Short Communication: Insulin Alters Hepatic Progesterone Catabolic Enzymes Cytochrome P450 2C and 3A in Dairy Cows¹

C. O. Lemley,* S. T. Butler,† W. R. Butler,‡ and M. E. Wilson*²

*Division of Animal and Nutritional Sciences, Davis College of Agriculture, Forestry and Consumer Sciences, West Virginia University, Morgantown 26506

†Teagasc, Moorepark Dairy Production Research Centre, Fermoy, Co. Cork, Ireland

‡Department of Animal Science, Cornell University, Ithaca, NY 14853

ABSTRACT

High proportions of embryonic and early fetal losses in dairy cattle are associated with low peripheral concentrations of progesterone, which could result from increased catabolism, decreased production, or both. Progesterone catabolism occurs primarily in the liver via the cytochrome P450 2C (CYP2C) and cytochrome P450 3A (CYP3A) subfamilies (EC 1.14.14.1; unspecific monooxygenases). Recent observations from our laboratory have shown that the fractional rate constant of progesterone decay can be dramatically reduced by insulin because of a decrease in hepatic CYP2C and CYP3A activity. Little information exists on the regulation of progesterone catabolic enzymes in dairy cows. We hypothesized that elevated insulin concentrations would down-regulate hepatic CYP2C and CYP3A mRNA; therefore, our objectives were to determine the relative abundance of hepatic CYP2C and CYP3A mRNA in dairy cows in response to elevated concentrations of insulin. In the first experiment, 17 mature Holstein cows were drenched daily with 500 mL of water ($n = 10$) or propylene glycol (a gluconeogenic substrate; $n = 7$) from 10 d before their expected calving date until d 25 postpartum. Cows drenched with propylene glycol had a 30% increase in peripheral concentrations of insulin. Liver biopsies were collected on d 25 postpartum to determine the relative abundance of CYP2C and CYP3A mRNA. In the second experiment, 19 mature, lactating Holstein cows were randomly assigned to a hyperinsulinemic-euglycemic clamp (0.3 or 1.0 μg of insulin/kg of BW per h; $n = 6$ each) or remained as controls (saline infused; $n = 7$) for 96 h beginning on d 10 postpartum. Insulin infusion resulted in a 2.6- or 8-

fold increase in peripheral concentrations of insulin, respectively. On d 14 postpartum, a liver biopsy was collected to determine CYP2C and CYP3A mRNA abundance. In experiment 1, the relative abundance of CYP2C mRNA in cows treated with propylene glycol did not differ from controls; however, the relative abundance of CYP3A mRNA in the propylene glycol group was 63% that of controls. For experiment 2, there was a dose-dependent decrease in the relative abundance of both CYP2C and CYP3A mRNA with increasing dosage of insulin. In conclusion, this study demonstrated that, in the cow, either providing a gluconeogenic feedstuff or treatment with insulin decreased the abundance of mRNA for enzymes responsible for hepatic progesterone catabolism.

Key words: progesterone catabolism, cytochrome P450, insulin, dairy cow

Dairy cow pregnancy rates to first insemination have steadily declined over the last 50 yr and current pregnancy rates are approximately 40% (Inskeep and Dailey, 2005). Low concentrations of progesterone in the blood, because of elevated rates of catabolism, deficiencies in luteal function, or both may be responsible for the increased pregnancy wastage. Starbuck et al. (2004) reported pregnancy retention to wk 7 of gestation as 96 vs. 80% in dairy cows that were classified during wk 5 as having high vs. low concentrations of progesterone, respectively. Larson et al. (2007) supplemented progesterone in the form of an intravaginal progesterone-releasing device (CIDR; resulting in an increase in progesterone of approximately 1 ng/mL) from d 3.5 to 10 postinsemination, which increased pregnancy rates by 37% in cows treated with progesterone (48%) compared with the control group (35%). In contrast, Villarroel et al. (2004) found no difference on pregnancy outcomes in repeat-breeder Holstein cows treated with a progesterone-releasing intravaginal device from d 5 to 19 postinsemination. Stevenson et al. (2007) found a trend for increased conception rates in Holstein cows treated with a CIDR for 7 d beginning between 4 and

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²Corresponding author: mwilso25@wvu.edu

9 d postinsemination ($n = 711$) compared with control cows ($n = 708$); moreover, of the 5 herds tested in this study, CIDR treatment increased conception rates in 2 of the herds and decreased conception rates in 1 of the herds. In general, progesterone supplementation during gestation remains a controversial area because of the variable results in pregnancy outcomes (Robinson et al., 1989; Stevenson and Mee, 1991; Diskin et al., 2006).

