Expression of Peroxisome Proliferator-activated Receptor PPARδ Promotes Induction of PPARγ and Adipocyte Differentiation in 3T3C2 Fibroblasts*

Claire Bastie, Dorte Holst, Danielle Gaillard, Chantal Jehl-Pietri, and Paul A. Grimaldi‡

From the Centre de Biochimie, INSERM U470, Parc Valrose, UFR Sciences, Université de Nice-Sophia Antipolis, 06108 Nice Cedex 2, France.

Nutritional long chain fatty acids control adipose tissue mass by regulating the number and the size of adipocytes. The molecular mechanisms implicated in this action of fatty acids remain poorly understood. It has been well established that peroxisome proliferator-activated receptor (PPAR) γ, activated by specific prostanooids, plays a central role in the control of adipocyte gene expression and terminal differentiation. Thus far, the role of PPARδ in the control of adipose tissue mass has remained unclear. Herein, we report that a transcriptionally ecotopically expressed PPARδ on the control of adipose-related gene expression and adipogenesis of 3T3C2 fibroblasts. Treatment of PPARδ-expressing fibroblasts with fatty acids alone did not stimulate adipogenesis, whereas exposure of cells to a combination of fatty acids and PPARγ activators promoted lipid accumulation and expression of a typical adipocyte program. At the molecular level, activation of PPARδ by fatty acids induced transcription of the genes encoding fatty acid transporter, adipocyte lipid-binding protein, and PPARγ. Subsequent activation of PPARγ by specific agonists appeared to be required to promote terminal differentiation. These data demonstrate that PPARγ gene expression is under the control of PPARδ activated by fatty acids and could explain, at least partially, the adipogenic action of nutritional fatty acids.

Fatty acids have been suggested to regulate adaptation to nutritional changes. In vivo studies have illustrated the effects of high fat diets on obesity. In adult animals, high fat feeding leads to the appearance of new fat cells due to proliferation and differentiation of preadipocytes (1–3). We have demonstrated that long chain fatty acids (LCFAs)1 exert a potent adipogenic action in Ob1771 preadipose cells by increasing both the number of cells committed to differentiate and the level of expression of adipose-related genes (4). This adipogenic action of LCFAs was likely related to a direct effect of the molecule rather than increased substrate availability, as bromopalmitate, a non-metabolized derivative of palmitate (5), was found to be more active than native LCFAs. Furthermore, the adipogenic action of LCFAs is restricted to a critical period, corresponding to the preadipose state, whereas LCFA treatment of terminally differentiating cells is without effect (4).

The cellular effects of fatty acids and some of their metabolites are related, at least in part, to activation of transcription factors called PPARs that regulate the expression of genes directly implicated in lipid metabolism in various tissues including liver, muscle, and adipose tissue. PPARs exert their effects by binding to a specific responsive DNA element, called peroxisome proliferator responsive element after heterodimerization with retinoid X receptors (6–8). Three different PPAR subtypes have been described, and their differential distribution suggests that they have specific roles in different organs. PPARα, mainly expressed in liver and brown adipose tissue, plays an important role in fatty acid catabolism (9). PPARγ, predominantly expressed in adipose cells, in combination with other transcription factors plays a crucial role in activation of genes of the adipose differentiation program and adipogenesis (10). PPARδ displays a high level of expression in lipid-mobilizing tissues, such as adipose tissue, small intestine, heart, and skeletal muscle and could regulate the expression of genes implicated in fatty acid uptake and activation. This is supported by transfection experiments demonstrating that its ectopic expression in fibroblasts confers fatty acid responsiveness to FAT, ALBP, and acyl-CoA synthetase genes (11). Naturally occurring and synthetic molecules that are ligands for these nuclear receptors control transcriptional activity of PPARs. Some compounds, such as carbacyclin, do not show any isoform specificity (12–14), whereas other compounds are more strictly isoform-specific. Thiazolidinediones and 15-deoxy-Δ12-14-prostaglandin J2 (15d-PGJ2) have been identified as specific PPARγ ligands and activators (15, 16). Peroxisome proliferators, such as fibrates, selectively activate PPARα (17). PPARδ is activated by LCFA Complex, including non-metabolized analogs such as bromopalmitate (11).

