Endocrine responses to short-term feed deprivation in weanling pigs

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Abstract

The study objective was to assess endocrine and tissue responses to feed deprivation in weaned pigs. In experiment 1, eight crossbred castrated male pigs were either fed on a continual basis (CON; $n=4$) or were feed deprived for 24 h and then re-fed until 30 h (FD; $n=4$). Relative serum concentrations of ghrelin tended to be lower in FD pigs at 12 h ($P=0.08$) when compared with CON pigs, but was higher at 24 h and 30 h compared with 12 h ($P<0.05$). Serum IGF-I was lower in FD pigs from 12 to 30 h as compared with CON pigs ($P<0.05$) and increased following re-feeding ($P<0.06$). Experiment 2 consisted of 32 pigs that were either fed for 72 or 96 h (CON72 and CON96; $n=16$), feed deprived for 72 h (FD72; $n=8$), or FD72 and then re-fed from 72–96 h (FD72/RF24; $n=8$). Ghrelin in the FD72 and FD72/RF24 groups was lower at

12 h ($P<0.03$) compared with CON pigs, but then increased from 12 h to 36 h ($P<0.01$). Serum IGF-I and leptin decreased following feed deprivation ($P<0.001$) and remained low until re-feeding. Cortisol was elevated from 12 h to 72 h during feed deprivation ($P<0.01$) but was not different from CON96 pigs following refeeding ($P>0.88$). Expression of ghrelin mRNA tended to be lower in the FD72 pigs' stomachs, pituitary glands, and hypothalami ($P=0.06$, 0.07 , and 0.08 respectively) compared with CON pigs. These results provide evidence that feed deprivation is accompanied by multiple changes in the endocrine and neuroendocrine axis which influences feed intake, somatotrophic response, and hypothalamic–pituitary–adrenal axis hormone concentrations.

Journal of Endocrinology (2003) **178**, 541–551

Introduction

Weaning is a time of high stress for piglets in modern swine production systems. While pigs in natural conditions undergo the weaning process gradually and can consume a solid diet as well as suckle from their dam, pigs that undergo weaning in most production systems are weaned abruptly and are required to make the transition to an exclusively solid diet quickly. The stress of separation from the sow, as well as the nutritional effects of feed deprivation, cause a period of growth stasis that can be detrimental. This transition is characterized by a period of voluntary feed deprivation and weight loss during the change of diet from liquid sow's milk to a solid pig starter diet (Riley 1989, Forbes 1995). Individual pig responses to these stressors are variable and are associated with post weaning growth rate (Giroux *et al.* 2000).

The transition in eating behavior that is necessary for the initiation of solid feed consumption by pigs is influenced by several factors, including: (1) access to creep feed while the pig is still suckling (Funderburke & Seerley 1990) (2) social hierarchy within a pen (3) feeder availability; and (4) size of the pig (smaller pigs eat less and

tend to have a lower daily weight gain than larger pigs; Georgsson & Svendsen 2002). Metabolic factors, feed intake, and stress hormones have not been intensively studied during the weaning transition. Thus, the present studies were designed to elucidate the endocrine dynamics that accompany the food deprivation response in pigs during weaning.

One to three days of feed deprivation is typically observed during weaning. By conducting the experiments on individually penned pigs after their transition to solid feed and at least five days following weaning and transport to new facilities, we minimized the effects of stressors such as transportation, psychological/behavioral response, and hierarchical order by our design. Although individual penning could be considered a stressor, past studies have provided evidence that penning pigs individually does not affect feed efficiency or plasma cortisol and may actually increase feed intake compared with groups consisting of four pigs (Bustamante *et al.* 1996). The practice of individual penning utilized in the present experiments did not prevent sight or smell contact with other pigs, but it did ensure an unabated access to feed.

Materials and Methods

Experiment 1

Animal care and procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia. Eight crossbred castrated male pigs (barrows) were utilized in a study to measure the serum hormone response to 24 h of feed deprivation (FD). Pigs (18 days of age) were weaned and transported to an environmentally controlled room where they were housed in individual pens that consisted of rubber slotted floors and steel partitions. They were allowed free access to feed and water until the start of experiment 1. The diet consisted of a commercial starter feed (MFA Muscle Pig 1 C) which met NRC (1998) requirements. Pigs were monitored for 5 days after weaning and prior to the start of the FD period to ensure that the transition to a solid diet was complete. Four days following weaning, all pigs were fitted with an indwelling jugular vein catheter using a non-surgical procedure (Carroll *et al.* 1999b). Body weights (BW) of pigs were recorded at weaning (18 days of age), 21 days of age, and after the 30-h experimental time point (24 days of age). Pigs were allotted (groups balanced on individual weights) to either the control (CON; $n=4$) or feed-deprived (FD; $n=4$) treatment groups at weaning. Blood was collected from all pigs at 0 h, 6 h, 12 h, 24 h, and 30 h of the experiment. Four blood samples, 15 min apart, were collected at each sampling-hour for assessment of serum ghrelin and insulin-like growth factor-I (IGF-I). Pigs in the CON treatment were allowed free access to feed and water throughout the blood sampling interval. Pigs subjected to the FD treatment had feed removed following the 0 h blood sample and were allowed access to water, but no feed for 24 h. Feed was returned to the FD group following the 24-h sampling interval and additional blood samples were collected at 30 h.

