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# Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival

(nuclear receptors/adipocytes/differentiation)

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ABSTRACT **Culture of mouse 3T3-L1 preadipocytes in medium containing delipidated bovine calf serum caused the cells to elongate and divide after reaching confluency. The continued proliferation correlated with sustained expression of the c-*myc* gene, which was repressed in control cells. Exposure of the cells to activators of peroxisome proliferator-activated receptor (PPAR), including clofibrate, WY-14,643, and 5,8,11,14-eicosatetraynoic acid, reversed and prevented the effects of culturing preadipocytes in delipidated serum. Continued exposure to PPAR activators led to adipose conversion, during which PPAR and its heterodimerization partner (retinoid X receptor) were induced. Retinoic acid (RA) had no effect on the growth or survival of preadipocytes grown in the presence of normal bovine serum. However, treatment of cells cultured in delipidated serum with RA caused death of the cells by apoptosis. Thus, preadipocyte phenotype and survival are regulated by activators of nuclear hormone receptors.**

3T3-L1 cells have been selected from Swiss 3T3 mouse fibroblasts for their ability to differentiate into adipocytes after exposure to fetal calf serum (FCS), dexamethasone (Dex), isobutylmethylxanthine (IBMX), and insulin (1, 2). Adipose conversion involves coordinated expression of a variety of transcription factors prior to the morphological changes and induction of structural proteins (3). Factors induced during adipocyte differentiation include C/EBP$\alpha$, -$\beta$, and -$\delta$ (4, 5) as well as Jun and Fos (6, 7). In addition, c-*myc* gene expression is repressed in the adipocyte, and overexpression of c-*myc* can inhibit differentiation (8). However, the biological basis of the adipose commitment of the 3T3-L1 cell is not well understood.

Retinoic acid (RA) and peroxisome proliferators are lipophilic substances that act through related nuclear receptors to directly regulate gene expression (9–12). Both RA receptors (RARs) and peroxisome proliferator-activated receptors (PPARs) heterodimerize with retinoid X receptor (RXR) *in vitro* (ref. 12 and references therein) and, in the case of PPAR, regulation of gene transcription is greatest when the activators of both receptors are present (13–16). RA prevents the adipose conversion of 3T3-L1 cells (17, 18). Inhibition of differentiation is an unusual effect of RA, which more commonly promotes differentiation (19). In contrast to the effects of RA, peroxisome proliferators such as clofibrate have been shown to potentiate adipose conversion of 3T3-L1 cells in the presence of Dex, FCS, and insulin (20).

PPAR is of particular interest in adipocytes because it regulates genes involved in lipid metabolism (21, 22) and is activated not only by peroxisome proliferators but by fatty acids (13, 23) as well. We have found that culturing 3T3-L1 preadipocytes in medium containing delipidated serum induces a proliferative and elongated phenotype that is reversed by PPAR activators. Furthermore, a variety of PPAR activators cause adipose conversion in the absence of Dex, IBMX, FCS, and insulin. PPARs and RXRs are both induced during adipose conversion, which is inhibited by RA. However, exposure of the cells cultured in delipidated medium to RA results in cell death by apoptosis. Thus, PPAR activators and retinoids co-regulate preadipocyte proliferation, differentiation, and survival.

## MATERIALS AND METHODS

**Cell Culture.** 3T3-L1 cells (ATCC) were cultured in growth medium containing Dulbecco's modified Eagle's medium and 10% bovine calf serum (HyClone) with a change of medium every 2 days. Bovine calf serum was delipidated by a modification of the method of Goodman (24). Briefly, serum was extracted two or three times with an equal volume of *n*-heptane by vigorous stirring at 4°C for 20 h. The aqueous phase was separated by centrifugation at 2000 × *g* for 2 h. Cells were initially cultured in normal growth medium for 2–3 days (40–50% confluence) and then switched to medium containing 10% delipidated serum.

The standard method of cell differentiation and inhibition by RA was as described (25). RA was added in ethanol at a final concentration of 10 $\mu$M. For experiments with PPAR activators, 3T3-L1 cells were cultured in growth medium until confluent and then switched to growth medium supplemented with various concentrations of clofibrate, WY-14,643 (pirinixic acid; kindly supplied by Wyeth–Ayerst), or 5,8,11,14-eicosatetraynoic acid (ETYA) in ethanol. Control cells were treated with the same volume of ethanol alone.