Peripheral concentrations of progesterone may be insufficient in high-producing dairy cows because of a higher rate of hepatic catabolism. The majority of progesterone is inactivated or catabolized by the cytochrome P450 2C (**CYP2C**) and cytochrome P450 3A (**CYP3A**) subfamilies, and their major metabolites are 21-hydroxyprogesterone and 6 β -hydroxyprogesterone, respectively (Murray 1991, 1992). Hepatocyte primary cell culture studies in rodents have shown a decrease in CYP3A1 mRNA and protein expression after exposure to physiological concentrations of insulin (Sidhu and Omiecinski, 1999). Similarly, Saad et al. (1994) exposed rat hepatocytes to increasing physiological concentrations of insulin and found a dose-dependent decrease in the formation of 6 β -hydroxytestosterone, a metabolic product primarily catalyzed by the CYP3A subfamily, with minor contributions from CYP2C13. Smith et al. (2006) observed a dose-dependent decrease in the fractional rate constant of progesterone decay in a murine hepatocyte cell line cultured with increasing amounts of insulin. Recently, our laboratory showed that the same hepatocyte cell line, cultured under identical experimental conditions, exhibited a dose-dependent decrease in both CYP2C and CYP3A activity after a 4-h insulin challenge, which is congruent with the observed decrease in progesterone decay (Lemley, 2007).

Miller et al. (1963) estimated a half-life of 33.8 min for progesterone in cows that were infused with radiolabeled progesterone, whereas Hawkins et al. (1995) determined a half-life of approximately 150 min for progesterone in cows by measuring steroid disappearance after ovariectomy. Studies by Sangsritavong et al. (2002) in the dairy cow determined a positive correlation between liver blood flow and metabolic clearance rate of progesterone. The relationship was determined to be metabolic clearance rate = $1.38(\text{liver blood flow}) + 0.10$ ($r^2 = 0.92$). However, these studies compared high-maintenance ($2\times$) vs. low-maintenance ($1/2\times$) diets, which differed in energy, protein, and DMI. Previous observations by Smith et al. (2006) in anestrus ewes orally gavaged with propionate (a gluconeogenic substrate) had elevated concentrations of insulin and decreased clearance of progesterone compared with ewes orally gavaged with an isocaloric amount of so-

dium acetate. In a similar experiment, Lemley (2007) showed that ovariectomized ewes supplemented with sodium propionate had elevated insulin concentrations at 15, 30, and 60 min postfeeding and a 50% reduction in CYP2C and CYP3A activity at 60 min postfeeding compared with ewes supplemented with an isocaloric amount of sodium acetate.

The data set in the current experiment is from liver tissue collected in previous studies (Butler et al., 2003, 2004, 2006). In the first experiment, 17 mature Holstein cows were drenched daily with 500 mL of water ($n = 10$) or propylene glycol (a gluconeogenic substrate; $n = 7$) from d 10 before their expected calving date until d 25 postpartum; milk yields from d 0 to 30 postpartum were not different and averaged 44.9 compared with 44.3 kg/d, respectively. Propylene glycol drenching caused an improvement in energy balance of -18% (-9.4 vs. -11.4 Mcal/d) and a 28% increase in plasma concentrations of insulin (0.68 vs. 0.53 ng/mL; Butler et al., 2006).

