High Feed Intake Increases Liver Blood Flow and Metabolism of Progesterone and Estradiol-17 β in Dairy Cattle

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ABSTRACT

Increased liver blood flow (LBF) resulting from elevated feed intake in lactating dairy cows may increase steroid metabolism. Continuous infusion of bromosulphthalein (BSP; specifically metabolized in liver) was used to measure LBF. Similarly, progesterone (P_4) and estradiol-17 β (E_2) were administered by continuous infusion. Circulating concentrations at steady state were used to calculate the metabolic clearance rate (MCR) of BSP, P_4 , and E_2 . Experiment 1: Variation in LBF was determined in three nonlactating and four lactating cows over 3 d at 3 to 5 h after feeding. Coefficients of variation ranged from 14 to 31% among cows within day and from 4 to 8% within cows across days. Experiment 2: Six nonlactating cows were used in a 3 $\times$ 3 Latin-square design with three feed regimens: no feed, 0.5 maintenance diet (M), and 1.5 M. Experiment 3: Eight lactating cows were used in a 4 $\times$ 4 Latin-square design with four feed regimens: no feed, 0.5 M, 1.5 M, and 2.2 M. In experiments 2 and 3, LBF and MCR of P_4 increased immediately after feed consumption and increases persisted longer at higher intakes. The LBF reached a maximum at 2 h after feeding and MCR of P_4 reached a maximum at 3 h after feeding with a positive correlation ($r = 0.92$) between LBF and MCR for P_4 . Experiment 4: A crossover design was used to determine MCR of E_2 in unfed or full-fed lactating dairy cows. The MCR of E_2 increased immediately after feeding and stayed elevated throughout the 4.5-h infusion period. Thus, LBF and steroid metabolism were acutely elevated by feed consumption in lactating and nonlactating cows. Higher rates of LBF and steroid metabolism in lactating than in nonlactating cows may indicate chronic effects of higher feed intakes as well.

(**Key words:** fertility, steroid metabolism, liver, dairy cattle)

Abbreviation key: BSP = bromosulphthalein, E_2 = estradiol-17 β , LBF = liver blood flow, M = maintenance

diet, MCR = metabolic clearance rate, P_4 = progesterone, PAH = para-aminohippurate, PCV = packed cell volumes.

INTRODUCTION

Milk production per cow has increased dramatically in the last two decades primarily due to improved genetics for milk production coupled with changes in nutritional management. However, a decline in reproductive efficiency in high producing dairy cows has also been noted (Faust et al., 1988; Darwash et al., 1997; Butler, 1998; Washburn et al., 2002). There are probably multiple reasons for the decline in reproductive efficiency, including differences in reproductive management and changes in the physiology of the lactating dairy cow. One feature of the high-producing lactating dairy cow is high dry matter intake (DMI). There is a close correlation ($r = 0.88$) between DMI and milk production in lactating dairy cows (Harrison et al., 1990). Detrimental effects of DMI on reproductive efficiency have been reported. In sheep, an elevation in DMI during early pregnancy increases the incidence of embryonic loss (Parr et al., 1987). In beef heifers, an increase in feed intake before AI decreases embryonic survival (Dunne et al., 1999). In a study with Meishan pigs, higher feed intake increased embryo survival at day 12 (Ashworth et al., 1999). In contrast, a statistical study summarizing 55 trials with swine found that high levels of feed after breeding led to an increase in embryonic mortality (den Hartog and van Kempen, 1980).

The decrease in reproductive efficiency in animals on a high plane of nutrition may be due to alterations in circulating hormone concentrations. There is an inverse relationship between level of feed intake and peripheral plasma progesterone (P_4) concentration in sheep (Williams and Cumming, 1982; Parr et al., 1993) and pigs (Prime and Symonds, 1993; Miller et al., 1999). Increasing DMI led to an increase in metabolic clearance rate (MCR) of P_4 (Parr et al., 1993; Miller et al., 1999). Similarly, increasing plane of nutrition in ovariectomized gilts increased MCR of P_4 and also increased blood flow to the hepatic portal vein by a similar percentage (Prime and Symonds, 1993). In grazing dairy cattle treated with an exogenous progesterone source

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(CIDR), progesterone concentrations were lower in cows fed ad libitum than feed-restricted cows (Rabiee et al., 2001a). However, CIDR-treated dairy cows with different levels of milk yield did not differ in circulating progesterone; although, mass of progesterone delivered from the CIDR was greater in the higher than the lower producing cows (Rabiee et al., 2001b). In sheep, Burrin et al. (1989) reported that increased feed intake led to increased liver blood flow (LBF) and liver oxygen consumption. In addition, sows with greater feed intake had greater blood flow in the hepatic portal vein (Symonds and Prime, 1989). Given that the liver is the major site of P_4 and estradiol-17 β (E_2) metabolism (Bedford et al., 1973; Parr et al., 1993; Freetly and Ferrell, 1994), it is logical that increases in DMI would increase MCR of these steroids due to an elevation in LBF.

Thus, we hypothesized that increased LBF as a result of elevated feed intake in lactating dairy cows will increase metabolism of P_4 and E_2 . We further hypothesized that there would be both an acute component, observed soon after meal introduction, and a chronic component, due to the persistently elevated feed intake of high producing dairy cows. To test these hypotheses we measured steroid metabolism by continuous infusion of P_4 and E_2 . We calculated the MCR for these compounds under various physiological conditions using the steady state circulating concentrations during these continuous infusions. Furthermore, we estimated LBF using the MCR of bromosulphthalein (BSP), a compound that has been previously used for this purpose in other species (Clarkson et al., 1976; Araya and Ford, 1982; West, 1995).

