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.

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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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 150*g* 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

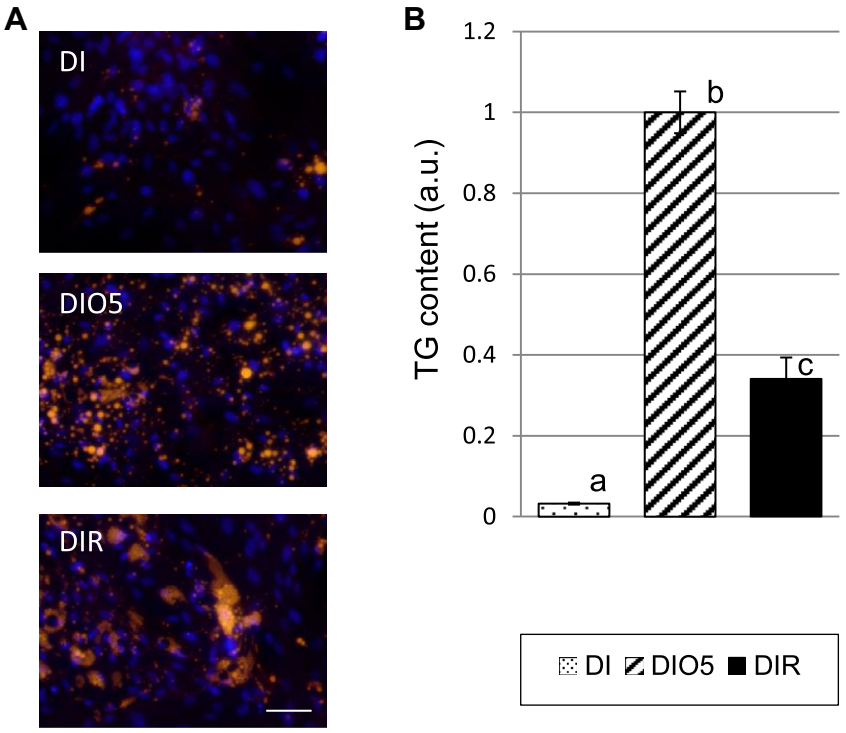

Fig. 1. Cytosolic triglyceride (TG) accumulation in porcine preadipocytes after being treated with dexamethasone and insulin (DI), DI and 5 mM octanoate (DIO5), or DI with 1 μM rosiglitazone (DIR) (A) AdipoRed O staining of adipocytes that were differentiated for 8 days with DI, DIO5, or DIR. Scale bar represents 100 μm. (B) The quantification of accumulated TG in preadipocytes after 8 days of treatment with DI, DIO5, or DIR. The data are expressed as means ± standard error of the mean (SEM) of three independent experiments. Values with different letters differ significantly ($P < 0.05$).

