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Endrin Inhibits Adipocyte Differentiation by Selectively Altering Expression Pattern of CCAAT/Enhancer Binding Protein-α in 3T3-L1 Cells

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ABSTRACT

The effects of selected chlorinated cyclodiene pesticides on the adipocyte differentiation process were examined using the 3T3-L1 adipocyte model in vitro. Endrin was found to cause a dose-dependent inhibition of adipocyte differentiation in 3T3-L1 cells. Aldrin and dieldrin were less potent than endrin in interfering with the adipogenic process. Endrin's inhibitory effect was effective only when the pesticide was present in the medium during the first 48 h after exposure of 3T3-L1 cells to adipogenic inducers. Immunoblots analysis revealed that endrin caused a dose-dependent, selective inhibition of the intracellular levels of CCAAT enhancer binding protein (C/EBP)α without altering the expression patterns of C/EBPβ or C/EBPδ along the differentiation. Supershift analysis showed that DNA-binding capacity of C/EBPα was affected most by endrin treatment. Endrin also caused a decrease in the elevation of the adipogenic factor peroxisome proliferator-activated receptor (PPAR)γ elicited by the adipogenic inducers. However, the cotreatment with troglitazone, a thiazolidinedione known to activate PPARγ, did not suppress the antiadipogenic action of endrin, indicating that its direct action site is not PPARγ receptor. Endrin also altered the pattern of activation of nuclear factor-κB, a factor activated by 12-*O*-tetradecanoylphorbol-13-acetate and tumor necrosis factor-α, which are known to interfere with adipocyte differentiation. Thus, endrin inhibited the normal decrease in nuclear factor-κB-DNA binding observed as cells are acquiring the adipocyte phenotype at a late stage of differentiation. Our results suggest that endrin inhibits adipocyte differentiation through the specific suppression of C/EBPα.

The polyhalogenated cyclic hydrocarbons include a variety of pesticides, such as endrin and contaminants of pesticides like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hassoun and Stohs, 1996a). It is well known that many of environmental xenobiotics are stable lipophilic compounds; therefore, adipose tissue constitutes the main place for their accumulation in the organisms. It must be emphasized that adipose tissues are not a mere inert storage site for those xenobiotics; rather, several recent studies have shown that they are biochemically very active tissues responding to many hormonal, nutritional, inflammatory, and growth-regulating factors (Spiegelman, 1998). Because of this, adipocytes are a major target for the toxic actions of these lipophilic chemicals.

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Several studies performed by our group have shown that TCDD and related compounds cause major alterations of the function of adipose tissue (Enan et al., 1992; Liu and Matsumura, 1995), as well as adipogenic cell lines in culture, including the suppression of adipocyte differentiation (Phillips et al., 1995).

Endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4α-5,6,7,8,8α-octahydroendo, endo-1,4:5,8-dimethano-naphthalene) is a highly toxic chlorinated cyclodiene pesticide. As reported by Hassoun and Stohs (1996a), endrin's toxic effects in laboratory animals are similar in some aspects to those caused by TCDD, including hepatotoxicity, induction of drug-metabolizing enzymes, thymic hypoplasia, weight loss, and, eventually, death. Several studies have given strong evidence that free radical-mediated lipid peroxidation is contributing to the toxicity of TCDD and endrin (Bagchi and Stohs, 1993; Hassoun and Stohs, 1996b). A recent study has shown that the enhanced production of reactive species observed after in vitro and in vivo endrin treatment may interfere with the protein kinase (PK)C signaling pathway. It has been suggested that this could lead to

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; C/EBP, CCAAT enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; IBMX, 3-isobutyl-1-methylxanthine; PKC, protein kinase C; EMSA, electrophoresis gel mobility shift assay; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

altered cell proliferation and differentiation (Bagchi et al., 1995, 1997).

Taking into account that cyclodiene pesticides accumulate mainly in adipose tissue and the adipocyte differentiation process plays an important role in obesity and other metabolic diseases, we considered it to be extremely worthwhile to determine the effects of these xenobiotics on this differentiation process. The studies were performed using 3T3-L1 cells, which have been shown to be a very adequate, yet convenient cell culture model for the investigation of the program of adipose differentiation (Green and Kehinde, 1974).

Adipocyte differentiation is a complex process that is mainly controlled by two families of transcription factors: the CCAAT enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPARs). Several members of the C/EBP family of basic leucine zipper proteins have been identified (Cao et al., 1991). Several reports have provided strong evidence that C/EBPα plays an important role in the transcriptional control of the adipocyte differentiation process. In addition, binding sites for C/EBPα have been identified in the promoters of several adipocyte-specific genes, such as aP2 (reviewed by MacDougald and Lane, 1995). C/EBPβ and C/EBPδ, which are induced significantly earlier than C/EBPα in the time course of differentiation, also play a role in the control of adipogenesis (Wu et al., 1995, 1996).

PPARγ is the only member of the PPAR family that has been shown to be expressed at high levels specifically in adipose tissue. Like C/EBPα, the retroviral expression of PPARγ is sufficient to induce the conversion of several fibroblast cell lines into adipocytes (Tontonoz et al., 1994). It has been shown that the simultaneous coexpression of PPARγ and C/EBPα in fibroblast has a synergistic effect on the induction of adipocyte conversion process.

To determine the mechanism or mechanisms by which endrin and its analogs were interfering with the adipocyte differentiation process, its effects on the expression of the different C/EBP proteins as well as PPARγ were studied. We also investigated the effects of endrin on the capacity of the different C/EBPs to bind to a ^{32}P-labeled oligonucleotide containing the C/EBP response element.

Adipocyte differentiation can be affected by a large number of mitogens and growth factors. Tumor necrosis factor (TNF)α and other cytokines have also been shown to be potent inhibitors of adipogenesis (Zhang et al., 1996). Nuclear factor-κB (NF-κB) is a transcription factor that specifically carries the TNFα message (Beg et al., 1993). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) treatment is known to increase the nuclear level of NF-κB by increasing PKC activity in cytosol (Steffan et al., 1995). As previously indicated, endrin has been shown to induce an increase in PKC activity, which could lead to an activation of NF-κB transcriptional activity. To test this possibility, electrophoresis gel mobility shift assay (EMSA) analysis for NF-κB-DNA binding was performed in control and endrin-treated cells.

Materials and Methods

Cell Culture and Differentiation. 3T3-L1 cells (Green and Kehinde, 1974) were obtained from American Type Culture Collection (Rockville, MD). Passages 3 through 9 were used in all studies. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf bovine serum. Confluent cells were induced to differentiate by incubation for 48 h with differentiation medium containing 1 μM dexamethasone, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/ml insulin, and 10% FBS in Dulbecco's modified Eagle's medium. After this time, cells were maintained in postdifferentiation medium containing 10 μg/ml insulin and 10% FBS, and the medium was changed every 2 days. To study the effects of some pesticides on differentiation, different concentrations of the pesticides were added along with the differentiation medium for 48 h. The same concentration of the pesticides was maintained when the medium was replaced. Pesticides, obtained from Polysciences (Niles, IL), were prepared as 1000× stocks in ethanol. The same quantity of this solvent was added to the control cell culture plates. Troglitazone was obtained from Dr. Ohsumi (Sankyo Company, Tokyo, Japan).

Oil Red O Staining. Eight or 10 days after the induction of differentiation, cells were stained with Oil Red O according to Kasturi and Joshi (1982). Briefly, cells were washed twice with PBS and fixed with 10% formalin in PBS for 1 h; then they were washed an additional three times with water and dried. Cells were stained with Oil Red O [6 parts of saturated Oil Red O dye (0.6%) in isopropanol and 4 parts of water] for 15 min. Excess of stain was removed by washing with 70% ethanol, and then stained cells were washed with water. In some experiments, spectrophotometrical 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. Then, the absorbance was measured at 520 nm.

