Fatty acids modulate adipocyte growth and development in pig: An approach from cell culture study

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Abstract— **We have previously demonstrated that octanoate is necessary for inducing terminal differentiation of pig preadipocytes (Nakajima, I. et al. (2003) *Biochem. Biophys. Res. Commun.* 309:702-708). The aim of this study is to further investigate the effect of different types of fatty acids (FA) on pig adipocyte differentiation. To assess the role of individual FAs on adipose conversion, preadipocytes (PSPA) which were isolated from subcutaneous tissue of Western crossbred pig were cultured for 10 days in the presence of one of the following FAs; octanoate (C8:0, 1 μM ~ 5 mM), oleate (C18:1, 1 μM ~ 0.2 mM), or arachidonate (C20:4, 1 μM ~ 0.1 mM). We observed that lipid accumulated dose dependently in PSPA cells when treated with either C8:0 or C18:1. PSPA cells treated with C18:1 showed the highest TG content with large intracellular lipid droplets among FA at 0.1 mM concentration. RT-PCR analyses revealed that the expression levels of adipogenic markers, such as PPAR β/δ, PPARγ and CD36, were the lowest in C20:4 treated cells. In contrast to these results of C8:0 and C18:1 treatment, treatment of C20:4 increased the total cell number as well as the proportion of cells in S and G2/M phases of the cell cycle, indicating C20:4 induced cell proliferation rather than adipocyte differentiation. Taken together, our results that the adipose conversion was promoted by the addition of either C8:0 or C18:1 to the culture media but not by the addition of C20:4 revealed distinct effects of individual FAs on adipose development in pigs.**

Keywords— **fatty acid, adipocyte, pig backfat**

I. INTRODUCTION

Backfat thickness is one of the most economically important traits of pig carcasses. Regulating the amount of fat deposition has long been a major goal in the continuing improvement of pork production. Investigation of the factors that affect the development of adipose tissue will provide useful information for controlling carcass fat content of pigs.

Dietary fat is an essential macronutrient for the growth and development of all organisms. In addition to its role as an energy source and its effects on membrane lipid composition, dietary fat has profound effects on gene expression, leading to changes in metabolism, growth and cell differentiation [1]. Some fatty acids (FAs) and/or their metabolites serve as hormones to control the activity or abundance of specific transcription factors [1]. We previously demonstrated that octanoate (C8:0), an eight-carbon middle chain FA, became an agent with antimitotic and adipogenic activities that were essential for the terminal differentiation of porcine preadipocytes [2]. Furthermore, these positive effects of C8:0 on pig adipocyte differentiation were opposite from the result of mouse 3T3-L1 adipocytes which attenuated its differentiation level [3], thus, suggesting that there are species-specific differences in response to FAs.

In this study, we further investigated the effects of other essential FAs (oleate, C18:1 and arachidonate, C20:4) in addition to C8:0 on the adipocyte differentiation of PSPA cells, which were isolated from fetal subcutaneous tissue of commercial crossbred pig [2]. PSPA cellular responses to individual FAs were examined by following points; (i) dose effect on TG content and cell number; (ii) gene expressions of transcription factor peroxisome proliferator-activated receptor (PPAR) superfamily and FA uptake-related proteins; (iii) cell cycle distribution by flow cytometric analysis; and (iv) light microscopic observation.

II. MATERIALS AND METHODS

A. Cell culture

A clonal porcine subcutaneous preadipocyte (PSPA) cell line was maintained in the preadipocyte condition by cultivation in Dulbecco's modified

Eagle's medium (DMEM, 1 g/l glucose) supplemented with 10% fetal bovine serum, 1000 IU/ml penicillin, and 1 mg/ml streptomycin. Cells were passaged every 4 days, with the density of inoculation kept constant ($1x10^4$cells/cm^2).