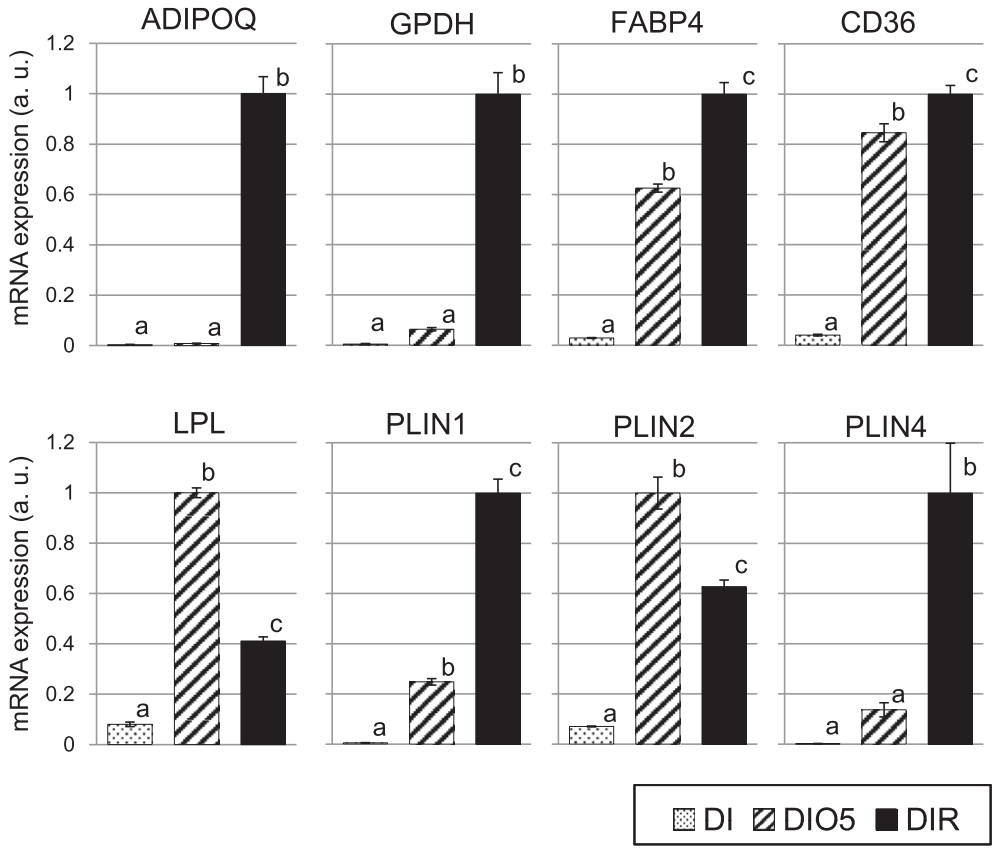

Fig. 2. The relative levels mRNA expressions of eight genes that are related to adipogenesis and lipid metabolism in preadipocytes after 8 days of treatment with DI, DIO5, or DIR. The expression of each gene was normalized to that of *HPRT1*. The data are expressed as the means ± SEM of three independent experiments. Values with different letters differ significantly ($P < 0.05$).

2.8. Lentivirus infection

293T cells were transiently cotransfected with each lentiviral expression plasmid, a packaging (pCAG-HIVgp), and a VSV-G- and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) (also provided by Dr. Miyoshi, RIKEN) with FuGENE HD (Promega Corporation). After incubating for 16-h, the cells were additionally cultured in a fresh medium containing 10 μM of forskolin for 48 h. The culture supernatants containing lentiviruses were harvested and concentrated by centrifugation using Lenti-X concentrator (Takara Bio Inc., Japan). Preadipocytes were infected with lentiviruses in the culture medium supplemented with 6 μg/mL of polybrene. After incubation for 12 h, the cells were refed with a fresh culture medium without viruses or polybrene.

2.9. Luciferase reporter assay

The cells were lysed with passive lysis buffer (Promega Corporation), and their luciferase and β-galactosidase activities were assayed using Bright-Glo Luciferase Assay System and Beta-Glo Assay System (Promega Corporation), respectively. Luciferase activity was quantified by normalization to each β-galactosidase activity.

2.10. Statistical analysis

Statistical analysis was performed using Student's *t*-tests or Tukey's multiple-comparison tests. *P* values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of lipid accumulation

Porcine preadipocytes were differentiated with dexamethasone and insulin (DI) with 5 mM octanoate (DIO5) or 1 μM rosiglitazone (DIR) for 8 days. Each treatment substantially induced cytosolic TG accumulation, as indicated in Fig. 1A. The quantification of cytosolic TG revealed that the total TG content was about three times higher in DIO5-treated cells than in DIR-treated ones, while the TG content was much less in DI-treated cells (Fig. 1B). The staining of TG with AdipoRed illustrated that the distribution pattern of lipid droplets differed between DIO5-treated and DIR-treated cells (Fig. 1A). In particular, DIO5-treated cells had more widely distributed and more diversely sized lipid droplets compared to DIR-treated cells, which had clustered and uniformly sized droplets.

