Study on the Lipolytic Function of *GPR43* and Its Reduced Expression by DHA

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ABSTRACT : G protein-coupled receptor 43 (*GPR43*) is a newly-discovered short-chain free fatty acid receptor and its functions remain to be defined. The objective of this study was to investigate the function of *GPR43* on lipolysis. We successfully cloned the *GPR43* gene from the pig (EU122439), and measured the level of *GPR43* mRNA in different tissues and primary pig adipocytes. The expression level of *GPR43* mRNA was higher in adipose tissue and increased gradually with adipocyte differentiation. Then we examined *GPR43* mRNA level in different types, growth-stages and various regions of adipose tissue of pigs. The results showed that the expression level of *GPR43* mRNA was significantly higher in adipose tissue of obese pigs than in lean pigs, and the expression level also gradually increased as age increased. We further found that the abundance of *GPR43* mRNA level increased more in subcutaneous fat than visceral fat. Thereafter, we studied the correlation between *GPR43* and lipid metabolism-related genes in adipose tissue and primary pig adipocytes. *GPR43* gene had significant negative correlation with hormone-sensitive lipase gene (*HSL*, r = -0.881, p<0.01) and triacylglycerol hydrolase gene (*TGH*, r = -0.848, p<0.01) in adipose tissue, and had positive correlation with peroxisome proliferator-activated receptor γ gene (*PPARγ*, r = 0.809, p<0.01) and lipoprotein lipase gene (*LPL*, r = 0.847, p<0.01) in adipocytes. In addition, we fed different concentrations of docosahexaenoic acid (DHA) to mice, and analyzed expression level changes of *GPR43*, *HSL* and *TGH* in adipose. The results showed that DHA down-regulated *GPR43* and up-regulated *HSL* and *TGH* mRNA levels; *GPR43* also had significant negative correlation with *HSL* (low: r = -0.762, p<0.01; high: r = -0.838, p<0.01) and *TGH* (low: r = -0.736, p<0.01; high: r = -0.586, p<0.01). Our results suggested that *GPR43* is a potential factor which regulates lipolysis in adipose tissue, and DHA as a receptor of GPR43 might promote lipolysis through down-regulating the expression of *GPR43* mRNA. (**Key Words :** *GPR43*, Pig, Mouse, Adipose Tissue, Adipocyte, Lipolysis, DHA)

INTRODUCTION

Adipose tissue plays an important role in regulating energy metabolism and functioning as an energy reserve organ. The key reaction of energy metabolism is adipose tissue lipolysis *in vivo*. During lipolysis, triglycerides (TGs) were broken down to energy-rich free fatty acids (FFAs) and glycerol (Gibbons et al., 2000). The effective regulation of lipolysis could decrease body fat and reduce the incidence of obesity and diabetes (Andrea and Philipp, 2005).

G-protein-coupled receptors (GPCRs) are key regulators in several physiological functions. Their roles in cellular signal transduction have made them the target of drug discovery efforts in various therapeutic areas (Howard et al., 2001). *GPR43* is one of the GPCRs which coupled to both

Gq and Gi/Go proteins. The pathways that were activated by this receptor include release of intracellular Ca^{2+} and ERK1/2 activation and inhibition of cAMP accumulation (Le Poul et al., 2003). Pharmacologically, Short chain fatty acids are most potent agonists for *GPR43* (Brown et al., 2003; Nilsson et al., 2003). *In vivo*, *GPR43* has been shown to be present in a variety of tissues, particularly evident in fat stores, inflammatory cells and the gastrointestinal tract. Recently, Hong et al. reported that *GPR43* expression was detected in adipose tissue and isolated adipocytes. Moreover, propionate and acetate stimulated adipogenesis through *GPR43*, it was suggested that *GPR43* might be involved in lipid metabolism (Hong et al., 2005).