To induce adipose conversion, cells were plated at $2.1x10^4$cells/cm^2 to obtain confluency within 3 days. After reaching confluence (day 0), adipose conversion was usually induced in high-glucose (4.5 g/l) DMEM containing 10% FBS, 5 μg/ml insulin, 0.25 μM dexamethasone, 33 μM biotin, 17 μM pantothenate, 5 mM octanoate. For fatty acid effects, the octanoate (C8:0) supplemented differentiation medium was selectively replaced with various concentrations of either oleate (C18:1) or arachidonate (C20:4). The medium was changed every other day and the cells were allowed to differentiate for 10 more days.

B. Triglyceride assay

Cultured cells on 24-well plates were washed with PBS, scraped off into 0.2 ml of 25 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and then homogenized. Triglyceride (TG) in the cell lysate was extracted with the same volume of chloroform-methanol (2:1, v/v) and quantified enzymatically using a Triglyceride E Test Wako Kit.

C. Cell number

Cell number was determined with a hemocytometer after trypsin-EDTA detachment.

D. Total RNA extraction and RT-PCR analysis

Total RNAs were isolated from cell using QuickGene RNA cultured cell kit S. Single-stranded cDNA was generated by M-MLV reverse transcriptase Rnase H minus with the primer 5' CTGCAGGAATTCGATATCGAAGCTTGC$(T)_{15}$VN 3'.

The synthesized cDNA was amplified with AmpliTaq Gold by PCR using paired forward and reverse primers with the ribosomal protein L7 (RPL7) as the internal control [4]. Primer sequences are described in Table 1. PCR reactions were conducted first for 9 min 95°C, followed by denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and 30 s of extension at 72°C for 25~31 cycles. The PCR products were size-fractionated by 4% agarose gel electrophoresis and stained with ethidium bromide. The signal intensity of the amplified fragments was analyzed by FluorChem 8900 scanner with AlphaEase FC software.

Table 1 The primer sequence used in PCR experiments

GENE		Primers
PPARα	f	TCACATGTGAACATGACCTAGAAGATGCCG
	r	AAGATGGCATCGTAGACGCCGTACTT
PPARβ/δ	f	AAGTGCCAGTACTGCCGCTTTCAGAAAT
	r	ATGCTCTTGGCGAACTCGGTCAGCT
PPARγ1	f	CCTTAAACGAAGAGTCATCTTTTAGCG
	r	GGCTCTTCGTGAGGTTTGTTGTACAG
PPARγ2	f	GGTGAAACTCTGGGAGATTCTCTTA
	r	GGCTCTTCGTGAGGTTTGTTGTACAG
FABP4	f	TTTGCTACCAGGAAAGTGGCTGGCAT
	r	GCAGTGACACCATTCATGACACATTCC
CD36/	f	GACATGATTAATGGTACAGATGCAGCCTCA
FAT	r	GGTTCAACATCCAAGTAAGTGCTATGCT
FATP	f	AAGTACAACTGCACGGTGGTCCAGTACAT
	r	TAGCTCCATGGTGTCCTCGTTGACCTTCA
CAV1	f	AAGGAGATAGACTTGGTCAACCGCGAT
	r	ACACGGCTGATGCACTGAATCTCAATCA
RPL7	f	GCAGAACCCAAATTGGCGTTTGTCATCAG
	r	GATGATGCCGTATTTACCAAGAGATCGAGC

f, forward primer; r, reverse primer. Other abbreviations used in Table 1 are mentioned somewhere in the text.

E. Analysis of cellular DNA content by flow cytometry

PSPA cells incubated in 6-well plates were harvested by trypsin-EDTA solution to produce a single cell suspension. The cells were then pelleted by centrifugation and fixed in 70% ice-cold ethanol at -20°C overnight. After fixation, cells were washed with PBS, resuspended in 1 ml PBS containing 0.5% RNase A for 30 min at RT, and incubated with 25 μg/ml) propidium iodide. Samples of stained 15,000 cells were analyzed using a Gallios flow cytometer (Beckman Coulter) in combination with FlowJo software to determine the cell cycle distribution.

