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Full Length Research Paper

Apolipoprotein D, apolipoprotein R and ST6GalNAc4 genes respond to clenbuterol administration in pig adipose tissue

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Pig fat accumulation can be reduced by feeding pigs with high dosages of clenbuterol, but the molecular mechanism has not yet been well characterized. cDNA microarray and real-time PCR were used to identify the molecules potentially responding to clenbuterol in adipose cells. The mRNAs of 17 genes were found to be differentially expressed more than 2 fold (ratios of the expression levels in the test pigs to those in the control pigs of more than 2 or less than 0.5) by microarray analysis, including three lipid metabolism related genes (apolipoprotein D, apolipoprotein R and ST6GalNAc4). Eight genes were verified by real-time PCR, with six of them reproducing the result from microarray analyses. The results showed that apolipoprotein D, apolipoprotein R and ST6GalNAc4 genes respond to clenbuterol administration in pig adipose tissue and their functions may relate to fat accumulation reduction.

Key words: Pig, clenbuterol, adipose, cDNA microarray, real-time polymerase chain reaction (PCR).

INTRODUCTION

Adipose tissue plays critical roles in the regulation of energy homeostasis (Gesta et al., 2007). Excessive accumulation of adipose tissue has many negative effects (Pospisilik et al., 2010). How to reduce adipose accumulation safely and effectively is of great importance both for human health and animal breeding. Clenbuterol, a type of β_2-agonist, can reduce body fat drastically (Plant, 2003; Sato, 2010). But clenbuterol is toxic both for animals and human and the usage has been banned for many years (Kuiper et al., 1998; Mitchell and Dunnavan, 1998; Shiu and Chong, 2001). Clenbuterol perturbs cell metabolism by binding to the β_2-adrenergic receptors and increasing the cyclic AMP concentration in cells. In adipocytes, stimulation of β-adrenergic receptors (by hormones) increases cyclic AMP levels and activates protein kinase A (PKA), which then stimulates lipolysis by phosphory-lating hormone-sensitive lipase and perilipin (Londos et al., 1985; Egan et al., 1990; Greenberg et al., 1991; Zhang et al., 2005). However, the molecular mechanism underlying the effect of clenbuterol on adipose accumulation is still not well understood. Unveiling the mechanism will potentially identify new molecules that might play important roles in regulating adipose metabolism.

DNA microarray technology has been widely used as a powerful tool for functional genomics study in both basic science research and biomedicine research (Petrik et al., 2006; Sawada et al., 2010). However, the application of this technology to animal genetics and breeding related research is rare. In this study, the genes responding to clenbuterol were profiled by cDNA microarray and real-time PCR techniques in fat tissue of Chinese miniature pigs treated with/without clenbuterol. These genes are potential candidates to be used in developing high lean pig breeding and also are potential drug targets for human obesity treatment.

MATERIALS AND METHODS

Animal sampling, clenbuterol treatment and body composition analysis

In total, 8 Chinese miniature pigs were used in the experiments. Four hogs and four sows, all at 4 weeks of age, were housed in the nutrition and metabolism laboratory at the China Agriculture

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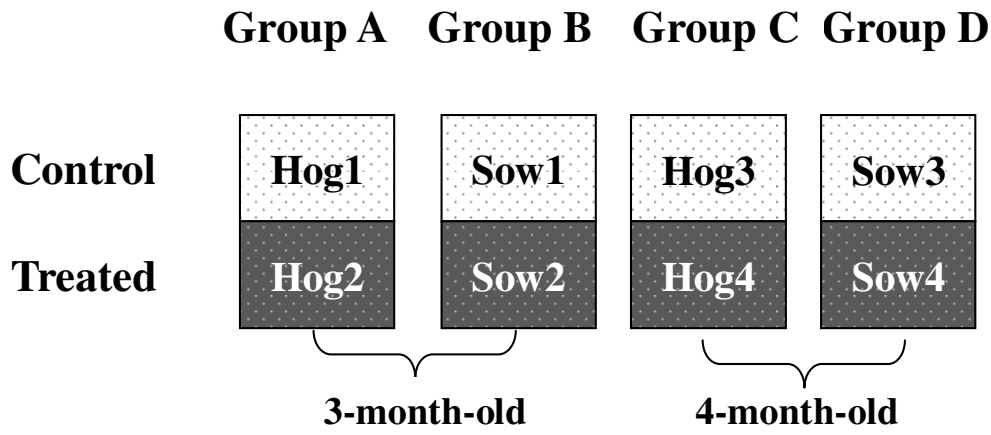

