In vitro and in vivo analysis of fatty acid effects on metabolism of 17 β -estradiol and progesterone in dairy cows

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ABSTRACT

Some studies have reported improved reproductive performance with dietary fat supplementation. This study examined effects of fatty acids with different lengths, or desaturation, or both, on metabolism of estradiol (E2) and progesterone (P4) in bovine liver slice incubations (experiments 1 and 2) and in vivo (experiment 3). In experiment 1, effects of fatty acids C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:1 (oleic acid), and C18:3 (linolenic acid) were evaluated at 30, 100, and 300 μ M on P4 and E2 metabolism in vitro. In experiment 2, stearic acid (C18:0) and C18:3 were evaluated in the same incubation conditions. In experiment 1, all of the fatty acids had some significant inhibitory effect on metabolism of P4, E2, or both (300 μ M C16:0 on E2; 100 μ M C16:1 on E2; 300 μ M C16:1 on both P4 and E2; 300 μ M C18:1 on P4; and 100 and 300 μ M C18:3 on both P4 and E2). In experiment 2, C18:3 (100 and 300 μ M) but not C18:0 decreased P4 and E2 metabolism. Overall, the most profound increase (~60%) in half-life of P4 and E2 was observed with incubations of 300 μ M C18:3 in both in vitro experiments. Based on these in vitro results, in experiment 3 linseed oil (rich in C18:3) was supplemented into the abomasum and acute effects on metabolism of E2 and P4 were evaluated. Cows ($n = 4$) had endogenous E2 and P4 minimized (corpus luteum regressed, follicles aspirated) before receiving continuous intravenous infusion of E2 and P4 to analyze metabolic clearance rate for these hormones during abomasal infusion of saline (control) or 70 mL of linseed oil every 4 h for 28 h.

Linseed oil infusion increased C18:3 in plasma by 46%; however, metabolic clearance rate for E2 and P4 were similar for control cows compared with linseed-treated cows. Thus, in vitro experiments indicated that E2 and P4 metabolism can be inhibited by high concentrations of C18:3. Nevertheless, in vivo, linseed oil did not acutely inhibit E2 and P4 metabolism, perhaps because insufficient C18:3 concentrations (increased to ~8 μ M) were achieved. Further research is needed to determine the mechanism(s) of fatty acid inhibition of P4 and E2 metabolism and to discover practical methods to mimic this effect in vivo.

Key words: polyunsaturated fatty acid, estrogen, progesterone, steroid metabolism

INTRODUCTION

Dietary fat supplementation is a strategy that has been widely used in livestock operations to improve productivity and reproductive performance. However, results of fat supplementation on reproductive performance in beef or dairy cattle have been conflicting (reviewed by Grummer and Carroll, 1991; Staples et al., 1998; Mattos et al., 2000; Williams and Stanko, 2000; Funston, 2004). For example, some studies found a lack of response in reproductive performance when supplemental fat was fed (Carroll et al., 1990; Carr et al., 1994). In contrast, other studies indicated that effects of fatty acids on reproduction include increased conception rate (Son et al., 1996; Staples et al., 1998), increased number or diameter of follicles (Mattos et al., 2002; Robinson et al., 2002), and increased circulating progesterone (P4; Hawkins et al., 1995; Lammoglia et al., 1997) or estradiol (E2; Robinson et al., 2002) concentrations.

The observed increase in circulating P4 and E2 concentrations may be a mechanism underlying improvements in fertility following fat supplementation. Fat supplementation could enhance circulating steroids by increasing steroid hormone production through various mechanisms, including increased availability of lipopro-

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tein-cholesterol, modulation of prostaglandin synthesis, or direct stimulation of ovarian steroidogenesis (Grummer and Carroll, 1991; Staples et al., 1998; Williams and Stanko, 2000). In addition to mechanisms that alter steroid production, the circulating steroid concentrations can be regulated by changes in the rate of steroid metabolism. It has been recognized that the rate of steroid metabolism may be a critical regulator of steroid hormone concentrations and possibly of reproductive performance in lactating dairy cows (Sangsritavong et al., 2002; Wiltbank et al., 2006). More than a decade ago, a model based on data from sheep and pigs was developed that connected increases in feed intake with elevated liver blood flow and subsequent augmentation of steroid hormone metabolism (Parr et al., 1987, 1993; Prime and Symonds, 1993; Miller et al., 1999). Thus, changes in steroid metabolism could alter the reproductive physiology of any species, but may particularly alter reproduction in species with extreme increases in feed intake, such as the lactating dairy cow (Wiltbank et al., 2006).

