Transcriptional Profiling of the Caloric Restriction in Key Metabolic Tissues of Pigs Differing in Feed Efficiency

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Summary

Residual feed intake is a measure of feed efficiency, where low RFI denotes high feed efficiency. Caloric restriction (CR) is associated with feed efficiency in livestock species and to human health benefits such as longevity and cancer prevention. We have developed pig lines that differ in RFI and we are interested in identifying the genes and pathways that underlie feed efficiency. Prepubertal Yorkshire gilts with low RFI (n=10) or high RFI (n=10) were fed *ad libitum* or at 80% of maintenance for 8 days. We measured serum metabolites and generated transcriptional profiles of liver and subcutaneous adipose tissue on these animals. Overall, 6,114 genes in fat and 305 genes in liver were differentially expressed (DE) in response to CR, and 311 genes in fat and 147 genes in liver were DE due to RFI differences. Pathway analyses of CR-induced DE genes indicated a dramatic switch to a conservation mode of energy usage by down-regulating lipogenesis and steroidogenesis in both liver and fat. Interestingly, CR altered expression of genes in immune and cell cycle/apoptotic pathways in fat, which may explain part of the CR-driven lifespan enhancement. *In-silico* analysis of transcription factors revealed ESR1 as a putative regulator of the adaptive response to CR, as several targets of ESR1 in our DE fat genes were annotated as cell cycle/apoptosis genes. The lipid metabolic pathway was overrepresented by down-regulated genes due to both CR and low RFI. We propose a common energy conservation mechanism, which may be controlled by PPARA, PPARG, and/or CREB in both CR and feed efficient pigs.

Introduction

Genetic mechanisms that control feed intake (FI) and feed efficiency are not well understood. Differences in feed efficiency arise due to factors such as variations in body composition, feeding patterns, digestibility, activity, thermoregulation, and tissue metabolic rates.

Residual feed intake (RFI) has been broadly accepted as a reliable method of measuring feed efficiency and is defined as the feed consumed above what is required for growth and maintenance. Pigs with low RFI (LRFI) consume less food than the population average without a significant loss in growth parameters such as body weight and composition, and therefore are more feed efficient. Our group has successfully developed pig lines that differ in RFI up to 124 g/day without a significant change in the body composition, with an estimated heritability for RFI of 0.33. The physiology underlying RFI differences has been studied mainly in poultry and in beef cattle, in which whole-genome SNP analyses and microarray approaches have been undertaken. For example, transcriptomic analysis of liver biopsies from Angus bulls identified 163 differentially expressed genes between animals with high and low RFI. These genes represented several cellular pathways such as growth, proliferation, protein synthesis, lipid metabolism, and carbohydrate metabolism.

Efficient feed utilization has also been achieved with caloric restriction (CR) in cattle and chickens. The motivation to understand biological mechanisms underlying response to CR extends beyond feed efficiency in livestock species. Caloric restriction prolongs lifespan in virtually all species, including mammals, and recent reports suggest that CR is the most compelling cancer-prevention regimen in the carcinogenesis models. Translation of the CR phenomenon to human health is critical considering that obesity, a major risk factor for several types of cancers and age-associated chronic diseases, is alarmingly increasing in the Western world.

Transcriptional profiling of CR to elucidate pathways involved in longevity promoting mechanisms in rodents has been investigated; however, the pig is a better suited model for the human energy homeostatic system than rodents and has contributed to improved knowledge of human metabolic disorders such as obesity and diabetes. Understanding efficient feed utilization in pigs will also lead to improved agricultural economy as pork is used as a major human food source worldwide and the cost of feed amounts to the largest variable cost in pork production, making up 68% of the total variable cost.

Materials and Methods

Transcriptional profiling: Low and high RFI gilts from the lines were allowed feed *ad libitum* or were feed restricted to 80% of maintenance for 7 days in a complete 2 x 2 factorial design. Five pigs were evaluated per treatment combination. Total RNA was isolated from liver and fat tissues and analyzed using hybridization to the Affymetrix Porcine Genechip™, which allows assessment of the level

of expression of over 24,000 transcripts for genes across the genome. A mixed linear model was fit to each tissue and each gene using SAS Proc Mixed to identify genes that differed in expression between treatments.

Blood parameter assays: Blood samples were collected at sacrifice and put on ice upon collection and kept at 4°C overnight to aid coagulation. Blood was centrifuged at 1000 x *g* for 15 min and the serum was isolated and stored at -20°C. Blood leptin and cortisol were measured using radioimmunoassays by collaborator. Blood glucose and TG levels were determined using Hexokinase (Roche Diagnostics) and TG (Diagnostic Chemicals Limited) kits.

