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Identifying lipid metabolism genes in pig liver after clenbuterol administration

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1. ABSTRACT

Clenbuterol is a repartition agent (beta 2-adrenoceptor agonist) that can decrease fat deposition and increase skeletal muscle growth at manageable dose. To better understand the molecular mechanism of Clenbuterol's action, GeneChips and real-time PCR were used to compare the gene expression profiles of liver tissue in pigs with/without administration of Clenbuterol. Metabolism effects and the global gene expression profiles of liver tissue from Clenbuterol-treated and untreated pigs were conducted. Function enrichment tests showed that the differentially expressed genes are enriched in glycoprotein protein, plasma membrane, fatty acid and amino acid metabolic process, and cell differentiation and signal transduction groups. Pathway mining analysis revealed that physiological pathways such as MAPK, cell adhesion molecules, and the insulin signaling pathway, were remarkably regulated when Clenbuterol was administered. Gene prioritization algorithm was used to associate a number of important differentially expressed genes with lipid metabolism in response to Clenbuterol. Genes identified as differentially expressed in this study will be candidates for further investigation of the molecular mechanisms involved in Clenbuterol's effects on adipose and skeletal muscle tissue.

2. INTRODUCTION

Beta 2-Adrenoceptor agonists have been shown to promote protein deposition and to increase fat lipolysis in various vertebrates (1). Clenbuterol an efficient beta2-adrenoceptor agonist that is membrane permeable due to its high lipid solubility (2), its ready passage of the blood-brain barrier (3), and its long half-life (about 26 hours) (4). Despite proven acute toxicity, Clenbuterol has been misused to increase the lean meat production of commercial animals (5-9).

Clenbuterol initiates repartition effects by activating the beta 2-adrenergic receptor/cAMP-dependent pathway. Initially, the beta 2-adrenoceptor agonist binds to beta 2-adrenergic receptor forming a complex, which activates the α subunit of a G protein (Gsα). The Gsα then activates adenylyl cyclase, which produces cyclic adenosine monophosphate (10). This classic second messenger, cAMP, binds to the regulatory subunit of PKA (protein kinase A), releasing the catalytic subunit, which finally moves to the nucleus

to phosphorylate intracellular proteins. These intracellular proteins may be enzymes or transcription factors, such as hormone sensitive lipase (HSL), Perilipin, acetyl-CoA carboxylase (ACC) or cAMP response element binding protein (CEBP), which are key regulators affecting lipogenesis and lipolysis.

The Clenbuterol-mediated decrease in body fat has been observed in many species (11-14). It has been speculated that this is the result of increased lipolysis in adipose tissue, decreased lipogenesis in both adipose and liver tissues, or the combination of the two (15-17).

Previous studies on Clenbuterol-mediated fat decrease mainly focused on physiological indicators or limited genes with known functions, and they did not provide a global view at the molecular level. To date, only some sporadic studies have been conducted on gene expression patterns in Clenbuterol treated animals. Using the Affymetrix platform Spurlock *et al.* (18) examined gene expression changes in mouse skeletal muscle after treatment with Clenbuterol. They showed that Clenbuterol has important effects on cell proliferation/differentiation and translation regulation. Our previous research used cDNA microarrays to analyze the gene expression profile of adipose tissue from pigs treated with Clenbuterol. We identified six lipid metabolism-related genes that warranted further study (19, 20).

Hepatocytes are responsible for many essential functions, including food intake, metabolism, and body weight (21). beta 2-Adrenergic receptors are abundant in the liver of most species (22). Notably, previous studies showed that the highest Clenbuterol concentration was in liver tissue after oral administration (8, 23). This prompted us to examine the gene expression profile in liver tissue after administration of Clenbuterol.

The object of this study was to identify important genes and signaling pathways in liver tissue from pigs treated with Clenbuterol. The global gene expression profiles of liver tissues from Clenbuterol-treated Chinese miniature pigs and untreated control pigs were determined by Porcine Genome Arrays, which contain over 24,123 probe sets (covering over 23,375 porcine transcripts) (http://www.affymetrix.com). Our study provides an insight into the regulation of the liver metabolism of beta 2-adrenoceptor agonists. We hope that our results will aid in the search for Clenbuterol alternatives that have similar repartition effects but no toxicity for domestic breeding.

3. MATERIALS AND METHODS

3.1. Animal management and tissue collection

Four Chinese miniature pigs were used in this experiment; two males and two females. They were randomly divided into two groups, and each group contained two pigs that were the same gender and were full-sibs. The one treated pig in each group was fed 25mg/kg Clenbuterol twice daily for the first four weeks and 50 mg/kg Clenbuterol for the next four weeks. The other pig (control) was fed the same diet but without Clenbuterol. Animals were managed as described previously (19). All animals were sacrificed according to accepted standards of handling, including preslaughter fasting and welfare issues. Liver tissue samples taken from each animal were flash frozen in liquid nitrogen and stored at -80°C prior to RNA isolation.

3.2. Histology

Frozen sections, 7-μm thick, were made from livers of Clenbuterol-treated and untreated pigs as indicated above. Glycogen was visualized by periodic acid-Schiff staining (24).

3.3. RNA isolation and Microarray hybridization

RNA from the liver tissue was extracted with TRIZOL reagent (Invitrogen, Gaithersburg, MD, USA), and the resulting total RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA quality was assessed using a spectrophotometer (Eppendorf). RNA with an OD 260/280 ratio greater than 1.8 was used for the microarray experiments.