There is evidence that fats or fatty acids may regulate circulating steroid concentrations. Feeding beef heifers from 100 d before parturition through the subsequent postpartum period approximately doubled the circulating P4 concentrations during their second or third postpartum estrous cycle (Hawkins et al., 1995) and increased the half-life of P4 (170 vs. 113 min; Hawkins et al., 1995). In addition, cows fed diets supplemented with C18:3 had increased E2 concentrations during the follicular phase (Robinson et al., 2002). The liver is the primary site for steroid hormone metabolism (Rendic and DiCarlo, 1997), with liver cytochrome P450 (**cyp**) enzymes catalyzing critical steps in this metabolism. Studies *in vitro* indicate that polyunsaturated fatty acids (**PUFA**) may act as competing substrate for binding sites of **cyp** (Hirunpanich et al., 2006, 2007; Yao et al., 2006). Thus, inhibition by PUFA of the key enzymes of steroid metabolism could increase circulating steroid hormone concentrations and partially overcome the negative effects of high steroid metabolism that have been documented in dairy cattle (Wiltbank et al., 2006).

Nevertheless, despite the substantial number of studies on the effects of fats on reproductive performance of dairy cows and the growing evidence for a role of steroid hormone metabolism in reproduction in dairy cows, no previous studies have been designed to specifically evaluate the effects of fats on E2 and P4 metabolism in dairy cattle. The present study evaluated whether fatty acids with different lengths or different amounts of desaturation could directly alter metabolism of steroids by slices from bovine liver. Further, an *in vivo* study evaluated the effect of linseed oil on circulating

E2 and P4 concentrations during continuous infusion of these steroids. The overall hypothesis tested in these experiments was that specific fatty acids reduce E2 and P4 metabolism *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents and Products

Progesterone and E2 were purchased from Steraloids Inc. (Newport, RI). Cell culture media (M-199, catalog # M3769) without phenol red was purchased from Sigma Co. (St. Louis, MO) and modified by adding HEPES (4.76 g/L), sodium bicarbonate (0.35 g/L), and L-glutamine (0.1 g/L), with final pH adjusted to 7.35. The intravaginal P4 devices [Eazi-Breed controlled internal drug-releasing intravaginal insert (**CIDR**) containing 1.39 g of P4] were purchased from InterAg Company (Hamilton, New Zealand), and PGF_{2α} (Lutalyse; 25 mg) was purchased from Pharmacia Animal Health (Peapack, NJ). Linseed oil was a gift of Archer Daniels Midland Company (Red Wing, MN).

Liver Incubations and Measurements

Bovine livers were obtained from an abattoir that primarily slaughters Holstein cattle; however, prior cow history was unknown for all livers used in this study. The livers were transported from the slaughterhouse in a thermos filled with physiological saline (36°C) and arrived at the laboratory by 4 h after slaughter. At the laboratory, the liver from each individual cow was thinly sliced (100–130 mg/slice) using a Stadie Riggs tissue slicer (Fisher Scientific Corp., Pittsburgh, PA). Each treatment (described below) was replicated in 3 different plates for each bovine liver. The steroid hormone being tested was dissolved in absolute ethanol before addition into the media with a final concentration of 0.25% (vol/vol). Each concentration of fatty acid was directly diluted into media with individual steroid hormones (unsaturated fatty acids were warmed up to allow dilution). Five milliliters of M-199 media containing the steroid hormone and fatty acids were put into 6-well tissue culture plates (Fisher Scientific Corp.). Plates were incubated at 39°C for 3 h in humidified air to equilibrate temperature before addition of liver slices. Liver slices were then added to the media containing one of the steroids [30 nM (~9.4 ng/mL) P4 or 3.5 nM (~953 pg/mL) E2] without any added fatty acids or with 10, 100, or 300 μM fatty acids. Media samples (100 μL) were taken before addition of a liver slice to account for time 0 (0 min). After addition of the liver slices, media samples were taken at 2, 5, 8, 15, and 30 min. Samples were then diluted 1:3.5 with

ELISA assay buffer and hormone concentrations were measured by ELISA as described below. The rate of disappearance of steroids during the period of incubation was used to calculate half-life. Metabolism of P4 and E2 follows first-order kinetics (Brueggemeier et al., 1984a, b; Freetly and Ferrell, 1994); therefore, half-life was calculated from a semilog plot between the natural log of steroid concentration versus time of incubation (Rivieri, 1999).

Experiment 1: In Vitro Inhibition of Steroid Metabolism by C18 and C16 Fatty Acids

Our objective was to investigate the effects of saturated and unsaturated fatty acids on P4 and E2 metabolism. Each steroid was investigated separately. Liver slices were incubated with 30 nM P4 or 3.5 nM E2 using 4 different liver preparations (each treatment analyzed in triplicate wells). The following fatty acid treatments were mixed in the media: C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:1 (oleic acid), or C18:3 at 0, 30, 100, and 300 μ M with each of the steroid hormones (P4 or E2).