Experiment 2

Thirty-two crossbred barrows obtained from a commercial producer were housed and managed as in experiment 1, except pigs were jugular vein catheterized 9 days post-weaning. Two experimental durations were studied in experiment 2. Starting 10 days post-weaning, 16 pigs were allotted to a 72-h study interval group and 16 pigs were allotted to a 96-h study interval group. Within the 72-h group, eight pigs were assigned free access to feed (CON 72), and eight pigs were feed deprived for 72 h starting at 0 h (FD72). Pigs in the 72-h group were killed at 72 h via captive bolt and tissues were collected. Within the 96-h group, eight pigs were allowed free access to feed (CON 96) and eight pigs were feed deprived for 72 h and then allowed free access to feed until 96 h (RF 24). Pigs in the 96-h group were killed at 96 h and tissues collected.

Feeder weights were taken to estimate feed consumption at weaning (18 days of age), 5 days post-weaning, the day prior to cannulation (approximately 48 h prior to 0 h), 0 h, and at 72 h or 96 h, depending on the treatment group. Body weights were determined at cannulation (approximately 20 to 24 h prior to 0 h) and at death (72 h or 96 h).

Blood samples were collected at 12-h intervals for all groups starting at -12 h and continuing until the pigs were slaughtered for determination of serum ghrelin, IGF-I, cortisol, leptin, and growth hormone (GH). Blood was refrigerated for 12 h, centrifuged, and serum was collected and stored at -80°C until quantification of hormones. Tissues from stomach (corpus), hypothalamus, pituitary, liver, fat, muscle, and adrenal gland were collected immediately after euthanasia, placed on dry ice, and transported to the laboratory where they were stored at -80°C until measurement of mRNA expression of hormones and receptors.

Hormonal analysis

Serum ghrelin was quantified using a commercially available RIA kit (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). The antibody utilized in this assay was a rabbit anti-human ghrelin. The kit protocol was followed with the exception that 25 μl serum were diluted with 75 μl assay buffer for use in the assay. An additional standard point (256 pg/tube) was added to the standard curve and the lowest standard dilution suggested by the kit protocol (1 pg/tube) was not used. Validation of this assay was conducted by verifying parallelism of dilutions of serum and plasma samples with the standard curve. Since this RIA was for use with human plasma, we verified that pig serum and plasma were both suitable matrices. Concentrations of ghrelin in pig serum and plasma were highly correlated ($r=0.997$; $P<0.0001$). Recovery of known amounts of unlabeled human ghrelin diluted in a pool of porcine serum yielded an average recovery of 96.72% of the added amount. All samples from experiment 1 were analyzed in duplicate for ghrelin within one RIA. The mean intra-assay coefficient of variation between duplicates was 5% and the minimum detectable mass was 8 pg/ml. The intra-assay front-to-back drift coefficient of variation for pool samples was 5%. Samples from experiment 2 were analyzed for serum ghrelin concentrations in two assays. Inter- and intra-assay coefficients of variation were 8% and 5% respectively.

Serum concentrations of IGF-I were determined using a non-extraction, coated tube two-site immunoradiometric assay kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The two antibodies utilized in this assay were goat and mouse anti-human IGF-I. Two additional points were added to the standard curve (2 and 4 ng/ml). Dilutions of a pool of porcine serum were analyzed with each assay to assure parallelism with the

standard curve. The recovery of known amounts of unlabeled IGF-I was 83%. Samples from experiments 1 and 2 were analyzed in individual assays and the intra-assay coefficients of variation were 4% and 6% respectively.

Serum concentrations of leptin were determined in samples collected from experiment 2 as previously described and validated (Berg *et al.* 2003). Dilutions of a pool of porcine serum were parallel to the standard curve. All samples were analyzed in a single assay. The intra-assay coefficient of variation was 13%.

Cortisol concentrations were determined using a Coat-A-Count solid phase RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA) that has previously been validated in our laboratory (Daniel *et al.* 1999). Serum samples from experiment 2 were analyzed in one assay. Minimum detectable mass was 2 ng/ml and the intra-assay coefficient of variation was 5%.

Serum GH was quantified using a commercially available kit specific for porcine growth hormone (Linco Research, St Charles, MO, USA). The antibody utilized with this kit was a guinea pig anti-porcine GH. Two additional points were added to the standard curve (0.25 and 0.5 ng/ml). The assay protocol was followed as directed in the kit. Dilutions of both the quality control solution and pig serum pool dilutions were parallel to the standard curve. Recovery of known amounts of unlabeled GH yielded an average recovery of 106% of the added amount. All samples from experiment 1 were analyzed for GH within one RIA. The mean intra-assay coefficient of variation was 6% and the sensitivity was 0.25 ng/ml. The mean intra- and interassay coefficients of variation for experiment 2 were 7% and 9% respectively.

Messenger RNA quantification

Tissue samples from stomach, hypothalamus, pituitary, adrenal gland, muscle, fat, and liver were homogenized in Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) and total RNA was extracted using previously described procedures (Carroll *et al.* 2002). Messenger RNA expression was quantified by slot blot analysis and incubation with biotinylated riboprobes. Tissues and probes used in each were: stomach (28S, ghrelin), muscle (28S, GHrec, IGF-Irec), adrenal gland (28S, ACTHrec), pituitary (28S, ghrelin, POMC, GH, IGF-Irec), hypothalamus (28S, NPY, AGRP, CRH, ghrelin, IGF-Irec), fat (28S, GHrec, leptin, IGF-Irec), and liver (28S, IGF-I, GHrec, IGF-Irec). Membranes were probed using biotinylated riboprobes and a commercially available mRNA detection kit according to manufacturer's instructions (BrightStar System, Ambion, Austin, TX) under conditions optimal for the specific probe. Hybridization signal intensities were quantified by densitometry and target mRNA values were expressed relative to 28S ribosomal RNA for each sample.