Figure 1. The groups of pig population. Eight pigs were broken into four groups. There were two pigs in each group with the same gender. Group A and B were slaughtered when they were 3-month-old. Group C and D were slaughtered at 4-month-old.

University. They were raised under exactly the same conditions and were fed the same diets until 8 weeks old (average body weight 17.4±1.2 kg, Table 3). They were broken into 4 groups named group A, B, C and D (Figure 1), each group consisting of two pigs with the same gender. For the following 4 weeks, one pig in each group was fed 25 mg/kg clenbuterol twice daily in the diets, while the other was fed the same diet without clenbuterol as the control. Pigs in group A and B were slaughtered for analysis (at 3 months old), while pigs in groups C and D were continued feeding with/without 50 mg/kg clenbuterol twice daily in their diets for another 4 weeks and then slaughtered for analysis. These two groups are referred to as the 4 month-old pigs. Approximately, 1 g biopsies were taken from the back fat adipose tissues of each pig. The adipose tissue samples were rinsed in sterile water, snap frozen in liquid nitrogen and stored at -80°C.

Body composition analysis was done for each group just after the slaughter. The fat thickness were measured at the 10th rib by using standard procedures and the carcass lean content for each pig was also calculated (National Pork Board, 2000).

HPLC analysis for clenbuterol residue in different tissues

The samples were sent to livestock and poultry quality inspection center of China Ministry of Agriculture and clenbuterol residue was analyzed according to NY/xq421-2003, which is the standard method for determination of clenbuterol residues in animal product with GC/MS.

RNA preparation, RNA labeling and DNA microarray hybridization

Total RNA of the adipose tissue was extracted with TRIZOL reagent (Invitrogen, Gaithersburg, MD, USA). Briefly, the sample was put into mortar with liquid nitrogen and grinded into power. RNA was extracted from 100 mg of sample with 1000 ml TRIZOL added and following the manufacturer's instructions. The RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufactures' protocol.

A cDNA microarray representing 3358 pig genes was developed by our group (Zhang, 2011). A cDNA microarray hybridization analysis was performed in accordance with the standard protocol provided anywhere (Zhang et al., 2011).

DNA microarray imaging and data analysis

Arrays were scanned with a ScanArray express scanner (Parckard Bioscience, Kanata, OT, U.S.) with the obtained images analyzed with GenePix Pro 4.0 (Axon Instruments, Foster City, CA). The resulting microarray data was normalized using the space and intensity-dependent normalization in the LOWESS program (Yang et al., 2002). Each gene was represented in triplicate on each slide. The ratio presented for each gene was the mean of the "median of ratios" of 3 spots for this gene. For each slide, the positive genes were defined as those with gene ratios more than 2 (including 2) or less than 0.5 (including 0.5). To avoid dye bias, the experiments were performed in duplicate by dye swap with Cy5-dCTP first used with the test pig and Cy3-dCTP used with the control pig and then Cy3-dCTP used with the test pig and Cy5-dCTP used with the control pig. The positive genes in both dye swap slides were identified as the differentially expressed genes. Differentially expressed genes in both the hogs and the sows of the same age were chosen for further analysis.