Experiment 2: In Vitro Inhibition of Steroid Metabolism by Stearic and Linolenic Acids

This additional in vitro experiment tested the effect of double bonds in C18 fatty acids on P4 and E2 metabolism. All of the experimental conditions including concentrations of P4 (30 nM) and E2 (3.5 nM) in the incubations were the same as in experiment 1. Five different liver preparations (each done in triplicate) were performed using increasing concentrations (0, 30, 100, or 300 μ M) of C18:0 (stearic acid) or C18:3.

Experiment 3: Effects of Intraabomasal Infusion of Linseed Oil on E2 and P4 Metabolism In Vivo

This study was conducted at the Dairy Cattle Center at the University of Wisconsin–Madison. Four lactating Holstein cows (BW = 694.6 ± 27.7 kg; BCS = 4; milk production = 27.5 ± 4.4 kg; DIM = 257 ± 51) were kept in stanchions for 10 d before the experiment (to acclimate and to determine normal feed intake) and throughout the experimental protocol. The animal protocol was as follows. Animals had ovarian function synchronized with a CIDR that was kept for 8 d, and 2 injections of PGF_{2 α} were given 12 h apart on the day of CIDR removal (d -2). One day after CIDR removal (d -1), catheters were placed in both jugular veins and all follicles greater than 6 mm in diameter were aspirated using an ultrasound-guided transvaginal needle and a 7.5-MHz convex-array transducer (Aloka 900V,

Corometrics Medical Systems Inc., Wallingford, CT) in order to minimize endogenous production of E2 and avoid ovulation. Follicle aspiration was also performed on d 4. Cows were milked at their stalls twice a day, and milking times did not coincide with steroid infusions (described below).

On d 0, the cows received a sequence of 3 i.v. saline infusions containing E2 (300 μ g/h) and P4 (9 mg/h) during a 31-h period (period 1). The first infusion lasted 9 h and the other 2 lasted 4 h each, with an interval of 6 h between the end of the first and the beginning of the second and 8 h between the end of the second and the beginning of the third infusion. On d 5, a second series of infusions was performed to complete the crossover design (period 2). An interval of 3 d between periods was performed to reduce carryover effects. Stock solutions of P4 and E2 were prepared using 99.8% absolute ethanol. While stirring, this solution was added to physiological saline [0.9% (wt/vol) sodium chloride] to produce a final concentration of 15.0 μ g/mL of P4 and 0.5 μ g/mL of E2 with a final ethanol concentration of 2% of total volume (vol/vol). Infusions were delivered using a peristaltic pump (Masterflex L/S, Cole Plamer, Vernon Hill, IL). Body temperature was monitored before and after infusion and did not change during the study.

Intraabomasal infusion (70 mL every 4 h) of either linseed oil (56.0% C18:3; 16.4% C18:2; 18.0% C18:1; 3.4% C18:0; 5.8% C16:0) or saline was performed during 28 h starting at 3 h after the initiation of the concurrent intravenous steroid infusion. Infusions were done via vinyl tubing that passed through a ruminal cannula plug that bypassed the omasum, ending in the lumen of the abomasum. Each cow underwent 2 experimental periods in a crossover design ($n = 4$). Hourly blood samples were collected from a catheter placed in the contralateral jugular vein (one jugular vein for infusions, one for collection of samples). Patency was maintained by flushing catheters with 5 mL of heparinized saline (10 IU/mL) during frequent sampling periods. An additional blood sample was also collected by venipuncture from coccygeal vessels at 24 h after start of abomasal infusion (d 1) to monitor fatty acid composition of plasma. This blood sample was collected in a special Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ) for plasma separation.

From 12 h before until the end of saline infusion, cows were fed a TMR at 80% of previously determined feed intake (3.0–3.4 kg of DM every 4 h) and were offered water ad libitum. Animals were fed every 4 h to ensure constant rumen repletion to help reduce the effects of acute feeding of large amounts of feed on hepatic metabolism. The BCS of the cows were taken before the experimental period and at the end of the

experiment. All animal procedures had prior approval by the Animal Care and Use Committee of the University of Wisconsin–Madison College of Agriculture and Life Sciences.

Hormone Assays

The samples taken during liver incubation (experiments 1 and 2) were diluted 3.5 times in assay buffer (0.04 M 3-[N-morpholino] propane sulfonic acid, 0.12 M sodium chloride, 0.01 M EDTA, 0.05% Tween 20, 0.005% chlorohexidinedigluconate, and 0.1% gelatin; pH 7.4), and the concentrations of P4 and E2 were determined by a competitive ELISA as described previously (Rasmussen et al., 1996). The intra- and interassay coefficients of variation for the E2 assay were 2 and 4%, respectively. In addition, the intra- and interassay coefficients of variation for the P4 assay were 3 and 7%, respectively.