The probes and procedures for detection of mRNA specific for pro-opiomelanocortin (POMC) and adrenocorticotropin (ACTH) receptor (Daniel *et al.* 1999), corticotropin releasing hormone (CRH) (Carroll *et al.* 2002), leptin (Lin *et al.* 2000), GH (Matteri & Carroll 1997), GH receptor (Carroll *et al.* 1999a), orexin (Dyer *et al.* 1999), and IGF-I (Matteri *et al.* 1997) have previously been described. PCR was used to amplify cDNA for agouti-related protein (AGRP), neuropeptide-Y (NPY), ghrelin, IGF-I receptor and 28S ribosomal RNA. The up- and downstream oligonucleotide primers for PCR amplification were 5' GCCCACTGAAGAAGACAAC 3' and 5' GTACCCAGCTTGCGGCAGTA 3' for AGRP (650 bp), 5' ACCCTCGCCCTGTCCCTGCT 3' and 5' ATGTGGTGATGGGAAATGAG 3' for NPY (269 bp), 5' TACTCGTGGCAGACTTGGC 3' and 5' TTAG GAGGCTGGGAGAACA 3' for ghrelin (353 bp), 5' GAGAGCAGAGTGGATAACAA 3' and 5' CGGGT TCACAGAGGCATACA 3' for IGF-I receptor (600 bp), and 5' GACCGTGAAGCGGGGCCTC 3' and 5' CAGGTTAGTTTTACCCTACT 3' for 28S rRNA (230 bp). The PCR products were cloned into vectors and the identities of the cDNA clones identified by dideoxy termination sequencing. Biotinylated riboprobes were synthesized from clones for use in chemiluminescence-based detection (BrightStar System, Ambion).

Statistical analysis

Hormonal data were analyzed by analysis of variance specific for repeated measures and mean comparisons were conducted using Fisher's Protected Least Significant Differences with the StatView statistical analysis program (SAS, Cary, NC, USA). Hormonal data that were expressed as a percentage of 0 h were log transformed prior to statistical analysis; however, results are presented as a percentage to facilitate interpretation of the data. Hormonal data in experiment 2 from the two CON groups were combined as were the two FD groups in samples collected before the 72-h time point. The change within group over time was also analyzed. Main effects were time (–12 h, 0 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h) and group (CON and FD). The effect of a.m. versus p.m. samples was included in the cortisol data. Effect of a.m./p.m. was not different in leptin, IGF-I, or GH data and thus was removed from the model. The data from CON96 and FD72/FR24 were compared using the 72-h, 84-h, and 96-h time points to detect hormonal response due to feed return. Messenger RNA measurements were analyzed using ANOVA with treatment as the main effect. There was no treatment difference between CON72 and CON96 treatments – therefore data from these groups were combined and compared with the FD72 and FD72/RF24 treatments. Calculations of correlations utilized Fisher's *r* to *z* test.

Results

Experiment 1

The initial weight of the pigs did not differ between CON and FD treatments (5.7 ± 0.5 kg and 5.8 ± 0.3 kg respectively; $P=0.7$). However, there was a treatment $\times$ time effect on BW ($P<0.05$) with FD pigs weighing less than CON pigs at the end of the experiment because FD pigs gained less weight than CON pigs from day 3 to day 6 post-weaning (0.4 ± 0.1 kg vs 1.0 ± 0.2 kg respectively; $P<0.05$).

Serum concentrations of ghrelin did not differ among the four sub-samples collected for each hour-point sampled ($P>0.9$), therefore the mean concentration of these sub-samples was utilized for statistical analysis. Serum concentrations of ghrelin at 0 h tended ($P=0.06$) to be different between treatments, therefore ghrelin concentrations were expressed as a percentage of the 0 h concentration and log-transformed prior to statistical analysis. Additionally, there was a time $\times$ treatment effect on relative concentrations of ghrelin ($P<0.01$; Fig. 1). Serum ghrelin tended to be lower in FD pigs than in CON pigs at 12 h ($P=0.08$). Subsequently, relative concentrations of ghrelin increased in FD pigs at 24 h and 30 h ($P<0.01$). Serum ghrelin did not change throughout the experiment in CON pigs ($P=0.91$).

Serum IGF-I did not differ among the four sub-samples collected at each hour-point sampled ($P>0.9$), therefore the mean concentration was used for statistical analysis. There was a time $\times$ treatment effect on serum IGF-I ($P<0.001$) such that IGF-I was lower in FD pigs at 12 h, 24 h, and 30 h than in CON pigs ($P<0.05$; Fig. 1). Serum IGF-I increased in FD pigs (at 30 h) following their refeeding at 24 h ($P<0.06$). Serum concentrations of IGF-I increased throughout the experiment in CON pigs ($P<0.01$).

