Suppression of Adipocyte Differentiation by Aldo-keto Reductase 1B3 Acting as Prostaglandin F\textsubscript{2\alpha} Synthase\textsuperscript{*}

Received for publication, October 20, 2009; and in revised form, January 19, 2010 Published, JBC Papers in Press, January 21, 2010, DOI 10.1074/jbc.M109.077164

Ko Fujimori\textsuperscript{i}, Toshiyuki Ueno\textsuperscript{j}, Nanae Nagata\textsuperscript{k}, Kaori Kashiwagi\textsuperscript{l}, Kosuke Aritake\textsuperscript{m}, Fumio Amano\textsuperscript{n}, and Yoshihiro Urade\textsuperscript{o}

From the \textsuperscript{i}Laboratory of Biodefense and Regulation, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094 and the \textsuperscript{j}Department of Molecular Behavioral Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan

Prostaglandin (PG) F\textsubscript{2\alpha} suppresses adipocyte differentiation by inhibiting the function of peroxisome proliferator-activated receptor \(\gamma\). However, PGF\textsubscript{2\alpha} synthase (PGFS) in adipocytes remains to be identified. Here, we studied the expression of members of the aldo-keto reductase (AKR) 1B family acting as PGFS during adipogenesis of mouse 3T3-L1 cells. AKR1B3 mRNA was expressed in preadipocytes, and its level increased about 4-fold at day 1 after initiation of adipocyte differentiation, and then quickly decreased the following day to a level lower than that in the preadipocytes. In contrast, the mRNA levels of Akr1b8 and 1b10 were clearly lower than that level of Akr1b3 in preadipocytes and remained unchanged during adipogenesis. The transient increase in Akr1b3 during adipogenesis was also observed by Western blot analysis. The mRNA for the FP receptor, which is selective for PGF\textsubscript{2\alpha}, was also expressed in preadipocytes. Its level increased about 2-fold within 1 h after the initiation of adipocyte differentiation and was maintained at almost the same level throughout adipocyte differentiation. The small interfering RNA for Akr1b3, but not for Akr1b8 or 1b10, suppressed PGF\textsubscript{2\alpha} production and enhanced the expression of adipogenic genes such as peroxisome proliferator-activated receptor \(\gamma\), fatty acid-binding protein 4 (aP2), and stearoyl-CoA desaturase. Moreover, an FP receptor agonist, Fluprostenol, suppressed the expression of those adipogenic genes in 3T3-L1 cells; whereas an FP receptor antagonist, AL-8810, efficiently inhibited the suppression of adipogenesis caused by the endogenous PGF\textsubscript{2\alpha}. These results indicate that AKR1B3 acts as the PGFS in adipocytes and that AKR1B3-produced PGF\textsubscript{2\alpha} suppressed adipocyte differentiation by acting through FP receptors.

Adipocytes are unique cells involved in the regulation of energy homeostasis (1, 2). The amount of adipose tissue is altered in various conditions (3). The excessive lipid accumulation or enlarged size of adipocytes is associated with diseases such as obesity and diabetes (4). Adipocyte differentiation (adipogenesis) is a complex process including the coordinated changes in hormone sensitivity and gene expression in response to various stimuli including lipid mediators. Prostaglandins (PGs)\textsuperscript{2} are a group of lipid mediators involved in the regulation of adipocyte differentiation. PGD\textsubscript{2} produced by lipocalin-type PGD synthase enhances adipocyte differentiation (5), whereas PGE\textsubscript{2} and PGF\textsubscript{2\alpha} suppress the differentiation through EP4 (6) and FP (7–10) receptors, respectively.

PGF\textsubscript{2\alpha} plays a variety of physiological functions in the body and is synthesized by the reduction of either the 9,11-endoperoxide moiety of PGH\textsubscript{2} or the 9-ketogroup of PGE\textsubscript{2} in an NAD(P)H-dependent manner, both of which reactions are catalyzed by aldo-keto reductases (AKRs) (11). PGF\textsubscript{2\alpha} synthase (PGFS; EC 1.1.1.188) was first isolated from mammals as an enzyme that catalyzes the reduction of PGH\textsubscript{2} to PGF\textsubscript{2\alpha} and PGD\textsubscript{2} to 9\(\alpha\),11\(\beta\)-PGF\textsubscript{2\alpha}, a stereoisomer of PGF\textsubscript{2\alpha} (11). PGF\textsubscript{2\alpha} synthase, which catalyzes the reduction of prostamide H\textsubscript{2} to prostamide F\textsubscript{2\alpha} in the mouse brain and belongs to the thiorodoxin-like superfamily was recently also found to convert PGH\textsubscript{2} to PGF\textsubscript{2\alpha} (12). PGF\textsubscript{2\alpha} binds to a specific cell surface G-protein-coupled receptor, the FP receptors (13, 14), which binding activates downstream signaling pathways including those responsible for activation of the \(G_{\text{q}}\) heterotrimeric G-protein, stimulation of phospholipase C to increase the intracellular calcium level, and activation of various kinases (13, 15–17).

