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Changes in the expression of uncoupling proteins and lipases in porcine adipose tissue and skeletal muscle during feed deprivation☆

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Abstract

The hormone-sensitive and lipoprotein lipases are critical determinants of the metabolic adaptation to starvation. Additionally, the uncoupling proteins have emerged with potential roles in the metabolic adaptations required by energy deficiency. The objective of this study was to evaluate the expression (mRNA abundance) of uncoupling proteins 2 and 3 and that of hormone-sensitive and lipoprotein lipase in the adipose tissue and skeletal muscle of the pig in relationship to feed deprivation. Thirty-two male castrates (87 kg ± 5%) were assigned at random to fed and feed-deprived treatment groups. After 96 hr, the pigs were euthanized and adipose and skeletal muscle tissue obtained for total RNA extraction and nuclease protection assays. Feed deprivation increased uncoupling protein 3 mRNA abundance 103–237% ($P < 0.01$) in longissimus and red and white semitendinosus muscle. In contrast, the increase in uncoupling protein 3 mRNA in adipose tissue was only 23% ($P < 0.06$), and adipose uncoupling protein 2 mRNA was not influenced ($P > 0.66$) by feed deprivation. The increased abundance of uncoupling protein 2 mRNA in the longissimus muscle of feed-deprived pigs was small (22%), but significant ($P < 0.04$). The expression of hormone-sensitive lipase was increased 46% and 64% ($P < 0.04$) in adipose tissue and longissimus muscle, respectively, by feed deprivation, whereas adipose lipoprotein lipase expression was reduced ($P < 0.01$) to 20% of that of the fed group. Longissimus lipoprotein lipase expression in the feed-deprived group was 37% of that of the fed group ($P < 0.01$), and similar reductions were detected in red and white semitendinosus muscle. Overall, these findings indicate that uncoupling protein 3 expression in skeletal muscle is quite sensitive to starvation in the pig, whereas uncoupling protein 2 changes are minimal. Furthermore, we conclude that hormone-sensitive lipase is upregulated at the mRNA level with prolonged feed deprivation, whereas lipoprotein lipase is downregulated.

Keywords: pig; uncoupling proteins; lipase; starvation

1. Introduction

Starvation causes a homeorrhetic response in which coordinate changes in the expression of key regulatory genes and in the activities of certain metabolic enzymes change energy metabolism in peripheral tissues to facilitate survival. Energy is provided by fatty acids mobilized from triglyceride stores in adipose depots by the action of hormone-sensitive lipase (HSL), the primary mediator of the lipolytic pathway [1]. Surprisingly, the uncoupling proteins (UCP) are perhaps linked to this metabolic adaptation, at least in rodent models. The UCP provide a channel for proton leak which allows energy to be dissipated as heat rather than captured in the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) [2]. In light of the original view that the UCP function only in heat production, increased UCP-2 and/or UCP-3 expression during starvation [3,4] presents an interesting paradox as starvation requires conservation of energy rather than increased heat production. This paradox has led to speculation that the UCP, and UCP-3 in particular, have roles apart from thermogenesis. Specifically, the UCP may be upregulated during metabolic circumstances such as starvation in which fatty acid oxidation is increased [5–7]. In rodents, the regulation of thermogenesis in brown adipose tissue is a critical component of the control of heat production and energy

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expenditure. However, the pig and human have only negligible numbers of brown adipocytes [8], and the metabolic adaptations to energy deficiency may differ from those of rodents. The primary objective of this study was to determine the extent to which prolonged feed deprivation (96 hr) altered the expression of UCP-2 and UCP-3 in the adipose tissue and skeletal muscle of the pig. In addition, we wanted to test the hypothesis that starvation alters the expression of HSL and lipoprotein lipase (LPL) in these tissues.