Results

Growth and FI related parameters. The effects of RFI group and 8-day CR on body weight, ADG, FI, backfat, and loin eye area are summarized in Table 1. Eighty percent of maintenance caloric requirement was roughly equivalent to 25% of the AL feed, which was ~2.85 kg/day.

Table 1. Body weight, average daily gain, feed intake, RFI, backfat depth, and loin eye area in pigs fed ad libitum or CR and that have high and low RFI

	HRFI		LRFI				
	Ad libitum feed	Caloric restriction	*Ad libitum* feed	Caloric restriction	*p* value RFI	*p* value CR	*p* value RFI x CR
Body weight (kg)							
Pre-treatment	84.21[a]	82.92[a]	79.66[a]	78.85[a]	6.9E-02	6.4E-01	9.2E-01
SE	2.99	2.85	2.99	2.99			
Post-treatment	88.72[a]	76.44[b]	89.17[a]	77.20[b]	3.5E-01	4.9E-13	7.9E-01
SE	0.61	0.55	0.61	0.60			
Average daily gain (kg/d)							
Pre-treatment	0.66[b]	0.75[a]	0.70[a,b]	0.75[a]	4.4E-01	9.2E-03	3.9E-01
SE	0.03	0.03	0.03	0.03			
During treatment	0.92[a]	-0.62[b]	1.04[a]	-0.50[b]	1.5E-01	1.3E-10	9.9E-01
SE	0.10	0.08	0.09	0.09			
Pre-treatment RFI (kg/d)	0.15[a]	0.15[a]	-0.14[b]	-0.15[b]	2.8E-06	9.2E-01	8.5E-01
SE	0.04	0.04	0.04	0.04			
Feed intake (kg/d)							
Pre-treatment	1.68[a]	1.80[a]	1.40[b]	1.44[b]	3.0E-05	1.5E-01	5.2E-01
SE	0.07	0.06	0.07	0.07			
During treatment	2.83[a]	0.61[b]	2.87[a]	0.80[b]	2.3E-01	1.3E-16	2.1E-01
SE	0.06	0.07	0.07	0.07			
Backfat depth (mm)							
Pre-treatment	15.46[a]	15.21[a]	12.07[b]	12.84[a,b]	8.8E-03	7.9E-01	6.1E-01
SE	0.99	0.91	0.99	0.99			
Post-treatment	17.03[a]	12.48[c]	14.69[b]	11.51[c]	1.5E-02	1.5E-06	1.9E-01
SE	0.55	0.51	0.56	0.55			
Loin eye area (mm^2)							
Pre-treatment	3324[a]	3234[a]	3011[a]	3349[a]	5.6E-01	4.6E-01	2.3E-01
SE	179.1	165.2	179.1	179.1			
Post-treatment	3399[a,b]	3138[b]	3541[a]	3302[a,b]	1.2E-01	1.7E-02	9.1E-01
SE	107.3	99.6	113.5	107.9			

Blood parameters. Several blood parameters were measured in the post-treatment serum samples and these results are shown in Table 2. Serum concentration of NEFA tended to be higher in the CR than the AL group (p=0.075). Thyroxine concentration was 22.6% lower in the CR than the AL pigs (p=0.023) and the triiodothyronine concentration tended to be 18.5% lower in the CR than the AL pigs (p=0.076). No significant effect of CR was noted for serum concentrations of glucose, insulin, TG, cortisol, and leptin. The serum triiodothyronine concentration in LRFI pigs was 31% higher than that of HRFI pigs (p=0.024). The effect of RFI was not significant for serum concentrations of glucose, insulin, TG, thyroxine, cortisol, and leptin. A significant interaction of RFI and CR was observed for the serum concentration of leptin (p=0.027), in which leptin concentration tended to decrease in response to CR in the HRFI group, but the opposite trend was present in the LRFI pigs