For each sample, 20μg total RNA was used for cDNA synthesis. cRNA synthesis, labelling reactions, and hybridization to the Porcine Genome GeneChip™ (Affymetrix, Santa Clara, CA. Cat. No.900623) were conducted according to the manufacturer's protocols (http://www.affymetrix.com). Microarray experiments were designed to comply with minimum information about a microarray experiment (MIAME) guidelines (25). Data derived from the four microarrays has been submitted to the Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE13786.

3.4. Data analysis

GeneChip Operating Software (GCOS,version 2.0) was used to process the hybridization images from the Affymetrix GeneChip Scanners. The outputs were manually inspected for the percentages of present calls and extreme values. Cluster (26)and PCA (27) were used to check the consistency for replicates, with the criteria of normalized maximum value/minimum value >5 across all the conditions.

Raw data extracted from the arrays were normalized using the gcRMA algorithm (28) at the probe level and then log2 transformed. Probe sets with at least one Present call across all the hybridizations were kept for further analysis. Differentially expressed genes were identified using the LIMMA package (29) and SAM method (30).

3.4.1. Validation by quantitative real-time RT-PCR

Differentially expressed genes identified from the microarray were validated by real-time RT-PCR using a fluorescent temperature cycler (ABI Prism 7900HT Sequence Detection System, Applied Biosystems). A standard two-step procedure was applied. RNAs isolated from livers of four pigs were reverse transcribed into single strand cDNA using oligodT primers and M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Real-time RT-PCR was performed with 15-μl reaction mixtures consisting of cDNA, 0.5μM specific primer sets for each target gene, and SYBR Green PCR Master Mix (Applied

Table 1. Summary of porcine liver gene expression profile changes between pigs treated with Clenbuterol-HCL and the untreated control group

Sample (4 months)		Porcine Genechips	Porcine Genome Array Number of probe sets	Genes After Image Processing	Genes Differentially Expressed (Gene with>2-fold Changes)	
Control	Treated				Upregulated	Downregulated
Male 1 Female 1	Male 2 Female 2	4	23937	15383	337	355

Table 2. BLASTN results of porcine target sequences against human Refseq

Method	Genes on Chips	Using Database	Annotated genes	Category
Blastp	15383	NCBI human protein Refseq	45	Relative to immunity
Blastn	15383	NCBI human Refseq	9838 (up 265,down 147)	Various

Biosystems, UK). Conditions were: 50°C for 2min, 95°C for 10min) followed by 40 cycles of 95°C for 15sec and 60°C for 1min. The specificity and quantification of each PCR product were checked by melting curve analysis, as follows: 95°C for 15sec, 60°C for 15sec, 95°C for 15sec. For all assays, expression levels were quantified by the relative standard curve by amplifying serial dilutions of plasmid by cloning the PCR product of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into pMD-19 T-vector (Takara, China). Each PCR reaction was run in quadruple independent samples. The beta-actin gene was used as internal control to normalize the initial RNA input, which was found to display remarkably stable expression across all experimental treatments. Primers for the RT-PCR are listed in Additional file 1 Table S1.

3.5. Clustering of differentially expressed genes

Hierarchical clustering of differentially expressed genes was analyzed with The Institute for Genomic Research (TIGR) Multiexperiments Viewer (MEV) v4.5.1 (31), which uses average linkage and a Euclidean distance metric to generate the hierarchical tree.

3.6. Gene function and pathway enrichment analysis

The lists of differentially expressed genes from microarray analysis were passed to the Database for Annotation, Visualization And Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) (32, 33) for functional analysis. DAVID groups candidate genes that share similar functional annotation and detects statistically significant enrichment of the functional groups. In additional to functional enrichment, differentially expressed genes that shared biological pathways were also identified using Biorag (http://www.biorag.org/pathway.php) (34). Biorag organizes candidate genes and detects biological pathway enrichment among them by searching known pathways from various sources (BIOCARTA, GENMAPP, and KEGG).

3.7. Prioritization of differentially expressed genes

To provide a shortened list of genes that are most likely to be involved in Clenbuterol-related metabolic processes, the differentially expressed genes were prioritized using Endeavour. Endeavour (http://www.esat.kuleuven.be/endeavour) is a supervised algorithm that, when given a set of causative genes, prioritizes candidates by identifying other genes with similar bioinformatic profiles. These profiles are based on literature, function, sequence, protein interaction, gene expression, pathways, and other bioinformatic information. To train the Endeavour algorithm, we used genes known to be involved in lipid metabolism. The differentially expressed genes identified by the microarray were then scored and ranked using this model.

4. RESULTS

4.1. Phenotypic changes

In our previous paper (19), we found significant differences in blood Clenbuterol concentrations and body compositions between Clenbuterol-treated and untreated animals of both 3 and 4 months, especially for animals of 4 months.

Therefore, we used frozen liver slices from 4-month-old pigs to visualize the effect of Clenbuterol on glycogen deposition (Figure 1). For untreated animals, glycogen was observed in the majority of hepatocytes. However, for treated animals, we found depletion glycogen deposition in hepatocytes. This confirmed the possible role of Clenbuterol in lipid metabolism.