Five hundred microliters of serum samples were extracted twice with ethyl ether (4 mL each time) for samples collected in experiment 3. The extraction was dried, resuspended in 200 μ L of assay buffer, and used directly in the ELISA assay for E2 analysis. Serum concentration of P4 was evaluated by a nonextraction radioimmunoassay (Count-a-count, Diagnostic Products Corp., Los Angeles, CA). A quality control sample was prepared from charcoal-stripped serum containing a known concentration of P4 (2.5 ng/mL) and E2 (500 pg/mL). Duplicate quality control samples in each assay were used for calculating the intra- and interassay coefficients of variation, which were 8.8 and 13.7%, respectively, for the E2 ELISA. For the P4 assay, all samples were analyzed in a single assay with an intraassay coefficient of variation of 1.5%.

Nonesterified Fatty Acids and Total Lipid Analysis

Plasma samples from all cows 24 h after initiation of treatment (intraabomasal infusion) were extracted according to Dole (1956). Method Ce 1h-05 of the American Oil Chemists' Society was used for quantification of fatty acids (Firestone and Sheppard, 1992). Neutral lipids were then separated by thin-layer chromatography and spots corresponding to the fatty acid fraction were removed, extracted with chloroform:methanol (2:1), and methylated. Fatty acid composition was determined by gas liquid chromatography. The method included fatty acid methyl ester derivatization and separation on a 100-m polar capillary column (Firestone and Sheppard, 1992). Plasma samples were also analyzed for NEFA (NEFA-C kit, Wako Fine Chemical Industries USA, Dallas, TX) according to Pires et al. (2007) to ensure treatment effectiveness.

Statistical Analysis

In experiments 1 and 2, data were analyzed separately using the MIXED procedure of SAS (SAS Institute, 2004). Differences in half-life of E2 and P4 were analyzed with time, treatment, and treatment by time interactions in the statistical models, using the *P*-value obtained for the difference of least squares means (pDiff) for means separation. Values reported are least squares means and standard errors of the means.

In experiment 3, the continuous infusion of P4 and E2 allowed calculation of steroid concentration and metabolic clearance rate (MCR) based on the circulating concentrations of these steroids at steady state (Longcope et al., 1981; Parr et al., 1993). A summarized description of the formulas for calculation of MCR can be found in Sangsritavong et al. (2002). The statistical model for analysis of steroid concentration and MCR included fixed effects of treatment, random effect of cow, and the residual error term. Means were calculated for treatments using the difference between the linseed group and the control group at each time. The concentration and MCR of E2 and P4 over time was analyzed by adding time and treatment by time interaction to the models, using repeated measures in time. The 2 analyzed variables, steroid concentrations and MCR, were evaluated by comparing the experimental groups once the infusions reached steady state; therefore, the initial times (0, 1, and 2 h) were not used in this analysis. Compound symmetry covariance structure was used for data collected during infusion.

Fatty acid from the total lipid list was analyzed by the MIXED procedure of SAS using cow as a random effect. For the statistical analysis of NEFA concentration in plasma, treatment was used as a fixed effect in the MIXED procedure of SAS and the output was the mean for each treatment. For all comparisons, significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

In Vitro Studies

Steroid metabolism was expressed as the mean half-life calculated by using the E2 or P4 concentration at multiple time points during the liver slice incubations (0, 2, 5, 8, 15, and 30 min). The mean half-life calculated from multiple replicates ($n = 4$ in experiment 1; $n = 5$ in experiment 2) were used to analyze the effects of fatty acid treatments (Tables 1, 2, and 3).

Progesterone Metabolism: Experiment 1. The half-life of P4 (Table 1) was affected by treatment ($P < 0.0001$), concentration of fatty acids ($P < 0.0001$), and the interaction between treatment and concentra-

Table 1. Progesterone half-life (min; least squares means $\pm$ SEM) during incubation of liver slices ($n = 4$ different cows, each done in triplicate incubations) with differing concentrations of C16:0, C16:1, C18:1, or C18:3 fatty acids

Item	C16:0	C16:1	C18:1	C18:3	<i>P</i> -value
0 μM	18.8 $\pm$ 3.5	18.8 $\pm$ 3.5 ^x	18.8 $\pm$ 3.5 ^x	18.8 $\pm$ 3.5 ^x	—
30 μM	18.8 $\pm$ 2.2	18.2 $\pm$ 2.5 ^x	20.0 $\pm$ 2.9 ^{xy}	20.9 $\pm$ 2.4 ^x	0.380
100 μM	20.6 $\pm$ 1.4 ^a	21.3 $\pm$ 2.4 ^{a,xy}	19.2 $\pm$ 2.5 ^{a,x}	24.6 $\pm$ 2.1 ^{b,y}	0.017
300 μM	19.4 $\pm$ 2.0 ^a	23.2 $\pm$ 3.5 ^{b,y}	23.2 $\pm$ 3.0 ^{b,y}	30.3 $\pm$ 3.2 ^{c,z}	<0.0001
<i>P</i> -value	0.686	0.016	0.049	<0.001	—

