ABSTRACT: The nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ) triggers adipocyte differentiation by regulating lipogenic genes. A ligand for PPARγ is necessary to activate PPARγ function. Fatty acids are potential ligands for PPARγ activation. The current experiment was designed to determine the potential for individual fatty acids to activate porcine PPARγ ectopically expressed in myoblasts. The expression of adipocyte fatty acid binding protein (aP2) and adiponectin in myoblasts stably expressing porcine PPARγ was increased when docosahexaenoic acid (DHA) was added to the adipogenic medium. The response was positively related to DHA concentration and suggests that DHA may bind to and activate porcine PPARγ, leading to increased expression of aP2 and adiponectin. The conditioned media collected from myoblasts expressing PPARγ between d 3 and 6 or between d 6 and 9, but not DHA itself, activated the aP2 gene promoter-driven luciferase activity. These results suggest that a metabolite of DHA is the ligand binding to and activating porcine PPARγ. The metabolite and pathway for its production are currently unknown.

Key words: adipocyte differentiation, adipocyte fatty acid-binding protein, adiponectin, docosahexaenoic acid, peroxisome proliferator-activated receptor γ, pig

INTRODUCTION

Adipocyte differentiation is a complicated process regulated by several transcription factors. Peroxisome proliferator-activated receptor γ (PPARγ) has been clearly demonstrated to be an important factor in porcine adipogenesis (Yu et al., 2006). Activation of PPARγ is mediated by binding of a ligand to its ligand-binding domain. Activated PPARγ modulates transcription of several adipogenic-related genes through binding to their PPARγ response elements during adipocyte differentiation (Tontonoz et al., 1994, 1995).

Preadipocyte replication and differentiation into adipocytes are stimulated when rodents are fed a high-fat diet (Ellis et al., 1990; Belzung et al., 1993). High-fat feeding increases expression of PPARγ and its target genes during adipocyte differentiation (Lopez et al., 2003). In porcine preadipocytes, long-chain fatty acids (FA) regulate differentiation and induce expression of adipogenic genes (Ding and Mersmann, 2001). The arachidonic acid (ArA) metabolite, 15-deoxy-Δ12,14-PG J2 (15d-PGJ2), enhances adipogenesis (Tontonoz et al., 1994). Putative metabolites of docosahexaenoic acid (DHA) activate PPARγ (Yamamoto et al., 2005). These results suggest that FA and some FA metabolites are able to regulate adipocyte differentiation. In previous studies, we demonstrated that porcine PPARγ stimulates adipocyte-like differentiation in myoblasts (Yu et al., 2006). Although various FA increase PPARγ transcripts during differentiation of porcine preadipocytes (Ding and Mersmann, 2001), no evidence is available to indicate directly which FA bind to and activate porcine PPARγ. Consequently, we treated C2C12 myoblasts expressing porcine PPARγ with several FA to determine which FA are capable of inducing PPARγ functions.

MATERIALS AND METHODS

Institutional Animal Use approval was not needed because the experiment used only cells in culture.
**Cell Culture**

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). The C2C12 myoblasts expressing porcine PPARγ or empty vector (Yu et al., 2006) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO2. Confluent cells were cultured in adipogenic differentiation medium (Dulbecco modified Eagle medium containing 10% fetal bovine serum, 1 µM dexamethasone, and 5 µg/mL of insulin; AM) with or without FA. Fatty acids, namely, palmitic acid (PA), oleic acid (OA), linoleic acid (LA), ArA, eicosapentaenoic acid (EPA), and DHA, were dissolved in dimethyl sulfoxide for dispersal. The dimethyl sulfoxide concentration in all treatment groups and control groups was 0.1%. Aliquots of FA were mixed with BSA (FA-free BSA), and the final molar ratio of FA:BSA was 4:1. The concentration of these FA in medium was 100 µM. For DHA, we designed an experiment to include concentrations of 12.5, 25, 50, 75, and 100 µM in the media to examine the effect of DHA closer to feasible physiological concentrations. Cells were cultured in adipogenic differentiation medium AM for 10 d with a medium change every 2 d. Rosiglitazone (1 µM), a PPARγ agonist, and GW9662 (1 µM), a PPARγ antagonist, were used to evaluate the interaction of DHA and PPARγ. To examine the effects of DHA on modulation of porcine PPARγ function, conditioned medium from myoblasts expressing porcine PPARγ and cultured in adipogenic medium without or with 100 µM DHA was collected from d 0 to 3, 3 to 6, and 6 to 9. These media were defined as conditioned media (CM) and DHA-CM, respectively. The CM was added to myoblasts expressing the adipocyte FA-binding protein (aP2) promoter region coupled to luciferase.