MATERIALS AND METHODS

General Animal Procedures

Ovarian function was synchronized using an intravaginal P_4 releasing device (Easi-breed CIDR, containing 1.9 g P_4 ; InterAg; Hamilton, New Zealand) given to each cow for 10 to 12 d before the infusion. At 48 h before the infusion, cows were treated with prostaglandin (PG) $F_{2\alpha}$ (Lutalyse; 25 mg; Pharmacia Animal Health, Peapack, NJ) followed by a second 25 mg PGF $_{2\beta}$ injection and removal of the CIDR 12 h later. At 24 h before the infusion, all follicles greater than 6 mm were aspirated and catheters inserted into both jugular veins (Bodensteiner et al., 1996; Bergfelt et al., 1997). Body temperature was monitored before and after infusion each day. Cows were eliminated from the experiments if body temperature was greater than 40°C for more than 24 h.

The steroids used for infusions were purchased from Steraloids Inc. (Newport, RI) and were first dissolved in 99.8% absolute ethanol. While stirring, this solution

was added to physiological saline (0.9% w/v sodium chloride). Bromosulphthalein powder was dissolved in normal saline solution to a final concentration of 3 mg/ml. In order to facilitate the dissolution of BSP, the solution was warmed to 40°C. Infusions were delivered using a peristaltic pump (Masterflex L/S; Cole Palmer, Vernon Hills, IL). Based on preliminary experiments, silicone tubing (Masterflex L/S 13; internal diameter 0.8 mm) had minimal P_4 or E_2 binding and could be used to deliver a constant 10 ml/min infusion rate without affecting the concentration of either steroid. At 12 h before each infusion, all feed was removed from each cow.

Liver blood flow measurement. Liver blood flow was measured by continuous infusion of BSP. The BSP dye was removed almost entirely by the liver and then excreted in the bile (Tietz, 1976; Price and Alberti, 1979). Serum BSP concentration was evaluated by the methods adapted from Clarkson (1961). One ml of serum was thoroughly mixed with 3 ml of 0.5 N ammonium hydroxide and evaluated on a spectrophotometer (Beckman DU 640) at wavelengths of 580 and 426 nm. Native BSP provides maximum absorbance at 580 nm, but this value must be corrected for any hemolysis or yellow pigmentation in the serum of each cow using the formula:

$$\begin{aligned} &\text{Corrected BSP reading at 580 nm} \\ &= [(\text{Value at 580 nm}) - (0.21 \times \text{Value at 426 nm})] \\ &\quad - 'A'. \end{aligned}$$

Where 'A' is calculated for the serum from each cow on each experimental day before infusion, using the formula:

$$\begin{aligned} 'A' &= \text{Serum reading at 580 nm before BSP infusion} \\ &\quad - (0.21 \times \text{serum reading at 426 nm}). \end{aligned}$$

The value 0.21 is a constant that must be determined for each particular spectrophotometer by comparison of the relative absorbance values of bovine hemoglobin in 0.5 N ammonium hydroxide at wavelengths of 580 and 426 nm. A standard curve of BSP in bovine serum (range of BSP concentrations from 0.39 to 50 $\mu\text{g/ml}$) was produced and used to calculate the BSP concentration in each serum sample based on the corrected BSP reading at 580 nm.

Liver blood flow (LBF) was calculated from the MCR of BSP at steady state using the following formulas and the assumption that 80% of LBF is cleared of BSP (Araya and Ford, 1982):

$$\text{MCR of BSP (L/h)} = \frac{\text{Infusion rate } (\mu\text{g/h})}{\text{BSP concentration } (\mu\text{g/l}) \text{ in jugular serum}}$$

$$\text{LBF (l/h)} = \frac{\text{MCR of BSP (l/h)} / 0.80}{1 - \% \text{ Hematocrit}}$$

Metabolic clearance rate for progesterone and estradiol-17 β . The continuous infusion of P₄ and/or E₂ allowed calculation of MCR based on the circulating concentrations of these steroids at steady state, a 40% clearance rate of P₄ across the head (difference between carotid artery and jugular vein; Parr et al., 1993), minimal clearance of E₂ across the head (Longcope et al., 1981), and the following formulas:

$$\text{MCR for P}_4 \text{ (l/h)} = \frac{\text{Infusion rate } (\mu\text{g/h})}{\text{P}_4 \text{ concentration } (\mu\text{g/l}) \text{ in jugular serum} / 0.60}$$

$$\text{MCR for E}_2 \text{ (l/h)} = \frac{\text{Infusion rate } (\mu\text{g/h})}{\text{E}_2 \text{ concentration } (\mu\text{g/l}) \text{ in jugular serum}}$$

Hormone analyses. Serum concentrations of P₄ and E₂ were determined by a competitive enzyme linked immunosorbent assay (ELISA) as described previously (Rasmussen et al., 1996). 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). The quality control sample was evaluated multiple times in each assay and the intra- and inter-assay coefficients of variation (CV) were 8% and 7% for P₄ and 2% and 4% for E₂.