2. Methods and materials

2.1. Animal protocol

The experiment was conducted at the Purina Mills, Inc. Research Center, Gray Summit, MO USA, and was approved by the institutional animal care and use committee. Thirty-two pigs (male castrates) weighing 87 kg (±5%) were allotted to two treatment groups: to be allowed ad libitum feed consumption or to be feed-deprived of feed, for 96 hours. The pigs allowed ad libitum consumption were fed a commercial-type corn–soybean meal diet, and all pigs had free access to water. The pigs were kept in individual pens in an environmentally controlled room. When the study was terminated, blood samples were obtained for serum recovery by jugular venipuncture, and the pigs killed by exsanguination following mechanical stunning. Samples of longissimus and semitendinosus muscle and subcutaneous adipose (from the depot located over the cervical spine) were removed immediately. The deep red and superficial white components of the semitendinosus were separated for independent analysis to address the possibility that muscle fiber-type influences the UCP response. All samples were cubed, frozen in liquid nitrogen, and stored at −80°C pending total RNA extraction.

2.2. Serum chemistry

With the exception of insulin, serum variables were quantified using an automated clinical chemistry analyzer (Hitachi Model 704, San Jose, CA USA) and commercially available assay kits. Glucose, triglyceride, and urea nitrogen kits were purchased from Sigma Chemical Co. (St. Louis, MO USA), and nonesterified fatty acids (NEFA) kits were purchased from Wako Chemical (Richmond, VA USA). Insulin concentrations were determined using a commercially available porcine insulin kit and protocol (ALPCO, Windham, NH USA).

2.3. Total RNA isolation

Total RNA was extracted from all tissues as described by Chomczynski and Sacchi [9]. Briefly, tissue was homogenized in guanidinium thiocyanate, followed by the addition of sodium acetate. The samples were then extracted sequentially with water-saturated phenol and chloroform:isoamyl alcohol, and the aqueous fractions precipitated with isopropanol. After the second ethanol precipitation, the RNA pellets were resuspended in Tris-EDTA buffer and quantitative and qualitative measures obtained spectrophotometrically. In addition, the actual RNA concentration of sample was determined using the RiboGreen® assay (Molecular Probes, Eugene, OR USA) and the manufacturer's protocol.

2.4. Riboprobe construction and ribonuclease protection assays

The relative mRNA abundance of the transcripts of interest was measured using ribonuclease protection assays. The reverse transcription-polymerase chain reaction was used to generate cDNA templates for porcine LPL, HSL, and UCP-2 and UCP-3. The antisense primers for each gene were added to the T7 promoter sequence (5′-GGATC CTAAT ACGACTCACT ATAGGGAGG-3′) to allow riboprobe transcription under the control of T7 polymerase. The primer sequences are shown in *Table 1*. As regards UCP-3, the short form of this transcript lacks 37 C-terminal amino acids as a result of premature termination of the mRNA elongation [10]. Accordingly, our probe was designed to target a region common to both forms so that we could detect both transcripts. The identities of the PCR products were confirmed by sequence analysis.

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 ribonuclease (RNAse) protection assays were performed with commercially available kits (Maxiscript T7 + RPA II, Ambion, Austin, TX USA). Twenty to 30 μg total RNA was used in solution hybridization for each sample. In addition, the 18S ribosomal RNA was measured as an internal marker for quality control purposes. All data are presented as arbitrary units of volume based on total RNA, rather than normalized to the 18S signal. Autoradiographs (*Figure 1*) were quantified using an image analysis system and software purchased commercially (Interactive Technologies International, St. Petersburg, FL USA).

2.5. Statistical analysis

The data were analyzed by analysis of variance [11] using the general linear models (GLM) procedure of SAS. The nuclease protection assays were conducted in replicate groups, and the gel term included in the model so that the associated variation could also be removed from the error term.

