Dietary Supplementation of High Levels of Saturated and Monounsaturated Fatty Acids to Ewes during Late Gestation Reduces Thermogenesis in Newborn Lambs by Depressing Fatty Acid Oxidation in Perirenal Brown Adipose Tissue

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Abstract
We hypothesized that dietary supplementation of (n-6) plus (n-3) PUFA during late gestation would increase uncoupling protein-1 (UCP1) gene expression and thereby increase thermogenic capacity of newborn lambs. Thirty twin-bearing ewes were fed rumen-protected fat (2, 4, or 8%) high in saturated and monounsaturated fatty acids (SMFA) or high in (n-6) and (n-3) PUFA. Lambs (n = 7–10 per ewe treatment group) were placed in a cold chamber at 0°C for 2 h. Rectal temperature was higher at birth and increased more with cold exposure in lambs from ewes fed 2 or 4% supplemental fat than in lambs from ewes fed 8% SMFA (fat type x fat level interaction, P = 0.001). Cytochrome c oxidase activity was greatest in brown adipose tissue (BAT) lambs from ewes fed 2% SMFA or 4% PUFA (fat type x fat level interaction, P = 0.01). BAT of lambs from ewes fed 2 or 4% PUFA had nearly 7-fold more (P = 0.05) UCP1 mRNA than BAT of lambs from ewes fed 8% PUFA. UCP1 expression decreased by over 80% by 24 h of age. Supplementation of 8% fat tended to depress palmitate esterification into lipids (P = 0.07) and decreased palmitate oxidation (P = 0.003) in lamb BAT in vitro, especially in those lambs from ewes fed 8% SMFA. Thus, supplementing the diets of ewes with 8% SMFA depressed cold tolerance in newborn lambs, which was reflected in their decreased ability to oxidize fatty acids in vitro. J. Nutr. 137: 43–48, 2007.

Introduction
Weather-related losses account for ~40% of total lamb deaths in the US. (1). To avoid hypothermia during cold stress, newborn lambs must be able to increase thermogenic rates 3- to 4-fold. Approximately one-half of this heat is generated from brown adipose tissue (BAT) nonshivering thermogenesis in newborn lambs (2). Supplementation of PUFA to ruminants has been shown to modify fatty acid composition of adipose tissues (3) and increase serum and muscle lipid concentrations (4). Moreover, PUFA supplementation to rodents in investigations of diet-induced thermogenesis was shown to promote BAT thermogenesis via increased norepinephrine turnover rates, O2 consumption, and activity of cytochrome c oxidase (COX) activity (5–8). Feeding rats a PUFA-supplemented diet increased adenylyl cyclase response to β-adrenergic agonists, affinity of the adrenoreceptors in perirenal adipose tissue, triiodothyronine secretion, uncoupling protein-1 (UCP1) gene expression, and GDP-binding activity (7–11).

Although a number of studies have investigated the effects of source and level of (n-3) and (n-6) PUFA on BAT thermogenesis in rodents (5–11), little research has been conducted to examine the effects of prenatal fat supplementation on BAT thermogenesis of newborn ruminant species. Lambs born to ewes fed 1.5× the metabolizable energy (ME) requirement during late gestation were heavier at birth and had increased BAT thermogenic capacity than lambs born to ewes fed 1 × ME requirement (12). Similarly, calves born to cows fed a diet containing 4.7 lipid/100 g diet enriched in 18:2(n-6) had higher rectal temperature responses to cold exposure than calves born to cows fed a diet containing 1.7 lipid/100 g diet (13). We hypothesized that supplementing the diets of gravid ewes with (n-3) plus (n-6) PUFA during late gestation would increase lamb BAT thermogenic capacity, thereby increasing rectal temperature response of the lambs to cold exposure.