4.2. Differentially expressed transcripts in liver tissue

Expression levels were compared between treated and untreated animals for 15383 probe sets whose signals were available for all four microarrays. Differentially expressed probe sets were identified if both of two criteries were met: 1) a more than 2-fold change in expression and 2), statistical significance reported by SAM and LIMMA.
A total of 692 probe sets were shown to be differentially expressed, of which 337 were upregulated, with a maximun fold change of 34.06; 355 were downregulated with a maximum fold change of 47.14. Details of differentially expressed probe sets are shown in Table 1.

Most of the target sequences in Affymetrix Porcine GeneChips are derived from unannotated pig sequences; therefore,we mapped the target sequences to human Refseq using BLAST with the threshold set to e-10, resulting in 7666 nonredundant transcripts, of which 377 were differentially expressed (237 upregulated and 140 downregulated transcripts, Table2; Details are in Additional file 2 Table S2).

4.3. Hierarchical Clustering of differentially expressed probe sets

Hierarchical clustering, which groups samples according to the degree of their expression similarity, has been widely used. The importance of clustering is twofold: it provides quality control and it might suggest functional relationships based on similar expression patterns when analyzed together with other evidence. Clustering was applied to the identified differentially expressed probe sets.

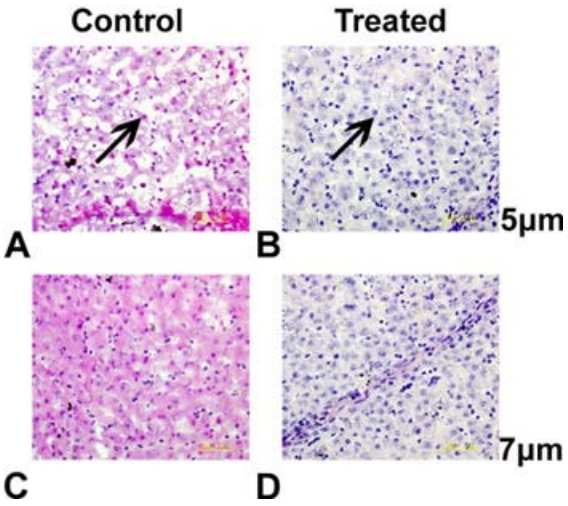

Figure 1. Periodic acid-Schiff staining of livers of pig that were untreated or treated with Clenbuterol. Histological visualization of hepatic glycogen in cryostat slices (5μm and 7μm) of livers of pig that were untreated (A and C) and treated with Clenbuterol-HCL (B and D), as indicated in Materials and Methods. Glycogen was visualized using the periodic acid-Schiff reagent.

The outputs displayed several unique distribution features: 1) the four samples were clearly clustered into treated and untreated groups; and 2) the two biological replicates in each group exhibited highly similar expression patterns, indicating good reproducibility in our experiments (Figure 2).

4.4. qPCR validation of differentially expressed transcripts/genes

Thirteen transcripts/genes that were differential expressed were chosen for real-time PCR validation. The transcripts/genes were selected on the basis of their previously reported associations with lipid or fatty acid metabolism (ectonucleotide pyrophosphatase, ENPP3; fatty acid synthase, FASN; fatty acid desaturase 2, FADS2; glutathione peroxidase 3, GPX3; acyl-CoA desaturase, SCD; CCAAT/enhancer binding protein (C/EBP), gamma, CEBPG; proteolipid protein), the MAPK signaling pathway (growth arrest and DNA-damage-inducible, Beta, GADD45B; fibroblast growth factor 13, FGF13; frizzled homolog 4, FZD4), cell growth (connective tissue growth factor, CTGF; v-fos FBJ murine osteosarcoma viral oncogene homolog, FOS) and cell adhesion (immunoglobulin superfamily, member 4, IGSF4). The results of the validation are shown in Table 3. Correlation analysis suggested that the two techniques generally agreed with each other (Spearman rho=0.711, p=0.002).

4.5. Functional enrichment of differentially expressed transcripts/genes

To get some insight into the functional profile of genes related to Clenbuterol, 377 differentially expressed transcripts/genes were analyzed using the DAVID program for functional enrichment. We found 24 enriched groups for upregulated transcripts/genes (Table 4) and nine for downregulated transcripts/genes (Table 5). There were some interesting groups among them, such as signal protein, fatty acid biosynthesis, amino acid metabolic process, cell adhesion, calcium, protease inhibitor, and apoptosis for the upregulated transcripts/genes, and developmental process, cell differentiation, organic acid transport, and signal transduction for the downregulated transcripts/genes. More details of the enrichment analyses are available in Additional file 3 Table S3 and Table S4.

4.6. Pathways enrichment of differentially expressed transcripts/genes

In addition to functional similarity, genes that are connected with each other to accomplish biological function are also important. Therefore, we performed pathway analysis for the differentially expressed genes using BioRag. BioRag searches currently available pathway information from Kegg, Biocarta, and GenMapp databases to identify pathway enrichment for candidate genes. As shown in Table 6, differentially expressed transcripts/genes were found to be enriched in 18 pivotal physiological pathways, some of which were likely to lead to adipose reduction. These included signal transmission (Cell adhesion molecules and Cell Communication pathway), cell proliferation and differentiation (MAPK signaling pathway, Wnt signaling pathway, and Signaling Pathway from G-Protein Families), and insulin signaling (Type I diabetes mellitus pathway and Insulin signaling pathway). Notably, 17 and 13 of the 377 differentially expressed transcripts/genes fell into the Cell adhesion molecules and MAPK signaling pathway, respectively, and most of them were upregulated. This suggested that signal transmission and intracellular signaling cascade regulation in the liver might be affected by Clenbuterol treatment.

