Peroxisome-proliferator-activated receptor δ mediates the effects of long-chain fatty acids on post-confluent cell proliferation

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Nutritional long-chain fatty acids control adipose tissue mass by regulating the number and the size of adipocytes. It is now established that peroxisome-proliferator-activated receptors (PPARs) play crucial functions in the control of gene expression and the level of cell differentiation. PPARγ, which is activated by specific prostanoids, is a key factor in activating terminal differentiation and adipogenesis. We have recently demonstrated that PPARδ, once activated by fatty acids, drives the expression of a limited set of genes, including that encoding PPARγ, thereby inducing adipose differentiation. Thus far, the mechanism of action of fatty acids in the control of preadipocyte proliferation has remained unknown. We show here that PPARδ is directly implicated in fatty acid-induced cell proliferation. Ectopic expression of PPARδ renders 3T3C2 cells capable of responding to treatment with long-chain fatty acids by a resumption of mitosis, and this effect is limited to a few days after confluence. This response is restricted to PPARδ activators and, for fatty acids, takes place within the range of concentrations found to trigger differentiation of preadipocytes both *in vitro* and *in vivo*. Furthermore, the use of a mutated inactive PPARδ demonstrated that transcriptional activity of the nuclear receptor is required to mediate fatty acid-induced proliferation. These data demonstrate that PPARδ, as a transcription factor, is directly implicated in fatty acid-induced proliferation, and this could explain the hyperplastic development of adipose tissue that occurs in high-fat-fed animals.

Key words: adipose tissue, hyperplasia, obesity, PPARδ.

INTRODUCTION

Fatty acids have been suggested to regulate the adaptation of adipose tissue to nutritional changes. It is well established that an increase in the number of adipocytes occurs during the development of high-fat-diet-induced obesity in rodents [1–3], and that the diet-induced increase in adipocyte number is a consequence of cellular proliferation rather than the filling of existing preadipocytes [4]. Furthermore, Faust et al. [2] found that the increased adipocyte number induced by high-fat feeding persisted even when animals were returned to a standard diet.

Although factors that stimulate the formation of new fat cells in animals receiving a high-fat diet are yet to be identified, some data strongly suggest that fatty acids play a crucial role in the regulation of this biological process. We have demonstrated that exposure of preadipose Ob1771 cells to long-chain fatty acids increases both post-confluent proliferation and terminal differentiation [5]. This adipogenic action of fatty acids is not a consequence of an increase in substrate availability, since 2-bromopalmitate, which is not metabolized in preadipocytes [6], was found to be very active in promoting both adipogenesis and post-confluent mitosis [5]. Several studies have shown that growth-arrested preadipocytes undergo some rounds of mitosis before terminal differentiation and that these rounds of DNA synthesis are critical for expression of the overall differentiation programme [7].

The cellular effects of fatty acids and some of their metabolites are related, at least in part, to the activation of nuclear receptors of the peroxisome-proliferator-activated receptor (PPAR) family. These transcription factors exert their effects by binding to a specific responsive DNA element, called the PPRE (PPAR-responsive element), that has been characterized in several of the genes encoding proteins involved in lipid metabolism [8–10]. The

PPARδ and PPARγ subtypes are up-regulated during adipocyte differentiation with distinct time courses. PPARδ is expressed during the first steps of the differentiation process, whereas PPARγ emerges during terminal differentiation [11,12]. It is now established that PPARγ, when activated by naturally occurring molecules, such as 15-deoxy-Δ12,14-prostaglandin J$_2$, or by drugs, such as thiazolidinediones, plays a crucial role in the control of terminal adipose differentiation [13–15]. Recent evidence from our laboratory has demonstrated that the positive action of fatty acids on terminal differentiation involves the activation of PPARδ. We have shown that exposure of PPARδ-expressing 3T3C2 fibroblasts to long-chain fatty acids led to a rapid induction of the genes encoding fatty acid translocase (FAT) and adipocyte lipid-binding protein (ALBP), and to a delayed induction of the PPARγ gene. Further treatment of these cells with a specific PPARγ activator resulted in expression of a typical adipose differentiation programme [16]. From these findings, we proposed that PPARδ could act as an early player in the induction of terminal differentiation by fatty acids through PPARγ expression, thereby regulating adipocyte size.