Preparation of Nuclear Extracts. Nuclei were isolated from 3T3-L1 cells according to a slightly modified method of Dignam et al. (1983). Briefly, cells were washed and scraped into PBS and then centrifuged for 10 min at 1850*g* at 4°C. The cell pellets were resuspended in 5 volumes 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) and centrifuged for 5 min at 1850*g* at 4°C. Then, the packed cells were resuspended in 3 volumes of hypotonic buffer and allowed to swell for 10 min on ice. After that, cells were homogenized with 10 strokes using a glass Dounce homogenizer and then centrifuged for 15 min at 3300*g* at 4°C. The pellets obtained were the nuclei. Nuclear extracts were prepared from these nuclei by a slight modification of the method of Lavery and Schibler (1993). Pelleted nuclei were resuspended in 1.1× extraction buffer (300 mM NaCl, 1 M urea, 1% Nonidet P-40, 1 mM dithiothreitol, and 25 mM HEPES, pH 7.9), mixed vigorously by vortex, 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°C in a microfuge. The supernatants were adjusted to 10% glycerol and then rapidly frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined as described by Bradford (1976).

Western Blot (Immunoblot) Analysis. Twenty micrograms of nuclear protein was fractionated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. After transfer, Poinceau S staining was performed to ensure equal loading of each sample. To block nonspecific binding membranes were incubated in TBST (150 mM NaCl, 10 mM Tris, 0.09% Tween 20, pH 8.0) containing 10% milk for 1 h at room temperature. The blots were then incubated in TBST containing 5% milk with primary antibody, polyclonal anti-C/EBPα or anti-C/EBPβ or anti-C/EBPδ antiserum (1:1000 dilution for anti-C/EBPα and anti-C/EBPβ and 1:500 for C/EBPδ; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h. After washing for three times with TBST, the membranes were incubated in TBST containing 5% milk with secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit; 1:2000, Amersham Life Sciences, Arlington Heights, IL) for 1 h. After three washes with TBST, the blots were developed by the enhanced chemiluminescence detection system (Pierce, Rockford, IL) and visualized by exposure to autoradiography film. Immunoblotting analyses for PPARγ were performed in similar conditions using 30 μg of nuclear extracts. Polyclonal anti-PPARγ antibody (1:500 dilution) and secondary antibody, horseradish peroxidase-conjugated an-

ti-goat IgG (1:3000) were purchased from Santa Cruz Biotechnology, Inc.

Electrophoretic Mobility Shift Assays. A double-stranded oligonucleotide corresponding to the C/EBP binding site in the C/EBPα promoter (Christy et al., 1991) or NF-κB response element (Stratagene, La Jolla, CA) was end-labeled using [γ-^{32}P]ATP (Amersham Life Sciences) and T4 polynucleotide kinase (Promega, Madison, WI) according to the 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 dithiothreitol, with 3 μg of poly[d(I-C)] (Boehringer Mannhein, Indianapolis, IN) and 5 μg of acetylated BSA for 30 min at 4°C. A 100-fold excess of specific competitor was added to some samples. Then, the radiolabeled double-stranded oligonucleotide (100,000 cpm) was added and incubated for an additional 20 min at 4°C. For supershift assay, the specific antibodies anti-C/EBPs (α, β, or δ; from Santa Cruz Biotechnology, Inc.) or combinations of them were added at the end of the second incubation period, mixed gently, and incubated for 20 min at 4°C. Oligonucleotide-C/EBP binding was determined by electrophoresis in a nondenaturing 4% polyacrylamide gel at 150 to 200 V for 3 to 4 h at 4°C. Gels were dried for 2 h at 80°C and exposed to X-ray film in the presence of an intensifying screen at −80°C.

Statistical Analysis. Data were evaluated statistically by one-way ANOVA followed by Fisher's PLSD test or Student's t test at the significant level of $p < .05$.

Results

Effect of Some Cyclodiene Pesticides on Cell Morphology During Adipocyte Differentiation. It is well known that 3T3-L1 preadipocytes are able to initiate their conversion to mature adipocytes after the addition to the incubation medium of the differentiation inducers dexamethasone, insulin, and isobutylmethylxantine in the presence of FBS for 48 h. During this differentiation process, cells start to exhibit the morphology of adipocytes, including intracellular accumulations of lipid droplets, which can be stained with Oil Red O.

To test the effects of several pesticides on the conversion of 3T3-L1 fibroblast to adipocytes, the xenobiotics were added to the culture medium at the same time of the differentiation inducers and incubated for 48 h. The same concentration of pesticide was added subsequently in 48-h intervals each time the medium was changed to a fresh one. Under these conditions, our results have shown that some cyclodiene pesticides are able to inhibit the adipocyte differentiation process, antagonizing the action of these inducers.

Figure 1, top, shows plates of control and endrin-treated cells, at different concentrations, stained with Oil Red O 8 days after differentiation induction. As can be observed, endrin clearly induced a dose-dependent inhibition on adipocyte differentiation.

The microscopical examination of endrin-treated cells along the differentiation process did not reveal any sign of cytotoxicity caused by pesticide treatments. Indeed, 48 h after the induction of differentiation, control and treated cells, even at high dose (30 μM), present a similar morphology. Only from the third or fourth day, when intracellular fat droplets start to appear in control cells, did differences induced by pesticide treatment start to be exhibited. However, to ensure that endrin- induced inhibition of adipocyte differentiation is not related with an extensive damage of the cultures by pesticide presence, a trypan blue exclusion test was performed. The test results showed that the percentage of cell death in control and endrin (10 and 30 μM)-treated cells was similar or even slightly lower in treated cells (9.28 ± 1.42% in control versus 9.51 ± 1.51% and 5.6 ± 1.61% in 10 and 30 μM endrin-treated cells, respectively; these results are expressed as mean ± S.E.M. of at least five independent experiments).

Figure 1, bottom, shows microphotographs of 3T3-L1 fibroblast and cells obtained 8 days after the induction of differentiation. In comparison with preadipocytes (Fig. 1, bottom, A), the control cells (Fig. 1, bottom, B) show the typical adipocyte morphology, in which intracellular fat droplets can be easily observed. When 10 μM endrin was present in the medium, the percentages of cells exhibiting adipocyte morphology are considerably reduced (Fig. 1, bottom, C), and when pesticide is added at 30 μM concentration, the conversion from preadipocytes to adipocytes was almost completely blocked, and cells retained the morphology of preadipocytes (Fig. 1, bottom, D) in agreement with the Oil Red O test results.

The effects of other endrin analogs, such as aldrin, dieldrin, and heptachlor epoxide, on adipocyte differentiation

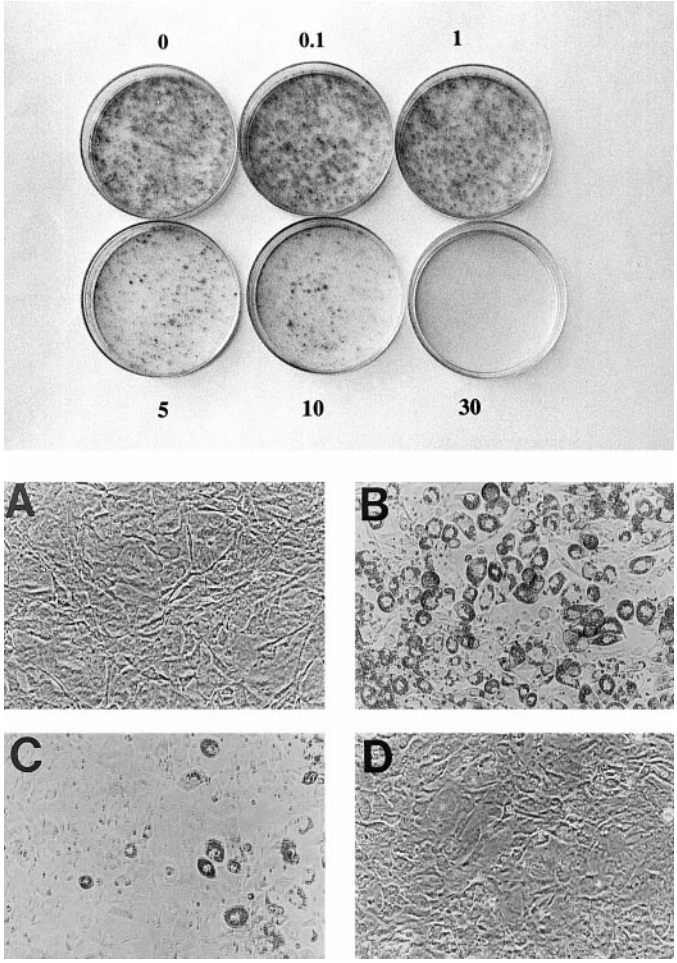

Fig. 1. Effect of endrin dose on adipocyte differentiation. Top, the indicated micromolar concentrations of endrin were added in ethanol along with differentiation medium, and the same concentrations of pesticide were added when the medium was replaced. Ten days after induction, dishes were stained with Oil Red O and photographed. Bottom, photomicrographs of 3T3-L1 cells taken 8 days after initiation of induction: A, preadipocytes; B, control cells converted to adipocytes; C, 10 μM endrin-treated cells; D, 30 μM endrin-treated cells. Original magnification, 20×.