3.2. Gene expression patterns in adipocytes differentiated with distinct treatments

We examined the expression levels of several adipocyte-related genes that were upregulated with adipocyte differentiation, using real-time RT-PCR methods (Fig. 2). The expression levels of all the genes that were examined were upregulated in both DIR- and DIO5-treated cells compared to DI-treated cells. However, the gene expression patterns in adipocytes differentiated with DIR and DIO5 varied substantially. The expression levels of adiponectin and glycerol-3-phosphate dehydrogenase (*GPDH*) were much higher in adipocytes that were differentiated with DIR than in those differentiated with DIO5. CD36 and aP2 gene expression levels were slightly higher in adipocytes that were differentiated with DIR than in those differentiated with DIO5. In contrast, LPL gene expression levels were higher in adipocytes that were differentiated with DIO5 than in those differentiated with DIR. Three dominantly expressed PAT family genes that encode lipid-droplet associated proteins were also examined. Perilipin 1 and 4 expression levels were higher in adipocytes differentiated with DIR, while Perilipin 2 expression levels exhibited an opposite trend.

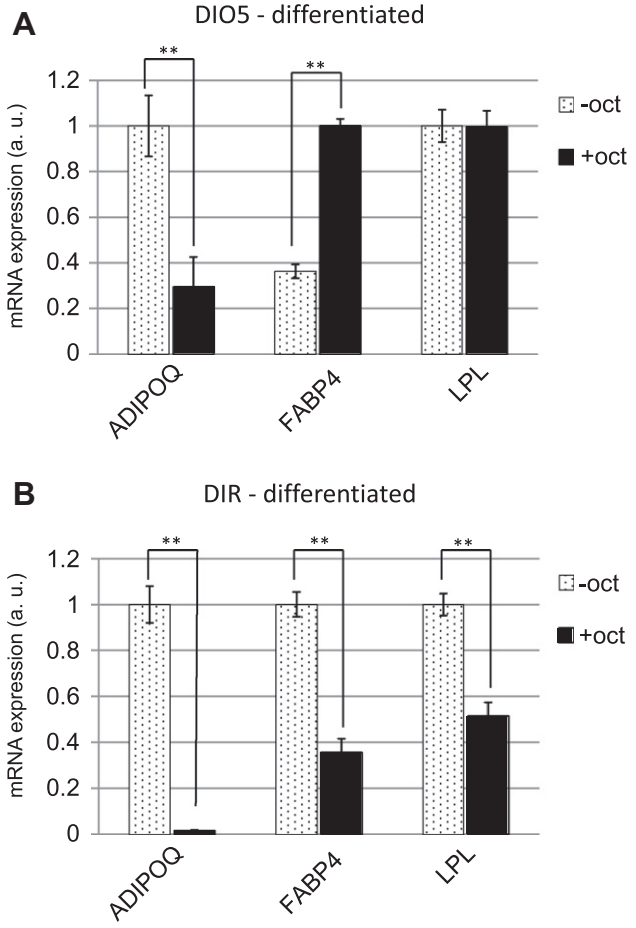

Fig. 3. The influence of octanoate on adipocytes that were differentiated for 6 days. The relative levels mRNA expression of the *ADIPOQ*, *FABP4*, and *LPL* genes in adipocytes that were differentiated for 6 days with DIO5 (A) or DIR (B) and then further cultured for 2 days with DI containing 5 mM octanoate (+oct) or no octanoate (−oct). The data are expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$.

3.3. Effects of addition and removal of octanoate on gene expression levels

We subsequently, examined the effects of addition and removal of octanoate on adiponectin, aP2, and LPL gene expression levels in the media after 6 days of differentiation with DIR or DIO5, respectively (Fig. 3). The addition of octanoate to adipocytes resulted in decreased expression of all of the examined genes. After the removal of octanoate, adiponectin gene expression was upregulated, while aP2 gene expression was downregulated. LPL gene expression was not much affected.

