Anim. Reprod., v.10, n.2, p.112-118, Apr./Jun. 2013

Lipoic acid decreases progesterone clearance in ovariectomized ewes

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Abstract

Lipoic acid is a naturally occurring compound that has been shown to modulate insulin sensitivity when supplemented to the diet. Elevated blood insulin concentrations have been shown to decrease progesterone catabolism in several species by modulating hepatic steroid metabolic enzyme activity and expression. We hypothesized that lipoic acid supplementation would decrease progesterone (P4) catabolism by the liver. Eight ovariectomized ewes were fed an alfalfa-grass ration at 95% of ad libitum for the duration of the experiment. Ewes were randomly assigned to the control group [an empty bolus administered by gavage (n = 4; CON)] or lipoic acid group [supplemented at 32 mg/kg BW administered by gavage (n = 4; LA)]. Progesterone was administered via CIDR devices on day 5 to all ewes. Blood samples were collected daily from day 6 to 9. On day 10, liver biopsies were obtained from each ewe to determine cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A) and aldo-keto reductase 1C (AKR1C) activity. On day 11, serial blood samples were collected after CIDR removal to determine P4 clearance. Ewes treated with LA had a lower rate constant ($P < 0.03$) for P4 clearance compared to CON ewes; however, no difference ($P > 0.05$) in hepatic enzyme activity was observed. There was also no difference ($P > 0.05$) in circulating concentrations of P4 in CON ewes compared to LA ewes on day 6 to 11. We conclude that while lipoic acid decreased P4 clearance from the blood, it did so without affecting hepatic enzyme activity; therefore the mechanism of action is yet to be elucidated.

Keywords: hepatic enzymes, lipoic acid, progesterone.

Introduction

Embryonic death in sheep is highest within the first 3 weeks of gestation and contributes greatly to economic losses suffered by producers (Moore, 1985). Over $1 billion is lost annually as a result of reproductive disease, infertility and pregnancy conditions causing abortions, stillbirths or dystocia in beef and dairy cattle (Bellows *et al*., 2002). Progesterone plays a critical role in the maintenance of pregnancy in mammals (Csapo, 1956); therefore factors contributing to early pregnancy losses may include low initial levels of circulating P4 during gestation, an overactive mechanism of P4 catabolism, improper luteal function or a combination of these factors (Inskeep and Dailey, 2005). Progesterone is critical during early gestation as it influences the production of endometrial secretions that aid in early embryonic development (Nephew *et al*., 1991). Consequently, low concentrations of P4 can lead to poor embryonic development (Nephew *et al*., 1994) and may enable an increase in embryo-toxic hormones (Inskeep, 2004).

Catabolism of progesterone occurs predominantly in the liver where there is an abundance of hepatic cytochrome P450 enzymes involved in steroid inactivation (Parr *et al*., 1993). Those contributing the greatest to P4 catabolism include cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), and aldo-keto reductase 1C (AKR1C; Murray *et al*., 1991, 1992; Penning *et al*., 2000; Lemley and Wilson, 2010). Administering ewes a gluconeogenic substrate to increase circulating concentrations of insulin was shown to decrease P4 clearance compared to energy control animals (Smith *et al*., 2006; Lemley *et al*., 2008b). In addition, challenging a mouse hepatocyte cell line with increasing physiological concentrations of insulin resulted in a dose-dependent decrease in CYP2C and CYP3A activity (Lemley *et al*., 2009).

Lipoic acid is a naturally occurring compound that has been found to increase insulin sensitivity and glucose uptake when supplemented in human and animal models (Jacob *et al*., 1996; Yaworsky *et al*., 2000; Moini *et al*., 2002). Jacob *et al*. (1996) observed that dosing obese Zucker rats with a racemic mixture of lipoic acid significantly enhanced insulin action in skeletal muscle by increasing oxidative and nonoxidative glucose metabolism. A study by Yaworsky *et al*. (2000) showed that lipoic acid rapidly stimulated glucose uptake in cell cultures by activating the insulin-signaling pathway. Consequently, we hypothesized that lipoic acid would decrease P4 clearance rates by decreasing hepatic steroid metabolic enzyme activity.

