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Biochemical Pharmacology 63 (2002) 997–1007

Biochemical Pharmacology

Effects of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (*p*,*p*′-DDT) on 3T3-L1 and 3T3-F442A adipocyte differentiation

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Received 28 November 2000; accepted 17 July 2001

Abstract

Based upon our initial observations that 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (*p*,*p*′-DDT) induces a concentration-dependent increase in 3T3-L1 adipocyte differentiation, the mechanism of the *p*,*p*′-DDT-induced adipocyte differentiation was studied, using 3T3-L1 and 3T3-F442A cells. Since, it is known that the differentiation of the 3T3-L1 adipocyte cell line involves the induction of the transcription factors CCAAT enhancer binding protein β (C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ), and C/EBPα, the possible role of these factors in *p*,*p*′-DDT-induced adipocyte differentiation had to be examined. It was found that *p*,*p*′-DDT-treated 3T3-L1 cells showed a concentration-dependent increase in the nuclear levels of both PPARγ and C/EBPα protein. On the other hand, treatment with *p*,*p*′-DDT (20 μM) did not affect the expression pattern of C/EBPβ protein during differentiation. Gel shift analysis of nuclear proteins for binding to the C/EBP recognition site of DNA showed an increase in binding activity at day 2 of differentiation in *p*,*p*′-DDT-treated cells. Supershift analysis revealed that this rise was caused mainly by a dramatic increase in the abundance of the C/EBPα–DNA complex. Similar increases were observed at days 4 and 7 after the induction of differentiation. Tumor necrosis factor α induced a strong inhibition of adipocyte differentiation, which was reversed by co-treatment with troglitazone, an activator of PPARγ. *p*,*p*′-DDT was unable to reverse the inhibitory effect of tumor necrosis factor α on adipocyte differentiation in 3T3-L1 cells. 3T3-F442A is another preadipocyte cell line that can be induced to differentiate into adipocytes in the presence of insulin and fetal bovine serum. *p*,*p*′-DDT (20 μM) induced an alteration in the morphology of these cells at day 2 after the induction of differentiation. These cells however, were unable to become fully differentiated adipocytes. These data showed, therefore, the ability of *p*,*p*′-DDT to alter the differentiation process of adipocyte cell lines through the modification of transcription factors regulating this event.

Keywords: *p*,*p*′-DDT; C/EBPα; PPARγ; Adipocyte differentiation; 3T3-L1; 3T3-F442A

1. Introduction

Adipocytes, the main cellular component of adipose tissue, play a critical role in the energy balance of organisms. Indeed, adipose tissue has been shown recently to secrete factors involved in the immune response (tumor necrosis factor α (TNFα, acylation stimulation protein)), the regulation of food intake and energy expenditure (leptin), vascular function (angiotensin and plasminogen activator inhibitor type 1), and hormonal function (estrogen). Hence, alterations in the growth and development of adipocytes are likely to lead to the development of pathological disorders such as obesity, non-insulin-dependent diabetes, hypertension, and atherosclerosis [1].

Adipocyte differentiation is a complex process that can be affected by various hormones, growth factors, and mitogens [2–5]. Previous experiments by our group have shown the ability of some environmental xenibiotics, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and endrin, to inhibit the differentiation process of adipocytes [6–8].

In its insecticidal form, DDT consists of a mixture of *p*,*p*′-DDT (85%) and *o*,*p*′-DDT (15%). DDT has been shown to disrupt normal endocrine functions, resulting in deleterious effects on growth, development, and reproduction [9,10]. *o*,*p*′-DDT and its metabolites are recognized as xenoestrogens, and have been shown to bind to the

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Abbreviations: TNF, tumor necrosis factor; *p*,*p*′-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; *o*,*p*′-DDT, 1,1,1-trichloro-2-*o*-chlorophenyl-2′-*p*-chlorophenyl-ethane; C/EBP, CCAAT enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; IBMX, isobutylmethylxanthine; DTT, dithiothreitol; EMSA, electrophoresis gel mobility shift assay; IL-1 and IL-6, interleukin-1 and -6.

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PII: S0006-2952(01)00933-9

estrogen receptor and to stimulate the proliferation of ER-positive breast cancer cells [11,12]. Previous studies from our group have also shown that *o,p′*-DDT promotes foci formation in MCF-7 human breast cancer cells, which was correlated with the activation of c-Neu tyrosine kinase [13,14]. *p,p′*-DDT, which is weakly estrogenic, has also been shown to stimulate breast epithelial cell proliferation. The mitogenic effects of *p,p′*-DDT are thought to be mediated through the activation of receptor tyrosine kinases (RTK)-Ras-MAPK (mitogen-activated protein kinase) and JAKs-STATs signaling pathways, which play important roles in regulating cell differentiation and proliferation in many biological systems [15].

DDT is highly lipophilic and, therefore, accumulates mainly in adipose tissue with a long half-life [16]. Little is known, however, about the effects of DDT on adipose tissue development and metabolism. The aim of the present study was to determine the effects of *p,p′*-DDT on adipocyte differentiation. Pesticide effects were tested in 3T3-L1 and 3T3-F442A cells, two *in vitro* models employed most frequently for adipocyte differentiation.

