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Differential Expression of Cytochrome P450 Genes Regulate the Level of Adipose Arachidonic Acid in *Sus Scrofa*

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ABSTRACT : We compared the fatty acid composition of adipose tissues prepared from Korean native and Yorkshire pigs that have different characteristics in growth and fat deposition. There was no significant difference in the content of most fatty acids between the two breeds, with the exception of arachidonic acid and cis-11,14,17-eicosatrienoic acid. We also investigated the transcriptional levels of genes encoding three different types of oxygenases, including cytochrome P450 (CYP), lipoxygenase and cyclooxygenase, which metabolize arachidonic acid. We found a significant difference in the expression of the CYP genes, *CYP2A13*, *CYP2U1* and *CYP3A4*, but no differences for the latter two genes between the two breeds. Our results suggest that the difference in arachidonic acid content between the two breeds was caused by differential expression of the CYP genes. Eventually, different levels of EETs and HETEs produced from arachidonic acid by the activity of CYP might contribute partly to the difference of fatness between the two breeds. (**Key Words :** Arachidonic Acid, Cytochrome P450, Gene Expression, Adipose Tissue, *Sus scrofa*)

INTRODUCTION

Both accumulation and distribution of adipose tissue in an animal are key determinants indicating metabolic status of the organism. These characters have been used as traits to select breeds having better economic values. In addition, adipose tissue produces and secretes various molecules that affect physiologically on itself and other tissues. But there has been little evaluation related to this property for selecting breeds having better economic values. Adipose tissue itself is a metabolically active tissue, along with liver and skeletal muscle. In adipose tissue, fatty acids, prostaglandins, and steroids are either synthesized or converted into different forms, then released into blood stream. These secreted bioactive molecules eventually join the networks of autocrine, paracrine and endocrine pathways (Mohamed-Ali et al., 1998). Because of this, the properties of adipose tissue may influence the overall metabolic homeostasis in organism. Pig (*Sus scrofa*) that has well-developed adipose tissue underneath the skin, could be a good model for adipogenesis research. Originally, Korean native pig (KNP) had a small body size with slow growth and higher back-fat thickness (Kim et al., 2005), which were distinctive characteristics compared to western commercial breeds, such as Yorkshire (YS). Recently, KNP has been restored from the hybrids to retain most original characteristics maintaining a relatively large size (Kim et al., 2007). When we measured fatty acid contents, there was little difference between two breeds. To better characterize the properties of the adipose tissue between these two breeds, we compared their fatty acid composition with gene expression.

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MATERIALS AND METHODS

Animals and tissue preparation

KNP and YS boars were raised under identical feeding conditions and management practices in the National Livestock Research Institute, Jeju Island, South Korea. Unrelated eight pigs (4 KNP and 4 YS) were slaughtered at ~6.5 month-old with a final weight of ~100 kg, and the back-fat tissues between the seventh and ninth ribs were collected. The tissue samples were immediately frozen in

liquid nitrogen and stored at -80°C until use.

Gas chromatography (GC)

To characterize the difference in fatty acid composition between the two breeds, we measured the fatty acid profiles based on a well-established method (Folch et al., 1957; Lepage et al., 1986) with minor modification. Briefly, 200 mg of frozen tissue was homogenized in 10 ml chloroform/methanol (2:1) solution using a homogenizer (PT-MR3100, Polytron, Switzerland). After overnight incubation, 5 ml of 0.88% NaCl was added and mixed well by shaking. After centrifugation, the chloroform fraction was collected and used for further analysis. Five ml of the chloroform fraction was evaporated in the dry-bath (Type 16500, Barnstead, USA) at 50°C under nitrogen gas flow. The hexane-dissolved samples were then injected into the gas chromatography instrument (GC: HP 5890II, Hewlett Packard Co., USA) equipped with a fused silica capillary column (SPTM-2330: 30 m×0.25 mm, i.d. ×0.20 μm in thickness) purchased from Supelco, USA. The injector and the flame ionization detector were maintained at 250°C during the procedure. The initial column temperature was 50°C (held for 2 min), and then was increased gradually by 4°C/min to 250°C (held for 15 min). Ultra-pure helium was used as a carrier gas. For quantitation purposes, we added tridecanoic acid (C13:0, Sigma, USA) to the sample as an internal marker. We used a standard fatty acid methyl ester mixture (18920-1AMP, Supelco, USA) to obtain retention times and to calibrate the chromatogram.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