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

^{x-z}Means within a column with different superscripts differ ($P < 0.05$).

tion of fatty acids ($P = 0.0037$). The saturated fatty acid C16:0 had no effect on P4 half-life ($P = 0.686$), whereas the unsaturated fatty acids C16:1, C18:1, and C18:3 increased the half-life of P4 ($P = 0.016$, $P = 0.049$, and $P < 0.001$, respectively). Fatty acids at 30 μM concentration showed no effect on P4 metabolism ($P = 0.380$). In contrast, fatty acids at 100 and 300 μM concentration had a significant effect on P4 metabolism. Among the treatments at the concentration of 100 μM , there was an increase in P4 half-life in the slices treated with C18:3 ($P = 0.017$) but not in those treated with the other fatty acids. In the presence of 100 μM or 300 μM fatty acids, the half-life of P4 was significantly greater in the presence of C18:3 than any of the other fatty acids. The 300 μM concentrations of C16:1 and C18:1 produced greater P4 half-life than C16:0 but did not reach the levels found with C18:3 ($P < 0.0001$). There was 61.1% inhibition of P4 metabolism with 300 μM C18:3 compared with controls without fatty acids (18.8 $\pm$ 3.5 vs. 30.3 $\pm$ 3.2 min).

Estradiol Metabolism: Experiment 1. The overall results from the incubations of liver slices with 3.5 nM E2 and different concentrations of fatty acids indicated an effect of treatment ($P < 0.0001$), concentration of fatty acid ($P < 0.0001$), and their interaction ($P = 0.0003$). There was an increase in E2 half-life (see Table 2) in the presence of C16:0 ($P = 0.0013$), C16:1 ($P = 0.0028$), and C18:3 ($P < 0.0001$), but not C18:1 ($P = 0.136$). The largest increase in half-life was observed

when slices were incubated with 300 μM C18:3 (21.3 $\pm$ 1.6 vs. 34.3 $\pm$ 2.4 min), with a 60.9% inhibition of E2 metabolism as compared with controls (slices incubated without fatty acids). Similarly, 100 μM C18:3 increased E2 half-life ($P < 0.05$) compared with no fatty acid (21.3 vs. 27.8 min; 30.3% inhibition) or other fatty acids at 100 μM . Thirty micromoles C18:3 did not affect E2 half-life compared with no fatty acid ($P > 0.05$) but was different from 30 μM C16:0. Nevertheless, the E2 half-life was greater with 300 μM C18:3 than with 30 or 100 μM C18:3 ($P < 0.05$). The fatty acid C16:1 increased the half-life of E2 when used at the higher concentrations (100 and 300 μM) as compared with controls and 30 μM ($P < 0.003$). The fatty acid C16:0 also increased E2 half-life at the 300 μM concentration. All other concentrations of C16:0 did not affect E2 metabolism in these in vitro conditions ($P > 0.05$).

One hundred micromoles of C18:3 had a greater half-life (27.8 $\pm$ 1.9 min) than 100 μM C16:0 (22.4 $\pm$ 0.9 min), C16:1 (24.6 $\pm$ 1.4 min), or C18:1 (23.9 $\pm$ 2.5 min). There was an effect of 300 μM fatty acids on E2 half-life ($P < 0.0001$), with greater inhibition of metabolism by C18:3 compared with the other fatty acids.

Progesterone Metabolism: Experiment 2. Results from the second experiment that directly compared steroid metabolism by liver slices incubated with C18:0 with those incubated with C18:3 are shown in Table 3. There was a greater effect of C18:3 on P4

Table 2. Estradiol half-life (min; least squares means $\pm$ SEM) during incubation of liver slices ($n = 4$ different cows, each done in triplicate incubations) with differing concentrations of C16:0, C16:1, C18:1, or C18:3 fatty acids

Item	C16:0	C16:1	C18:1	C18:3	<i>P</i> -value
0 μM	21.3 $\pm$ 1.6 ^x	21.3 $\pm$ 1.6 ^x	21.3 $\pm$ 1.6	21.3 $\pm$ 1.6 ^x	—
30 μM	19.7 $\pm$ 1.7 ^{a,x}	21.8 $\pm$ 1.1 ^{ab,x}	23.8 $\pm$ 2.3 ^b	23.2 $\pm$ 1.1 ^{b,x}	0.032
100 μM	22.4 $\pm$ 0.9 ^{a,x}	24.6 $\pm$ 1.4 ^{a,y}	23.9 $\pm$ 2.5 ^a	27.8 $\pm$ 1.9 ^{b,y}	0.005
300 μM	25.7 $\pm$ 2.3 ^{a,y}	26.4 $\pm$ 1.6 ^{a,y}	24.6 $\pm$ 1.7 ^a	34.3 $\pm$ 2.4 ^{b,z}	<0.0001
<i>P</i> -value	0.0013	0.003	0.136	<0.0001	—

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).