Adipocyte differentiation is a process in which committed preadipocytes undergo growth arrest and subsequent terminal differentiation into adipocytes. After reaching confluence, 3T3-L1 cells become contact-inhibited and growth-arrested, and they start to express some of the early markers of adipocyte differentiation [17]. Treatment of these growth-arrested cells with differentiation medium leads them to re-enter the cell cycle, and undergo several rounds of DNA replication and proliferation. This mitotic clonal expansion of committed cells is essential to completing terminal differentiation into mature adipocytes. Retinoblastoma (Rb) proteins modulate the activity of E2F, a transcription factor that plays a critical role in regulating cell-cycle progression. Recent reports have suggested that Rb proteins play a key role in regulating the mitotic clonal expansion necessary for 3T3-L1 adipocyte differentiation [17,18]. As clonal expansion ceases, the coordinate transcriptional activation of adipocyte-specific genes is initiated. Expression of these genes is accompanied by dramatic biochemical and morphological changes that lead to the expression of the adipocyte phenotype. Two families of transcription factors, the C/EBPs and PPARγ, have been identified as ''master regulators'' of adipocyte gene transcription [1,19]. It has been observed that the overexpression of C/EBPβ, PPARγ, or C/EBPα stimulates adipogenesis, which suggests that these transcription factors play an important role in the regulation of the adipogenic process [20–22]. To elucidate the mechanisms by which *p,p′*-DDT alters adipocyte differentiation, we investigated its effects on the pattern of expression of these three adipogenic proteins during differentiation. We also performed an electrophoresis gel mobility shift assay (EMSA) to study the effects of *p,p′*-DDT on the binding activity of the C/EBP proteins (α, β, and δ) to their response element.

2. Materials and methods

2.1. Cell culture and differentiation

3T3-L1 cells [23] were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf bovine serum. Confluent cells were induced to differentiate by incubating them for 48 hr with differentiation medium containing 1 μM dexamethasone, 0.2 mM isobutylmethylxanthine (IBMX), 10 μg/mL of insulin, and 10% fetal bovine serum in DMEM. After this time, cells were maintained in post-differentiation medium containing 10 μg/mL of insulin and 10% fetal bovine serum, and the medium was changed every 2 days. 3T3-F442A cells were a gift from Howard Green (Harvard Medical School). Differentiation of 3T3-F442A cells was initiated by incubating them with medium containing 10 μg/mL of insulin and 10% fetal bovine serum. For both cell types, *p,p′*-DDT was added at the same time that differentiation medium was added and maintained for 48 hr, and then added each time the medium was replaced. *p,p′*-DDT was obtained from AccuStandard, Inc. and was prepared as 1000 × stocks in ethanol. The same quantity of ethanol was added to the control cell culture plates and caused no apparent effects on differentiation.

2.2. Oil Red O staining

Four or five days after the induction of differentiation, cells were stained with Oil Red O according to Kasturi and Joshi [24]. In some experiments, spectrophotometric quantification of the stain was performed by dissolving the stained oil droplets in the cell monolayers with 4% Nonidet P-40 in isopropanol for 5 min. Absorbance of the extract was measured at 520 nm.

2.3. Preparation of nuclear extracts

Nuclei were isolated from 3T3-L1 or 3T3-F442A cells according to the method of Dignam *et al.* [25], which was modified slightly as follows. Briefly, cells were washed and scraped into PBS and then centrifuged at 1850 *g* for 10 min at 4°. The cell pellets were resuspended in 5 vol. of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol (DTT)) and centrifuged at 1850 *g* for 5 min at 4°. The packed cells were resuspended in 3 vol. of hypotonic buffer and allowed to swell for 10 min on ice. Next, cells were homogenized with 10 strokes using a glass Dounce homogenizer, and centrifuged at 3300 *g* for 15 min at 4°. The pellets obtained were the nuclei. Nuclear extracts were prepared from these nuclei by a slight modification of the method of Lavery and Schibler [26]. Pelleted nuclei were resuspended in a 1.1 × extraction buffer (300 mM NaCl, 1 M urea, 1% Nonidet P-40, 1 mM DTT, and 25 mM HEPES, pH 7.9), mixed vigorously by vortexing, and

incubated for 30 min on ice. The extracts were clarified by pelleting the insoluble debris through centrifugation at 15,000 *g* for 20 min at 4° in a microfuge. The supernatants were adjusted to 10% glycerol, and then were rapidly frozen in liquid nitrogen and stored at −80°. Protein concentration was determined as described by Bradford [27].

2.4. Western blot (immunoblot) analysis

Nuclear protein was fractionated by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Then membranes were incubated in TBST buffer (150 mM NaCl, 10 mM Tris, 0.09% Tween 20, pH 8.0) containing 10% milk for 1 hr at room temperature in order to block non-specific binding. The blots were incubated in TBST buffer containing 5% milk with one of three primary antibodies (polyclonal anti-C/EBPα, anti-C/EBPβ, or anti-PPARγ antiserum (1:1000 dilution for anti-C/EBPα and anti-C/EBPβ and 1:500 for PPARγ, Santa Cruz Biotechnology, Inc.)) for 2 hr. Protein/antibody complexes were detected by incubating with a secondary antibody for 90 min (horseradish peroxidase-conjugated anti-rabbit (1:2000, Amersham Life Sciences) for C/EBPα and β, or horseradish peroxidase-conjugated anti-goat IgG (1:3000, Santa Cruz Biotechnology, Inc.) for PPARγ). The blots were developed by the enhanced chemiluminiscence detection system (Pierce) and visualized by exposure to X-ray film.

2.5. EMSA

A double-stranded oligonucleotide containing a C/EBP binding site in the C/EBPα promoter [28] was 5′-end-labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Promega) and purified according to standard methods. Ten micrograms of nuclear extracts was incubated in a buffer containing 25 mM HEPES, pH 7.9, 10% glycerol, and 0.5 mM DTT, with 3 μg of poly-deoxy-inosinic-deoxy-cytidylic acid (poly [d(I-C)]; Boehringer Mannhein) and 5 μg of acetylated bovine serum albumin for 30 min at 4°. A 100-fold excess of unlabeled oligonucleotide was added to some samples. Then the radiolabeled double-strand oligonucleotide (100,000 cpm) was added and incubated for an additional 20 min at 4°. For the supershift assay, the specific antibodies, anti-C/EBPs (α, β, or δ, obtained from Santa Cruz Biotechnology) or combinations of them, were added at the end of the second incubation period, mixed gently, and incubated for 20 min at 4°. Oligonucleotide-C/EBP protein binding was determined by electrophoresis on a nondenaturing 4% polyacrylamide gel at 175 V for 3 hr at room temperature. Gels were dried for 2 hr at 80° and exposed to X-ray film in the presence of an intensifying screen at −80°.