PPARδ and γ isoforms are up-regulated during adipose differentiation with distinct time courses. PPARδ is expressed during the initial steps of the differentiation process, whereas PPARγ is expressed during terminal differentiation (11, 18). Expression of PPARγ promotes adipogenesis in NIH-3T3 fibroblasts exposed to strong specific activators (19), whereas expression of PPARδ and its activation by fatty acids is not sufficient to induce lipid accumulation in the same cells (12). However, circumstantial evidence argues against the sole and direct involvement of PPARγ in the adipogenic action of fatty acids and favors a role for PPARδ. First, it has been shown that PPARγ is not directly activated by fatty acids (20, 21), whereas LCFA's are strong activators of PPARδ (11, 14). Second, PPARγ is expressed at very low levels in preadipose cells, i.e. during

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Centre de Biochimie, INSERM U470, Parc Valrose, UFR Sciences, Université de Nice-Sophia Antipolis, 06108 Nice Cedex 2, France. Tel.: 33 492 07 64 34; Fax: 33 492 07 64 02; E-mail: grimaldi@talao.unice.fr.

1 The abbreviations used are: LCFA, long chain fatty acid; ALBP, adipocyte lipid binding protein; C/EBP, CCAAT/enhancer binding protein; cPGI2, carbacyclin; 15d-PGJ2, 15-deoxy-Δ12-14-prostaglandin J2; FAT, fatty acid transporter; GAPDH, glyceraldehyde phosphate dehydrogenase; PPAR, peroxisome proliferator-activated receptor; SDM, standard differentiation medium.

21920 This paper is available on line at http://www.jbc.org
the critical period when LCFA's exert their adipogenic action (4, 11). Finally, fatty acids promote adipose conversion of calvaria-derived osteoblasts or osteoma clonal cells that express PPARδ but not PPARα and PPARγ (22).

To delineate more precisely the potential role of PPARδ in the control of fatty acid-induced adipogenesis, we have forced its expression in 3T3C2 fibroblasts, which are normally totally refractory to adipose differentiation (23), and we examined their response to LCFA's and various PPAR activators used alone or in combination. We found that expression of PPARδ in fibroblasts is capable of promoting adipose differentiation in response to treatment by combination of PPARδ and PPARγ activators. Investigation at the molecular level has revealed that activation by LCFA's of ectopically expressed PPARδ leads to induction of endogenous PPARδ and that, in turn, activation of the latter by its specific agonists promotes adipogenesis.

EXPERIMENTAL PROCEDURES

Plasmids—The retroviral construct containing PPARδ cDNA was derived from pS05-FAAR (11) and cloned into the BamHI site of pBizeoneo (Dr. K. Kristiansen, University of Odense, Denmark).

Cell Culture—Cells were grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum, 200 units/ml penicillin, and 50 µg/ml streptomycin (standard medium). For differentiation, cells were shifted to standard medium supplemented with 1 nM insulin and 1 nM triiodothyronine (standard differentiation medium). For experiments in serum-free medium, cells were first inoculated in standard medium at a density of 10^5 cells/cm^2 and washed 24 h later with Dulbecco's modified Eagle's medium/Ham's F12 (50:50). Cells were grown to confluence in 4-F medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 supplemented with insulin (5 µg/ml), transferrin (10 µg/ml), fetuin (0.5 µg/ml), and fibroblast growth factor (25 ng/ml). At confluence, cells were shifted to the same medium without fibroblast growth factor and supplemented with growth hormone (2 nM) and triiodothyronine (0.2 nM) (referred to as 5F medium). Medium was changed every other day.

Stable Cell Lines—BOSC23 cells were transduced at 50–70% confluence by lipofection (DOTAP, Roche Molecular Biochemicals) with 1 µg of pBizeoneo or pBizeoneoPPARδ expression vector. After 8 h, cells were refed with fresh standard medium, and viral supernatants were collected 48 h later. 3T3C2 cells maintained in standard medium were infected with equal titers of recombinant virus for 6 h. Cells were maintained for 48 h in fresh medium and then replated with a 1:10 dilution in standard medium containing 0.4 mg/ml Geneticin. Stable cell populations were obtained after 7–10 days of selection. After confluence, cells were maintained in standard differentiation medium with or without various inducers. Fatty acids, bromopalmitate, and BRL 49653 were dissolved in Me2SO, carbacyclin in ethanol, and 15d-PGJ2 in ethyl acetate. Red Oil O staining was performed as described previously (23). Total RNA was prepared from virally infected 3T3C2 fibroblasts by SDS gels and blotted to nitrocellulose membranes. PPARδ and GAPDH probes.