Quantitative real-time PCR

To confirm the transcriptional differences observed by the microarray, fluorescent real-time PCR was done on an ABI Prism 9700 sequence detection system (Applied Biosystems, Foster, CA, USA) using SYBR green technology as described by Li (2005). Briefly, the PCR reaction mixture (20 µl) contained 10 µl of DyNAmo SYBR Green qPCR mix, 5 µl of primer (0.3 µM forward and 0.3 µM reverse) and 5 µl of cDNA template (<10 ng/µl). The PCR protocol included uracil-N-glycosylase (UNG) enzyme incubation at 50°C for 2 min and an initial denaturation at 95°C for 10 min. This was followed by 40 cycles of 10 s each at 94°C for DNA denaturation, 20 s at different temperatures for annealing of primers, 20 s at 72°C for primer extension and 1 s at a different elevated temperature for data acquisition. The PCR primer sequences used for the real-time PCR are shown in Table 1. The size of the PCR products was between 100 to 250 bp for the benefit of fluorescent signals accuracy. The annealing temperature of each gene was about 57±2°C so the PCR reaction for different genes could be done on the same plate. All primers were tested by none fluorescent PCR at first and the product size was confirmed by agarose gel. The quantification was normalized to an endogenous RNA control Glyceraidehyde-3-phosphate dehydrogenase (Gapdh, a house-

Table 1. Primers used for the real-time PCR analysis.

Genes and clone number	Primer sequence (5' to 3')	PCR product size (bp)
GAPDH	For. ATGGTGAAGGTCGGAGTGA Rev. ATGGGTAGAATCATACTGGA	154
Apoliprotein D (rpfat_18926)	For. AGATCCCAGTGAGCTTTGAG Rev. CGTAGTTCTCATAGTCGGTG	233
PRKAR1A (rpfat_17661)	For. GGCGACGAGGTGCTATCAG Rev. ATGGCATCAAAAATATCAC	159
COL1A1 (rpfat_8523)	For. TCAAGATGTGCCACTCCGACT Rev. GCCTGTCTCCATGTTGCAGAA	104
COL1A2 (rpfat_16033)	For. ATATGCACCTTGGACATCGGT Rev. CACGATGCTCTGATCAATCCT	241
COL3A1 (rpfat_19990)	For. CCTGCTGGAAAGAATGGTGAC Rev. ACGTTCACCGGTTTCACCTT	132
COL1A2 (rpfat_17393)	For. CCTGGCTCTAGAGGTGAACG Rev. AGCAGGACCAGGATTACCAG	247
COL3A1 (rpfat_18309)	For. TTTCTTTTATGGCTCCCCCTG Rev. GCGTGTTCGATATTCGAAGAC	101
COL3A1 (rpfat_19990)	For. CTGCTGGAAAGAATGGTGAC Rev. ACGTTCACCGGTTTCACCTT	132
SCD (rpfat_16685)	For. AAGGAACTAGAAGGCTGCTC Rev. TGTAGAGCAGCAGCCATCAC	156
PHPT1 (rpfat_15312)	For. GAAGACACAGTTGAGGACAC Rev. GGACATTGTTCGGAGGATAG	110
HSL (rpfat_11096)	For. TCCGAATGGAGTCTGCACTGT Rev. CTTCCACTCTGACCTCCAACG	128
PMP22 (rpfat_18575)	For. CATGAACATTTGCACCACTTG Rev. GTCAGCACCTAATGGTATGGA	133

For.: Forward; Rev.: reverse.

Table 2. Clenbuterol residue in porcine blood and adipose.

Pig number (ng/ml)	3-month-old group				4-month-old group			
	Hog 1	Hog 2	Sow 1	Sow 2	Hog 3	Hog 4	Sow 3	Sow 4
Blood	0.00	10.00	0.00	26.44	0.00	60.57	0.00	176.08
Adipose	0.00	0.00	0.00	1.62	0.00	5.32	-	-

-:not determined.

keeping gene) which its expression level was unchanged to clenbuterol stimulation according to none fluorescent PCR analysis.

RESULTS

Clenbuterol administration dramatically reduced adipose accumulation

HPLC analyses of the blood samples showed that the clenbuterol concentrations in the test pigs fed with clenbuterol were about 20 ng/ml in 3-month-old pigs and about 100 ng/ml in the 4-month-old pigs. Clenbuterol could not be detected in the control pigs fed without clenbuterol (Table 2). There was no difference in body weight between the test pigs and control pigs (Table 3), but there was significant difference in body compositions (Table 4). For 3-month-old pigs, the lean meat percentage in the carcass was improved by about 2%, the back fat thickness was reduced about 0.2 cm and the eye muscle area was reduced by 4.7 cm^2 when treated with clenbuterol. For 4-month-old pigs, the clenbuterol effects were more significant with the lean meat percentage in the carcass increased by 10.99%, the back fat reduce by

Table 3. Body weight of pigs treated with/without clenbuterol.