**Northern Blot Analysis.** Northern blot analyses were performed as described (25) with cDNA probes for aP2 (26); C/EBP$\alpha$ (27); PPAR$\alpha$ (11); RXR$\alpha$, -$\beta$, and -$\gamma$ (28); Nuc-1 (29); c-myc; and $\beta$-actin labeled with $^{32}$P using random hexamers.

**Cell Viability.** At 40–50% confluence, cells cultured in 60-mm$^2$ dishes were switched from control to delipidated medium containing either 10 $\mu$M RA or ethanol alone. Approximately every 24 h, floating and adherent cells were pooled and viability was assessed by trypan blue exclusion. Similar results were observed when cells were exposed to RA throughout the experiment or only during the initial 48 h. For determination of [$^3$H]thymidine incorporation, cells were cultured in 96-well plates and [$^3$H]thymidine (5 $\mu$Ci per well; 1 Ci = 37 GBq) was added for 1 h, after which the cells were harvested and incorporated radioactivity was measured by scintillation counting. Genomic DNA was isolated from

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Abbreviations: FCS, fetal calf serum; Dex, dexamethasone; IBMX, isobutylmethylxanthine; RA, retinoic acid; RAR, RA receptor; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; ETYA, 5,8,11,14-eicosatetraynoic acid.

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adherent and floating cells by a method that selected for fragmented DNA (30) and was analyzed in 1.2% agarose gels.

## RESULTS

**Characteristics of Preadipocytes Grown in Delipidated Serum.** Preadipocytes were cultured in medium containing bovine calf serum, which was extracted with *n*-heptane to remove lipophilic substances, including fatty acids and retinoids (24). Under these conditions, preadipocytes continued to proliferate after control cells had reached confluence, and the number of cells was increased ≈3-fold after 4 days (Fig. 1*A*). [$^3$H]Thymidine incorporation of the cells in delipidated serum was ≈5-fold greater than that of control cells, which decreased by 88% after confluence (Fig. 1*B*). This was paralleled by the continued expression of c-myc (Fig. 1*C*), which was dramatically reduced in control preadipocytes shortly after confluence as described (8). The increase in cell density was apparent on inspection of the culture dishes (Fig. 2 *A–D*), and the cell shape was noted to be elongated as well (Fig. 2*B*). These morphological changes could be prevented by addition of a chloroform extract of the material removed from the delipidated serum (Fig. 2*E*).

**Activators of PPAR Prevent and Reverse the Effects of Culturing Preadipocytes in Delipidated Serum.** Since PPAR is activated by lipophilic agents, including fatty acids (13, 22, 23), we tested whether PPAR activators could substitute for the extracted substance(s) in this system. Remarkably, treatment with WY-14,643, a peroxisome proliferator and activator of PPAR, prevented and reversed the changes induced by the delipidated serum (Fig. 2 *F* and *G*). Continued exposure to WY-14,643 ultimately caused adipose conversion (Fig. 2*H*). Indeed, we found that clofibrate as well as the polyunsaturated fatty acid ETYA, another activator of PPAR, also caused adipose conversion of 3T3-L1 cells (Fig. 3 *C*, *E*, and *G*). The concentrations required to induce >90% of the cells to differentiate after 7 days were 3 mM clofibrate, 450 μM WY-14,643, and 50 μM ETYA. The relative potencies of these compounds paralleled those for activation of PPAR (13). Adipose conversion due to PPAR activators, like that