3. Results

The serum chemistry data are summarized in *Table 2*. As expected, serum NEFA concentrations were markedly increased ($P < 0.01$) by feed and deprivation, and insulin and

Table 1
Primer design and sequence reference information for porcine uncoupling proteins and lipases

Gene	Primer (5′ to 3′)[a]	Reference No.[b]	Product (bp)[c]
UCP-2 (sense)	ACA AGA CCA TTG C[A/C]C GAG AG	HSU76367	175
UCP-2 (antisense)	AGA A[A/G]T GAA GTG GCA AGG GAG	MMU94593	
UCP-3 (sense)	TGG TGA AGG TCC GAT TTC A[A/G]	AF001787	255
UCP-3 (antisense)	AGG CAG AGA CAA AGT G[A/G]C AG	AF053352	
LPL (sense)	TTC AAC CAT AGC AGC AAA ACC	X62984	232
LPL (antisense)	ATT CAA CCG CCA TCC AGT C		
HSL (sense)	ACA AAC GCA ATG AAA CAG	AJ000482	160
HSL (antisense)	TCA ATC TCG GTG ATA TTC C		

[a] Brackets indicate positions for which degenerate primers were designed based on the reference sequences provided.
[b] EMB or GenBank accession numbers.
[c] The porcine LPL and HSL sequences obtained shared 100% homology with the corresponding EMB or GenBank sequences. The UCP-2 sequence was approximately 93% homologous with the corresponding human and mouse sequences, and UCP-3 shared 89% and 83% homology with the human and mouse sequences, respectively. The riboprobe (PCR product with T7 promoter) and the protected fragment differ in length by 29 nt (i.e., the length of the T7 promoter).
UCP–uncoupling protein. LPL–lipoprotein lipase. HSL–hormone-sensitive lipase. PCR–polymerase chain reaction.

urea nitrogen were reduced by about 30% ($P < 0.01$) and 11% ($P < 0.04$), respectively. However, serum glucose and triglyceride concentrations in the fed and feed-deprived groups were not different at the completion of the study.

Feed deprivation resulted in marked changes in gene expression (mRNA abundance) in both adipose tissue and skeletal muscle. Increases in UCP-3 mRNA abundance of 103% to 237% ($P < 0.01$) were detected in longissimus and red and white semitendinosus muscle (*Figure 2*). The increase in UCP-3 mRNA in adipose tissue was small in comparison (only 23%), but significant ($P < 0.06$). The abundance of UCP-2 mRNA was not influenced ($P > 0.66$) by feed deprivation in adipose tissue, but was slightly increased ($P < 0.04$) in longissimus muscle (*Figure 3*).

The expression of HSL was increased by feed deprivation 46% and 64% ($P < 0.04$) in adipose tissue and longissimus muscle, respectively (*Figure 4*), and as shown in *Figure 5A*, the abundance of LPL mRNA in adipose tissue was reduced ($P < 0.01$) to only 20% of that of the fed group. Longissimus muscle LPL mRNA abundance in the feed-deprived group was 37% of that of the fed group ($P < 0.01$). The reduction in LPL mRNA caused by feed deprivation was also evident in the deep red and superficial white portions of the semitendinosus muscle (*Figure 5B*); in both components, the abundance of LPL mRNA in the feed-deprived group was significantly less ($P < 0.05$) than that in the fed group.