^{x-z}Means within a column with different superscripts differ ($P \leq 0.05$).

Table 3. Effect of C18:0 or C18:3 fatty acids on the half-life (min; least squares means $\pm$ SEM) of progesterone or estradiol

Item	Progesterone			Estradiol		
	C18:0	C18:3	P-value	C18:0	C18:3	P-value
0 μ M	18.2 $\pm$ 1.3	18.2 $\pm$ 1.3 ^a	—	24.0 $\pm$ 2.6	24.0 $\pm$ 2.6 ^a	—
30 μ M	19.9 $\pm$ 1.2	22.4 $\pm$ 2.6 ^b	0.154	25.2 $\pm$ 2.9	29.2 $\pm$ 2.9 ^{ab}	0.226
100 μ M	20.7 $\pm$ 1.3	25.4 $\pm$ 3.2 ^{bc}	0.011	28.5 $\pm$ 3.5	33.0 $\pm$ 2.8 ^b	0.164
300 μ M	21.8 $\pm$ 1.7	27.3 $\pm$ 3.2 ^c	0.003	28.7 $\pm$ 4.3	39.9 $\pm$ 5.0 ^c	0.002
P-value	0.208	<0.0001		0.385	0.0003	

^{a-c}Means within a column with different superscripts differ ($P < 0.05$).

metabolism than C18:0 ($P < 0.0001$). Treatment ($P = 0.0009$) and concentration ($P < 0.0001$) effects were significant, whereas the treatment by dose interaction was not significant ($P = 0.123$). There was a greater P4 half-life with 100 and 300 μ M C18:3 than controls ($P < 0.05$). The 100 and 300 μ M C18:3 also increased P4 half-life compared with C18:0 at 100 or 300 μ M ($P = 0.003$).

Estradiol Metabolism: Experiment 2. There was an overall effect of C18:3 ($P = 0.0003$) but not C18:0 on E2 metabolism (Table 3). Similar to the results found with P4 incubations, there was an effect of treatment ($P = 0.005$) and dose ($P = 0.0007$) but no treatment by dose interaction ($P = 0.12$). Treatment with 300 μ M C18:3 produced a greater E2 half-life compared with no fatty acid or with 30 or 100 μ M C18:3 ($P < 0.05$). However, 30 μ M C18:3 was not different from controls or 100 μ M C18:3. In addition, there was a longer E2 half-life in the presence of 300 μ M C18:3 than 300 μ M C18:0 ($P = 0.002$).

Discussion of In Vitro Experiments. Previous studies (Hirunpanich et al., 2006, 2007; Yao et al., 2006) also found that PUFA had inhibitory effects on a variety of cyp enzymes but little, if any, effect of saturated fatty acids on cyp activity (Hirunpanich et al., 2006; Yao et al., 2006). To our knowledge, this is the first report of direct effects of fatty acids on P4 and E2 metabolism in vitro in any species.

The mechanism(s) by which C18:3 inhibited P4 and E2 metabolism could not be determined from our experimental design. It seems unlikely that new transcription or translation of enzymes was involved, given the short incubations (30 min) in our experiments and the effect of PUFA on activity of recombinant cyp enzymes in previous studies. Competitive inhibition at specific cyp seems likely given the overlap in cyp activity for metabolizing steroid hormones and fatty acids (particularly PUFA) and previous studies of cyp-associated drug–drug interactions (Rifkind et al., 1995; Yamazaki and Shimada, 1999; Yao et al., 2006). For instance, Yao et al. (2006) found that a variety of PUFA but not saturated fatty acids inhibited the activity of recombi-

nant cyp1A2, cyp2C9, and cyp3A4. All 5 PUFA that were examined in that study caused almost complete inhibition of cyp3A4 activity at 100 μ M concentrations and of cyp1A2 activity at 200 μ M. These cyp enzymes are also involved in E2 (Lee et al., 2003; Zhu and Lee, 2005) and P4 (Yamazaki and Shimada, 1997) metabolism. Similarly, Hirunpanich et al. (2007) showed a lower ratio of formation of 6 β -hydroxy testosterone when rat liver microsomes, rich in metabolizing enzymes, were incubated with PUFA. Fatty acids are also high-affinity substrates for glucuronidation (Little et al., 2002, 2004), the key conjugation process for detoxification of many compounds including steroids (Zhu et al., 1996). In addition, hepatic steroid metabolizing enzymes (cyp, 5 α -reductase) require the cofactor NADPH for activity (Swinney, 1990), and NADPH-dependent reactions may be rate-limiting for these enzymes (Bjorkhem, 1972). Fatty acids can alter lipid and glucose metabolism in bovine hepatocytes (Mashek and Grummer, 2003) and reduce NADPH in rats fed n-3 PUFA (Nace and Szepesi, 1976; Ikeda et al., 1998), and these changes could alter P4 and E2 metabolism. Thus, the interaction between PUFA and steroid metabolism could occur at multiple enzymatic locations and through multiple mechanisms. The most critical and rate-limiting of these interactions in inhibition of P4 and E2 metabolism by PUFA remains to be determined in future research.