AKRs are monomeric soluble oxidoreductases dependent on NAD(P)H; and they are found in various organisms, such as mammals, amphibians, plants, yeast, protozoa, and bacteria, in which they play important physiological roles (18). AKRs metabolize a variety of substrates including steroid hormones, monosaccharides, and PGs (19). Most AKRs are recognized as potential therapeutic targets of compounds developed with the desired specificity and clinical efficacy by means of structure-based drug designing. For example, aldose reductase inhibitors are useful as drugs for diabetes, because aldose reductase converts glucose into sorbitol, a hyperosmotic sugar, which plays an important role in diabetic retinopathy, neuropathy, and nephropathy (20). AKRs are classified into 14 families compris-

\textsuperscript{*}This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K. F. and K. A.) and by grants from the Research for Promoting Technological Seeds from Japan Science and Technology Agency, the Suzuken Memorial Foundation, the Sumitomo Foundation, the Gushinkai Foundation (to K. F.), and the Takeda Science Foundation (to K. F. and Y. U.).

\textsuperscript{i}The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

\textsuperscript{1}To whom correspondence should be addressed. Tel./Fax: 81-726-690-1055; E-mail: fujimori@gly.oups.ac.jp.

\textsuperscript{2}The abbreviations used are: PG, prostaglandin; PGFS, PGF\textsubscript{2\alpha} synthase; AKR, aldo-keto reductase; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer-binding protein; aP2, fatty acid-binding protein 4; SCD, stearoyl-CoA desaturase; COX, cyclooxygenase; pAb, polyclonal antibody; NC, negative control; siRNA, small interfering RNA.
AKR1B3 as PGFS in Adipocytes

Expression of Akr family and Pparγ genes in 3T3-L1 cells. A, Oil Red O staining of 3T3-L1 cells. 3T3-L1 cells were cultured with dexamethasone, 3-isobutyl-1-methylxanthine, and insulin for 2 days and then in medium with insulin alone for 6 more days. Undifferentiated (U) and differentiated (D) 3T3-L1 cells were stained with Oil Red O to visualize the accumulation of lipid droplet in the cells. Bar, 0.5 μm (Inset, 0.1 μm). B, Measurement of Oil Red O dye extracted from lipid droplet-laden cells. *, p < 0.01 as compared with undifferentiated cells. C, Expression level of Akr1b3, Cox-2, and Pparγ genes during differentiation of 3T3-L1 cells. Expression levels of each gene were measured by quantitative PCR. The data are presented as the mean ± S.D. from three independent experiments. Protein levels of Akr1b3, Cox-2, and Pparγ were detected during the adipogenesis of 3T3-L1 cells. *, p < 0.01 as compared with undifferentiated cells. D, Crude cell extracts (20 μg) were loaded in each lane in Western blot analysis. The PGF2α level was measured by enzyme immunoassay. Cells were cultured with A23187 (5 μM) for 10 min at 37°C after which the medium was collected to measure the PGF2α level. *, p < 0.01 as compared with undifferentiated cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse adipocytic 3T3-L1 cells were obtained from the Human Science Research Resources Bank (Osaka, Japan). Human embryonic kidney 293 cells were from Invitrogen. Both of these cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum and antibiotics, and maintained in a humified atmosphere of 5% CO2 at 37°C. Adipocyte differentiation of the 3T3-L1 cells was initiated by incubation for 2 days in Dulbecco’s modified Eagle’s medium containing insulin (10 μg/ml; Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). On day 2, the medium was replaced with Dulbecco’s modified Eagle’s medium containing insulin (10 μg/ml) alone and changed every 2 days. Oil Red O staining was carried out as described previously (5). Spectrophotometric measurement for Oil Red O staining was performed by dissolving the stained lipid droplets in isopropl alcohol, and then the absorbance was measured at 520 nm.

RNA Preparation and Quantification of RNA Level—Total RNA was extracted with Sepasol-RNAI (Nacalai Tesque, Kyoto, Japan), followed by further purification with an RNeasy Purification System (Qiagen, Hilden, Germany) (26). The first-strand cDNAs were synthesized from 1 μg of total RNA with random hexamer and ReverTra Ace Reverse Transcriptase (Toyobo, Osaka, Japan), followed by further purification with an RNeasy MinElute Columns (Qiagen, Valencia, CA). The cDNAs were diluted and further utilized as the templates for quantitative PCR analysis.