4.7. Prioritization of differentially expressed transcripts/genes

There are three drawbacks to the function or pathway enrichment approach: 1) they are less effective because they use partial information only, 2) they do not provide quantitative measurements for the importance of candidate genes, and 3) they identified a group of genes that responded to Clenbuterol, but were not necessarily related to Clenbuterol-mediated lipid metabolism. To better identify transcripts/genes related to Clenbuterol-mediated lipid metabolism, we used Endeavour, which integrates a large number of different data sources, to generate biologically relevant prioritizations for candidate genes. Using genes known to be involved in lipid metabolism as a training set, all differentially expressed genes were subjected to Endeavour prioritization. Candidate genes ranked in Top 20 are listed in Table 7.

5. DISCUSSION

5.1. Phenotype comparison

Consistent with other studies, we observed significant differences in physiological performance between Clenbuterol treated and untreated pigs (19). Beta 2-Adrenegic receptors exist in many organs that are related to body growth, including skeletal muscle, adipose tissue, neuroendocrine organs, and especially, the liver. It is

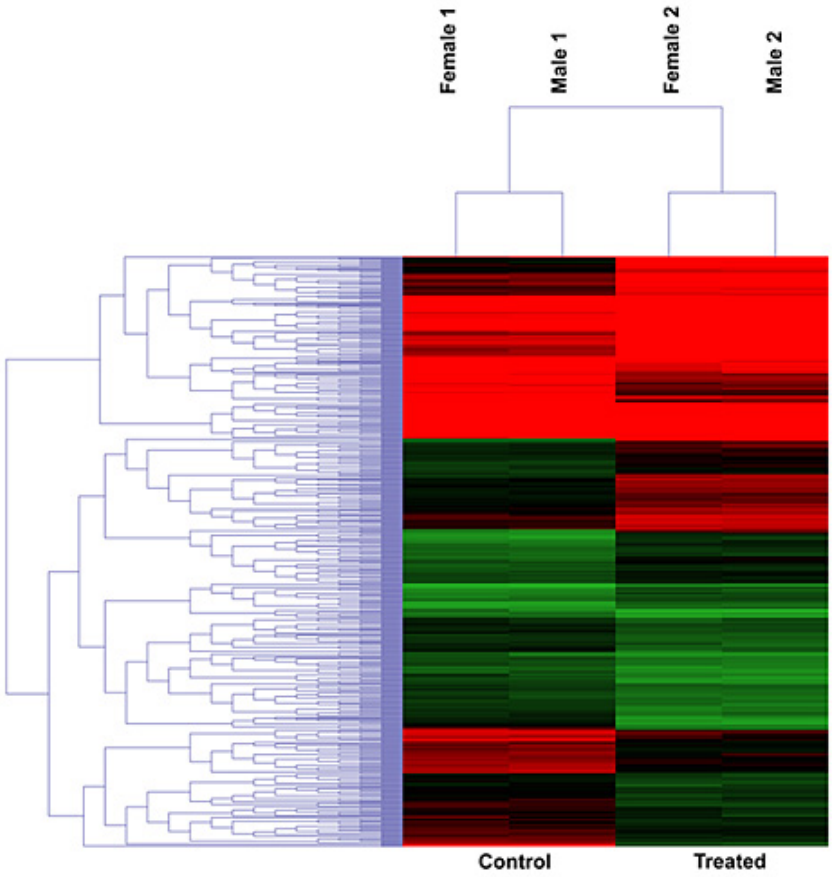

Figure 2. Hierarchical clustering of differentially expressed genes. Hierarchical clustering of microarray data for 692 microarray probes showing differential expression in all four contrasts performed between the with and without Clenbuterol-HCL administration groups. Individuals are scattered along the x-axis and probes are distributed along the y-axis. For each individual and probe set, colors represent log2 (Clenbuterol-HCL administration group fluorescence/control group fluorescence) according to the upper scale bar. Hierarchical clustering was performed using the Euclidean metric.

reasonable to suspect that previous reported reduced lipid droplets in adipocytes might be a consequence of reduced fatty acid synthesis in the liver, caused by beta 2-adrenegic receptor-mediated decrease of corresponding enzyme activities.

In the human liver, glycogenolysis relies primarily on a beta 2-adrenegic receptor/cAMP-dependent mechanism (35). Here, using a periodic acid-Schiff staining technique, we observed glycogen deposition in most hepatocytes of untreated pigs and depleted glycogen deposition in Clenbuterol-treated pigs, suggesting that the beta 2-adrenegic receptor/cAMP-dependent mechanism also operates in pig liver.

5.2. Function and pathway enrichments of differentially expressed genes

In this study, we detailed the global change in gene expression in pig livers after Clenbuterol administration. This is, to our knowledge, the first study to focus on the effects of a beta 2-adrenegic receptor agonist on liver gene expression in the pig model. A total of 692 probe sets were found to be differentially expressed, which represented 377 transcripts/genes. Some of these transcripts/gene could be cross-validated by previous studies of beta 2-adrenegic receptors; however, most of them were firstly identified in this study. After enrichment analysis, some interesting function and pathway enrichments involving the differentially expressed genes were identified.