Pig number	3-month-old group				4-month-old group			
	Hog1	Hog2	Sow1	Sow2	Hog 3	Hog 4	Sow3	Sow 4
Clenbuterol (mg/kg BW [a])	0	25	0	25	0	50[b]	0	50[b]
2-month BW (kg)	18.6	17.4	17.5	16.2	16.8	17.2	17.5	18.3
3-month BW (kg)	33 .2	32.6	30.6	29.8	30.5	32	31	32.2
4-month BW (kg)	-	-	-	-	45.6	46.6	46	46.4
Increased BW (kg)	14.6	15.2	13.1	13.6	28.8[c]	29.4[c]	28.5[c]	28.1[c]

a, BW : Body weight; b, 25 mg/kg body weight for the 3rd month and 50 mg/kg body weight for the 4th month; c, increased body weight in the 3rd month and increased body weight in the 4th month.

Table 4. Body composition of pigs treated with/without clenbuterol.

Clenbuterol administration* (mg/kg body weight)	Hog2 and Sow2 3-month-old	Hog1 andSow1 3-month-old	Hog4 and Sow4 4-month-old	Hog3 andSow3 4-month-old
	0	25	0	50
Slaughter rate (%)	63.75	60.86	66.80	64.63
Lean meat percentage of carcass(%)	52.40	55.24	41.45	52.44
Thickness of back fat (m)	2.336	2.118	3.200	2.818
Eye muscle area (cm^2)	19.488	24.199	23.083	25.270

*Twice daily. The carcass lean meat percentage, the thickness of back fact and eye muscle area were all changed statistically between test pigs and control pigs in both 3 month old pigs and 4 month old pigs ($P < 0.05$).

1.02 cm and the eye-muscle area reduced by 2.18 cm^2 (Table 4).

Seventeen genes were up-regulated in adipose tissue by clenbuterol treatment

Genes whose expression levels were changed by more than or equal to two fold in clenbuterol treatment group versus control group were selected as differentially expressed genes. There were 56 differentially expressed genes (35 up-regulated, 21 down-regulated) in the 3 month-old group and 58 differentially expressed genes (41 up-regulated, 17 down regulated) in the 4 month-old group (Data not shown). 17 genes were differentially expressed in both groups (Table 4). The purpose of this study was to found the key molecules by which clenbuterol reduce pig fat accumulation. Therefore, the overlapping differentially expressed genes in 3 and 4-month old group were considered as the stable responding genes for clenbuterol stimulation in pig adipose tissue and thus, selected for the first stage analysis.

Among the 17 genes, two genes related to signal transduction were found to be up-regulated

These genes are cAMP dependent protein kinase type I regulatory gene (PRKAR1A) and RAB30. Up-regulation of PRKAR1A indicates increasing protein kinase A (PKA) activity. Three genes directly related to lipids metabolism were found to be up-regulated: apolipoprotein D (apoD), apolipoprotein R (apoR) and ST6GALNAC4. Furthermore, eight clones on the microarray were from three genes related to collagen protein synthesis which were up-regulated more than two folds: type I collagen alpha 1 (COL1A1), type I collagen alpha 2 (COL1A2) and type III collagen alpha 1 (COL3A1). Eight differentially expressed genes were analyzed by real-time PCR to validate the microarray data (Table 5). Two of them were not detected by the real-time PCR, while the other six were also found to be differentially expressed more than 2 fold in the real-time PCR.

DISCUSSION

To identify the key molecules in regulating fat metabolism, pigs treated with or without clenbuterol were used as the animal model. The body composition of the pigs was the most affected by clenbuterol treatment. The difference between test and control pigs could come from individual difference since there are only two pigs in each group. However, the same trend was observed in all four comparison groups suggesting that clenbuterol does play a role in regulating adipose metabolism. The sample quality was sufficient for further analysis to identify genes with differential expression levels that impact adipose

Table 5. Differentially expressed genes in adipose with the administration of clenbuterol.