In the present study, we examined whether PPARδ could also be implicated in the effects of fatty acids on post-confluent cell proliferation. For this purpose we used 3T3C2 fibroblast populations infected with a retroviral vector expressing native or mutated inactive PPARδ. An inactive form of PPARδ was obtained by changing the glutamate residue at position 411 to a proline in the loop preceding the hormone-dependent activation function-2 (AF-2) domain. This AF-2 domain is involved in the transcriptional activity of several members of the nuclear receptor family. It has been demonstrated that deletion or mutation of this domain leaves receptors able to bind their ligands, but unable to release co-repressors [17,18], leading to a loss of transcriptional activity of the nuclear receptor. Our results

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demonstrate that expression of an active PPARδ confers fatty acid-responsiveness, inducing cell proliferation in fibroblasts.

MATERIALS AND METHODS

Plasmids

Mutations were performed directly in the pSG5-FAAR plasmid [11] by the method described by Viville [19] using the oligonucleotide 5′-CATCATCTGGGCGTGTGGGGTGACCAGC-TG-3′. The construct was verified by sequencing. The mutated PPARδ coding sequence was excised from the pSG5 constructs by *Bam*HI and cloned into the *Bam*HI site of pBizeoneo to generate the pBizeoneoPPARδE411P plasmid.

Cell culture

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 8% (v/v) fetal calf serum, 200 units/ml penicillin and 50 μg/ml streptomycin. Cell counting experiments were performed in quadruplicate using a Coulter ZBIC instrument (Coulter Electronics). DNA synthesis was assessed by monitoring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) using immunofluorescence with the *in situ* cell proliferation FLUOS kit, as described by the manufacturer (Roche Molecular Biochemicals). Slides were mounted with Vectashield mounting medium (Vector Laboratories) containing DAPI (4,6-diamidino-2-phenylindole) for total nuclei labelling. The same fields were recorded under two excitation wavelengths, 488 and 360 nm, for visualization of BrdU-positive cells and total number of nuclei respectively. Pictures were analysed with NIH Image computer software to calculate mitotic indexes.

Stable cell lines

Establishment of 3T3C2Biz and 3T3C2BizPPARδ cell populations has been described previously [16]. 3T3C2BizPPAR-δE411P cell populations were obtained as follows. BOSC23 cells were transfected at 50–70% confluence by lipofection (Fugene-6; Roche Molecular Biochemicals) with the pBizeoneo-PPARδE411P expression vector. After 8 h, cells were re-fed with fresh standard medium, and viral supernatants were collected 48 h later. 3T3C2 cells maintained in standard medium were infected with equal titres 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. Fatty acids, 2-bromopalmitate, BRL 49653 and clofibrate were dissolved in DMSO. Control experiments were performed to exclude any side effects of the vehicle.

RNA and protein analysis, and enzymic assays

Total RNA was prepared and analysed by Northern blot, as described previously [5]. Blots were subjected to digital imaging (FujixBAS 1000). Glyceraldehyde-3-phosphate dehydrogenase mRNA, which is expressed in a constitutive manner, was monitored as an internal standard.

Transient transfection of HEK293 cells was performed by lipofection (Fugene-6). Typically, 1 ml of transfection medium contained 1 μg of 3xPPRE ACO-TK-luciferase [20], 35 ng of pCMV-β-galactosidase, 25 ng of expression vector for RXRα (retinoid X receptor) and 25 ng of expression vector for native or mutated PPARδ. After 6 h, cells were re-fed with fresh medium containing or not 50 μM 2-bromopalmitate. Luciferase and galactosidase activities were assayed 48 h later using the luciferase

assay system (Promega) and the Galacto-Light assay system (Tropix) respectively. Each transfection was performed in triplicate, and the fluorescence of the samples was measured using a 1450 MicroBeta luminometer (Wallac).

Materials

Culture media, fetal calf serum and geneticin were purchased from Life Technologies. Other chemical products were purchased from Sigma-Aldrich. Radioactive materials, random priming kit and nylon membranes were from Amersham. BRL 49653 was a gift from SmithKline-Beecham Pharmaceuticals (Harlow, Essex, U.K.).