were also studied. All compounds were tested at three concentrations (1, 10, and 30 μM) in parallel with same concentrations of endrin. Figure 2A shows the Oil Red O-stained cells plates after 8 days of treatment in the absence or presence of 10 and 30 μM endrin, dieldrin, or aldrin. Spectrophotometrical quantification of the stain was also performed, and the results are shown in Fig. 2B. As can be observed, endrin was found to be more potent than dieldrin or aldrin in interfering with adipocyte differentiation. Endrin caused a significant inhibition even at 1 μM concentration (77.66% of control value, which is considered as 100%) and progressive inhibition at higher doses (59.87, 28.78, and 7.05% of control for 5, 10, and 30 μM, respectively). In comparison, aldrin showed an inhibitory effect on adipocyte differentiation only at the highest dose studied (30 μM), inducing a decrease of 11.75% on lipid accumulation as estimated by Oil Red O stain. With regard to the effect of dieldrin, the conversion of preadipocytes to adipocytes at 30 μM was only 6.71% of control values. However, the treatment with lower doses (1 and 10 μM) did not show any significant inhibitory effect on adipocyte differentiation. Heptachlor showed no inhibitory effect even at the highest dose tested (data not shown).

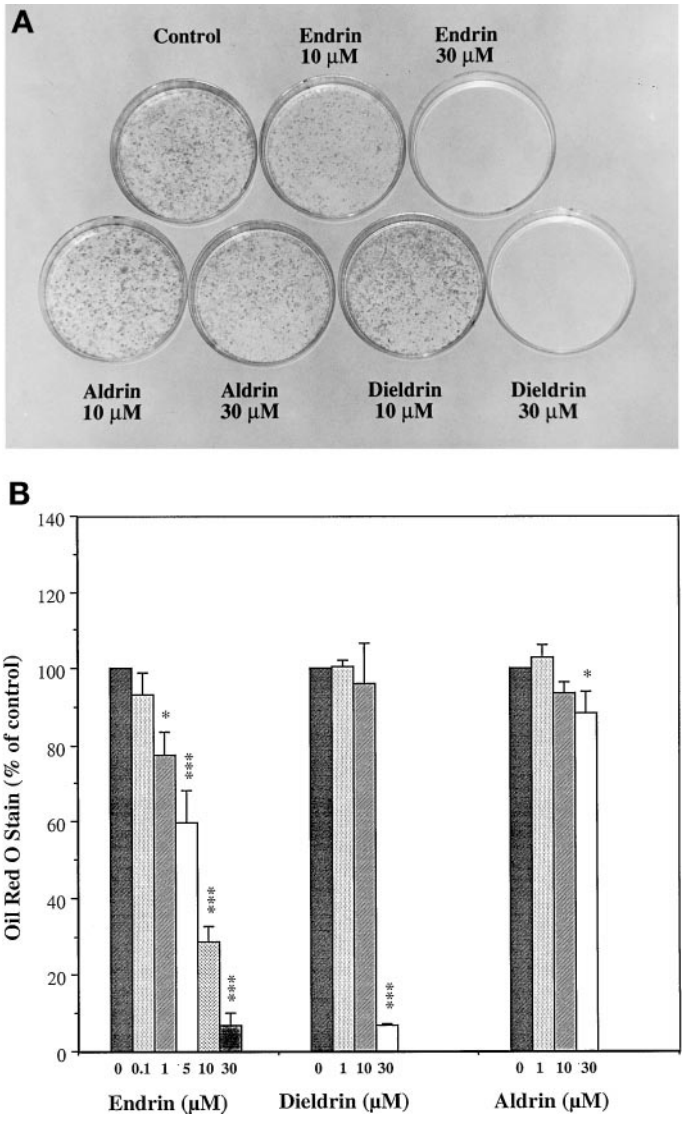

Fig. 2. Effect of several cyclodiene pesticides on adipocyte differentiation. A, cells were treated with the indicated concentration of each pesticide and stained with Oil Red O 8 days after the induction of differentiation. B, quantitative evaluation of the effects on adipocyte differentiation of several doses of cyclodiene pesticides performed by spectrophotometrical analysis of Oil Red O stained-plates, as described in *Materials and Methods*. Results are expressed as a percentage of the value found in the control adipocytes. Values are given as mean ± S.E.M. of at least three independent experiments. $^{*}p < .05$, $^{***}p < .001$.

Effects of Time of Endrin Treatment on Adipocyte Differentiation. As previously indicated, the incubation of confluent cells with differentiation inducers for 48 h initiates the adipocyte differentiation program in this cell line. Some chemical agents, such as retinoic acid, which is known to interfere with this overall process, has been shown to be ineffective if they are added after this critical initiation period (Schwarz et al., 1997). To test whether the endrin effect on adipocyte differentiation depends on the period of differentiation process in which pesticide is added, we conducted an experiment in which endrin (10 and 30 μM) was added to the incubation medium either 2 days before the addition of differentiation medium (day −2), at the same time as the addition of differentiation inducers (day 0), or 1, 2, or 4 days after the differentiation medium. Results shown in Fig. 3 indicate that endrin effects on adipocyte differentiation depend on the period of the time of addition. Suppression of fat colonies was more pronounced when it was added before or at the same time of the addition of differentiation medium. When endrin was added 1 or 2 days after the addition of the differentiation inducers, the inhibitory effect of endrin was not so pronounced, especially in the case of day 2 experiment. However, the addition of endrin to the medium 4 days after the initiation of differentiation, even at 30 μM concentration, could not reverse the differentiation process that was already initiated. In this case, the range of differentiation of endrin-treated cells as estimated by Oil Red O stain was the same as that found in control even though this pesticide was present for a long exposure period (data not shown).

It appears to be reasonable to conclude, therefore, that the first 48 h in the presence of the differentiation inducers is the critical period for initiating the differentiation process and that endrin must work mainly during this first 48 h to inhibit the adipocyte differentiation process. On the other hand,

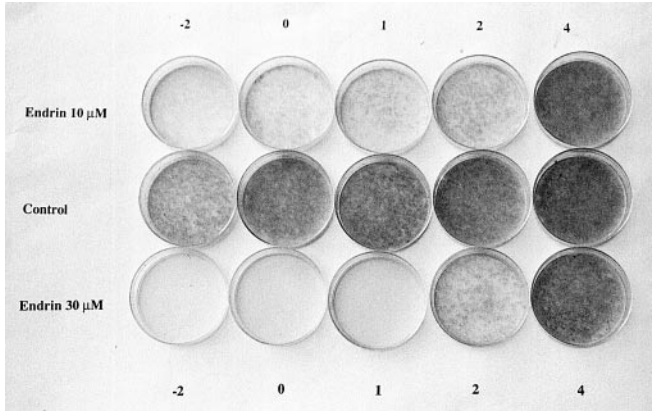

Fig. 3. Effect of time of endrin treatment on adipocyte differentiation. Pesticide (10 and 30 μM) was added to the cell culture plates 2 days before the addition of differentiation medium (−2), at the same time as differentiation medium (0) or 1, 2, or 4 days after differentiation medium addition. Then, the pesticide was added each time the medium was replaced. At 8 days after induction, dishes were stained with Oil Red O and photographed.

another test result showed that if endrin is removed from the medium after 48 h treatment, the degree of adipogenesis in the treated samples was similar to that seen in control cells (data not shown). Therefore, the continued presence of endrin in the medium is also required to prevent differentiation.