Retroviral Infection—To explore the potential action of PPARδ in promoting fatty acid responsiveness, the mouse PPARδ coding sequence was expressed in Swiss 3T3C2 fibroblasts by retroviral infection using the pBizeoneo vector. This vector contains an internal ribosome entry site from the encephalomyocarditis virus that allows transcription of a same RNA transcript encoding both PPARδ and neomycin gene products. Transcription of this bicistronic RNA is driven from the Moloney murine leukemia virus long term repeat. As shown in Fig. 1A, 3T3C2Biz, i.e., infected with the empty original pBizeoneo vector, expressed a low, but significant, level of PPARδ mRNA at the expected size of 3.5 kilobase pairs. In two independent 3T3C2BizPPARδ cell populations, i.e., infected with the retroviral vector containing the PPARδ-coding sequence, the viral transcript (about 5 kilobase pairs) is considerably better expressed than the endogenous PPARδ mRNA. Western blot analysis shown in Fig. 1B revealed a faint PPARδ signal in nuclear extracts from the control 3T3C2Biz cells. The signals detected in nuclear extracts from two independent retrovirally infected 3T3C2BizPPARδ cell populations were considerably more intense and nearly similar to that detected in nuclear extract from differentiated Ob1771 cells. Further experiments showed that the amount of PPARδ protein remains unchanged in 3T3C2BizPPARδ cells for 1 week after confluence whatever the culture condition (not shown).

Expression of PPARδ Confers Fatty Acid Responsiveness to 3T3C2 Fibroblasts—To confirm that the PPARδ protein expressed in 3T3C2BizPPARδ cells is functional, we investigated the response of these cells to short term exposure to various compounds described as PPAR agonists. 3T3C2BizPPARδ and 3T3C2Biz cells were grown to confluence and exposed for 24 h to the various activators either in the presence (Fig. 2A and B) or absence (Fig. 2C) of serum.

As shown in Fig. 2A, 3T3C2BizPPARδ cells maintained in standard medium do not express FAT or ALBP mRNA. Exposure to the thiazolidinedione BRL 49653, a selective PPARγ agonist, to clofibrate or Wy 14,643, selective PPARα agonist, or to bromocristane, a middle chain fatty acid, are without effect on FAT and ALBP gene expression. By contrast, treatments with long chain fatty acids, such as palmitate, oleate, or bromopalmitate, induce a strong expression of FAT and ALBP genes. The effects of long chain fatty acids are confined to PPARδ-expressing cells as 3T3C2Biz cells remain insensitive to the above treatments.
Adipogenic Potential of PPARδ

Fig. 2. Activation of FAT mRNA expression by fatty acids and cPGI2 in 3T3C2BizPPARδ cells. 3T3C2BizPPARδ and 3T3C2Biz cells were grown to confluence in standard medium and then exposed for 24 h to the indicated compounds. Total RNA was extracted and analyzed by Northern blot. GAPDH mRNA was monitored as internal standard. Results are representative of three independent experiments.

Fig. 3. Exposure to PPARδ and PPARγ activators promotes adipogenesis in 3T3C2BizPPARδ cells. 3T3C2BizPPARδ and 3T3C2Biz cells were maintained from confluence to day 8 post-confluence in SDM with or without 1 μM BRL 49653 and treated or not from day 0 to day 4 by 100 μM α-linolenate or 25 μM bromopalmitate. At day 8, cells were fixed and stained with Oil Red O as indicated under “Experimental Procedures.”

to such treatments (Fig. 2A, lower panel) and are dose-dependent in 3T3C2BizPPARδ cells as illustrated in Fig. 2B. Bromopalmitate, a non-metabolized LCFA, is more active than linolenate. To investigate further the selectivity of the process of FAT mRNA induction by PPARδ activation, 3T3C2BizPPARδ cells were maintained in serum-free medium with or without the PPAR pan-activator cPGI2 or a specific PPARγ ligand, 15d-PGJ2. As shown in Fig. 2C, cPGI2 activates FAT mRNA expression at low concentrations (73% of maximal effect for 30 nM), whereas 15d-PGJ2 is completely inactive whatever the concentration used.

