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 ± 911 l/h). MCR of P₄ tended (P<0.10) to be lower in cows fed the high cornstarch diet (25 ± 5 l/h×BW⁰.⁷⁵) versus the high fiber diet (40 ± 6 l/h×BW⁰.⁷⁵). 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.


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 vitro 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 6β-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 (3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase activities) to P₄ inactivation (Penning et al. 2000). Saad et al. (1994) found a 40% decrease in the formation of 6β-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 anestrous ewes orally gavaged with sodium propionate (glucoseogenic 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 mono-oxygenase (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 isoenenergetic 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 described in 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 used for sampling (Fig. 1). Diets 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 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 CIDsRs 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

### Table 1 Experimental diet composition and average (± s.e.m.) nutrient composition for high cornstarch (n=4) or high fiber diet (n=4)

<table>
<thead>
<tr>
<th>Ingredient (% of DM)</th>
<th>High cornstarch</th>
<th>High fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>31.1 ± 1</td>
<td>36.7</td>
</tr>
<tr>
<td>Chopped alfalfa hay</td>
<td>17.8 ± 7</td>
<td>15.3</td>
</tr>
<tr>
<td>Ground corn</td>
<td>12.5 ± 6</td>
<td>6.1</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>1.8 ± 1</td>
<td>1.5</td>
</tr>
<tr>
<td>Corn gluten feed</td>
<td>5.4 ± 5</td>
<td>5.1</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>7.3 ± 5</td>
<td>5.2</td>
</tr>
<tr>
<td>Soybean meal (47% CP)</td>
<td>8.5 ± 5</td>
<td>5.0</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>–</td>
<td>23.1</td>
</tr>
<tr>
<td>Refined cornstarch</td>
<td>15.1 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>Mega-L (Ca soaps of fatty acids)</td>
<td>1 ± 1</td>
<td>0.8</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.29 ± 0</td>
<td>0.8</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.1 ± 0</td>
<td>0.14</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.04 ± 0</td>
<td>0.04</td>
</tr>
<tr>
<td>Trace mineral salt</td>
<td>0.13 ± 0</td>
<td>0.13</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.08 ± 0</td>
<td>0.08</td>
</tr>
<tr>
<td>Nutrient composition (% of DM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>28.4 ± 1</td>
<td>39.9 ± 0.7</td>
</tr>
<tr>
<td>ADF</td>
<td>16.1 ± 0.6</td>
<td>26.0 ± 0.4</td>
</tr>
<tr>
<td>NFC</td>
<td>50.7 ± 3</td>
<td>35.3</td>
</tr>
<tr>
<td>Starch</td>
<td>33.8 ± 1.5</td>
<td>19.1 ± 0.6</td>
</tr>
<tr>
<td>CP</td>
<td>14.7 ± 0.5</td>
<td>14.9 ± 0.4</td>
</tr>
<tr>
<td>Ether extract</td>
<td>2.3 ± 0.1</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Energy content, NEL Mcal/kg DM²</td>
<td>1.58 ± 0.02</td>
<td>1.58</td>
</tr>
</tbody>
</table>

**Notes:**
- DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrates; NEL, net energy used for lactation.
- Vitamin 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.
- Based on tabular values (NRC 2001).
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 lutalyse 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 (P4; open circles) concentrations. CIDRs were removed after the start of P4 infusion, i.e. at 0 h post-feeding. MCR of P4 was determined at 1, 2, 3, and 4 h post-feeding, and P4 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:

\[
EB = NEc – (NEI + NEr),
\]

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

\[
NEI = MY \times ((0.0929 \times \% \text{ fat}) + (0.0563 \times \% \text{ protein}) + (0.0395 \times \% \text{lactose})),
\]

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

(NEc, net energy consumed; NEI, net energy used for lactation; NEr, net energy required; NRC 2001).