5.2.1. Glycogen and fatty acid metabolism

It has been generally accepted that epinephrine (a catecholamine) and beta-adrenergic receptors are primary mediators of glycogen regulation. For most species, catecholamine in the liver affects glycogenolysis via the beta 2-adrenegic receptor/cAMP signaling pathway under normal physiological condition (36). The key enzymes of glycogen breakdown, such as glycogen phosphorylase and glucose-6-phosphatase, can be activated by a cAMP-dependent protein kinase. Subsequently, glucogenolysis and gluconeogenesis are elevated to increase hepatic glucose production. Glucose 6-phosphate is formed directly from glycogen hydrolysis; thus, the glucose that is derived from glycogen can enter the glycolysis pathway. Liver possesses glucose-6-phosphatase, which can convert

Table 3. Comparison of fold changes in expression obtained by real-time RT-PCR and microarray analyses of 14 differentially expressed genes

Gene[1]	Microarray data	Real-time PCR data	Real-time PCR p-value
ENPP3	-47.12	-1.704	0
FASN	2.84	4.693	-0.0002
proteolipid protein	1.79	2.293	0.0119
FADS2	3.82	6.023	0.0002
CEBPG	-2.22	-1.945	0.002
GPX3	-2.46	-2.71	0.0217
SCD	3.15	4.665	0.0174
GADD45B	3.48	1.96	0.009
FGF13	7.46	4.28	0.0002
FZD4	3.05	-1.328	0
CTGF	2.7	-1.762	0.0015
FOS	3.02	1.091	0.2817
IGSF4	-2.15	-1.32	0.1205

[1]The full names of each gene are listed in main text, Microarray values were normalized to all spots on the microarray, where real-time PCR values, were normalized to housekeeping the beta-actin gene.

Table 4. GO-categories over-represented among the transcripts significantly upregulated after Clenbuterol administration

Annotation Cluster[1]	Enrichment Score
Glycoprotein protein	7.24
Immune response	5.25
Signal peptide	4.64
Immunoglobulin	2.91
Response to stimulus	2.59
Binding	2.28
Endoplasmic reticulum	2.05
Plasma membrane	2.03
Heparin-binding	2
Fatty acid biosynthesis	1.65
Amino acid metabolic process	1.6
Thrombospondin	1.48
Prostaglandin metabolic process	1.47
Transmembrane	1.41
Extracellular matrix	1.28
Cell adhesion	1.24
Organismal development	1.24
Calcium	1.23
Coagulation	1.18
Immunoglobulin	1.17
Protease inhibitor	1.17
Cadherin	1.08
Amino acid biosynthetic process	1.04
Apoptosis	0.99

[1]Annotated clusters are listed by enrichment scores. The cluster names were chosen by us to indicate the nature of the clustered biological process, because the DAVID algorithm did not assign clusters names after clustering.

glucose 6-phosphate to glucose, thus glucose released from liver glycogen can also be exported to other tissues.

Consistent with previous studies, we found that "signal protein" groups related to glucose metabolism were enriched. For example, the signal facilitated glucose transporter (GLUT2), which functions as a glucose sensor in the liver to facilitate glucose entry into cells (37), and glucuronidase, beta (GUSB), which catalyzes breakdown of complex carbohydrates, were both up-regulated. After increases in hepatic glucose production, beta 2-adrenoceptor agonists inhibit insulin secretion, and the glucose uptake induced by insulin is thus inhibited in skeletal muscle and white adipose tissues.

In addition, the enrichment of genes involved in "fatty acid metabolism" support the hypothesis that Clenbuterol can enhance lipolysis, or perhaps, the breakdown of triglycerides into glycerol and nonesterified fatty acid (NEFAs). It was reported that several beta-adrenoceptor agonists acutely elevate the plasma nonesterified fatty acid concentration in pigs (14) and in cattle (12, 38). We found upregulated genes that are responsible for unsaturated fatty acids synthesis (Fatty acid desaturase 2, (FADS2); stearoyl-CoA desaturase, (SCD)), fatty acid oxidation (hepatic lipase (LIPC); acyl-Coenzyme A oxidase 1, palmitoyl, (ACOX1)), and removal of cholesterol (cytochrome P450, family 2, (CYP2J2); cytochrome P450, family 7, subfamily A, polypeptide 1, (CYP7A1)). Our results suggest that Clenbuterol can decrease body weight by increasing fatty acid catabolism directly.

In pathway mining analysis, we also identified enriched pathways related to Type I diabetes mellitus and the Insulin signaling pathway. It has been suggested that beta 2-adrenoceptor agonists affect glucose homeostasis through modulating of insulin secretion.

5.2.2. Cell development, differentiation, and intracellular signaling regulation

Hepatic effects of Clenbuterol beyond glycogen regulation and lipid metabolism have been observed. Up-regulated genes in enrichment group "cell apoptosis", such

Table 5. GO-categories over-represented among the transcripts significantly downregulated after Clenbuterol administration

Annotation Cluster[1]	Enrichment Score
Developmental process	1.37
Development	0.99
Cell differentiation	0.91
Immune system	0.9
Neurological system	0.9
Organic acid transport	0.88
Plasma membrane	0.88
Signal transduction	0.88
Cell growth	0.84

[1]Annotated clusters are listed by enrichment scores. The cluster names were chosen by us to indicate the nature of the clustered biological process, because the DAVID algorithm did not assign clusters names after clustering.