2.6. Statistical analysis

The means of more than two groups were compared by ANOVA (GraphPad Prism, GraphPad Software Inc.).

3. Results

3.1. Stimulation by p,p′-DDT of the morphological differentiation of 3T3-L1 cells

Approximately 4 days after incubation in the presence of dexamethasone, IBMX, insulin, and fetal bovine serum, 3T3-L1 cells started to exhibit the morphology

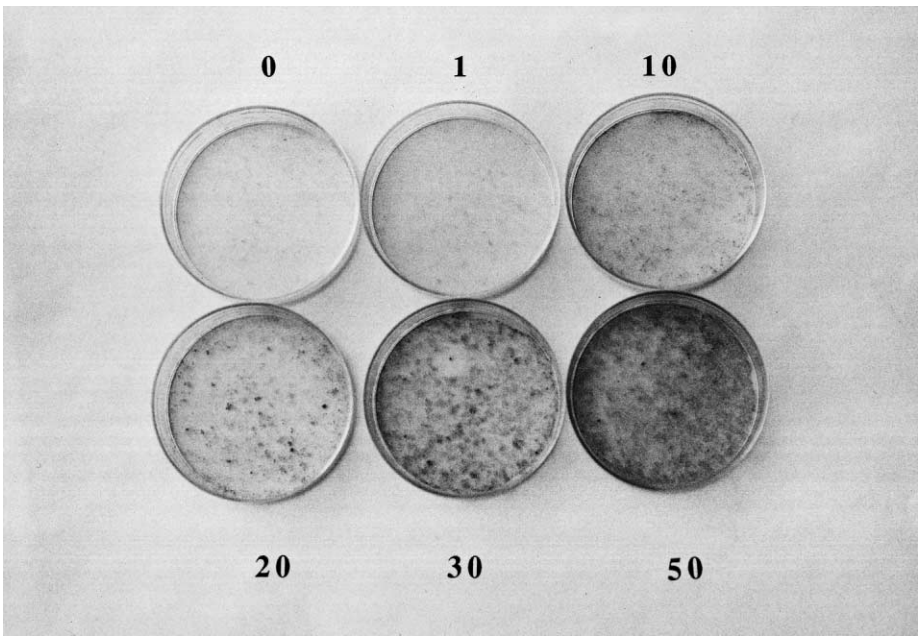

Fig. 1. Effect of *p,p′*-DDT on the differentiation of 3T3-L1 cells. The indicated micromolar concentrations of *p,p′*-DDT were added in ethanol along with the differentiation medium, and the same concentrations of pesticide were added when the medium was replaced. Four days after induction, dishes were stained with Oil Red O and photographed.

of adipocytes, including intracellular accumulations of lipid droplets which can be stained with Oil Red O.

As observed in Fig. 1, on day 4 after the induction of differentiation, *p,p′*-DDT-treated cells showed a significant increase in the abundance of cells exhibiting the characteristic phenotype of the adipocytes, in comparison to the untreated cells, as estimated by the Oil Red O staining. This observation suggested that *p,p′*-DDT accelerated the morphological conversion of 3T3-L1 preadipocytes into adipocytes in a concentration-dependent manner.

3.2. Effects of p,p-DDT on the expression of C/EBP and PPARγ proteins

Two families of transcription factors, the C/EBP proteins and PPARγ, have been shown to be implicated in the transcriptional up-regulation of adipogenesis [19]. We tested the effects of *p,p′*-DDT treatment on the temporal expression patterns of C/EBPα, C/EBPβ, and PPARγ during the differentiation of 3T3-L1 preadipocytes.

As observed in Fig. 2A, the C/EBPβ protein isoforms were highly expressed in the early stages of differentiation (day 2), but their levels decreased by late differentiation (day 6), and became almost undetectable by day 8 (data not shown). *p,p′*-DDT treatment (20 μM) did not cause dramatic alterations in the expression of the translational products of C/EBPβ, LAP 32 kDa and LIP 20 kDa proteins, in the course of differentiation.

Fig. 2B shows the expression pattern of C/EBPα during differentiation in control and *p,p′*-DDT-treated cells. In agreement with previous findings, our data showed that in control cells C/EBPα is induced relatively late in the course