Effect of Endrin on Cellular Levels of C/EBP Isoforms in Differentiating 3T3-L1 Cells. The important role of C/EBP proteins in the transcriptional control of adipogenesis has been extensively described (Lin and Lane, 1992, 1994; Yeh et al., 1995; Wu et al., 1996). To determine whether endrin altered C/EBP proteins expression during the differentiation, immunoblots on nuclear extracts of control and pesticide-treated cells were performed. Figure 4A shows the representative Western blots analyzing the effect of endrin on the three C/EBP proteins described (C/EBPα, C/EBPβ, and C/EBPδ). The results of densitometric evaluation of Western blots from at least two independent experiments were summarized and are shown in Fig. 4B. The pattern of changes of temporal expression of both translational C/EBPα proteins ($p42^{C/EBP\alpha}$ and $p30^{C/EBP\alpha}$) in control cells is in close agreement with the previous observations reported from this laboratory (Liu et al., 1996). Endrin treatment at 10 μM concentration induced a significant decrease in the titer of both p42 and p30 C/EBPα proteins, starting from day 1 of treatment.

With regard to C/EBPβ, two translational products are observed: the C/EBPβ isoform designated as liver activator protein and a smaller-molecular-weight protein of 20 kDa. As described previously (Liu et al., 1996), the expression of both C/EBPβ isoforms presented high values at early stages of the differentiation process (1 and 2 days), and from day 4, C/EBPβ protein levels start to decrease. Endrin treatment did not induce significant alterations in this temporal expression pattern of either of C/EBPβ proteins. C/EBPδ protein was also expressed at high levels during the 48-h induction period (days 1 and 2), and similar to what was observed with C/EBPβ protein, endrin treatment did not modify C/EBPδ cellular levels.

Previously, we showed that intracellular accumulation of fat droplets, characteristic of the conversion to adipocytes, evaluated by Oil Red O stain, was inhibited by endrin in a dose-dependent manner. Figure 5A shows that the inhibition of C/EBPα protein was also endrin concentration dependent. C/EBPα protein levels were also determined after the treatment of cells with different concentrations of dieldrin and aldrin. As predicted from the previous results of Oil Red O testing, the treatment with 30 μM dieldrin caused a most significant decrease in the C/EBPα protein expression (Fig. 5B). In addition, as showed in Fig. 5C, a direct correlation between C/EBPα inhibition and the decrease in intracellular accumulation of fat droplets after endrin and dieldrin treatment can be observed.

EMSA on Effect of Endrin on DNA-Binding Capacities of C/EBP Isoforms. To determine whether the effect of

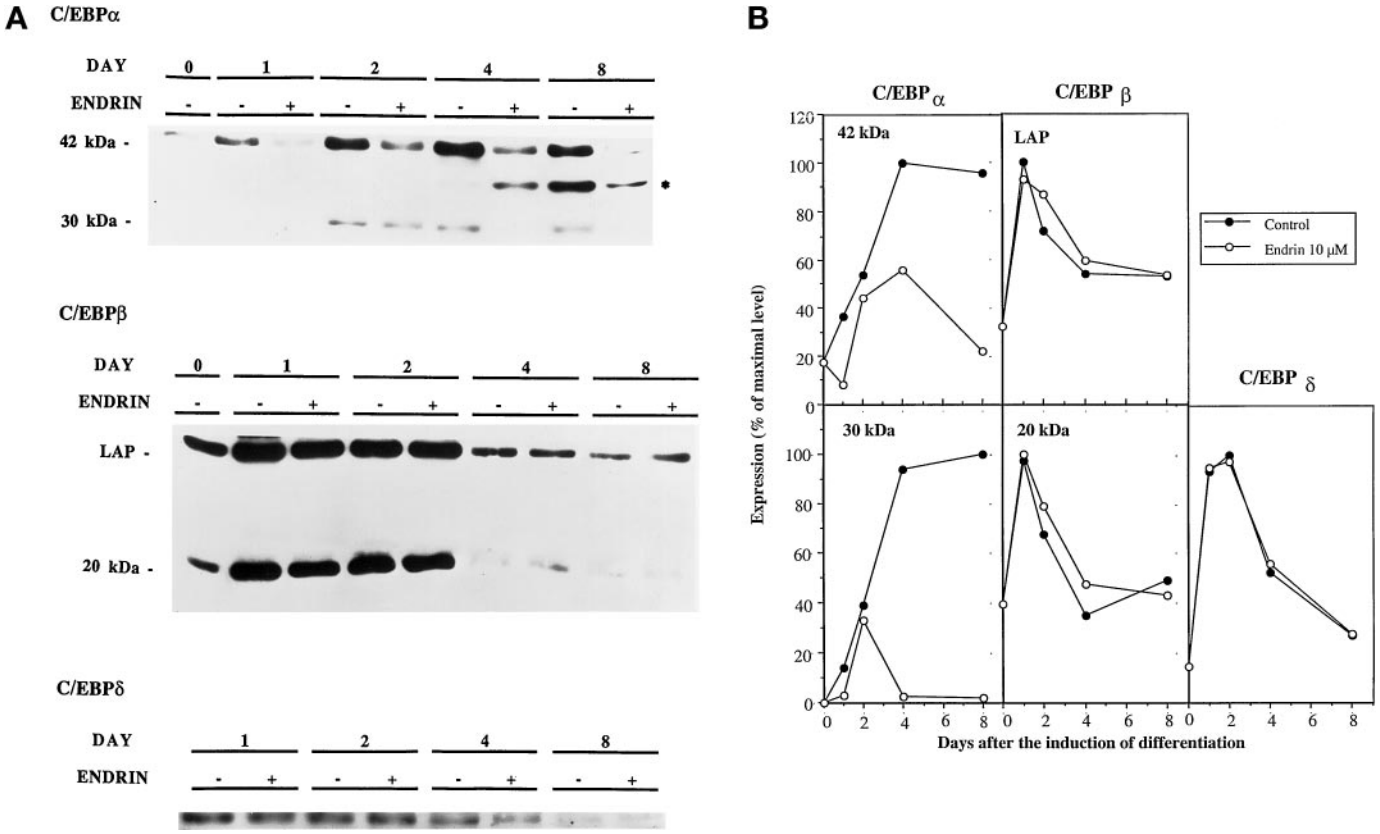

Fig. 4. Effects of endrin on temporal expression pattern of C/EBP proteins (α, β, and δ) during differentiation of 3T3-L1 cells. Nuclear extracts were prepared from control and 10 μM endrin-treated cells at different days after differentiation induction (0, 1, 2, 4, and 8). The extracts (20 μg) were subjected to gel electrophoresis and immunoblotted using antisera against C/EBPα, C/EBPβ, or C/EBPδ. A, representative Western blot analysis of endrin effects on C/EBPα, C/EBPβ, and C/EBPδ during the course of differentiation. *, a nonspecific band. B, graphic representation of the effects of endrin (10 μM) on the C/EBP (α, β, and δ) expression during the course of differentiation. Quantification of the Western blots was performed by a densitometric evaluation using an AMBIS radioanalytical-imaging instrument. The values (mean of at least two independent experiments) are given as a percentage of the maximal level of each C/EBP isoform.

