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Chronic leptin administration increases serum NEFA in the pig and differentially regulates PPAR expression in adipose tissue[1]

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Abstract

Two in vivo studies were conducted with pigs to determine the effects of exogenous leptin on the expression of peroxisome proliferator activated receptors (PPAR), and on serum concentrations of selected metabolites and hormones. Initially, leptin was administered i.m. to young pigs for 15 days at 0 (control), 0.003 (low), 0.01 (medium) and 0.03 (high) mg · kg^{-1} · day^{-1}. There was no leptin effect on serum glucose ($P > 0.84$), triglycerides ($P > 0.69$), non-esterified fatty acids (NEFA, $P > 0.53$), or glycerol ($P > 0.33$). Leptin at the intermediate and high doses depressed adipose expression of both PPARγ1 ($P < 0.06$) and PPARγ2 ($P < 0.01$). In a second study, we used a paired-feeding experimental design to determine the effects of a higher dose of leptin (0.05 mg · kg^{-1} · day^{-1}) on serum metabolites and PPAR expression in selected tissues. At this dose, leptin increased ($P < 0$ .0001) serum NEFA concentrations relative to both the ad libitum and pair-fed control groups. However, in this study, there was no difference in the expression of PPARγ1 in adipose tissue, but PPARγ2 mRNA was upregulated by leptin ($P < 0.08$). In contrast, leptin had no impact on the expression of PPARα in liver, skeletal muscle or adipose tissue. Adipose tissue explants were also incubated with leptin to assess the effect on PPARγ expression, in vitro. The abundance of PPARγ1 mRNA ($P < 0.05$) was increased after 24 hr of exposure, but the effect of leptin on γ2 was not significant ($P > 0.24$). The lipolytic effect of leptin was also evaluated in vitro using isolated adipocytes. In keeping with the increase in serum NEFA concentrations in vivo, leptin stimulated lipolysis in vitro, increasing glycerol concentrations in the medium to about 219% of that in basal (non-treated) culture medium after 8 hr of incubation. Collectively, the data presented herein indicate that leptin modulates lipid metabolism in the pig, but that PPARα expression is not a parallel target of leptin as it is in rodent models. The regulation of PPARγ by leptin seems complex in that it varied in relation to dose in vivo, and may be impacted by in vitro vs. in vivo circumstances.

Keywords: Leptin; Pig; Lipolysis; Adipocyte; Non-esterified fatty acids PPAR

1. Introduction

The preponderance of literature to date indicates that leptin regulates fat accretion by influencing a combination of central and peripheral pathways. Leptin receptors have been identified in adipose tissue [1,2], and in vitro studies have shown that leptin reduces acetyl-CoA carboxylase expression and activity [3], and depresses fatty acid synthase expression [4]. Furthermore, leptin antagonizes insulin-stimulated lipogenesis while also attenuating the inhibition of lipolysis by this anabolic hormone [5]. Of considerable interest regarding the control of adiposity, leptin stimulates fatty acid oxidation in vivo [6,7] and in vitro [8], and enhances lipolysis and energy expenditure via increased triglyceride-fatty acid cycling [9].

The peroxisome proliferator activated receptors alpha (PPARα) and gamma (PPARγ) are expressed in adipocytes, and seem to be major regulators of lipid metabolism. The stimulation of PPARα expression in adipocytes by leptin is associated with the enhanced fatty acid oxidation and lipoatrophy, and is perhaps a critical component of the mechanism by which leptin stimulates glycerol release in vitro without a corresponding release of NEFA [8]. Rats rendered hyperleptinemic via administration of an adenovirus construct show evidence of lipid depletion from the adipocytes of the white adipose tissue and a concomitant down-regu-

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lation of PPARγ [10]. However, central administration of leptin causes apoptosis in adipose tissue [11], a process associated with increased expression of PPARγ [12].

The lipolytic response and the regulation of PPAR by leptin may vary with species. Although Newby et al. [13] concluded that leptin did not exert a lipolytic effect on ovine adipocytes, Ramsay [14] has shown that leptin acts acutely on porcine adipocytes (stromal vascular cultures) to stimulate lipolysis, and that chronic exposure attenuates the ability of insulin to antagonize the lipolytic action of isoproterenol. However, the effect of leptin on lipid metabolism and PPAR expression in the pig or other non-rodent models has not been studied previously in vivo. In the current study, we tested the hypothesis that prolonged administration of leptin to young growing pigs would invoke a lipolytic response and alter the expression of both PPARα and PPARγ in peripheral tissues, independent of any effect on feed intake. We also tested the hypothesis that leptin acts directly (in vitro) to stimulate lipolysis and regulate PPARγ expression.