In Vivo Study

Dietary unsaturated fatty acids normally undergo extensive bacterial biohydrogenation in the rumen and consequently may not reach the circulation (Demeyer and Doreau, 1999). Therefore, in these experiments, fat was supplemented directly into the abomasum by using a cannulation system that bypassed the rumen (Drackley et al., 1992). Linseed oil was chosen as the supplemented fat because of its high C18:3 content (56% C18:3; 9.2% saturated fats).

After initiation of infusion, concentrations of P4 increased rapidly, reaching about 2.0 ng/mL in both treatment groups (Figure 1). Infusion of fat was initi-

Figure 1. Progesterone concentration (ng/mL; lines) and metabolic clearance rate (L/h; bars) during experiment 3. There were 3 steroid infusion periods: 9, 4, and 4 h. The x-axis corresponds to time (h) from the start of the intraabomasal infusions (0 h) with either saline (control; light gray bar and line) or linseed oil (black bar and line). Metabolic clearance rate values are mean $\pm$ SEM of the 2 last sample collections during each individual period.

ated at 3 h after the start of steroid infusion and there were no acute changes in P4 concentration during the infusion. The second steroid infusion began at 12 h after initiation of fat infusion, and P4 concentrations in both groups reached approximately 2.5 ng/mL by 2 h after infusion. There were again no effects of fat infusion on P4 concentrations. At 24 h, a third steroid infusion was performed. Steady-state concentration was achieved by about 2 h after this infusion and, again, there was no effect of time or treatment on P4 concentrations or MCR ($P > 0.05$).

Estradiol reached steady state after the first hour of intravenous infusion with serum concentrations at 205.5 ± 36.3 pg/mL for controls and 197.9 ± 16.5 pg/mL for linseed oil-treated cows at the time fat infusion began (Figure 2). There were no differences caused by fat infusion in E2 concentrations at any time point during the 3 steroid infusions. There was also no effect of fat on E2 MCR during any of the 3 steroid infusions (Figure 2).

Plasma NEFA concentrations were 90.3 ± 6.8 and 108.0 ± 8.0 μ M (μ Eq/L) for the control and the linseed-infused group, respectively ($P = 0.19$). As expected, there were changes in percentage of specific fatty acids in plasma because of linseed oil infusion (Table 4). There was an increase ($P = 0.004$) of 45.8% in C18:3n3

(4.8 ± 0.4 to $7.0 \pm 0.3\%$). There was also a tendency ($P = 0.082$) for an increase in the C18:3 *trans* isomer and for decreases in C18:1 ($P = 0.065$), C18:0 ($P = 0.084$), and C16:1 ($P = 0.092$) in cows infused with linseed oil (Table 4). There were no detectable effects of linseed oil infusion on other fatty acids (Table 4).

Despite dramatic and acute inhibitory effects of C18:3 on E2 and P4 metabolism by liver slices *in vitro*, there was no detectable effect of intraabomasal infusion of fat on steroid metabolism *in vivo*. Fat infusion *in vivo* was effective in changing plasma concentration of C18:3n3, an expected change in fat composition because of linseed oil infusion. Nevertheless, we were not able to replicate the *in vitro* inhibition of P4 and E2 metabolism during the *in vivo* infusion of a total of 490 mL of linseed oil into the abomasum during a 28-h period.

There are multiple potential reasons for the discrepancy between the *in vitro* and *in vivo* results. The most obvious is that the circulating concentrations of C18:3 during linseed oil infusion may not have achieved sufficient concentrations to inhibit the key enzymes involved in steroid metabolism. Inhibition of P4 or E2 metabolism *in vitro* required concentrations of 100 to 300 μ M, whereas circulating PUFA concentrations were much lower *in vivo*. Although total plasma NEFA was approximately 100 μ M in this experiment, the C18:3

Table 4. Percentage of plasma fatty acids in cows treated with saline (control; n = 4) or linseed oil (n = 4)