Expression levels were quantified by using a LightCycler system (Roche Diagnostics) with SYBR Green Realtime PCR Master Mix Plus (Toyobo) and primer sets (supplemental Table S1). The expression level of the target genes was estimated by the use of concentration known standard DNA and normalized to that of TATA-binding protein (TBP).

Suppression by RNA Interference—The following Stealth siRNA for Akrb3, 1b8, and 1b10 and Stealth negative control (NC) siRNA were obtained from Invitrogen: Akrb3 siRNA-1, 5′-CACAACGUCAGAAGACUCUGUAGAA-3′; Akrb3 siRNA-2, 5′-CAAGCCAGAGGACUCUGUAGAA-3′; Akrb3 siRNA-3, 5′-GCCACUAUCCUAGCCUCACACAGA-3′; Akrb10 siRNA-1, 5′-CACGUCCACUUGAAGACUCUGUAGAA-3′; Akrb10 siRNA-2, 5′-CAAGCGAGGAAGCGUGUAGAA-3′; Akrb10 siRNA-3, 5′-GAGACUUGUAACUGCUUGACUA-3′. For 2 days during their differentiation, 3T3-L1 cells were transfected with each siRNA or NC siRNA (20 nM) using TransIT TKO transfection reagent (Mirus Bio, Madison, WI). Transfec-
AKR1B3 as PGFS in Adipocytes

**FIGURE 2. Suppression of Akr1b family gene expression by siRNA.** A, siRNA-mediated suppression of Akr1b family gene expression. Preadipocytic 3T3-L1 cells were transfected with either of three siRNAs for each Akr1b family genes or NC (N.C). siRNA (Invitrogen). Transfection was performed every 2 days. Eight days after the initial transfection, cells were harvested for extraction of RNA. Each gene expression was then measured by quantitative PCR. The data are presented as the mean ± S.D. from three independent experiments. *, p < 0.01 as compared with NC siRNA. B, suppression of the AKR1B3 protein level by Akr1b3 siRNAs. 3T3-L1 cells were knocked down by each of three Akr1b3 siRNAs as described in A. Crude cell extracts (30 μg) were loaded in each lane, and protein levels of AKR1B3 and actin as an internal control were detected in Western blot analysis. C, PGF2α production was measured by enzyme immunoassay. Cells were transfected with each siRNA as described in A. At 8 days after the initial transfection, the medium was incubated for 5 min at 37 °C, after which the medium was collected to measure the PGF2α level. *, p < 0.01 as compared with NC siRNA. D, Oil Red O staining of lipid droplet in the cells. Bar, 0.5 μm (inset, 0.1 μm). E, measurement of Oil Red O dye extracted from lipid droplet-laden cells. *, p < 0.01 as compared with NC siRNA.

blots were incubated with anti-AKR1B3 polyclonal antibody (pAb; 1:5,000; provided from Dr. Antoine Martinez; Unite’ Mixte de Recherche, France), anti-cyclooxygenase-1 (COX-1) (1:200; C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-COX-2 (1:200; C-20; Santa Cruz Biotechnology), or anti-peroxisome proliferator-activated receptor (PPAR) γ (1:200; H-100; Santa Cruz Biotechnology) pAb and anti-β-actin monoclonal antibody (1:1000; AC-15; Sigma), and then incubated with anti-rabbit, anti-goat, or anti-mouse IgG antibody conjugated with horseradish peroxidase (GE Healthcare and Santa Cruz Biotechnology). Immunoreactive signals were detected by the use of an Immobilon Western Detection reagent (Millipore). Protein concentrations were measured with Pierce BCA Protein Assay reagent (Thermo Scientific, Rockford, IL).

**Incubation with an Agonist or Antagonist of FP Receptor**—3T3-L1 cells were incubated with Fluprostenol (5 nM; Cayman Chemical, Ann Arbor, MI), an FP receptor agonist or with AL-8810 (10 nM; Cayman Chemical), an FP receptor antagonist, for 2 days in the presence of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine. Then the agonist-treated cultures were incubated with the agonist and insulin for 6 more days; and the antagonist-treated cultures, with antagonist and insulin for 2 more days. Extraction and expression analysis of RNAs, and staining of lipid droplet with Oil Red O were carried out as described above.

**Measurement of PGF2α**—Production of PGF2α in adipocytes was measured by using PGF2α EIA Kit (Cayman Chemical) according to the manufacturer’s instruction.

**Statistical Analysis**—The data were presented as mean ± S.D. and statistically analyzed by the use of the unpaired t test or Welch t test when variances were heterogeneous. p values <0.05 were considered significant.