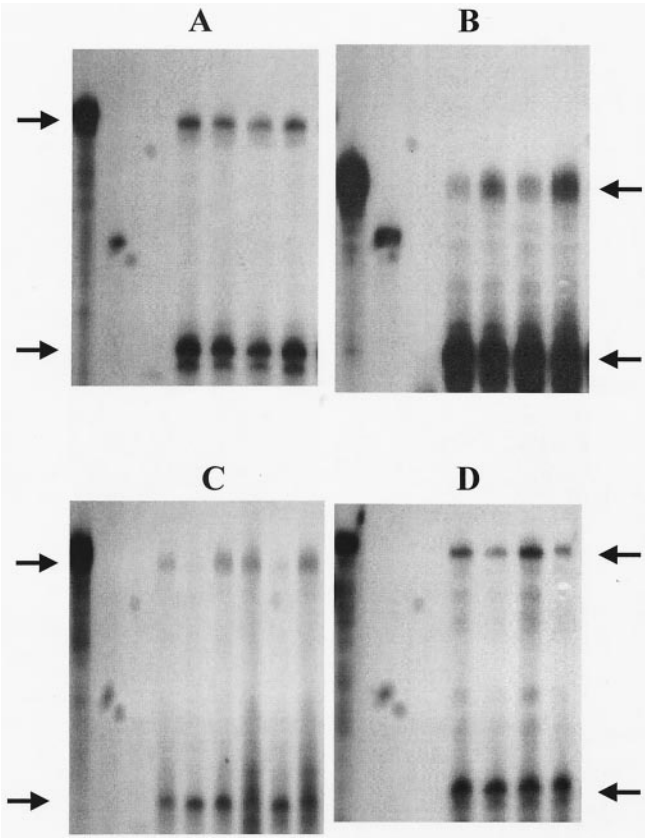

Figure 1 Nuclease protection assays were established for uncoupling protein 2 (A), hormone-sensitive lipase (B), lipoprotein lipase (C), and uncoupling protein 3 (D). The riboprobes were constructed using the primers detailed in *Table 1*. Total RNA was extracted from adipose tissue and skeletal muscle, and 20–30 μg hybridized with the radiolabeled (^{32}P-UTP) riboprobe. Autoradiographs were exposed for 18 to 24 hr. Within each radiograph, the upper bands (top arrows) represent the protected fragments of the transcripts of interest and the lower bands (bottom arrows) the 18S ribosomal RNA fragment. The undigested transcript and 18S riboprobes are shown in the first two lanes, and the digested controls (i.e., nonprotected) probes are in the third lane. The remaining lanes are tissue samples.

4. Discussion

Damon et al. [12] documented the expression of UCP-2 and UCP-3 in the adipose tissue and skeletal muscle of young piglets, and also established that UCP-3 expression in skeletal muscle is responsive to triiodothyronine in vivo, albeit dependent upon fiber type. The data provided herein also document the presence of these transcripts in porcine

Table 2
Serum variable concentrations in pigs allowed ad libitum feed consumption or deprived of feed[a]

	Fed	Feed-Deprived	SEM	$P > F$
UN (mmol/L)	11.1	9.8	0.4	0.04
Glucose (mmol/L)	5.2	5.3	0.1	0.36
TG (μmol/L)	539	531	40.3	0.87
NEFA (mEq/L)	0.74	5.78	0.30	0.01
Insulin (pmol/L)	19.1	12.9	1.6	0.01

[a] Pigs were either allowed ad libitum consumption or deprived of feed for 96 h prior to serum recovery.

UN–urea nitrogen. TG–triglyceride; based on triolein as assay standard. NEFA–nonesterified fatty acids.

adipose tissue and skeletal muscle, and relate changes in their expression energy deficiency. The UCP-2 response to starvation was absent in adipose tissue and was minimal in skeletal muscle, whereas marked UCP-3 responses were found in skeletal muscle, but only a marginal response in adipose tissue. Thus, as regards starvation, it is predominantly UCP-3 that is responsive in the pig, and the response is largely limited to skeletal muscle. Our results in the pig are contrary, in part, to those obtained recently in rats [6,13] in which feed deprivation caused marked increases in both UCP-2 and UCP-3 mRNA in the skeletal muscle. However, our findings seem consistent with those of Khalfallah et al. [14] who reported that increased UCP expression in human skeletal muscle was limited to UCP-3 in a model system in which elevated circulating NEFA concentrations (a circumstance also caused by starvation) were achieved by lipid infusion. As regards the influence of muscle fiber type on the UCP-3 response, our data substantiate the findings of