**Quantitative Reverse Transcription-PCR**

Total RNA was isolated from cells by guanidinium-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The quality of the RNA was determined by examination of the 18S and 28S ribosomal RNA bands after electrophoresis. The cDNA was synthesized by using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative reverse transcription-PCR was performed by using DNA Engine Opticon-2 (MJ Research, Waltham, MA) and FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). β-Actin expression was determined as the internal control gene. The sequence of primers for quantitative reverse transcription-PCR is listed in Table 1. The PPARγ was of porcine origin so porcine primers were used, whereas mouse primers were used for the other genes expressed by the rodent-derived C2C12 cells. The mRNA expression of each gene was normalized to its β-actin mRNA expression. The relative expression of target genes was determined by using the relative standard curve. Details for all these procedures were described previously (Chen et al., 2008). Threshold cycle (Ct) values were obtained, and relative gene expression was calculated by using the formula (1/2)^Ct target genes–Ct β-actin (Schmittgen et al., 2000).

**Northern Blot**

The mRNA expression of adipogenic marker genes in the PPARγ antagonist and DHA experiments was determined by Northern blot analysis. Ribonucleic acid (20 µg) was electrophoresed and transferred to nylon membranes. The probes for the genes measured were generated by PCR. The membrane was prehybridized at 42°C for 1 h, and the denatured cDNA probe was added to hybridize with the targeted gene transcripts overnight at 42°C. Phosphorimage analysis (ImageQuant TL v2005 software, GE Healthcare, Piscataway, NJ) was performed to quantify hybridization results. The densitometric value for an individual transcript in a sample lane was normalized to the densitometric value for the glyceraldehyde-3-phosphate dehydrogenase mRNA in the same lane. Detailed probe information and procedures are described in Yu et al. (2006).

**Transient Transfection**

For luciferase assays, a mouse 5.4-kb aP2 promoter coupled to the luciferase gene was cloned into the pGL3 reporter plasmid (Promega, Madison, WI). It

<table>
<thead>
<tr>
<th>Gene1</th>
<th>Primer2</th>
<th>Annealing temperature, °C</th>
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<tbody>
<tr>
<td>Porcine PPARγ (AF103946)</td>
<td>S 5’-TGACCATGGTTGACACCG-3’ A 5’-AAGCATGAACTCCATAGTG-3’</td>
<td>58</td>
</tr>
<tr>
<td>Mouse aP2 (NM 024406)</td>
<td>S 5’-CCGCAGACGACAGGA-3’ A 5’-CTCATGCCCTTTCATAAACT-3’</td>
<td>58</td>
</tr>
<tr>
<td>Mouse adiponectin (NM 009605)</td>
<td>S 5’-GATGGCCAGGATGCGACTCC-3’ A 5’-CTTGCCAGTCGTCGGCGCTCAT-3’</td>
<td>58</td>
</tr>
<tr>
<td>Mouse SREBP-1c (NM 011480)</td>
<td>S 5’-GGACGACGGAGCCATGG-3’ A 5’-GGAAAGTCACTGCTTGGTGTGGA-3’</td>
<td>58</td>
</tr>
<tr>
<td>Mouse β-actin (NM 007393)</td>
<td>S 5’-AGGTGACAGCATGTTCTCTG-3’ A 5’-GCTGCTCACACACCTC-3’</td>
<td>60</td>
</tr>
</tbody>
</table>

1PPARγ = peroxisome proliferator-activated receptor γ; aP2 = adipocyte fatty acid binding protein; SREBP-1c = sterol regulatory element-binding protein-1c.

2S = sense; A = antisense.