Materials and Methods

Animals and diets. This research protocol was approved by the Texas A&M University Laboratory Animal Care Committee. Thirty ewes...
thought to be twin-bearing were allotted to 1 of 6 groups \((n = 5)\), beginning 40 ± 15 d prior to expected parturition. Ewes were assigned randomly to treatments in a 3 × 2 factorial arrangement with factors being level of rumen-protected fat (2, 4, or 8%), and type of rumen-protected fat, high in saturated and monounsaturated fatty acids (SMFA), or high in \((n-3)\) plus \((n-6)\) PUFA. Energy Booster 100 (Milk Specialties) was used as the source of rumen-protected SMFA in the study. Casein-formaldehyde protected flaxseed oil (Rumentek Industries Pty) was the source of PUFA. All diets were isonitrogenous (13.3%) and isocaloric (11.07 MJ/kg) (Table 1).

**Ewe treatments.** Ewes were fed individually in an open-sided barn. Food intake and body weight were measured at 7-d intervals. Blood samples were obtained by jugular venipuncture in heparinized vacutainer tubes between 0700 and 0800, before feeding, at d 0 and d 28. Ewes were monitored at 4-h intervals prior to expected lambing, and immediately following parturition, lambs were separated from ewes, dried of amniotic fluid, weighed, and moved to a warm chamber (25°C). Total lambs collected for the ewes supplemented with 2, 4, or 8% PUFA were 10, 9, and 9, respectively, and total lambs collected for the ewes supplemented with 2, 4, or 8% SMFA were 8, 8, and 7, respectively.

**Lamb treatments and sampling.** Lambs were fed pooled bovine colostrum (30 mL/kg of body wt) at 2 h of age and fed saline (60 mL) at 4 h of age. At 4 h of age, blood was collected by jugular venipuncture, lambs were placed in a cold chamber (0°C), and at 6 h of age. At 8, 14, and 20 h of age, lambs were fed 30 mL colostrum/kg body wt. Remaining lambs of each twin pair were returned to the cold chamber at 22 h of age and rectal temperature was measured for 2 h at 15-min intervals. Lambs were killed at 24 h of age and vital organs, including total perirenal BAT, were removed and weighed. Portions of the BAT were snap-frozen in liquid nitrogen and stored at −80°C, whereas other portions were used fresh for isolation of mitochondria and incubations in vitro.

**Fatty acid analysis.** Lipids were extracted from 1 mL of plasma from ewes and lambs and 100 mg of perirenal BAT by the method of Folch et al. (14). After methylation (15), the FAME were analyzed as described previously (16). Identities of FAME were established by comparison to authentic standards (GLC 96; Nu-Chek Prep). Individual FAME were quantified as mmol/L plasma or mmol/mL 100 g BAT.

**Isolation of mitochondria.** Mitochondria were isolated from fresh BAT by differential centrifugation as described by Cannon and Lindberg (17). Aliquots of homogenate and mitochondria preparations were frozen at −80°C for subsequent determination of COX activity (18) and protein (19). Total mitochondrial protein was determined based on mitochondrial recovery from preparations.

**GDP-binding assay.** Mitochondrial GDP-binding assays were performed according to Nizielski et al. (20). Freshly prepared mitochondria were incubated for 5 min in triplicate with a medium containing \([U-14C]sucrose (0.92 GBq/L), 0.115 \mu M [3H]GDP (9.17 GBq/L), in 2 \mu M unlabeled GDP. Scatchard analyses of GDP binding were performed using a pooled mitochondrial sample from each group with 0, 1, or 500 \mu M unlabeled GDP. A competition assay was conducted by adding 200 \mu M GDP to maximally displace \([3H]GDP from GDP binding sites to assess nonspecific binding.

**UCP1 gene expression.** Approximately 100 mg of snap-frozen perirenal BAT was weighed, covered with liquid nitrogen, and pulverized with mortar and pestle. Samples were treated with TRIReagent (Sigma Chemical) for RNA extraction according to manufacturer's instructions.