GenBank access number (Clone no.)	Gene name	Potential function	Induction fold change	
			microarray	real-time PCR
NM_001647 (rpfat_18926)	Apolipoprotein D	Lipids metabolism	2.20±0	8.81±0.12
L06820 (rpfat_18262)	Apolipoprotein R	Lipids metabolism	2.47±0.23	-
AB035172 (rpfat_16328)	ST6GalNAc4 mRNA for N--acetylgalactosaminide	Glycolipid metabolism	2.53±0.20	-
X05942 (rpfat_17661)	cAMP dependent protein kinase type I regulatory	Signal transduction	2.0±0	7.0
U57092 (rpfat_19360)	RAB30	Signal transduction	2.23±0.18	-
Z74615 (rpfat_8523)	mRNA for prepro--alpha1(I) collagen (COL1A1)	Cell structure and mobility	2.86±0	16.79
Z74616 (rpfat_16033)	Prepro--alpha2(I) collagen	Cell structure and mobility	4.47±1.41	3.03±0.89
AB008683 (rpfat_12534)	COL1A2 mRNA for alpha2(I) collagen	Cell structure and mobility	4.56±0.71	-
V00503 (rpfat_17393)	mRNA encoding pro--alpha--2 chain of type I procollagen	Cell structure and mobility	4.20±1.07	2.52±0.90
X06700 (rpfat_18309)	mRNA 3' region for pro--alpha1(III) collagen	Cell structure and mobility	3.58±0.42	ND
X14420 (rpfat_19990)	Pro--alpha--1 type 3 collagen	Cell structure and mobility	3.31±0.02	ND
NM_000090 (rpfat_12646)	Collagen, type III, alpha 1	Cell structure and mobility	2.57±0.07	-
AF017305 (rpfat_18326)	Deubiquitinating enzyme UnpEL (UNP) mRNA	Protein metabolism	2.35±0.03	-
AW656523 (rpfat_19130)	108820 MARC 1PIG cDNA 5', mRNA sequence.		2.90±0.68	-
AC087859.3 (rpfat_17641)	*H. sapiens* chromosome 3 clone RP11-34L16 map 3p, complete sequence		3.74±0.37	-
rpig_3584	Unknown EST		2.35±0.13	-
rpfat_8229	Unknown EST		2.41±0.02	-

-: Not analyzed with the real-time PCR; ND: not detected by the real-time PCR analysis.

accumulation. To minimize the individual differences, only the genes that showed changes in all four comparison groups are used for further analysis.

It has been suggested that clenbuterol works by binding to the β-androgenic receptor and transmitting signals into the cell alone the G-protein mediated cAMP signaling pathway. In this study, 17 genes were found to be consistently differentially expressed with clenbuterol treatment in the microarray analysis. Among the 17 genes, two genes related to signal transduction were found to be up-regulated, the cAMP dependent protein kinase type I regulatory gene (PRKAR1A) and RAB30. Up-regulation of PRKAR1A correlates with increasing protein kinase A (PKA) activity reported previously (Domina et al., 2005). RAB30 is a member of the RAS oncogene family and involved in signaling pathway that controls the expression of a subset of yet-to-be-defined genes that are crucial for cell growth and differentiation. Until now, there has been no reported physiologic function of RAB30.

Only 3 genes (apolipoprotein D, apolipoprotein R and ST6GalNAc4) directly involved in lipid metabolism were found to be differentially expressed. ApoD is a component of high density lipoproteins. Apo-D is also closely associated with the enzyme lecithin: cholesterol acyltransferase, an enzyme involved in lipoprotein metabolism. ApoR is a 23 kDa protein found on very low-density lipoproteins (VLDL), on chylomicrons and in the d > 1.21 g/ml fraction of pig plasma (Cooper and Attie, 1992). The physiologic function of apo D and apo R in adipose tissue are unknown. The protein encoded by ST6GALNAC4 is a type II membrane protein that catalyzes the transfer of sialic acid from CMP-sialic acid to galactose-containing substrates (Kang et al., 2004). This protein is a member of glycosyltransferase family 29. ST6GALNAC4 is involved in glycolipid metabolism. The functions of three genes in adipose metabolism were not well studied previously and might be worthy further characterization in regards to the reported findings here.