To examine the expressional difference in CYP genes, we performed RT-PCR analysis of these three CYP transcripts. Four μg of total RNA was reverse-transcribed in a mixture of 0.5 μg anchored Oligo(dT)$_{20}$ Primer (Invitrogen Inc., Carlsbad, CA, USA) and 200 U SuperScript II reverse transcriptase (Invitrogen Inc., Carlsbad, CA, USA). One μl of RT reaction was used directly in PCR for two transcripts, the CYP target transcript and an internal control (beta actin) transcript. Primer sets were designed using Primer3 (Rozen et al., 2000): *ACTB* (CTCCGATCTGTGCAGGGTAT/TGGGG CCTAACGTTCTCAC), *CYP2A13* (AGTTTCGGGACTT CCTCCTC/TAGCCAGACCTTCTCCGAAA), *CYP2U1* (GCGGACCCTTGTTCAGTATG/CTCAGGGCTCAAGTC AAAGG), and *CYP3A4* (GGCTTACACAACCGGAAAA A/TGCCCGTATGTAACACTCCA). The PCR reaction was performed in an AccuPower HotStart PCR Premix (Bioneer, Seoul, South Korea) containing 1 U of HotStart Taq DNA polymerase, 1×PCR buffer and 250 μM of each dNTP in 20 μl reaction volume. In general, the PCR was performed using 6-10 pmole of the target gene primer set and 1.5-2.0 pmole of the beta actin primer set. After an initial activation step at 95°C for 15 min, each PCR cycle was set as denaturation at 94°C for 45 sec, annealing at 52-54°C for 45 sec, and extension at 72°C for 45 sec. After the last cycle, PCR reaction was incubated at 72°C for 5 min. PCR analysis was performed for 25, 28, and 30 cycles to avoid PCR saturation, and PCR products were analyzed by 2% agarose gel electrophoresis. After staining with ethidium bromide, gel image was obtained and analyzed with Quantity One software 4.6.3 (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

GC analysis revealed distinctive peaks for 27 identifiable fatty acids. Among them, there were significant differences in amounts of arachidonic acid (AA) and cis-11,14,17-eicosatrienoic acid between the two breeds (*t*-test $p<0.05$). KNP adipose tissue contained more arachidonic acid, and YS contained more cis-11,14,17-eicosatrienoic acid than the counterpart (Table 1). However, there were no significant differences in contents for the other fatty acid, including in the total amount of fatty acids. Cis-11,14,17-eicosatrienoic acid is a rare and a biologically inactive fatty acid. In contrary, AA is known to be a precursor for important bioactive molecules involved in inflammation (Parnes et al., 2002) and blood pressure regulation (Moreno et al., 2001). Our data analyses showed that the contents of precursor fatty acids for AA including linoleic acid, γ-linolenic acid, and cis-8,11,14-eicosatrienoic acid did not differ between the two breeds. This result suggests that differing ability to utilize AA between breeds results in the difference of AA contents in adipose tissue.

AA is an essential fatty acid that is abundant in cell membrane-phospholipids, and phospholipase A_2 liberates it from the cell membrane (Kudo et al., 2004). The released AA could be further metabolized by three different types of oxygenases: cytochrome P450 (CYP), cyclooxygenase (COX), and lipoxygenase (LOX) (Roman et al., 2002). CYP can produce epoxy-modified arachidonic acids (EETs) and hydroxy-modified arachidonic acids (HETEs), while COX can produce prostaglandins and thromboxanes, and LOX can produce leukotrienes and hydroperoxides (HPETEs). Therefore, it could be inferred that the different amounts of arachidonic acid between the two breeds be caused by different levels of activity in the genes encoding these metabolizing enzymes.