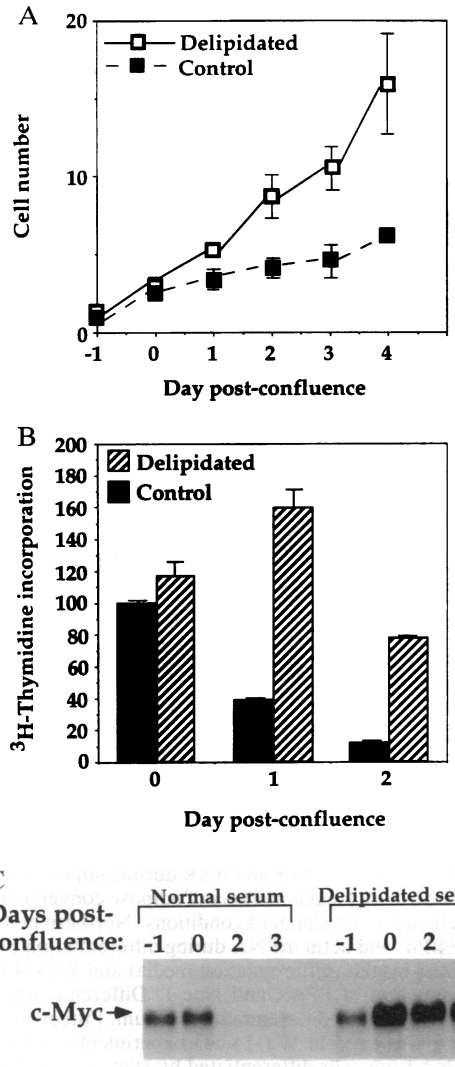


FIG. 1. Delipidated serum causes proliferation of preadipocytes beyond confluence. (*A*) Cell growth. Cells were switched to medium containing 10% delipidated serum or control just prior to confluence, and viable cell number was determined each day thereafter. Results are normalized to viable cell number at day 0. (*B*) Thymidine incorporation. Cells were incubated with [$^3$H]thymidine under each of the above conditions. Results are expressed as percentage of day 0 control. Shown is the mean ± SE of two experiments, with each data point done in triplicate in each experiment. (*C*) c-myc expression. Northern blot analysis for c-myc mRNA from cells cultured as in *A*. Loading was judged to be equal by ethidium bromide fluorescence of rRNA as well as by hybridization of an actin probe.

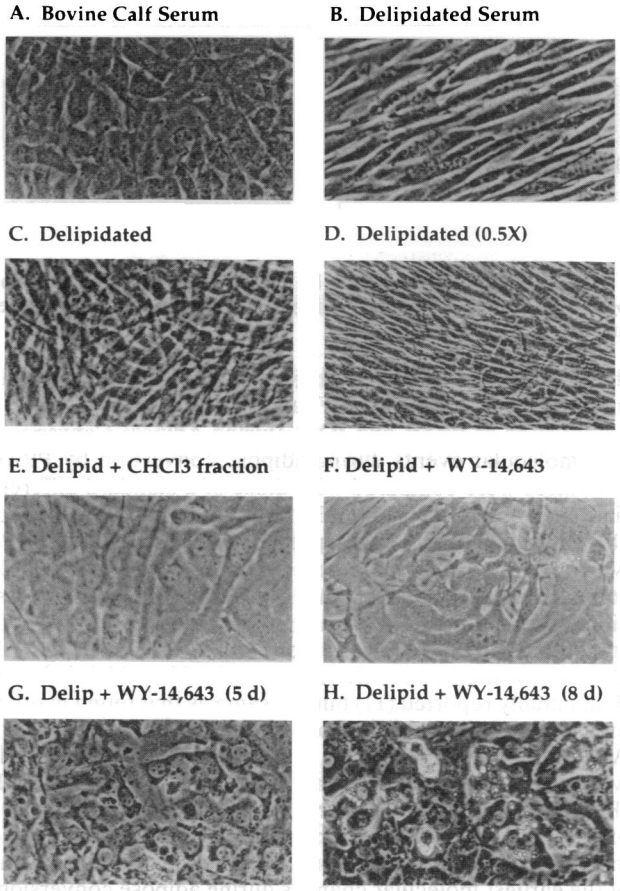


FIG. 2. Morphological effects of culturing preadipocytes in delipidated serum, and reversal by PPAR activators. 3T3-L1 preadipocytes were cultured under the conditions shown. Cells shown in *B* were less confluent at the time of change to medium containing delipidated serum (delipid) and resembled those shown in *C* after additional incubation time. Cells shown in *E* are representative of those near a drop of $CHCl_3$ extract of the heptane-soluble fraction of normal serum. $CHCl_3$ alone had no effect. The concentration of WY-14,643 used in *F–H* was 0.30 mM. Cells in *F* were exposed to delipidated serum and WY-14,643 simultaneously. Cells in *G* and *H* resembled those in *B* before addition of WY-14,643 for 5 and 8 days, respectively. Phase-contrast microscopy. (*A–C* and *E–H*, ×150; *D*, ×75.)