UCP1 gene expression was evaluated by quantitative real-time PCR, utilizing a fluorescent reporter and 5’ exonuclease assay system (TaqMan, PE Biosystems). Reverse transcription (RT) of total RNA and PCR amplification was performed using the TaqMan One-Step RT-PCR Master Reagents kit, TaqMan fluorescent probe, and sequence detection primers (PE Biosystems). A TaqMan probe specific for the target was designed to contain a fluorescent 5’ reporter dye and 3’ quencher dye. Each 1-step RT-PCR (20 \mu L) contained the following: 2 \times Master mix (10 \mu L), 40 \times MultiScribe and RNase Inhibitor mix (0.5 \mu L), target forward primer (900 nmol/L), target reverse primer (900 nmol/L), fluorescent labeled target probe (250 nmol/L) designed from the mRNA sequence, and total RNA (100 ng). Forward and reverse primers were 5’-GGT TGG GTG CTT GAC ATC AT-3’, 5’-CTG GCT CCT GTA GGC CCT TTG-3’, respectively. Probe sequence was 5’-6FAM-CCT GCC GCT TGG ACA CGG CCT-TAMRA-3’. PCR amplification was carried out in the ABI PRISM 7900 Sequence Detection System (PE Biosystems). Thermal cycling conditions were 48°C for 30 min, 95°C for 10 min, followed by 40 repetitive cycles of 95°C for 15 s and 60°C for 1 min. As a normalization control for RNA loading, parallel reactions in the same multi-well plate were performed using 18S ribosomal RNA as target (18S Ribosomal control kit, PE Biosystems). Reactions were made according to manufacturer’s instructions with 40 ng total RNA used in normalization reactions.

Quantification of gene amplification was made following RT-PCR by determining the threshold cycle \((C_T)\) number for fluorescent 5’ reporter dye fluorescence within the geometric region of the semilog plot generated during PCR. Within this region of the amplification curve, each difference of 1 cycle is equivalent to a doubling of the amplified product of the PCR.

Relative quantification of target gene expression across treatments was evaluated using the comparative \(C_T\) method (21,22). The \(\Delta C_T\) value was determined by subtracting ribosomal \(C_T\) value for each sample from target \(C_T\) value of that sample. Calculation of \(\Delta \Delta C_T\) involved using the highest sample \(\Delta C_T\) value (sample with the lowest target expression) as an arbitrary constant to subtract from all other \(\Delta C_T\) sample values. Fold changes in the relative gene expression of target were calculated as \(2^{-\Delta \Delta C_T}\).
Adipose tissue metabolism. Two-hour in vitro incubations were performed with fresh perirenal BAT samples (23), taken from lambs after cold exposure from 4-6 h of age. Flasks contained 0.75 mM/L sodium palmitate, 30 g/L bovine serum albumin and 3.06 GBq/L [1-14C]palmitate plus 3.06 GBq/L [3-3H]palmitate in Krebs-Henseleit bicarbonate buffer system. Flasks contained 0 or 10^-9 to 10^-6 mol/L norepinephrine plus 5 mmol/L theophylline (24). After 2-h incubations, CO2 was detected as described previously (25) and neutral lipids were extracted (23), completely evaporated, resuspended in 10 mL of scintillation cocktail (Biosafe II, Research Products International), and radioactivity counted with the scintillation counter (LS8300, Beckman Instruments).

Cellularity. Samples of frozen BAT were sliced in 1-mm thick sections to facilitate tissue fixation (26,27). Fixed cells were used for cell size and number determination using a Coulter Counter, Model ZM equipped with a channelizer, Model 256 (Coulter Electronics).

Statistical analyses. Data from ewes and lambs were analyzed as a 3 (level of rumen-protected fat) × 2 (type of rumen-protected fat) factorial experiment design using the general linear model procedure of SAS (SAS Institute). With the exception of lamb BAT and plasma fatty acids, all lamb samples were obtained at 6 and 26 h of age, so age of lamb also was tested as a main effect. Post-hoc separation of least-squares means was accomplished using the pdiff statement in SAS. Lamb rectal temperature data were analyzed as a split-plot design with a factorial arrangement of treatments using the general linear model procedure of SAS. Replication was the time in the chamber, split-plot was level of fat, and whole-plot was the type of fat. Fat type, level of supplemental fat maintained at higher rectal temperature than lambs born to ewes fed 8% fat at 4 and 22 h postnatally (level of fat main effect, P = 0.001). Type of supplemental fat had no effect on rectal temperature, but there was a fat type × fat level interaction (P < 0.001). Lambs from ewes fed 8% SMFA exhibited a strong depression in cold-induced thermogenesis (Fig. 1).