In this study, we examined the expression characteristic of *GPR43* in different tissues and primary adipocytes of pig both *in vitro* and *in vivo*, and analyzed the correlation between *GPR43* and lipid metabolism-related genes. Furthermore, we studied the function of *GPR43* on mouse adipose tissue which treated with DHA. Our data could

Table 1. PCR parameter of primers

Gene	Primer sequences	Tm (°C)	Cycles	Length (bp)
GPR43 (for cloning)	F: CCTTGATCCTCATGGCTTAC	60	30	488
	R: ATCGGTGAAGTTCTCGTAGC			
GPR43 (for RT-PCR and real time PCR)	F:ACCCATCCACATCCTCCTGC	60.4	30	151
	R:GCTGCTGTAGAAGCCGAAACC			
TGH	F:CTTGGCTCCTTGAGATTTG	53.3	30	455
	R:AGTTGGCAATGTTGTCCTG			
HSL	F: GGAGCACTACAAACGCAAC	57.9	30	357
	R: TCCCGTAGGTCATAGGAGAT			
PPARγ	F:ACCACTCGCATTCCTTTGAC	52.1	30	261
	R:CCACAGACTCGGCACTCAAT			
LPL	F: ACGCAGCCTTACGGAACT	54.3	30	388
	R: TTGTTTAGTTGGGGATTTGCT			
β-actin (for RT-PCR and real time PCR)	F: ACTGCCGCATCCTCTTCCTC	53.8	30	399
	R:CTCCTGCTTGCTGATCCACATC			

F: Forword; R: Reverse.

provide some insight into understanding the role of *GPR43* on lipolysis.

MATERIALS AND METHODS

Materials

Experimental animals : Large White pig is a kind of lean pig. Guanzhong black pig is a kind of obese pig, which belongs to local strain of Chinese pig. Both of them were provided by Shaanxi Guangming pig farm. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Northwest Agriculture and Forestry. Subcutaneous fat and visceral fat tissue were quickly excised after the animal were slaughtered, then frozen in liquid nitrogen, and stored at -80°C for later use. A range of non-adipose tissues were also collected and stored: muscle, heart, liver, spleen, lung and kidney.

Two-week-old male Kunming mice were obtained from the Fourth Military Medical University (Xi'an, China). They were housed individually in cages at a constant temperature (25°C) and under a 12 h light/12 h dark cycle. The animals had free access to water and standard diet for 1 week of an acclimatization period. Then the mice were divided into two groups, the low concentration group and high concentration group, each group had thirty-five mice. Before operation, mice were fasted for 12 h, then both low and high concentration groups were given 6.25 g/kg and 12.5 g/kg DHA respectively by intragastric administration. At 0, 1, 2, 4, 8, 16, 24 h, five mice of each group were killed by decapitation. Subcutaneous adipose tissues were rapidly separated, immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

Cell culture : Primary pig adipocyte was obtained from new born pig subcutaneous adipose tissue, grown and differentiated as previously described (Hongmei et al., 2007). In brief, tissues were initially washed with PBS containing high concentration of mycillin and then digested with collagenases at 37°C for 1 h. Cells were then filtered through 40 micron nylon membrane to remove tissue debris and concentrated by centrifugation. Isolated cell pellets were resuspended in DMEM/F12. Finally, cells were seeded in culture plates at a density of 5×10^4 cells/cm^2 and cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h, cells were switched to differentiation medium consisting of DMEM/F12, supplemented with 10% (v/v) FBS, hydrocortisone (50 ng/ml), insulin (10 μg/ml), transferrin (10 μg/ml), and penicillin and streptomycin (MDI). Primary adipocyte was grown for preparation of total RNA at day 0, 2, 4, 6, 8 and 10 respectively. The medium was changed every two days.

Gene cloning : Total RNA was extracted from adipose tissue using Trizol reagent (Invitrogen, USA). First strand cDNA was prepared with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Since there was no pig *GPR43* gene sequence available, we used human and mouse sequences as reference to design primers for amplification of pig *GPR43* cDNA. The PCR reaction conditions were summarized in Table 1. The 25 μl PCR reaction contained ddH$_2$O 14.3 μl, 1 μl tissue-specific cDNA, 2.5 μl MgCl$_2$ (25 mmol/L), 0.2 μl Taq DNA polymerase (Fermentas, LT), 2.5 μl dNTPs (2.5 mmol/L), 2.5 μl 10× buffer and 1 μl of each primer (10 μmol/L). The PCR product was examined by agarose gel electrophoresis (AGE). PCR fragment was purified from agarose gel and cloned into pMD-18T vector (TaKaRa, Japan) for sequencing.