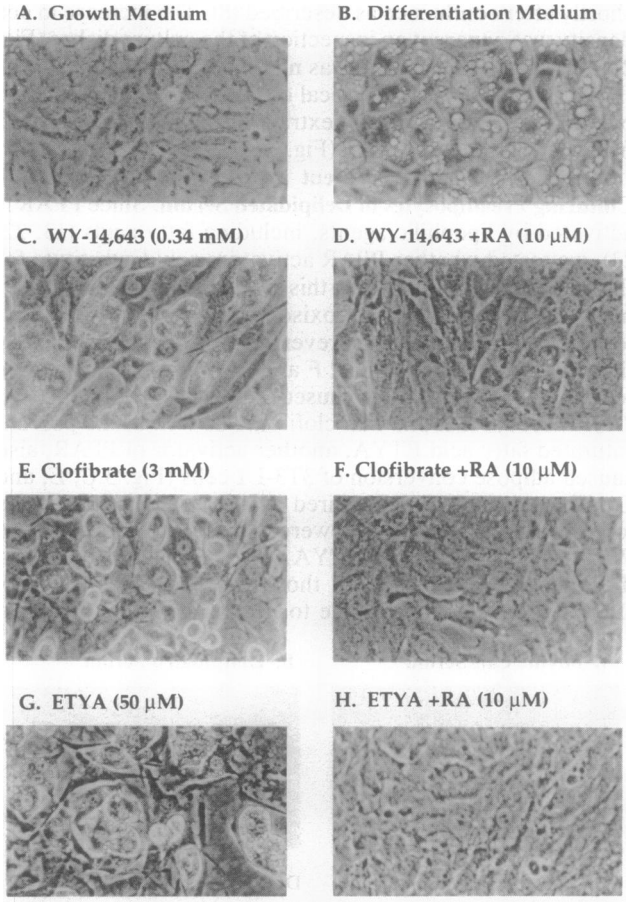


FIG. 3. Adipose conversion by PPAR activators. Cell morphology. Cells were cultured in conditions noted. Phase-contrast images are shown. (×150.)

due to standard differentiating conditions, was completely inhibited by RA (Fig. 3 *D*, *F*, and *H*).

**Induction of PPAR and RXR During Adipose Conversion.** The molecular events during adipose conversion by PPAR activators were compared with those of a standard differentiation protocol. WY-14,643, clofibrate, and ETYA (not shown) induced adipocyte-specific genes *aP2* and *C/EBPα* with a similar magnitude and time course as that due to standard conditions (Fig. 4*A*). In addition, PPARα expression increased ≈10-fold during adipose conversion by both standard differentiation conditions and WY-14,643 (Fig. 4*B*). The size of the PPAR mRNA was ≈7.0 kb, which is larger than initially reported (11) but the same as that found in other mouse tissues (unpublished observations) and in the rat (31). A second PPAR, Nuc-1 (29), was induced as well. The cells were also found to express the α, β, and γ subtypes of RXR (Fig. 4*C*). RXRα mRNA increased within 4 h after initiation of the standard differentiation protocol; this is the most rapid induction that has been demonstrated for any RXR and is one of the earliest molecular changes during adipose conversion. RXRγ was also induced, although considerably later than RXRα. The size of the RXRγ mRNA was ≈6 kb, which is larger than that previously reported (28) and raises the possibility that an unusual RXRγ isoform is expressed in fat.

**RA Causes Apoptosis of Preadipocytes Cultured in Delipidated Medium.** 3T3-L1 preadipocytes express RARα, -β, and -γ subtypes (unpublished data; see also ref. 32). However, while RA inhibited adipose conversion, it did not cause an appreciable phenotypic change in the cells cultured under usual conditions. Nevertheless, we were interested in the effects of RA on cells cultured in delipidated serum because this medium is depleted of retinoids. Remarkably, the cells