Materials and Methods

Animals and diets

Animal care and use was approved by the Institutional Animal Care and Use Committee (#A0956)

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Received: January 21, 2013
Accepted: May 23, 2013

at North Dakota State University (Fargo, ND, USA). Eight mature Katahdin-cross, previously ovariectomized ewes were used for this trial. For ovariectomy procedures, ewes were sedated with 0.4 ml xylazine (20 mg/ml; Rompun®, Bayer HealthCare, Shawnee Mission, KS, USA) and 2.0 ml ketamine hydrochloride (100 mg/ml) intramuscularly. An abdominal incision approximately 5-8 cm in length was made on the midline anterior to the udder. Ovaries were extracted from the abdominal cavity and surgically removed. The ovarian pedicle was sutured at the site of removal. After the body cavity and skin were sutured, ewes were injected with short-acting penicillin (300,000 units/ml; Agri-Cillian, Agri Laboratories, Ltd., St. Joseph, MO, USA) per manufacturer's recommendations. Ewes were allowed to recover for 28 days prior to initiation of treatment.

For the current trial, ewes were housed at the Animal Nutrition and Physiology Center in Fargo. Ewes were penned individually in 0.91 x 1.20-m pens in a temperature controlled (12°C), well-ventilated facility with ad libitum access to fresh water. Lighting was automatically timed to mimic the natural photoperiod during the fall season in North Dakota. Ewes were weighed, individually penned, and fed an *ad libitum* diet of alfalfa-grass (97.7% dry matter; 7.3% crude protein) mix hay for 1 week prior to the start date (day 0). Intake was determined by measuring weight of initial feed offered subtracted by weight of residual feed after a 24 h period and averaged for the 1 week period. Subsequently, intake was restricted to 95% of intake from day -14 to -7 with a 1 week acclimation period to this diet. From day -7 until completion of the 11 day trial ewes continued to receive 95% of intake.

Ewes were randomly assigned to one of two groups; control (CON; empty bolus; n = 4), or lipoic acid (LA; 32 mg/kg BW; n = 4; racemic mixture of lipoic acid, MTC Industries, Inc., Edgewood, NY, USA). The dose of lipoic acid was based on a previous study done in cattle (Schmidt *et al*., 2006) investigating the effects of lipoic acid supplementation on metabolic hormones and acute-phase proteins during a challenge with infectious bovine rhinotracheitis virus. This study reported augmentation of immune response in cattle supplemented with lipoic acid compared to control animals. Ewes were weighed on day -2 and day -1 and BWs averaged to determine lipoic acid dosages. Lipoic acid was measured into clear gelatin capsules that held a maximum of 1 g (Torpac, Inc., Fairfield, NJ, USA). Prior to feeding, LA ewes were dosed with lipoic acid at 7:30 a.m. on day 0. Lipoic acid was administered to LA ewes by gavage using a plastic tube with a wooden rod for capsule projection to encourage swallowing. Similarly, CON ewes were gavaged with empty capsules. The number of capsules administered to the control group was based on the weight of a full capsule of lipoic acid and BW of control ewes. Leftover feed that was uneaten by the animal was collected and weighed daily to determine feed intake.

Sample collection

On day 5, serum samples were collected via jugular venipuncture (BD Vacutainer, Franklin Lakes, NJ, USA) at 7:30 a.m. prior to feeding and lipoic acid supplementation. Blood samples were immediately put on ice and cooled for 2 h before centrifugation. Samples were centrifuged at 2,500 x g for 20 min and serum was pipetted and stored at -20°C until further analysis. Following day 5 blood sampling, a controlled internal drug release [CIDR; EAZI-BREED CIDR, 0.3 g progesterone (P4), Pfizer Animal Health, New York, USA] was inserted vaginally into ewes. From day 6 to 9 blood samples were collected daily at 7:30 a.m. and handled as described previously.