**LBF, MCR of P4, and insulin concentrations**

On day 11, directly after the 1600 h milking, cows were infused with 50 µg/ml P4 at an average infusion rate of 2.45 ± 0.09 ml/min. A stock solution of 1 mg/ml P4 in ethanol was diluted into one liter saline bags to a final concentration of 50 µg/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 P4. Steady-state concentrations of P4 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 P4 was calculated using the equation:

\[
\text{MCR of P4 (l/h) = infusion rate (µg/h)/BSP concentration in plasma (µg/l)}.
\]

The value A is given by absorbance at 580 nm before BSP infusion (−0.2 × absorbance at 426 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 µg/ml) in cow plasma was used to calculate peripheral concentrations. The MCR of BSP was calculated using the equation:

\[
\text{MCR of BSP (l/h) = infusion rate (µg/h)/BSP concentration in plasma (µg/l)}.
\]

BSP concentrations were corrected for any hemolysis in the plasma using the equation:

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

The value A is given by absorbance at 580 nm before BSP infusion (−0.2 × absorbance at 426 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 µg/ml) in cow plasma was used to calculate peripheral concentrations. The MCR of BSP was calculated using the equation:

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

\[ t_{1/2} = \frac{\ln(2)}{k} \]

P₄ concentrations were determined using RIA (Sheffel et al. 1982) with a sensitivity of 10 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%; 5α-pregnane-3,20-dione, 15%; 17α-hydroxy-4-pregnen-3,20-dione, 3%; testosterone, 2%; corticosterone, 1%; 20α-hydroxy-4-pregnen-3-one, 0.8%; 17α-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 µM ketoconazole. CYP2C enzymatic reactions contained CYP3A-inhibited microsomes, 2.5 mM omeprazole, and 250 µM NADPH. CYP3A activity was measured as the nifedipine-dependent oxidation of NADPH. CYP3A enzymatic reactions contained fresh microsomes, 200 µM nifedipine, and 250 µM NADPH. AKR1C activity was determined in cytosolic cellular fractions using the specific substrate 1-acenaphthol following the methods of Palackal et al. (2002) and Savlik et al. (2007). Briefly, AKR1C enzymatic reactions contained 150–650 µg of cytosolic protein, 250 µM 1-acenaphthol and 500 µM NADP. The 1-acenaphthol-dependent reduction of NADPH was standardized using cytosolic protein. All solutions were added to u.v. star 96-well plates (PGC Sciences, 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/mM/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 µg RNA/µl 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′-GGATGTCTCACGTCAACATTT-3′), CYP2C (accession no. XM_587518; forward: 5′-TATGGACTCTGCTGTCCTG-3′; reverse: 5′-CATCTGTGTTAGGCATGCAG-3′), and CYP3A (accession no. BT030557; forward: 5′-GTTGCCAATCTCTGTGCTTCA-3′; reverse: 5′-CCAGTTCACAAAGGGAGTTA-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, Hillerod, 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 ferriycyanide. 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 PDIFF 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≤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 consuming the high cornstarch diet versus the high fiber diet. Plasma insulin concentrations at the time of liver biopsy (377±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>
<thead>
<tr>
<th>Dependent variable</th>
<th>High cornstarch</th>
<th>High fiber</th>
<th>S.E.M.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI (kg/day)</td>
<td>24.77</td>
<td>24.44</td>
<td>0.82</td>
<td>0.516</td>
</tr>
<tr>
<td>Milk yield (kg/day)</td>
<td>37.19</td>
<td>36.82</td>
<td>2.31</td>
<td>0.677</td>
</tr>
<tr>
<td>Fat percent (%)</td>
<td>3.54</td>
<td>4.14</td>
<td>0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein percent (%)</td>
<td>2.91</td>
<td>2.73</td>
<td>0.05</td>
<td>0.005</td>
</tr>
<tr>
<td>EB (kg/day)</td>
<td>1.08</td>
<td>1.01</td>
<td>0.06</td>
<td>0.056</td>
</tr>
<tr>
<td>Protein yield (%)</td>
<td>4.83</td>
<td>4.92</td>
<td>0.05</td>
<td>0.070</td>
</tr>
<tr>
<td>Lactose yield (%)</td>
<td>1.80</td>
<td>1.81</td>
<td>0.12</td>
<td>0.825</td>
</tr>
<tr>
<td>Energy balance (Mcal/day)</td>
<td>4.09</td>
<td>1.94</td>
<td>1.07</td>
<td>0.028</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>High cornstarch</th>
<th>High fiber</th>
<th>S.E.M.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average insulin (nM)</td>
<td>0.18</td>
<td>0.14</td>
<td>0.01</td>
<td>0.048</td>
</tr>
<tr>
<td>Biopsy insulin (nM)</td>
<td>0.22</td>
<td>0.17</td>
<td>0.03</td>
<td>0.198</td>
</tr>
<tr>
<td>CYP2C (pmol/min × μL reductase)</td>
<td>14.83</td>
<td>3.84</td>
<td>0.20</td>
<td>0.008</td>
</tr>
<tr>
<td>CYP3A (pmol/min × μL reductase)</td>
<td>4.24</td>
<td>9.31</td>
<td>1.41</td>
<td>0.027</td>
</tr>
<tr>
<td>AKR1C (pmol/min × mg protein)</td>
<td>178.9</td>
<td>221.2</td>
<td>15.2</td>
<td>0.069</td>
</tr>
</tbody>
</table>