Experiment 1: Repeatability of Measurements of LBF and Steroid Clearance

To determine reproducibility of measurements, LBF and steroid clearance were measured on 3 consecutive d in nonlactating, nonpregnant Holstein cows (n = 3; BW $\pm$ SEM = 686 $\pm$ 17 kg) and lactating Holstein cows (n = 4; BW $\pm$ SEM = 688 $\pm$ 47 kg; milk yield = 42.0 $\pm$ 1.8 kg/d). Nonlactating cows were fed at a maintenance level (M; 7.5 kg DM/d) with the lactating cow diet (19% CP, 1.72 Mcal NE_L/kg DM) for 3 weeks before infusions. At the start of each experimental day, all cows were given the lactating cow ration, nonlactating cows were fed at maintenance level and lactating cows were fed ad libitum. At 3 h after feeding, cows were infused at 10 ml/min with saline containing BSP (3 mg/ml, w/v), P₄ (20 μ g/ml, w/v), and E₂ (1 μ g/ml, w/v); final infusion contained 30% ethanol by volume. Cows were infused continuously for 2 h, and blood samples were taken before, at 1 h, and at 2 h after the start of infusion. Packed cell volumes (PCV) were determined from samples taken before infusion and at the end of infusion. After centrifugation of blood at 3000 $\times$ g for 20 min, serum samples were analyzed for BSP, P₄, and E₂ concentrations.

Experiment 2: Effects of DMI on LBF and P₄ Metabolism in Nonlactating Cows

Nonlactating Holstein cows (n = 6; BW = 681 $\pm$ 38 kg) were fed at maintenance with the lactating cow diet for 2 wk before the experiment. The lactating cow diet contained 55% alfalfa silage, 14.5% corn silage, 21% medium ground corn grain, 5.5% roasted soybeans, 1.1% corn gluten meal, and 2.25% soybean meal with balanced vitamin and mineral (CP = 19%; ME = 1.72 Mcal NE_L/kg DM). Cows were randomly allocated into three treatment groups in a 3 $\times$ 3 Latin-square design. The three treatments were unfed, fed at 0.5 times maintenance 0.5 M (3.54 kg of DM), or fed at 1.5 M times maintenance (10.62 kg DM). Follicles were aspirated and corpora lutea regressed as described above. Catheters were placed into both jugular veins 1 d before infusion started and were maintained throughout the experiment. Interim experiments indicated that BSP might decrease steroid metabolism (Sangsritavong and Wiltbank, unpublished). Therefore, BSP and P₄ infusions were done on different days with a 1 d interval between infusions to avoid any potential interference of BSP on P₄ metabolism. Infusions were delivered at 10 ml/min using silicone tubing (BSP = 3 mg/ml, w/v; P₄ = 20 μ g/ml, w/v, 1% ethanol). Infusions started 1 h before feeding and continued for another 4 h after feeding. Blood samples (2 samples at 30-s intervals each hour) were taken before feeding and 1, 2, 3, and 4 h after feeding throughout the infusion period. After centrifugation of blood at 3000 $\times$ g for 20 min, serum samples were kept at -20°C until analyzed for P₄ and BSP.

Experiment 3: Effects of DMI on LBF and P₄ Metabolism in Lactating Cows

Lactating cows (n = 8; BW = 645 $\pm$ 18 kg; milk production = 38.9 $\pm$ 1.3 kg/d) were allocated into a 4 $\times$ 4 Latin-square design with cows either: unfed, fed 0.5 M (3.54 kg DM), fed 1.5 M (10.62 kg DM), or fed 2.5 M (17.70 kg DM). Diet was the same as used in experiment 2. Two weeks before the experiment, the animals were trained into a limited-time feeding program. Feed was only available from 8:00 am to 4:00 pm, during the training period. Follicle aspiration was repeatedly performed every 4 d throughout the infusion period to avoid ovulation. To avoid health problems, catheters were kept for only 8 d. After removal of catheters, cows received another CIDR to prevent ovulation and were kept on the limited-time feeding program throughout a 2-wk resting period. Catheters were again inserted in the jugular veins, follicles aspirated, and infusions continued for another 8 d, similar to the initial infusion period. During this second period, one cow developed

an infection and was dropped from the experiment. Infusions were prepared as described in experiment 2. The concentration of BSP was 3 mg/ml (w/v), and P_4 was 20 μ g/ml (w/v) in physiological saline with 1% ethanol by volume. Infusions were delivered at 10 ml/min using silicone tubing. The BSP and P_4 were infused on different days in order to avoid interference of BSP on P_4 metabolism. Because serum concentration of BSP reached steady state by 40 min, BSP infusion started 1 h before feeding and continued for another 4 h after feeding. To assure that P_4 was at steady state, 100 mg of P_4 in 5 ml absolute ethanol was given as a bolus into the jugular vein at the start of the infusion followed by 3 h of infusion before feeding. The infusion continued for another 4 h after feed was offered (unfed, 0.5 M, 1.5 M or 2.5 M). Blood samples were taken hourly (2 samples at 30-s intervals at each hour) throughout the infusion period. After centrifugation of blood at $3000 \times g$ for 20 min, serum samples were kept at -20°C until analyzed for P_4 and BSP.

Experiment 4: Effect of DMI on Estradiol-17 β Metabolism in Lactating Cows

Lactating cows ($n = 3$; BW = 670 ± 19 ; milk production = 38.7 ± 3.7 kg/d) were randomly allocated into two treatment groups with a crossover experimental design. They were either unfed or fed ad libitum and the diet used in this experiment was the same diet as experiment 2. One d before infusion, catheters were placed into both jugular veins and all follicles were aspirated. Follicle aspiration was repeatedly performed in all cows every other day to minimize endogenous E_2 . For infusion, E_2 was dissolved in absolute ethanol before adding into physiological saline, to make a final concentration of 3 μ g/ml (w/v) with 1% ethanol by volume. Infusion was delivered at 10 ml/min with silicone tubing. To assure that E_2 had reached steady state before treatments were applied, 10 mg of E_2 in 5 ml absolute ethanol was injected as a bolus into the jugular vein followed by a 3 h infusion before feeding. Infusion continued for another 4.5 h after treatments were applied (fed or unfed). Blood samples were taken hourly (2 samples at 30-s intervals at each hour) throughout the infusion period with an added sample taken at 4.5 h after feeding. After centrifugation of blood at $3000 \times g$ for 20 min, serum samples were kept at -20°C until analyzed for E_2 .

