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Nutritionally induced adipose hypertrophy in young pigs is transient and independent of changes in the expression of the *obese* and peroxisome proliferator activated receptor genes☆

M.E. Spurlock[a,*], C.A. Bidwell[a], K.L. Houseknecht[a], J.L. Kuske[a], C. Camacho-Rea[a], G.R. Frank[b], G.M. Willis[b]

[a]*Purdue University, West Lafayette, IN 47907-1151, USA*
[b]*Purina Mills, Inc., St. Louis, MO, USA*

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Abstract

Previous studies have shown that piglets weaned to a liquid milk replacer (MR), rather than a typical dry diet (DD) regimen, have improved growth rates and deposit more energy as body fat. In the present study, we used this model to determine whether changes in the expression of genes linked to the regulation of adiposity were related to the accelerated fat accretion. We also determined whether the increase in body fat was sustained throughout a substantial proportion of the growth curve. At weaning (19 ± 2 days of age), 96 piglets were placed in 12 replicate pens per diet (4 pigs per pen, 2 barrows and 2 gilts), and fed a liquid MR or conventional DD regimen for 5 weeks. Thereafter, 6 barrows and 6 gilts pigs from each diet were killed for determination of whole body chemical composition (less gastrointestinal contents). The remaining pigs were assigned randomly to weight target groups (60, 85, and 110 kg), placed in individual pens, and fed a conventional dietary regimen until killed at their respective weight targets for tissue sampling and determination of whole body chemical composition. Over the 5-week period in which the MR was fed, the growth rate of the pigs consuming the MR exceeded that of the pigs fed the DD by 36% ($P < .05$). Fat gain in these pigs was increased to 1.8 times that of the pigs fed the DD, and percentage body fat was 45% greater ($P < .05$). Acetyl Co-A carboxylase (ACC) activity (per mg of adipose extract protein) was not different between the two diet groups at the conclusion of the 5-week period, or at 110 kg body weight. During the MR period, actual protein gain was increased ($P < .05$) 22% in the pigs fed the MR as well. By 110 kg of body weight, body fat was reduced ($P < .05$) by 7.7% (total fat mass) and 8.3% (percentage of body weight basis) in the pigs fed MR vs. the DD group. The expression of the peroxisome proliferator activated receptors (PPAR) α and γ was not influenced by diet or by body weight. Expression of the *obese* gene was independent of diet, but was greater ($P < .09$) in pigs at 110 kg body weight than at 60 kg. These data provide additional evidence that piglets weaned to liquid diets have greater rates of growth and deposit more body fat, but that this difference subsides quickly when a typical dry dietary regimen is imposed. Furthermore, the biochemical changes responsible for the increased adiposity are independent of changes in the expression of the *obese* or PPAR genes, at least at the mRNA level.

Keywords: Pig; Adipose tissue; Body composition; Growth; *Obese* gene; Peroxisome proliferator activated receptor

1. Introduction

The weight gain of piglets weaned to a liquid MR diet is greater than that of piglets weaned to conventional dry diets [1–3]. The improved gain is achieved in part by an increase in the accretion of body fat [1,3]. The expression of several genes is presently of considerable interest relative to the accretion (or loss) of body fat. Leptin, the product of the *obese* gene, acts upon the adipocyte to regulate lipid metabolism. Expression of the *obese* gene is highly correlated with adiposity in the pig [4,5]. Leptin antagonizes insulin-stimulated glucose transport and lipogenesis [6], reduces acetyl Co-A carboxylase activity [7], and acts directly upon the adipocyte to stimulate lipolysis [8–11]. Additionally, the peroxisome proliferator activated receptors (PPARα and

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* Corresponding author. Tel.: +1-765-494-4808; fax: +1-765-494-9346.

E-mail address: spurloc0@purdue.edu (M. Spurlock).

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Table 1
Nursery diet compositions and chemical analysis[1]

	Milk replacer	Phase 1	Phase 2	Phase 3
Crude protein, %	27.6	25.8	26.5	25.6
Crude fat, %	14.3	8.6	6.6	6.2
Metabolizable energy, MJ/kg	17.5	17.0	15.8	15.9
Crude protein from plants:				
Soy, %	0.0	55.0	74.0	74.0
Other, %	0.0	68.0	93.0	100.0
Total animal and milk products, %	100.0	32.0	7.0	0.0

[1] Dry matter basis. The liquid mild replacer diet was fed for 5 weeks. For the piglets weaned to the dry diet regimen, Phase 1 was fed for 1 week, followed by Phases 2 and 3 for 2 weeks each.