Taken together, these data strongly suggest that 3T3C2BizPPARδ cells are responsive to compounds already described as PPARδ activators, whereas selective activators of either PPARα or PPARγ are without effect.

Adipose Differentiation of 3T3C2BizPPARδ Cells—To examine whether or not ectopic expression of PPARδ promoted lipid accumulation in fibroblasts, 3T3C2BizPPARδ and 3T3C2Biz cells were cultured after confluence in standard differentiation medium supplemented with various PPAR activators. After 8 days, cells were fixed and stained for accumulated lipid with Oil Red O. As expected, no lipid accumulation was detected in control 3T3C2Biz cells regardless of the activator or combination of activators used (Fig. 3, lower panel). A similar lack of differentiation was found for 3T3C2BizPPARδ cells maintained in control medium with or without 1 μM BRL 49653. Exposure of post-confluent 3T3C2 cells to bromopalmitate or linolenate resulted in cell death after day 5. However, treatment from day 0 to day 4 post-confluence was not toxic and led to a very faint staining due to limited lipid accumulation. A strong staining was observed in cells exposed to a mixture of linolenate or bromopalmitate (for the 1st 4 days) and 1 μM BRL 49653.

Microscopic examination of 3T3C2BizPPARδ cells confirmed these observations as no lipid accumulation was seen in cells maintained in standard differentiation medium (SDM) or treated with BRL 49653 alone (Fig. 4A). Exposure to α-linolenate or bromopalmitate failed to promote lipid droplet appearance except in less than 1% of the cells. Treatment of the cells with a combination of LCFA (days 0–4) and BRL 49653 (days 0–8) led to a classical morphology of cultured adipocytes. The effects of prostaglandins activating both PPARδ and PPARγ, i.e. cPGI2, or specifically PPARγ, i.e. 15d-PGJ2, have been studied in cells maintained in serum-free medium already used to study the control of Ob1771 differentiation by prostaglandins (27). As shown in Fig. 4B, 3T3C2BizPPARδ cells did not accumulate lipids when maintained for 8 days post-confluence in 5% medium. Chronic exposure to cPGI2 promoted lipid accumulation in about 50% of the cells, whereas treatment with 15d-PGJ2 failed to promote adipogenesis. 3T3C2Biz did not accumulate lipid under all culture conditions tested (not shown).

To confirm that lipid accumulation occurring in 3T3C2BizPPARδ cells in certain conditions was related to an actual differentiation process, GPDH activity, an indicator of terminal differentiation, was determined (Fig. 5A). No GPDH activity was detected in cells maintained for 8 days post-confluence in...
Chronic treatment with BRL 49653 was not required since cells were maintained for 8 days post-confluence in SDM or serum-free medium as described under "Experimental Procedures." Cells were maintained for 8 days post-confluence in SDM supplemented with 1 μM BRL 49653 without any further treatment (lane A) or exposed from day 0 to day 4 to 100 μM clofibrate (lane B), 100 μM α-linolenate (lane C), or 25 μM bromopalmitate (lane D). RNA was prepared and was analyzed by Northern blot (20 μg of total RNA per lane). After normalization to GAPDH signals, results are expressed by taking the maximal value obtained for each probe as 100.

5F medium. Chronic exposure to cPGI2 resulted in a dose-dependent activation of GPDH expression. This adipogenic action of cPGI2 took place at very low concentrations and reached a maximum of two (A) or four (B) separate experiments.

FIG. 4. Morphological differentiation of 3T3C2BizPPARδ cells in serum-supplemented (A) and serum-free medium (B). Cells were maintained for 8 days post-confluence in SDM or serum-free medium as described under "Experimental Procedures." A, cells were maintained with or without BRL 49653 and treated or not from day 0 to day 4 by α-linolenate or bromopalmitate. B, cells were maintained for 8 days after confluence in 5F medium with or without either cPGI2 or 15d-PGJ2. Magnification is × 60.