Experiment 2

There were no differences among treatment groups in feed consumption or body weights prior to the initiation of the feed deprivation ($P>0.99$). However, there was an increase in daily feed consumption throughout the three time points measured from weaning to initiation of feed deprivation ($P<0.001$). During the feed deprivation period for FD72 and FD72/RF24 there was no difference in daily feed consumption between the CON72 and CON96 groups. When feed was returned to the FD72/RF24 pigs, their 24-h feed intake did not differ from that observed in the CON96 pigs ($P=0.48$; Table 1). Final BW of pigs differed among treatments ($P=0.02$). Pigs in the CON72 and CON96 groups were heavier ($P<0.01$) than FD72 pigs. Pigs in the FD72/RF24 group were intermediate in BW and did not differ from the other three treatment groups ($P>0.14$). Pig weight gain or loss

Figure 1 Effect of *ad libitum* feeding (CON) or feed deprivation (FD) followed by re-feeding on serum ghrelin (top panel) expressed as a percentage of the 0 h concentration and on serum IGF-I concentrations (bottom panel) in experiment 1. Values are means $\pm$ S.E.M. of $n=4$ pigs per group. Pigs in the FD treatment were given access to *ad libitum* feed after the 24 h sample collection. *Denotes difference between FD and CON treatments ($P=0.08$); †denotes a difference in FD pigs from the 12-h time point to the 24-h time point ($P<0.01$); **denotes difference between FD and CON treatments ($P<0.01$); ‡ denotes difference in FD pigs from the 24-h time point to the 30-h time point ($P<0.06$).

from -12 h to euthanasia differed among treatments ($P<0.0001$). Pigs in the FD72 treatment lost an average of 1.2 ± 0.2 kg during the experimental period while the FD72/RF24 pigs gained 0.4 ± 0.2 kg, presumably from gut fill after re-feeding for 24 h. Weight gain did not differ between the two control groups ($P=0.35$; Table 1).

Prior to 72 h, the CON72 and CON96 pigs were treated identically as were the FD72 and FD72/RF24 pigs. Serum ghrelin did not differ between the two CON groups ($P=0.53$), or between the two FD groups ($P=0.41$), therefore, the CON72 and CON96 data were combined as were the FD72 and FD72/RF24 data. Analysis of data from 72 h to 96 h included only the CON96 and FD72/RF24 treatments. The main effect of 'feeding' (CON vs FD) was assigned and tested on combined treatment data. There was a time $\times$ feeding effect on serum ghrelin concentrations ($P<0.001$). The -12 h ghrelin concentration tended ($P=0.06$) to differ between the CON and FD groups, therefore, serum

Table 1 Body weight (BW) and feed consumption for pigs fed *ad libitum* for 72 h (CON72), or 96 h (CON96) compared with pigs feed-deprived for 72 h (FD72) or feed-deprived for 72 h and re-fed for 24 h (FD72/RF24) in experiment 2. Values are means $\pm$ S.E.M.

	CON72	FD72	CON96	FD72/RF24
Body weight (kg)				
Cannulation BW	10.09 $\pm$ 0.72	10.01 $\pm$ 0.67	10.05 $\pm$ 0.73	10.05 $\pm$ 0.65
72-h BW	11.84 $\pm$ 0.76 ^a	8.85 $\pm$ 0.59 ^b		
96-h BW			12.06 $\pm$ 0.85	10.46 $\pm$ 0.77
Average daily gain	0.58 $\pm$ 0.07 ^a	-0.38 $\pm$ 0.05 ^b	0.50 $\pm$ 0.05 ^a	0.10 $\pm$ 0.05 ^c
Feed/gain	1.03 $\pm$ 0.24	NE	1.02 $\pm$ 0.09	1.36 $\pm$ 0.83
Final weight gain	1.76 $\pm$ 0.21 ^a	-1.16 $\pm$ 0.16 ^b	2.01 $\pm$ 0.20 ^a	0.41 $\pm$ 0.19 ^c
Daily feed consumption (kg)				
Day 18–day 23	0.11 $\pm$ 0.03	0.14 $\pm$ 0.04	0.18 $\pm$ 0.02	0.10 $\pm$ 0.02
Day 23–day 26	0.24 $\pm$ 0.05	0.24 $\pm$ 0.03	0.25 $\pm$ 0.05	0.25 $\pm$ 0.04
Day 26–day 28	0.39 $\pm$ 0.06	0.45 $\pm$ 0.04	0.45 $\pm$ 0.08	0.39 $\pm$ 0.02
Day 28–Euthanasia	0.51 $\pm$ 0.04	0.00 $\pm$ 0.00	0.50 $\pm$ 0.06	0.56 $\pm$ 0.06*

a,b,c Means in a row with different superscripts are different ($P < 0.05$).

*FD72/RF24 consumption during 72–96 h realimentation.

NE, Not estimable.

Figure 2 Effect of feed deprivation for 72 h (FD72) followed by re-feeding until 96 h (FD96/RF24) on serum ghrelin (expressed as a percentage of the 0 h concentration) compared with *ad libitum* feeding for 72 h (CON72) or 96 h (CON96) in experiment 2. Values are means $\pm$ S.E.M. of $n=8$ pigs per group. Pigs in the CON72 and FD72 groups were killed for tissue collection at 72 h and thus were not included in the subsequent data. * Denotes difference from the 0 h concentration within the same treatment ($P < 0.05$); † denotes a difference in FD pigs from the 12-h time point to the 36-h and 48-h time points ($P < 0.01$).

ghrelin was expressed relative to the mean of the -12 h and 0 h concentrations which were assigned a value of 100% and log transformed prior to statistical analysis. Concentrations of ghrelin in the FD groups differed throughout time ($P < 0.001$; Fig. 2) and when presented as values relative to 0 h decreased ($P < 0.03$) to 89% at 12 h, then increased ($P < 0.01$) to 112% at 36 and 48 h, then decreased ($P < 0.001$) to 75% at 72 h. When relative concentrations of ghrelin in CON96 and FD72/RF24 groups were compared at 72 h, 84 h, and 96 h, there was no effect of time or treatment ($P=0.32$ and 0.30 respectively; Fig. 2).