In the second experiment, mature, lactating Holstein cows ($n = 19$) on d 8 postpartum were randomly assigned to a hyperinsulinemic-euglycemic clamp ($n = 12$) or remained as controls ($n = 7$). Control and insulin-treated cows had indwelling jugular catheters fitted to facilitate infusion of treatments (saline or both insulin and glucose, respectively) and collection of blood samples. Insulin was infused at a constant rate of 0.3 ($n = 6$) or 1.0 ($n = 6$) $\mu\text{g/kg}$ of BW per h, and euglycemia was maintained by infusion of glucose at a variable rate for 96 h starting on d 10 postpartum at 1500 h and ending on d 14 at 1500 h. Infusing cows with 0.3 μg of insulin/kg of BW per h resulted in a 2.6-fold increase (0.73 vs. 0.28 ng/mL) in plasma insulin concentrations (Butler et al., 2004). Throughout the 96-h infusion period, average milk production (39.3 vs. 41.1 kg/d) was similar and energy balance was improved in cows receiving insulin compared with control cows (-11.1 vs. -19.1 Mcal/d). Cows subjected to infusions of 1.0 μg of insulin/kg of BW per h exhibited an 8-fold increase in plasma insulin concentrations (2.33 vs. 0.27 ng/mL) relative to saline-infused cows (Butler et al., 2003). Throughout the 96-h infusion, milk production was reduced in cows receiving 1.0 μg of insulin/kg of BW per h compared with control cows (30 vs. 40 kg/d), and energy balance was significantly improved in treated cows (approximately -5 vs. -18 Mcal/d).

Liver biopsies from water- or propylene glycol-drenched cows were collected on d 25 postpartum, whereas liver biopsies from insulin- or saline-infused cows were collected on d 14 postpartum immediately before the termination of treatment. Liver samples were stored at -80°C until total cellular RNA was extracted (Butler et al., 2003, 2004). Real-time PCR was

performed as previously described (Costine et al., 2007). Briefly, samples were diluted to 1 μg of RNA/ μL and reverse transcribed by using Moloney murine leukemia virus reverse transcription (Promega, Madison, WI) following the manufacturer's protocol. β -Actin was used as a reference gene, and bovine CYP2C and CYP3A genes were chosen because of their homology to the human progesterone catabolic enzymes CYP2C19 and CYP3A4 (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>). Primers for β -actin (accession no. NM_001009784; forward: 5'-ATGAGCTGCCCCGATGGTC-3'; reverse: 5'-GGATGTCCACGTCACTTC-3'), CYP2C (accession no. XM_587518; forward: 5'-TATGGACTCCTGCTCCTGCT-3'; reverse: 5'-CATCTGTGTAGGGCATGCAG-3'), and CYP3A (accession no. BT030557; forward: 5'-GTGCCAATCTCTGTGCTTCA-3'; reverse: 5'-CCAGTTCCAAAAGGCAGGTA-3') were synthesized (Integrated DNA Technologies Inc., Coralville, IA). 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, respectively. The relative abundances of mRNA for CYP2C and CYP3A were corrected for PCR efficiency, standardized by using β -actin, and expressed relative to a pooled sample, as described by Costine et al. (2007).

The effects of propylene glycol treatment on the relative abundance of CYP2C and CYP3A mRNA were tested with ANOVA by using the GLM procedure of SAS (SAS software, version 9.1, SAS Institute Inc., Cary, NC). The effects of insulin infusion rate on the relative abundance of CYP2C and CYP3A mRNA were analyzed by using linear regression with the GLM of SAS (SAS software version 9.1). Treatment with propylene glycol did not affect ($P > 0.1$) CYP2C mRNA expression; however, the propylene glycol treatment caused a reduction ($P < 0.05$) in CYP3A mRNA expression relative to controls (Figure 1). During insulin administration, the expression of CYP2C mRNA exhibited a dose-dependent decrease ($P < 0.01$) with increased rates of insulin infusion [CYP2C mRNA = $-1.75(\text{insulin infusion rate}) + 1.93$; Figure 2]. Similarly, CYP3A mRNA expression decreased ($P < 0.05$) in a dose-dependent manner with increasing insulin infusion rate [CYP3A mRNA = $-0.49(\text{insulin infusion rate}) + 1.08$; Figure 3].