FIG. 5. GPDH activity is induced in 3T3C2BizPPARδ cells by PPARδ and PPARγ activators. A, cells were grown in serum-free medium and exposed from confluence to day 8 to increasing concentrations of cPGI2 (●), PGD2 (○) or 15d-PGJ2 (■). B, cells were shifted in SDM at confluence, and GPDH activity was determined at day 8. Conditions are as follows: a, control medium; b, 1 μM BRL 49653 days 0–8; c, 1 μM BRL 49653 days 0–4; d, 1 μM BRL 49653 days 4–8; e, 25 μM bromopalmitate days 0–4; f, 25 μM bromopalmitate days 4–8; g, 25 μM bromopalmitate days 0–4 and 1 μM BRL 49653 days 4–8; h, 25 μM bromopalmitate days 0–4 and 1 μM BRL 49653 days 0–8; i, 1 μM BRL 49653 days 0–4 and 25 μM bromopalmitate days 4–8. 100 corresponds to 1250 milliunits (mU) of GPDH activity. These results are representative of two (A) and four (B) separate experiments.

FIG. 6. Effects of various PPAR activators on gene expression in 3T3C2BizPPARδ cells. A, 3T3C2BizPPARδ cells were maintained from confluence to day 8 in SDM in the absence or presence of 1 μM BRL 49653 without any further treatment (lane A) or exposed from day 0 to day 4 to 100 μM clofibrate (lane B), 100 μM α-linolenate (lane C), or 25 μM bromopalmitate (lane D). RNA was prepared and was analyzed by Northern blot (20 μg of total RNA per lane). B, after confluence cells were cultured in SDM in the presence of 1 μM BRL 49653 and exposed from day 0 to day 4 to increasing concentrations of bromopalmitate. RNA was prepared and was analyzed by Northern blot (20 μg of total RNA per lane). After normalization to GAPDH signals, results are expressed by taking the maximal value obtained for each probe as 100. •, PPARγ mRNA; ■, ALBP mRNA; △, GPDH mRNA; ○, 3T3C2BizPPARδ cells; □, □, □, 3T3C2Biz cells. Results are representative of three separate experiments.

Adipogenic Potential of PPARδ 21923

Altogether, these observations indicated that ectopic PPARδ expression promotes adipogenesis in fibroblasts, but this requires exposure to both PPARδ and PPARγ activators, whereas treatments with activators of either PPARδ or PPARγ are not sufficient to induce GPDH expression and lipid accumulation. These results also suggest that ectopic expression of PPARδ in 3T3C2 fibroblasts and its activation by fatty acids confer responsiveness to thiazolidinedione treatment, which in turn induces both GPDH expression and lipid accumulation.

Activation of PPARδ by Fatty Acids Leads to PPARγ Gene Expression—To characterize the differentiation phenotype of the cells at the molecular level, the level of expression of genes encoding adipose markers, including PPARγ, was analyzed by Northern blot for cells maintained for 8 days post-confluence in various conditions (Fig. 6A). Cells maintained in SDM, or treated with 1 μM BRL 49653, 100 μM clofibrate, or a combination of both compounds did not express detectable amounts of any mRNA related to adipose differentiation. Cells exposed for 4 days from confluence to either 25 μM bromopalmitate or 100 μM α-linolenate expressed relatively high signal for PPARγ mRNA and weak signals for ALBP and FAT mRNA. In these cells, GPDH mRNA remained undetectable. In good agreement with the previous data of this study, cells chronically treated with BRL 49653 and exposed for 4 days post-confluence to bromopalmitate or linolenate express a high level of the complete panel of adipose markers including GPDH. In such conditions, the levels of FAT and ALBP mRNA expression were found to be similar to those of differentiated Ob1771 cells, whereas those of GPDH and PPARγ mRNA were 50 and 70%, respectively, of the signals found in differentiated cells.

The dose dependence of bromopalmitate adipogenic action was next examined. For that purpose, cells were maintained for 8 days post-confluence in SDM supplemented with 1 μM BRL 49653 and exposed from confluence to day 4 to various concentrations of bromopalmitate (Fig. 6B). Northern blot analysis revealed that the bromopalmitate effect on the expression of mRNA for PPARγ, ALBP, and GPDH took place between 5 and
50 μM. Adipose marker mRNAs remained undetectable in 3T3C2-Biz cells under all culture conditions.