The CON72 and CON96 pigs did not differ in expression of ghrelin mRNA ($P > 0.62$), therefore their data were combined into one group and compared with

the FD72 group. There was a tendency for stomach ($P=0.06$), pituitary ($P=0.07$), and hypothalamic ($P=0.08$) ghrelin mRNA to be lower in the FD72 pigs compared with the CON pigs. Serum ghrelin concentrations at 72 h were positively correlated with ghrelin mRNA levels in stomach tissue ($r=0.5$; $P < 0.05$), but not in the pituitary ($P=0.4$) or hypothalamus ($P=0.2$). Expression of ghrelin mRNA in stomach and pituitary tissue was positively correlated with pituitary expression of GH ($r=0.6$ and 0.8 respectively; $P < 0.01$) when data from the CON72 and FD72 treatments were analyzed collectively. This relationship was not observed when comparing the CON96 and FD72/RF24 treatments. Upon refeeding, the FD72/RF24 pigs tended ($P=0.09$) to have greater expression of ghrelin mRNA in stomach tissue than FD72 pigs.

There was a time $\times$ feeding effect on serum IGF-I ($P < 0.001$; Fig. 3). At 24 h, IGF-I was lower in the FD pigs than CON pigs ($P=0.02$) and continued to decrease at each time point through 36 h ($P < 0.05$). At 48 h onward, serum concentrations of IGF-I did not decrease significantly in the FD pigs; however IGF-I continued to decline slowly to its lowest concentration of 13 ng/ml at 72 h. On the other hand, IGF-I in the CON pigs increased throughout the experiment ($P < 0.001$) to a peak of 215 ng/ml at 60 h. Following the return of feed at 72 h there was a time $\times$ treatment effect on IGF-I. Concentrations of IGF-I in the FD72/RF24 pigs tended to increase ($P=0.08$) at 84 h and were significantly greater at 96 h ($P < 0.02$). Concentrations of IGF-I in the CON96 pigs remained constant during this interval ($P > 0.6$). Expression of IGF-I receptor mRNA in adipose tissue was lower in the FD72 pigs compared with the CON pigs ($P < 0.01$). There was no change in IGF-I ($P > 0.5$), IGF-I receptor ($P > 0.3$), or GH receptor mRNA ($P > 0.30$) in the liver due to FD; however, GH receptor mRNA in fat

Figure 3 Effect of feed deprivation for 72 h (FD72) and FD72 followed by re-feeding until 96 h (FD72/RF24) on serum concentrations of IGF-I compared with *ad libitum* feeding for 72 h (CON72) or 96 h (CON96) in experiment 2. Values are means $\pm$ S.E.M. of $n=8$ pigs per group. Pigs in the CON72 and FD72 groups were sacrificed for tissue collection at 72 h and thus were not included in the subsequent data. *Denotes difference from the 0 h concentration within the same treatment ($P<0.05$); † denotes difference in FD pigs from the 72-h time point to the 96-h time point ($P<0.05$).

Figure 4 Effect of feed deprivation for 72 h (FD72) and FD72 followed by re-feeding until 96 h (FD72/RF24) on serum concentrations of leptin compared with *ad libitum* feeding for 72 h (CON72) or 96 h (CON96) in experiment 2. Values are means $\pm$ S.E.M. of $n=8$ pigs per group. *Denotes difference from the 0 h concentration within the same treatment ($P<0.05$); † denotes change in hormone concentrations of the FD72/RF24 treatment compared with the 72-h time point ($P<0.05$).

tissue from both the FD72 and FD72/RF24 pigs was lower than in the CON72 and CON96 pigs ($P<0.01$). Expression of IGF-I receptor mRNA in fat tissue also followed this same trend, with FD72 and FD72/RF24 pigs having a lower expression than CON72 and CON96 pigs ($P<0.01$).

There was a time $\times$ feeding effect on serum concentrations of leptin ($P<0.001$; Fig. 4). Pigs in the FD group had lower serum leptin than CON pigs ($P<0.05$). By 12 h, leptin was lower in FD pigs than CON pigs ($P<0.01$) and remained lower throughout the 72 h time period. Leptin decreased in FD ($P<0.01$) and CON ($P<0.08$) pigs from -12 h to 0 h, and again from 0 h to 12 h ($P<0.001$) in the FD pigs. Leptin remained unchanged after 12 h in FD pigs ($P>0.20$). There was a general trend for leptin to be greater at -12 h, and at 12 h than at 48 h, 60 h, or 72 h ($P<0.07$) in CON pigs.

Figure 5 Effect of feed deprivation for 72 h (FD72) and FD72 followed by re-feeding until 96 h (FD72/RF24) on serum concentrations of cortisol compared with *ad libitum* feeding for 72 h (CON72) or 96 h (CON96) in experiment 2. Values are means $\pm$ S.E.M. of $n=8$ pigs per group. *Denotes difference from the 0 h concentration within the same treatment ($P<0.05$); ‡ denotes change in hormone concentrations of CON96 and FD72/RF24 treatments compared with the 72-h time point ($P<0.05$); † denotes change in hormone concentrations of the FD72/RF24 treatment compared with the 72-h time point ($P<0.05$).

The -12 h and 12 h time points corresponded to p.m. blood sampling periods. Serum leptin in CON pigs appeared to be influenced by diurnal rhythms but there was no difference, however, between a.m. and p.m. samples ($P=0.2$). After refeeding, leptin increased in FD72/RF24 pigs ($P<0.001$) to 84 h. Leptin did not change from 84 h to 96 h ($P=0.6$) and was not different from CON96 pigs ($P=0.6$). Leptin mRNA expression in fat tissue was lower in the FD72 pigs compared with CON pigs ($P=0.03$) and remained low in the FD72/RF24 pigs at 96 h ($P<0.05$).