PPARγ) are members of the nuclear hormone receptor superfamily and have been linked to myriad biological processes, including glucose homeostasis, adipocyte differentiation, and lipid metabolism [12]. The former PPAR regulates the expression of acyl Co-A oxidase, the rate-limiting enzyme for peroxisomal fatty acid oxidation. Of particular interest, Wang et al. [13] reported that leptin stimulates PPARα expression concomitantly with the induction of lipolysis and the stimulation of acyl Co-A oxidase. The lipolytic and anti-lipogenic activities of leptin reported thus far have spawned the hypothesis that leptin production by the adipocyte increases in response to feed intake and insulin to impose a limitation on additional lipid storage in "well-nourished" adipocytes [6]. Whereas PPARα is associated with lipid disposal, PPARγ has been linked to adipocyte differentiation and lipid filling [14,15] Although there is presently limited data pertaining to the metabolic regulation of the PPAR genes, the γ2 isoform is down regulated by feed deprivation [16], a metabolic circumstance in which lipid deposition in the adipocyte is attenuated. In the present study, we sought to establish the impact of prolonged feeding of a liquid MR on body composition, and to relate this effect to changes in the expression of genes linked to the control of adiposity.

2. Materials and methods

2.1. Experimental design and diets.

The experiment protocol was approved by the Animal Care and Use Committee, Purina Mills, Inc. Ninety-six weanling pigs (barrows and gilts, 19 $\pm$ 2 days of age, 6.95 $\pm$ .19 kg body weight) were allotted to two dietary treatment groups based on weight, sex and litter of origin. At weaning, the pigs were placed in 12 replicate pens per diet (4 pigs per pen, 2 barrows and 2 gilts), and allowed ad libitum intake of either a commercial-type dry starter diet or a liquid MR (Table 1) for 5 weeks. The milk replacer was diluted to 25% with water and fed by gravity from buckets suspended above the pens into cup-type drinkers mounted in each pen. Fresh dilutions were provided in the morning and late afternoon each day. The pigs fed the dry starter regimen were allowed ad libitum water consumption. At the end of the 5-week period, 12 pigs from each diet (1 pig per pen, 6 total of each sex) were killed for determination of whole body chemical composition. Of the remaining pigs, 36 per diet were placed in individual pens and fed a common commercial-type growing-finishing dietary regimen. The first 10 pigs (5 barrows and 5 gilts) per group to reach their pre-assigned target weights (60, 85, and 110 kg live weight, $\pm$ 2.5%) were killed for tissue sampling and determination of whole body chemical composition.

2.2. Chemical composition and tissue sampling

All pigs were killed by exsanguination following mechanical stunning. Immediately thereafter, samples (5–10 g) of adipose tissue were collected from the depot located over the cervical spine, cubed, and frozen in liquid nitrogen pending RNA extraction. The gastrointestinal tract was removed from the body cavity and cleaned of feed residue. Whole carcasses were then ground, mixed, and reground, and samples submitted to a commercial laboratory for determination of chemical composition (nitrogen, fat, and moisture [17]. The composition of gain in the experimental pigs was calculated relative to composition data obtained in a previous study performed with similar management procedures and genetic lines.

2.3. Total RNA isolation

Total RNA was extracted from the subcutaneous adipose tissue using the method reported by Chomczynski and Sacchi [18]. Tissue was homogenized in 4M guanidinium thiocyanate followed by addition of 0.1 volume of 2M sodium acetate (pH 5.0). The samples were extracted sequentially with water-saturated phenol and chloroform:isoamyl alcohol (24:1) and the aqueous fractions precipitated with isopropanol. After a second precipitation in ethanol, the RNA pellets were resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0) and analyzed by spectrophotometry for quantification (A_{260}) and qualitative (A_{260}:A_{280}) determinations. Additionally, the actual RNA concentration of sample was determined using the RiboGreen® assay (Molecular Probes, Eugene, OR) and the manufacturer's protocol.

2.4. Ribonuclease protection assays

Construction of the transcription plasmids for the *obese* and PPARγ mRNAs has been described previously [19,16]. Radiolabeling of the riboprobes was accomplished by in vitro transcription with T7 RNA polymerase in the presence of ^{32}P-UTP. The in vitro transcription and RNAse protection assays were performed using a commercially available kit (Maxiscript T7 + RPA II, Ambion, Austin, TX). The riboprobe for porcine PPARα was prepared from a clone