**RESULTS**

**Identification of Akr1b Family Genes in Adipocytes**—Mouse 3T3-L1 cells were caused to differentiate into adipocytes by treatment with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine. After 2 days, the medium was replaced to fresh medium with insulin only, and these cells were then cultured for a further 6 days, with a change in the medium every 2 days.
agonist Fluprostenol. 3T3-L1 cells were caused to differentiate for 8 days in the presence of Fluprostenol (5 nM).

The protein level of PPARγ was measured by quantitative PCR. The data are presented as the mean ± S.D. from three independent experiments. *, p < 0.01 as compared with undifferentiated cells. B, Oil Red O staining of 3T3-L1 cells treated with the FP receptor agonist Fluprostenol. 3T3-L1 cells were caused to differentiate for 8 days in the presence of Fluprostenol (5 nM). Lipid droplets in the cells were visualized by staining with Oil Red O. Bar, 0.5 μm (inset, 0.1 μm). C, Oil Red O was extracted from lipid droplet-laden cells and quantified by colorimetric intensity. *, p < 0.01 as compared with vehicle control. D, down-regulation of adipogenic gene expression in Fluprostenol-treated 3T3-L1 cells. 3T3-L1 cells were transfected with the Akr1b3 siRNA together with or without various concentrations of Fluprostenol. Transfection was carried out every 2 days and Fluprostenol was added for every transfection. At 8 days after the initial transfection, RNA was extracted, and the transcription level of adipogenic genes Pparγ, aP2, and Scd were measured by quantitative PCR. The data are presented as the mean ± S.D. from three independent experiments. #, p < 0.01 as compared with vehicle control; *, p < 0.01 and **, p < 0.05 as compared with vehicle control. The protein level of PPARγ was detected by the use of crude cell extracts (30 μg/lane, inset).

The accumulation of lipid droplets in the differentiated 3T3-L1 cells was observed by staining with Oil Red O and measured spectrophotometrically (Fig. 1, A and B).

Next, we examined the time courses of the changes in the expression profiles of Akr1b3, 1b8, 1b10, Pparγ, COX-1, and COX-2 mRNAs during differentiation of the cells. Expression of Akr1b3 mRNA was detected in preadipocytes and enhanced ~4-fold by 1 day after initiation of adipocyte differentiation, and then quickly decreased on the next day to a level lower than that of the preadipocytes and maintained its low level during their differentiation into adipocytes (Fig. 1C). The protein level of Akr1b3, examined by Western blot analysis, well resembled the mRNA expression (Fig. 1C). On the other hand, Akr1b8 and 1b10 mRNAs were expressed very weakly in preadipocytes at levels about 10- and 100-fold, respectively, lower than that of Akr1b3 (supplemental Fig. S1). When we measured the PGF2α level during adipogenesis, the PGF2α production after incubation of the cells with A23187 for 10 min rapidly increased to a peak at 3 h after initiation of adipogenesis and decreased to a level lower than that for the undifferentiated cells within 12 h (Fig. 1C). The production pattern of PGF2α resembled the changes in the COX-2 level (Fig. 1C), but not COX-1 (supplemental Fig. S1) during adipogenesis. On the contrary, the PPARγ mRNA level was low or negligible in preadipocytes and during the early phase of adipogenesis, but remarkably increased during its late phase, which was consistent with the protein level shown by Western blot analysis (Fig. 1C). These results indicate that Akr1b3 was abundantly expressed in preadipocytes and that its expression and PGF2α production were induced at the early phase of adipogenesis but decreased during adipogenesis, along with enhanced expression of Pparγ.

Suppression of Akr1B Enzymes by Their Specific siRNAs in Adipocytes—To investigate the effect of gene knockdown of members of the Akr1b family on adipocyte differentiation, we transfected 3T3-L1 cells with one of three siRNAs; i.e. 1, 2, or 3, for each of Akr1b3, 1b8, and 1b10, respectively. The mRNA levels of all three Akr1b enzymes were significantly decreased by more than 50% by their respective siRNAs as compared with their levels when treated with NC siRNA (Fig. 2A). Each siRNA for its Akr1B family member did not repress the expression of other Akr1b family genes (data not shown). Moreover, each of three Akr1b3 siRNAs suppressed the Akr1b3 protein level as compared with that of NC siRNA, although the actin level, an internal control, was not altered in all samples (Fig. 2B). Among them, we used the most suppressive siRNAs (Akr1b3-2, 1b8-3, and 1b10-3) for further study. The siRNA for Akr1b3, but not for Akr1b8 or 1b10 decreased PGF2α production by 3T3-L1 cells to about 38% of that with NC siRNA (Fig. 2C). Moreover, the lipid accumulation in Akr1b3 siRNA-transfected cells was clearly increased as compared with that in NC siRNA-transfected cells (Fig. 2, D and E).
AKR1B3 as PGFS in Adipocytes