3.4. PPAR and LXR reporter analysis

To explore the underlying mechanisms of the regulation of gene expression by octanoate, we examined PPAR and LXR transcription activities with PPRE- and LXRE-luciferase reporter systems. On the third day of differentiation, PPRE-reporter activity was upregulated in both DIO5- and DIR-treated preadipocytes, and this effect was more apparent in DIO5-treated cells (Fig. 4A, left). However, LXR-reporter activity was upregulated in DIO5-treated cells but not in DIR-treated cells (Fig. 4A, right). In addition, we examined the changes in reporter activity after the addition of octanoate or the

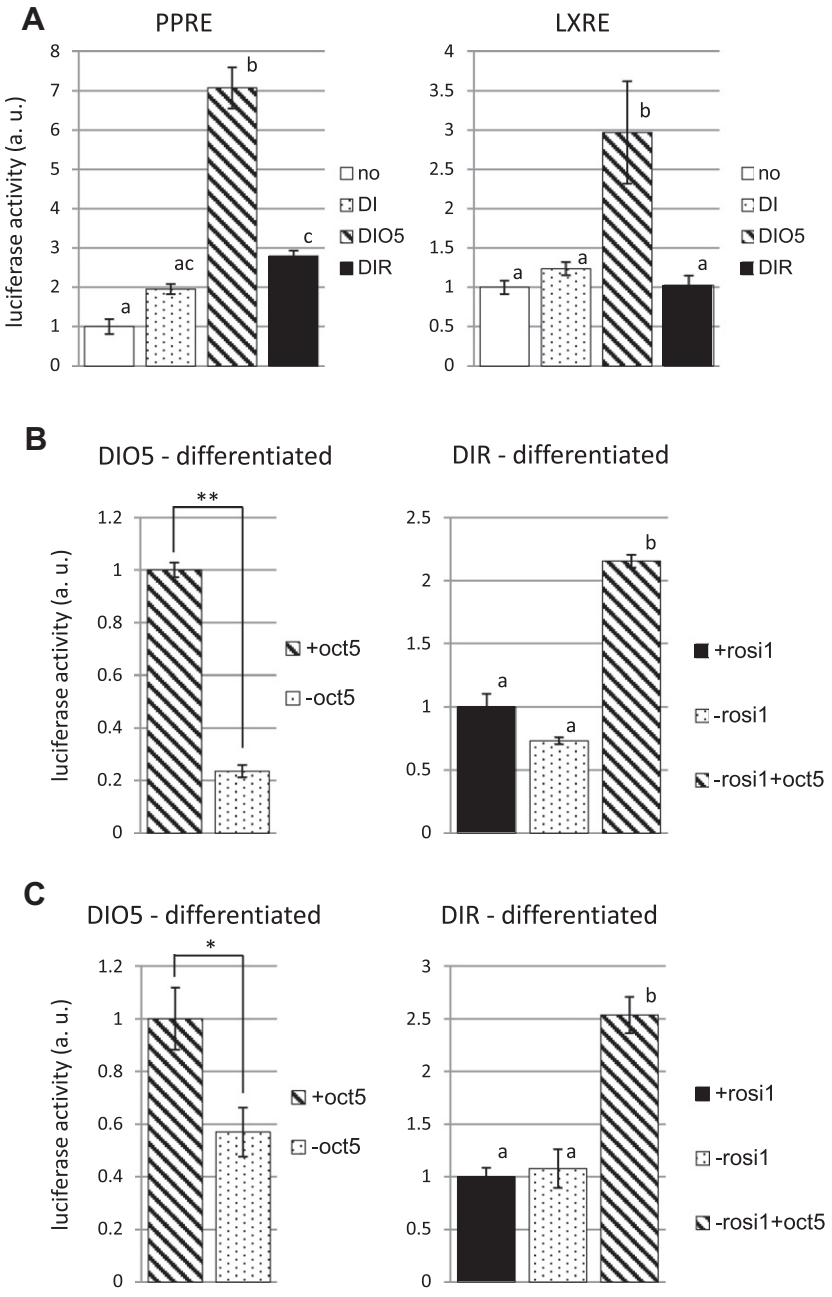

Fig. 4. Reporter gene analysis of the peroxisome proliferator-activated receptor (PPAR) response element (PPRE) and LXR response element (LXRE). Reporter activities were normalized to constitutively expressed beta-galactosidase activities. (A) PPRE and LXRE reporter activities in preadipocytes that were treated with no additives (no), DI, DIO5, or DIR for 3 days. The data are expressed as means ± SEM of three independent experiments. Values with different letters differ significantly ($P < 0.05$). (B) The left columns represent PPRE reporter activities in adipocytes that were differentiated with DIO5 for 6 days and then further cultured for 2 days with DI containing 5 mM octanoate (+oct) or no octanoate (−oct). The right columns represent the PPRE reporter activities in adipocytes differentiated with DIR for 6 days and then further cultured for 2 days with DI (−rosi) or DI containing 1 μM of rosiglitazone (+rosi) or 5 mM of octanoate (−rosi + oct). The data are expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$. Values with different letters differ significantly ($P < 0.05$). (C) LXRE reporter activities. Please see (B) for explanation. The data are expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$. Values with different letters differ significantly ($P < 0.05$).

removal of it from the adipocytes that were differentiated for 6 days. Both PPRE- and LXRE-reporter activities were decreased by the removal of octanoate from DIO5-induced adipocytes and increased by the addition of 5 mM octanoate to DIR-induced adipocytes (Fig. 3B and C).

4. Discussion