2. Materials and methods

2.1. Animals and experimental design

Two experiments were carried out in which leptin was injected i.m. twice daily into young growing pigs. The animals, handling procedures, and experiment protocols have been described in detail in the companion publication [15]. The Animal Care & Use Committee, Purdue University, approved all animal care and handling procedures. Briefly, in the first experiment, male castrates weighing approximately 27 kg were assigned to four treatment groups based on the dose of leptin (0, 0.003, 0.010, and 0.030 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}, designated as control, low, intermediate, and high doses, respectively). The experiment was conducted in three replicate groups over time, with 3 pigs per treatment in each replicate group (n = 9). Animals were weighed and assigned to treatments after being deprived of feed overnight. Thereafter, blood samples were obtained via jugular venipuncture for serum recovery, and each pig was placed in an individual metabolism crate in an environmentally controlled room to allow an assessment of nitrogen retention. A standard corn-soybean meal diet containing 18% crude protein and 0.65% lysine was offered at approximately 4% of body weight (approximately 90% of the estimated ad libitum intake quantity). Half of the feed was provided in the morning (by 0900) and the remainder in the evening (about 1800).

A recombinant human leptin analog (LY355101) was provided by Eli Lilly & Co., Indianapolis, IN, and was demonstrated to be effective in the pig in a preliminary study in which feed intake was reduced in a dose-dependent manner [16]. The protein was injected i.m. for a total of 15 days, with half the daily dose given at each feeding. Blood samples were obtained from all pigs 24 hr after initiation of the leptin injection regimen, and on days 6 and 15. On day 8, the pigs were re-weighed so the quantity of leptin injected could be adjusted for weight gain.

In the second experiment, 30 male castrates weighing approximately 53 kg were randomly assigned to three treatments (n = 10). One group was allowed ad-libitum intake and injected with vehicle. A second group was injected with leptin and allowed ad-libitum intake, whereas the final group was injected with vehicle and pair-fed to match the intake of the group injected with leptin. Leptin was injected i.m. daily (AM and PM) at a dose of 0.025 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} at each injection to achieve a dose of 0.05 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} for 14 days. Blood samples were collected prior to the initiation of treatment protocols for determination of basal hormone and metabolite concentrations, and on days 7 and 14 of treatment.

2.2. Ribonuclease protection assays

Ribonuclease protection assays were performed to assess the relative abundance of the transcripts for the obese gene and PPARγ1-γ2 in subcutaneous adipose tissue, and PPARα in liver, adipose tissue and skeletal muscle. Total RNA was extracted as described by Chomczynski and Sacchi [17]. The riboprobes and nuclease protection assays for the obese and PPARγ transcripts have been described previously [18,19]. The riboprobe for porcine PPARα was prepared from a clone provided by Dr. Harry Mersmann, USDA-ARS, Baylor College of Medicine, which has been described previously [20]. Primers were designed to amplify an approximate 180 bp fragment from the cloned insert. The forward and reverse primers, and the procedures for the riboprobe construction and the nuclease protection assay have been published previously [21]. Twenty μg of total RNA were used for each assay in the in vivo studies whereas 15 μg were used in the in vitro PPAR assay. Total RNA was quantified using the ribogreen assay kit (Molecular Probes, Eugene, OR). Protected fragments were resolved on 5% acrylamide gels containing 8M urea. Autoradiographs were prepared from dried gels, and signal intensity was quantified using a Digital Science Imaging System (V. 2.0.1, Kodak, New Haven, CT).

2.3. Acetyl Co-A carboxylase (ACC) activity

In the second in vivo experiment, the activity of ACC in adipose tissue samples was determined based on the incorporation of $NaH^{14}CO_3$ into malonyl Co-A, as we have described previously [21]. The protein concentration of the enzyme preparations was determined using the Bicinchoninic Acid (BCA) method and the kit purchased commercially (Pierce Chemical Co., Rockford, IL). The activity is reported as nmol per min per mg of protein.

2.4. Adipocyte isolation and lipolysis

For the determination of the in vitro lipolytic effect of leptin, adipocytes were isolated from subcutaneous adipose tissue obtained from male castrates at slaughter (approximately 120 to 135 kg body weight) by collagenase digestion as described previously [22–24]. The isolated cells were diluted to approximate 25 to 30% cell suspensions with DMEM containing 3% fatty acid free serum albumin. The incubation vials were gassed initially (and at 2-hr intervals) with oxygen containing 5% CO_2 and incubated in a gyratory waterbath (37°C) for the selected duration. The incubations were terminated at 8 hr by transferring the contents of the vials to 12 × 75 mm tubes containing 100 μL 35% perchloric acid. The killed cell suspensions were centrifuged, the supernatant fraction recovered and neutralized with KOH, and then assayed for glycerol. The experiment was replicated 3 times with tissue from different pigs. Within each experiment (pig), each treatment was replicated 3 to 4 times. Lipolysis was based on glycerol release into the medium as measured using the procedure of Eggstein and Kuhlmann [25] adapted to a microtiter plate protocol. The data are expressed relative to the non-stimulated control within each experiment to adjust for variation in the number of cells incubated from experiment to experiment. The viability of each adipocyte preparation was confirmed by verification of a lipolytic response to isoproterenol (10^{-7} M) during the final 2 hr of incubation.