Next, we measured the expression levels of adipogenic genes in 

Akr1b3 siRNA-transfected 3T3-L1 cells. The PPARγ mRNA level was increased ~2.4-fold by transfection with Akr1b3, but not Akr1b8 and 1b10, as compared with that of NC siRNA (Fig. 3), whose results were well consistent with the result of the PPARγ protein level obtained by Western blot analysis (Fig. 3, inset). Expression of adipogenic genes such as fatty acid-binding protein 4 (aP2) and stearoyl-CoA desaturase (SCD) was enhanced ~2.6- and 1.5-fold, respectively, only with the siRNA for Akr1b3, but not that for Akr1b8 or 1b10 (Fig. 3). Moreover, we obtained almost the same results when we used another Akr1b3 siRNAs, instead of Akr1b3-2 (data not shown). These results suggest that Akr1B3 was involved in suppression of adipogenic gene expression through production of PGF2α.

Expression of FP Receptor in Adipocytes—We next investigated expression of the FP receptor for PGF2α, during differentiation of 3T3-L1 cells from preadipocytes to adipocytes. FP receptor mRNA was found in preadipocytes and its expression was increased about 2-fold at 3 h after the initiation of adipocyte differentiation, maintained at almost the same level for 1 day, and then increased ~3-fold at 2 days after the initiation of adipocyte differentiation (Fig. 4A).

When 3T3-L1 cells were treated with Fluprostenol (5 nM), a stable PGF2α analog acting as an agonist for FP receptors (10), the accumulation of lipid droplet in the cells was almost completely suppressed as shown by Oil Red O staining (Fig. 4, B and C). Moreover, the expression of adipogenic genes such as Ppar, aP2, and SCD was decreased to about 17, 55, and 10%, respectively, by treatment of the differentiated cells with Fluprostenol (0.5 nM) (Fig. 4D and inset). To prove the involvement of Akr1B3-produced PGF2α in FP receptor-mediated suppression of adipogenesis, we measured the expression levels of adipogenic genes in 3T3-L1 cells that had been transfected with Akr1b3 siRNA and further differentiated for 8 days with or without Fluprostenol. siRNA-mediated suppression of Akr1B3 mRNA activated the transcription of Ppar, aP2, and SCD genes ~6.7-, 1.7-, and 1.6-fold, respectively, as compared with that in the cells transfected with NC siRNA (Fig. 4D and inset). This Akr1B3 siRNA-mediated enhancement of adipogenic gene expression was suppressed by adding 0.05–5 nM Fluprostenol in a concentration-dependent manner in Akr1b3-knockdown (PGF2α-decreased) cells (Fig. 4D and inset), indicating that Akr1B3-produced PGF2α suppressed the adipocyte differentiation via the FP receptor.

Enhancement of Adipogenesis in 3T3-L1 Cells by an FP Antagonist—AL-8810 is a selective antagonist for the FP receptor (27). When PGF2α-mediated FP receptor signaling was inhibited by AL-8810 (10 nM) in 3T3-L1 cells, the lipid accumulation was accelerated (Fig. 5, A and B). When the cells were treated with AL-8810 for 4 days, expression of PPARγ-target genes such as aP2 and SCD was enhanced about 1.5- and 1.2-fold, respectively, as compared with those of the vehicle control, although the PPARγ level was not clearly changed (Fig. 5C and inset). These results coincided well with the results showing that adipogenic gene expression was enhanced by siRNA-mediated suppression of the PGF2α-synthesizing enzyme, Akr1B3 (Fig. 3). These results, taken together, indicate that inhibition of PGF2α-mediated FP receptor signaling accelerated the adipogenesis of 3T3-L1 cells.