RESULTS

Effects of PPARδ expression and activation by fatty acids on 3T3C2 cell proliferation

To investigate the role of PPARδ in the control of cell proliferation by fatty acids, 3T3C2Biz cells, infected with the empty pBizeoneo retroviral vector, and 3T3C2BizPPARδ cells [16] were maintained from seeding to 8 days post-confluence in standard medium supplemented or not with 25 μM 2-bromopalmitate. As shown in Figure 1, the growth rate of the control 3T3C2Biz cells was not affected by the fatty acid treatment, and the cell density was not significantly affected by the culture conditions. These cells displayed a growth pattern very similar to that of the parent 3T3C2 cells (results not shown). 3T3C2BizPPARδ cells showed a decreased growth rate when compared with control cells (doubling times of approx. 30 h and 22 h respectively). Furthermore, the cell density at confluence was markedly lowered in PPARδ-expressing cells, representing 40% decrease in cell number per dish when compared with 3T3C2Biz control cells. Exposure of 3T3C2BizPPARδ cells to 2-bromopalmitate did not affect either growth or cell density at confluence. After confluence, 3T3C2BizPPARδ cells maintained in standard medium underwent growth arrest, whereas exposure to the fatty acid derivative
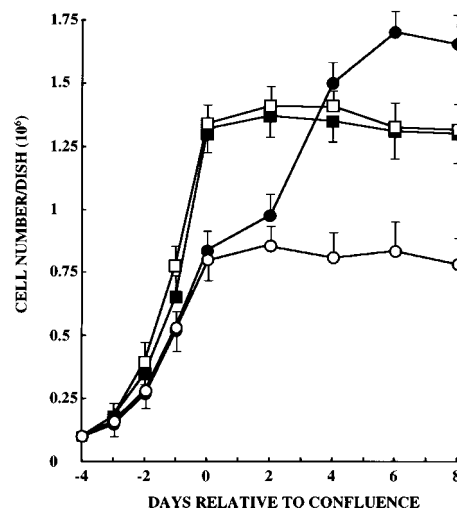

Figure 1   Effects of 2-bromopalmitate on the proliferation of 3T3C2Biz and 3T3C2BizPPARδ cells

3T3C2Biz ($\square$, $\blacksquare$) and 3T3C2BizPPARδ ($\bigcirc$, $\bullet$) cells were maintained in standard medium containing ($\blacksquare$, $\bullet$) or not ($\square$, $\bigcirc$) 25 μM 2-bromopalmitate. Cell number per dish was determined on the indicated days, as described in the Materials and methods section. Values represent means $\pm$ ranges from two separate experiments (two dishes each).
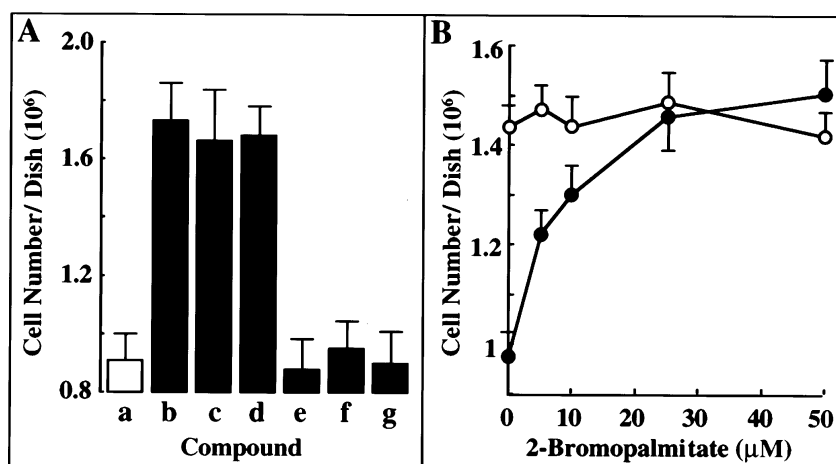
Figure 2   Effects of various PPAR activators on 3T3C2BizPPARδ cell proliferation