2.5. Adipose explant culture

To establish whether leptin acts directly on adipose tissue to regulate PPARγ expression, adipose tissue explants were prepared from adipose tissue as described by Donkin et al. [26]. Briefly, inner layer subcutaneous adipose tissue was obtained from male castrate pigs weighing approximately 50 kg. The tissue was placed immediately in a transport medium (M199 with Hanks salts, Sigma Chemical Co., St. Louis, MO) containing 0.1 g/L L-glutamine, 25 mM HEPES, 0.36g/L $NaHCO_3$, 1% antibiotic-antimycotic, 0.5% BSA, and adjusted to pH 7.4. The tissue was rinsed quickly in 70% ethanol to remove bacterial contamination, and trimmed to remove dead cells from the surface layer. The trimmed tissue was cut into thin slices with a microtome, and the slices cut into pieces of approximately 80 mg. Each piece was cultured in 3 mL of the buffered Hanks solution described above, except that the BSA concentration was increased to 3.0%. The explants were cultured in different treatment conditions as follows: control (explants without leptin and cultured for either 12 or 24 hr; leptin (explants with 100 nM leptin and cultured for 12 or 24 hr). At the termination of the experiment, explants were frozen in liquid nitrogen and stored at −80°C pending RNA extraction.

2.6. Statistical analyses

For the in vivo studies, all data were analyzed using a mixed model analysis of a split plot design [27] as described for these experiments in a separate publication [15]. The lipolysis data (medium glycerol concentrations) were analyzed by analysis of variance and mean separation (Least Significant Differences) when protected by a significant F-test [28]. The in vitro PPARγ expression data were analyzed by analysis of variance with treatment and time as the main effects, and treatment × time as the interaction effect. Mean separation was performed with the least squares mean separation when protected by a significant F-test (SAS, 1999).

3. Results

3.1. Experiment 1

The serum metabolite data are summarized in Table 1. There was no effect of leptin on serum concentrations of glucose ($P > 0.84$), triglycerides ($P > 0.69$), NEFA ($P > 0.53$) or glycerol ($P > 0.33$), nor was there an interaction of leptin treatment with day. However, triglyceride concentrations were reduced ($P < 0.0001$), and NEFA concentrations increased ($P < 0.0008$), on day 15 relative to previous measurements. The expression data (relative mRNA abundance) for PPARγ and the *obese* gene are summarized in Table 2. Although the expression of the *obese* gene was not influenced ($P > 0.27$) by exogenous leptin, both the γ1 and γ2 isoforms of PPAR were reduced approximately 50% ($P < 0.06$ and 0.01, respectively) by the two higher doses of leptin.

3.2. Experiment 2

The serum metabolite concentrations obtained in the second in vivo experiment are summarized in Table 3. The concentration of glucose was not different among treatments after 1, 7 or 14 days ($P > 0.42$), nor was there a significant treatment by day interaction ($P > 0.79$). Likewise, there was no effect of treatment on the serum triglyceride concentrations ($P > 0.36$). However, serum triglyceride concentrations did decline with time ($P < 0.001$) as the experiment progressed (day 1 > day 7 > day 14). Serum NEFA concentrations were higher ($P < 0.0001$) in pigs treated with leptin than in either the ad libitum-fed or the pair-fed groups (60% and 106% increase over the ad-libitum and pair-fed groups, respectively). The NEFA concentrations were also higher ($P < 0.0001$) on day 14 than on previous days, but the treatment by day interaction was not significant ($P > 0.26$). There were no differences in serum concentrations of glycerol across treatments ($P > 0.29$). However, as with NEFA concentrations, there was a significant increase ($P < 0.0001$) in the glycerol concentration on day 14 vs. days 1 and 7.

Table 1
Serum metabolite concentrations in pigs injected with vehicle or a recombinant human leptin analog for 15 days

		Leptin Dose, mg · kg^{-1}day^{-1}						Significance (P > F)		
Metabolite	Day	0	0.003	0.010	0.030	Mean	SEM[1]	Trt[2]	Day	Trt*Day
Glucose (mg/dL)	105.01	108.44	113.72	108.68	108.96	3.77				
	6	110.50	100.82	100.36	104.66	104.08	3.84			
	15	110.58	102.51	108.48	113.38	108.74	3.86	0.84	0.60	0.91
	Mean	108.70	103.92	107.52	108.91		4.40			
Triglycerides (mg/dL)	1	27.99	20.61	26.75	34.18	27.38[a]	2.52			
	6	21.31	18.06	20.40	24.94	21.17[b]	2.55			
	15	15.80	17.67	12.93	13.55	14.99[c]	2.54	0.69	0.0001	0.23
	Mean	21.70	18.78	20.02	24.22		3.58			
NEFA (μM)	1	27.01	28.01	29.18	21.92	26.54[a]	23.41			
	6	24.82	28.10	27.66	30.01	27.65[a]	23.45			
	15	56.79	61.01	114.69	118.19	87.67[b]	23.42	0.53	0.0008	0.61
	Mean	36.21	39.07	57.17	56.71		24.28			
Glycerol (μM)	1	113.93	71.82	116.88	58.98	90.40	19.96			
	6	83.11	59.22	63.10	64.24	67.42	20.57			
	15	122.67	82.41	205.18	98.25	127.13	20.27	0.33	0.12	0.80
	Mean	106.57	71.15	128.39	73.82		23.70			

[a,b,c] Means in the same column with different superscripts significantly differ at P < 0.01
[1] Pooled standard error
[2] P > F for treatment (n = 8–9)

The expression (mRNA abundance) data are summarized in Table 4. There was an upregulation of PPARγ2 by leptin (61% and 39% increase over the ad-libitum and pair-fed groups, respectively, $P < 0.08$). However, the expression of PPARα in skeletal muscle ($P > 0.61$), adipose tissue ($P > 0.26$) and liver ($P > 0.24$) was unresponsive to exogenous leptin. Likewise, the activity of ACC (Fig. 1) was not altered by leptin, but was clearly reduced ($P < 0.0001$) by the reduction in feed intake caused by leptin and pair-feeding (i.e., leptin administration and paired feeding caused a similar reduction in activity).