Liver biopsy procedure

On day 10, liver biopsies were performed percutaneously 1 h post gavage and feeding to determine hepatic enzyme activity. Ewes were placed in restraining pens and wool was removed from the animals' right side over the rib cage. Ultrasonography was performed on the first ewe to determine a location devoid of major branches of the hepatic portal vein and hepatic artery. The point of insertion was at the 10th intercostal space intersected by a line drawn from the point of the elbow to the point of the hip. The area was cleaned three times with betadine scrub and sprayed with 70% ethanol. Ewes were administered 10 cc of 2% lidocaine hydrochloride subcutaneously and intramuscularly at the point of insertion. The skin was punctured using a scalpel and liver samples 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). The biopsy tool was guided to the liver and a 0.5 to 1 g sample was collected. Approximately 200 mg of liver was submerged in a 100 mM potassium phosphate buffer containing 1 mM EDTA to assess CYP2C and CYP3A activity in fresh liver samples. The remaining portion of the liver was snap frozen in liquid nitrogen and stored at -80°C for later analysis of AKR1C activity. Upon completion of the liver biopsy procedure, the incision site was closed with a sterile surgical staple and sprayed with Blu-Kote (H.W. Naylor Company Inc., Morris, NY, USA).

Progesterone clearance

On day 11 the CIDRs were removed and serial blood samples were obtained via jugular catheters to determine P4 clearance. The catheterization site was prepared, cleaned with alcohol and injected with 1 ml lidocaine prior to insertion of a 14 gauge catheter needle and catheter (I-CATH, Charter Med Inc., Winston-Salem, NC, USA). After placement was confirmed, catheters were flushed with 3 ml of heparin saline,

stitched into place and covered with co-flex vet wrap. Serial blood samples were collected through jugular catheters at 0 (i.e., just prior to CIDR removal), 2, 5, 10, 15, 30, 60, 120, and 360 min post CIDR removal. Gavage of treatment and feeding was performed coinciding with the 0 min sample. At each sample time, the first 1 ml of blood was discarded, followed by a 3 to 4 ml blood sample which was placed into a Vacutainer for serum collection (Red Tops, BD Diagnostics, Franklin Lakes, NJ, USA) that was immediately placed on ice. Following each sample collection, the catheter was flushed with 2 ml heparin saline. Catheters were removed after the 120 min sample and jugular venipuncture was used for the 360 min sample. Blood samples were cooled for 2 h followed by centrifugation to obtain serum as described previously.

Hepatic enzyme activity

Activity of CYP2C and CYP3A was determined in fresh liver samples following the protocol by Lemley *et al*. (2008a). Briefly, liver samples were submerged in phosphate buffer and homogenized using a Polytron homogenizer. Microsomes were collected and concentrated using differential centrifugation techniques. Fresh homogenized tissue was spun for 10 min at 10,000 x g. The pellets were discarded and the supernatants were centrifuged at 100,000 x 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 was measured as the non-ketoconazole-inhibitable, omeprazole-dependent oxidation of NADPH. 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.

Activity of AKR1C was measured in cytosolic cellular fractions using the specific substrate 1-acenapthenol (Lemley *et al*., 2010). Briefly, AKR1C enzymatic reactions contained 150-160 µg of cytosolic protein, 250 µm 1-acenapthenol, and 500 µm NADP. The 1-acenapthenol-dependant reduction of NADP was standardized using cytosolic protein concentrations. Solutions were added to 96-well plates (PGC Scientifics, Frederick, MD, USA) and the oxidation of NADPH (CYP2C and CYP3A) or reduction of NADP (AKR1C) was determined by measuring the amount of light absorbed at 340 nm for 5 min at 37ºC. 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*cm) was used to calculate oxidized NADPH or reduced NADP per unit time.

Progesterone analysis

Serum progesterone concentrations were determined as described by Galbreath *et al*. (2008) using solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 1000, Diagnostic Products Corp., Los Angeles, CA, USA); 50 µl of serum were assayed in duplicate. Within each assay, low, medium, and high P4 pools were run in duplicate. The intraassay coefficient of variance was 4.7%.