To examine the differential expression of genes for AA metabolism, we used global gene expression profile data obtained from the same adipose tissues for the fatty acid analysis using a commercial microarray (GeneChip® Porcine Genome Array, Affymetrix Inc., Santa Clara, CA, USA) system. We collected porcine probe sets that have

Table 1. Composition of fatty acids in adipose tissue from KNP and YS

Fatty acid	KNP				YS				p-value
	#1	#2	#3	#4	#1	#2	#3	#4	
Capric acid (C 10:0)	0.60	0.14	0.00	0.61	0.00	0.00	0.40	0.00	0.2573
Lauric acid (C 12:0)	0.90	0.87	1.01	0.70	0.63	0.72	0.83	0.73	0.1189
Myristic acid (C 14:0)	9.5	10.2	9.3	9.6	8.0	9.3	11.6	8.8	0.7856
Myristoleic acid (C 14:1)	0.39	0.45	0.00	0.00	0.00	0.00	0.34	0.48	0.9778
Pentadecenoic acid (C 15:1)	0.59	0.44	0.00	0.64	0.44	0.66	0.74	1.12	0.1634
Palmitic acid (C 16:0)	152	154	116	147	125	153	193	142	0.5563
Palmitoleic acid (C 16:1)	15.1	17.4	13.7	14.8	10.0	13.2	15.7	16.0	0.3846
Margaric acid (C 17:0)	2.7	1.9	2.0	2.8	2.3	3.6	3.7	4.9	0.0921
Heptadecenoic acid (C 17:1)	2.4	1.8	2.4	2.8	2.1	3.0	3.0	4.3	0.1934
Stearic acid (C 18:0)	79	73	58	72	71	96	107	59	0.3524
Oleic acid (cis 9-C 18:1)	254	245	184	293	213	286	322	272	0.3983
Vaccenic acid (trans11-C 18:1)	1.9	1.8	17.5	2.4	1.4	2.0	3.1	2.0	0.4062
Linolelaidic acid (tC 18:2)	0.42	0.77	0.00	0.47	0.76	0.85	0.79	0.9	0.0788
Linoleic acid (C 18:2n6)	83	82	68	75	62	97	101	107	0.2576
Alpha-linolenic acid (C 18:3n3)	0.55	1.03	0.00	1.44	0.98	1.46	1.34	1.46	0.1725
Gamma-linolenic acid (C 18:3n6)	6.2	5.6	13.1	5.6	5.3	7.1	6.6	7.6	0.6464
Eicosanoic acid (C 20:0)	1.1	0.8	5.6	1.9	6.2	0.8	1.8	0.6	0.9856
Eicosenoic acid (C 20:1)	0.85	1.17	1.08	0.78	0.00	1.45	0.53	0.75	0.4179
Eicosadienoic acid (C 20:2)	8.0	6.6	0.0	11.4	0.0	8.1	9.2	7.1	0.9067
cis-11,14,17-Eicosatrienoic acid (C 20:3n3)	1.26	1.03	1.05	0.98	3.67	2.96	1.80	2.35	0.0262
cis-8,11,14-Eicosatrienoic acid (C 20:3n6)	3.7	3.1	16.8	4.2	2.4	4.0	4.8	4.0	0.4119
Arachidonic acid (C 20:4)	1.45	1.60	0.94	1.30	0.76	0.80	0.77	0.89	0.0325
Heneicosanoic acid (C 21:0)	0.85	0.46	2.36	0.68	0.56	0.73	0.38	0.37	0.2747
Behenic acid (C 22:0)	0.53	0.45	2.98	0.73	0.00	0.57	0.91	0.50	0.3520
Tricosanoic acid (C 23:0)	3.02	3.67	0.94	3.09	4.34	2.43	0.31	1.67	0.6518
Lignoceric acid (C 24:0)	0.26	0.34	3.08	0.45	1.40	1.04	1.71	0.97	0.7463
Nervonic acid (C 24:1)	0.73	0.68	1.60	0.26	0.00	0.00	0.76	0.68	0.2430
Total	632	617	523	655	522	697	795	648	0.4037

Table 2. Expression level of genes encoding oxygenases that metabolize arachidonic acid from gene expression profile