3.2.1. *In vitro lipolysis*

Dose titration experiments with isolated adipocytes indicated that the lipolytic response to leptin (i.e., glycerol release) was similar from 20 to 100 ng per mL (data not shown). As shown in Fig. 2, leptin at 40 ng per mL stimulated glycerol release, and by 8 hr, the glycerol concentration in the medium of cells treated with leptin was about 200% of that in the basal (non-stimulated) cells.

3.2.2. *Regulation of PPARγ in vitro*

Fig. 3A and Fig. 3B show the PPARγ1 and PPARγ2 mRNA expression, respectively, in adipose explants. Leptin increased PPARγ1 expression at 24 and 48 hr; explants treated with 100 nM leptin had increased expression (130% increase) of PPARγ1 (pooled means of 12 and 24 hr cultures, $P < 0.05$). Though PPARγ2 was also up regulated in the leptin treated explants (65% increase), the difference was not statistically significant ($P > 0$ .24).

Table 2
Relative abundance of PPARγ and *Obese* mRNA transcripts in tissues of pigs injected with vehicle or recombinant human leptin analog for 15 days

	Leptin Dose, mg · kg^{-1} · day^{-1}					
	0	0.003	0.010	0.030	SEM[1]	P > F[2]
PPARγ2	23.10[a]	26.00[a]	11.68[b]	12.00[b]	3.30	0.01
PPARγ1[3]	52.41[a]	51.85[a]	33.96[b]	32.74[b]	6.5	0.06
Obese[4]	14.97	15.70	10.60	11.94	3.09	0.27

[1] Pooled standard error
[2] P > F for treatment (n = 8–9)
[3] Means differ at P < 0.06.
[4] Analysis based on log transformed data.
[a,b] Means with different superscripts significantly differ at P < 0.05

4. Discussion

Reidy and Weber [9] determined in rabbits that a single i.v. leptin bolus stimulated lipolysis (increased serum NEFA concentrations) and enhanced triglyceride-fatty acid cycling. Though an assessment of such cycling was beyond the objectives of the present study, our data corroborate and extend their findings in part. We show herein that despite maintenance of a positive energy balance in growing pigs, chronic leptin administration at 0.05 mg · kg^{-1} · day^{-1} (Experiment 2) increases serum NEFA concentrations for at least 2 weeks, but has no effect on serum triglyceride or glucose concentrations. Although not studied previously in

Table 3
Serum metabolite concentrations in pigs injected with recombinant leptin for 14 days

		Treatment[1]					Significance (P > F)		
	Day	Ad libitum	Leptin-treated	Pair-fed	Day Mean	SEM[2]	Trt	Day	Trt*Day
Glucose (mg/dL)	1	105.80	100.39	113.72	103.85	3.00			
	7	92.99	98.51	98.62	96.71	3.00			
	14	91.51	108.56	95.06	98.37	3.00	0.42	0.22	0.79
		96.76	102.48	99.69		3.00			
Triglycerides (mg/dL)	1	18.97	16.98	18.49	18.14[a]	1.17			
	7	14.57	11.96	16.93	14.48[a,b]	1.17			
	14	11.19	11.54	12.18	11.64[b]	1.19	0.36	0.001	0.65
		14.91	13.49	15.87		1.15			
NEFA[3] (μM)	1	28.61	31.76	20.77	27.05[a]	23.02			
	7	31.21	66.32	27.97	41.84[b]	23.02			
	14	189.04	301.42	14.77	211.74[c]	24.50	0.0001	0.0001	0.26
		82.95[b]	133.17[a]	64.50[b]		23.28			
Glycerol (μM)	1	71.39	41.28	43.33	51.99[b]	10.92			
	7	49.75	45.97	35.11	43.61[b]	10.92			
	14	143.52	176.74	121.12	147.13[a]	11.14	0.29	0.0001	0.46
		88.22	87.99	66.52		10.90			

[1] The ad libitum group was allowed ad libitum intake and injected with vehicle twice daily for 14 days. The leptin group was injected with a recombinant human leptin analog (0.05 mg · kg^{-1} · day^{-1} and also allowed ad libitum intake. The pair-fed group was injected with vehicle and their intake was limited to that of the pigs injected with leptin; (treatment n = 10).
[2] Pooled standard error.
[3] Analysis based on log transformed data due to heterogeneity of variances.
[a,b,c] Means in the same column with different superscripts significantly differ at P < 0.01.