Calculations and statistical analyses

Statistical *analyses* of treatment differences and P4 clearance were conducted using the MIXED and GLM procedures of SAS (SAS Inst. Inc., Cary, NC, USA). Comparisons of means with a $P < 0.05$ were considered statistically significant and $P > 0.05$ to <0.1 as tendancies. Data are reported as least squares means. The fractional rate constant of P4 clearance at time points 0, 2, 5, 10, 15, 30, 60, 120, and 360 min following CIDR removal was determined using the equation: $N(t)=N0e^{-kt}$ where N is the reactant (P4) at time t, N0 is the initial value, k is the first-order fractional rate constant, t is time and e is the base of natural logarithms. The fractional rate constant (k) and progesterone intercept (N0) were calculated for each individual animal using Sigma Plot (Systat Software Inc., San Jose, CA, USA).

Results

There were no significant differences ($P > 0.05$) in feed intake or BW between treatment groups throughout the trial. While there was no effect of treatment, there was a difference ($P < 0.02$) across days in P4 concentrations. Day 6 (prior to CIDR insertion) had decreased ($P < 0.05$) P4 compared to all other days. Moreover, concentrations of P4 on day 7 were greater ($P < 0.05$) than days 8 through 11, which were similar (Fig. 1).

When examining the effect of lipoic acid supplementation and progesterone within day, we observed a treatment by time interaction ($P < 0.02$; Fig. 2). At time 0 (prior to LA supplementation), concentrations of progesterone tended to be lower ($P < 0.1$) in LA supplemented ewes (3.33 ± 0.17 ng/ml) compared to CON ewes (4.07 ± 0.17 ng/ml). In LA ewes, P4 remained relatively steady until greater ($P < 0.02$) P4 was observed at 120 min compared to time 0. In CON ewes, P4 decreased ($P < 0.01$) 30 min after placebo bolus administration, thereafter increasing from 0 min through 120 min (Fig. 2). Moreover, at 60 min after bolus, CON ewes tended ($P < 0.08$) to have greater P4 than LA ewes. On the day of CIDR removal (day 11) there was a treatment by time interaction ($P < 0.02$) such that progesterone concentrations were greater prior to and for 10 min after CIDR removal in CON versus

LA ewes. While P4 concentrations readily dropped after 10 min in CON ewes, P4 in LA ewes remained relatively constant until 60 min after CIDR removal (Fig. 3). The fractional rate constant for P4 clearance on day 11 was lowest ($P < 0.03$) in LA treated ewes compared to CON ewes (Fig. 4). There was no difference ($P > 0.05$) in the average intercept for P4 clearance between CON and LA supplemented ewes (Fig. 4). No differences ($P > 0.05$) were found in CYP2C or CYP3A hepatic enzyme activity in liver biopsies obtained one-hour post-gavage. There was a tendency ($P < 0.08$) for increased AKR1C activity in LA ewes compared to CON ewes (Table 1).