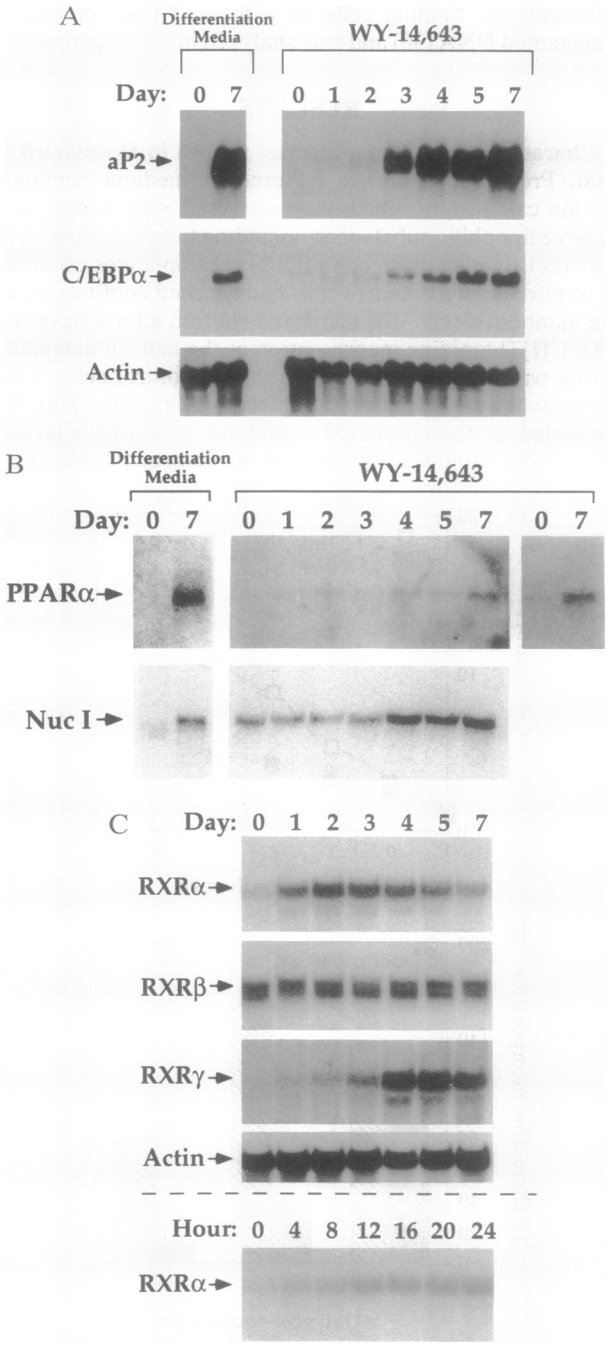


FIG. 4. Induction of PPAR and RXR during adipose conversion. (*A*) Comparison of molecular markers of adipose conversion induced by PPAR activators and standard conditions. Northern blot analysis of aP2, C/EBPα, and actin mRNA during differentiation by IBMX, Dex, FCS, and insulin (differentiation media) and WY-14,643 (0.45 mM). (*B*) Induction of PPAR and Nuc-1. Differentiation by WY-14,643 (0.45 mM) and by differentiation medium. Fifteen micrograms of total RNA was used in WY-14,643 experiments, while 4 μg of poly(A)$^+$ RNA from cells differentiated by standard conditions was used. (*C*) Induction of RXR. Fifteen micrograms of total RNA per lane. Loading was judged equal by actin hybridization as well as ethidium bromide fluorescence of rRNA.

developed cytoplasmic blebbing, vacuolization, and condensation within 3–4 days of exposure to RA (Fig. 5 *C* and *D*), changes characteristic of cells undergoing apoptosis (33). Quantitatively, viable cell number was decreased by >90% 5 days after exposure to RA (Fig. 5*E*). Fig. 5*F* shows that chromosomal DNA from the dying cells displayed the periodic (≈200 bp) fragmentation pattern frequently seen in

