Biochemical and Biophysical Research Communications 432 (2013) 92–98

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of actions of octanoate on porcine preadipocytes and adipocytes differentiated *in vitro*

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ARTICLE INFO

Article history:
Received 16 January 2013
Available online 29 January 2013

Keywords:
Adipocyte differentiation
Gene expression
Lipogenesis
Pig
Reporter assay

ABSTRACT

Octanoate is used to induce adipogenic differentiation and/or lipid accumulation in preadipocytes of domestic animals. However, information on detailed actions of octanoate and the characteristics of octanoate-induced adipocytes is limited. The aim of this study was to examine these issues by comparing the outcomes of the effects of octanoate with those of rosiglitazone, which is a well-defined activator of peroxisome proliferator-activated receptor (PPAR)-γ. The adipocytes that were differentiated with 5 mM of octanoate had dispersed and diversely sized lipid droplets compared to those that were differentiated with 1 μM of rosiglitazone. The gene expression levels of adiponectin, glycerol-3-phosphate dehydrogenase, perilipin 1, and perilipin 4 were much higher in the adipocytes that were differentiated with rosiglitazone than in those differentiated with octanoate, while the gene expression levels of lipoprotein lipase and perilipin 2 were decreased in rosiglitazone-differentiated adipocytes compared to octanoate-differentiated adipocytes. However, the expressions of aP2 and CD36 genes were comparably induced. Luciferase reporter assays revealed that PPAR and liver-X-receptor activities were upregulated by octanoate more effectively than by rosiglitazone. Overall, these results suggested that the action of octanoate was complicated and may be dependent on the targeted genes and cellular status.

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1. Introduction

The adiposity of pigs is important for meat production. Excess subcutaneous fat tissue is a factor that reduces the efficiency of meat production and conversely, intramuscular fat favorably influences the quality of the meat for edible purposes [1]. Because the fat accumulation in these two depots is generally correlated with carcass quality [2], methods that control fatness in a depot-specific manner are highly valuable. Pigs are increasingly used as model animals for human medical research because of their similarity to humans with respect to physiology and anatomy [3]. Pigs whose adiposity is artificially controlled are potential animal models for obesity or lipodystrophy. In particular, pigs are much more susceptible to arteriosclerosis than mice [4], which makes an obese pig a desirable model animal for the study of metabolic syndromes. Therefore, a deep understanding of porcine adipogenesis and lipid accumulation may lead not only to production of higher quality meat at a lower cost, but also to development of excellent medical model pigs. Thus, extensive *in vitro* research of porcine adipogenesis is a prerequisite to the realization of these perspectives. With this goal in mind, we attempt to characterize the actions of octanoate, which is a medium-chain fatty acid (MCFA) that can potentially influence adiposity.

MCFAs comprise fatty acids with 6–10 carbons. Animals that are fed MCFA triglyceride (MCT)-based diets have increased insulin sensitivity and improved serum triglycerides (TG) and lipid profiles [5]. Furthermore, MCT-based diets reduce adipogenesis by downregulating peroxisome proliferator-activated receptor (PPAR)-γ signals [5]. Consistent with this, octanoate, which is a MCFA with C8, not only attenuates the differentiation of mouse 3T3-L1 preadipocytes [6], but also inhibits TG synthesis in 3T3-L1 and human adipocytes [7]. In contrast, octanoate efficiently stimulates the accumulation of TG in bovine or porcine preadipocytes [8,9]. Furthermore, 3T3-L1 preadipocytes are induced by octanoate to accumulate lipids when a standard dexamethasone, isobutylmethylxantine and insulin (DMI) protocol is not used [10,11]. Although these contradictory findings suggest that octanoate influences adipogenesis and lipid metabolism in a context-dependent manner, its detailed effects and underlying mechanisms are largely unknown. Therefore, we compared the morphology, gene expression patterns, and PPAR or liver-X-receptor (LXR) reporter activities of preadipocytes that were differentiated with octanoate and those that were differentiated with rosiglitazone, a well-defined PPARγ agonist that can efficiently activate PPARγ and subsequently induce adipocyte differentiation. Furthermore, we examined the influence of the addition and removal of octanoate on the gene expression and reporter activities of post differentiated preadipocytes.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.080

2. Materials and methods

Protocols for the use of animals in this study were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences.

2.1. Cell isolation

Mesenteric adipose tissue was obtained from 1-year-old female pigs (Landrace or crossbred). Mature fat cells were isolated essentially as described [12]. Briefly, adipose tissue was minced finely and then incubated with gentle shaking in a digestion buffer that was comprised of Dulbecco's modified Eagle medium (DMEM), 100 mM of HEPES, and 2% (w/v) bovine serum albumin (A6003, Sigma–Aldrich Co. LLC, St. Louis, MO, USA) that was supplemented with 0.2% (w/v) type-I collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) for 1 h at 37 °C. The digested tissue was filtered through a 150-μm stainless mesh and centrifuged at 150g for 5 min to separate the floating adipocytes from the pellet of stromal vascular cells. The floating adipocytes were collected and subjected to ceiling culture [13].

2.2. Preparation of preadipocytes by ceiling culture

Primary preadipocytes were established by the ceiling culture method [13]. Floating adipocytes were washed twice with digestion buffer and seeded in 12.5-cm^2 culture flasks (Falcon 353107; BD, Franklin Lakes, NJ, USA) completely filled with DMEM containing 20% (v/v) fetal bovine serum (Biowest SAS, Nuaillé, France) at a density of about 10^5 cells/flask. The flasks were incubated upside down in a humidified atmosphere of 5% CO_2 in air at 37 °C for 7 days. After we confirmed the presence of fibroblastic cells attached to the inner ceiling surface, the flask was turned upside down. The cells were subsequently cultured in DMEM containing 10% FBS until they were subjected to the differentiation procedure.