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has been demonstrated that this promoter sequence is effectively activated by PPARγ through binding to the aP2 PPAR response element (Ross et al., 1990). Confluent myoblasts expressing porcine PPARγ were cotransfected with the pGL3 reporter plasmid (with or without the aP2 promoter and luciferase gene) by lipofection (Lipofectamine 2000, Invitrogen). Myoblasts expressing porcine PPARγ transfected with the pGL3 reporter plasmid (without the aP2 promoter) were the control group. In addition, phRG-TK plasmid (an internal control) was transfected into all cells for normalization. After 6 h of transient transfection, media were changed to adipogenic medium without (AM) or with 100 µM DHA (AM + DHA), or CM or DHA-CM. Forty-eight hours after treatment, cells were harvested and luciferase was assayed (Dual-Glo luciferase assay system, Promega).

**Statistical Analysis**

The treatment effects were analyzed by using an ANOVA procedure to determine the main effects of FA on the porcine PPARγ function. Tukey’s test was used to evaluate differences among means (SAS Inst. Inc., Cary, NC). A significant difference indicated that the P-value was ≤0.05.

**RESULTS**

**The Effect of FA on Adipogenic Gene Expression**

In myoblasts transfected with empty vector and exposed to AM containing any of several FA, the endogenous PPARγ was not induced (Figure 1A). In myoblasts transfected with porcine PPARγ, the enhanced PPARγ expression was not changed by any of the FA treatments (Figure 1A). In cells containing empty vector, the mRNA for the adipogenic transcription factor, sterol regulatory element-binding protein 1 (SREBP-1c), was increased when PA, OA, LA, or EPA was added to the adipogenic medium (Figure 1B). However, ArA or DHA treatment reduced the expression of SREBP-1c compared with other FA and the control (Figure 1B). In cells ectopically expressing PPARγ, PA, OA, LA, and EPA, but not ArA or DHA, increased expression of SREBP-1c (Figure 1B). In cells containing empty vector, only PA, ArA, and DHA increased expression of aP2, whereas in cells ectopically expressing PPARγ, the LA, EPA, and especially ArA and DHA increased aP2 mRNA concentration (Figure 2A). No FA increased expression of adiponectin in cells containing empty vector, whereas all FA increased adiponectin mRNA in cells ectopically expressing PPARγ (Figure 2B). The effect of DHA was greater than for any other FA. These results demonstrated that individual FA have different regulating effects on individual adipogenic genes.

**Promotion of Adipogenic Gene Expression by DHA Is Mediated by PPARγ**

The expression of aP2 mRNA in either C2C12 myoblasts or PPARγ expressed C2C12 was increased in a dose-dependent manner by DHA treatment (Figure 3A). The effectiveness with which the expression of aP2 mRNA was increased by DHA treatment was greater in the porcine PPARγ expressing C2C12 than in the wild-type C2C12 myoblasts (Figure 3A). A similar tendency was observed for regulation of the expression of adiponectin by increasing concentrations of DHA (Figure 3B). The aP2 expression induced by treatment with the PPARγ agonist, rosiglitazone, was inhibited by addition of the PPARγ antagonist, GW9662 (Figure 4A). In a similar fashion, the DHA-induced aP2 expression was inhibited by addition of GW9662 (Figure 4A), suggesting that this DHA effect is specifically mediated through PPARγ. The responses of lipoprotein lipase mRNA to addition of DHA were similar to those of the aP2 gene (Figure 4B), supporting the concept that the DHA-induced expression of adipogenic genes was specifically mediated by PPARγ. Therefore, we tested whether DHA directly stimulated PPARγ-targeted gene expression through binding to and activation of the PPARγ ligand-binding domain. In the reporter luciferase assay, the mouse aP2 promoter was not activated in myoblasts expressing porcine PPARγ when AM or AM + DHA was added for 48 h (Figure 5). The result indicated that after this relatively short-term exposure, DHA was not able to directly regulate PPARγ-targeted genes. However, an increased level of luciferase activity was detected when cells were incubated with DHA-CM; CM was collected from d 3 to 6 or 6 to 9 of DHA treatment. Conditioned medium prepared from cells incubated without DHA (i.e., CM) did not stimulate luciferase activity. In contrast, luciferase activity was not stimulated when cells were treated with the PPARγ antagonist, GW9662 (Figure 5). In the presence of the PPARγ antagonist, the aP2 promoter was not affected by DHA-CM, indicating that factors from the DHA-CM affected the aP2 promoter through PPARγ.