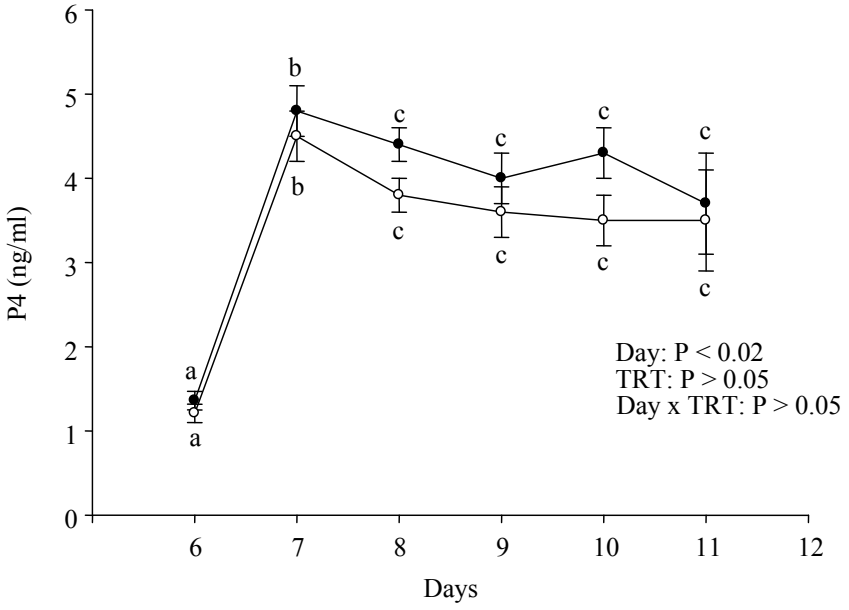

Figure 1. Serum progesterone concentrations on day 6 through 11 in ewes supplemented with 32 mg/kg lipoic acid BW by gavage daily (LA, ○) or with a control bolus (CON, ●). Blood samples were collected prior to feeding. [abc]LSMeans ± SEM with different superscripts differ ($P < 0.05$) across days.

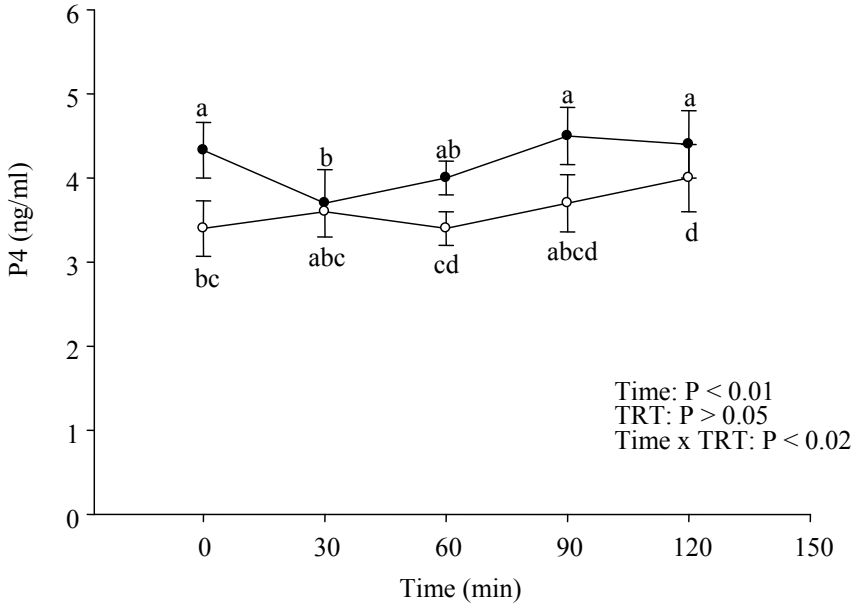

Figure 2. Serum progesterone concentrations on day 10 in ewes supplemented with 32 mg/kg BW lipoic acid by gavage daily (LA, ○) or with a control bolus (CON, ●). Blood samples were collected prior to feeding (0 min) and up to 120 min post-feeding. [abcd]LSMeans ± SEM with different superscripts differ ($P < 0.05$) across days.