endrin on C/EBPs also includes changes in their capacity of binding to their specific response element DNA, EMSA experiments were carried out. The oligonucleotides used as the probe corresponded to the consensus C/EBP-binding site sequence in the C/EBPα promoter, which is known to bind with all of the three different C/EBP isoforms (C/EBPα, C/EBPβ, and C/EBPδ). Nuclear extracts (10 μg) were prepared from control and 10 μM endrin-treated cells at different stages of differentiation process and subjected to EMSA. Figure 6A shows the effects of endrin on binding capacity of C/EBP proteins to ^{32}P-labeled C/EBP response element oligonucleotide probe at different times during the differentiation process. Lane 1 shows DNA-binding activity of C/EBPs in 3T3-L1 fibroblast. After the exposure of 3T3-L1 preadipocytes to the adipogenic inducers for 1 or 2 days (lanes 2 and 4, respectively), a heterogeneous group of C/EBP complexes with capacity to bind to their DNA recognition sequences were observed. At days 4 and 8 after the induction of differentiation, the quantity of these DNA-C/EBP complexes was significantly higher (lanes 6 and 8). Endrin treatment (10 μM) did not induce any detectable alteration in C/EBP binding capacities on day 1 (lane 3 versus lane 2). A slight in-

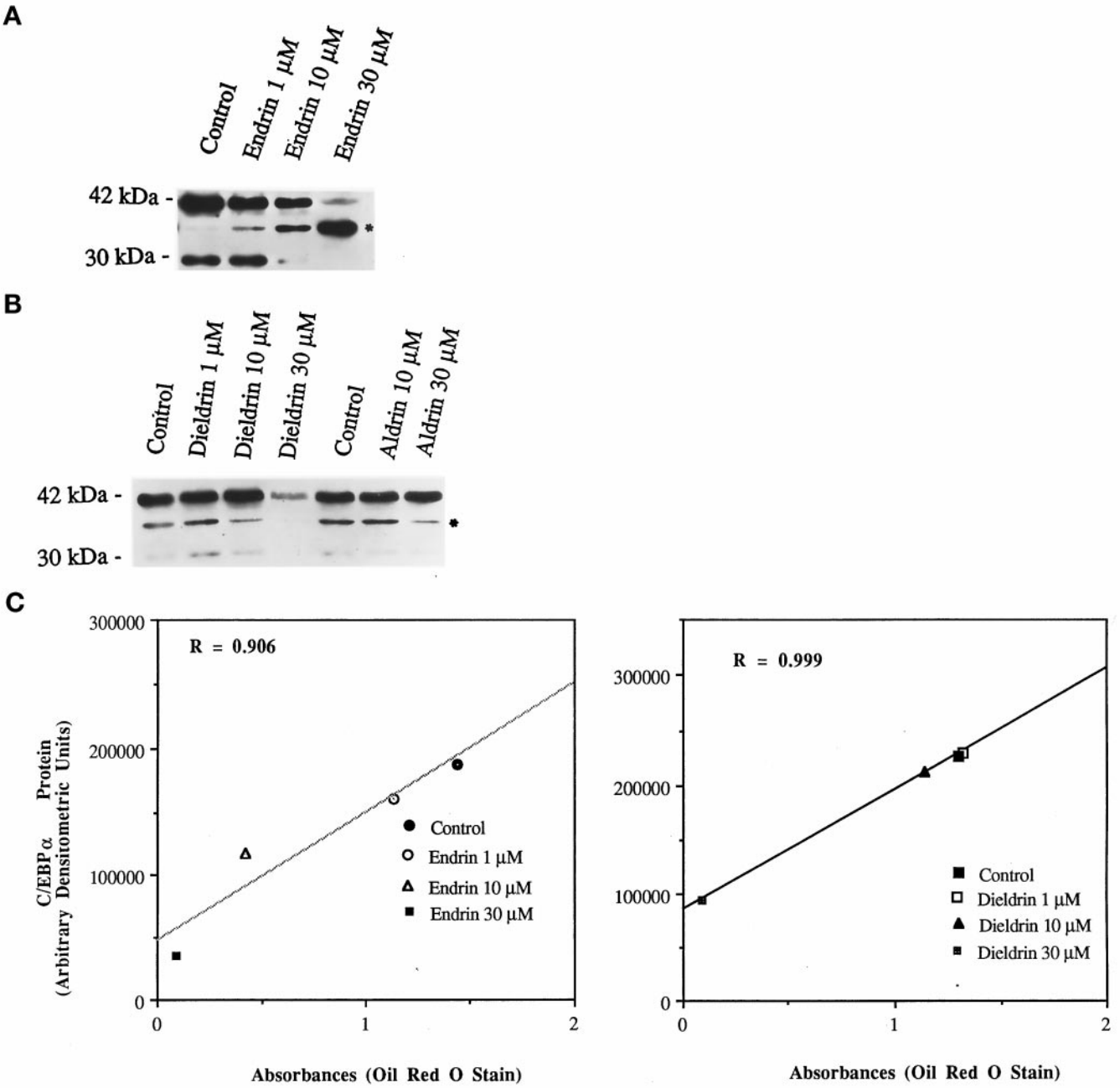

Fig. 5. Effect of the dose of different pesticides on C/EBPα protein expression in 3T3-L1 cells. A, immunoblotting analysis of nuclear extracts prepared from control and endrin-treated cells (1, 10, and 30 μM) 8 days after induction of differentiation. B, C/EBPα protein levels in cells treated with different doses of dieldrin and aldrin. Western blot analysis was performed in nuclear extracts of control and treated cells at day 6 of differentiation. $*$, a nonspecific band seen in all lanes. C, graphic representation of the correlation between the decrease of C/EBPα protein levels and the inhibition on adipocyte differentiation induced by pesticide treatment. C/EBPα levels were evaluated by laser densitometry, and the inhibition on differentiation was quantified by spectrophotometric analysis after Oil Red O stain. Data are mean of at least two independent experiments for each pesticide.

crease (lane 5 versus lane 4) or an absence of effects (experiments not shown) in C/EBP-DNA complexes in endrin-treated samples was observed on day 2. However, pesticide-treated samples showed a significant decrease in DNA-binding capacities of the C/EBP isoforms at days 4 and 8 after differentiation induction (lane 7 versus lane 6 and lane 9 versus lane 8, respectively). Furthermore, at both day 4 and 8, this reduction in the C/EBP binding capacity induced by endrin was mainly caused by a dramatic decrease in the abundance of the upper band species of DNA-C/EBP complexes.