Fatty acids. Ewes at d 0 had lower plasma concentrations of 18:2(n-6) than at d 28 (regardless of supplementary fat type) (Table 3). After 28 d, ewes fed diets supplemented with PUFA had greater plasma concentrations of 18:2(n-6), 18:3(n-3), and 20:5(n-3), but less 18:1(n-9) and 20:4(n-6) than ewes fed the SMFA-enriched diets. Plasma 18:3(n-3) increased as the level of dietary fat increased in PUFA-fed ewes but did not change in the SMFA-fed ewes.

The only fatty acid in lamb plasma that was affected by supplemental fat type or level was 18:1(n-9) (Table 3). The concentration of 18:1(n-9) increased from 0.65 to 0.83 mmol/L in plasma of lambs from ewes fed 2 to 8% SMFA and decreased from 0.65 to 0.52 mmol/L in lambs from ewes fed 2 to 8% PUFA (fat type × fat level interaction, P = 0.05). The plasma concentrations of 18:2(n-6) and 18:3(n-3) in lambs were <15% of the

Results

Ewes. There was no effect of type of dietary fat on final body weight, weight gain, food intake, or body condition score of ewes (Table 2). Ewes fed 8% added fat had lower weight gains than ewes fed 2 or 4% added fat. Ewes fed 8% PUFA had lower gain:feed ratios than ewes fed 2 or 4% PUFA. The gain:feed ratios for ewes fed the SMFA diets were not influenced by level of added fat.

Lambs. Feeding ewes 8% PUFA tended (fat type × fat level interaction P = 0.07) to reduce lamb body weight, but neither type nor level of supplemental fat affected perirenal BAT mass in lambs (Table 2). Lambs born to PUFA-fed ewes had lighter livers than lambs born to SMFA-fed ewes (19.7 vs. 22.3 g/kg body wt; P = 0.02; data not shown). Neither type nor level of prenatal fat supplementation influenced heart, spleen, kidney, or adrenal gland weights of newborn lambs (data not shown). Lambs killed at 24 h of age (after 2 cold exposures) had heavier spleen and kidney weights than lambs killed at 6 h of age (both P = 0.01), whereas BAT weights were 10% less (P = 0.03) in lambs killed at 24 h of age than in lambs killed at 6 h of age.

Rectal temperature of lambs increased in response to cold exposure at both 4 and 22 h of age, and rectal temperature reached a plateau by 15 min (Fig. 1). Because there was no difference in rectal temperature response to cold temperature between the 4- and 22-h sampling periods, data were pooled across postnatal ages. Lambs born to ewes fed 2 or 4% supplemental fat maintained at higher rectal temperature than lambs born to ewes fed 8% fat at 4 and 22 h postnatally (level of fat main effect, P = 0.001). Type of supplemental fat had no effect on rectal temperature, but there was a fat type × fat level interaction (P < 0.001). Lambs from ewes fed 8% SMFA exhibited a strong depression in cold-induced thermogenesis (Fig. 1).