the pig, the effect of chronic leptin administration (35 days) on lipid oxidation and plasma metabolites has been studied in mature (weight stable) rats [6,7]. In these rodent studies, daily peripheral injections of leptin caused a transient reduction in food intake. Additionally, there was a sustained stimulation of lipid utilization that was reflected in reduced plasma NEFA and triglyceride concentrations, and in lower respiratory quotients relative to controls, for a significant

Table 4
Relative abundance of selected transcripts in tissues of pigs injected with recombinant leptin for 14 days

	Treatment[1]				
	Ad libitum	Leptin-treatment	Pair-fed	SEM[2]	P > F[3]
PPARγ1	3.29	5.32	3.82	1.08	0.19
PPARγ2	1.98[a]	4.60[b]	3.08[a,b]	1.12	0.08
Obese	67.11	55.15	54.14	22.67	0.69
PPARα					
Muscle[4]	0.33	0.29	0.28	0.03	0.61
Adipose	20.73	20.14	31.64	11.24	0.26
Liver	0.79	1.12	0.91	0.14	0.24

[1] The ad libitum group was allowed ad libitum intake and injected with vehicle twice daily for 14 days. The leptin group was injected with a recombinant human leptin analog (0.05 mg · kg^{-1} · day^{-1} and also allowed ad libitum intake. The pair-fed group was injected with vehicle and their intake was limited to that of the pigs injected with leptin; (Treatment n = 10 pigs).
[2] Pooled standard error
[3] P > F for treatment (n = 10)
[4] longissimus dorsi.
[a,b] Means with different superscripts significantly differ at P < 0.05

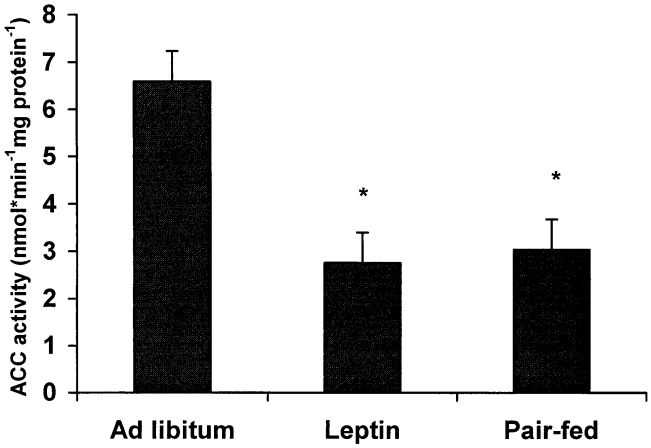

Fig. 1. Activity of acetyl Co-A carboxylase in the adipose tissue of pigs injected with a recombinant human leptin analog for 14 days. One group of pigs was fed ad libitum and injected with saline whereas another group was injected with 0.05 mg · kg^{-1} leptin per day, half the dose in the morning and the remainder in the evening. The pair-fed group had their feed intake limited to that of the leptin-treated pigs. Means with asterisks (*) differ from the ad libitum fed group at $P < 0.0001$; $n = 10$ pigs per treatment.

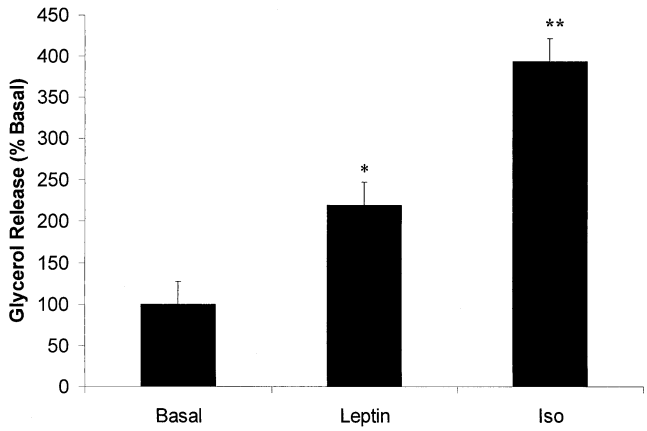

Fig. 2. Stimulation of lipolysis by leptin in isolated porcine adipocytes. Adipose tissue was obtained from male castrates weighing 120 to 135 kg, and adipocytes isolated by collagenase digestion. Thereafter, the cells were incubated with leptin (40 ng per mL) in DMEM for 8 hr. Isoproterenol (10−7 M) was used as the positive control and was added for the final 2 hr of incubation. The bars represent the pooled least squares means and SE expressed as a percent of the basal treatment ($n = 3$). Significance relative to the basal treatment is indicated by the asterisks (* $P < 0.037$; ** $P < 0.01$).

portion of the experimental period. Thus, whereas leptin stimulated lipid mobilization in these young growing pigs, any stimulation of lipid oxidation was insufficient to cause net reductions in serum NEFA and triglyceride concentrations.