The current finding in dairy cows describes a role for insulin in down-regulating the mRNA for those cytochrome P450 responsible for progesterone catabolism. The propylene glycol treatment caused a 30% increase in plasma insulin concentration and a 40% reduction in CYP3A mRNA expression. By infusing 1.0 μg of insulin/kg of BW per h, CYP2C and CYP3A were decreased by 88 and 45%, respectively. Although CYP2C was unaffected in dairy cows treated with propylene glycol, there was a greater response in CYP2C mRNA expres-

Figure 1. Relative abundance of cytochrome P450 2C (CYP2C) and cytochrome P450 3A (CYP3A) mRNA in cows drenched with 500 mL of propylene glycol (open bars) or water (hatched bars). An asterisk (*) indicates a difference between treatments ($P < 0.05$).

sion in cows subjected to the hyperinsulinemic-euglycemic clamp compared with CYP3A mRNA expression. Potential mechanisms for the observed decrease in cytochrome P450 expression after insulin exposure were recently reviewed by Kim and Novak (2007). Kim and Novak (2007) suggested that insulin binding to its receptor (tyrosine kinase receptor family) alters endogenous and exogenous compound metabolism through the phosphatidylinositol-3-kinase pathway of insulin signaling. The insulin-mediated down-regulation in cytochrome P450 mRNA expression can be blocked by cul-

Figure 2. Relative abundance of cytochrome P450 2C (CYP2C) mRNA in cows infused with different amounts of insulin (0, 0.3, or 1.0 $\mu\text{g/kg}$ of BW per h). The CYP2C mRNA exhibited a dose-dependent decrease ($P < 0.01$) with increasing insulin infusion rate [CYP2C mRNA = $-1.75(\text{insulin infusion rate}) + 1.93$].

Figure 3. Relative abundance of cytochrome P450 3A (CYP3A) mRNA in cows infused with different amounts of insulin (0, 0.3, or 1.0 µg/kg of BW per h). The CYP3A mRNA exhibited a dose-dependent decrease ($P < 0.05$) with increasing insulin infusion rate [CYP3A mRNA = $-0.49(\text{insulin infusion rate}) + 1.08$].

turing hepatocytes with a phosphatidylinositol-3-kinase inhibitor (Kim and Novak, 2007); however, inhibition of the p38 mitogen-activated protein kinase, mitogen-activated protein kinase kinase, or Src kinase in rat hepatocytes failed to alter cytochrome P450 mRNA expression. Martinez-Jimenez et al. (2006) determined that insulin caused a down-regulation of the mRNA encoding a peroxisomal proliferator-activated receptor- γ coactivator 1 α , which was congruent with the decrease in cytochrome P450 mRNA expression. In general, insulin signaling was shown to regulate coactivators (i.e., peroxisomal proliferator-activated receptor- γ coactivator 1 α) that are directly involved in enhancing transcription factors on promoter regions of cytochrome P450 genes. Our current understanding of the intracellular signaling pathways associated with the insulin-mediated decrease in cytochrome P450 expression has not been addressed in ruminants and may be due to the lack of an appropriate ruminant hepatocyte cell line.

Decreasing the expression of the progesterone catabolic enzymes during early pregnancy, and thereby extending the biological half-life of the steroid, may be a useful approach to increasing peripheral concentrations of progesterone. Elevated concentrations of progesterone have been associated with increased pregnancy retention during early gestation (Starbuck et al., 2004). Dairy cows that have low concentrations of progesterone may have a higher metabolic clearance rate relative to others in the herd, and future work should focus on identifying methods to decrease excessive hepatic clearance without compromising DMI. The cur-

rent work is limited by lacking essential information on plasma progesterone concentrations as well as enzymatic activities for CYP2C and CYP3A; cows studied were early postpartum prior to first ovulation. Future studies should focus on determining the abundance or activity of these enzymes, or both and their effectiveness in decreasing the metabolic clearance rate of progesterone.

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