There was a time $\times$ feeding $\times$ a.m./p.m. effect on serum cortisol ($P<0.0001$; Fig. 5). The -12 h and 0 h cortisol levels were not different between treatments ($P>0.3$). Cortisol increased in FD pigs by 12 h ($P<0.04$) and continued to increase up to 24 h ($P<0.01$) after which cortisol remained elevated up to 72 h. Cortisol in pigs in the CON treatment remained relatively unchanged throughout the experiment; however patterns of cortisol in pigs in both treatment groups demonstrated a diurnal rhythm. Samples taken in the a.m. were higher in cortisol than samples taken during the p.m. ($P<0.001$). This diurnal variation accounted for the variation observed in CON pigs, which appeared to increase as the experiment progressed. There were differences at each consecutive time point from 24 h to 96 h in CON pigs with the a.m. cortisol concentrations consistently greater than the p.m. concentrations. This same pattern was observed in the FD pigs from 36 h to 60 h. Following the return of feed to the FD72/RF24 pigs at 72 h, cortisol decreased to concentrations which were not different from the CON96 pigs at 84 h ($P=0.8$) or 96 h ($P=0.5$).

There were no differences detected in serum GH concentrations between feeding group ($P=0.13$), time

($P=0.99$), or the interactive effects ($P=0.4$). There was a greater amount of variation observed among times in both the CON and FD groups. There also was no difference during the 72 h to 96 h time points for the feeding ($P=0.3$), time ($P=0.1$), and interactive effects ($P=0.11$). Pituitary GH mRNA levels did not differ among treatments ($P>0.5$), nor did IGF-I receptor mRNA levels ($P>0.3$). Expression of mRNA for GH receptor was lower in the FD72/RF24 pigs compared with the CON and FD pigs ($P<0.05$). Expression of IGF-I receptor mRNA followed this same pattern with FD72/RF24 pigs expressing lower levels than CON or FD pigs ($P=0.05$).

Expression of mRNA encoding hypothalamic proteins AGRP ($P=0.2$), CRH ($P=0.6$), NPY ($P=0.6$), or orexin ($P=0.5$) were not affected by FD, nor was hypothalamic IGF-I receptor mRNA ($P=0.5$). Hypothalamic ghrelin mRNA expression was positively correlated ($P<0.01$) with hypothalamic expression of AGRP mRNA ($r=0.8$ and 0.7) and NPY mRNA ($r=0.8$ and 0.7) at both 72 h and 96 h respectively. Expression of mRNA for POMC tended to be lower in the FD72 pigs compared with the CON72 pigs ($P=0.06$). There were no treatment effects on ACTH receptor mRNA in the adrenal gland.

Discussion

The interval of feed deprivation utilized in the present experiments is not unlike that traditionally observed during weaning in production systems. The fact that there was an effect on live weight gain during the 24 h feed deprivation period verifies that there was a quantifiable effect of this length of feed deprivation on BW. In newly weaned pigs, the increase in weight gain around weaning is delayed or even reversed and feed intake is less than that needed for maintenance (Riley 1989).

Ghrelin is a recently discovered hormone that has previously been shown to be produced predominantly by cells located in oxyntic glands of the stomach in rats and humans (Kojima *et al.* 1999). It is thought that ghrelin has two primary roles, one of which may be as a GH releasing agent (Seoane *et al.* 2000, Tolle *et al.* 2001, Arvat *et al.* 2000) and the second of which is the regulation of appetite (Tschop *et al.* 2000, Wren *et al.* 2000). These two functions are thought to be independent of each other (Tschop *et al.* 2000, Nakazato *et al.* 2001). Ghrelin has also been isolated in domestic animals, namely the Shiba goat and cow (Hayashida *et al.* 2001). Serum ghrelin in both experiment 1 and experiment 2 followed the same general pattern during feed deprivation. This was characterized by a brief suppression of ghrelin at 12 h and then an increase from 12 h to its plateau at 36 h and 48 h. This is consistent with studies that show ghrelin increases in the peripheral circulation during fasting (Tschop *et al.* 2000) in rodents, and after an overnight fast in anorectic humans (Cuntz *et al.* 2001). Serum ghrelin in experiment 2 decreased from

48 h to 72 h. This was consistent with the lower mRNA expression for ghrelin we detected in the stomach at 72 h. Studies utilizing rodents have shown that ghrelin mRNA from stomach tissue increases following a 48-h fast (Toshinai *et al.* 2001). This is consistent with the higher serum concentration of ghrelin we observed at 48 h in pigs. Concentration of ghrelin in stomach tissue is reduced after fasting in rats (Toshinai *et al.* 2001) and the low levels of serum ghrelin we observed at 72 h may reflect the inability of ghrelin synthesizing cells in the stomach to produce sufficient ghrelin to maintain serum concentrations at a high level.

To our knowledge, these specific hormonal responses to absolute food deprivation of the duration utilized in experiment 2 (72 h) have not previously been reported in pigs. There may be differences in response between absolute feed deprivation and feed restriction. Since ghrelin stimulates gastric acid secretion and motility in rats (Matsuda *et al.* 2000), it is logical to assume a chronic and total lack of feed would eventually reduce the need for gastric acid secretion and gut motility. Central and intraperitoneal injections of ghrelin have been reported to be anxiogenic in mice, and intraperitoneal injections increase both CRH mRNA in the hypothalamus and serum corticosterone levels (Asakawa *et al.* 2001). Thus, a decrease in serum ghrelin during a prolonged interval of feed deprivation may prevent acid-induced gastrointestinal disease and may represent an adjustment to the stressor.