The time course of adipose-related mRNAs was next examined in 3T3C2BizPPARδ cells exposed to bromopalmitate for the 1st 4 days in the presence (Fig. 7A) or absence (Fig. 7B) of BRL 49653. PPARγ mRNA emerged at day 4 and accumulated thereafter to reach a maximum at day 8 in cells exposed to BRL 49653. The treatment with thiazolidinedione was not strictly required for PPARγ gene expression but led to an increased RNA level as cells maintained in the absence of BRL 49653 expressed about 60% of the maximal level observed in treated cells. The expression of GPDH mRNA appeared to be strictly dependent on exposure to the thiazolidinedione since no expression was detected in cells treated only with the fatty acid. In cells maintained in conditions permissive for terminal differentiation, GPDH induction occurred very late. The FAT gene expression pattern was found to be different as this mRNA emerged early after confluence and reached a maximal expression after 3 days of bromopalmitate treatment in cells maintained with or without BRL 49653. In both culture conditions, withdrawal of the fatty acid resulted in a rapid down-regulation of FAT mRNA that completely disappeared in cells maintained in SDM and was lately re-induced in cells exposed to BRL 49653.

**DISCUSSION**

The knowledge of mechanisms implicated in the adaptation of adipose tissue to high fat feeding remains a critical issue. It is now established that PPARγ when activated by naturally occurring molecules, such as 15d-PGJ2, or drugs, such as thiazolidinediones, plays a crucial role in adipogenesis (10, 28, 29). The role of PPARδ, that is expressed at relatively high levels in adipose tissue and emerges early during adipocyte differentiation, had remained unclear. The findings of the present work support the idea that PPARδ could act as an early player in the induction by fatty acids of adipose cell commitment to differentiation.

It has already been demonstrated that forced expression of PPARδ in fibroblasts confers fatty acid responsiveness to ALBP and FAT genes (11). Consistent with these data, 3T3C2Biz-PPARδ cells, which express the nuclear receptor to levels closed to those found in differentiated adipocytes (Fig. 1), display a similar response to LCFAs. This biological response appeared to be primarily due to PPARδ activation as specific activators of either PPARγ, such as 15d-PGJ2 or BRL 49653, or PPARδ, such as fibrates, are inactive (Fig. 2A and C). Interestingly, FAT gene up-regulation occurred within low ranges of concentrations of bromopalmitate and higher concentrations of linolenate. In this respect, 3T3C2BizPPARδ cells are similar to Ob1771 preadipose cells in which it has been demonstrated that FAT and ALBP genes are rapidly induced by treatment with long chain fatty acids (30–32) and that bromopalmitate exert more potent effects than native fatty acids (Ref. 5 and Fig. 2B).

The main goal of this work was to investigate the role of PPARδ on adipogenesis. It has been shown that PPARδ-expressing NIH3T3 fibroblasts do not undergo adipose differentiation upon treatment with bromopalmitate alone (12). Our findings are not contradictory with these data, as exposure of 3T3C2BizPPARδ cells to linolenate or bromopalmitate alone does not promote lipid accumulation or GPDH induction (Figs. 3 and 4). Not surprisingly, in the absence of PPARγ expression (Fig. 6), exposure of the cells to specific activators of PPARγ alone also failed to induce terminal differentiation. However, several lines of evidence indicate that activation of both PPARδ and PPARγ promote adipogenesis and expression of a typical adipose differentiation program in 3T3BizPPARδ cells. This terminal differentiation can be attained by exposure of the cells to low concentrations of cPGI2, a strong PPAR pan-activator, whereas 15d-PGJ2 and its precursor PGD2 were ineffective. Notably, a similar response has been observed for Ob1771 preadipose cells in which cPGI2 exerted adiogenic action in the same range of concentrations, whereas 15d-PGJ2 and PGD2 were inactive (33).

The relationship between PPARδ and PPARγ was shown, as exposure of 3T3C2BizPPARδ cells to either linolenate or bromopalmitate is sufficient to induce expression of the PPARγ gene. In sharp contrast, terminal differentiation requires PPARγ gene expression, *i.e.* as a result of PPARδ activation, and its subsequent activation by specific ligands such as thiazolidinediones. A similar strict requirement for PPARγ activators was already reported for terminal differentiation of PPARγ-expressing fibroblasts (19). These observations illustrate a major difference between PPAR-expressing fibroblasts and actual preadipocytes that undergo terminal differentiation when maintained in standard medium suggesting that preadipocytes synthesize and accumulate natural PPARγ activators.