DISCUSSION

Here, we identified Akr1B3 as the PGFs in adipocytic 3T3-L1 cells. PGFs had originally been purified from bovine lung (11) and characterized to be a bifunctional protein carrying both activities of PGH2 9,11-endoperoxide reductase to produce PGF2α from PGH2 and PGD2 11-ketoreductase, producing 9α,11β-PGF2α from PGD2, in the presence of NADPH (28). Human 3α-hydroxysteroid dehydrogenase type 2, which is classified as AKR1C3, was then identified as a PGF (19) and its expression was demonstrated to be low in adipose tissue of obese subjects (29). However, the expression of Akr1C1B, a mouse homolog of human Akr1C3, was negligible in 3T3-L1 cells (data not shown). By using their purified recombinant proteins, we recently demonstrated that several members of the AKR1B family, such as human AKR1B1, mouse AKR1B3, and bovine AKR1B5, have PGFS activity higher than that of AKR1C3 (30). Among those members of the AKR1B family, AKR1B7 was demonstrated by Lambert-Langlais et al. (31) to be involved in the production of PGF2α in adrenal glands for inhibition of glucocorticoid secretion. Tirard et al. (32) previously reported that overexpression of Akr1b7 suppressed lipid accumulation in transfected 3T3-L1 cells. Those researchers reported that AKR1B7 was involved in the regulation of adipogenesis in 3T3-L1 cells, because they detected AKR1B7 expression in epididymal fat and demonstrated that overexpression of antisense AKR1B7 cDNA in 3T3-L1 cells accelerated adipogenesis (32). However, the expression level of Akr1b7 in 3T3-L1 cells was not shown.
cells was under the detection limit in our experimental condition (data not shown). The discrepancy between our and their studies may be due to the difference of 3T3-L1 cells maintained in both laboratories. Another possible reason is that the antisense RNA for Akr1b7 used in their study might also have inhibited other members of the AKR1B family including Akr1b3, because the Akr1b family shows high homologies (72% identity between Akr1b3 and Akr1b7 at the DNA level). Recently, prostamide/PGFS was identified as a novel PGFS with a turnover number much higher than that of AKR1B7 in vitro (12). However, the gene expression of prostamide/PGFS was not detected in 3T3-L1 cells (data not shown).

Akr1b3 expression was high in the preadipocytes and at the early phase of adipogenesis of 3T3-L1 cells, with a peak at day 1 after differentiation into adipocytes (Fig. 1C), a time course similar to the time course of the CCAAT/enhancer-binding proteins, C/ebpβ and C/ebpδ, critical transcriptional factors in the early phase of adipogenesis (33). Therefore, AKR1B3 is a novel marker of the early phase of adipogenesis. As summarized in Fig. 6, AKR1B3-produced PGF$_{2\alpha}$ suppresses adipocyte differentiation in the preadipocytes and the early phase of adipogenesis in 3T3-L1 cells by acting in an autocrine manner mediated by FP receptors to inhibit PPARγ activity. Suppression of adipogenesis by AKR1B3-mediated PGF$_{2\alpha}$ was also observed in adipocyte differentiation of mouse mesenchymal stem cells. On the contrary, in the mid and late phases of adipogenesis, AKR1B3 and COX-2 levels were decreased to reduce PGF$_{2\alpha}$ production and accelerate adipogenesis. However, the level of FP receptors was increased ~2-fold in the mid- and late-phase adipogenesis of 3T3-L1 cells (Fig. 4A), suggesting that PGF$_{2\alpha}$ suppressed adipogenesis in a paracrine manner at these phases of adipogenesis.

PG synthesis is coordinately regulated through coupling of terminal PG synthases with each or both COX-1 and COX-2 (34). Both COXs were expressed in the undifferentiated 3T3-L1 cells (Figs. 1C and supplemental S1), indicating that both COXs would probably have the ability to couple with AKR1B3 for PGF$_{2\alpha}$ production. In fact, functional expression of AKR1B3 with either of these COX enzymes in 293 cells (supplemental Fig. S2A) produced PGF$_{2\alpha}$ with preferential production in the combination of AKR1B3 with COX-1 and moderate production with COX-2 (supplemental Fig. S2B). This assay system has been well established to elucidate the functional coupling between COXs and various terminal PG synthases, such as hematopoietic PGD synthase, cytosolic PGE synthase, prostacyclin synthase, and thromboxane A$_2$ synthase (34). However, the production of PGF$_{2\alpha}$ in 293 cells after overexpression of exogenous AKR1B3 with either COXs (36–74 pg/ml, supplemental Fig. S2B) was markedly lower than that produced in 3T3-L1 cells (231 pg/ml, Fig. 2C), being consistent with the previous report that the ectopic expression of membrane-type PGFS with COX in 293 cells resulted in very low PGF$_{2\alpha}$ production (35). Moreover, in the heterologous expression system in 293 cells, the intracellular localization of the FLAG-tagged AKR1B3 protein did not change after an increase in the intracellular calcium level, being different from that of other cytosolic terminal PG synthases such as hematopoietic PGD synthase and cytosolic PGE synthase, both of which show membrane trafficking after the increase in the intracellular calcium level (36–38). Therefore, some AKR1B3-associated proteins that are abundant in 3T3-L1 cells, but not in 293 cells, may be involved in the efficient functional coupling of AKR1B3 to COX within cells. In further study, membrane trafficking of AKR1B3 should be analyzed, and the predicted AKR1B3-associated proteins have to be identified to elucidate the mechanism of PGF$_{2\alpha}$ production within cells. In the present study, we attempted to use anti-AKR1B3 pAb for immunofluorescence staining of AKR1B3 within 3T3-L1 cells. However, the pAb showed such high nonspecific binding to the cells that we could not use this pAb for histochemical analysis. Therefore, a novel specific antibody for AKR1B3 is needed to investigate the precise cellular localization of AKR1B3 and the immunoprecipitation of AKR1B3-associated proteins in 3T3-L1 cells.