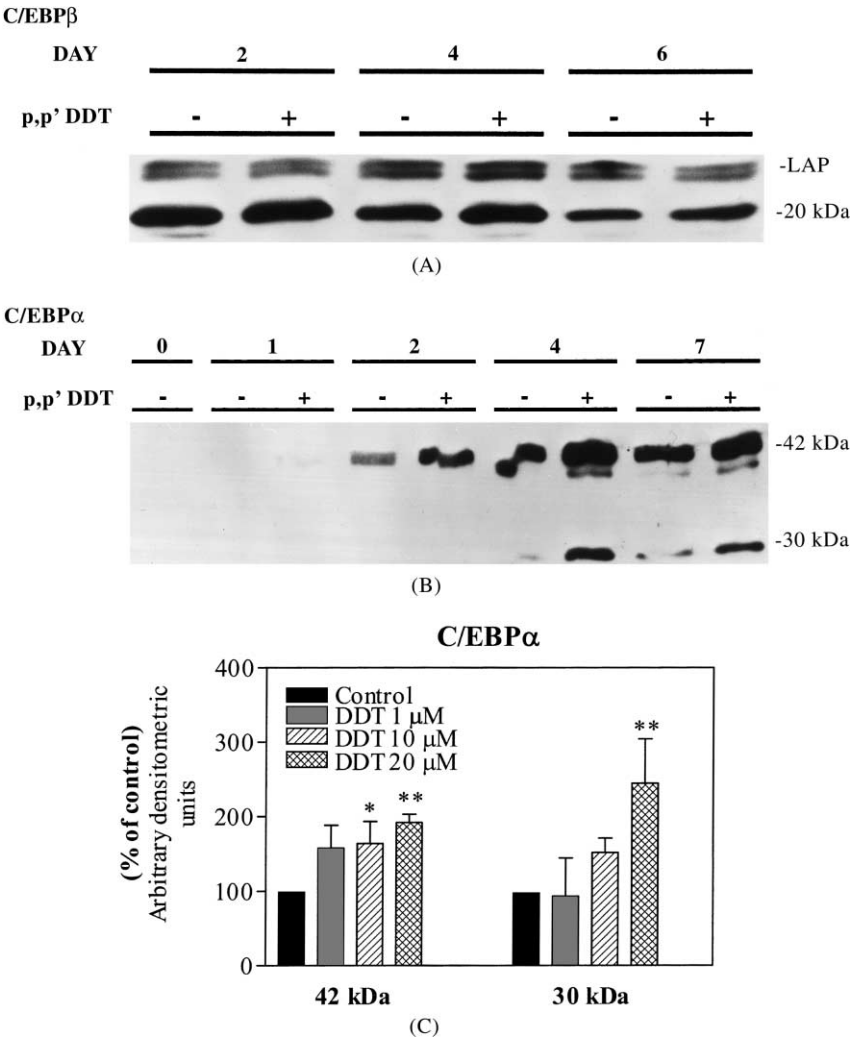

Fig. 2. Effects of *p,p′*-DDT on the expression of C/EBPα and C/EBPβ proteins during differentiation of 3T3-L1 cells. Nuclear extracts were prepared from control and *p,p′*-DDT-treated cells at different days after differentiation induction (0, 1, 2, 4, and 7). The extracts (20 μg) were subjected to gel electrophoresis, and immunoblotted using antisera against C/EBPα and C/EBPβ. (A) Western blot analysis of the effects of *p,p′*-DDT (20 μM) on C/EBPβ during the course of differentiation. (B) Representative western blot analysis of the effects of *p,p′*-DDT (20 μM) on C/EBPα during the course of differentiation. Blots are representative of at least two independent experiments. (C) Concentration response study of *p,p′*-DDT on C/EBPα protein expression in 3T3-L1 cells. Nuclear extracts were prepared from control and *p,p′*-DDT-treated cells (1, 10, and 20 μM) 3 days after induction of differentiation. Quantification of the western blots was performed by a densitometric evaluation, using an AMBIS radioanalytical-imaging instrument. The values (means ± SD of at least three independent experiments) are given as a percentage of control of each C/EBP isoform.(*) $P < 0.05$, and (**) $P < 0.01$, compared with the control cells.

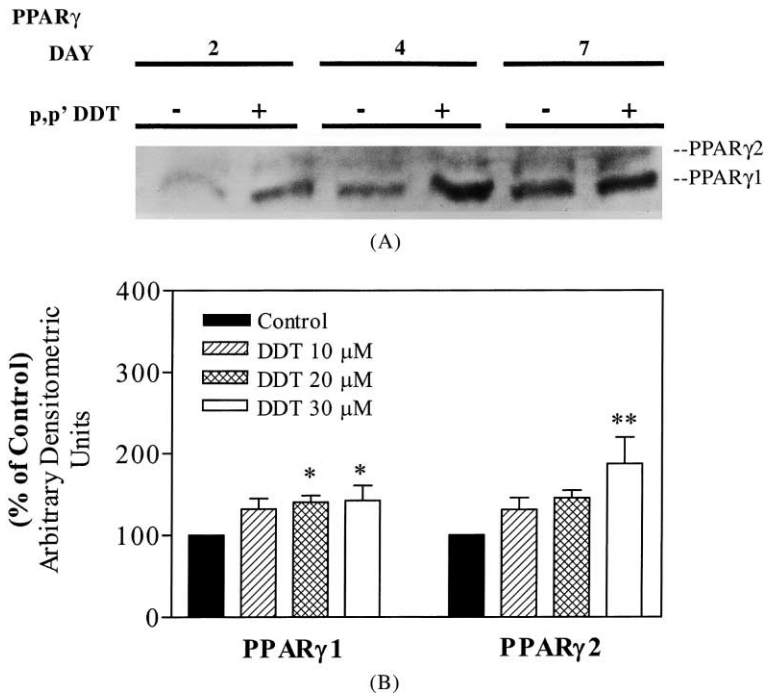

Fig. 3. Effects of *p,p′*-DDT on the expression of PPARγ protein isoforms. Immunoblotting analyses were performed in nuclear extracts (40 μg) prepared from control and pesticide-treated cells at different days in the differentiation process. (A) Western blot showing the effect of 20 μM *p,p′*-DDT on PPARγ protein isoforms at different stages of the differentiation process. (B) Effect of increasing concentrations of *p,p′*-DDT on PPARγ protein isoform levels at day 4 after the induction of differentiation. Quantification of the western blots was performed by a densitometric evaluation, using an AMBIS radioanalytical-imaging instrument. The values (means ± SD of at least three independent experiments) are given as a percentage of the control of each PPARγ isoform. (*) $P < 0.05$, and (**) $P < 0.01$, compared with the control cells.

of differentiation, i.e. by day 4 [7]. Treatment with 20 μM *p,p′*-DDT accelerated and stimulated significantly the expression of both translational products of C/EBPα, p30 and p42 kDa. This increase of C/EBPα induced by *p,p′*-DDT was also shown to be concentration-dependent (Fig. 2C).