Gene	Probe set	e-value	Bit score	KNP[a]	YS[a]	p-value
Cytochrome P450 (CYP)						
CYP1B1	Ssc.8767.1.A1_at	2.4E-60	234	9.7±0.19	9.7±0.16	0.9766
CYP2A13	Ssc.15742.2.S1_a_at	0	1,527	8.3±0.90	11.2±0.54	0.0355
CYP2U1	Ssc.27105.1.A1_at	1.4E-08	62	8.0±0.21	9.0±0.19	0.0110
CYP3A4	Ssc.204.1.S1_at	7E-130	466	5.8±0.84	8.9±0.48	0.0179
CYP3A7	Ssc.8670.1.A1_at	0.007	46	8.0±0.28	7.8±0.26	0.6583
CYP4B1	Ssc.19211.1.S1_at	0	1,186	11.8±0.41	11.9±0.74	0.9298
CYP4F3	Ssc.15069.1.S1_at	0	946	11.4±0.20	11.1±0.33	0.5487
CYP27A1	Ssc.3804.1.A1_at	0	1,115	11.2±0.37	10.4±0.21	0.1061
CYP51A1	Ssc.5712.1.S1_at	0	2,060	11.4±0.23	11.2±0.14	0.4193
Lipoxygenase (LOX)						
ALOX5AP	Ssc.5822.1.S1_at	1E-129	464	9.5±0.09	9.4±0.19	0.5755
Cyclooxygenase (COX)						
PTGS1	Ssc.1986.1.S1_at	1.2E-49	199	8.6±0.13	8.3±0.15	0.2106
PTGS2	Ssc.23994.1.A1_at	2.8E-10	68	7.6±0.47	8.4±0.41	0.2344

[a] Mean (n = 4)±SEM (log_2 scale).

ALOX5AP: arachidonate 5-lipoxygenase-activating protein, *PTGS*: prostaglandin G/H synthase.

Fold change (KNP/YS) of *CYP2A13*: -5.8, *CYP2U1*: -1.5, *CYP3A4*: -6.4 in array.

List of genes encoding oxygenases that were not detected or barely detected (below background level = 5.79±0.04) in array.

CYP: *CYP1A1*, *CYP1A2*, *CYP2B7*, *CYP2C9*, *CYP2C18*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP2J2*, *CYP4A11*, *CYP4F2*, *CYP7A1*, *CYP7B1*, *CYP8B1*, *CYP11A1*, *CYP17A1*, *CYP19A1*, *CYP24A1*, *CYP26A1*, *CYP26B1*, *CYP27B1*, *CYP39A1*.

LOX: *ALOX12*, *ALOX15*.

Using the probe set ID, the GenBank accession number and UniGene ID can be found at the Affymetrix Netaffx analysis center (https://www.affymetrix.com/analysis/netaffx/index.affx).