**DISCUSSION**

Adipocyte differentiation is regulated by several transcription factors, including PPARγ, CCAAT/ enhancer-binding protein α, and SREBP-1c (Rosen and Spiegelman, 2000). These factors promote cell morphologic conversion, lipogenic gene expression, and triacylglycerol accumulation. However, PPARγ is the most important transcription factor in regulating adipogenesis (Rosen et al., 1999; He et al., 2003). In pigs, we demonstrated that ectopic expression of porcine PPARγ stimulates adipogenesis in myoblasts (Yu et al., 2006). The structure of the PPARγ protein has 2 functional domains, the ligand-binding domain and the DNA-binding domain. The ligand-binding domain
is responsible for ligand binding to activate PPARγ to trigger target gene expression (Nolte et al., 1998). Thiazolidinedione antidiabetic drugs are high-affinity ligands for mouse PPARγ (Kletzein et al., 1992) and also promote porcine adipocyte differentiation (Tchoukalova et al., 2000). Desvergne and Wahli (1999) suggested that, among the natural ligands for PPARγ activation, OA, ArA, and EPA are capable of activating human PPARγ. Several FA enhance triacylglycerol accumulation in 3T3-L1 preadipocytes (Madsen et al., 2005) and in differentiating porcine preadipocytes in culture (Ding and Mersmann, 2001; Ding et al., 2002).

**Figure 1.** Effect of individual fatty acids on expression of peroxisome proliferator-activated receptor γ (PPARγ; A) and sterol regulatory element-binding protein-1c (SREBP1c; B). The C2C12 myocytes with empty vector (Empty) and C2C12 cells expressing porcine PPARγ were maintained in adipogenic medium without or with 100 µM fatty acids (PA = palmitic acid; OA = oleic acid; LA = linoleic acid; ArA = arachidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid) for 10 d. All culture media, including the control media, contained dimethyl sulfoxide (DMSO). The mRNA levels were measured by quantitative reverse transcription-PCR. The expression of PPARγ and SREBP-1c were determined and normalized to the mRNA for β-actin. The error bars indicate the means ± SE for cells from 3 independent replicates (n = 3). a–e Means without a common letter differ, P ≤ 0.05.

**Figure 2.** Effect of individual fatty acids on the expression of adipocyte fatty acid binding protein (aP2; A) and adiponectin (B). The C2C12 myocytes with empty vector (Empty) and C2C12 cells expressing porcine peroxisome proliferator-activated receptor γ (PPARγ) were maintained in adipogenic medium without or with 100 µM fatty acids (PA = palmitic acid; OA = oleic acid; LA = linoleic acid; ArA = arachidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid) for 10 d. All culture media, including the control media, contained dimethyl sulfoxide (DMSO). The mRNA levels were measured and expressed as indicated in Figure 1. The error bars indicate the means ± SE for cells from 3 independent replicates (n = 3). a–e Means without a common letter differ, P ≤ 0.05.
Figure 3. Effect of docosahexaenoic acid (DHA) on the expression of adipogenic marker genes. After confluence, C2C12 expressing empty vector (Empty) and C2C12 expressing porcine peroxisome proliferator-activated receptor γ (PPARγ) were cultured for 10 d with increasing concentrations of DHA. The mRNA levels were measured by quantitative reverse transcription-PCR. The relative mRNA concentration average of the C2C12 expressing empty vector (Empty) with no DHA treatment was designated as 1 (equal to 100% of the control group). Other data were expressed relative to this value. The expression of adipocyte-specific genes, adipocyte fatty acid-binding protein (aP2; A) and adiponectin (B), were increased as the DHA increased with the expression level in PPARγ much greater than that in the empty vector. The error bars indicate the means ± SE from cells for 3 independent replicates (n = 3). * Means without a common letter differ, P < 0.05.