The expression of both isoforms of PPARγ protein (PPARγ1 and PPARγ2) was also accelerated by *p,p′*-DDT after day 2 of differentiation (Fig. 3A). In addition, the higher the concentration of *p,p′*-DDT used, the higher the increase in PPARγ levels (Fig. 3B).

3.3. Effects of p,p′-DDT on the binding patterns of C/EBP proteins to DNA

To determine if *p,p′*-DDT treatment affects the ability of different C/EBP proteins to bind to DNA, electromobility shift assays were performed on different days of differentiation.

Our results showed an increase in the abundance of C/EBP–DNA complexes in nuclear extracts from *p,p′*-DDT-treated cells in comparison with control cells at day 2 of differentiation (Fig. 4A, lane 9 vs. 1, respectively).

The three C/EBP isoforms (α, β, and δ) have been shown to be able to bind to the same consensus C/EBP binding site. Supershift studies were performed, therefore, to identify which of the components of the C/EBP complexes was affected by *p,p′*-DDT treatment. Incubation of nuclear extracts with an antibody specific for C/EBPα supershifted the upper part of the C/EBP–DNA complexes, indicating that they corresponded to C/EBPα–DNA binding. A dramatic increase in the abundance of the C/EBPα–DNA band was observed in cells treated with *p,p′*-DDT at day 2 of differentiation (Fig. 4A, lane 10 vs. 2). When C/EBPβ and C/EBPδ were supershifted at the same time using antibodies specific against them, the remaining C/EBPα–DNA complexes could be observed. Again, an increase in *p,p′*-DDT-treated cells could be observed (Fig. 4A, lane 14 vs. 6). The intensity of the C/EBPα–DNA complexes observed after the supershift of β and δ at day 2 of differentiation was very slight, which was in agreement with the low levels of expression of this protein at this stage of differentiation.

With regard to C/EBPβ–DNA binding, when C/EBPβ was supershifted by using its specific antibody, a slight increase was observed in *p,p′*-DDT-treated samples at day 2 (Fig. 4A, lane 11 vs. 3). However, when C/EBPβ–DNA complexes were analyzed after the removal of C/EBPα and C/EBPδ with antibodies against these isoforms, no significant differences between control and treated samples were observed (Fig. 4A, lane 7 vs. 15). Furthermore, *p,p′*-DDT did not induce significant alterations in C/EBPδ–DNA binding (Fig. 4A, lane 12 vs. 4 in which C/EBPδ complexes have been supershifted by using antibody against C/EBPδ; compare gel shift profiles in lane 13 vs. 5 in which C/EBPδ complexes can be observed after α and β C/EBP isoforms have been supershifted). When

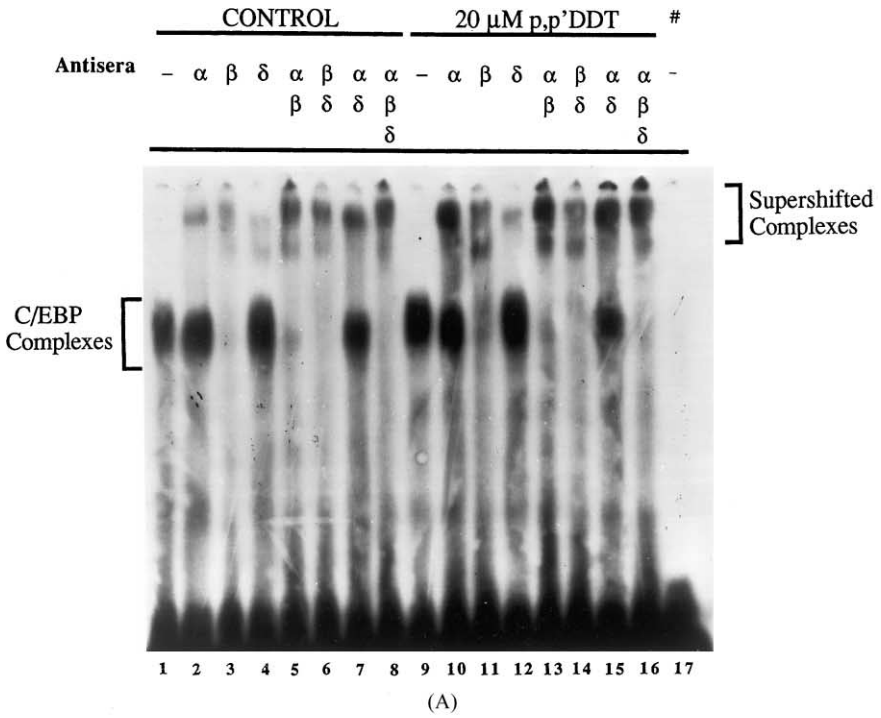

Fig. 4. Effects of *p,p′*-DDT treatment on the DNA binding capacity of C/EBP isoforms in 3T3-L1 cells. Gel shift analyses were performed on nuclear extracts prepared from control cells and cells treated with 20 μM *p,p′*-DDT, as described in Section 2. For supershift complexes, nuclear extracts (10 μg) were incubated with the specific antibody for each C/EBP protein (α, β, or δ), or with different combinations of these three, as indicated. Supershift analysis of C/EBP–DNA complexes was performed in nuclear extracts prepared from control and *p,p′*-DDT-treated cells at 2 (A) and 4 (B) days after the induction of differentiation. The # symbol indicates that a competition assay, using 100-fold excess of unlabeled oligonucleotide, was performed to obtain non-specific binding activity. Each lane is representative of two independent experiments.

antibodies against the three C/EBP proteins were used, almost all of the C/EBP–DNA complexes were supershifted (Fig. 4A, lane 16 vs. 8). An excess of 100-fold unlabeled oligonucleotide was added to calculate the non-specific binding activity (lane 17).