Statistical Analyses

In experiment 1, the concentration of circulating steroids and LBF were analyzed using Proc Mixed in SAS with autoregressive-1 (ar-1) as the covariate structure for repeated measurements, using the model:

$$Y_{icd} = \mu + \alpha_i + C_{c(i)} + D_d + \varepsilon_{icd}$$

α = treatment (nonlactating cows, lactating cows),
 C = cow (1,2,3) for nonlactating cows and (1,2,3,4) for lactating cows,
 D = day of sampling (1,2,3),
 ε_{icd} = random residual effect.

In both experiments 2 and 3, LBF and circulating concentrations and MCR of P_4 were analyzed using Proc Mixed in SAS (ar-1) with the model:

$$Y_{scit} = \mu + S_s + C_{c(s)} + \alpha_i + \tau_t + \beta(\chi_{sci}) + \alpha\tau_{it} + \tau\beta_{t(\chi_{sci})} + \alpha\tau\beta_{it(\chi_{sci})} + \varepsilon_{scit}$$

S = square (1,2,3) in experiment 2 and (1,2,3,4) in experiment 3,
 C = cow (1,2,3,...,6) in experiment 2 and (1,2,3,...,8) in experiment 3,
 α = treatment (1,2,3) in experiment 2 and (1,2,3,4) in experiment 3,
 τ = time of sampling (1,2,3,4,5),
 β = basal values before treatments,
 $\chi_{sci} = Y_{scit}$ at $t = 1$, and
 ε_{scit} = random residual effect.

In experiment 4, circulating concentrations and MCR of E_2 were analyzed using Proc Mixed in SAS (ar-1) with the model:

$$Y_{pcit} = \mu + P_p + C_{c(p)} + \alpha_i + \tau_t + \beta(\chi_{pci}) + \alpha\tau_{it} + \varepsilon_{i(\chi_{pci})} + \varepsilon_{pcit}$$

P = period (1,2),
 C = cow (1,2,3),
 α = treatment (1,2),
 τ = time of sampling (1,2,3,4,5,6),
 β = basal values before treatments,
 $\chi_{pci} = Y_{pcit}$ at $t = 1$, and
 ε_{pcit} = random residual effect.

In all experiments, the time that steady state was achieved was determined by analyzing the data in the control (unfed) group using Proc Mixed in SAS (ar-1). Steady state was defined as the points that serum BSP and steroid concentrations were constant until the end of the infusion period.

RESULTS

Experiment 1

Table 1 summarizes the results for LBF in lactating and nonlactating cows. Liver blood flow was relatively constant within an individual cow (average CV = 0.06) when measured on 3 consecutive days. However, there

Table 1. Liver blood flow in lactating and nonlactating cows measured by continuous infusion of BSP at 3 to 5 h after feeding on 3 consecutive days (experiment 1).

Liver blood flow (liters/hour)					
Lactating cows					
Cow. No.	Day 1	Day 2	Day 3	Average	CV ²
3989	997	1014	917	976	0.05
4075	1278	1338	1420	1345	0.05
4180	1227	1243	1317	1262	0.04
4202	1195	1038	1215	1149	0.05
CV ¹	0.14	0.14	0.20		
			LSM ± SEM *1183 ± 90.8		
Nonlactating cows					
Cow No.					
4431	908	1004	1007	971	0.07
4223	751	785	700	745	0.06
4146	535	522	603	554	0.08
CV ¹	0.26	0.31	0.31		
			LSM ± SEM 757 ± 104.8		

¹CV between animals within day.²CV within the animal between days.*LBF in lactating cows were greater ($P = 0.02$) than in nonlactating cows.

was substantial variation in LBF between animals (average CV = 0.22). Lactating cows had greater ($P = 0.02$) LBF than nonlactating cows (1183 vs. 757 l/h). Lactating cows had lower circulating P₄ (2.43 vs. 3.53 ng/ml, $P = 0.009$) and E₂ (265 vs. 351 pg/ml, $P < .0001$) as compared to nonlactating cows, even though infusion rates for steroids were identical (Tables 2 and 3).

Experiment 2

Before the start of the experiment, the nonlactating cows received the lactating cow diet at a maintenance level (~7 kg DM/d), and they consumed all of this feed within 1 h. During the experiment, cows offered 0.5

M (3.54 kg DM) consumed all feed by 0.5 h. The cows offered 1.5 M (10.62 kg DM) consumed 74% of their feed by 2 h, and all feed was consumed by 4 h. Table 4 summarizes the results for LBF in nonlactating cows after consumption of differing amounts of diet. There was no significant change in LBF in unfed cows during the 4 h infusion period. The LBF increased in the first hour in cows fed diets with either 0.5 or 1.5 times maintenance requirements for energy. In cows fed 0.5 M, LBF was maximal by 2 h after feeding, and then decreased back toward basal levels. In cows fed 1.5 M, LBF was greatest at 4 h after feeding and was significantly greater than for unfed cows or cows fed 0.5 M.