Table 6. Over-represent cellular pathways that are involved in the response to Clenbuterol administration (selected pathways of interest)

Pathway name[1]	Included genes	p-value	Gene name
Cell adhesion molecules (CAMs)	17	0	HLA-B;CADM1;ITGAV;CDH1;HLA-DMB; CLDN7;VCAM1;HLA-DRA;HLA-DQA2; HLA-DQB2;HLA-DRB5;SELL;HLA-G
Antigen processing and presentation	15	0	HLA-B;HSPA9;CD74;HLA-DMB;HLA-DRA; HLA-DQA2;HLA-DQB2;PDIA3; HLA-DRB5;IFI30;HLA-G
MAPK signaling pathway	13	0	CACNA2D1;MAP3K7;DUSP1; MAPK7;HSPB2;DUSP6;PAK1 HSPA9;FOS;MKNK1;GADD45B; GADD45G;FGF13
Type I diabetes mellitus	11	0.00001	HLA-B;HLA-DMB;HLA-DRA HLA-DQA2;HLA-DQB2;HLA-DRB5 HLA-G
Hs_Cell_cycle_KEGG	8	0	GNAI1;CDKN1A;CALM1;MCM6 CDH1;RGS2;ATP1B1;RGS1
Insulin signaling pathway	6	0.00199	PPP1CA;CALM2;PPP1CC; MKNK1;FASN
Cell Communication	6	0.00199	COL1A1;DSC2;DSG2;COL5A2 LMNA;THBS1
Wnt signaling pathway	5	0.00568	TCF7L2;DVL3;MAP3K7;FZD4;SFRP1
Arachidonic acid metabolism	5	0.00568	GPX3;CYP2J2;CYP2C9;PTGDS CYP2C18
Notch signaling pathway	5	0.00568	HES1;DVL3;PSEN2;DTX3L;NUMB
Regulation of actin cytoskeleton	5	0.00568	PPP1CA;ITGAV;PPP1CC;PAK1 FGF13
Cytokine-cytokine receptor interaction	5	0.00568	KIT;CXCR4;CCL2;CCL19;CXCL3
Hs_TGF_Beta_Signaling_Pathway	4	0.00017	ZNF423;SERPINE1;FOS;THBS1
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa (alpha)	4	0.00111	ME1;DUSP1;ACOX1;CITED2
Hematopoietic cell lineage	3	0.00568	KIT;HLA-DRA;HLA-DRB5
Control of skeletal myogenesis by HDAC&calcium/calmodulin-dependent kinase (CaMK)	3	0.00111	CALM2;IGF1;MAPK7
Signaling Pathway from G-Protein Families	3	0.00111	GNAI1;CALM2;FOS
Hs_Proteasome_Degradation	3	0.00017	HLA-B;PSMB3;HLA-G

[1]Pathways listed are those that include three or more of the 412 differentially expressed genes. WNT, wingless and Int; TGF, transforming growth factor

as apoptosis inhibitor 5 (API5), BCL2-associated athanogene 3 (BAG3), and heat shock 70kDa protein 9B (HSPA9B), inhibit cell apoptosis by binding caspases, controlling cell cycle, or preventing apoptosome formation, respectively. Our results were consistent with a previous experiment in mouse, which demonstrated that Clenbuterol reduced liver apoptosis while propranolol, a beta 2-adrenoceptor antagonist, blocked the protective effects (39). However, the relationship between reduced apoptosis and the decrease of fat mass after Clenbuterol treatment remains unknown. Perhaps apoptotic inhibition of cell proliferation contributes to slower metabolism of hepatocytes (11).

We also found that most of the down-regulated transcripts/genes were enriched in “development” and “cell differentiation” groups. The genes in these groups are mainly cell growth or transcription factors, such as, IGF1 family related genes (insulin-like growth factor 1, IGF1; insulin-like growth factor binding protein 5, IGFBP5), and TNF family related genes (TNF receptor-associated factor 5, TRAF5; lipopolysaccharide-induced TNF factor, LITAF). IGF1 is mostly synthesized in the liver and circulates in the plasma. In addition to its role in growth and differentiation, IGF-1 also indirectly affect carbohydrate and lipid metabolism by adjusting insulin secretion (40). Liver-derived IGF-1 is something of an enigma, because hepatocytes produce IGF-1 but have no detectable IGF-1 receptor expression (41), and the function of IGF-1 in the liver remains unclear. We found IGF1 was downregulated in the liver after Clenbuterol treatment. The mechanism underlying beta 2-adrenoceptor agonist-mediated downregulation of IGF-1 expression in liver is undefined; however, the relationship between IGF-1 and

Table 7. Prioritized list of differentially expressed genes related to Clenbuterol-mediated lipid metabolism