Real-time quantitative PCR : *GPR43* mRNA from samples of different types, growth-stages and various regions adipose tissue of pigs were determined by real time quantitative PCR. The 20 μl real-time reaction contained 12.5 μl SYBR Premix EX Taq (Takara, Japan), 0.5 μl Forward Primer (10 μmol/L), 0.5 μl Reverse Prime (10
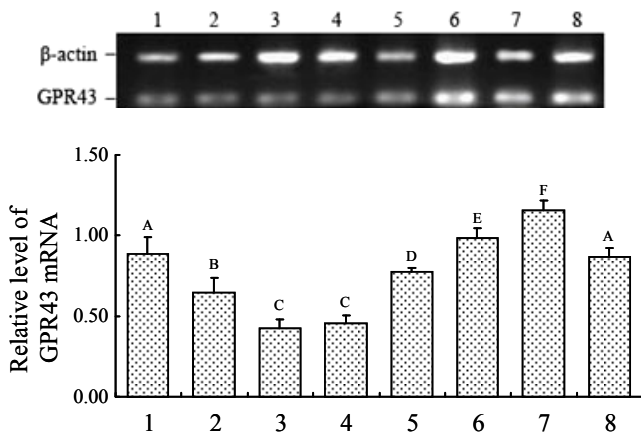
Figure 1. The expression of *GPR43* mRNA in different tissues from pigs. β-actin was used as an internal control. 1: subcutaneous adipose, 2: abdominal adipose, 3: muscle, 4: heart, 5: liver, 6: spleen, 7: lung, 8: kidney. Values are means±SEM, n = 5. Means without a common letter differ, p<0.01.

μmol/L), 1 μl cDNA, ddH$_2$O 10.5 μl. Reactions were incubated in an TP800 Real-time System (Takara, Japan) for 10 s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for30 s, 95°C at 15 s, 60°C at 30 s, 95°C at 15 s.

Using the method of $2^{-\Delta\Delta Ct}$ to analysis the expression of gene. The method of $2^{-\Delta\Delta Ct}$ always been used to relative quantification of gene expression. The expression level of genes were evaluated by utilizing the comparative critical threshold (Ct). The Ct values for each *GPR43* reaction were subtracted from the respective Ct value of the β-actin control, resulting in the ΔCt value. The largest ΔCt value was arbitrarily used as a constant that was subtracted from all other ΔCt values to determine ΔΔCt value. Fold changes were then generated for each *GPR43* by calculating $2^{-\Delta\Delta Ct}$.

Quantitative PCR : Also, we measured levels of pig and mouse *GPR43, PPARγ, LPL, HSL, TGH* and β-actin mRNA by use of a simple and similar real-time PCR technique in accordance with the principle of real-time quantitative PCR. The primers and PCR amplification conditions were listed in Table 1. Briefly, for each sample, we prepared 6 tubes for PCR reaction, and the reaction was set as 10, 15, 20, 25, 30 and 35 cycles, respectively. The PCR products were examined by agarose gel electrophoresis, then the expression of *GPR43* be corrected by β-actin. In six different cycles, we selected correction value used for statistical analysis while the difference was the biggest between different samples at the same cycle.

Data analysis : Software SPSS 13.0 was used for statistical analysis. The expression of genes was analyzed with one-way ANOVA and LSD multiple comparison. Pearson's correlation coefficients were used to determine statistical linear associations between *GPR43* and other genes involved in lipid metabolism. All data from samples was shown as means±standard error (SEM).

