, which could improve reproductive performance during early lactation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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