Gene	Global prioritization	Description	
	Rank	Score	
HNF4A	1	1.15E-11	Hepatocyte nuclear factor 4-alpha
FASN	2	3.66E-11	Fatty acid synthase
CEBPG	3	1.96E-08	CCAAT/enhancer-binding protein gamma
ACOX1	4	1.30E-07	Acyl-coenzyme A oxidase 1, peroxisomal
SCD	5	2.95E-06	Stearoyl-CoA desaturase
IGF1	6	4.08E-06	Insulin-like growth factor I
APOB	7	6.6E-06	Apolipoprotein B-100 precursor
SLC2A2	8	2.23E-05	Solute carrier family 2, facilitated glucose transporter member 2
LIPC	9	0.000163	Hepatic triacylglycerol lipase precursor
FADS2	10	0.000701	fatty acid desaturase 2
CHUK|ERLIN1	11	0.000743	SPFH domain-containing protein 1
GUSB	12	0.000789	Beta-glucuronidase precursor
CYP2C9	13	0.00221	Cytochrome P450 2C9
FOSL2	14	0.00409	Fos-related antigen 2
FOS	15	0.00412	Proto-oncogene protein c-fos
PTGDS	16	0.00669	Prostaglandin-H2 D-isomerase
DUSP6	17	0.00995	Dual specificity protein phosphatase 6
ACAT1	18	0.0101	Acetyl-CoA acetyltransferase, mitochondrial
CLTC	19	0.0102	Clathrin heavy chain 1
LMNA	20	0.0103	Lamin-A/C (70 kDa lamin)
F9	21	0.0139	Coagulation factor IX
PPP1CA	22	0.0139	Serine/threonine-protein phosphatase
CYP7A1	23	0.0167	Cytochrome P450 7A1
COL1A1	24	0.0167	Collagen alpha-1 (I) chain
MKNK1	25	0.0172	MAP kinase-interacting serine/threonine-protein kinase 1
CPS1	26	0.0177	Carbamoyl-phosphate synthase
SERPIND1	27	0.0177	Heparin cofactor 2
ASAH1	28	0.0183	Acid ceramidase
GADD45G	29	0.0184	Growth arrest and DNA-damage-inducible protein
MAP3K7	30	0.0218	Mitogen-activated protein kinase kinase kinase 7

lipid metabolism has been suggested. In a transgenic mouse model where the IGF-1 gene was specifically inactivated in hepatocytes, serum levels of insulin, leptin, and cholesterol increased, which resulted in a state of insulin resistance and caused a decrease in fat mass (41). This could partially explain the fat loss in our model.

Tumor necrosis factor (TNF) is a proinflammatory cytokine that is strongly correlated with lipid metabolism. Beta 2-Adrenoceptor agonists have been shown to inhibit the production of TNFa in human peripheral blood mononuclear cells and renal tissue (42). TNFa is 7.5 times more abundant in the adipose tissue of obese subjects than in lean subjects and can be reduced with weight loss interventions (43, 44). Although we did not identify the pig TNF gene from the microarray probe sets using BLAST searching, we expected that pig TNF would be downregulated because we observed downregulation of TNF receptor-associated factor 5 (TRAF5) and lipopolysaccharide-induced TNF factor (LITAF).

We detected 13 differentially expressed genes (Table 5) in the mitogen-activated protein kinase (MAPK) signaling pathway. The MAPK signaling pathway is of particular interest because it is a key upstream mediator of many growth and transcription factors. Some of the factors have already been found to be enriched in “development” and “cell differentiation”. Activation of the MAPK signaling pathway following beta 2-adrenoceptor agonist administration has been previously described (45). Generally, beta 2-adrenergic receptors are mediated through the Gs proteins and the cAMP dependent protein kinase (PKA) system after beta 2-adrenoceptor agonist treatment. In other circumstances, beta 2-adrenergic receptors couple to a Gi protein, and the beta gamma-subunit of the Gi protein stimulates the ERK-MAPK signaling cascade (46). By altering the activity of transcription factors, the MAPK signaling pathway leads to altered transcription of genes that are important for the cell cycle and metabolism. It has been reported that peroxisome proliferator-activated receptor g (PPARg) can be phosphorylated by the extracellular signal-regulated protein kinase (ERK), which is a member of the MAPK family. The transcriptional activity of phosphorylated PPARg was significantly reduced and resulting in the inhibition of adipogenesis in NIH 3T3 cells (47). The activation of the MAPK signaling pathway by extracellular stimuli in our model suggests cross-talk between MAPK and Clenbuterol-affected signaling pathways enables complex regulation of liver metabolism and adipocyte differentiation.

5.2.3. Cell adhesion molecules

In this study, we identified a novel enriched group “Cell adhesion molecules (CAMs)” by both function and pathway analysis, which has not been reported to be related to beta 2-adrenegic receptor agonist function. Most of the upregulated genes in this group, such as integrin, alpha V (ITGAV) (48), cadherin 1, type 1 (CDH1), and cell adhesion molecule with homology to L1CAM (CHL1) (49), are known to facilitate signal transduction. CAMs can be classified into Cadherins, Immunoglobulin-like adhesion molecules, Integrins, and Selectins. CAM functions between neighboring cells, or is located in the plasma membrane, and produces intracellular signals to dynamically interact with the cytoskeleton. Therefore, CAMs can be viewed as adhesive/signaling/trafficking

molecules that transmit chemical and physical information across the plasma membrane in both directions. Our results indicated that communication and transmission between cells are very active after Clenbuterol administration, and the beta 2-adrenoceptor agonist-mediated G protein signal might not necessarily be the only method of Clenbuterol-induced signaling among cells. However, there has been no report concerning the relationship between beta 2 - adrenoceptor agonists and CAMs; therefore, further experimentation is required to investigate this issue.