RESULTS AND ANALYSES

Gene clone and bioinformatics analysis

To amplify the pig *GPR43* gene, we designed one pair of primers based on human and mice gene sequences as there was no pig *GPR43* gene sequences available. Total RNA was prepared from adipose tissue of an adult Large White pig and used for RT-PCR. Agarose gel analysis of PCR product indicated a specific band and the sequencing result showed that the pig *GPR43* partial cDNA consists of 488 bp. The pig *GPR43* cDNA sequence was submitted to GeneBank (accession no. EU122439). Aligned with other species, pig *GPR43* cDNA shares the highest homology with human (89%), followed by mouse (84%) and rat (83%).

### Different expression pattern of *GPR43* mRNA in pig different tissue

In order to study the expression pattern of *GPR43* mRNA in pig different tissues, we redesigned primers of *GPR43* gene base on pig gene sequence that we had obtained, and amplified it in pig different tissues. As was shown in Figure 1, *GPR43* expressed in multiple tissues including subcutaneous adipose, visceral adipose, muscle, heart, liver, spleen, lung and kidney, and showed its highest expression abundance in lung, which was significantly higher than the other tissues (p<0.01). The expression abundance in subcutaneous adipose was significantly higher than visceral adipose (p<0.01).

Level of *GPR43* mRNA during differentiation of pig primary cultured adipocytes

To investigate the involvement of *GPR43* in pig primary cultured adipocytes differentiation *in vitro*, *GPR43, LPL* and *PPARγ* mRNA levels were analyzed at different cultured time using quantitative PCR. As showed in Figure 2, the levels of *GPR43* mRNA increased after adipocyte differentiation with a peak at day 6, the expression tendency of *LPL, PPARγ* were same as *GPR43*. This suggested that *GPR43* might be involved in the regulation of pig adipocytes differentiation.

Real-time quantitative PCR determine the expression characteristic of *GPR43* mRNA in different types, growth-stages and various regions adipose tissue of pig

The amplification curve and melt curve are necessary for relative quantitation using real-time quantitative PCR. In this experiment, we used β-actin as house keeping gene, to determine the expression level of *GPR43* in different types (obese, lean), different growth-stages (5-month-old,

A **B**
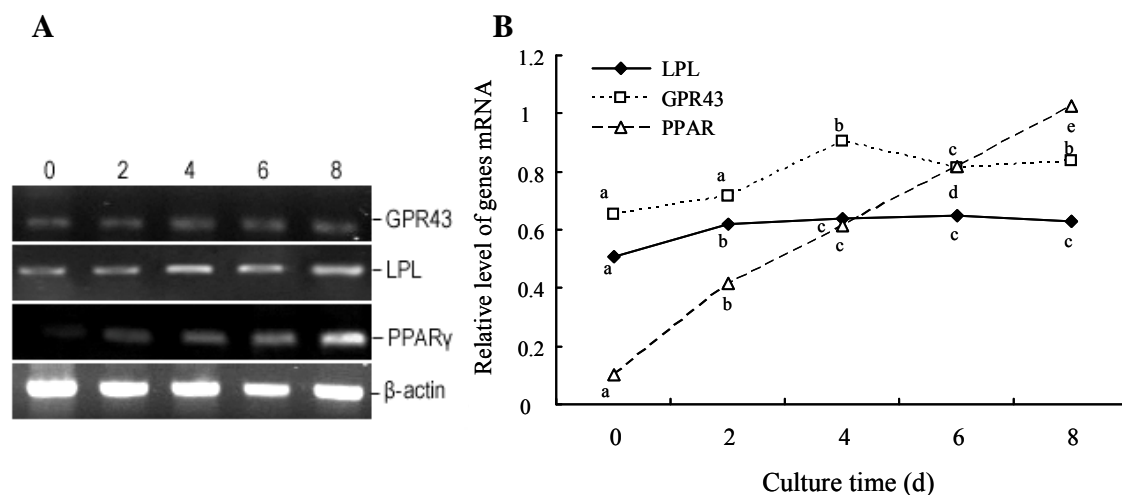
Figure 2. The expression levels of *GPR43*, *PPARγ* and *LPL* mRNA in the primary cultured adipocytes of pig. A: Electrophoresis results of *GPR43*, *PPARγ* and *LPL*. B: Quantitative PCR Analysis of the expression levels of *GPR43*, *PPARγ* and *LPL*. Values are means±SEM of experiments from four samples, each has three repetition. Means without a common letter differ, p<0.05.