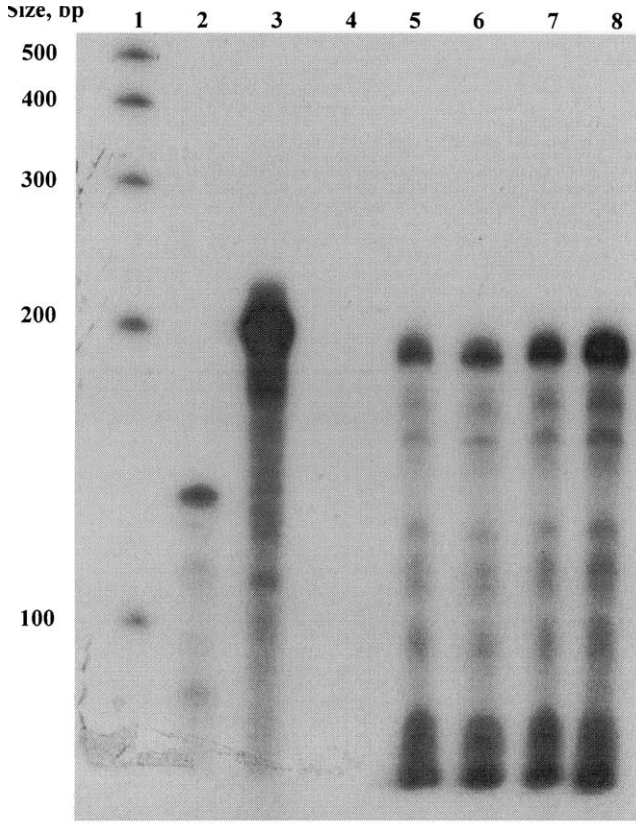

Fig. 1. Ribonuclease protection assay for PPARα in porcine adipose tissue. The riboprobe for this transcript was prepared from a clone provided by Dr. Harry Mersmann, USDA-ARS, Baylor College of Medicine, that has been described previously [20]. A protected fragment (180 bp) resulted when 10–30 μg total RNA were hybridized in solution with the riboprobe, subjected to ribonuclease treatment, and separated on polyacrylamide gels containing 8 M urea. Signal intensity increased with increasing quantities of total RNA, thus demonstrating the ability of the assay to establish relative differences in the abundance of the PPARα transcript in total RNA preparations. Lane Identification: (1)100 bp ladder, (2)18S probe; (3) PPARα probe; (4) digested (unprotected) 18S and PPARα probes, (5–8) protected fragments from hybridization of the 18S and PPARα probes with 10, 15, 20, and 30 μg total RNA, respectively.

provided by Dr. Harry Mersmann, USDA-ARS, Baylor College of Medicine, that has been described previously [20]. Briefly, primers were designed to amplify an approximate 180 bp fragment from the cloned insert. The resulting PCR product was sequenced to confirm its identity, and the T7 promoter ligated using the Lig'NScribe® kit (Ambion, Austin, TX) to allow transcription of the antisense riboprobe. An example nuclease protection assay is shown in Fig. 1. Ribosomal RNA (18S) was used as the internal marker. Twenty μg of total RNA were used for solution hybridization and X-ray film was exposed overnight (16–18 hr). Autoradiographs were quantified using an image analysis system and software purchased commercially (Interactive Technologies International [ITTI], St. Petersburg, FL). The abundance of the 18S rRNA was not influenced by diet or body weight. Accordingly, all data are presented as the ratio of the specific transcript signal to that of the 18S.

2.5. Acetyl Co-A carboxylase activity

Acetyl Co-A carboxylase activity was measured as described by Liu et al. [21] Briefly, 1 g of adipose tissue was homogenized at 4°C for 2 min in 3 volumes of 0.02 M sodium phosphate, pH 7.0, 0.15 M NaCl, and 1mM dithiothreitol. The fat was removed from the homogenate by centrifugation at 10,000 X g for 20 min. The infranatant fraction was immediately used for determinations of ACC activity. Duplicate 0.1 ml aliquots were preincubated 30 min at 37°C in 0.2 ml of buffer containing 1M Tris-HCL, 100 mM dithiothreitol, 100 mM potassium citrate, 1M $MgCl_2$, 5.0 mg BSA, and 1.7 ml H_2O. The reaction was initiated by addition of 0.1 ml of incubation media containing 100 mM ATP, 100 mM acetyl Co-A, and 200 mM Na-[H^{14}]CO_3 and allowed to continue for 10 min. The reaction was stopped with 0.1 ml of 4N HCl and then dried by airflow. The dried residue was dissolved in 5 ml scintillation cocktail for counting. The activity is expressed as nmol bicarbonate incorporated into malonyl Co-A per minute per milligram of protein. The protein concentration was determined using the bicinchoninic acid (BCA) kit obtained from Pierce Chemical Co., Rockford, IL. Bovine serum albumin was used as the standard.

2.6. Statistical analyses

Least squares means ± SEM are reported for all variables. The data collected at the completion of the 5-week period in which the MR was fed were analyzed as a randomized complete block design with terms for replicate and litter of origin included in the statistical model. For the subsequent growth portion of the study, the data were analyzed as a randomized complete block design as above, but with treatments arranged as a 2 X 3 factorial (2 nursery diets and 3 target weights). The treatment df were partitioned to test the main effects of diet and body weight, and their interaction. Means were separated based on the least significant differences procedure when protected by a significant F-test [22].