PPARδ activation leads to FAT, ALBP, and PPARγ gene expression in 3T3C2BizPPARδ cells. However, time courses of FAT and PPARγ induction appeared different. FAT gene is rapidly induced during fatty acid treatment of 3T3C2BizPPARδ cells, is down-regulated after fatty acid withdrawal, and increased thereafter in cells exposed to thiazolidinedione (Figs. 2 and 7). From these findings it could be proposed that the first wave of FAT expression is under the control of PPARδ activated by fatty acids, whereas the second wave, which occurs only in cells exposed to thiazolidinedione, is under the control of endogenous ligand activated-PPARγ. A similar pattern of expression was observed for the ALBP gene (not shown). These transcriptional regulations probably imply association of PPARδ and PPARγ, respectively, activated by fatty acid and thiazolidinedione, to the PPAR-responsive elements identified in ALBP (34) and FAT (35) gene promoters. The expression

**FIG. 7. Time course of adipose marker expression in 3T3C2BizPPARδ cells.** Cells were maintained from confluence in SDM in the presence (A) or absence (B) of 1 μM BRL 49653 and treated with 25 μM bromopalmitate from day 0 to day 4. RNA was prepared at various days and analyzed by Northern blot. After standardization to GAPDH mRNA, results are presented by taking the maximal value obtained for each probe as 100 and are representative of three separate experiments. □, △, FAT mRNA; ○, □, PPARγ mRNA; ●, ○, GPDH mRNA.
profile of PPARγ in response to PPARδ activation is clearly different. PPARγ mRNA emerged only after 3 or 4 days of fatty acid treatment and accumulated after fatty acid withdrawal, suggesting that transcription of the PPARγ gene does not require permanent activation of PPARδ. Taken together, these data do not favor a direct action of PPARδ on the PPARγ promoter and are more suggestive of an unidentified indirect mechanism. Molecular mechanisms regulating transcription of the PPARγ gene have been documented. It is clearly established that the gene is up-regulated during adipose differentiation and that C/EBPβ play an important role in such process (29). Expression of C/EBPβ and δ into fibroblasts induces PPARγ levels seen in adipocytes (36, 37). A direct modulation of PPARγ2 promoter activity by C/EBPα and C/EBPδ was demonstrated by co-transfection studies, and two functional C/EBP recognition elements were identified in the mouse PPARγ2 promoter (38). These observations and the findings of the present study illustrate the multiplicity of mechanisms underlying PPARγ gene expression. Such redundancy is not surprising since this transcription factor is a major actor in the regulation of the adipose tissue mass.

Another feature of this paper is the demonstration that PPARδ and PPARγ play different roles in the regulation of adipose differentiation. Our data suggest that fatty acids selectively activate PPARδ leading to induction of certain adipose-related genes, such as FAT and PPARγ, but not to terminal differentiation that is under the control of ligand-activated PPARγ. From this model, it can be expected that selective PPARδ agonists should not act as insulin-sensitizing agents in obese diabetic animals since the high level of circulating fatty acids of such animals should maximally activate PPARγ.

In conclusion, PPARδ appears to be the primary target of LCFAs and controls PPARγ gene expression. This nuclear receptor could act as an early actor in the increase of fat cell number occurring during high fat feeding and pathological states characterized by high concentrations of circulating fatty acids.

Acknowledgments—We thank Ellen Van Obbergen-Schilling, Barry Rosen, and Gérard Ailhaud for critical comments and review of the manuscript.