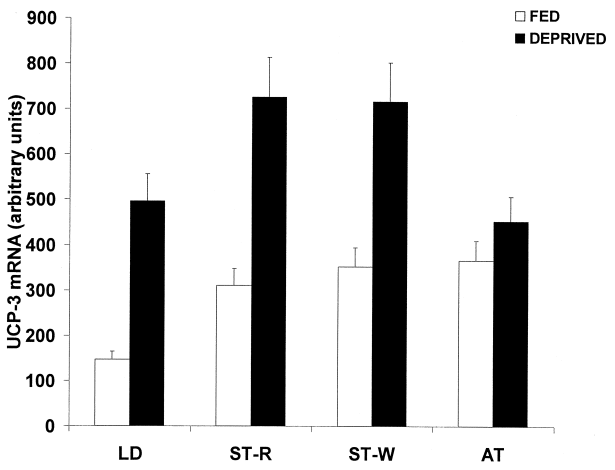

Figure 2 Expression of uncoupling protein (UCP)-3 in the skeletal muscle and adipose tissue of pigs allowed ad libitum feed intake or deprived of feed for 96 hr. Total RNA was extracted from longissimus (LD), red (ST-R), and white (ST-W) semitendinosus muscle, and adipose tissue (AT), and the abundance of the UCP-3 transcript determined by nuclease protection assay. The UCP-3 mRNA was increased ($P < 0.01$) in all muscle tissues and in adipose tissue ($P < 0.06$). Data presented are the least squares means and standard errors ($n = 13$–16).

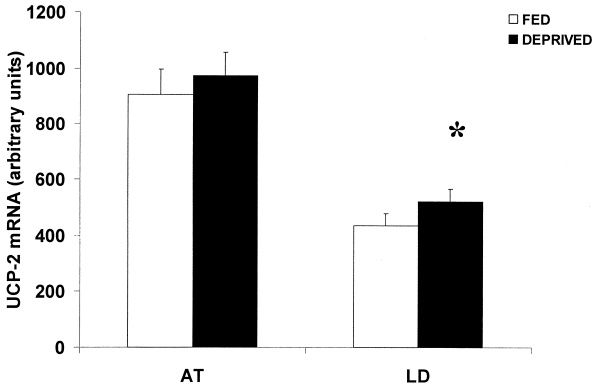

Figure 3 Expression of uncoupling protein (UCP)-2 in longissimus muscle (LD) and adipose tissue (AT) of pigs allowed ad libitum feed intake or deprived of feed for 96 hr. Total RNA was extracted and the abundance of the UCP-2 transcript determined by nuclease protection assay. The UCP-2 mRNA was slightly increased in LD (*$P < 0.04$) but was unchanged in AT ($P > 0.66$). Data presented are the least squares means and standard errors ($n = 13$–16).

Weigle et al. [7] and Samec et al. [6] that UCP-3 is responsive to energy deficiency in skeletal muscle, irrespective of fiber type. We documented marked increases in both red (oxidative) and white (glycolytic) portions of semitendinosus muscle, and in longissimus muscle with predominantly glycolytic myofibers.

Recent findings have linked increased UCP-3 mRNA abundance to an increased influx of fatty acids, perhaps in relationship to corresponding increases in peroxisomal and mitochondrial fatty acid oxidation. Hwang and Lane [15]

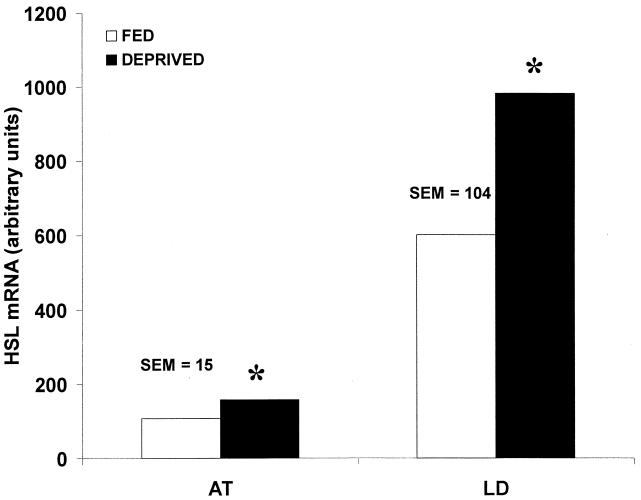

Figure 4 Expression of hormone-sensitive lipase (HSL) in adipose tissue (AT) and longissimus muscle (LD) of pigs allowed ad libitum feed intake or deprived of feed for 96 hr. Total RNA was extracted and the abundance of the hormone-sensitive lipase (HSL) transcript determined by nuclease protection assay, using 20 μg (AT) or 30 μg (LD) total RNA. The HSL mRNA abundance was increased in both AT and LD muscle (*$P < 0.04$). Data presented are the least squares means and standard errors ($n = 13$–16). The results for adipose tissue and skeletal muscle were obtained at different times and with different probes and assay conditions, and are thus not intended to illustrate relative tissue abundance of the HSL transcript.