3. Results

The intake, growth performance, and body composition data are summarized in Table 2. Over the 5-week nursery period, the caloric intake of the pigs fed the MR exceeded that of the pigs fed the DD regimen by 71% ($P < .01$). However, intake of the standard growing-finishing diets following the nursery phase was not influenced by nursery diet. The growth rate of the pigs weaned to MR exceeded that of the pigs fed the DD by 36% ($P < .05$) over the 5-week nursery period. From the diet transition through the 85 kg weight target, the growth rate of the pigs fed the MR was lower than that of the conventional DD group, but the difference was insignificant by 110 kg (Diet X Body Weight, $P < .08$). The length of time

Table 2
Growth rates and composition of gain in pigs fed a liquid milk replacer (MR) or dry diet (DD) for 5 weeks, and then changed to a common dry diet thereafter

	Weaning period[1]			Growth period[2,3]									
	5 Weeks		SEM	60 kg		85 kg		110 kg		SEM	Significance[4]		
	MR	DD		MR	DD	MR	DD	MR	DD		Diet	BW	Diet × BW
Caloric intake, MJ/day[5]	12.8[a]	7.5[b]	.4	—	—	—	—	—	—	—	—	—	—
Feed intake, kg/day[6]	—	—	—	1.73[a]	1.76[a]	2.03[b]	2.15[b,c]	2.20[c]	2.18[b,c]	.06	0.23	0.001	0.45
Gain, g/day	620[a]	456[b]	23	699[a]	837[b]	835[b]	952[d]	874[b,c]	916[c,d]	22	0.001	0.001	0.08
Protein, g/day	97[a]	75[b]	3	127[a]	149[b]	144[b]	164[c]	149[b]	155[b,c]	4	0.001	0.001	0.099
Fat, g/day	72[a]	27[b]	3	123	170	180	215	193	228	11	0.001	0.001	0.846
Body protein, %	15.9[a]	16.4[b]	.2	16.9	16.9	16.6	16.7	16.7	16.6	.2	.808	.484	.940
Body protein, kg	4.4[a]	3.6[b]	.2	10.1	10.0	14.0	14.2	18.2	17.8	.2	.877	.001	.391
Body fat, %	13.2[a]	9.1[b]	.6	15.3	15.8	18.2	18.6	19.8	21.6	.6	.080	.001	.539
Body fat, kg	3.6[a]	2.0[b]	.5	9.0[a]	9.4[a]	15.2[b]	15.7[b]	21.7[d]	23.5[e]	.5	.434	.001	.083

[1] Treatment means were compared using the t-test procedure. Different superscripts indicate a significant difference ($P \leq .05$). Protein and fat gains were calculated relative to the composition of a comparable set of piglets at the time of weaning.

[2] Growth period data were analyzed as a completely randomized design with treatments arranged as a 2 × 3 factorial (2 weaning period diets, 3 body weight targets). Treatment df was partitioned to test the main effects and the interaction.

[3] Gain variables for the growth period pertain to the time period from the diet change (i.e., conclusion of the 5 week MR period) of the indicated target weights.

[4] Growth period analysis: Probability of a greater F for the effect of diet, body weight (BW), and the interaction. Means without a common superscript differ ($P \leq .05$).

[5] Pen consumption expressed per pig (4 pigs per pen).

[6] Individual pig data. A standard corn-soybean meal dietary regimen was fed to both groups throughout the growing-finishing periods. The 2 sequential diets contained approximately 13.6 MJ metabolizable energy per kg, and contained 1.00 and 0.76% lysine. The diet changes was made at approximately 60 kg body weight 4.

(days from weaning) required for the pigs to reach the 60 kg weight target was greater in the pigs fed the DD vs. those fed MR (79 vs. 76 ± 1 days, respectively, $P < .08$). However, the days required to reach the 85 kg (103 vs. 105 ± 1 days, $P > .28$) and 110 kg (128 vs. 128 ± 2 days, $P > .97$) weight targets were similar in the pigs fed the MR and DD regimens, respectively.

The fat deposition rate in the pigs fed the MR diet exceeded ($P < .05$) that of the group fed the DD by 167% during the 5-week period; actual fat accretion and the body fat percentage were 80 and 45% greater ($P < .05$), respectively, in these pigs. However, despite the greater fat deposition in these pigs, the activity of acetyl Co-A carboxylase (Fig. 2A) was not influenced by diet ($P > .16$). Although rate of protein accretion and actual protein gain were increased ($P < .05$) by feeding the MR, the greater increase in fat accretion overshadowed this enhancement on a percentage basis.