(**A**) 3T3C2BizPPARδ cells were grown to confluence in standard medium and then exposed for 4 days to control medium (a), 25 μM 2-bromopalmitate (b), 100 μM palmitate (c), 100 μM α-linolenate (d), 50 μM 2-bromo-octanoate (e), 1 μM BRL 49653 (f) or 100 μM clofibrate (g). (**B**) 3T3C2Biz (○) and 3T3C2BizPPARδ (●) cells were grown to confluence in standard medium and then exposed to increasing concentrations of 2-bromopalmitate for 4 days. In both experiments, cell number was determined at day 4, and values represent means ± ranges from two separate experiments (two dishes each).
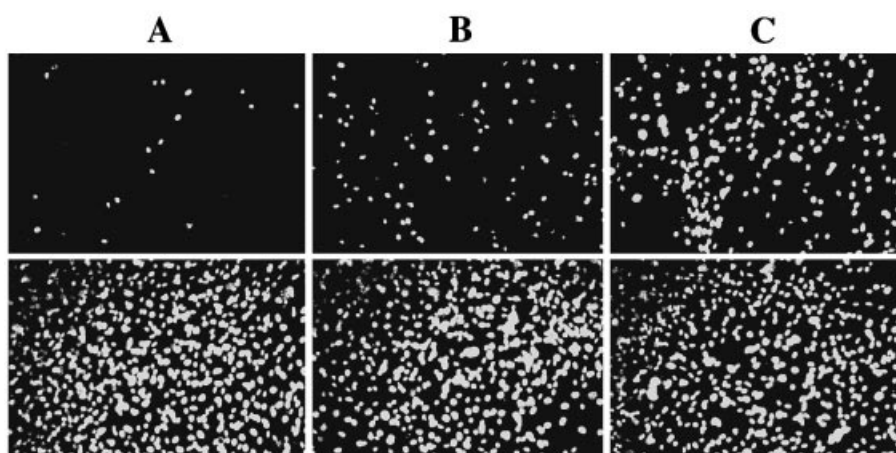
Figure 3   Immunodetection of DNA synthesis in 2-bromopalmitate-treated 3T3C2BizPPARδ cells

Cells were grown to confluence and then maintained in standard medium (**A**), or exposed to 5 μM (**B**) or 50 μM (**C**) 2-bromopalmitate for 4 days. Cells were exposed to BrdU for the last 24 h of the experiment and then treated as described in the Materials and methods section for detection of BrdU-positive nuclei (upper panels) and total nuclei (lower panels). Original magnification was 400 × .

promoted growth resumption, which took place between day 2 and day 6 post-confluence and resulted in a 2-fold increase in cell number at day 8.

Effects of PPARδ activators on post-confluent proliferation

To investigate the direct involvement of PPARδ in the control of proliferation of post-confluent cells by fatty acids, 3T3C2BizPPARδ cells were grown to confluence in standard medium and then exposed for 4 days to various fatty acids or specific activators of either PPARγ or PPARα (Figure 2A). Exposure to the long-chain fatty acids 2-bromopalmitate, palmitate or α-linolenate promoted post-confluent proliferation, resulting in an increase in cell number of approx. 2-fold. By contrast, a derivative of a short-chain fatty acid, 2-bromo-octanoate, did not exert any proliferative effect. Interestingly, treatment with BRL 49653, a specific activator of PPARγ [21,22], or with clofibrate, a specific activator of PPARα [23], used at concentrations known to be maximally effective, were without effect on the proliferation of 3T3C2BizPPARδ cells. Next, the dose-dependence of the proliferative action of the fatty acids was examined. For that purpose, confluent 3T3C2Biz and 3T3C2BizPPARδ cells were exposed for 4 days to increasing concentrations of 2-bromopalmitate. As shown in Figure 2(B), the action of 2-bromopalmitate on the proliferation of PPARδ-expressing cells was dose-dependent between 5 and 50 μM, whereas control 3T3C2Biz cells remained insensitive to the fatty acid whatever the concentration used.

To confirm that the increase in cell number that occurs after confluence in PPARδ-expressing fibroblasts treated with fatty
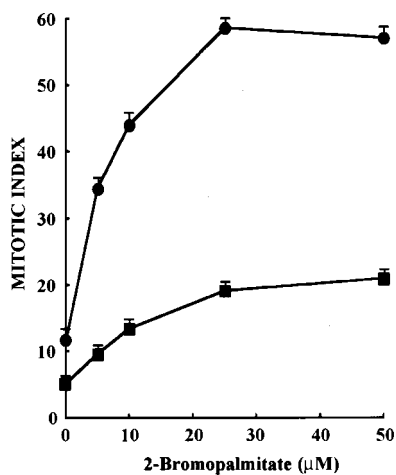
Figure 4   Dose–response to 2-bromopalmitate of DNA synthesis in confluent 3T3C2BizPPARδ cells

Cells were maintained from confluence in standard medium containing the indicated concentrations of 2-bromopalmitate, and then incubated with BrdU for the last 24 h of the experiment. Detection of BrdU-positive cells and total nuclei was performed at the end of the labelling period, i.e. on day 4 (●) and day 5 (■). Values represent means ± S.D. from six independent fields from three separate dishes.