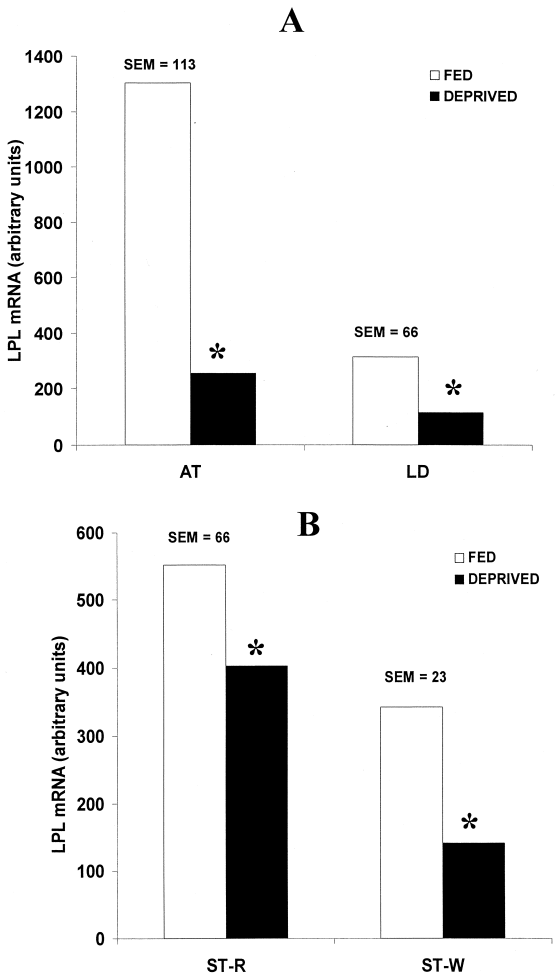

Figure 5 Expression of lipoprotein lipase (LPL) in adipose tissue (AT) and longissimus (LD) muscle (Panel A), and in red (ST-R) and white (ST-W) semitendinosus muscle (Panel B) of pigs allowed ad libitum feed intake or deprived of feed for 96 hr. Total RNA was extracted and the abundance of the LPL transcript determined by nuclease protection assay. The LPL mRNA was decreased (*$P < 0.01$) in both AT and LD muscle, and similar reductions ($P < 0.01$) were evident in semitendinosus muscle, irrespective of oxidative (red) or glycolytic (white) potential. Data presented are the least squares means and standard errors ($n = 13$–16).

showed that culturing C_2C_{12} myotubes in medium enriched with oleic acid is sufficient to induce the expression of UCP-3. We and others have shown previously [16,17] that NEFA concentrations are greatly increased in the serum or plasma of pigs deprived of feed for 72 hr. Similar NEFA results were obtained in the present study, and it is likely that the increase in UCP-3 expression is tightly linked to the increase in the circulating NEFA. Mechanistically, some NEFA are ligands for the nuclear transcription factors, that is, peroxisome proliferator activated receptors (PPAR). The gamma isoform of PPAR (PPARγ) is expressed in human skeletal muscle [18], and we confirmed this in several muscles of the pig (unpublished data). It is not yet known whether the UCP-3 gene contains a PPARγ response element, as has been shown for UCP-1 [19], but considering the marked influx of NEFA into skeletal muscle during starvation, it is possible that altered expression and/or activity of PPARγ contributes to the UCP-3 response to starvation. Also of importance, Ding et al. [20] established that the alpha isoform of PPAR (PPARα) is expressed in porcine skeletal muscle, albeit to a lesser extent than in adipose tissue. This is particularly intriguing because this factor regulates the expression of acyl Co-A oxidase and carnitine palmitoyltransferase, genes that control mitochondrial and peroxisomal fatty acid oxidation [21,22]. Thus, in skeletal muscle, it is also possible that PPARα drives the increase in fatty acid oxidation caused by starvation, and that UCP-3 is a target gene. In light of the marginal increase in adipose UCP-3 mRNA caused by feed deprivation in the present study, it is interesting to note that PPARγ mRNA in this tissue is either unresponsive (γ1) or reduced (γ2) when pigs are deprived of feed [23]. It will be important to establish if PPARγ and PPARα are differentially regulated in adipose tissue and skeletal muscle during starvation. Collectively, our findings in the pig reinforce the hypothesis that UCP-3 facilitates the increased metabolism of fatty acids by skeletal muscle that is necessitated by energy deficiency, and also indicate that UCP-2 likely plays a minimal role in the pig's adaptation to fatty acids as the primary energy source.