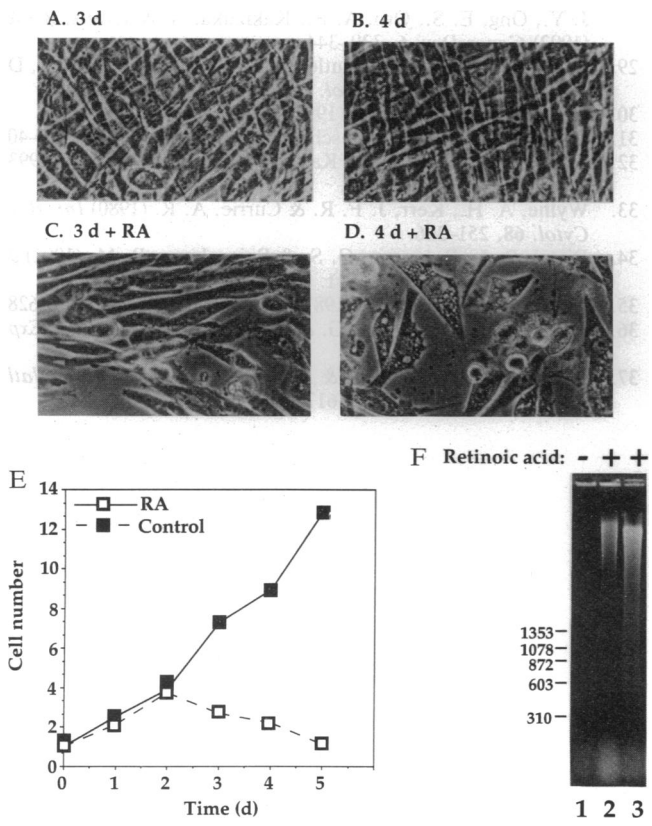


FIG. 5. RA induces apoptosis of preadipocytes cultured in delipidated serum. (*A–D*) Morphological changes in RA-treated cells. Cells were cultured for 3 days (*A* and *C*) and 4 days (*B* and *D*) in delipidated serum-containing media. In *C* and *D*, RA was present for the first 48 h. (*E*) Cell death due to RA. Cell number after incubation in delipidated serum-containing medium in the presence or absence of RA, normalized to cell number at day 0. (*F*) Chromosomal DNA fragmentation induced by RA. Cells were cultured in delipidated serum-containing medium for up to 5 days, with 10 μM RA added for the first 48 h (lanes 2 and 3). Lanes: 1, 5 days, no RA; 2, 4 days, plus RA; 3, 5 days, plus RA. Little genomic DNA is seen in lane 1 because the method used selected for fragmented DNA, which was nearly absent in these growing cells. Migration of DNA size markers is shown on the right. Numbers on left are bp.

apoptosis (compare lanes 1 and 3). The $ED_{50}$ for induction of apoptosis by RA was ≈0.5 μM. 9-*cis*-RA, a ligand for RAR as well as RXR, had similar effects (data not shown).

## DISCUSSION

The effects of incubation in delipidated serum reveal that usual culture conditions constrain the 3T3-L1 preadipocyte in a state that is intermediate between proliferative and terminally differentiated cells. Since this is prevented by activators of PPAR, we speculate that the delipidation process might have removed an endogenous activator of PPAR, perhaps the PPAR ligand itself. The correlation between the potency of PPAR activators and their abilities to induce adipose conversion further suggests that PPAR is mechanistically involved. Nevertheless, PPAR is also expressed in a variety of nonadipose tissues where it undoubtedly mediates other effects.

The simultaneous induction of PPAR and the accumulation of lipid within the adipocyte might serve to maintain the adipocyte phenotype, since PPAR is activated by fatty acids (13, 22, 23). Indeed, adipose-specific transcription of the *aP2* gene is stimulated by fatty acids (34). During adipose conversion, the induction of two RXRs may ensure that PPAR is in the heterodimer form. The main RXR partner is likely to vary during the course of differentiation since RXRα is rapidly induced, while RXRγ is induced to an even greater extent at later times. Understanding the role of endogenous activators of PPAR, RXR, and RAR in regulating adipocyte differentiation may allow the rational design of agents to prevent and treat obesity.

The preadipocyte cultured in delipidated serum displays a remarkable susceptibility to RA-induced cell death by apoptosis. It is known that RA sets in motion complex differentiation programs involving programmed cell death, such as limb development (35), and some HL-60 leukemic cells die via apoptosis after being induced to differentiate by RA (36). The present report provides an example of RA-induced apoptosis of a clonal, dividing cell population. It is of interest that 9-*cis*-RA also causes apoptosis of the preadipocytes, whereas it protects T cells from a similar fate (37). Relatively high concentrations of RA are required for induction of apoptosis in preadipocytes, suggesting that the RA may be acting via conversion to other retinoids. Nevertheless, the concentrations of RA that caused apoptosis were not toxic to adipocytes or to preadipocytes cultured in normal serum. It is possible that RA induces gene products that prevent differentiation in the presence of PPAR activators but are deleterious to cells cultured in delipidated serum. In any case, the diverse effects of RA on cells cultured in the absence and presence of PPAR activators indicate that convergence of these two nuclear receptor-mediated pathways regulates the fate of the preadipocyte.

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