The microarray analysis indentifies 17 genes to be up-regulated by the administration of clenbuterol in both the 3 and 4-month-old pigs, with no

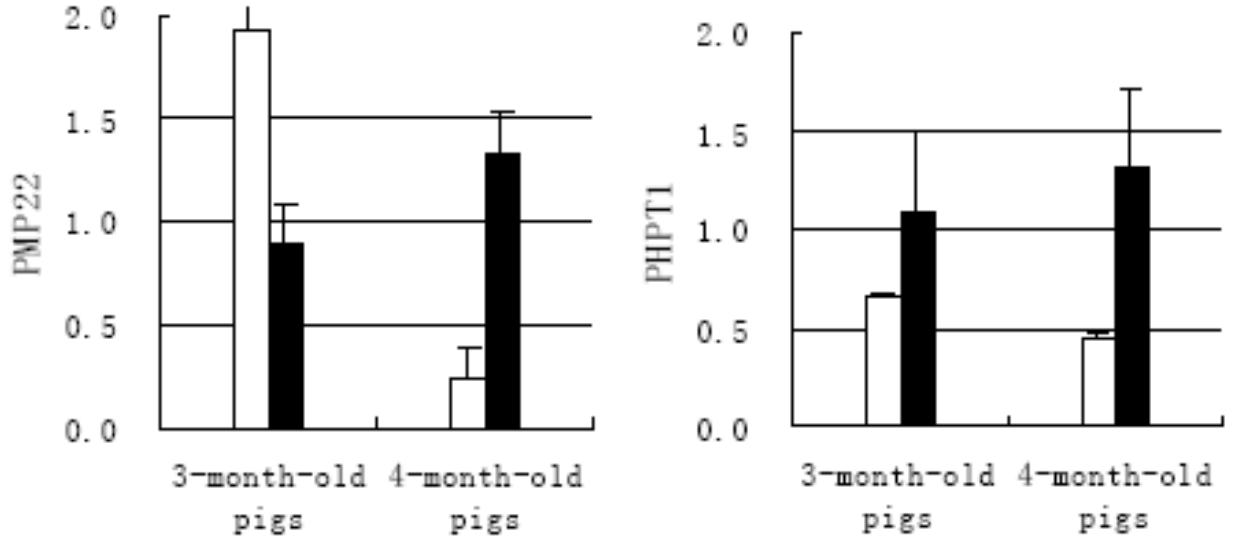

Figure 2. the real-time PCR analysis of the mRNA expression ratios of negative genes in the microarray results. Microarray results: clear bars; real-time PCR results: solid bars. The present values are expressed as the ratio of the group mean levels in the test pigs to those in the control pigs.