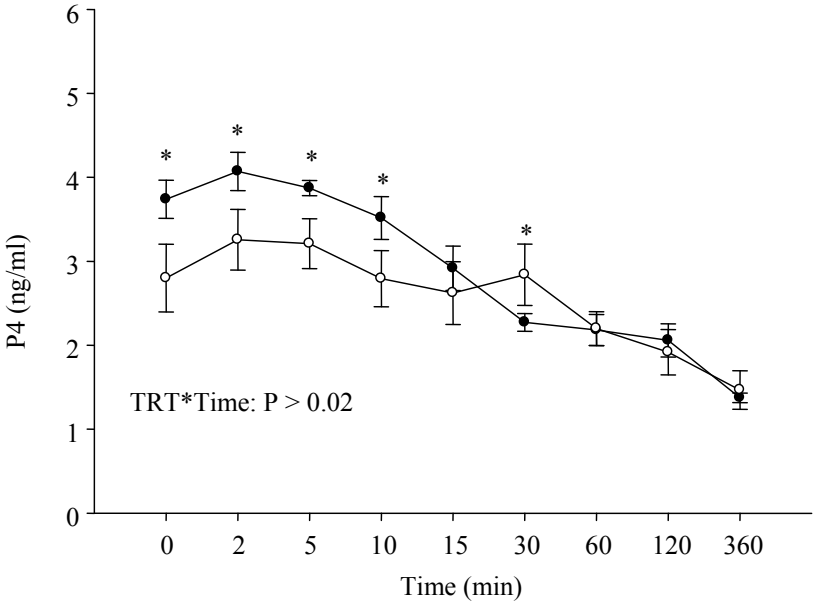

Figure 3. Serum progesterone concentrations over time (P < 0.02) on day 11 after CIDR removal in ewes supplemented with 32 mg/kg BW lipoic acid by gavage daily (LA, ○) or with a control bolus (CON, ●). A treatment x time interaction (P < 0.02) was observed. Significant differences (P < 0.05) between LSmeans in the same time point are denoted by an asterisk (*). Blood samples were taken in relation to CIDR removal at 0 min. Values are LSMeans ± SEM.

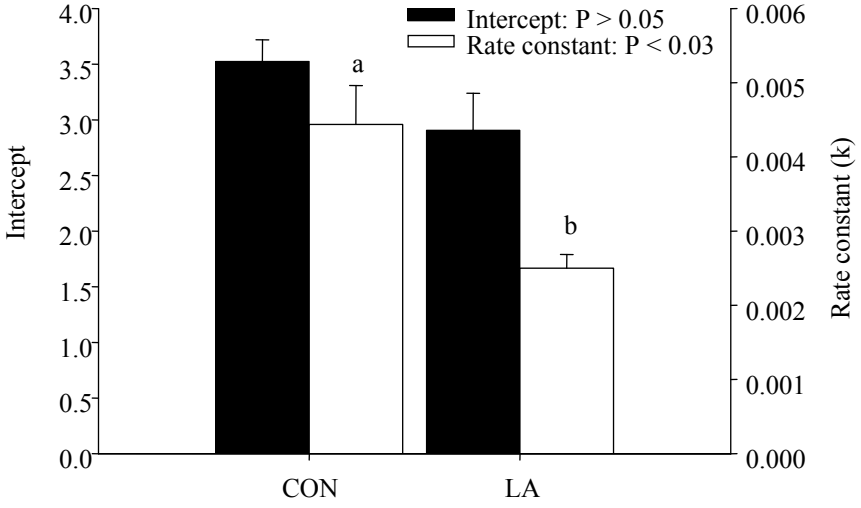

Figure 4. Average intercept (P > 0.05) and average fractional rate constant (^{ab}P < 0.03) for P4 clearance in ewes supplemented with 32 mg/kg BW lipoic acid by gavage daily (LA) or control (CON) after CIDR removal on day 11. Values are means ± SEM.

Table 1. Hepatic cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A) and aldo-keto reductase 1C (AKR1C) activity on day 10 in ewes supplemented with 32 mg/kg BW lipoic acid by gavage daily (LA) or control (CON). Hepatic enzyme activity was determined in liver biopsies taken ~1 h post-feeding.

	CON	LA	SEM	P-value
CYP2C, pmol/min*mU	25.59	24.13	4.82	0.84
CYP3A, pmol/min*mU	10.12	6.27	2.78	0.37
AKR1C, pmol/min*mg	169.26	309.48	46.42	0.08

Discussion

In the current study, ovariectomized ewes were used to determine progesterone clearance and hepatic enzyme activity after oral supplementation with lipoic acid. Ewes treated with lipoic acid showed no differences in circulating progesterone, average intercept for P4 clearance or hepatic enzyme activity when compared to control ewes. However, supplemented ewes did have a lower fractional rate constant (k) of progesterone clearance compared to control ewes on day 11.