When the pigs fed the MR were switched to the DD, the rate of fat gain was less ($P < .05$) than that of the pigs fed the DD. From the diet switch through 60 and 85 kg body weight, fat gain (g per day) in these pigs was 72% and 84%, respectively, of that of the pigs fed the conventional diet regimen. This reduction in fat gain was sufficient to negate the difference in body fat content (actual mass and percentage basis) by 60 kg of body weight, relative to the pigs fed the conventional regimen. From 85 to 110 kg, fat gain in the pigs fed the MR was still about 85% of that of the pigs fed the conventional nursery regimen ($P < .05$). This prolonged reduction in fat accretion caused body fat to be 7.7% and 8.3% lower ($P < .05$) on a total fat mass and percentage bases, respectively, at 110 kg body weight. From the diet switch through 60 and 85 kg body weight, protein gain (g per day) was less ($P < .05$) in the pigs fed the MR. However from 85 to 110 kg, protein gain was similar in the two groups, and there were no differences in protein (total mass or percentage basis) at 60, 85, or 110 kg body weight.

The expression of the PPAR and *obese* genes at the conclusion of the 5-week MR portion of the study is summarized in Fig. 2B. All transcripts were clearly detected in the adipose tissue of these pigs, but there was no effect of diet ($P > .56$) on the abundance of any of the transcripts. Expression data for the growth portion of the study are presented in Fig. 3 and Fig. 4. Neither weaning diet nor body weight influenced the abundance of the PPARγ ($P > .31$) or PPARα ($P > .19$) transcripts, and there was no interaction of diet and body weight target ($P > .53$). *Obese* expression (Fig. 6) was not influenced by weaning diet ($P > .98$). However, there was an increase in *obese* mRNA abundance due to body weight ($P < .09$), with the pigs at 110 kg having greater expression than those at 60 kg. *Obese* and PPARα expression were only correlated weakly ($r = 0.25$, $P < 0.10$) over the 60–110 kg body weight range.

4. Discussion

The data presented herein substantiate the results of previous studies [1–3] in which a liquid MR diet sustained