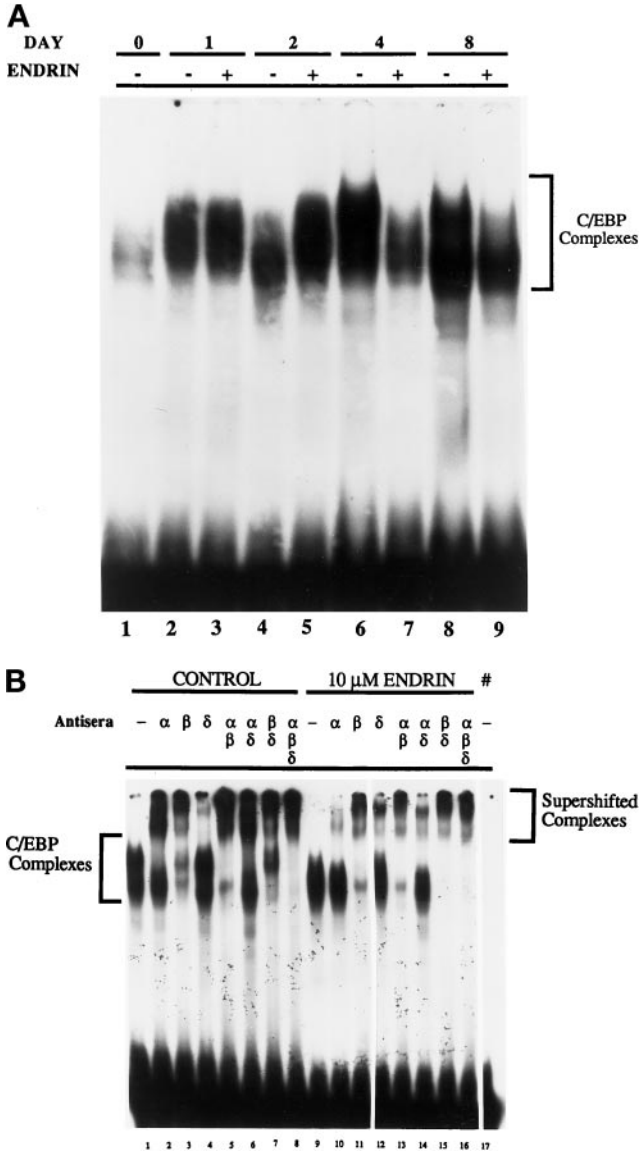

Fig. 6. Effects of endrin treatment on DNA-binding capacity of C/EBP isoforms in 3T3-L1 cells. Gel shift analyses were performed in nuclear extracts prepared from control and 10 μM endrin-treated cells as described in *Materials and Methods*. A, endrin effects on temporal DNA-binding pattern of C/EBP proteins. B, supershift analysis of C/EBP-DNA complexes in nuclear extracts prepared from control and endrin-treated cells at day 4 after the induction of differentiation. 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. #, a competition assay using 100-fold excess of unlabelled oligonucleotide was performed to obtain nonspecific binding activity. Each shift is representative of an experiment that was repeated two independent times.

Because the three C/EBP isoforms (α, β, and δ) are capable of binding to the same consensus C/EBP binding site, supershift studies were performed to identify the components of the C/EBP complexes affected by endrin treatment. To supershift complexes, nuclear extracts from both control and treated cells were incubated with specific antibodies against either one of these three C/EBP isoforms (α, β, or δ) or in combination. Supershift analyses were carried out at day 4 after the induction of differentiation, and the results are shown in Fig. 6B. Lanes 1 and 9 show the C/EBP-DNA complexes obtained without the incubation with the antibodies in control and treated samples, respectively, and the above-mentioned decrease induced by endrin on binding capacity was measured against these standards.

Incubation of nuclear extracts with antibody against C/EBPα caused the supershift of the upper species of C/EBP complexes, showing that this corresponds to the oligonucleotide-C/EBPα complexes (Fig. 6B, lane 2). In addition, C/EBPα binding capacity can be evaluated by studying the gel shift profiles when the β and δ C/EBP isoforms have been removal by supershifting using specific antibodies (Fig. 6B, lane 7). With regard to endrin effects, supershift studies on pesticide-treated nuclear extracts showed that endrin treatment at day 4 induced a dramatic decrease of oligonucleotide-C/EBPα complex abundance (Fig. 6B, lane 2 versus lane 10 and lane 7 versus lane 15).

In contrast, the DNA-binding of C/EBPβ at day 4 did not show any significant differences between control and endrin-treated samples (e.g., Fig. 6B, lane 3 versus lane 11 in which C/EBPβ complexes have been supershifted by using antibody against C/EBPβ; compare gel shift profiles in lane 6 with lane 14 in which C/EBPβ complexes can be observed after α and δ C/EBP isoforms have been supershifted).

With regard to C/EBPδ binding capacity, supershift analysis in the presence of its antibody gave the appearance that endrin induced a slight increase in C/EBPδ binding capacity after 4 days of treatment (Fig. 6B, lane 4 versus lane 12). However, when DNA-C/EBPδ complexes were analyzed after removal of C/EBPα and C/EBPβ with antibodies against these isoforms, no clear-cut differences between control and endrin-treated samples could be observed (Fig. 6B, lane 5 versus lane 13).

Incubation of nuclear extracts at the same time with antibody against all three C/EBP isoforms (α, β, and δ) caused a total removal of C/EBP complexes because all of them were supershifted (Fig. 6B, lane 8 versus lane 16). As expected, when a 100-fold excess of a cold oligonucleotide was used as competitor, all DNA-C/EBP complexes were totally eliminated (Fig. 6B, lane 17).

Supershift studies performed at day 8 after the induction of differentiation also confirmed that endrin caused a decrease in C/EBPα-DNA binding without altering C/EBPβ or δ-DNA complexes (data not shown).

It appears to be safe to conclude that DNA-binding activity of C/EBPα is selectively affected by endrin treatment, causing a significant decrease in the abundance of C/EBPα-DNA complexes. Because it has been already shown that endrin induces a decrease in the intracellular level of C/EBPα (Fig. 4A), such a decrease is likely caused by the reduction of the titer of C/EBPα protein.

Effects of Endrin and Dieldrin on Expression of PPARγ Protein Isoforms. In recent years, many studies

have demonstrated that in addition to C/EBPs, PPARγ also plays a fundamental role in the transcriptional control of adipogenesis (Tontonoz et al., 1995). For this reason, we tested the effects of endrin on PPARγ protein isoform levels. Immunoblottings were performed in nuclear extracts from control and treated cells at early stages after the induction of differentiation (1 and 2 days) because PPARγ is induced very early in the differentiation of cultured adipocyte cell lines. As previously described (Hu et al., 1996), two closely spaced bands of PPARγ (γ1, lower band; γ2, upper band) could be recognized in a Western blot analysis (Fig. 7A). As expected, 3T3-L1 preadipocytes/fibroblasts did not express recognizable amounts of PPARγ protein (Fig. 7A, lane 1). One day after the addition of differentiation inducers, the two isoforms of PPARγ could be detected in control samples (lane 2). At the same time, 10 μM endrin-treated nuclear extracts showed a reduction in the expression of PPARγ protein in comparison to control cells (lane 3).

At day 2 of differentiation, the abundance of PPARγ protein isoforms increased significantly in control cells (lane 4). Also, an increase can be observed in PPARγ levels in endrin-treated cells in comparison to the levels after pesticide treatment at day 1 (lane 5). However, PPARγ protein levels in endrin-treated samples at 2 days still stayed considerably reduced in comparison to what was observed in control samples (lane 4 versus lane 5). These endrin-induced reductions in PPARγ protein isoform levels at early stages of differentiation process could also be observed at day 4 of differentiation (data not shown).

Dieldrin effects on PPARγ protein expression were also tested in nuclear extracts at day 6 of differentiation. Western blotting analysis showed the ability of 30 μM dieldrin to reduce the cellular levels of both isoforms of PPARγ protein (Fig. 7B).

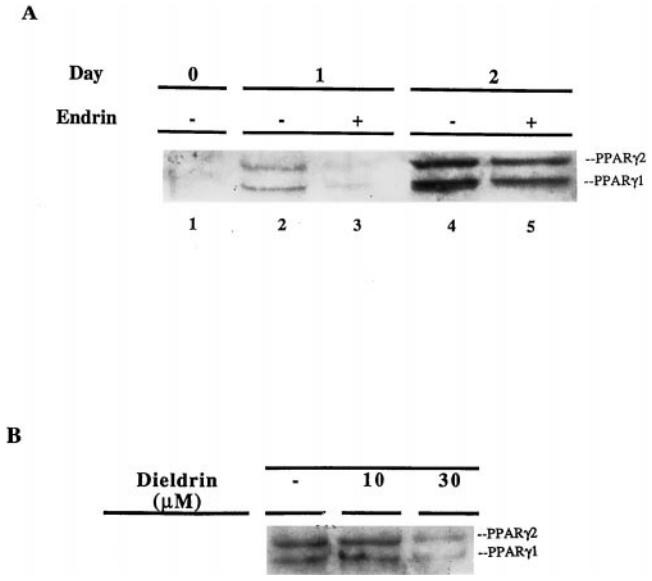

Fig. 7. Effects of endrin and dieldrin on the expression of PPARγ protein isoforms. Immunoblotting analyses were performed in nuclear extracts (30 μg) prepared from control and pesticide-treated cells at different days of differentiation process. A, Western blot showing the 10 μM endrin-induced inhibition on PPARγ protein isoforms at early stages of differentiation process (1 and 2 days after the induction of differentiation). B, PPARγ protein isoforms levels in nuclear extracts prepared from control and 30 μM dieldrin-treated cells 6 days after the induction of differentiation.