It is also of interest to note that, as in the rabbit study [9], the increase in serum NEFA was not paralleled by an increase in serum glycerol. This is opposite that reported by Wang et al. [8] for their in vitro model, in which it is presumed that fatty acids did not accumulate in the medium with glycerol because they were oxidized internally. The absence of a glycerol response to leptin in vivo likely reflects the ability of cells other than adipocytes to metabolize glycerol in vivo due to glycerokinase activity [29,30]. In our study, pigs receiving leptin were also consuming substantially less feed than the group allowed ad libitum intake [15]. Rather than accumulate in the blood, we speculate that the glycerol released by the lipolytic action of leptin was metabolized quickly to provide glucose. Despite the lower feed intake in the pigs injected with leptin, and those pair-fed with them, serum glucose concentrations were similar across treatments. As discussed in our companion publication [15], this perhaps explains the finding that serum urea nitrogen (an indicator of protein catabolism during intake restriction, and of dietary nitrogen intake in periods of ad libitum intake) was higher in the pair-fed pigs than in those injected with leptin. Thus, in the absence of the leptin-induced glycerol release, the pair-fed group may have used dietary and tissue-derived amino acids to maintain blood glucose. This is consistent with the interpretation of Chen and Heiman [6,7] that leptin spares glucose utilization. The glycerol or NEFA response that was intensified on the final day of the study in the in vivo experiments is likely explained by the stress associated with handling prior to and during the euthanasia procedure. It is important to note that the treatment by day interaction term was insignificant, and that the lipolytic response to leptin and stress are likely mediated by different pathways which seemed additive.

The lipolytic action of leptin on isolated pig adipocytes is not surprising given the similar findings reported for other models by Wang et al. [8] and Frühbeck et al. [31,32], and for the pig by Ramsay [14]. However, other researchers [13] have been unable to demonstrate a similar lipolytic effect in ovine adipocytes, and it is possible that species differences in lipolytic sensitivity exist. Adenosine acts upon adipocytes to suppress lipolysis through stimulation of the receptor-coupled inhibitory G protein (G_i), which reduces adeny-

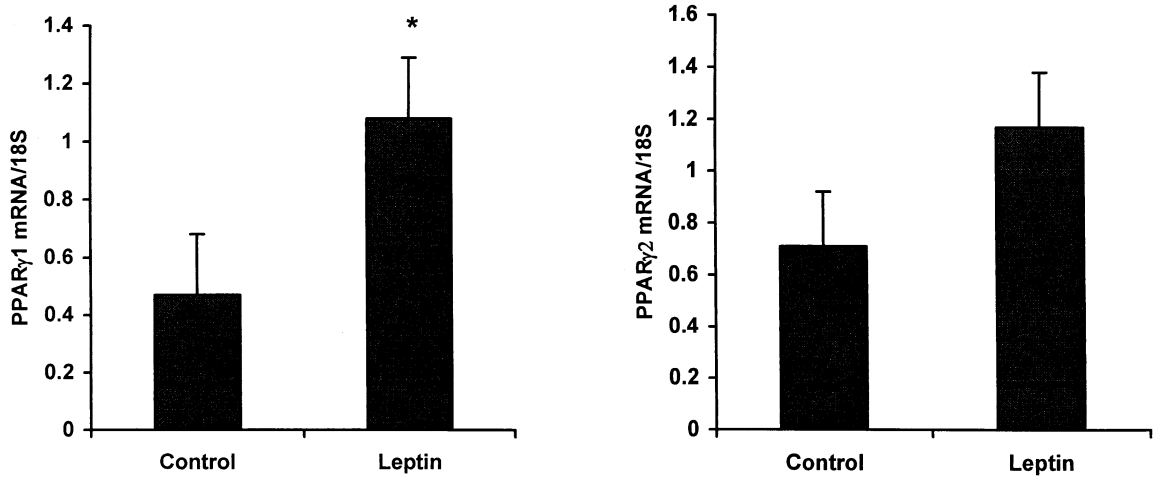

Fig. 3. (A) and (B) Expression of PPARγ1 and PPARγ2 mRNA in adipose explants treated with leptin. Explants were cultured without (Control) or with 100 nM recombinant human leptin analog (Leptin) for 12 or 24 hr after which they were assayed by RPA for the abundance of PPAR transcripts. The bars represent pooled least squares means across time (due to a non-significant treatment*time interaction). Significance relative to control is indicated by asterisk (*). Expression of PPARγ1 transcripts was increased by leptin relative to control ($P < 0.05$; $n = 5$ pigs per treatment).

lyl cyclase activity. Frühbeck et al. [31] provided convincing evidence that the lipolytic action of leptin is linked to an antagonism of the inhibitory activity of adenosine on the adipocyte. Whether species differences at this level of adenosine signaling contribute to the apparent species difference in lipolytic sensitivity to leptin remains to be determined.

Leptin has been suggested to stimulate peroxisomal fatty acid oxidation in adipose tissue and skeletal muscle [33,34]. This stimulation seems to be accomplished, at least in part, through an induction of PPARα expression [8]. Presumably, a greater activity of this transcription factor drives the expression of acyl Co-A oxidase (ACO), the rate-limiting enzyme in the peroxisomal fatty acid oxidation. In the present study, we found no evidence that leptin impacted PPARα expression in adipose tissue, skeletal muscle or liver. Thus, any stimulation of peroxisomal fatty acid oxidation in vivo by leptin was independent of increased PPARα expression in the pig, and was also insufficient to negate a measurable increase in serum NEFA concentrations or to lower circulating triglyceride concentrations.