While the present study did not show any differences in hepatic enzyme activity, previous studies have shown a reduction in CYP2C and CYP3A activity in the presence of elevated insulin or by supplementing animals with a gluconeogenic substrate (Smith *et al*., 2006; Lemley *et al*., 2008a, b). Smith *et al*. (2006) found that gavaging ewes with sodium propionate resulted in elevated insulin concentrations and decreased progesterone clearance compared to ewes dosed with an isocaloric amount of sodium acetate. A study by Lemley *et al*. (2008b) examined hepatic CYP2C and CYP3A activity in ovariectomized ewes dosed with sodium propionate, a gluconeogenic substrate, and found treatment ewes had elevated insulin concentrations and a 50% reduction in hepatic CYP2C and CYP3A activity compared to ewes dosed with an isocaloric level of sodium acetate. Although lipoic acid is not known to be a gluconeogenic substrate, it does modulate the action of insulin which is what led us to hypothesize that it would have a similar effect on progesterone clearance, as would a gluconeogenic substance (Jacob *et al*., 1996; Yaworsky *et al*., 2000; Moini *et al*., 2002).

While we did not measure insulin or glucose concentrations in the current study, we speculate that lipoic acid may have altered these substrates based on studies indicating that lipoic acid modulates the action of insulin in human and animal models (Jacob *et al*., 1995; Packer *et al*., 2001). In a study by Jacob *et al*. (1996), it was indicated that treatment with lipoic acid to obese Zucker rats improved glycogen synthesis, insulin-stimulated glucose oxidation and resulted in greater muscle glycogen concentration when compared to the control group. In this same study, a long term adaptation of the body to lipoic acid treatment decreased plasma insulin levels 15-17% compared to the control group (Jacob *et al*., 1996). A study by Yaworsky *et al*. (2000) found that treatment of lipoic acid on 3T3-L1 adipocytes resulted in an increase in GLUT1 and GLUT4 to the plasma membrane, an increase in insulin receptor substrate-1 and antiphosphotyrosine-associated activity, and activation of kinases in target cells which can result in increased glucose uptake. These studies indicate that lipoic acid does have an effect on the action of insulin in glucose uptake, although the exact mechanism of action is still unknown. Because we found no differences in hepatic enzyme activity it is possible that the rate of progesterone clearance in our study was impacted by enhanced insulin effectiveness rather than alterations in hepatic catabolic enzyme activity. Measuring insulin and glucose concentrations would have provided further insight into the mechanistic action of lipoic acid as to whether it mimics the action of insulin or simply enhances the effect of insulin to take up glucose.

Taking into account that the liver is the primary site of progesterone clearance, several researchers have correlated hepatic blood flow with the metabolic clearance rate of progesterone (Parr *et al*., 1993; Sansritavong *et al*., 2002). Parr *et al*. (1993) observed a doubling of progesterone clearance in ewes that had a 40% increase in liver blood flow due to feeding above maintenance diets. Therefore, apart from hepatic progesterone catabolic enzyme activity, the rate of delivery of substrate (i.e., progesterone) can impact the overall metabolic clearance rate. Although liver blood flow was not calculated in the current experiment, treatment differences in the fractional rate constant of progesterone decay could be the result of a lowered hepatic blood flow following lipoic acid supplementation.

Exploring methods of decreasing the rate of progesterone clearance is a worthwhile area of study, as this will likely decrease early embryonic losses by maintaining adequate progesterone concentration during the critical early embryonic period. While lipoic acid does not alter hepatic progesterone catabolic enzyme activity, supplementing with lipoic acid results in a decreased fractional rate constant of progesterone clearance in the ovariectomized ewe; however, the mechanism of action of lipoic acid has yet to be determined.

Acknowledgments

This research was supported by the NDSU Advance FORWARD program sponsored by the National Science Foundation, *HRD-0811239* (ELB), the Gordon A. Larson Agricultural Research Fund (ELB) and the North Dakota State University Development Foundation.

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