As shown in this study, PGF$_{2\alpha}$ suppressed the adipocyte differentiation of 3T3-L1 cells by binding to the FP receptor, a specific cell surface G$_{\text{q}}$ heterotrimeric protein-coupled receptor (7–10). AKR1B3-mediated PGF$_{2\alpha}$ production was high in the early phase of adipogenesis and almost stopped in the differentiated adipocytes (Fig. 1C), whereas the expression of FP receptors was detected throughout adipogenesis (Fig. 4A); and the FP receptor antagonist AL-8810 accelerated the adipogenesis (Fig. 5, A and B). These results indicate that PGF$_{2\alpha}$ acts as an anti-adipogenic mediator even in the differentiated adipocytes through FP receptors. 3T3-L1 cells also produce PGE$_2$ with a time course similar to that of PGF$_{2\alpha}$ and at an ~10-fold response.

3 K. Fujimori, unpublished data.

4 K. Fujimori and K. Kashiwagi, unpublished data.
larger amount than that of PGF$_{2\alpha}$ during adipocyte differentiation, although the PGE$_2$ synthase has not yet been identified in adipocytes. PGE$_2$ has also been reported to suppress adipogenesis by inhibiting PPARγ function through EP4 receptors (6). Therefore, these results, taken together, suggest that the PGF$_{2\alpha}$-FP receptor (7–10) and PGE$_2$-EP4 receptor-mediated signalings cooperate to suppress adipogenesis in 3T3-L1 cells. On the other hand, as we have already demonstrated (5), 3T3-L1 cells also produce PGD$_2$ by the action of lipocalin-type PGD synthase; and the PGD$_2$ production is increased during adipogenesis. Further studies will be needed to elucidate the whole regulation mechanisms of adipogenesis mediated by PGF$_{2\alpha}$, PGE$_2$, and PGD$_2$ in adipocytes.

In summary, we identified AKR1B3 as the PGFS in adipocytes, whose expression was high in preadipocytes and in the early phase of adipocyte differentiation (until 1 day). AKR1B3-synthesized PGF$_{2\alpha}$ suppressed adipocyte differentiation through FP receptors by down-regulating adipogenic gene expression. Further characterization of the in vivo function of AKR1B3 and PGF$_{2\alpha}$ in adipocytes is essential to understand the development of metabolic disorders such as diabetes and obesity.

Acknowledgments—We acknowledge Dr. Makoto Murakami (Tokyo Metropolitan Institute for Medical Science, Japan) for kindly giving COX-1 and COX-2 expression vectors and Dr. Antoine Martinez (Unite’ Mixte de Recherche, France) for generously providing AKR1B3 pAb. We thank Masumi Sakata and Yumiko Hoshikawa (Osaka Bioscience Institute) for secretarial assistance.