F. Immunocytochemistry

After culture on Lab Tek microslides, cells were fixed with 3.7% formaldehyde incubated at 4°C for overnight. Fixed cells were permeabilized with 1%

Triton X in PBS for 5 min at RT and rinsed, and then reincubated in 1% bovine albumin in PBS for 30 min at 37°C to prevent non-specific antibody binding. The cells were then reacted with anti-proliferating cell nuclear antigen (PCNA) antibody for 2~3 hr at 37°C and with AlexaFluor 555-conjugated goat anti-rabbit antibody for 30 min at 37°C. Lipids and nuclear were also counterstained by the addition of BODIPY® 493/503 and DAPI, respectively. Stained cell monolayers were observed under Zeiss Axiophot microscope.

G. Statistics

Data were analyzed for statistical significance using Student's *t*-test.

III. RESULTS

A. Dose dependent effects of FAs on PSPA cell growth and differentiation

To investigate the effects of FAs on adipocyte differentiation, confluent PSPA cells cultured under differentiation medium were exposed to various concentrations of one of the following FAs; C8:0, C18:1 or C20:4. After 10 days treatment of FAs, TG content and cell number were determined as indicators of adipocyte differentiation. We observed that treatment with C8:0 reduced cell number and conversely enhanced lipid accumulation in dose-dependent responses. The exposure to C18:1 also resulted in a net increase of TG content with increasing concentration, though it did not affect cell growth like C8:0. When compared at 0.1 mM added FAs, the highest TG content could be observed in C18:1 treated cells. On the other hand, C20:4 treated PSPA cells did not change TG accumulation level, while they proliferated in dose-dependent fashion, indicating that C20:4 act as a negative factor to adipocyte differentiation in pigs.

B. Effects of FAs on the induction of adipocyte-specific gene expressions

The expression of various genes related to lipid-activated transcription factor, such as that of PPARα, β/δ, γ1 or γ2 and to cellular uptake of FAs such as that of fatty acid binding protein (FABP) 4, fatty acid translocase (CD36/FAT), fatty acid transport protein (FATP) or caveolin (CAV) 1, has been used to characterize further the effects of individual FAs on the differentiation process. For that purpose, RNAs from cells exposed to 0.1 mM added each of FAs, were studied by RT-PCR analysis. There were no significant differences in these above gene expression levels between C8:0 and C18:1 treated adipocytes. By contrast, mRNA expression level of PPARs, FABP4, and CD36/FATP were low in cells exposed to C20:4. Taken together, these experiments indicated that exposure to C20:4 led to a negative effect on the terminal differentiation of pig adipocytes.

C. Effect of FA on cell cycle distribution by flow cytometry

We next tested the effect of FAs on cell growth which was analyzed using flow cytometry and their cell cycle distributions were determined. The percentage of cells in S+G2/M phase was 9.3%, 9.9%, 24.0% with C8:0, C18:1, C20:4 treated cells, respectively. In addition, the percentage of cells in G0/G1 phase was 84.4%, 83.6%, 68.6% with C8:0, C18:1, C20:4 treated cells, respectively. These data clearly demonstrated that the cell population of proliferating cells were much higher in C20:4 added cells than the other FA treated cells, which mostly stayed at G0/G1 phase.

D. Morphological observation

The effect of individual FA was verified by the morphological method. PSPA cells were maintained for 10 days after confluence with 0.1 mM FA, and then the cells were stained with an antibody against PCNA which is a marker of S phase cell, hydrophobic fluorochrome BODIPY® 493/503 which detects intracellular lipid droplets, and DAPI which detects the cell nuclear. In this experiment, the signal of PCNA, which protein is known to exist in nuclear at S phase and is tightly associated with DNA replication, was often detected in nuclear of C20:4 added cells. On the other hand, in the presence of C18:1, intracellular lipid droplets were observed to be larger than other FA treated cells.