5.3. Prioritized list of differentially expressed genes related to Clenbuterol's repartitioning effect

We retrieved 339 genes involved in the lipid metabolism pathway from the literature (Additional file 4 Table S5). All these genes have been experimentally validated. Based on the Endeavour global prioritization score, we selected the 30 top-ranked genes from the differentially expressed list as the most likely candidates (Table7).

Notably, candidate genes highly ranked by Endeavour include many lipid metabolism enzymes, such as Fatty acid desaturase 2 (FADS2), stearoyl-CoA desaturase (SCD), hepatic lipase (LIPC), and acyl-Coenzyme A oxidase 1, palmitoyl, (ACOX1). All of them were included in the enrichment group "fatty acid metabolism", as previously mentioned SCD, which is expressed in the liver, has the dual function of a triglyceride hydrolase and a ligand/bridging factor for receptor-mediated lipoprotein uptake. SCD catalyzes the rate-limiting step in the synthesis of unsaturated fatty acids. SCD-deficient mice show significantly reduced lipid synthesis and increased lipid oxidation, thermogenesis, and insulin sensitivity in various tissues, including the liver (50). It has been suggested that SCD could guard against dietary unsaturated fat deficiency. All of these genes are up-regulated in our model. This analysis supports the notion that the body might synthesis more unsaturated fatty acid via upregulation of these genes to cause weight loss after Clenbuterol administration. These highly ranked genes should be considered as candidates for developing treatments for obesity.

More importantly, nearly ten genes in the top 30 candidates belong to the MAPK signally pathway, which is consistent with the pathway mining results. The important functions of the ranked genes (e.g., Dual specificity protein phosphatase 6, DUSP6, and Growth arrest and DNA-damage-inducible protein, GADD45G) from the MAPK signaling pathway have been discussed above. In addition, the prioritized genes (Mitogen-activated protein kinase kinase kinase 7 (MAP3K7), MAP kinase-interacting serine/threonine (MKNK1), and Proto-oncogene protein, FOS; Protein phosphatase 1 (PPP1CA)) are also pivotal components in the classical MAP kinase or p38 MAP kinase pathway. Differentially expressed genes in Table 7 with obvious connection to MAPK are excellent candidates for further functional study in this context.

5.4. Comparison of the cDNA microarray data of adipose tissue derived from identically managed animals

Compared with our previous work using a pig fat cDNA microarray, we found that the changes in the gene expression profiles between adipose and liver tissue experiments were not identical. The main role of white adipose tissue is triacylglycerol (TAG) storage and fatty acid (FA) release. After Clenbuterol treatment, the direct action on adipose tissue should be to activate lipolytic enzymes and coordinate endocrine regulating signals to initiate lipolysis. Indeed, differentially expressed genes identified in adipose tissue were mainly associated with lipid metabolism and signal transduction. In this study, we found more differentially expressed genes in liver tissue that were associated with glucogenolysis, cell development/differentiation, and intracellular signaling cascades. The liver has a key role in lipid metabolism via mobilization of multiple functions, such as de novo lipogenesis

Secondly, the genome wide Porcine Genome Array contains over 24,123 probe sets, which is far more than the 3358 probe sets presented on our pig fat cDNA microarray. The Porcine Genome Array can globally evaluate gene expression changes after Clenbuterol treatment. Changes in the mRNA abundance of multiple genes associated with various functions can be detected, not just genes related to lipid metabolism.

The beta 2-adrenegic receptor has been considered as the primary mediator of glycogen regulation and indirectly affects hepatic glucagon release. Moreover, beta 2-adrenergic receptor agonists can reduce liver apoptosis and reduce the proliferation of preadipocytes. The enriched groups detected in this study indicated that Clenbuterol might decrease fat synthesis and hepatic glycogen levels, by mobilizing enzymes for fatty acid biosynthesis and transcription, and increasing the expression of growth factors regulated by intracellular signaling transduction pathways. For the adipose tissue data, we concluded that increased lipolysis in adipose tissue was mainly caused by increased concentrations of plasma nonesterified fatty acids and increased expressions of genes in the cAMP signaling pathway. In addition, several collagen genes and calcium-related genes were found to be differentially expressed in both liver and adipose tissue.

The inconsistent response of these two tissues after treatment with Clenbuterol might be partially due to differences in the abundance of beta 2- adrenergic receptors in pig liver and adipose tissue. Our results suggest that the effect of Clenbuterol on fat metabolism is more complex than previously thought. The results from adipose and liver tissues complement each will promote further research,

6. CONCLUSIONS

Global gene expression profiling of pigs after administration of Clenbuterol identified differences in liver gene expression and enrichment of functional categories. This study complements our previous study, which detailed

changes in adipose gene expression after Clenbuterol treated (19). We provide evidence indicating that Clenbuterol signaling involves cross-talk among metabolism, signal transduction, and apoptosis, as well as with cell differentiation/growth. This finding suggests that lipid mobilization is not the only reason for the reduced fat accumulation after Clenbuterol administration. Changes in MAPK signaling pathway, cell adhesion molecules, and cell communication might indirectly influence metabolism in pig livers. The differentially expressed genes and pathways detected here will help us to further understand the repartition mechanisms of beta 2-adrenergic receptor agonists on fat reduction and skeletal muscle growth.

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