Effects of Endrin and Dieldrin on NF-κB Activation. The effects of cyclodiene pesticides on NF-κB activation were determined by EMSA analysis with the NF-κB probe containing its specific DNA-response element. Figure 8A shows the effects of 10 μM endrin treatment on NF-κB-DNA binding at different days after the induction of differentiation. No significant differences were found in NF-κB-oligonucleotide complexes observed in nuclear extracts prepared from control and endrin-treated cells at days 1 and 2 (lane 2 versus 3 and

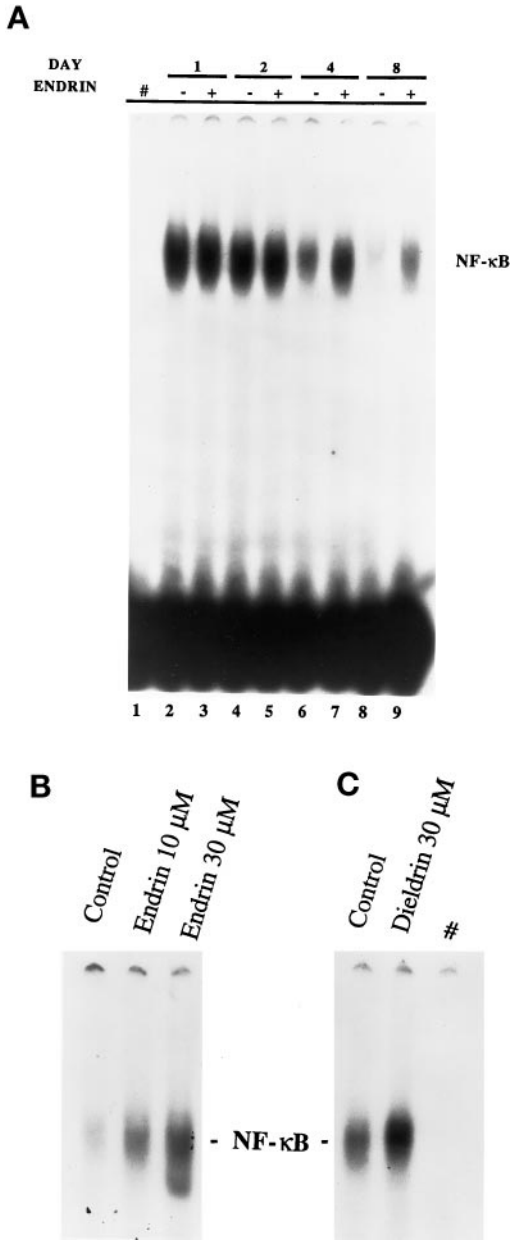

Fig. 8. Effects of endrin and dieldrin on the activation of NF-κB. Nuclear extracts were prepared from control and pesticide-treated cells at different days after the induction of differentiation. Then, 10 μg of the extracts was analyzed for NF-κB-specific DNA binding by EMSA. A, endrin (10 μM) effects on NF-κB-DNA binding in the time course of differentiation. B, dose-response studies with endrin at day 8 after the induction of differentiation. C, NF-κB-DNA binding in nuclear extracts from control and 30 μM dieldrin-treated cells at day 6 of differentiation. #, a 100-fold excess of unlabeled oligonucleotide was used to compete away specific NF-κB-DNA complexes (Fig. 8A, lane 1). Each shift is representative of an experiment that was repeated three independent times.

lane 4 versus 5, respectively). At day 4 after the induction of differentiation, a decrease in the abundance of the NF-κB-DNA complexes was observed in control cells in comparison to what was observed at day 1 and 2. However, in endrin-treated cells at day 4, the NF-κB-oligonucleotide complexes remained almost at the same level as at days 1 or 2 (lane 6 versus 7). On day 8, a dramatic failure in NF-κB-DNA binding was observed in control extracts (lane 8), whereas in the endrin-treated cells, the abundance of the NF-κB complexes was still significantly higher than that in control cells (lane 9 versus 8), although a decrease in their binding levels was noted. Figure 8B shows that the action of endrin on NF-κB at day 8 of differentiation is dose dependent, and the highest abundance of NF-κB-DNA complexes was found in cells treated with 30 μM endrin, a dose that almost totally suppressed the adipocyte conversion process. A similar increase in NF-κB-DNA binding was observed in cells treated with an inhibitory dose of dieldrin (30 μM) in comparison to control cells at day 6 after the induction of differentiation (Fig. 8C).

Effects of Troglitazone on Antiadipogenic Action of Endrin. Troglitazone is an antidiabetic drug known to act as a ligand for PPARγ. Like other thiazolidinediones, troglitazone has been shown to induce adipocyte differentiation (Spiegelman, 1998). We tested the possible antagonistic effect of troglitazone on the actions of endrin. The results clearly showed that endrin suppressed the process of adipogenic transformation even in the presence of troglitazone (Fig. 9) at the dose known to cause a relevant increase on the differentiation of the adipocytes.

Discussion

Adipocyte differentiation is a complex process that includes a cascade of events triggered by the action of insulin, aided by a cAMP-elevating agent and dexamethasone in the presence of FBS in the case of 3T3-L1 cells. Although not all of the interacting pathways have been totally elucidated, experts in this field generally acknowledge two major nuclear factor families controlling the process of adipocyte differentiation: C/EBPs and PPARγ. By far, C/EBPα appears to be a critical and indispensable nuclear transcription factor triggering the entire process of adipocyte differentiation (e.g., Lin and Lane, 1994; Yeh et al., 1995). Several studies have provided evidence that this transcription factor not only is required but also is sufficient to trigger differentiation of preadipocytes in the absence of the cocktail of differentiation inducers (Freytag et al., 1994; Lin and Lane, 1994). In support of the above conclusion, the suppression of C/EBPα expression with antisense treatment caused inhibition of the terminal adipocyte differentiation, which seems to indicate that the sustained expression of C/EBPα is required (Lin and Lane, 1992). This continued expression of C/EBPα during the terminal differentiated state has been attributed to an autoactivation of the transcription of its own gene, which contains C/EBP binding site in its proximal promoter. Our results showed that endrin specifically caused a dramatic decrease in C/EBPα-DNA binding, which otherwise would have activated the transcription of many specific adipocyte genes, including the C/EBPα gene. In analogy to the blocking action of antisense oligonucleotide against C/EBPα gene (Lin and Lane, 1992), the alterations induced by endrin on C/EBPα-mediated transcription should therefore be sufficient to cause the suppression of the acquisition of the adipocyte phenotype observed in the pesticide-treated cells. Prevention of adipogenesis by other compounds such as TCDD and retinoic acid has also been related to an antecedent inhibition of C/EBPα expression (Liu et al., 1996; Schwarz et al., 1997).

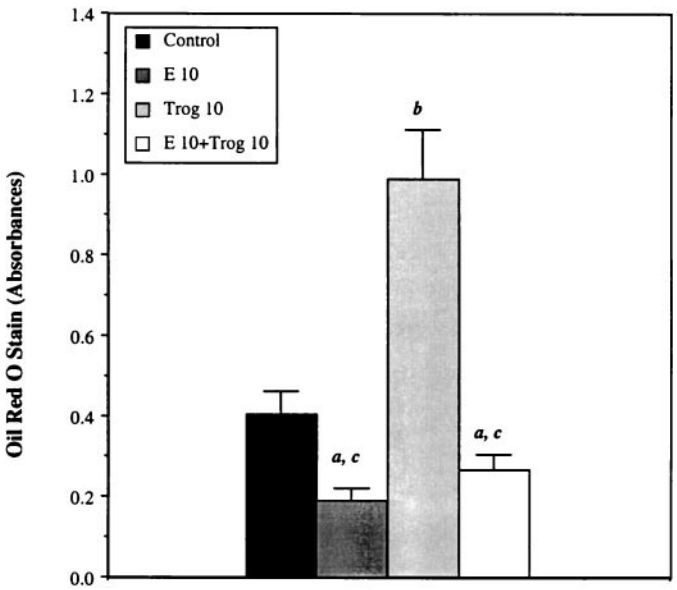

Fig. 9. Effects of troglitazone on antiadipogenic action of endrin. Cells were incubated in the presence of 10 μM endrin (E 10) or 10 μM troglitazone (Trog 10) or both (E 10+Trog 10) along with the differentiation medium and then each time that the medium was replaced. Cells were stained with Oil Red O 8 days after the induction of differentiation. Quantitative evaluation was performed by spectrophotometrical analysis of Oil Red O stained-plates, as described in *Materials and Methods*. Values are given as mean ± S.E.M. of at least three independent experiments. $^{a}p < .05$ compared with control cells. $^{b}p < .001$ compared with control cells. $^{c}p < .001$ compared with troglitazone-treated cells.