Supershift studies performed at days 4 (Fig. 4B) and 7 (data not shown) after the induction of differentiation also confirmed that *p,p′*-DDT caused a dramatic increase in the abundance of C/EBPα–DNA complexes (Fig. 4B, lane 6 vs. 2) with very minor increases in C/EBPβ and C/EBPδ

binding activities (Fig. 4B, lane 7 vs. 3 and 8 vs. 4, respectively).

3.4. Effects of p,p′-DDT on the antiadipogenic effects of TNFα in 3T3-L1 cells

Thiazolidinediones, a group of antidiabetic drugs that act like ligands for PPARγ, are potent stimulants of adipocyte differentiation [29]. It has been shown that these drugs are able to induce adipogenesis of post-confluent 3T3-L1 cells in the absence of the differentiation medium by the activation of an endogenous low level of PPARγ in the preadipocytes [18].

TNFα is a catabolic proinflamatory cytokine that potently inhibits adipocyte differentiation [30]. It has been suggested that the effects of TNFα are mediated by a negative regulation of the expression of PPARγ. Thiazolidinediones have been shown to prevent the antiadipogenic effects of TNFα and other inflammatory cytokines in 3T3-L1 cells [31].

We tested the ability of *p,p′*-DDT to block the inhibitory effect of TNFα on differentiation in 3T3-L1 cells. Fig. 5

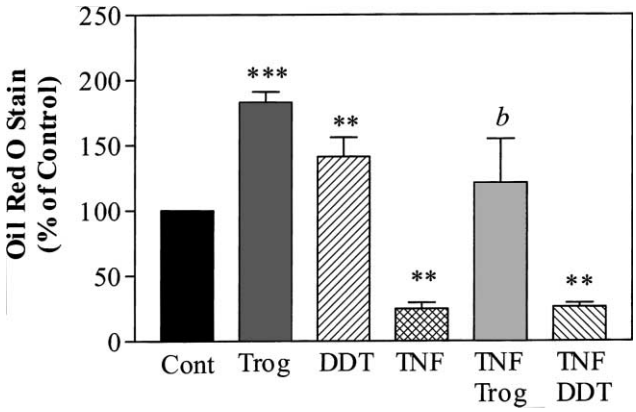

Fig. 5. Effects of *p,p′*-DDT and troglitazone (Trog) on the antiadipogenic effects of TNFα. Cells were treated with TNFα (5 ng/mL) in the absence or presence of 10 μM *p,p′*-DDT or 10 μM Trog at the same time that the differentiation medium was added, and then every time that the medium was replaced. After 8–10 days of differentiation, cells were fixed and stained with Oil Red O. Quantitative evaluation of the effects on adipocyte differentiation was performed by spectrophotometric analysis of Oil Red O-stained plates, as described in Section 2. Values are the means ± SEM of at least three independent experiments. (**) $P < 0.01$, and (***) $P < 0.001$, compared with control; (*b*) $P < 0.01$, compared with TNFα-treated cells.