Serum ghrelin tended to increase following re-feeding; thus the return to baseline concentrations of circulating ghrelin may be due to the need to re-initiate gastric activity. The fact that ghrelin was able to increase following refeeding diminishes the likelihood that the biosynthetic capacity was at its maximum level at 72 h; however, due to the similarity in response of the CON96 and FD72/RF24 pigs during this phase of the study, we cannot say with certainty that the increased ghrelin in the FD72/RF24 pigs was solely due to feed return and consumption. Ghrelin mRNA expression tended to increase after refeeding.

Other metabolic factors may, therefore, be the causative agents that act to reduce ghrelin during the later phases of feed deprivation. Exogenous ghrelin treatment has been reported to acutely increase plasma glucose and lower insulin (Broglia *et al.* 2001), and alternatively, exogenous insulin as well as leptin has been reported to increase expression of ghrelin mRNA (Toshinai *et al.* 2001).

Leptin produced from adipocytes is thought to play a role in signaling body composition status to the brain (reviewed by Keisler *et al.* 1999). We observed a decrease in serum leptin following feed removal at 12 h. This was similar to other studies that found leptin was depressed following a short-term fast (Bergendahl *et al.* 2000). The fact that leptin mRNA expression in fat tissue was lower in feed-deprived animals is consistent with other studies utilizing pigs (Spurlock *et al.* 1998, Barb *et al.* 2001). The

decrease in leptin in recently weaned pigs utilized in experiment 2 was rather dramatic and occurred within 12 h. This may be due to differences in percentage body fat and/or differences in metabolism between adult and young animals. Upon refeeding, serum leptin concentrations increased within 12 h. Leptin mRNA expression was not increased following refeeding for 24 h. There is evidence that insulin may be the acute regulator of leptin in rodents (Saladin *et al.* 1995) and pigs (Spurlock *et al.* 1998). It has also been hypothesized that regulation of leptin secretion is accomplished via neural inputs into adipose tissue (Youngstrom & Bartness 1995). Although diurnal patterns of leptin are thought to exist in humans (Sinha *et al.* 1996), the present study failed to support this in pigs.

Leptin reduces expression of NPY and AGRP in neurons in the arcuate nucleus (Elias *et al.* 1999). We did not demonstrate a correlation between serum leptin (at the time of tissue collection) and hypothalamic content of AGRP and NPY mRNA; however AGRP and NPY mRNA were positively correlated at 72 h, and hypothalamic content of ghrelin mRNA was highly correlated with both AGRP and NPY mRNA. The relative roles of stomach, pituitary, and hypothalamic ghrelin have yet to be defined, but ghrelin does seem to act, in part, by activation of these neuropeptide systems (Wren *et al.* 2001). Ghrelin receptors are expressed on hypothalamic NPY/AGRP neurons and may play a role in the central regulation of energy balance (Howard *et al.* 1996). It is interesting to note that administration of an NPY-Y1 antagonist reverses a ghrelin-induced increase in food intake (Shintani *et al.* 2001) but administration of a ghrelin agonist Growth Hormone Releasing Peptide-2 does increase weight gain in NPY (–/–) mice (Tschöp *et al.* 2002) while increasing AGRP mRNA. These seemingly contradictory results may be explained if different subtypes of ghrelin receptors specific for appetite and GH release exist.

Orexin mRNA expression was not affected by feeding regimen. Fasting upregulates orexin gene expression in the hypothalamus, and orexin is thought to stimulate appetite through NPY (Sakurai *et al.* 1998). It is likely that orexin stimulates vagal activity and modulates the response to gastrointestinal motility and secretion as well as plays a role in other cephalic phase reflexes (Kirchgessner 2002). Orexin is not stimulated under all conditions of hunger and may play a role in short-term feeding behavior stimulated by a fall in serum glucose (Cai *et al.* 2001). Tissue collected in the present study (at 72 h and 96 h) may have been after changes in orexin expression had already taken place and animals readjusted to long-term feed deprivation, rather than short-term. The pattern of serum ghrelin expression observed in the present studies substantiates the possibility of a diminution of hunger-related hormones after an acute hunger phase has passed.

The post-translational processing of POMC gives rise to a range of bioactive peptides, including ACTH and melanocyte stimulating hormones (MSH) that bind to their receptors and suppress feed intake. Humans with an inactivating defect of the POMC gene (Krude 1998) or a specific MSH receptor (Huszar *et al.* 1997) are typically obese. Feed deprivation down-regulates expression of POMC in rodents (Brady *et al.* 1990). This is consistent with our results that support a strong tendency for pituitary POMC expression to be lower in the FD72 pigs compared with their CON72 contemporaries. Schwartz and co-workers (2000) previously noted that leptin acts to up-regulate POMC in an effort to decrease feed intake by the animal. Glucocorticoids may also differentially regulate POMC expression in various tissues. Glucocorticoids inhibit POMC expression in the pituitary; however they up-regulate POMC expression in the hypothalamus (Pritchard *et al.* 2002). Our studies found a negative correlation between pituitary POMC expression at 72 h and serum concentrations of cortisol at 72 h. However, it may be an oversimplification to emphasize POMC gene expression alone since there are other regulating factors that may differentially affect post-translational modification events of POMC that are as yet unknown.