10-month-old) and various regions (Subcutaneous fat and visceral fat). The Figure 3 demonstrated that *GPR43* mRNA level was significantly higher in adipose tissue of obese pigs than that of lean pigs (p<0.01), and the expression level also gradually increased as age increased (p<0.01), in addition, the abundance of *GPR43* mRNA level increased more in subcutaneous fat than visceral fat (p<0.01). These results suggested that *GPR43* might be involved in the regulation of adipose tissue development.
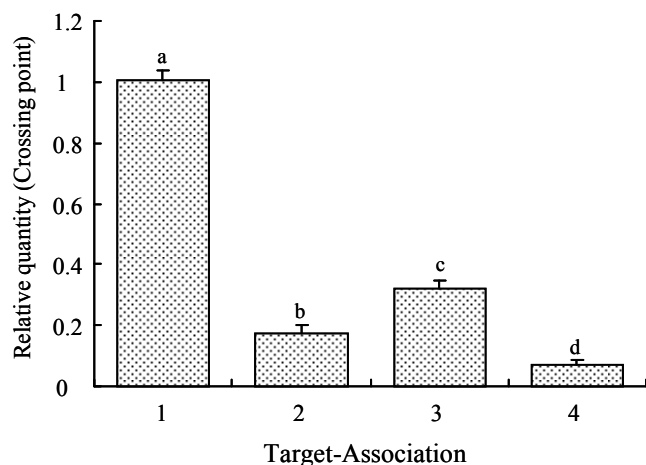

Figure 3. The expression rule of *GPR43* mRNA in pig adipose tissues. The bar chart was mapped by real-time system. '1' represent quantity of *GPR43* mRNA in subcutaneous adipose tissue of 10 months lean-type pigs. '2' represent quantity of *GPR43* mRNA in subcutaneous adipose tissue of 5 months lean-type pigs. '3' represent quantity of *GPR43* mRNA in subcutaneous adipose tissue of 5 months obese-type pigs. '4' represent quantity of *GPR43* mRNA in abdominal adipose tissue of 5 months lean-type pigs. Values are means±SEM, n = 5. Means without a common letter differ, p<0.05.

Correlation between *GPR43* and some lipid metabolism-related genes expressed in adipose tissues and primary cells

To support the hypothesis that *GPR43* is involved in adipocyte differentiation and adipose tissue development, we next investigated the relationship between GRP43 expression and the expression levels of other lipolysis genes in adipose tissue (Figure 4) by quantitative PCR. *HSL* and *TGH* are the main genes that play an important role in lipid metabolism in adipose tissue. Applying Pearson's correlation analysis to the data obtained from the adipose tissue samples, we revealed that the expression level of *GPR43* gene had a negative correlation with *HSL* gene (r = -0.881, p<0.01) and *TGH* gene (r = -0.848, p<0.01) expression (Table 2). In primary adipocytes, we examined the expression of *LPL* and *PPARγ* genes and compared their expression levels with *GPR43* gene expression (Figure 2). *GPR43* expression showed a postive correlation with *PPARγ* gene (r = 809, p<0.01) and *LPL* gene (r = 0.847, p<0.01) gene expression (Table 2).