Table 2. Serum P₄ concentrations in lactating and nonlactating cows during continuous infusion of P₄ at 3 to 5 h after feeding (experiment 1).

Serum progesterone concentrations (ng/ml)					
Lactating cows					
Cow No.	Day 1	Day 2	Day 3	Average	CV ²
3989	2.98	2.60	2.77	2.78	0.06
4075	2.18	2.40	2.53	2.37	0.07
4180	2.43	2.39	2.39	2.40	0.01
4202	1.97	2.15	2.32	2.15	0.08
CV ¹	0.20	0.08	0.08		
			LSM ± SEM 2.43 ± 0.17		
Nonlactating cows					
Cow No.					
4431	3.18	3.17	2.96	3.10	0.04
4223	3.59	3.87	3.30	3.59	0.07
4146	4.22	3.96	3.69	3.96	0.06
CV ¹	0.14	0.12	0.11		
			LSM ± SEM *3.53 ± 0.20		

¹CV between animals within day.²CV within the animal between days.*P₄ concentrations in nonlactating cows were greater ($P = 0.009$) than in lactating cows.

Table 3. Serum E₂ concentrations in lactating and nonlactating cows during continuous infusion of E₂ at 3 to 5 h after feeding (experiment 1).

Serum estradiol-17 β concentrations (pg/ml)					
Lactating cows					
Cow No.	Day 1	Day 2	Day 3	Average	CV ²
3989	283	249	244	259	0.08
4075	296	246	239	260	0.11
4180	292	254	298	271	0.08
4202	265	277	253	265	0.04
CV ¹	0.05	0.05	0.10		
				LSM $\pm$ SEM	265.44 $\pm$ 4.99
Nonlactating cows					
Cow No.					
4413	351	347	318	339	0.05
4223	369	337	379	362	0.06
4146	366	345	356	356	0.03
CV ¹	0.02	0.01	0.09		
				LSM $\pm$ SEM	*351.19 $\pm$ 5.77

¹CV between animals within day.²CV within the animal between days.*E₂ concentrations in nonlactating cows were greater ($P < 0.0001$) than in lactating cows.

Serum P₄ concentrations were low in all cows before infusion (0.15 ± 0.01 , 0.20 ± 0.02 , and 0.18 ± 0.02 in unfed, 0.5 M, and 1.5 M, respectively). Infusion of P₄ in the absence of BSP infusion was found to not reach steady state as rapidly as was observed in our first experiment. Serum P₄ concentrations continued to increase from 0 h to 2 h in cows that were unfed, and therefore we concluded that P₄ concentrations had not achieved steady state until 3 h. All calculations are based on an assumption of steady state, and therefore only values at 3 and 4 h after feeding are summarized in Table 5. Nonlactating cows that were unfed had significantly greater serum P₄ concentrations than cows given feed 3 or 4 h previously at either 0.5 M or 1.5 M. Serum P₄ concentrations in 0.5 M and 1.5 M were similar at 3 h after feeding. At 4 h after feeding, the cows given 0.5 M had significantly greater serum concentration of P₄ than cows given 1.5 M. The MCR

of P₄ was greater in fed (0.5 M or 1.5 M) compared to unfed cows. Cows given 1.5 M had significantly greater MCR for P₄ than cows given 0.5 M at 4 h after feeding.

Experiment 3

After being trained in the limited-time feeding program, cows had a rapid rate of feed consumption. The trained cows consumed 12.2 ± 0.6 kg DM during the first 2 h after the feed had been offered compared to 7.8 ± 1.3 kg DM for untrained cows ($n = 7$; BW = 687 ± 23 kg; milk production = 37.9 ± 2.1 kg/d). At 4 h after feed was offered, total feed intake in trained cows was 15.3 ± 0.5 kg DM vs. 10.1 ± 1.2 kg DM for untrained

Table 4. Least-squares means (LSM) of effects of dry matter intake on liver blood flow in nonlactating cows (experiment 2).

Hour(s) after feeding	LSM Liver Blood Flow (liters/h)		
	Unfed	0.5 M (3.54 kg DM)	1.5 M (10.62 kg DM)
0	747	753	757
1	729 ^a	978 ^{ab}	1085 ^{ab}
2	727 ^a	1059 ^{ab}	1127 ^{ab}
3	746 ^a	1032 ^{ab}	1148 ^{ab}
4	743 ^a	939 ^{ab}	1207 ^{ac}
Pooled SE	47	47	47

Within treatment ^{a,b,c} indicates differences from time zero.* $P < 0.01$.** $P < 0.0001$.^{a,b,c}Indicate differences ($P < .05$) within the row.**Table 5.** Least-squares means (LSM) for effects of dry matter intake on serum concentrations and metabolic clearance rates (MCR) of P₄ in nonlactating cows (experiment 2).

Hour(s) after feeding	Unfed	0.5 M (3.54 kg DM)	1.5 M (10.62 kg DM)
LSM Serum P ₄ Conc. (ng/ml)			
3	4.11 ^a	3.12 ^b	2.64 ^b
4	4.14 ^a	3.33 ^{ab}	2.70 ^c
Pooled SE	0.23	0.23	0.23
LSM MCR for P ₄ (liters/h)			
3	1776 ^a	2414 ^b	2807 ^b
4	1779 ^a	2225 ^{ab}	2704 ^c
Pooled SE	147	147	147
LSM MCR for P ₄ (liters/min/kg BW ^{0.75})			
3	.224 ^a	.303 ^b	.352 ^b
4	.224 ^a	.280 ^{ab}	.341 ^c
Pooled SE	.019	.019	.019

Within treatment ^{a,b} indicates differences ($P < 0.05$) within the same column.^{a,b}Indicate differences ($P < .05$) within the row.