IV. DISCUSSION

In the present study, we showed that each FA is able to provide different signals in stimulating PSPA cells. Among three of FAs we examined, the C20:4 functioned as negative agent to induce cell growth rather than lipid synthesis. In other word, most of the PSPA cells still remain under cell cycle process due to less effect of C20:4 to enter terminal adipocyte differentiation. Our results that adipogenesis in pig preadipocytes was not stimulated by C20:4 was inconsistent with the report of mouse Ob1771 cells which explained that C20:4 functioned as a promoter agent for adipose conversion [5]. We have also observed opposite effects with C8:0 on adipocyte differentiation between pig and mouse [2]. Therefore, we strongly suggest there are species differences in the effect of individual FA on adipocyte differentiation and it is worth studying with pig cell culture model to identify the roles of FA in pigs.

The precise mechanism by which C20:4 suppreses adipogenesis is unclear. The TG amount was nearly equal between the cells exposed to C8:0 and C20:4. However, gene expression patterns of adipogenic markers were much lower in C20:4 treated cells. It seems that C20:4 treated cells are not just resembling undifferentiated preadipocytes maintained in growth medium, because they showed higher FATP mRNA expression than other FA treated adipocytes. The mRNA expression level of PPARβ/δ in C20:4 treated cells was also lowered than preadiopcytes. PPARs are nuclear receptors for FAs [6]. It remains to be determined whether this negative effect of C20:4 are related with the expression pattern of PPARβ/δ. In addition, it remains to be studied the way of cellular uptake of C20:4 are related with the high expression of FATP mRNA. Since C20:4 is known as a precursor for eicosanoids, it may play important roles in the metabolic processing in the body.

C18:1 is a monounsaturated FA and a major dietary fat, often constituting at least a third of the total FA intake. In consistent with our present results, Ding and Mersmann [7] reported that C18:1 increased adipocyte differentiation and the mRNA concentration of adipogenic markers in porcine stromal-vascular cell in a dose-dependent fashion. Although TG content was 3-fold higher in 0.1 mM C18:1 added PSPA cells than in C8:0 added cells, anti-proliferative effect shown in dose dependent manner by C8:0 was not observed with C18:1. Therefore, it is suggested that C18:1 acts positive but different from that of C8:0 in pig adipocyte differentiation.

V. CONCLUSIONS

Our results demonstrated that the adipose conversion was induced by the addition of either C8:0 or C18:1 to the media but not by C20:4, indicating that individual FA can regulate factors such adipocyte size and numbers differently in pigs, thus leading to controlling adipose tissue mass.

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REFERENCES

1. Jump DB (1999) Regulation of gene expression by dietary fat. Annu Rev Nutr 19:63-90
2. Nakajima I, Muroya S, Chikuni K (2003) Growth arrest by octanoate is required for porcine preadipocyte differentiation. Biochem Biophys Res Commun 309:702-708
3. Han J, Farmer SR, Kirkland JL et al. (2002) Octanoate attenuates adipogenesis in 3T3-L1 preadipocytes. J Nutr 132:904-910
4. Venuti JM, Morris JH, Vivian JL et al. (1995) Myogenin is required for late but not early aspects of myogenesis during mouse development. J Cell Biol 128:563-576
5. Gaillard D, Negrel R, Lagarde M et al. (1989) Requirement and role of arachidonic acid in the differentiation of pre-adipose cells. Biochem J 257:389-397
6. Xu HE, Lambert MH, Montana VG et al. (1999) Molecular recognition of fatty acids by peroxisome proliferators-activated receptors. Molecular Cell 3:397-403
7. Ding SH, Mersmann HJ (2001) Fatty acid modulate porcine adipocyte differentiation and transcripts for transcription factors and adipocyte-characteristic proteins. J Nutr Biochem 12:101-108