Effect of *GPR43* on DHA treated mice adipose tissue

We next examined the effect of *GPR43* on DHA treated mice adipose tissue. As showed in Figure 5, quantitative PCR analysis showed that the level of *GPR43* mRNA was down-regulated, on the contrary, the levels of *HSL* and *TGH* mRNA were up-regulated at different concentration groups. Applying Pearson's correlation analysis to the data obtained from the adipose tissue samples, we revealed that the expression level of *GPR43* still had a negative correlation with *HSL* gene (low: r = -0.762, p<0.01; high: r = -0.838, p<0.01) and *TGH* gene (low: r = -0.736, p<0.01; high: r = -0.586, p<0.01) expression (Table 3). The result was same as above.
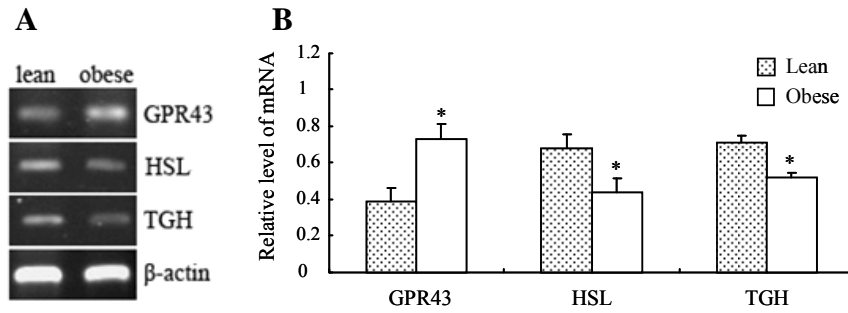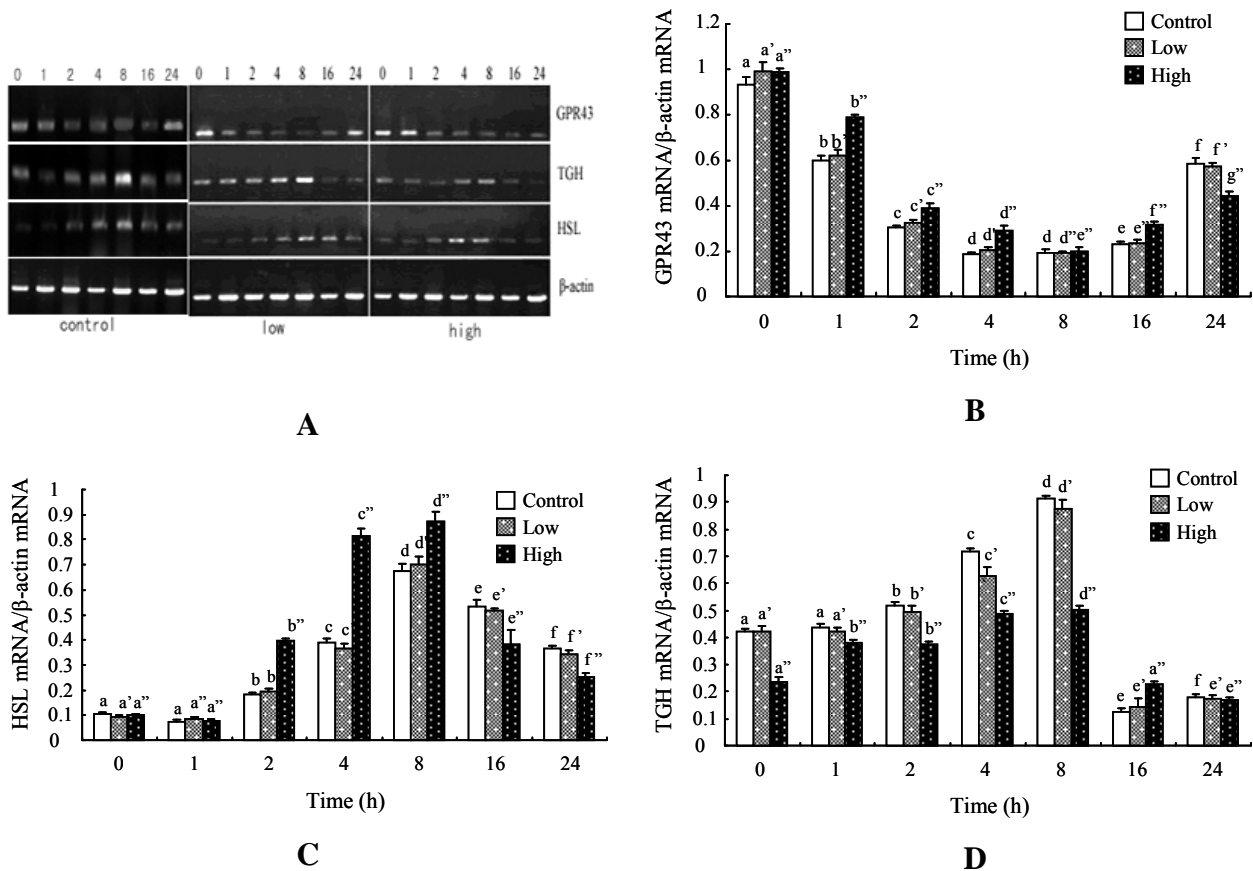
Figure 4. The expression levels of *GPR43*, *HSL* and *TGH* mRNA in the adipose tissue of lean and obese type pigs. A: Electrophoresis results of *GPR43*, *HSL* and *TGH*. B: RT-PCR Analysis of the expression level of *GPR43*, *HSL* and *TGH*. Values are means±SEM of experiments from six animals, each has three repetition. * p<0.05.