Table 6. Least-squares means (LSM) for effects of dry matter intake on liver blood flow in lactating cows (experiment 3).

Hour(s) after feeding	LSM Liver Blood Flow (liters/h)			
	Unfed	0.5 M (3.54 kg DM)	1.5 M (10.62 kg DM)	2.2 M (15.2 kg DM)
0	1561	1577	1572	1521
1	1574 ^a	1816 ^{***b}	1897 ^{***b}	1937 ^{***b}
2	1527 ^a	1765 ^{ab}	1871 ^{***bc}	2017 ^{***c}
3	1583 ^a	1711 ^{***a}	1835 ^{***ab}	1967 ^{***b}
4	1547 ^a	1685 ^{ab}	1817 ^{***b}	1984 ^{***c}
Pooled SE	57	60	56	62

Within the same treatment ^{***} indicates differences from time zero.

^{*} $P < 0.05$.

^{**} $P < 0.0001$.

^{a,b,c}Indicate differences ($P < .05$) within the row.

cows. On the days that cows received only 0.5 M, the feed had been consumed by 15 min after being offered. Cows receiving 1.5 M finished their feed within 2 h. However, cows that received 2.5 M failed to consume all of the feed within the 4-h period. Orts were weighted at the end of each infusion; DMI was calculated, and the actual consumption was found to average 2.2 times maintenance in the group designated as 2.5 M.

Lactating cows (experiment 3) had much greater LBF than nonlactating (experiment 2) cows (1569 vs. 751 L/h average at 0 h). Unfed cows had no change in LBF during the 4-h period, whereas, all fed cows had an increase in LBF by 1 h after provision of feed (Table 6). Cows fed 0.5 M increased LBF abruptly after feeding; LBF reached a maximum at 1 h, and it declined back to values similar to unfed cows at 3 and 4 h after feeding. Similar to the nonlactating cows, the higher levels of DMI caused a more prolonged elevation in LBF, with greater LBF in both the 1.5 M and 2.2 M groups than in unfed cows (Table 6). Despite numerical differences, there were no significant differences in LBF between 1.5 M and 2.2 M groups during the first 3 h after feeding. However, LBF in the 2.2 M group was greater at 4 h.

In general, DMI decreased serum P_4 concentration (Table 7). Cows receiving any amount of feed had lower serum P_4 concentrations than unfed cows between 1 h and 4 h after feeding. Serum P_4 concentrations were not significantly different between the 1.5 M and 2.2 M groups throughout the infusion period. The decrease in serum P_4 concentrations was greater in the 2.2 M cows than the 0.5 M cows at 4 h after feeding. The MCR for P_4 showed similar patterns to the serum P_4 concentrations (Table 7). There was a high correlation ($r = 0.92$) between LBF and MCR for P_4 from both nonlactating (experiment 2) and lactating (experiment 3) cows, but no significant differences in the slope (P

$= 0.74$) and intercept ($P = 0.53$) between these two experiments. Figure 1 shows the correlation between LBF and MCR for P_4 from the combined data.

Experiment 4

Serum concentrations of E_2 were relatively constant in unfed cows throughout the infusion period. When cows were fed, serum E_2 concentrations decreased by 1 h after feeding and stayed depressed during the 4.5-h infusion period. The MCR for E_2 was increased immediately after feeding and remained elevated for the 4.5 h after feeding (Table 8).

DISCUSSION

Bromosulphthalein has been used to study liver function as well as to measure LBF in many species, mostly by means of a single injection (Center et al., 1983a, 1983b; Clarkson, 1961; Clarkson and Richards, 1967; West, 1995). This study is the first that we have found in which continuous infusion of BSP was used as a tool to measure LBF in dairy cattle. This methodology was highly repeatable on multiple days in an individual cow. Our values for LBF of ~1000 to 1200 l/h in fed nonlactating cows and ~1600 to 2000 l/h in fed lactating cows were similar to results using the more technically challenging method of measuring dilution of para-aminohippurate (**PAH**) in the liver vasculature (Lomax and Baird, 1983; Reynolds et al., 1988; Reynolds et al., 2000b; Weighart et al., 1986). The values in our first experiment may be lower than in other experiments, possibly due to nonspecific inhibition of liver cytochrome P-450 enzymes by the high concentration of ethanol (30%) in the infusion solution (Reinke et al., 1980). Previous experiments using PAH dilution have been limited in the numbers of cows that were examined (Lomax and Baird, 1983, 7 cows;

Table 7. Least-squares means (LSM) for effects of dry matter intake on serum concentrations and MCR of P₄ in lactating cows (experiment 3).