acids is related to DNA synthesis, we measured the percentage of DNA-synthesizing cells by BrdU labelling. For that purpose, confluent cells were treated with various concentrations of 2-bromopalmitate and exposed for the final 24 h of the experiment to BrdU. Typical images of labelling of cells exposed to BrdU

from day 3 to day 4 are presented in Figure 3. The number of BrdU-positive cells was very low in standard medium conditions (Figure 3A), whereas exposure to the fatty acid resulted in a dose-dependent increase in labelling (Figures 3B and 3C). The percentage of BrdU-positive cells for each condition was measured by a computer-assisted method. As shown in Figure 4, 2-bromopalmitate treatment increased the number of BrdU-positive cells in a dose-dependent manner. This effect occurred within the same range of 2-bromopalmitate concentrations as that described for the increase in cell number (see Figure 2B). In good agreement with the results of Figure 1, 2-bromopalmitate promoted intense DNA synthesis between day 3 and day 4, whereas the percentage of positive cells decreased thereafter (almost 60 % BrdU-positive cells at day 4, compared with approx. 20 % at day 5).

Taken together, these observations clearly indicate that expression of PPARδ confers to fibroblasts the ability to respond to long-chain fatty acids with post-confluent proliferation.

The proliferative activity of fatty acid-activated PPARδ is related to its transcriptional activity

To examine whether or not the effect of ligand-activated PPARδ on cell proliferation is related to its potency in activating transcription, we introduced a mutation in a region known to be crucial for nuclear receptor activity. The glutamate residue at position 411 in the PPARδ sequence was changed to a proline by site-directed mutagenesis. The mutated nuclear receptor (PPARδE411P) was first assayed for its ability to transactivate a synthetic PPAR-responsive promoter [20] in HEK293 cells. Cells were co-transfected with pBizeoneo, pBizPPARδ or pBizPPARδE411P together with an expression vector for the obligate partner RXRα, and then exposed for 48 h to 50 μM 2-bromopalmitate. As shown in Figure 5(A), 2-bromopalmitate
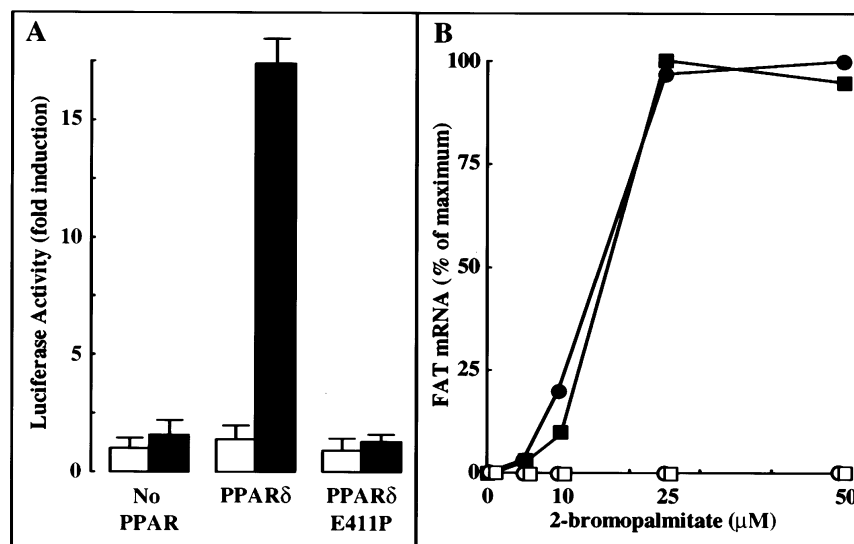
Figure 5   The Glu-411 → Pro mutation inhibits the transcriptional activity of PPARδ