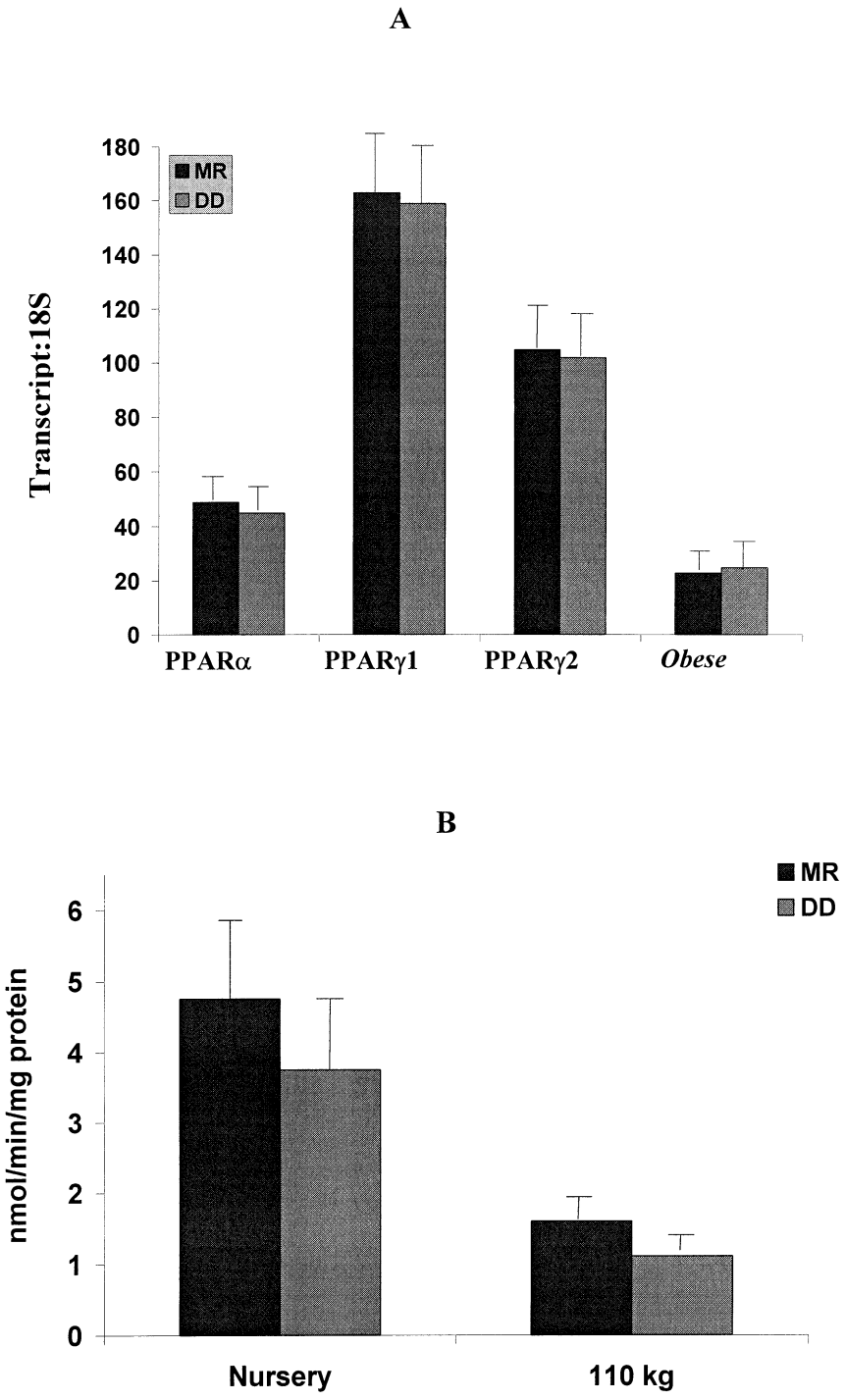

Fig. 2. (A) Relative abundance of PPARα, PPARγ1, PPARγ2, and *obese* mRNA in the adipose tissue of piglets fed a liquid milk replacer (MR) or dry diet (DD) regimen for 5 weeks following weaning. Total RNA was extracted from adipose tissue and 20 μg used in ribonuclease protection assays for each of the transcripts noted. There was no effect of diet ($P > .56$) on the relative abundance of any of the transcripts measured. The data shown are the least squares means and SE ($n = 12$). (B) Acetyl Co-A carboxylase activity in adipose extracts of pigs fed the MR or DD regimen for 5 weeks in the nursery (left bars), and at 110 kg body weight (right bars). There was no effect of diet ($P > .19$) either in the 5-week nursery period, or at the 110 kg weight target. The data presented are the least squares means and SE ($n = 8$).

a greater rate of growth in newly weaned piglets. Whereas protein gain has also been enhanced in pigs fed MR, we provide strong evidence that the greater rate of growth is accompanied by a marked, but transient, increase in body fat. Previous work has shown that feeding dietary fat tends to increase adiposity in pigs [23]. In the present study, the activity of ACC, the rate-limiting enzyme for de novo fatty acid synthesis, was similar in both diet groups just prior to