Table 2. Correlation between *GPR43* and lipid metabolism related genes in pig

Gene		*HSL*	*TGH*	*PPARγ*	*LPL*
GPR43	Tissue	Adipose tissue	Adipose tissue	Adipocyte	Adipocyte
	Pearson's correlation	-0.881**	-0.848**	0.809**	0.847**
	Significance level	0.002	0.007	0.001	0.003
	Sample size	18	18	12	12

Values are means±SEM of experiments, each sample has three repetition. ** p<0.01.

Figure 5. The effect of DHA on *GPR43*, *HSL* and *TGH* expression in mice adipose tissue. A: Electrophoresis results. B: RT-PCR Analysis of the expression level of *GPR43*. C: RT-PCR Analysis of the expression level of *HSL*. D: RT-PCR Analysis of the expression level of *TGH*. Values are means±SEM of experiments from six animals, each has three repetition. Means without a common letter differ, p<0.05.

Table 3. Correlation between *GPR43* and other lipolytic-related genes in mouse

Gene		HSL		TGH	
		Low	High	Low	High
GPR43	Pearson's correlation	-0.762**	-0.838**	-0.736**	-0.586**
	Significance level	0.006	0.008	0.0069	0.005
	Sample size	15	15	15	15

Values are means±SEM of experiments, each sample has three repetition. ** p<0.01.

DISCUSSION

GPR43 gene was originally identified in human by Sawzdargo et al. which located at chromosome 19q13.1 (Sawzdargo et al., 1997). It was initially believed that *GPR43* was only expressed in a range of peripheral blood leukocytes, with particularly high levels shown in monocytes and neutrophils (Senga et al., 2003). However, Hong et al. demonstrated that *GPR43* was highly expressed in adipose tissue and adipocytes (Hong et al., 2005). In this study, we found that *GPR43* also expressed in pig adipose tissue and primary adipocytes. We cloned *GPR43* gene from pig adipose tissue and examined its expression pattern in adipose tissues and primary adipocytes. It was found that *GPR43* might influence the regulation of lipolysis and adipocyte differentiation.

In this experiment, we examined *GPR43* mRNA levels in different types, growth-stage and various regions adipose tissue. The result showed that *GPR43* mRNA level was significantly higher in adipose tissue of obese pigs than that of lean pigs. This result was similar to the previous research, which showed that *GPR43* mRNA was up-regulated in adipose tissues of mice fed a high-fat chow compared with those on normal chow (Hong et al., 2005). Given the fact that the fat deposition capacity of obese pig significantly higher than lean pig (Gongshe, 1997), so we suggested that *GPR43* might be involved in adipose tissue development. Then we found that the expression level of *GPR43* in pig adipose gradually increased as age increased, and the abundance of *GPR43* mRNA level increased more in subcutaneous fat than visceral fat. Previous research showed that lipolysis gradually decreased as age increased in pig (Mersmann, 1998), and lipolysis capacity of subcutaneous fat better than visceral fat (Bjorntorp, 2000), suggesting that *GPR43* might negatively regulate lipolysis in adipose tissue.