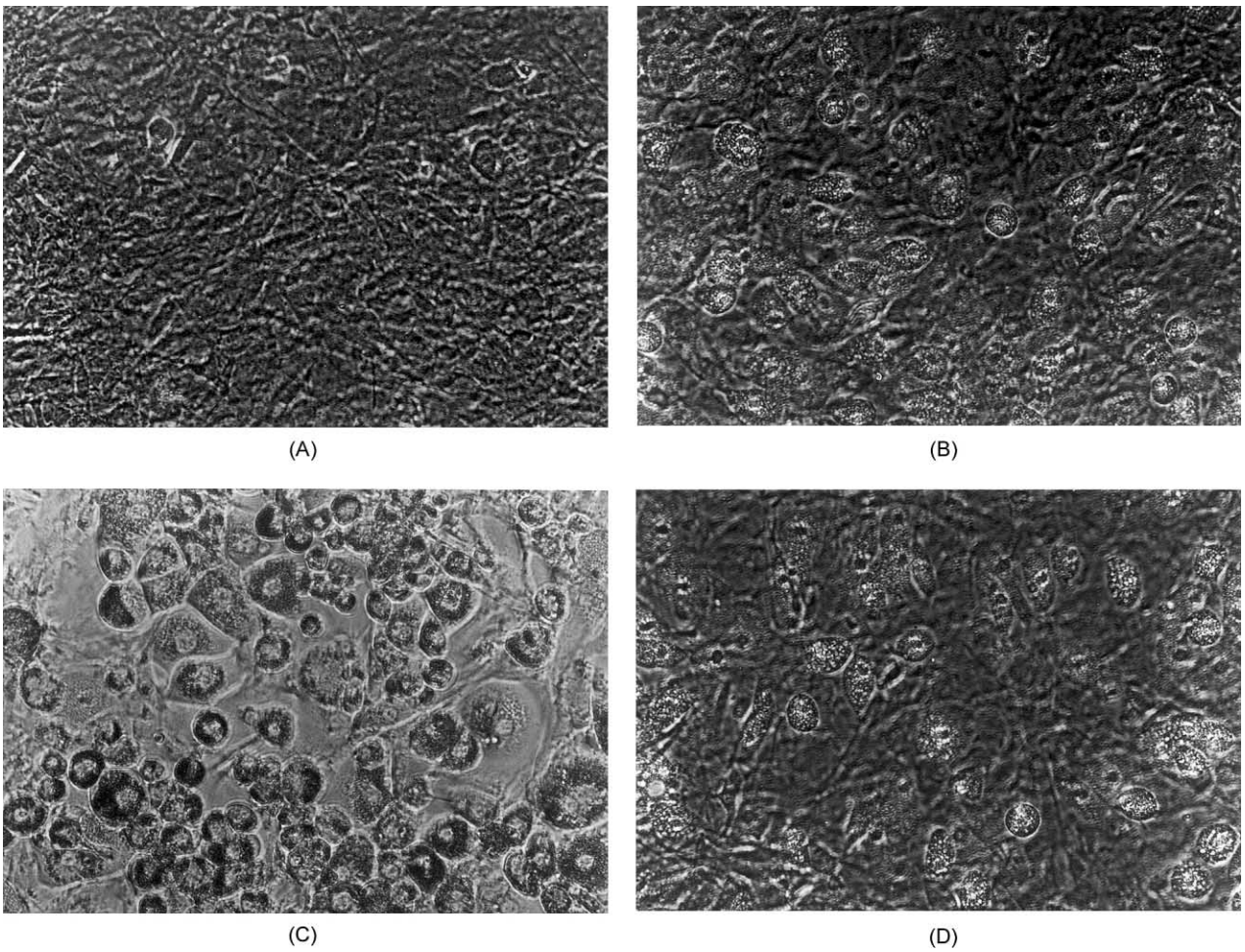

Fig. 6. Effect of *p,p′*-DDT on 3T3-F442A adipocyte differentiation. Photomicrographs of 3T3-F442A cells were taken 2 or 8 days post-initiation of differentiation. (A) Control cells at day 2; (B) 20 μM *p,p′*-DDT-treated cells at day 2; (C) control cells converted to adipocytes at day 8; and (D) 20 μM *p,p′*-DDT-treated cells at day 8. Original magnification, 20×.

shows that troglitazone, but not *p,p′*-DDT (10 μM) prevented the negative actions of TNFα on adipocyte differentiation.

3.5. Effects of p,p′-DDT on the differentiation of 3T3-F442A preadipocytes

3T3-F442A is another preadipocyte cell line frequently used to study the adipocyte differentiation process. The adipogenic process of 3T3-F442A preadipocytes does not require stimulation with IBMX and dexamethasone. Cells are able to differentiate into mature adipocytes in the presence of only FBS and insulin [1].

We studied the effects of *p,p′*-DDT on this adipogenic cell line. Cells were treated with 20 μM *p,p′*-DDT, a concentration that increased differentiation in 3T3-L1 cells. Two days after the treatment of confluent cells with insulin and FBS, dramatic differences were observed between the morphology of control and *p,p′*-DDT-treated cells (Fig. 6). As expected, the control cells did not exhibit the characteristic phenotype of adipocytes at this early stage of differentiation (Fig. 6A). However, *p,p′*-DDT-treated plates clearly showed an abundant number of cells having a more spherical shape (Fig. 6B), suggesting that *p,p′*-DDT was accelerating some differentiation process, which resulted in an adipocyte-like morphology in 3T3-F442A cells at an earlier stage of their differentiation. At day 8 after the induction of differentiation, control cells presented typical adipocyte morphological characteristics (Fig. 6C), including the accumulation of intracellular fat droplets. Cells treated with *p,p′*-DDT did not fully differentiate, and most of these cells exhibited the same characteristics shown at day 2 of differentiation (Fig. 6D).

C/EBPα is believed to terminate the mitotic clonal expansion preceding entry into the terminally differentiated state, and seems to also be involved in the maintenance of the fully differentiated state [1,19]. Immunoblot analysis for C/EBPα at day 4 of differentiation showed that *p,p′*-DDT-treated 3T3-F442A cells, which failed to become truly differentiated adipocytes, had lower levels of C/EBPα protein than the normally differentiated control cells (Fig. 7).

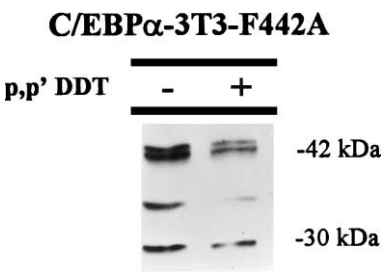

Fig. 7. Western blot analysis of the effects of *p,p′*-DDT (20 μM) on the C/EBPα protein level in 3T3-F442A cells. Nuclear extracts were prepared from control and treated cells 4 days after the induction of differentiation. Blots are representative of two independent experiments.

4. Discussion

In the present paper, we have shown (a) the ability of *p,p′*-DDT to alter adipocyte differentiation and (b) that these effects correlate with changes induced in the expression of C/EBPα and PPARγ, the main transcription factors regulating the adipogenic process.

Several studies have provided evidence that C/EBPα is not only required, but its up-regulation alone is sufficient to trigger differentiation of preadipocytes in the absence of exogenous hormonal inducers [20,32,33]. Our data have shown that, *p,p′*-DDT induces a dramatic increase in C/EBPα protein levels from day 2 of differentiation in 3T3-L1 cells. C/EBPα inhibits cell proliferation and, therefore, has been suggested to play a key role in terminating mitotic clonal expansion [19,34]. *p,p′*-DDT-treated cells also showed an increase in the binding activity of C/EBPα to its DNA response element. C/EBPα has been shown to bind the promoter and coordinately activate the transcription of many adipocyte-specific genes such as *aP2*, *PEPCK*, *leptin*, and *GLUT4* and to maintain the fully differentiated state by autoactivating transcription of its own gene [35,36]. The increase induced by *p,p′*-DDT on C/EBPα–DNA binding would be expected to lead to a rise in the activation of the transcription of many adipocyte genes including C/EBPα itself, resulting in the accelerated conversion into mature adipocytes observed in the *p,p′*-DDT-treated cells.

Ectopic expression of PPARγ has also been shown to be capable of triggering the entire program of adipogenesis in fibroblasts [37]. It has been demonstrated that when PPARγ and C/EBPα are co-expressed in fibroblasts, they act synergistically to induce adipogenesis [38]. *p,p′*-DDT also caused a stimulation in the expression of PPARγ, which together with the increase in the expression of C/EBPα would be expected to contribute to the adipogenic effects of the pesticide in 3T3-L1 cells. The remaining question then is how does *p,p′*-DDT influence the expression of PPARγ and C/EBPα.