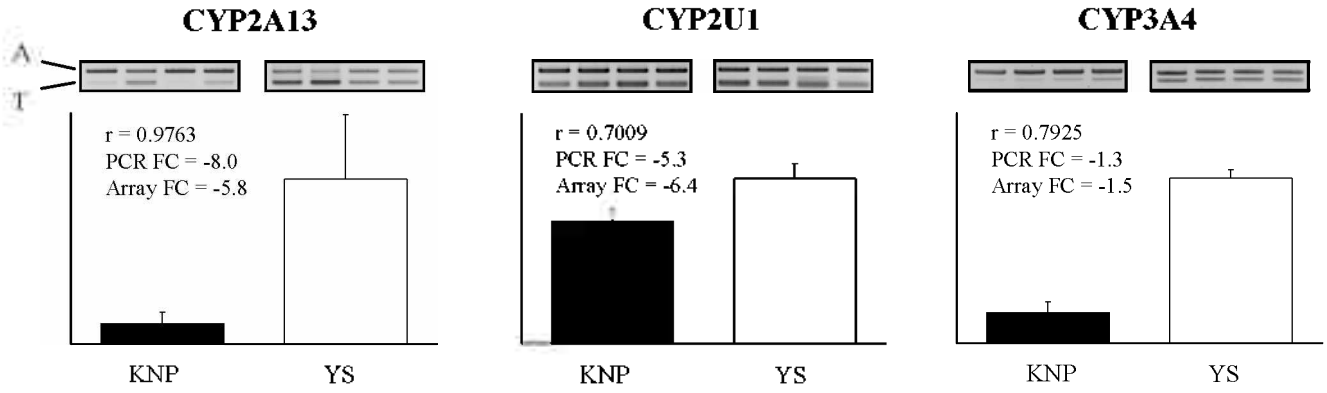

Figure 1. RT-PCR analysis for expression of CYP genes. Differentially expressed CYP genes in the microarray data were independently confirmed using RT-PCR analysis for *CYP2A13*, *CYP2U1* and *CYP3A4*. The graph shows average ratio of target gene and beta-actin of four pigs in a breed (either KNP or YS) with an error bar indicating standard error of the mean. The size of graph was adjusted to maintain the relative distance between KNP and YS by making an equal length of three CYP genes in YS. The quantities for graph came from the agarose gel images. Upper and lower bands indicate amplicons of β-actin (A) and target CYP (T) respectively. Fold changes (FC) between KNP and YS were obtained similarly in both RT-PCR (PCR FC) and microarray (Array FC). Correlation coefficient values (r) indicated that individual variation was well maintained between methods.

sequence homology with human CYP, LOX, and COX, due to poor annotation for the pig genes (Tsai et al., 2006). By comparing $\log_2$ transformed signals between the two breeds (n = 4 in each group), we found that expression of *CYP2A13*, *CYP2U1*, and *CYP3A4* were significantly different (*t*-test $p<0.05$) between breeds (Table 2). These three genes were consistently up-regulated in YS, while there were no significant differences in expression of LOX and COX genes between breeds (Table 2). We further measured transcripts for these three CYP genes using RT-PCR. The β-actin transcript was used as a reference. The ratios of each CYP target and β-actin band intensities were then compared between the two breeds. The gel images and quantified graph revealed significant differences in expression for *CYP2A13*, *CYP2U1*, and *CYP3A4* (Figure 1). We found consistently strong correlation between RT-PCR and array results.

CYP3A4 has weak epoxygenase activity for AA, but its catalytic activity produces 5,6-, 8,9-, 11,12-, and 14,15-EETs (Ayajiki et al., 2003). CYP2U1 metabolizes AA into two bioactive derivatives, 19- and 20-HETE (Chuang et al., 2004). To date, there is no known activity of CYP2A13 for AA. Because these arachidonic acid-derived eicosanoids have short biological half-lives (Madsen et al., 2005), they mainly play roles as autocrine and paracrine factors in the tissue (Roman et al., 2002). EETs are known to bind to peroxisome proliferator-activated receptor γ (PPARγ) and to increase its transcriptional activity (Liu et al., 2005). PPARγ is a transcription factor that regulates genes in adipose tissue, including those for triacylglyceride storage (Sharma et al., 2007). EETs and 20-HETEs stimulate angiogenesis by modulating several signaling cascades (Amaral et al., 2003; Jiang et al., 2004; Fleming et al., 2006; Michaelis et al., 2006; Wang et al., 2006). Both EETs and 20-HETEs are vasoactive molecules that work as antagonists of each other. The majority of EETs are vasodilators; on the other hand, 20-HETEs are vasoconstrictors (Escalante et al., 1990; Roman et al., 2000; Roman et al., 2002; Kroetz et al., 2002; Kroetz et al., 2005).

Our data can infer that the transcriptional up-regulation of CYP genes in YS decreases the level of AA content in adipose tissue, and eventually causes AA metabolic difference, which explains partly difference of fatness between the two breeds. In conclusion, we observed different contents of AA in back-fat underneath the skin between KNP and YS, and differential gene expression of CYP genes that could affect on the local availability of EETs and HETEs. These bioactive molecules derived from AA could affect properties of fat metabolism and fatness by modulating adipose vascular structure and blood flow that determine distinct characteristics between the two breeds.

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