Our studies indicate that PPARγ may be a regulatory target of leptin, but that the regulation may depend on dose and on factors associated with in vivo vs. in vitro administration. Leptin administered peripherally, and at low doses compared with those used in rodent studies, down regulates both PPARγ1 and γ2 in adipose tissue by approximately 50%, relative to control animals (Experiment 1). Zhou et al. [10] reported a significant down regulation of PPARγ expression (mRNA and protein), a reduction in adipose leptin protein content, and an induction of a preadipocyte marker, Pref-1, in epididymal fat of rats rendered hyperleptinemic by adenoviral transfection. Our findings with PPARγ parallel this observation, although *obese* mRNA abundance was not significantly reduced by exogenous leptin. A determination as to whether this relates to altered differentiation status is beyond the current study. However, given the critical role of PPARγ in the maintenance of adipocyte differentiation, it is possible that a significant reduction in expression of this critical transcription factor might lead to dedifferentiation of existing adipocytes [10].

In the second in vivo experiment, leptin administration caused an increase in total serum leptin and a reduction in feed intake that is indicative of the central activity of leptin [15]. Herein we also report an up-regulation of PPARγ2 by leptin, an effect that was also obtained in part in adipose explants. Few studies have determined the expression of the separate PPARγ transcripts, and there is currently little information by which to ascribe cellular events to PPARγ1 or γ2, specifically. The up-regulation of PPARγ has been linked to the induction of adipose apoptosis by leptin administered centrally to rats [12], but this has not yet been reported in other mammalian models. Whereas our results in general are consistent with other studies indicating PPARγ regulation by leptin, it is possible that variations in the dose administered and in vivo vs. in vitro circumstances may lead to differential regulation of these transcription factors. This would perhaps reconcile the results obtained in the two in vivo experiments, as well as the in vivo vs. in vitro findings. It is also important to note that leptin did not impact the expression of PPARα in any of the tissues examined. Thus, this transcription factor, which is commonly associated with fatty acid oxidation, may not be a regulatory target of leptin in the pig.

Others have shown that leptin is a potent regulator of the ACC activity in skeletal muscle [35] and pre-adipocytes [3]. However, we found no evidence that leptin reduced ACC activity in adipose tissue beyond the reduction caused by the lower feed intake, as reflected in the pair-fed control group. Thus, in the pig, and under the conditions of leptin administration used in Experiment 2, it seems unlikely that leptin attenuates lipid accretion by regulating ACC activity. It is possible that a more pronounced hyperleptinemia, or longer duration of treatment would lower adipose ACC activity, and we are currently investigating that possibility.

In summary, we provide evidence in the pig that prolonged administration of a sufficient dose of leptin causes a sustained increase in serum NEFA, but in contrast with other models reported to date, we see no net reduction in serum triglyceride. Also in contrast with other published works, our data indicate that PPARα expression is not a major regulatory target of leptin in the pig, and that regulation of PPARγ by leptin seems complex. Collectively, these findings reinforce that comparative studies in multiple species are necessary to fully understand the biological roles of hormones and cytokines, and to make applications to human and animal health.

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References

[1] Siegrist-Kaiser CA, Pauli V, Juge-Aubry CE, Boss O, Pernin A, Chin WW, Cusin I, Rohner Jeanrenaud F, Burger AG, Zapf J, Meier CA. Direct effects of leptin on brown and white adipose tissue. J Clin Invest 1997;100:2858–64.

[2] Lin J, Barb CR, Matteri RL, Kraeling RR, Chen X, Mersmann RJ, Rampacek GB. Long form leptin receptor mRNA expression in the brain, pituitary, and other tissues in the pig. Dom Animal Endocrinol 2000;19:53–61.

[3] Bai Y, Zhang S, Kim KS, Lee JK, Kim KH. Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. J Bio Chem 1996;271:13939–42.