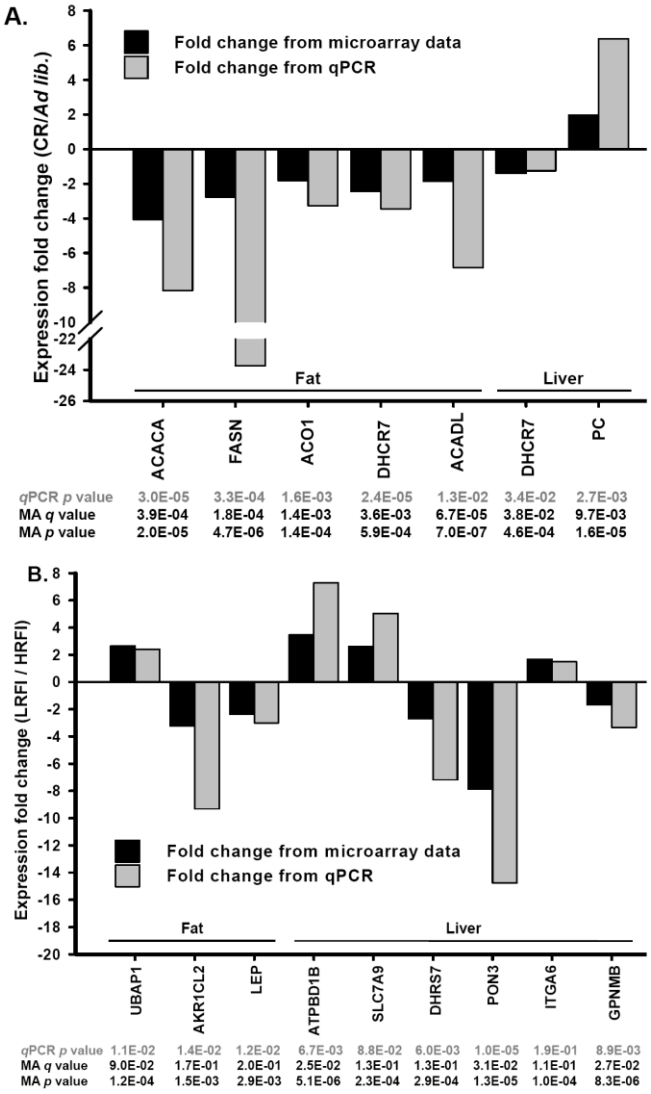
Table 2. Blood parameters in pigs fed *ad libitum* or CR and that have high or low RFI

	HRFI		LRFI				
	Ad libitum feed	Caloric restriction	*Ad libitum* feed	Caloric restriction	RFI *p* value	CR *p* value	RFI x CR *p* value
Glucose (mg/dl)	85.9[a]	92.4[a]	99.1[a]	98.7[a]	1.1E-01	6.0E-01	5.8E-01
SE	7.1	6.7	7.1	7.1			
Insulin (ng/ml)	0.21[a]	0.19[a]	0.11[a]	0.19[a]	3.3E-01	6.0E-01	3.8E-01
SE	0.05	0.05	0.05	0.05			
NEFA (mEq/L)	0.19[a]	0.29[a]	0.22[a]	0.26[a]	9.7E-01	7.5E-02	4.1E-01
SE	0.04	0.03	0.04	0.04			
TG (mg/dl)	28.0[a]	32.2[a]	28.3[a]	23.9[a]	4.9E-01	9.8E-01	4.9E-01
SE	6.0	5.5	6.0	6.0			
T4 (µg/dl)	5.11[a]	4.28[a,b]	5.09[a]	3.61[b]	4.7E-01	2.3E-02	4.9E-01
SE	0.47	0.43	0.47	0.47			
T3 (ng/dl)	103.5[a,b]	77.9[b]	127.4[a]	110.2[a,b]	2.4E-02	7.6E-02	7.2E-01
SE	12.80	11.92	12.80	12.80			
Cortisol (ng/ml)	19.78[a]	22.31[a]	13.55[a]	22.09[a]	7.5E-01	5.8E-01	7.7E-01
SE	12.5	11.8	12.5	12.5			
Leptin (ng/ml)	4.37[a]	3.31[a]	2.84[a]	4.05[a]	5.4E-01	9.2E-01	2.7E-02
SE	0.76	0.43	0.45	0.63			

Caloric restriction and RFI effects on expression of genes in fat and liver. Of 24,123 probe sets evaluated by microarray analysis, 20,058 and 18,787 provided data indicating that the transcripts represented by these probe sets were expressed in fat and liver tissue, respectively. In response to an 8-day CR treatment, 6,114 transcripts in fat were DE (q≤0.05, p≤0.024), of which 2,845 were up-regulated and 3,269 down-regulated. In liver, 305 transcripts were identified to be DE (q≤0.05, p≤0.0009), of which 156 were up-regulated and 149 down-regulated. We considered transcripts with false discovery rate less or equal to 20% (q≤0.2) to be DE as a result of difference between RFI groups. Due to RFI difference between groups, 311 transcripts in fat (p<0.003) and 147 transcripts in liver (p<0.0015) were declared to be DE (q≤0.2). An improved annotation of probesets on the Affymetrix GeneChip® Porcine Genome Array assigned gene names (BLASTN expectation score <1E-10) to 84% and 81% of all DE transcripts due to either CR or RFI in liver and in fat, respectively.