The HSL is the major enzyme mediator of the lipolytic pathway, and is activated (i.e., phosphorylated) as a consequence of β-adrenoceptor stimulation (see Ramsay [1] for a review). Little is known regarding the regulation of HSL mRNA abundance, but there is evidence that increased lipolysis in human and rodent adipocytes and adipose tissue, respectively, is supported by an increase in HSL mRNA abundance [24,25]. We provide strong evidence herein that prolonged feed deprivation results in an increase in HSL mRNA in both adipose tissue and skeletal muscle tissue in the pig. Although adipocytes reside within the muscle and likely contribute to the HSL signal, it seems unlikely that this is the sole explanation for the marked increase caused by feed deprivation. The expression of HSL in the myofiber itself is indicated by documented expression in primary muscle cell cultures [26]. Coordinate regulation of HSL and UCP-3 expression in skeletal muscle is possible, and it also seems possible that fatty acids liberated by HSL activity within the muscle contribute to the induction of UCP-3 expression.

Lipoprotein lipase catalyzes the release of fatty acids from triacylglycerol esters, and thus facilitates cellular uptake of free fatty acids from its circulating triglyceride substrate. Sugden et al. [11] reported that LPL activity in rat adipose tissue was reduced by feed deprivation, whereas skeletal muscle activity was increased. Furthermore, in adipose tissue, Lee et al. [28] showed that the decreased activity of LPL after long-term starvation (3 days) paralleled a 50% decrease in LPL mRNA and a 50% reduction in LPL synthesis. We sought to determine the extent to which LPL mRNA abundance is influenced by starvation in porcine adipose tissue and skeletal muscle. As reported recently by McNeel and Mersmann [17], we also found that LPL

mRNA in porcine adipose tissue was reduced by feed deprivation (96 hr) to about 20% of that of the fed group. However, we found LPL mRNA abundance to be reduced in longissimus muscle as well, a result that we also confirmed in red and white semitendinosus muscle. We reported previously [16] that serum triglyceride concentrations are reduced in fatter (136 kg) pigs deprived of feed, whereas leaner (60 kg) pigs have increased concentrations. McNeel and Mersmann [17] also reported that in very young pigs, circulating triglyceride concentrations were actually increased by feed deprivation, but LPL mRNA in skeletal muscle was not evaluated. In this study (87-kg pigs), the triglyceride concentration was independent of nutritional status. Reductions in muscle LPL mRNA (and presumably catalytic activity) may have been required to maintain triglyceride homeostasis during prolonged starvation. Reduced LPL expression in muscle may lead to an attenuation of triglyceride hydrolysis to favor the use of NEFA derived from adipose lipolysis. However, the relationship between skeletal muscle LPL mRNA abundance and catalytic activity remains to be determined in the pig during starvation.

In summary, we provide clear evidence that increased UCP-3 expression in skeletal muscle, irrespective of oxidative or glycolytic potential, is a component of the metabolic adaptation to starvation in the pig. We also document marked increases in HSL mRNA abundance and reductions in that of LPL in response to starvation in adipose tissue and skeletal muscle. Although the biochemical role of UCP-3 during starvation is currently unknown, it is likely that this UCP facilitates the utilization of fatty acids for fuels, possibly by protecting against unfavorable changes in the ratio of ATP to ADP that could stem from excessive fatty acid oxidation.

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