TABLE 2 Growth and food intake of ewes and body and BAT weights of lambs born to ewes fed diets containing SMFA or PUFA supplements

<table>
<thead>
<tr>
<th>Group/item</th>
<th>SMFA</th>
<th>PUFA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewes, n</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Weight gain, kg/d</td>
<td>0.55</td>
<td>0.59</td>
<td>0.72</td>
</tr>
<tr>
<td>Food intake, kg/d</td>
<td>3.3</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Gain/feed</td>
<td>0.16 (n=6)</td>
<td>0.14 (n=6)</td>
<td>0.15 (n=6)</td>
</tr>
<tr>
<td>Lambs, n</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Final body wt, kg</td>
<td>4.9</td>
<td>4.2</td>
<td>4.7</td>
</tr>
<tr>
<td>BAT mass, g/kg body wt</td>
<td>4.2</td>
<td>4.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

1 Means in a row with superscripts without a common letter differ, P < 0.05.
2 There was an age effect on BAT mass (P = 0.03). BAT mass was greater at 6 h of age (4.0 g/kg body wt) than at 24 h of age (3.6 g/kg body wt).

Figure 1 Rectal temperatures of newborn lambs from ewes fed diets containing 2% SMFA (red triangles), 4% SMFA (green squares), 8% SMFA (black circles), 2% PUFA (blue diamonds), 4% PUFA (blue circles), and 8% PUFA (purple squares) were measured in a cold chamber (0°C) for 2 h. Values (means ± SEM) were pooled over time of measurement (4-6 and 22-24 h of age). Data were analyzed as a split-plot design with a factorial arrangement of treatments using the general linear model procedure of SAS. Replication was the time in the chamber, split-plot was level of fat, and whole-plot was the type of fat. Effect of type of prenatal fat (SMFA vs. PUFA) P-value = 0.19. Effect of level of prenatal fat P-value = 0.001. Time in chamber effect P-value = 0.001. Fat type × fat level interaction P-value = 0.001.

Maternal nutrition and brown adipose tissue thermogenesis 45
concentrations observed in their respective ewes. Conversely, plasma concentrations of 20:5(n-3) and 22:6(n-3) were similar between lambs and ewes.

Perirenal BAT from lambs born to PUFA-fed ewes had higher concentrations (P < 0.002) of 20:5(n-3) and 22:6(n-3) than BAT from lambs born to SMFA-fed ewes (Table 3). The concentration of 20:5(n-3) increased as the level of dietary fat increased in lambs born to PUFA-fed ewes but was not affected by level of supplemental fat in the lambs born to SMFA-fed ewes.

**TABLE 3 Concentrations of unsaturated acids of ewes and lambs born to ewes fed diets containing SMFA or PUFA supplements**

<table>
<thead>
<tr>
<th>Group/fatty acid</th>
<th>SMFA</th>
<th>PUFA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2%</td>
<td>4%</td>
<td>8%</td>
</tr>
<tr>
<td><strong>Ewe plasma, d 0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>0.65</td>
<td>0.65</td>
<td>0.05</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Lamb BAT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>73.9</td>
<td>73.9</td>
<td>0.02</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>2.11</td>
<td>2.11</td>
<td>2.11</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
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</table>

**Dietary effects.** Lammoglia et al. (13) fed cows isocaloric diets containing 1.7 (basal diet) or 4.7 g lipid/100 g diet (supplemented with safflower seed) and calves born to cows fed the supplemental safflower seed had higher rectal temperature response to cold stress than calves of cows fed the basal diet. This indicated that either the supplemental fat or the enrichment of the diet with 18:2(n-6) enhanced BAT thermogenesis in response to cold exposure. Early research by Nedergaard et al. (28) documented that feeding dams diets enriched with 18:2(n-6) increased BAT thermogenesis in rats. Similarly, BAT of rats fed 18:1(n-9), 18:2(n-6), or 18:3(n-3)-enriched diets had greater rates of β-oxidation and higher activities of carnitine palmitoyl transferase and COX activities than BAT from rats fed lard-enriched diets (6). Others (5,12) have demonstrated that 18:3(n-3) is even more potent than 18:2(n-6) is promoting BAT thermogenesis in rats. Whole body oxygen consumption in rats fed 18:1(n-9) or 18:2(n-6) was 2-fold higher than in rats fed lard but was 3-fold higher in rats fed 18:3(n-3) (5).