(**A**) HEK293 cells were grown to confluence in standard medium, and then transfected as described in the Materials and methods section with luciferase and galactosidase reporter plasmids, an RXRα expression vector and, where indicated, expression vector for either native or mutated PPARδ. Cells were then maintained in standard medium (open bars) or exposed to 50 μM 2-bromopalmitate (solid bars), and luciferase and galactosidase activities were determined after 48 h. Results are presented by taking as 1.0 the value obtained with cells transfected in the absence of PPARδ expression vector and maintained in control medium, and are means ± S.D. from three separate experiments. (**B**) 3T3C2BizPPARδ (●, ■) and 3T3C2BizPPARδE411P (○, □) cells maintained in standard medium were exposed at day 1 post-confluence to various concentrations of 2-bromopalmitate. RNA was prepared 48 h later and analysed by Northern blot, as described in the Materials and methods section, for FAT (●, ○) and ALBP (■, □) mRNAs. The glyceraldehyde-3-phosphate dehydrogenase mRNA signal was used as internal standard. Results are presented by taking as 100 % the maximal signal for each probe. Results are representative of three independent experiments.
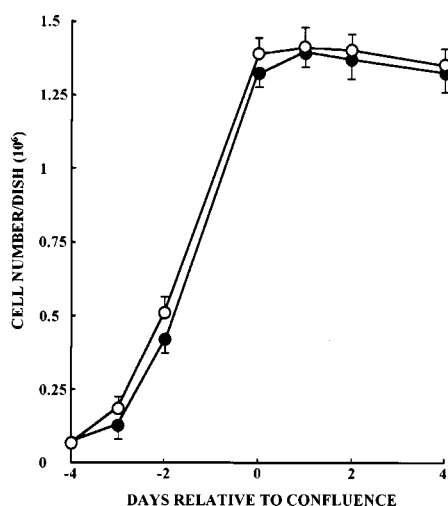
Figure 6   2-Bromopalmitate does not affect 3T3C2BizPPARδE411P cell growth

Cells were plated and maintained in standard medium supplemented (●) or not (○) with 25 μM 2-bromopalmitate. Cell number was determined as in Figure 1. Values represent means ± S.D. from triplicate dishes.

resulted in a minor induction of luciferase activity in cells transfected with the empty pBizeoneo vector, whereas transfection with the PPARδ expression vector promoted induction of luciferase upon fatty acid treatment. As expected, the glutamate → proline substitution at position 411 completely abolished the ability of PPARδ to transactivate the PPAR-responsive reporter gene.

Stable cell populations were obtained by infection of 3T3C2 cells with the retroviral expression vector containing the mutated PPARδ. Western blot analysis with an antiserum directed against the A/B domain of the receptor revealed that the amount of PPARδ protein in nuclear extracts from 3T3C2BizPPARδE411P cells was very similar to that observed in nuclear extracts from 3T3C2BizPPARδ cells (results not shown). We have demonstrated previously that expression of PPARδ confers to fibroblasts fatty acid-responsiveness of FAT gene induction [11,16]. To examine the effect of the glutamate → proline substitution in PPARδ on FAT and ALBP gene induction, 3T3C2BizPPARδE411P cells were grown to confluence and then exposed for 24 h to increasing concentrations of 2-bromopalmitate. In this experiment, 3T3C2BizPPARδ and 3T3C2Biz cells were used as positive and negative cell lines respectively. In good agreement with our previous observations [16], exposure of 3T3C2BizPPARδ cells to the fatty acid led to a dose-dependent induction of the FAT and ALBP genes, whereas 3T3C2Biz cells remained insensitive to 2-bromopalmitate (results not shown). FAT and ALBP mRNAs remained undetectable in 3T3C2BizPPARδE411P cells whatever the fatty acid concentration used, indicating that, in the 3T3C2 cell context, the mutation at position 411 abolished the transcriptional activity of PPARδ (Figure 5B).

The effects of 2-bromopalmitate exposure on cell growth and post-confluent proliferation were next investigated for 3T3C2BizPPARδE411P cells by an experimental protocol identical with that used for 3T3C2Biz and 3T3C2BizPPARδ cells (see Figure 1). As shown in Figure 6, the growth pattern of 3T3C2BizPPARδE411P cells was very similar to that of the control 3T3C2Biz cells, as the growth rate of the cells was not

significantly affected by fatty acid treatment before or after confluence. This finding indicated that the glutamate → proline mutation in PPARδ abolished the ability of the nuclear receptor to mediate the fatty acid effect on post-confluent cell proliferation.

DISCUSSION

Knowledge of the molecular mechanisms implicated in the overdevelopment of adipose tissue by hypertrophy and hyperplasia in animals receiving a high-fat diet is a critical issue in the design of pharmaceutical strategies aimed at lessening nutritional obesity.