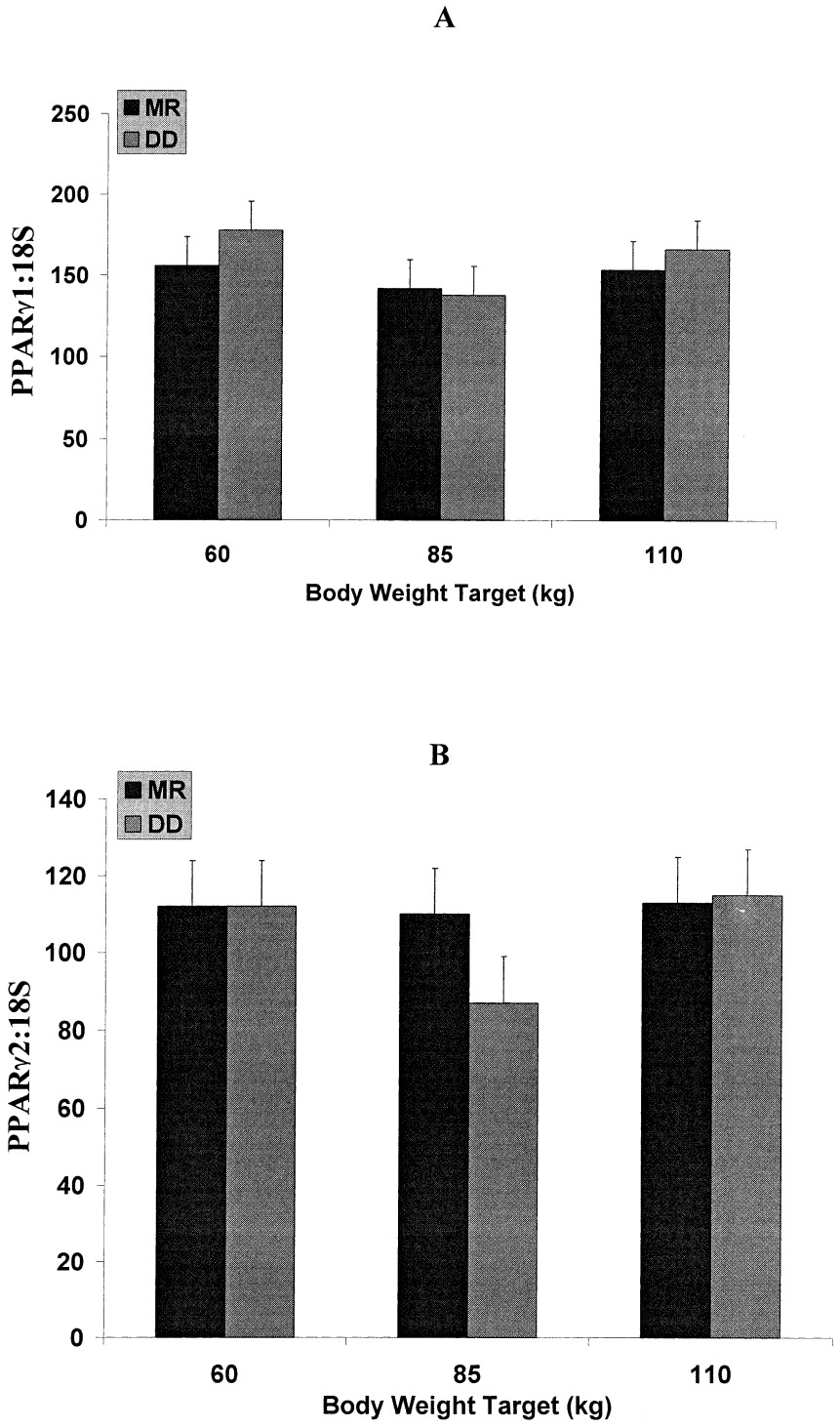

Fig. 3. Changes in the abundance of PPARγ1 (A) and PPARγ2 (B) mRNA in the adipose tissue of pigs fed a liquid milk replacer (MR) or conventional dry diet (DD) regimen for 5 weeks post-weaning, and then placed on a common dry dietary regimen for the remainder of the study. Samples were collected at the 60, 85, and 110 kg weight targets. Weaning diet had no effect ($P > .49$) on the expression of either PPARγ isoform, nor was there an effect of weight target ($P > .31$). The data presented are the least squares means and SE ($n = 10$).

the diet change at 5 weeks postweaning. This, coupled with the evidence that dietary fat augments body fat accretion, makes likely that the greater intake of dietary fat in the pigs fed the MR contributed substantially to their greater body fat content. It also seems logical that the diet change after the MR triggered the relative adjustments in fat deposition that ultimately normalized body fat. It should also be noted that the increased adiposity associated with feeding the MR was actually reversed at 110 kg body weight with the pigs fed the MR actually having significantly less body fat. The