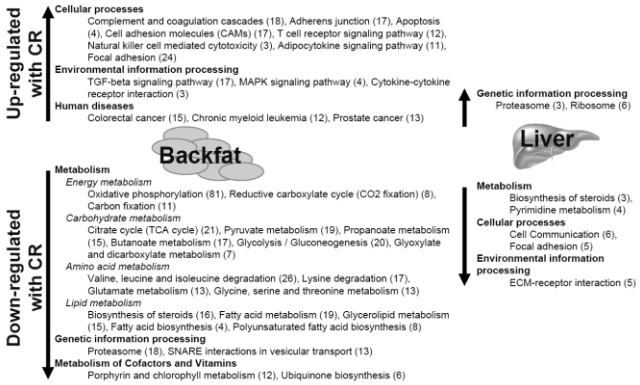
Expression patterns were verified by *q*PCR for seven genes in either fat or liver, which were predicted to be DE due to CR (q≤0.05); these were selected to represent the lipid biosynthetic pathways in subcutaneous adipose tissue and gluconeogenesis in the liver (Figure 1A). Similarly, seven of nine tested genes (q≤0.2) representing lipid and amino acid metabolic processes in fat and cell proliferation and energy metabolism in liver were validated as DE in LRFI vs. HRFI pigs (Figure 1B). For all tested genes, expression differences were consistent in direction with the microarray results (Figures 1A and 1B). Statistical significance of effects of CR treatment or RFI (p<0.05) was confirmed by *q*PCR for all genes, except for SLC7A9 (p=0.09) and ITGA6 (p=0.2) in liver of HRFI vs. LRFI pigs (Figure 1B). The results obtained from microarray were statistically confirmed for 88% of the tested genes in liver and fat.

Figure 1A and 1B.



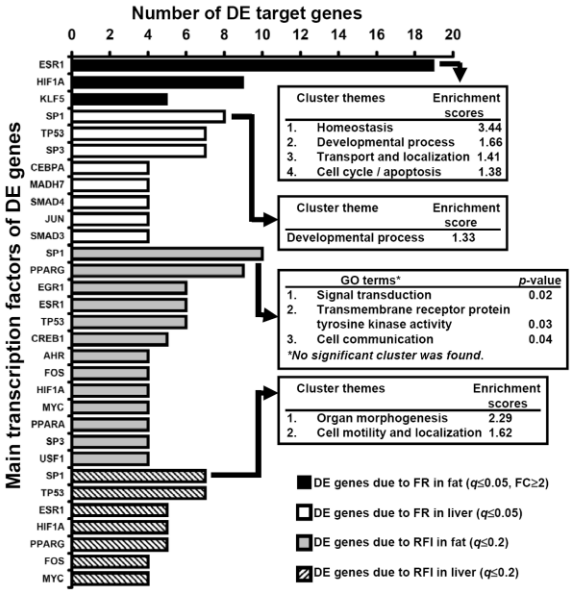
***Pathway Analyses*.** To identify biological processes that respond to CR or RFI, the KEGG database was used to identify specific pathways that were overrepresented by genes that responded to CR or RFI ($p \leq 0.1$). KEGG annotations that were overrepresented by CR-induced DE genes in fat or liver are summarized in Figure 2.

Figure 2.



Key transcriptional regulators of response to CR or RFI. Most of the changes in RNA levels we observed were likely due to changes in levels of transcription. To understand the main transcriptional regulation involved in the response to CR or RFI, genes that were DE in liver and fat were analyzed for their connections to common TF or nuclear receptor regulators by using Pathway Studio 5.0. The analysis of CR-induced DE genes in adipose tissue was limited to genes with fold change equal to or more than two, because of the large number of DE genes. Connections between TF and their targets within the four DE gene lists (*i.e.* DE genes due to CR or RFI in fat or liver) were populated based on literature evidence of at least one of four interaction categories provided by Pathway Studio 5.0, which were promoter binding, binding, regulation, and direct regulation. Common regulators with the highest number of target genes across all four categories were determined and their target genes were functionally annotated by GO biological processes terms ($p \leq 0.05$) and pathway clusters (Figure 3).

Figure 3.



Conclusions

Our main findings demonstrate specific transcriptional responses to CR and RFI across liver and adipose tissues with congruent changes in serum metabolites. We document metabolic pathways and transcription factors that appear to govern these responses. Our results provide potential candidate markers for breeding feed efficient pigs. The triiodothyronine difference between the pig lines points to further studies to determine the characteristics of the thyroid axis in the feed efficient pigs.