REFERENCES

1. Faust, I. M., Johnson, P. R., Stern, J. S., and Hirsch, J. (1978) Am. J. Physiol. 235, E279–E286
2. Klyde, J. B., and Hirsch, J. (1979) J. Lipid Res. 20, 705–715
3. Shillabeer, G., and Lau, D. C. (1994) J. Lipid Res. 35, 592–600
4. Amri, E. Z., Ailhaud, G., and Grimaldi, P. A. (1994) J. Lipid Res. 35, 938–937
5. Grimaldi, P. A., Knobel, S. M., Whitesell, R. R., and Abumrad, N. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10930–10934
6. Green, S., and Wahli, W. (1994) Mol. Cell. Endocrinol. 100, 149–153
7. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 841–850
8. Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996) Endocrinology 137, 354–366
9. Lee, S. S. T., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salgues, P. M., Wespahel, H., and Gonzalez, F. J. (1995) Mol. Cell. Biol. 15, 3012–3022
10. Spiegelman, B. M., and Flier, J. S. (1996) Cell 87, 377–389
11. Amri, E. Z., Bonino, F., Ailhaud, G., Abumrad, N. A., and Grimaldi, P. A. (1995) J. Biol. Chem. 270, 2367–2371
12. Brun, R. P., Tontonoz, P., Forman, B. M., Ellis, R., Chen, J., Evans, R. M., and Spiegelman, B. M. (1996) Genes Dev. 10, 974–984
13. Hertz, R., Berman, I., Keppler, D., and Bar-Tana, J. (1996) Eur. J. Biochem. 235, 242–247
14. Forman, B. M., Chen, J., and Evans, R. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4312–4317
15. Lehman, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkinson, W. O., Willson, T. M., and Kliever, S. A. (1995) J. Biol. Chem. 270, 12953–12956
16. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Cell 83, 803–812
17. Göttlicher, M., Widmark, E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4653–4657
18. Chawla, A., Schwarz, E. J., Dimaculangan, D. D., and Lazar, M. A. (1994) Endocrinology 135, 798–200
19. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156
20. Kliever, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangendorf, D. J., Umesono, K., and Evans, R. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7355–7359
21. Nagey, L., Tontonoz, P., Alvarez, J. C. A., Chen, H., and Evans, R. M. (1998) Cell 93, 229–240
22. Diascro, D. D., Vogel, R. L., Johnson, T. E., Whirtherup, K. M., Fitzenberger, S. M., Rutledge, S. J., Prescott, D. J., Rodan, G. A., and Schmidt, A. (1998) J. Bone Miner. Res. 13, 96–106
23. Green, H., and Ichinose, O. (1974) Cell 1, 113–116
24. Grimaldi, P., Dijian, P., Negrel, R., and Ailhaud, G. (1982 EMBO J. 1, 687–692
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
26. Dignam, J. D., Lebovitz, and R. M., Roeder, R. G. (1983) Nucleic Acids Res. 11, 1575–1579
27. Guillard, D., Negrel, R., Lagarde, M., and Ailhaud, G. (1989) Biochem. J. 257, 389–397
28. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) Genes Dev. 8, 1224–1254
29. Mandrup, S., and Lane, M. D. (1997) J. Biol. Chem. 272, 5367–5370
30. Amri, E. Z., Bertrand, B., Ailhaud, G., and Grimaldi, P. (1991) J. Lipid Res. 32, 1449–1456
31. Amri, E. Z., Ailhaud, G., and Grimaldi, P. (1991) J. Lipid Res. 32, 1457–1463
32. Sfeir, Z., Ibrahimi, A., Amri, E. Z., Grimaldi, P., and Abumrad, N. A. (1997) Prostaglandins Leukot. Essent. Fatty Acids 57, 17–21
33. Ailhaud, G. (1999) Clin. Chim. Acta, in press
34. Ross, S. R., Graves, R. A., Greenstein, A., Platt, K. A., Shyu, H. L., Mellovit, B., and Spiegelman, B. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9590–9594
35. Tontonoz, P., Nagy, L., Alvarez, J. G. A., Thomazy, V. A., and Evans, R. M. (1998) Cell 93, 241–252
36. Wu, Z., Xie, Y., Bucher, N. L. R., and Farmer, S. R. (1995) Genes Dev. 9, 2350–2356
37. Wu, Z., Bucher, N. L. R., and Farmer, S. R. (1996) Mol. Cell. Biol. 16, 4128–4136
38. Clarke, S. L., Robinson, E. C., and Gimble, J. M. (1997) Biochem. Biophys. Res. Commun. 240, 99–103
39. Berger, J., Liebowitz, M. D., Doebber, T. W., Elbrecht, A., Zhang, B., Zhou, G., Biswas, C., Cullinan, C. A., Hayes, N. S., Li, Y., Tanen, M., Venter, J., Wu, M. S., Berger, G. D., Mosley, R., Marquis, R., Santini, C., Sahon, S. P., Tolman, R. L., Slith, R. G., and Moller, D. E. (1999) J. Biol. Chem. 274, 6718–6725