The results of this report indicated that, although octanoate and rosiglitazone have apparently similar potentials to induce lipid accumulation in porcine preadipocytes, they resulted in different patterns of gene expression of adiponectin, *GPDH*, and perilipin family genes. We thought that this difference might reflect differences between adipocyte differentiation (adipogenesis) and lipid accumulation (lipogenesis). Rosiglitazone certainly induced adipogenesis, but the effects of octanoate involved lipogenesis more than adipogenesis. Although it is difficult to clearly separate adipogenesis and lipogenesis, adipogenesis is considered to involve the development of adipocytes, which accompanies not only lipid accumulation, or lipogenesis, but also the induction of adipocyte-specific gene expression, such as with adiponectin. We showed

that, in rosiglitazone-treated cells, the expression of adipocyte-specific genes, such as adiponectin and *GPDH*, were highly upregulated compared to octanoate-treated ones, while the aP2 and CD36 genes, which are involved in lipid metabolism, were comparably expressed in both cell populations. In addition, the distinct expression pattern of perilipin family genes supported this concept. The expression of perilipin 2 was rather ubiquitous, while those of perilipin 1 and 4 were largely restricted to adipose tissue [16]. Consistent with this, perilipin 2 is a major lipid droplet coat protein that is found in hepatocytes and muscles [17,18]. Therefore, the expression pattern of perilipins in octanoate-differentiated cells was considered to be of an intermediate nature between that of adipocytes and other lipid-laden tissues.

Another difference was detected in the morphology of lipid droplets. The rosiglitazone treated cells had clustered and uniformly sized droplets. However, octanoate-differentiated cells had many dispersed and randomly sized droplets. Although this difference was considered to be primarily caused by the distinct expression pattern of the perilipin gene family, it might also have reflected the difference between adipogenesis, in which unilocular lipid droplets are formed ultimately, and mere lipid accumulation. However, this could not be determined because it was difficult to form the unilocular lipid droplets in our culture system.