Figure 4. Docosahexaenoic acid (DHA) mimics the effect of a peroxisome proliferator-activated receptor γ (PPARγ) agonist on the expression of lipogenic genes. The C2C12 myocytes with empty vector (Empty) and C2C12 cells expressing porcine PPARγ were maintained in adipogenic medium with addition of 1 µM rosiglitazone, a PPARγ agonist, 1 µM GW9662, a PPARγ antagonist, and 100 µM DHA as indicated. Cells were treated for 10 d with a medium change every 2 d. The mRNA levels were measured by Northern blot. The expression of the adipocyte-specific genes, adipocyte fatty acid-binding protein (aP2; A) and lipoprotein lipase (LPL; B), were determined and normalized to the mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The error bars indicate the means ± SE for cells from 3 independent replicates (n = 3). ND = not detected. * Means without a common letter differ, P ≤ 0.05.
In porcine preadipocytes, OA is especially effective in promoting differentiation. Arachidonic acid acutely increases differentiation, and DHA does not change differentiation (Ding et al., 2002). In these cells, both ArA and DHA decrease expression of the SREBP-1c mRNA and protein after 24 h in serum-free medium. The long-term effects of highly unsaturated long-chain FA on porcine adipocyte differentiation are not known because these FA are toxic [i.e., cause cell lysis in serum-free medium; Ding, McNeel (Baylor College of Medicine), and Mersmann, unpublished data]. In the current study, addition of individual FA to myoblasts expressing porcine PPARγ increased expression of aP2 and adiponectin. Furthermore, the effects of DHA were found to be concentration dependent. The expression of SREBP-1, a transcription factor involved in FA metabolism and adipocyte differentiation, is decreased in differentiating porcine preadipocytes exposed to DHA (Ding et al., 2002), in porcine liver after feeding DHA oil (Hsu et al., 2004), and in C2C12 myoblasts transfected with PPARγ. Polyunsaturated FA can also suppress the expression of FA synthase and stearoyl CoA desaturase 1 in liver (Jump and Clarke, 1999). In addition, DHA regulates hepatic SREBP-1c degradation through a proteasome-dependent pathway (Botolin et al., 2006). These results demonstrate that individual FA have a role in regulating expression of genes associated with lipid metabolism.

The synthetic PPARγ antagonist, GW9662, is used to block PPARγ-mediated adipogenesis during adipocyte differentiation (Nakano et al., 2006). In the current study, the DHA-induced expression of aP2 and lipoprotein lipase was reduced in the presence of GW9662, suggesting that the DHA-induced increase in transcripts was mediated through PPARγ. To understand the interaction between DHA and PPARγ, we performed reporter-gene analysis to demonstrate whether DHA can directly bind to and activate PPARγ. Mouse
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aP2 promoter-luciferase activity was not detected when DHA was present in adipogenic medium. These observations are similar to the results of Yamamoto et al. (2005). Pawar and Jump (2003) found that DHA had weak affinity for PPARα and that this weak affinity was similar to that of DHA with PPARγ. We concluded, based on these results, that the DHA could not directly activate porcine PPARγ in myoblasts. Recombinant human PPARγ protein binds DHA with 5 times greater affinity than OA [McNeel (Baylor College of Medicine) and Mersmann, unpublished data]. This observation suggests that DHA binds to porcine PPARγ (the protein is 93% homologous to the human protein), but cannot activate the protein. Conditioned medium obtained from myoblasts incubated from d 3 to 6 or 6 to 9 in DHA-CM was very effective in activating the reporter gene. These results imply that DHA was metabolized and that metabolites, rather than the parent DHA, activated porcine PPARγ. Yamamoto et al. (2005) reported that several putative metabolites of PUFA have high affinity for PPARγ.

The ArA metabolite, 15d-PGJ2, is derived from ArA by the conversion of cyclooxygenase 1 and 2 to PGH2. Prostaglandin D2 is generated from PGH2 by PGD synthase and further chemical dehydration to form 15d-PGJ2. It is believed that 15d-PGJ2 is an endogenous ligand for PPARγ (Forman et al., 1995). The 15d-PGJ2 is generated and secreted into the medium during induction of differentiation (Bell-Parikh et al., 2003). Note that, contrary to our observations, in 3T3-L1 cells ArA suppressed the expression of lipogenic genes via a prostanoid pathway (Mater et al., 1998). Although there is no direct evidence to explain how DHA regulates adipogenesis, we speculate that myoblasts metabolize DHA, perhaps through a prostanoid pathway, to produce one or more prostaglandins with a high affinity for binding to porcine PPARγ and with the structural capacity to activate it.

In conclusion, we demonstrated that DHA was a potent ligand precursor for porcine PPARγ activation. The DHA itself does not directly activate PPARγ. Therefore, metabolism of DHA to form a potent ligand for porcine PPARγ is a critical step in the DHA-mediated modification of adipocyte differentiation. This potential ligand has not been identified and characterized.

LITERATURE CITED