2.3. Differentiation of preadipocytes

The preadipocytes were seeded in 96-well plates that were precoated with gelatin (G1890, Sigma–Aldrich Co. LLC) and grown until they reached confluence. At confluence (day 0), adipogenic differentiation was induced by culturing the cells for 8 days in DMEM containing 5% newborn calf serum (Life Technologies Corporation, Grand Island, NY, USA), 5 μg/mL of insulin (Roche, Basel, Switzerland), 0.25 μM of dexamethasone (D4902, Sigma–Aldrich Co. LLC), 100 units/mL of penicillin, 100 mg/mL of streptomycin, 250 ng/mL of amphotericin B (Nacalai Tesque, Inc.) (DI), and 5 mM of octanoate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (DIO5) or 1 μM of rosiglitazone (DIR). The cells were then subjected to RNA extraction or lipid staining. In later experiments, the cells were cultured in DIO5 or DIR medium for 6 days, and, the media was subsequently, changed to DI, DIR, or DIO5, as appropriate. The cells were cultured for another 2 days and subjected to RNA extraction or a reporter assay.

2.4. TG analysis

Differentiated cells on 96-well plates were washed with PBS(−) and lysed in PBS(−) containing 1% Triton X-100. TG in the cell lysate was extracted with chloroform–methanol (2:1, v/v) after a small portion was separated for protein quantification with the bicinchoninic method. TG quantification was performed enzymatically with a Triglyceride E Test Kit (Wako Pure Chemical Industries, Ltd.).

2.5. AdipoRed stain

The lipid droplets of differentiated cells were stained with AdipoRed reagent (Lonza Group, Ltd., Basel, Switzerland) according to the manufacturer's instructions. The nuclei were simultaneously stained with 5 μg/mL of Hoechst 33342.

2.6. Total RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with a CellAmp Direct RNA Prep Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized at 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 5 min using a PrimeScript RT reagent kit (Takara Bio Inc., Japan).

Real-time quantitative RT-PCR of the transcripts of interest was performed using a LightCycler instrument (Roche). PCR amplification was performed in a 20-μL reaction mixture consisting of 1 μL of cDNA, 0.4 μM of each primer, and 10 μL of SYBR premix Ex Taq II (Takara Bio Inc.). Cycling conditions were 95 °C for 3 min, which was followed by 60 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. The primers used are indicated in Table 1. The relative quantification of target gene expression was normalized against the expression of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene.

2.7. Vector construction

The lentiviral plasmids pCS-PPRE-tkminP-luc-SV40-βGal and pCS-LXRE-tkminP-luc-SV40-βGal were constructed based on pCS-CDF-CG-PRE and pCSII-CMV-MCS-IRES-Bsd (Both were provided by Dr. Hiroyuki Miyohi, RIKEN BioResource Center, Tsukuba, Japan). The peroxisome proliferator response element (PPRE) or LXR response element(LXRE) -tkminP-luc region was generated by subcloning 3X PPRE (5′-AGG GGA CCA GGA CAA AGG TCA CGT TCG GGA-3′, three copies) [14] or 3X LXRE (5′-TCG AGC TTT GGT CAC TCA AGT TCA AGT TAC-3′, three copies) [15] and the minimal promoter of thymidine kinase gene (*tkminP*) into the luciferase reporter vector, pGL4 (Promega Corporation, Madison, WI, USA). PPRE- or LXRE-tkminP-luc fragments were substituted for the CMV promoter of the plasmid mentioned above. The SV40 promoter and β*Gal* gene from pSV-β-Galactosidase Control Vector (Promega Corporation) were substituted for the IRES-Bsd sequence.

Table 1
Sequences of primers used for the quantitative real-time polymerase chain reaction analysis (qPCR).

Accession no.	Gene name		Primer sequence 5′–3′
NM_214370	ADIPOQ	Sense	GACAAGGCTGTACTCTTCAC
		Antisense	GAGTCATTGACATTGTCAGC
NM_214286	LPL	Sense	GGCCGAGAGTGAAAACATCC
		Antisense	GTTGGACCAGCTGAAGTATG
NM_001190240	GPDH	Sense	GTGGCTGATGAGAAGTTCTG
		Antisense	CAGATCTCTACCGTGTCCAC
NM_001038638	PLIN1	Sense	TGCAATGCTTACGAGAAGGG
		Antisense	TCTTTTCTTCCAGGTGGTCC
NM_214200	PLIN2	Sense	AAGGGCGTCAAGACCATCAC
		Antisense	TCTGTCTAGTCCCTTACAGG
XM_003123070	PLIN4	Sense	CAGGGCAGCTACTTTGTGCG
		Antisense	CCTGCTGGGGCTTCTCAATC
NM_001002817	FABP4	Sense	CCTGATCATCACTGTGAATG
		Antisense	ATGGTGGTTGTCTTTCCATC
NM_001044622	CD36	Sense	AGAGAACGACACCTTCACTG
		Antisense	GGTATGGAACCAGGTTCAAG
NM_001032376	HPRT1	Sense	TACTGTAATGACCAGTCAACG
		Antisense	GCAACCTTGACCATCTTTGG

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