[4] Fukuda H, Iritani N. Transcriptional regulation of leptin gene promoter in rat. FEBS Let 1999;455:165–69.
[5] Müller G, Ertl J, Gerl M, Preibisch G. Leptin impairs metabolic actions of insulin in isolated rat adipocytes. J Bio Chem 1997;272: 10585–93.
[6] Chen Y, Heiman ML. Chronic leptin administration promotes lipid utilization until fat mass is greatly reduced and preserves lean mass of normal female rats. Reg Pep 2000;92:113–9.
[7] Chen Y, Heiman ML. Increased weight gain after ovariectomy is not a consequence of leptin resistance. Am J Physiol Endocrin Metab 2001;280:E315–22.
[8] Wang MY, Lee Y, Unger RH. Novel form of lipolysis induced by leptin. J Bio Chem 1999;274:17541–4.
[9] Reidy SP, Weber JM. Accelerated substrate cycling: a new energy-wasting role for leptin in vivo. Am J Physiol (Endocrinol Metab) 2002;282:E312–7.
[10] Zhou Y, Wang Z, Higa M, Newgard CB, Unger RH. Reversing adipocyte differentiation: implications for treatment of obesity. Proceeding of the National Academy of Science 1999;96:2391-2395.
[11] Qian H, Hausman G J, Compton MM, Azain MJ, Hartzell DL, Baile CA. Leptin regulation of peroxisome proliferator-activated receptor-gamma, tumor necrosis factor, and uncoupling protein-2 expression in adipose tissues. Biochem Biophys Res Comm 1998;246:660–7.
[12] Qian H, Azain MJ, Compton MM, Hartzell DL, Hausman GJ, Baile CA. Brain administration of leptin causes deletion of adipocytes by apoptosis. Endocrinol 1998;139:791–4.
[13] Newby D, Gertler A, Vernon RG. Effects of recombinant ovine leptin on in vitro lipolysis and lipogenesis in subcutaneous adipose tissue from lactating and non-lactating sheep. J Animal Sci 2001;79:445–52.
[14] Ramsay TG, Porcine leptin alters insulin inhibition of lipolysis in porcine adipocytes in vitro. J Animal Sci 2001;79:653–7.
[15] Ajuwon KM, Kuske JL, Ragland D, Adeola O, Houseknecht KL, Hancock DL, David B, Anderson DB, Spurlock ME. The regulation of IGF-1 by leptin in the pig is tissue specific and independent of changes in growth hormone. 2002; (Submitted for publication).
[16] Wuethrich AJ, Hancock DL, Heiman MI, Muegge JD, Roth JL, Anderson DB. The effect of injecting LY355101, a leptin analog, on feed intake of finishing swine. J Animal Sci 2000;78(Suppl 1):19 abstract.
[17] Chomczynski P, Sacchi N. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Analytic Biochem 1987;162:156–9.
[18] Houseknetcht KL, Baile CA, Matteri RL, Spurlock ME. The biology of leptin: a review. J Animal Sci 1998;76:1405–20.
[19] Spurlock ME, Ranalletta MA, Cornelius SG, Frank GR, Willis GM, Ji S, Grant AL, Bidwell CA. Leptin expression in porcine adipose tissue is not increased by endotoxin but is reduced by growth hormone. J Interferon Cytokine Res 1998;18:1051–8.
[20] Ding ST, Schinckel AP, Weber TE, Mersmann HJ. Expression of porcine transcription factors and genes related to fatty acid metabolism in different tissues and genetic populations. J Animal Sci 2000; 78:2127–34.
[21] Spurlock ME, Bidwell CA, Houseknecht KL, Kuske JL, Camacho-Rea C, Frank GR, Willis GM. 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. J Nutr Biochem 2002;13:112–20.
[22] Liu CY, Boyer JL, Mills SE. Acute effects of beta-adrenergic agonists on porcine adipocyte metabolism in vitro. J Animal Sci 1989;67: 2930–6.
[23] Spurlock ME, Cusumano JC, Mills SE. (-)-[3H]-dihydroalprenolol binding to beta-adrenergic receptors in porcine adipose tissue and skeletal muscle membrane preparations. J Animal Sci 1993;71:1778–85.
[24] Spurlock ME, Cusumano JC, Mills SE. The affinity of ractopamine, clenbuterol, and L-644, 969 for the beta-adrenergic receptor population in porcine adipose tissue and skeletal muscle membrane. J Animal Sci 1993;71:2061–5.
[25] Eggstein M, Kuhlmann E. Triglycerides and glycerol determination-after alkaline hydrolysis. In: Bermeyer HU, editor. New York: Academic Press, 1974. p. 1825. Methods of enzymatic analysis, 3.
[26] Donkin SS, Chiu PY, Yin D, Louveau I, Swencki B, Vockroth J, Evock-Clover CM, Peters JL, Etherton TD. Porcine somatotrophin differentially down-regulates expression of the GLUT4 and fatty acid synthase genes in pig adipose tissue. J Nutr 1996;126:2568–77.
[27] SAS. SAS User's guide, version 8. Cary, NC: NC Statistical Analysis Systems Institute, Inc, 1999.
[28] Steel RGD, Torrie JH. Principles and Procedures of Statistics: Biometrical Approach. 2nd ed. New York: McGraw Hill Publishing Co, 1980.
[29] Guo Z, Jensen MD. Blood glycerol is an important precursor for intramuscular triacylglycerol synthesis. J Biolog Chem 1999;274: 23702–6.
[30] Watford M. Functional glycerol kinase activity and the possibility of a major role for glyceroneogenesis in mammalian skeletal muscle. Review. Nutr Rev 2000;58:145–8.
[31] Frühbeck G, Aguado M, Martinez JA. In vitro lipolytic effect of leptin on mouse adipocytes: evidence for a possible autocrine/paracrine role of leptin. Biochem Biophys Res Comm 1997;240:590–4.
[32] Frühbeck G, Gomez-Ambrosi J, Salvador J. Leptin-induced lipolysis opposes the tonic inhibition of endogenous adenosine in white adipocytes. FASEB J 2001;15:333–40.
[33] Sarmiento U, Benson B, Kaufman S, Ross L, Qi M, Scully S, DiPalma C. Morphologic and molecular changes induced by recombinant human leptin in the white and brown adipose tissues of C57BL/6 mice. Lab Invest 1997;77:243–56.
[34] Muoio DM, Dohm GL, Fiedorek FT Jr., Tapscott EB, Coleman RA, Dohn GL. Leptin directly alters lipid partitioning in skeletal muscle. Diabetes 1997;46:1360–3.
[35] Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 2002;415:339–43.