On the other hand, because C/EBPα is activated at a relatively late point of differentiation (days 3–4) in comparison to C/EBPβ and C/EBPδ in 3T3-L1 cells, there have been some questions regarding the leading role of the α isoform in this process. According to Yeh et al. (1995), the β and δ isoforms play an early preparatory roles in the differentiation pathway, relaying the effects of dexamethasone and cAMP to C/EBPα gene-protein expression, leading to the activation of the α isoform. Thus, the main role of C/EBPβ and C/EBPδ is to prime the activation of C/EBPα, which, in effect, "turns on the battery" of many adipocyte-specific genes. Our results showed that the pattern of expression of C/EBPβ protein observed after IBMX stimulation was similar in control and endrin-treated cells. Also, endrin did not affect the levels of C/EBPδ. The lack of alteration in both C/EBPβ and C/EBPδ DNA binding suggests that cAMP and glucocorticoids pathways are not direct targets of the actions of endrin in 3T3-L1 differentiation process.

Endrin has been shown to be effective in preventing the adipocyte differentiation process only when it is added during the 24 to 48 h of exposure of the preadipocytes to the differentiation medium. As in the case of endrin, other drugs that are known to suppress the adipocyte differentiation program, such as TCDD and retinoic acid, have already also been shown to be ineffective if they are added after this critical period (Phillips et al., 1995; Xue et al., 1996). Our data suggest that 72 to 96 h after the induction of differentiation process, cells must have reached a stage at which point they are firmly committed to adipocyte differentiation.

To test the possibility that there could be another factor affected by endrin at an earlier stage of differentiation, we focused our attention on PPARγ. This receptor is also an important member of the nuclear transcription factor/receptor family that is known to mediate adipogenic signaling (Tontonoz et al., 1994). The expression of PPARγ is known to antecede the induction of C/EBPα in the cascade of events leading to adipocyte differentiation (reviewed by Tontonoz et al., 1995). Our results showed that endrin was able to inhibit the induction of PPARγ proteins elicited by hormonal inducers at an early stage of differentiation (days 1 and 2). TCDD, although it is known to mainly affect C/EBPs, was also observed to prevent mRNA PPARγ expression (Liu et al., 1996). As for the recognition of this receptor action, there is a family of chemical agents that are known to specifically affect PPARγ, making it possible to recognize the role of this specific pathway from others. Thiazolidinediones, which have been developed as antidiabetic drugs, act as direct ligands for PPARγ, and therefore, they cause a potent and effective stimulation of adipogenesis (Lehmann et al., 1995). Our data show that troglitazone, one of the thiazolidinediones, however, did not significantly prevent the inhibitory effects of endrin on adipocyte differentiation. This fact clearly indicates that endrin is not acting like a direct antagonist of PPARγ ligands because the treatment with even high doses of PPARγ stimulants could not reverse its actions on differentiation process.

Another possibility we considered is that NF-κB could be the key component directly affected by endrin. NF-κB has shown to be activated on treatment of cells with phorbol esters like TPA and cytokines such as TNFα (Beg et al., 1993). Both compounds are well known to interfere with adipocyte differentiation process. The potent inhibition of the adipocyte differentiation process induced by TNFα has been attributed to an antecedent inhibition of both PPARγ and C/EBPα expression (Ron et al., 1992; Zhang et al., 1996). Little is known, however, about the relationship between NF-κB and the factors implicated in the regulation of the differentiation of the adipocytes. It is worthy to note that the decrease in NF-κB activation observed from day 4 after the induction of differentiation occurs about the same time of the increase in the expression of C/EBPα protein as well as with a rise in its binding to DNA. Furthermore, the inhibition of the normal decrease of NF-κB-DNA complexes at days 4 and 8 induced by endrin also occurs at the same time as the decrease in the expression of C/EBPα and its binding to its response element observed in pesticide-treated cells. These facts are indicative of the relationship between these two nuclear transcription factors. It must be pointed out, however, that NF-κB is known to be a negative regulator, unlike C/EBPα or PPARγ, meaning that only its overexpression above the constitutive level could cause inhibition of differentiation. In this case, what we observed is the failure of endrin-affected cells to lower its level from normal at the proper time. Such an observation points to the possibility that the failure of NF-κB to down-regulate at days 4 and 8 in cells affected by endrin is not the cause for the inhibition of differentiation but rather is the direct result of endrin-induced suppression of C/EBPα. Consistent with this idea, some research groups have demonstrated the existence of functional and physical associations between NF-κB and C/EBP family members (Stein et al., 1993). Moreover, it appears that these physical associations can result in the synergistic transcriptional activation of a number of genes (Ray and Ray, 1995). It has also been suggested that the functional interactions between C/EBP and NF-κB might profoundly affect cellular growth control because the expression of C/EBP isoforms, particularly the α isoform, could modify the ability of NF-κB to control cellular proliferation (Stein et al., 1993). Two pieces of evidence further supporting this hypothesis are 1) the observation that troglitazone could not antagonize the inhibitory action of endrin on differentiation and 2) endrin did not cause direct down-regulation of TNFα receptor in our preliminary studies (data not shown). These results indicate that TNFα or any other proinflammatory cytokines pathway whose actions are mediated by NF-κB and, in turn, antagonized by thiazolidinediones (Ohsumi et al., 1994) is not directly affected by endrin.

These data, as well as observations by others, clearly support our hypothesis that the effect of endrin on C/EBPα itself is likely to be sufficient to explain its inhibitory action on adipocyte differentiation, which is already known to be accompanied by changes in a number of interactive systems, such as PPARγ and NF-κB, as explained above.

The key remaining question is, then, what is the direct cause for endrin-induced suppression of C/EBPα. As for the possible mechanism by which endrin causes suppression of insulin-induced rise in the titer of C/EBPα, our current thinking is that its action to induce EGF-like mitogenic signaling in 3T3-L1 cells, as in the case of TCDD, is the most likely cause. Cyclodiene insecticides such dieldrin, aldrin, chlordane analogs, and endrin have already been shown to increase PKC activities (Bagchi et al., 1997), to inhibit cell communication (Tsushimoto et al., 1983), and to mimic the epidermal growth factor in both 3T3-F442A and 3T3-L1 cells in their action to block insulin-induced rise in C/EBPα titer (Liu et al., manuscript in preparation). Additionally, the action of endrin in this regard is very similar to that of TCDD, which clearly acts in this manner by activating the epidermal growth factor signaling pathway (Phillips et al., 1995; Liu et al., 1996). It is well known that in many cell types, cell proliferation and differentiation are two mutually exclusive programs. Indeed, the increased growth factor signaling is already known to cause inhibition of C/EBPα gene expression (Mischoulon et al., 1992). Certainly, additional confirmatory work would be needed, however, to firmly establish the connection between the pesticide-induced mitogenic signaling and their inhibitory action on C/EBPα.

In summary, we clearly established that endrin causes selective inhibition of the rise in the level of C/EBPα protein that accompanies the early commitment of 3T3-L1 cells to differentiate into adipocytes. Such an effect of endrin is accompanied by shifts of PPARγ and NF-κB levels, which are consistent with the expected consequence of the change in C/EBPα.

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