Fatty acid (%)	Control	Linseed oil	P-value
C12:0	0.3 ± 0.1	0.2 ± 0.1	0.383
C14:0	1.0 ± 0.2	0.7 ± 0.1	0.296
C14:1	0.3 ± 0.0	0.3 ± 0.0	0.391
C15	0.5 ± 0.0	0.4 ± 0.0	0.391
C16	10.2 ± 0.3	9.1 ± 0.3	0.107
C16:1	1.3 ± 0.0	1.2 ± 0.1	0.092
C18	14.8 ± 0.5	14 ± 0.6	0.084
C18:1 isomer	7.7 ± 0.3	7.2 ± 0.4	0.065
C18:2 isomer	0.4 ± 0.0	0.4 ± 0.0	0.423
C18:2n6	38.4 ± 0.9	39.5 ± 1.2	0.508
C18:3n6	0.5 ± 0.1	0.5 ± 0.2	0.718
C18:3n3	4.8 ± 0.2	7.0 ± 0.2	0.004
C18:3 <i>trans</i>	0.2 ± 0.0	0.3 ± 0.00	0.082
C20:1	1.8 ± 0.1	1.5 ± 0.1	0.155
C20:2	0.2 ± 0.0	0.1 ± 0.0	0.423
C20:3n3	1.7 ± 0.1	1.5 ± 0.1	0.27
C20:3n6	2.1 ± 0.2	2.0 ± 0.2	0.353
C20:4n6	1.9 ± 0.1	1.8 ± 0.1	0.391
C20:5n3	0.3 ± 0.0	0.3 ± 0.0	1.000
C22	1.4 ± 0.1	1.2 ± 0.1	0.161
C22:5n3	0.6 ± 0.1	0.6 ± 0.0	0.391
CLA ¹	0.3 ± 0.1	0.2 ± 0.0	0.444
Others	9.5 ± 0.4	9.8 ± 0.8	0.635

¹CLA = conjugated linoleic acid.

concentrations never exceeded 8% of fatty acids. Thus, circulating C18:3 concentrations were less than 8 μ M, clearly lower than what is required for inhibition of steroid metabolism. Our level of linseed oil infusion was selected to be near the recommended dose used in

previous studies to avoid decreased DMI (Rindsig and Schultz, 1974; Drackley et al., 1992; Oldick et al., 1997). Longer term treatment with fat may produce more substantial changes in circulating PUFA and, potentially, steroid hormone metabolism. For example, Hawkins et

Figure 2. Estradiol concentration (pg/mL; lines) and metabolic clearance rate (L/h; bars) during experiment 3. There were 3 steroid infusion periods: 9, 4, and 4 h. The x-axis corresponds to time (h) from the start of the intraabomasal infusions (0 h) with either saline (control; light gray bar and line) or linseed oil (black bar and line). Metabolic clearance rate values are mean $\pm$ SEM of the 2 last sample collections during each individual period.

al. (1995) observed a fat-induced decrease in clearance of P4 following removal of the ovary containing the corpus luteum. However, in that experiment, calcium soaps of long-chain fatty acids were supplemented for 112 to 113 d before ovariectomy.

The duration, dose, and timing (during cycle, before parturition, and so on) to achieve improved reproduction because of fat supplementation is not known. All of the previous studies have supplemented fat for at least 30 d (see review by Funston, 2004). For example, Robinson et al. (2002) demonstrated that the effect of C18:3 was detected only during the second cycle after the first postpartum estrus, with the most dramatic effects observed during the fourth cycle. They observed that preovulatory E2 concentration was greater and the E2 peak was higher in cows fed a diet supplemented with C18:3 compared with those fed an isoenergetic control ration. This study reported a negative effect of PUFA supplementation on circulating P4 in contrast with what might be expected from our in vitro results and results of Hawkins et al. (1995). Similar to our results, Sklan and Tinsky (1993) observed no effect of fatty acids on circulating concentration of steroids, specifically E2. Nevertheless, a higher E2 concentration during the estrous cycle was observed by Lammoglia et al. (1997) after supplementation of rice bran, rich in C18:3, in beef cows. Culture of granulosa cells from these supplemented cows had similar production of E2 (Lammoglia et al., 1997) compared with controls, suggesting that inhibition of E2 metabolism, rather than greater follicular steroidogenesis, may be responsible for the increased circulating E2.

One further consideration in evaluating our in vivo experiment is that a saline control was chosen rather than isoenergetic controls such as glucose or tallow. We were concerned about potential confounding from isoenergetic controls. For instance, glucose infusion would have increased insulin concentrations, which can have dramatic effects on cyp enzymes (Smith et al., 2006; Lemley et al., 2008). There is also evidence that tallow can alter steroid metabolism (Hawkins et al., 1995). Therefore, we selected a saline control for this study; however, isoenergetic controls may be more appropriate for future studies.

In conclusion, supplementation with PUFA could potentially alter metabolism of E2 or P4 by liver cells. However, the concentrations of PUFA that are needed to produce this inhibition seem to be fairly high, at least as analyzed by acute, direct effects of PUFA on E2 and P4 half-life during liver slice incubations. Supplementation with linseed oil, as observed in this study, can change circulating amounts of PUFA in the plasma but was still ineffective in acutely inhibiting E2

or P4 metabolism in vivo. Thus, it remains to be shown whether normal dietary supplementation strategies in dairy cattle can achieve sufficient change in PUFA levels to alter in vivo steroid metabolism.

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