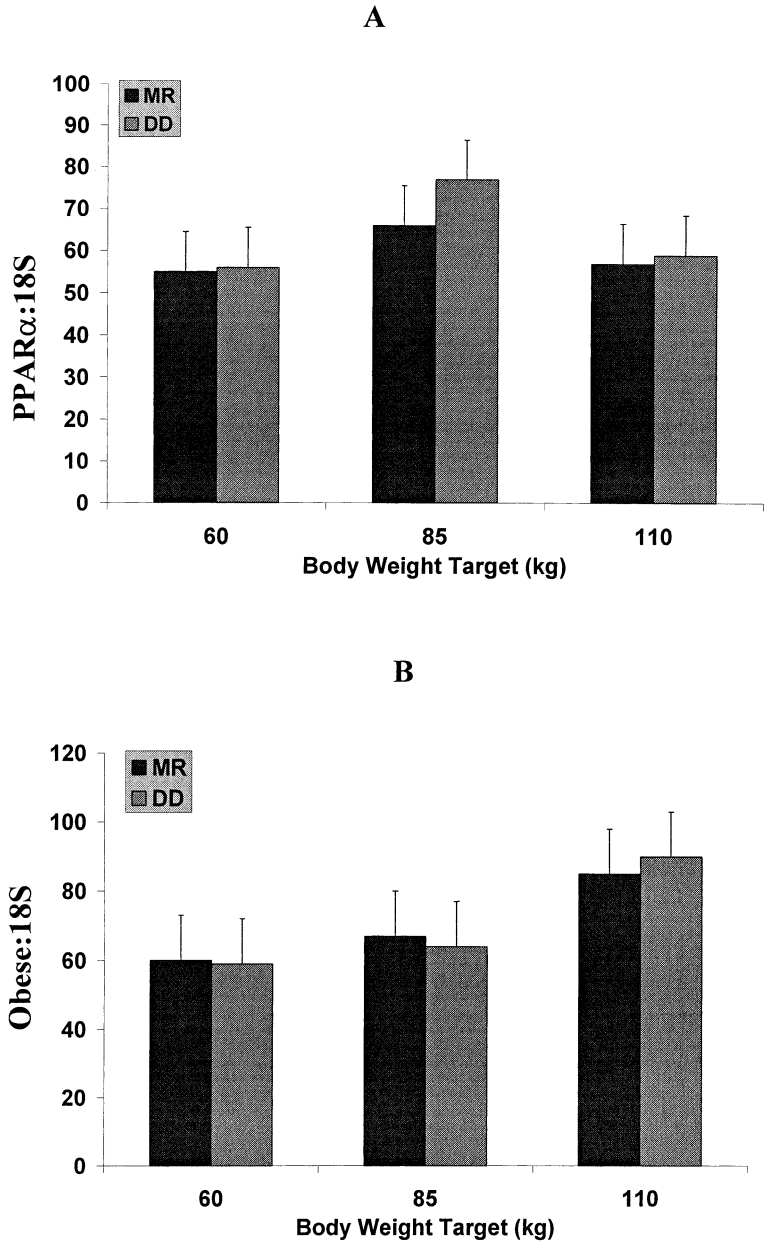

Fig. 4. Changes in the abundance of PPARα (A) and *obese* (B) mRNA in the adipose tissue of pigs fed a liquid milk replacer (MR) or conventional dry diet (DD) regimen for 5 weeks post-weaning, and then placed on a common dry dietary regimen for the remainder of the study. Samples were obtained at the 60, 85, and 110 kg weight targets. There was no effect of diet ($P > .50$) or weight target ($P > .19$) on PPARα expression. *Obese* mRNA increased ($P < .09$) with body weight such that the pigs at 110 kg had greater expression than those at 60 kg. There was no effect of diet ($P > .98$) on obese mRNA abundance. The data presented are the least squares means and SE ($n = 10$).

underlying cause of this apparent decompensation in adiposity is not yet clear, nor is it apparent whether it would be sustained past 110 kg body weight.

A substantial body of literature has linked PPARγ with adipocyte differentiation. Wright et al. [24], using a novel antagonist to PPARγ, provided strong evidence that PPARγ is essential to adipocyte differentiation. Evidence that PPARγ is related to obesity was provided by Kubota et al. [25] when it was discovered that the presence of a single functional allele for PPARγ precluded the onset of obesity and insulin resistance in the adipose tissue of mice fed a diet high in fat. In sharp contrast, the presence of both functional alleles allowed full manifestation of the obesity and insulin resistance. We have shown previously that PPARγ1 and γ2 are differentially regulated in porcine adipose tissue; γ2 expression is attenuated by feed deprivation [16] and upregulated by feeding safflower oil [26]. In the present study, we found neither the γ1 nor γ2 isoforms of this PPAR to be responsive (at the mRNA level) to a nutritional circumstance in which fat deposition was markedly increased. Likewise, the greater accumulation of body fat that characterized the pigs at 110 kg vs. those at 60 kg was not

associated with any difference in either γ1 or γ2 mRNA abundance. Although we did not attempt to distinguish the role of adipocyte hyperplasia vs. hypertrophy in the increased adiposity, it seems likely that the greater fat deposition was accomplished largely by hypertrophy. With little recruitment of new adipocytes from precursor pools, an increase in PPARγ mRNA is perhaps unlikely.

In contrast to the association of PPARγ with lipid accumulation, PPARα is associated with lipid oxidation and removal from the adipocyte. In particular, this PPAR regulates the expression of acyl Co-A oxidase and carnitine palmitoyl transferase, both of which regulate fatty acid oxidation. The expression of PPARα in the adipose tissue of the pig has been documented Ding et al. [20], and Wang et al. [13] showed that leptin stimulates lipolysis and the expression of PPARα, acyl Co-A oxidase, and carnitine palmitoyl transferase in adipocytes, in vitro. Because *obese* expression increases with adiposity in the pig [4,5], and because leptin exerts lipolytic and antilipogenic actions upon the adipocyte [6], we hypothesized that PPARα expression would increase in parallel with adiposity. Our results indicate that PPARα expression was independent of diet and body weight, and thus support the finding of Ding et al. [20] that PPARα expression was similar in genetic lines of pigs with quite different genetic potentials for fat accretion. Although *obese* and PPARα expression were weakly correlated, it is apparent that appreciable differences in adiposity are not linked with the abundance of PPARα mRNA.

We have shown previously [4] that *obese* expression is highly correlated with body weight, and others [5] reported that circulating concentrations of leptin are higher in fatter genotypes of pigs relative to contemporary lean genotypes. In keeping with these findings, *obese* expression was greater in pigs at 110 kg than at 60 kg in the present study. However, despite the greater adiposity of the pigs fed the MR during the 5-weeks postweaning, *obese* expression was not increased. In our earlier study [4], significant differences in *obese* expression were associated with large differences in body fat mass (2 vs. 26 kg body fat at 23 and 107 kg body weight, respectively). Thus, the difference in adiposity in the MR and DD pigs, though substantial, was insufficient to stimulate *obese* expression. Adipocyte size in heavier pigs *per se* may be more fundamentally linked to *obese* expression than is the rate of lipid accretion.

In summary, pigs weaned to the MR diet grow more rapidly, but accumulate considerably more body fat than pigs weaned to DD regimens. However, the increase in body fat is transient, and independent of changes in the expression of the PPAR and *obese* genes.

Acknowledgments

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