Hour(s) after feeding	Unfed	0.5 M (3.54 kg DM)	1.5 M (10.62 kg DM)	2.2 M (15.2 kg DM)
LSM serum P ₄ conc. (ng/ml)				
0	2.59	2.60	2.58	2.59
1	2.69 ^a	2.37 ^{*b}	2.40 ^b	2.32 ^{*b}
2	2.76 ^a	2.25 ^{**b}	2.02 ^{***b}	2.19 ^{**b}
3	2.58 ^a	2.35 ^{*a}	1.95 ^{***b}	1.96 ^{***b}
4	2.59 ^a	2.37 ^{*a}	2.18 ^{**ab}	2.01 ^{***b}
Pooled SE	0.09	0.10	0.09	.010
LSM MCR of P ₄ (liters/hour)				
0	2881	2830	2838	2836
1	2753 ^a	3226 ^{ab}	3025 ^{ab}	3138 ^b
2	2669 ^a	3295 ^{ab}	3717 ^{***c}	3324 ^{***b}
3	2843 ^a	3178 ^{ab}	3801 ^{***b}	3771 ^{***b}
4	2804 ^a	3066 ^a	3409 ^{**ab}	3692 ^{**b}
Pooled SE	135	144	135	144
LSM MCR of P ₄ (liters/min/kg BW ^{0.75})				
0	0.374	0.366	0.369	0.367
1	0.357 ^a	0.416 ^{*b}	0.392 ^{ab}	0.406 ^b
2	0.345 ^a	0.422 ^{*b}	0.483 ^{***c}	0.429 ^{**b}
3	0.368 ^a	0.408 ^{*a}	0.493 ^{***b}	0.489 ^{***b}
4	0.364 ^a	0.396 ^a	0.443 ^{**ab}	0.477 ^{***b}
Pooled SE	.017	.019	.017	.019

Within treatment "*" indicates differences from time zero.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.0001$.

^{a,b,c}Indicate differences ($P < .05$) within the row.

Reynolds et al., 1988, 4 cows; Weighart et al., 1986, 5 cows; Reynolds et al., 2000b, 4 cows) due to technical difficulties in cannulating and maintaining cannulas in both the mesenteric vein and hepatic vein in cows. In other species (sheep and dogs), the BSP procedure has been directly compared to the PAH procedure and mean LBF by BSP was $95 \pm 4\%$ of LBF measured by PAH dilution (Katz and Bergman, 1969). Thus, the BSP procedure should allow monitoring of changes in LBF in cows under a variety of physiological conditions.

The acute elevation of LBF after meal consumption has generally been acknowledged in most species, including dairy cattle (Lomax and Baird, 1983). In our study, LBF increased immediately after feeding and reached a maximum at 2 h in both lactating and non-lactating cows. Huntington (1990) compared the results from 16 studies in which cattle of various sizes and physiological conditions had hepatic portal blood flow measured. The author found a highly significant ($r^2 = 0.98$) linear relationship between LBF and the intake of metabolizable energy. Teleologically, an increase in LBF in response to increased metabolizable energy intake would serve to aid in transport of digested nutrients from the gut through the liver and on to the rest of the body (Huntington, 1990). In sheep,

the initial increase in blood flow to the salivary glands and smooth muscle of the ruminoreticulum occurred so quickly after feeding (30 to 60 s) that it was thought to be due to a neural mechanism (Barnes et al., 1983). The subsequent increase in ruminoreticular blood flow peaked at 2 to 4 h and was tentatively ascribed to changes in chemical stimulation resulting from fermentation (Barnes et al., 1983; Barnes et al., 1984; Sellers et al., 1964). In experiments 2 and 3, we found that feeding caused a similar acute increase (1 to 2 h) in LBF regardless of the amount of feed that was consumed. In addition, feeding greater quantities of feed prolonged the elevation of LBF, perhaps due to greater duration and/or quantity of ruminal fermentation. However, it is interesting to note that the absolute increase in LBF in response to feeding (+300 to 450 l/h) was similar in lactating and nonlactating cows, although the percentage increase was much greater in nonlactating (40 to 60%) than lactating cows (20 to 30%). The lower percentage increase was due to the greater basal LBF in lactating than nonlactating cows (1570 vs. 750 l/h).

The high basal LBF in lactating cows is probably due to chronic effects of a high plane of nutrition. Following parturition and initiation of lactation, there is a significant increase in the splanchnic tissue mass (Reyn-

Figure 1. Correlation of least-squares means (LSM) of liver blood flow (LBF) ($\text{l/min/kg BW}^{0.75}$) and LSM of metabolic clearance rates (MCR) for P_4 ($\text{l/min/kg BW}^{0.75}$), for nonlactating (□, experiment 2) and lactating (○, experiment 3) cows. The regression equation for the line is $Y = 1.38X + 0.10$; $r = 0.92$.

olds et al., 2000a) concomitant with an increase in LBF (Reynolds et al., 2000b). This is probably due to both hypertrophy and hyperplasia of the liver and the organs of the gastrointestinal tract in response to the increase in nutrient intake (Miller et al., 1999; Sainz and Bentley, 1996). The greater LBF is probably primarily due to greater blood flow from the splanchnic organs into the hepatic portal vein, because the hepatic artery appears to contribute only 20% or less of the total LBF in ruminants (Barnes et al., 1983). The finding that lactating cows have a basal LBF that is about double that found in nonlactating cows has important physiological implications for digestive physiology and, of particular interest in this manuscript, implications for reproductive physiology.

The methodology used to measure MCR of P_4 and E_2 was based on the circulating steroid concentration at steady state during continuous infusion of steroid. This allows a simple calculation of the MCR with no need for the more complex modeling equations that are required to accurately determine the half-life of a hormone after a single bolus injection. We chose to use continuous infusion of steroids rather than delivery from a CIDR or implant because these devices can have some variation in mass of progesterone delivered due to differences in milk yield (Rabiee et al., 2001b)

Table 8. Least-squares means (LSM) for effects of dry matter intake on E_2 metabolism (experiment 4).