PPARγ is a member of the nuclear receptor superfamily of transcription factors and previous studies showed that *PPARγ* has a important role in adipocyte differentiation and regulates gene expression in many functional pathways (Grimaldi, 2001). In this study, the correlation between *GPR43* and *PPARγ* in pig adipocyte was positive. This result consisted with previous research, which showed that SCFAs stimulated differentiation in 3T3-L1 adipocytes via

GPR43, with up-regulation of *PPARγ* (Hong et al., 2005). *LPL* hydrolyzes triglycerides (*TGs*) in chylomicrons and *VLDL*, thereby generating free fatty acids that enter either storage or oxidative pathways (Guo et al., 2008). Thiazolidinediones affected adipocyte *LPL* production through activation of *PPARγ*, promoting the hydrolysis of triglyeride (Schoonjans et al., 1996). Therefore, based on the observation that *GPR43* expression was positively correlated with *LPL* in primary adipocytes, it suggested that *GPR43* might be involved in lipid degradation.

Many studies have shown that SCFAs are endogenous ligands for *GPR43* (Brown et al., 2003; Kotarsky et al., 2003; Nilsson, 2003) and Isoproterenol-stimulated lipolysis is reduced in a dose dependent manner by acetate or propionate treatment *in vitro* (Hong et al., 2005), indicated that *GPR43* might play a important role in lipolysis. *HSL*, a key enzyme in fatty acid mobilization, and a rate-limiting enzyme of lipolysis (Holm et al., 1988). *TGH* is another enzyme that catalyzes the lipolysis of intracellular stored triacylglycerol. during the differentiation of 3T3-L1 adipocytes, lipolysis rate dramatically decreased as *TGH* mRNA and protein levels increased (Wei et al., 2005). At present, one of the best known mechanisms that activate lipolysis in the adipocyte is the cAMP dependent pathway. cAMP activates PKA which activates *HSL* by promoting its phosphorylation (Carmen and Victor, 2006). And another group of researchers thought that cAMP acts as a secondary messenger to activate protein kinase which activates HSL by phosphorylation to make it functional in lipid hydrolysis (Zou et al., 2007). We found that *GPR43* gene had a negative correlation with *HSL* gene and *TGH* gene. It is known that *GPR43* can stimulate increasing of IP3, and decreasing of cAMP content when *GPR43* expression in recombinant cho cells (Le Poul et al., 2003), suggesting that *GPR43* might inhibit lipolysis through cAMP decrease *HSL*, *TGH* also be decreased when the expression of *GPR43* up-regulation.

DHA is one of the polyunsaturated fatty acids which is necessary for the growth and development of the body and have an important role in regulation of fat deposition (Kim et al., 2006). When fed pigs with high DHA may lead to lower body fat deposition (Liu et al., 2005). And if feeding mice diets enriched in for 3 weeks might increase the expression of *TGH* (Dolinsky et al., 2003), in addition, add

polyunsaturated fatty acids to diet of type 2 diabetic patients, *HSL* activitiy was significantly increased (Rivellese et al., 2007). Those studies are similar to our results. We found that DHA promoted the expression of *HSL* and *TGH* in mice adipose tissue. This fully shows DHA may promote lipolysis to regulate fat deposition. Meanwhile, we also detected the expression of *GPR43*, and found that the expression of *GPR43* was significantly decreased with the extension of time. Through the correlation analysis, we also found that *GPR43* had a negative correlation with *HSL* and *TGH*, this result conformed our above research. Mohamed Soliman et al. reported that the stimulatory effect of SCFAs was abolished by LCFAs (Soliman et al., 2007), *GPR43* as a short-chain fatty acid receptor also be inhibited. These results demonstrated that DHA as a long-chain fatty acid may promote adipose lipolysis through inhibiting the expression of *GPR43*.

In conclusion, in the present study, we found that *GPR43* had a negative effect on lipolysis, the role of DHA through down-regulating *GPR43* might promote lipolysis. Our data could provide a new insight into the function of *GPR43* in lipid metabolism and its possible application to the therapy of obesity.

ACKNOWLEDGMENTS

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