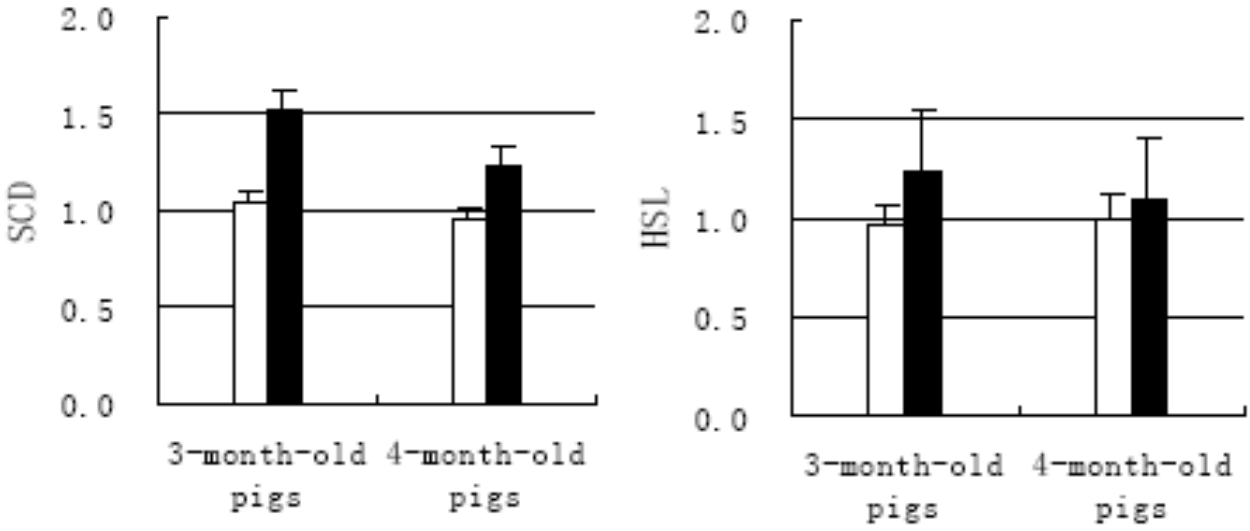

Figure 3. The real-time PCR analysis of the mRNA expression ratios of key genes in adipose metabolism. Microarray results: clear bars; real-time PCR results: solid bars. The present values are expressed as the ratio of the group mean levels in the test pigs to those in the control pigs.

genes found to be down-regulated in both groups. To further investigate whether some genes were down-regulated by the administration of clenbuterol, phosphohistidine phosphatase 1 (PHPT1) and peripheral myelin protein 22 (PMP22), which were found to be down-regulated in only one of the groups in the microarray results, were chosen for further analysis by real-time PCR (Figure 2). The PCR results also showed that the mRNA expressions of these two genes were not changed significantly (ratios between 0.5 and 2) which confirms that the unusual microarray result that no genes were suppressed.

Furthermore, some genes highly related to fat accumulation were not found to be differentially expressed by the administration of clenbuterol in the microarray data, such as stearoyl-CoA desaturase (SCD) and hormone-sensitive lipase (HSL). Real-time PCR analysis confirmed these results (Figure 3). SCD is a microsomal fatty acid modifying enzyme that catalyzes the introduction of the cis double bond between carbons 9 and 10 of saturated

fatty acyl-CoA substrates, resulting in the production of monounsaturated fatty acids. Therefore, SCD is the enzyme responsible for conversion of saturated fatty acids into monounsaturated fatty acids (MUFA) in mammalian adipocytes (Enoch et al., 1976). HSL hydrolyzes stored triglycerides to free fatty acids and is the rate-limiting enzyme of lipolysis. Both of these enzymes were not found to be differentially expressed by the microarray or the real-time PCR. It was reported that the HSL expression level does not change but that its translocation rate increases when lipolysis is enhanced (lipolysis may be induced by this hormone) (Egan et al., 1992; Londos et al., 1999 a, b). Such changes could not be detected by the techniques used in these tests.

The microarray analysis and the real-time PCR analysis showed that the mRNA of three collagen synthesis genes (COL1A1, COL1A2 and COL3A1) was increased in adipose cells by the administration of clenbuterol. Collagen protein expression increased in muscle tissue (including skeletal muscle and heart muscle) in mice fed with clenbuterol at high dosage, which indicates that collagen protein synthesis may be enhanced by clenbuterol (Patiyal and Katoch, 2005; Bonnet et al., 2005). Our data indicates that the collagen protein expression level also increased when the adipose cells were treated with clenbuterol.

Although, the biological functions of the differentially expressed genes are not completely known, higher expressions of these molecules in adipose tissue might contribute to the reduction of fat accumulation. It is possible to identify the key molecules which regulates adipose metabolism in these genes.

Conclusion

In our study, cDNA microarray and real-time PCR were used to identify 17 genes differentially expressed in pig adipose tissue when treated with clenbuterol. Eight of the 17 genes and four of the negative genes were verified by real-time PCR and 10 genes got similar magnitudes as in the microarray results. Apolipoprotein D, apolipoprotein R and ST6GalNAc4 genes respond to clenbuterol administration in pig adipose tissue and their function may contribute to adipose tissue reduction.

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