Hour(s) after feeding	Unfed	Full fed
LSM E_2 conc. (pg/ml)		
0	1071	1064
1	1108 ^a	914 ^{*b}
2	1049 ^a	750 ^{*b}
3	1192 ^a	740 ^{*b}
4	1052 ^a	747 ^{*b}
4.5	1135 ^a	736 ^{*b}
Pooled SE	59	59
LSM MCR of E_2 (liters/hour)		
0	1781	1774
1	1772	2071
2	1902 ^a	2632 ^{*b}
3	1637 ^a	2717 ^{*b}
4	1861 ^a	2742 ^{*b}
4.5	1712 ^a	2736 ^{*b}
Pooled SE	214	214
LSM MCR of E_2 (liters/min/kg $\text{BW}^{0.75}$)		
0	0.225	0.224
1	0.224	0.262
2	0.240 ^a	0.333 ^{*b}
3	0.207 ^a	0.343 ^{*b}
4	0.235 ^a	0.347 ^{*b}
4.5	0.216 ^a	0.346 ^{*b}
Pooled SE	0.026	0.026

Within treatment “*” indicates differences from time zero.

* $P < 0.05$.

** $P < 0.0001$.

^{a,b}Indicate differences ($P < .05$) within the row.

or type of feed (Rabiee et al., 1999). We also chose to not use ovariectomized animals because the liver enzymes involved in steroid metabolism may decrease when steroids are absent (MCR for $\text{P}_4 = 0.20 \text{ l/min/kg BW}^{0.75}$ in intact nonpregnant ewes; Bedford et al., 1972 and $0.08 \text{ L/min/kg BW}^{0.75}$ in ovariectomized ewes; Freetly and Ferrell, 1994). We attempted to eliminate endogenous P_4 and E_2 by regressing the corpus luteum and aspirating all follicles larger than 6 mm. This strategy was successful based on the low circulating P_4 and E_2 concentrations found before each infusion. However, one potential technical problem in our study is that sampling was done from the jugular vein rather than from the artery. We used a P_4 extraction rate of 40% between carotid artery and jugular vein based on previous literature (Parr et al., 1993).

The acute and chronic changes in MCR of P_4 appeared closely related to differences in LBF as previously reported in sheep and gilts (Parr et al., 1993; Miller et al., 1999). In gilts, Prime and Symonds (1993) reported that increased feed intake from 1 to 3 kg/d resulted in an increased portal blood flow by 45% and MCR of P_4 increased by 47%; similar results were also reported by Symonds and Prime (1989). In our studies, LBF increased by 57% after feeding in nonlactating

cows and MCR of P_4 increased by 55% (unfed vs. 1.5 M). Similarly, in lactating cows, LBF increased by 24%, and MCR of P_4 increased by 28% (unfed vs. 2.2 M). Our study cannot rule out the possibility that changes in MCR of P_4 may have been the results of changes in hepatic mixed-function oxidation enzymes or supply of NADPH, both critical aspects of steroid metabolism that have been reported to be regulated (Jung and Brand, 1975; Thomas et al., 1987). If all P_4 in blood passing through the liver was metabolized, then this would account for 57% and 42% of the total MCR of P_4 in lactating and nonlactating cows, respectively. This is comparable to the 45 to 53% in nonlactating ewes reported by Bedford et al. (1972). Therefore, a considerable amount of P_4 is metabolized by extra hepatic tissue, namely kidney, brain, ovary, and adrenal (Bedford et al., 1973; Bedford et al., 1974; Parr et al., 1993).

In contrast to the MCR of P_4 , metabolism of E_2 appeared to primarily occur in the splanchnic region. In this study, if all E_2 in the LBF is metabolized, this would account for 88% in fasted and 71% in well-fed lactating cows of the total MCR of E_2 . This is similar to previously reported values in ewes (82%; Freetly and Ferrell, 1994) and rhesus monkeys (80%; Longcope et al., 1981). The observed MCR of E_2 per metabolic weight (fasted vs. well-fed; 0.23 vs. 0.36 l/min/kg $BW^{0.75}$) are similar to values that can be calculated from previous data in gilts (0.11 to 0.24 l/min/kg $BW^{0.75}$; Christenson et al., 1985) or female rhesus monkeys (0.13 to 0.45 l/min/kg $BW^{0.75}$; Hotchkiss, 1983).

A decrease in circulating steroids could dramatically alter reproduction in high producing lactating dairy cows because circulating steroids are involved in almost every aspect of reproductive physiology. For example, the decrease in circulating P_4 due to elevated steroid metabolism could be partially responsible for the reduction in fertility (Lucy, 2001; Washburn et al., 2002) or the increased pregnancy loss (Vasconcelos et al., 1997) in lactating dairy cows. Low circulating P_4 either before or after (Ahmad et al., 1996; Folman et al., 1973; Fonseca et al., 1983; Larson et al., 1997; Mann et al., 1995) AI was associated with reduced fertility. Supplementation of P_4 may partially reduce these problems (Folman et al., 1990; Lynch et al., 1999; Wehrman et al., 1993). In addition, we previously speculated that increased steroid metabolism could be the explanation for the relationship between double ovulation rate and milk production in lactating cows (Wiltbank et al., 2000).

In conclusion, there is an acute increase in MCR of both P_4 and E_2 in response to feeding, and this appears to be related to acute changes in LBF. In lactating cows, a continuous high plane of nutrition appears to

chronically elevate LBF and the MCR of P_4 and E_2 . These results may have practical reproductive implications such that a similar production level of P_4 or E_2 may result in much lower circulating steroid concentrations in lactating dairy cows. This may be related to many of the changes in reproductive measures that have been observed in lactating dairy cows such as reduced fertility, reduced expression of estrus, and increased double ovulation.

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