Feed deprivation increased serum cortisol in the current study. A feedback relationship between glucocorticoids and NPY is thought to exist since glucocorticoid administration increases NPY expression in rats (Hisano *et al.* 1988) and we have previously reported that glucocorticoid administration to neonatal pigs increases weight gain (Gaines *et al.* 2002). The hypothalamic releasing factor, CRH, is the primary regulator of ACTH and subsequent cortisol release, but may also elicit direct anxiogenic and anorectic effects (Eaves *et al.* 1985). Urocortin is a neuropeptide related to CRH and binds to a CRH receptor subtype with high affinity. This hormone is more potent than CRH at suppressing appetite but does not elicit anxiogenic activities to the same extent as CRH (Spina *et al.* 1996). Peripherally administered ghrelin increases CRH mRNA and serum corticosterone, but not urocortin mRNA expression in the hypothalamus of mice (Asakawa *et al.* 2001). This separation of the anxiogenic and anorectic actions may play a very important role during the acute hunger phase of feed deprivation since a chronic stressor such as tail pinch or starvation has been shown to increase ghrelin expression, and appetite (Rowland 1976, Asakawa *et al.* 2001). An effect of feed deprivation on hypothalamic expression of CRH at the tissue collection times in these studies was not detected. The responses described may have taken place prior to the 72-h tissue collection period of this study. Due to the lack of feed during the feed deprivation period, an adjustment or downregulation of physiological responses to the stressor may have taken place. Expression of numerous appetite regulating peptides in the hypothalamus at 72 h were positively correlated with hypothalamic expression of

CRH mRNA including AGRP, ghrelin, NPY, and orexin.

An effect of FD on serum GH was not detected in experiment 2. Serial blood samples collected in experiment 1 were too few in number to reach any reliable conclusions and serial blood sampling in experiment 2 was not conducted. The fact that an effect of feed deprivation on GH secretion was not detected is not unusual in pigs (Booth 1990, Barb *et al.* 2001). The pulsatile nature of GH secretion may necessitate more frequent sampling over a longer period of time.

Expression of GH receptor in adipose tissue was lower in FD72 and FD72/RF24 pigs compared with CON72 and CON96 pigs. There were no differences in hepatic expression of GH receptor mRNA in the current study. This contradicts previous findings that indicate feed restriction decreases hepatic expression of GH receptor mRNA and increases expression of GH receptor mRNA in muscle (Weller *et al.* 1994). The differences in study findings may be due to total versus partial feed restriction or the specific muscle sampled (Katsumata *et al.* 2000).

A decrease in serum IGF-I was observed within 12 h in the FD group of pigs. This pattern of IGF-I suppression is similar to that observed during weaning (Matteri *et al.* 2000). There were no differences in mRNA expression for IGF-I in liver tissue. Matteri and coworkers (2000) reported similar results following weaning and hypothesized that various tissues may contribute to the total circulating quantity of IGF-I. The relative roles of the IGF-binding proteins are not yet fully known and may contribute to apparent discrepancies observed between liver IGF-I expression and serum concentrations of this hormone in young pigs (Lee *et al.* 1993). Another possible explanation is that alterations in IGF-I gene expression occur over a longer period of time than observable in the present studies. This may be due to IGF-I being more sensitive to a long-term (weeks) nutritional perturbation, rather than a short-term (days) feed restriction (Weller *et al.* 1994). Metabolic and endocrine differences in response to feed deprivation also depend on the age of the animals, since young animals have less energy reserves and have greater growth requirements (Barb *et al.* 1997). Serum IGF-I increased throughout experiment 2 in CON72 and CON96 pigs, in agreement with the postnatal increases in IGF-I observed by Lee *et al.* (1993).

Receptor numbers and mRNA expression of IGF-I in the liver decrease throughout the postnatal period of a pig's life and are not different than other non-hepatic tissues such as skeletal muscle, lung, or kidney (Lee *et al.* 1993). The mechanism that accomplishes an increase in serum IGF-I, while a decrease in mRNA for IGF-I occurs has not been defined, but may involve the transition from IGF-binding protein 2 to IGF-binding protein 3 and/or a decrease in plasma clearance rates. Fat tissue content of IGF-I receptor mRNA was lower in FD72 and FD72/RF24 pigs compared with CON72 and CON96 pigs in

the current study. There is limited information available regarding the regulation of IGF-I receptors in adipose tissue; however the imposition of feed deprivation in trout also decreases adipose tissue IGF-I receptors (Planas *et al.* 2000). Feed deprivation has also been reported to modestly increase IGF-I receptors in the pituitary glands of rats and decrease hypothalamic content of IGF-I (Olchovsky *et al.* 1993); however feed deprivation in the current study did not alter mRNA for IGF-I receptors in either the hypothalamus or pituitary gland of pigs.

In summary, several physiological systems play a role in the transition from a liquid milk diet to a diet consisting of solid food and water. Methods to minimize the stress or stimulate feeding behavior earlier following weaning may decrease the interruption in weight gain around weaning and may reduce the days-to-market weight. Based on the current findings, administration of appetite-inducing factors such as ghrelin may aid in the transition to solid food consumption and merits further investigation. Achieving a positive energy balance more quickly may also lessen the susceptibility to pathological or thermal stressors since immune function and thermogenesis is dependent on sufficient energy stores.

Acknowledgements

The authors thank Jim Ortals, Kurt Holiman, and Paul Little for their technical assistance. Mention of a trade name or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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Received 15 May 2003
Accepted 23 May 2003
Made available online as an
Accepted Preprint 4 June 2003