Table 3 Average insulin concentrations over the 10-h blood sampling period, insulin concentrations at the time of liver biopsy (377±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)
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 P4 and negatively correlated with the MCR of P4 on a per BW basis. Average insulin was positively correlated with P4 half-life and negatively correlated with CYP2C activity. P4 half-life was negatively correlated with CYP2C activity, while CYP3A activity and AKR1C activity were positively correlated with the average MCR of P4.

Discussion

In the current study, a high cornstarch diet improved EB and increased peripheral insulin concentrations. Hepatic P4 catabolic enzyme activities were decreased after feeding a high cornstarch diet, and the MCR of P4 tended to be lower while the half-life of P4 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 μg/kg BW×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 P4 rise. Mean P4 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 P4 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 P4 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 P4 clearance.

Researchers have found no change or increased first service conception rates after supplementing cows with P4 in the form of a CIDR, which can increase peripheral concentrations of P4 by ~1 ng/ml (Larson et al. 2007, Stevenson et al. 2007). However, supplementation with P4 partially masks the high rates of P4 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) infusions of P4 (k4) in dairy cows fed a glucogenic or a high fiber diet (closed bars, n=11). The equation T1/2=ln(2)/k was used to calculate the half-life of progesterone. Asterisk (*) represents P<0.01.
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 mRNAs (Lemley et al. 2008b). 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 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

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)

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

P values are presented in parentheses.
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.

**Funding**

This project was supported by National Research Initiative Competitive Grant no. 2008–35203–04503 from the USDA Cooperative State Research, Education, and Extension Service and Hatch project 468 (NE 1007).

**Acknowledgements**

The authors would like to thank the employees of the NDSU Dairy Research and Teaching Center. This work is published with the approval of the Director of West Virginia Agriculture and Forestry Experiment Station as scientific paper no. 3066.

**References**


Lemley CO, Koch JM, Blemings KP, Krause KM & Wilson ME 2008a Concomitant changes in progesterone catalytic enzymes, cytochrome P450 2C and 3A, with plasma insulin concentrations in ewes supplemented with sodium acetate or sodium propionate. *Animal* 2 1223–1229.


Smith D 1969 Removing and analyzing total nonstructural carbohydrates from plant tissue, p 1. In Wisconsin Agricultural Experiment Station Research Report 41. Madison, WI: Wisconsin Agricultural Experiment Station Res. Rep. 41.


Received in final form 5 March 2010
Accepted 11 March 2010
Made available online as an Accepted Preprint 11 March 2010