The role of PPARs in the control of genes encoding proteins involved in lipid synthesis and adipocyte differentiation is now well documented, and recent findings have uncovered a link between these nuclear receptors, their respective ligands and adipose differentiation. However, the molecular mechanisms behind the growth resumption of preadipocytes that occurs in high-fat-fed animals have remained unknown. In order to explore the possible involvement of PPARδ in the control of fatty acid-induced post-confluent proliferation, we investigated the effects of fatty acids on 3T3C2 fibroblasts expressing or not this nuclear receptor. Expression of PPARδ protein in 3T3C2 fibroblasts clearly affects cell growth and leads to a net decrease in cell saturation density at confluence (Figure 1). This effect is related to the expression of an active form of PPARδ, since 3T3C2Biz cells, infected with an empty retroviral vector, as well as 3T3C2BizPPARδE411P cells, which express an inactive nuclear receptor, did not display such a phenotype (Figures 1 and 5). This decrease in cell growth is not unexpected, as it has been shown previously that expression of transcription factors that regulate adipose differentiation seriously antagonizes cell proliferation. For example, some reports have described how expression of C/EBPα (CCAAT enhancer binding protein α) [24,25] or PPARγ [26] suppresses the proliferation of 3T3-L1 adipoblasts and fibroblasts. A similar inhibition of cell proliferation has been described for transcription factors involved in myogenic differentiation [27]. This finding suggests that PPARδ, which emerges around confluence in preadipose cells [11], could be one of the players implicated in the growth arrest required for cell commitment.

Another very interesting finding of the present study is the demonstration that PPARδ expression renders the fibroblastic 3T3C2 cells able to proliferate after confluence in response to fatty acid treatment. This fatty acid-induced cell proliferation that takes place after confluence is reminiscent of the process of clonal expansion that occurs in actual preadipose cell lines. First, as previously described for Ob1771 cells [5], this proliferative effect is restricted to molecules known to be PPARδ activators, such as native or non-metabolizable long-chain fatty acids, whereas short-chain fatty acids and specific activators of either PPARγ or PPARα (thiazolidinediones and fibrates respectively) are not effective (Figure 2A). Secondly, 2-bromopalmitate exerted a proliferative action on 3T3C2-PPARδ cells within the same range of concentrations (i.e. 1–25 μM) as that causing induction of Ob1771 post-confluent mitosis [5] (Figures 2B and 4). It is worth noting that the induction of adipogenesis with both Ob1771 cells [5] and 3T3C2-PPARδ fibroblasts [16] by the fatty acid derivative takes place within the same concentration range. Thirdly, as described previously for Ob1771 [5,28] and 3T3-F442A [29] cells, the proliferation of 3T3C2BizPPARδ cells is limited to the first days of the confluence phase, since the fatty acid-induced cell growth stopped after day 4 or 5 (Figures 1 and 4). Interestingly, we demonstrated that this period of time corresponds to the induction of PPARγ gene expression in these

cells [16], suggesting that accumulation of this transcription factor crucial for terminal differentiation could be implicated in the arrest of post-confluent cell proliferation that precedes adipogenesis. Consistent with such an anti-proliferative action of PPARγ, it has been demonstrated that specific PPARγ agonists inhibit the growth of PPARγ-expressing fibroblasts [26] and human colorectal cancer cells [30,31], and that loss-of-function mutations in this nuclear receptor are associated with human colon cancer [32].

The finding that PPARδ exerts growth-promoting activity is in good agreement with the recent observations describing a role for this receptor in intestinal tumorigenesis. He et al. [33] have demonstrated that the expression of PPARδ is increased in human colorectal cancer cells, and is down-regulated by the tumour suppressor adenomatous polyposis coli and up-regulated by β-catenin. In this colorectal cancer cell model, as in preadipose cells, it could be proposed that the growth-promoting activity of ligand-activated PPARδ is counterbalanced by PPARγ.

The mechanism by which fatty acid activation of PPARδ results in growth resumption of confluent cells remains an open question. The finding that the mutated PPARδE411P, which is unable to transactivate PPAR-responsive genes (Figure 5), is not able to mediate fatty acid effects on 3T3C2 post-confluent proliferation (Figure 6) strongly suggests that a protein up-regulated by PPARδ is implicated in the control of cell proliferation. In 3T2C2BizPPARδ cells, as well as in Ob1771 cells, fatty acids induce the expression of a limited set of genes [34], and further studies are required to identify such a protein.

Altogether, the findings of the present work clearly establish that PPARδ is one of the players in the signalling pathway leading to the proliferation of cells exposed to high concentrations of fatty acids. These data strongly support the hypothesis that PPARδ is a primary factor in the hyperplastic development occurring in the adipose tissue of high-fat-fed animals.

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