In addition, we examined the effects of octanoate on the transcriptional activity of major nuclear receptors to induce lipid accumulation using a luciferase reporter system. We observed that 5-mM octanoate treatment upregulated PPRE-reporter activity and 1-mM octanoate treatment had little effect on the reporter activity (data not shown). Guo et al. reported that PPRE activity was decreased by about 50% by 1-mM octanoate treatment [7], which was apparently inconsistent with our data. However, the conditions of their analysis were considerably different from our conditions. They used 293T cells that were transfected with PPARγ, and the status of the endogenous ligand of PPARγ was completely different from that of the adipocytes that used by us. Moreover, Malapaka et al. concluded that octanoate had no significant binding with PPARγ, but their analysis was limited to concentrations that were less than 1 mM [19]. If an extrapolation of their data is allowed, 5 mM of octanoate can yield significant binding to PPARγ. In addition, they indicated that the C10-MCFA, decanoate, binds to PPARγ in a fashion that is distinct from that of rosiglitazone or long-chain fatty acids. Octanoate, which is a C8-MCFA, may have similar features, which enabled us to postulate interactive effects with endogenous ligands. Overall, despite having no direct evidence, we suggest that 5-mM octanoate can activate the PPRE reporter by binding to PPARγ. However, contrary to this speculation, we also detected the poor induction and inhibition of adiponectin and *GPDH* gene expression, which is generally upregulated by PPARγ, in 5-mM octanoate-treated cells. This inconsistency may be explained by the promoter-selective actions of PPARγ, which are mediated by the binding of coactivators and corepressors to PPARγ. Guan et al. demonstrated that thiazolidinediones, not endogenous ligands, can activate a specific PPARγ-target gene (*Gyk*) expression by inducing dissociation of a corepressor complex and recruitment of a coactivator complex [20]. Moreover, decanoate has proven to be insufficient for the initiation of adipogenesis in 3T3-L1 cells, although it can moderately upregulate PPARγ-target gene expression [19]. Collectively, it was suggested that the effects of 5-mM octanoate on PPARγ-target gene expression is selective and/or limited, which yielded apparently inhibitory effect on expressions of some genes by overriding the action of other potent ligands, presumably due to the induction of unique conformational change which is observed in the case of decanoate [19].

Furthermore, we suggested that 5 mM of octanoate induced the transactivation of another nuclear receptor, LXR, by the LXRE-luciferase reporter assay. Several previous reports [21,22] have demonstrated that LXR can induce lipid accumulation in adipocytes, although contradictory reports also exist [23]. It is easily conceivable that LXR activation contributes to enhanced lipid accumulation and the expression of some genes in our system. However, polyunsaturated fatty acids have exhibited inhibition of LXR activity [24], and no reports have demonstrated the effect of MCFA on LXR activity. Therefore, whether LXR activation was caused by the direct interaction of octanoate and LXR or by indirect effects via PPARγ activation remains unclear. Taking into account these complicated actions of octanoate, the inconsistent influence of octanoate on various adipocytes, as mentioned in the introduction, may be explained by variations in the internal activities of PPAR and LXR, which correspond to the integration of the expression levels of PPAR and/or LXR and the levels of endogenous ligands.

Overall, we have reported here that octanoate can induce lipid accumulation and the expression of some related genes in porcine preadipocytes, and this is likely due to the distinct regulation of PPAR and LXR in the case of rosiglitazone. This difference may reflect the physiological status of adipose tissue and, particularly, the differences among adipogenesis, the normal growth of adipose tissue, and the overload of lipids, which occurs in obesity and ectopic fat accumulation, as exemplified in the expression of adiponectin and perilipin family genes. Although other studies are necessary to elucidate the detailed mechanisms involved, the present data will promote a better understanding for porcine adipose physiology. Consequently, the use of pigs as model animals for human metabolic disorders can be refined.

Acknowledgments

The authors would like to thank the staff of the Swine Management Section of the National Institute of Livestock and Grassland Science for taking care of the pigs, and N. Watanabe and K. Iijima for technical assistance. Moreover, we are grateful to Dr. H. Kitani and Dr. T. Takenouchi for their technical assistance and valuable advice in using lentiviral vectors.

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