REFERENCES

1. Kershaw, E. E., and Flier, J. S. (2004) J. Clin. Endocrinol. Metab. 89, 2548–2556
2. Spiegelman, B. M., and Flier, J. S. (2001) Cell 104, 531–543
3. Evans, R. M., Barish, G. D., and Wang, Y. X. (2004) Nat. Med. 10, 355–361
4. Berg, A. H., and Scherer, P. E. (2005) Circ. Res. 96, 939–949
5. Fujimori, K., Aritake, K., and Urade, Y. (2007) J. Biol. Chem. 282, 18458–18466
6. Tsuboi, H., Sugimoto, Y., Kainoh, T., and Ichikawa, A. (2004) Biochem. Biophys. Res. Commun. 322, 1066–1072
7. Casimir, D. A., Miller, C. W., and Ntambi, J. M. (1996) Differentiation 60, 203–210
8. Liu, L., and Clipstone, N. A. (2007) J. Cell. Biochem. 100, 161–173
9. Miller, C. W., Casimir, D. A., and Ntambi, J. M. (1996) Endocrinology 137, 5641–5650
10. Serrero, G., and Lepak, N. M. (1997) Biochem. Biophys. Res. Commun. 233, 200–202
11. Watanabe, K., Yoshida, R., Shimizu, T., and Hayashi, O. (1985) J. Biol. Chem. 260, 7035–7041
12. Moriuichi, H., Koda, N., Okuda-Ashitaka, E., Daiyasu, H., Ogasawara, K., Toh, H., Ito, S., Woodward, D. F., and Watanabe, K. (2008) J. Biol. Chem. 283, 792–801
13. Breyer, R. M., Bagdassarian, C. K., Myers, S. A., and Breyer, M. D. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 661–690
14. Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) Physiol. Rev. 79, 1193–1226
15. Fujino, H., and Regan, J. W. (2001) J. Biol. Chem. 276, 12489–12492
16. Pierce, K. L., Fujino, H., Srinivasan, D., and Regan, J. W. (1999) J. Biol. Chem. 274, 35944–35949
17. Watanabe, T., Nakao, A., Emerling, D., Hashimoto, Y., Tsukamoto, K., Horie, Y., Kinoshita, M., and Kurokawa, K. (1994) J. Biol. Chem. 269, 17619–17625
18. Penning, T. M., and Drury, J. E. (2007) Arch. Biochem. Biophys. 464, 241–250
19. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997) Biochem. J. 326, 625–636
20. Jez, J. M., Flynn, T. G., and Penning, T. M. (1997) Biochem. Pharmacol. 54, 639–647
21. Hayashi, O., Watanabe, K., Fujii, Y., Nakayama, K., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., and Nakanishi, S. (1988) Proc. Clin. Biol. Res. 274, 577–587
22. Watanabe, K., Fujii, Y., Nakayama, K., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S., and Hayashi, O. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 11–15
23. Kubata, B. K., Kabututu, Z., Nozaki, T., Munday, C. J., Fukuzumi, S., Ohkubo, K., Lazarus, M., Maruyama, T., Martin, S. K., Duszenko, M., and Urade, Y. (2002) J. Exp. Med. 196, 1241–1251
24. Kubata, B. K., Duszenko, M., Kabututu, Z., Rawer, M., Szallies, A., Fujimori, K., Inui, T., Nozaki, T., Yamashita, K., Horii, T., Urade, Y., and Hayashi, O. (2000) J. Exp. Med. 192, 1327–1338
25. Kabututu, Z., Manin, M., Pointud, J. C., Maruyama, T., Naga, N., Lambert, S., Lefrancçois-Martinez, A. M., Martinez, A., and Urade, Y. (2009) J. Biochem. 145, 161–168
26. Fujimori, K., Kadoyama, K., and Urade, Y. (2005) J. Biol. Chem. 280, 18452–18461
27. Griffin, B. W., Klimko, P., Crider, J. Y., and Sharif, N. A. (1999) J. Pharmaco- col. Exp. Ther. 290, 1278–1284
28. Watanabe, K., Iguchi, Y., Iguchi, S., Arai, Y., Hayashi, O., and Roberts, L. J., 2nd (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 1583–1587
29. Svensson, P. A., Gabrielson, B. G., Jernås, M., Gummesson, A., and Sjöholm, K. (2008) Cell Mol. Biol. Lett. 13, 599–613
30. Madore, E., Harvey, N., Parent, J., Chapdelaine, P., Arosh, J. A., and Fortier, M. A. (2003) J. Biol. Chem. 278, 11205–11212
31. Lambert-Langlais, S., Pointud, J. C., Lefrançois-Martinez, A. M., Volat, F., Manin, M., Couderé, F., Val, P., Sahut-Barnola, I., Ragazzon, B., Louiseit, E., Delarue, C., Lefebvre, H., Urade, Y., and Martinez, A. (2009) PLoS One 4, e7309
32. Tirard, J., Gout, J., Lefrançois-Martinez, A. M., Martinez, A., Begeot, M., and Naville, D. (2007) Endocrinology 148, 1996–2005
33. Letterova, M. I., and Lazar, M. A. (2009) Trends Endocrinol. Metab. 20, 107–114
34. Ueno, N., Takegoshi, Y., Kamei, D., Kudo, I., and Murakami, M. (2005) Biochem. Biophys. Res. Commun. 338, 70–76
35. Nakashima, K., Ueno, N., Kamei, D., Tanioka, T., Nakatani, Y., Murakami, M., and Kudo, I. (2003) Biochim. Biophys. Acta 1633, 96–105
36. Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fuseki, M., Ueno, A., Oh, S., and Kudo, I. (2000) J. Biol. Chem. 275, 32783–32792
37. Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M., and Kudo, I. (2000) J. Biol. Chem. 275, 32775–32782
38. Ueno, N., Murakami, M., Tanioka, T., Fujimori, K., Tanabe, T., Urade, Y., and Kudo, I. (2001) J. Biol. Chem. 276, 34918–34927