Therefore, we predicted that supplementing ewe diets with PUFA, and especially with 18:2(n-3), would increase newborn lamb thermogenesis, as assessed by rectal temperature response to cold exposure. Instead, we observed that thermogenesis was greatest in lambs of ewes fed either 2 or 4% supplemental fat.
lamb adipocyte volume, $\mu$m$^3$ 21.6 20.8 19.4 23.2 18.4 20.8 20.5 20.6 1.5 0.83 0.68 0.38 0.97

Adipocytes, n $\times 10^5/100$ mg BAT 1.93 1.85 1.47 2.02 1.71 1.79 1.86 1.77 0.32 0.33 0.55 0.75 0.96

1 Means for diet or age in a row with superscripts without a common letter differ, $P < 0.05$.
2 Lambs were exposed to cold at 4–6 h and 22–24 h of age.
3 Mean of UCP1 expression at 2% prenatal SMFA supplementation was used baseline for source and level effects, whereas UCP1 expression at 6 h was used as baseline for age effects.

### Age effects

Age effects. BAT mass decreased by 10% between 6 and 22 h of age, suggesting some lipid depletion during this period. This result is consistent with Wu et al. (32), who reported that lamb BAT mass was 25% less for 1-d-old lambs than for newborn lambs. We recently reported nearly complete delipidation of lamb brown adipocytes after exposure to 6°C for 48 h (33). Similarly, adipocyte volume decreased in BAT from Brahman calves exposed to 4°C for 48 h, although this did not occur in BAT from cold-exposed Angus calves (33). The lambs of the current study were only briefly exposed to the cold (2 2-h exposures at 0°C), which apparently was not sufficient to elicit a measurable decrease in adipocyte volume. However, BAT GDP-binding activity did increase between 2 and 22 h of age. Unmasking of GDP-binding sites occurs in response to acute cold stress (34). When rats are moved from 27°C to 4°C, unmasking of GDP-binding sites in BAT mitochondria more than doubled within 20 min (35). GDP is a potent inhibitor of H$^+$ transport, and as such antagonizes uncoupling protein activity (36). It is likely that the increase in GDP-binding activity that we observed, although modest (15%), similarly was in response to cold exposure.

Unlike GDP-binding activity, there was a marked decline in UCP1 gene expression by 24 h of age. UCP1 mRNA was nearly undetectable in perirenal BAT by 48 h age in newborn lambs held at 28°C and UCP1 gene expression was low even in lambs held at 6°C for 48 h (33). Similarly, there was a decline in UCP1 gene expression to nearly undetectable levels in BAT of newborn calves after 7 d of cold exposure (37). The decline in lamb BAT UCP1 gene expression, even after 2 periods of cold exposure, may have been related to the insensitivity of the BAT to noradrenaline. Stimulation of rat brown adipocytes with catecholamines or CAMP causes an acute elevation of UCP1 mRNA, with a half-life of 5 to 13 h (38,39). Klein et al. (40) previously reported that 10$^{-6}$ mol/L noradrenaline stimulated fatty acid oxidation in fetal and newborn ovine isolated brown adipocytes by over 3-fold. We demonstrated a doubling in palmitate oxidation and palmitate esterification into total lipids
at 10⁻⁶ mol/L norepinephrine in BAT of cold-exposed, newborn calves (33). However, we were unable to demonstrate any effect of norepinephrine on palmitate oxidation or esterification into lipids in lamb BAT in vitro in the current investigation, and UCPI gene expression also may have been refractory to sympathetic regulation in these lambs. Whether this was caused by the prenatal exposure to supplemental fat is not known.

We conclude that adding 2 or 4% SMFA or PUFA to the diets of ewes during late gestation did not depress and may have improved cold tolerance in newborn lambs. However, supplementing ewe diets with 8% SMFA caused a marked depression in rectal temperature in response to cold exposure. Similar depressions were observed in palmitate oxidation in BAT from lambs fed 8% SMFA, indicating high levels of SMFA depress the ability of BAT from their lambs to oxidize fatty acids. These effects were independent of COX activity, GDP binding or UCPI gene expression.

**Literature Cited**