C/EBPβ and C/EBPδ, which are known to be increased by IBMX and dexamethasone, respectively, act as earlier transcriptional activators than C/EBPα and PPARγ in the sequence of events leading to adipocyte differentiation in the 3T3-L1 model [39,40]. It has been observed that conditions that cause expression of either C/EBPβ or δ do indeed accelerate adipogenesis in 3T3-L1 preadipocytes [21]. However, the lack of dramatic changes in the expression or binding activity of C/EBPβ or C/EBPδ in *p,p′*-DDT-treated cells argues against their involvement in mediating the adipogenic effects of the pesticide.

PPARγ agonists such as the thiazolidinediones, a new class of antidiabetic drugs, have been shown to act strongly as adipogenic agents for fibroblasts [29]. Thus, thiazolidinediones are capable of reversing the inhibitory actions of agents such as TNFα, interleukin-1 and -6 (IL-1 and IL-6) [31]. Our results, however, suggest that it is very

unlikely that *p*,*p*′-DDT acts as a direct ligand activating PPARγ. The main reasons for such a conclusion are: (a) the antiadipogenic effect of TNFα could be prevented by PPARγ activators such as troglitazone, but *p*,*p*′-DDT was unable to antagonize TNFα actions, and (b) thiazolidinediones are potent inducers of adipogenesis in 3T3-F442A cells [5], but these cells are unable to become fully differentiated adipocytes in the presence of *p*,*p*′-DDT.

Several studies have shown evidence that the expression and activity of PPARγ and C/EBPs can be regulated by the MAPK cascade [41–44]. It has also been suggested that signal transducer and activator of transcription (STAT)5 and STAT1 could be potential inducers of genes that contribute to the development and maintenance of the adipocyte phenotype [45]. Studies in human breast epithelial cells have shown the ability of *p*,*p*′-DDT treatment to induce an increase in MAPK phosphorylation and activation, and also to stimulate STAT1 tyrosine phosphorylation [15]. These observations suggest the possibility that the stimulation in the expression and activity of C/EBPα and PPARγ observed in response to *p*,*p*′-DDT treatment in 3T3-L1 cells could be mediated by changes in the phosphorylation of MAPKs and/or STATs induced by the pesticide. On the other hand, DDT has also been shown to stimulate human breast cells to enter the cell cycle by allowing the release of E2F after the hyperphosphorylation and inactivation of pRb105 [11,12]. E2F and the Rb proteins have been implicated in regulating the mitotic clonal expansion necessary for adipocyte differentiation in 3T3-L1 cells [17,18]. This suggests the possibility that *p*,*p*′-DDT might stimulate the mitotic clonal expansion prior to terminal differentiation. In support of this hypothesis, Brown and Lamartiniere [46] demonstrated that *o*,*p*′-DDT given to pubertal rats acts as a morphogen, increasing mammary gland differentiation by promoting cell proliferation. However, further experiments are necessary to test these possibilities.

p,*p*′-DDT elicits a different effect on adipocyte differentiation in 3T3-L1 cells than in 3T3-F442A cells, another adipogenic cell line. These preadipocyte cell lines present some fundamental differences in the requirements needed to become fully differentiated adipocytes. In contrast to 3T3-L1, 3T3-F442A cells do not require pharmacological doses of IBMX and dexamethasone for conversion to fat cells. Previous experiments of our group have shown that the level of C/EBPβ is consistently elevated in the 3T3-F442 cell line, even before the addition of the differentiation inducers.[1] Additionally, a differential sensitivity between these two cell lines to the action of some xenobiotics, such as TCDD, on the adipogenic conversion has been demonstrated. TCDD induced a strong inhibition on the differentiation of 3T3-L1 cells, while differentiation of 3T3-F442A preadipocyte cells was not suppressed by TCDD (see footnote). Other differences between these cell lines such as their ability to express and produce leptin have been observed. 3T3-F442A cells express leptin at a much higher level than do 3T3-L1 adipocytes. It has been observed that, the level of leptin expression is related to the differentiation method used. When 3T3-L1 cells are differentiated in the presence of indomethacin/insulin, leptin expression increases dramatically. However, when 3T3-F442A preadipocytes are differentiated using the same method, leptin expression levels drop precipitously [47]. The different effects of *p*,*p*′-DDT on adipocyte differentiation observed in 3T3-L1 and 3T3-F442A cells could be due, therefore, to differences in cell physiology and differentiation requirements between the two cell lines.

In conclusion, our results showed, for the first time, the ability of *p*,*p*′-DDT to alter the differentiation process of adipocytes in two preadipocyte cell lines. The stimulation of the adipogenic process observed in *p*,*p*′-DDT-treated 3T3-L1 cells was mediated by an increase mainly in C/EBPα and to a lesser extent in PPARγ, two adipogenic transcription factors. The mechanisms leading to the stimulation of these nuclear factors by *p*,*p*′-DDT remain unclear. More studies are necessary to elucidate the cause for the aberrant differentiation observed in *p*,*p*′-DDT-treated 3T3-F442A cells. Further studies are also required to determine if the levels of DDT that alter adipocyte differentiation in these *in vitro* models are able to induce biologically significant effects *in vivo*.

Acknowledgments

This work was supported by Research Grants R01-ES05233, ES03575, and ES05707 from the National Institute of Environmental Health Sciences, and by a postdoctoral fellowship from the Departamento de Educación y Cultura del Gobierno de Navarra (1997) and from the Ministerio de Educación y Cultura of Spain (1998) to Maria J. Moreno-Aliaga.

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