Biochemical Pharmacology, Vol. 55, pp. 1077–1090, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00683-7

Interruption of Estradiol Signal Transduction by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Through Disruption of the Protein Phosphorylation Pathway in Adipose Tissues from Immature and Mature Female Rats

Essam Enan,†‡ Faten El-Sabeawy,† Francisco Moran,† James Overstreet† and Bill Lasley†*

Department of Environmental Toxicology, and Institute of Toxicology and Environmental Health, University of California, Davis, CA, U.S.A.

ABSTRACT. At doses of 10–115 μg/kg, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) decreased body and adipose tissue weights of mature female rats. Doses below 10 μg TCDD/kg decreased body and adipose tissue weights of immature, but not mature females. Doses of 2 and 10 μg TCDD/kg decreased adipose tissue epidermal growth factor receptor (EGFR) binding activity 5 and 7 days later in immature and mature females, respectively. At these times, there was a decrease in the activities of tyrosine kinase (TK), mitogen-activated protein kinase (MAP2K), and protein kinase A (PKA). In mature females, estradiol (E_2, 15 μg/kg) increased TK and PKA activities and decreased MAP2K activity. In immature females, E_2 decreased TK and PKA activities but not MAP2K activity. TCDD abolished the stimulatory effect of E_2 on TK and PKA in mature females, and in immature females TCDD potentiated the negative effect of E_2 on all three kinases. TCDD decreased binding of [^{3}H]E_2 to cytosolic and nuclear estrogen receptors (ERs) of mature and immature females, and antagonized the stimulatory effect of E_2 on ER binding activity. E_2 increased DNA binding activity of the estrogen response element (ERE) and activator protein-1, and TCDD antagonized this effect. Geldanamycin, an inhibitor of Src tyrosine kinase, reduced the effects of TCDD on body and adipose tissue weights. Geldanamycin antagonized the effects of TCDD on EGFR binding activity and TK activity. In cell-free preparations, TCDD antagonized E_2 action on TK activity in mature females, as well as E_2 action on PKA activity in immature females. We hypothesize that TCDD antagonizes E_2 action in female adipose tissues through disruption of common cytosolic signal transduction pathways. BIOCHEM PHARMACOL **55**;7:1077–1090, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. TCDD; AP-1; ERE; kinases; adipose tissue; female rats

The dioxins are part of a diverse group of chemical compounds that are commonly referred to as environmental antiestrogens and endocrine disruptors. The compound TCDD§ is often studied as a representative chemical for many dioxin-type compounds. Exposure to TCDD results in a number of toxic and biologic responses including a wasting syndrome, reduction of glucose transporting activity, changes in protein phosphorylation/dephosphorylation cascades, immunotoxic effects, reproductive toxicity, hepatoxicity and porphyria, hypo- and hyperplastic responses, carcinogenesis, and the induction of enzymes including specific forms of cytochrome P450 [1–11]. TCDD and related aryl hydrocarbons have been proposed to act after initial binding to the AhR protein, which is present in the target tissues. These compounds first interact with a soluble form of AhR, which, upon ligand binding, undergoes a "transformation" process and becomes localized to the nucleus [1, 12]. There, AhR associates with the nuclear protein Arnt [13, 14], and the complex binds to dioxin-response elements (DRE) [15] to activate transcription. The interaction between TCDD and AhR is similar in many respects to the mechanism by which steroid hormones initiate their interaction with target cells, and many of the effects of TCDD resemble those evoked by steroid hormones. For example, several classes of steroid hormones and TCDD induce cytochrome P450-dependent monooxygenases and their associated cytochrome P450 isozymes [15–17], although steroid hormones and TCDD induce different forms of cytochrome P450. Nevertheless, the mechanism of toxic action of TCDD is complex and also

‡ Corresponding author: Essam Enan, Ph.D., Institute of Toxicology and Environmental Health, University of California, Davis, CA 95616. Tel. (916) 754-9736; FAX (916) 752-5300; E-mail: eeenan@ucdavis.edu.

§ *Abbreviations:* TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AP-1, activator protein-1; ERE, estrogen response element; EMSA, electrophoretic mobility shift assay; TK, tyrosine kinase; MAP2K, mitogen-activated protein kinase (p^{42}); PKA, cyclic AMP-dependent protein kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; E_2, estradiol; AhR, aryl hydrocarbon receptor; ER, estrogen receptor; and DTT, diothiothreitol.

Received 14 May 1997; accepted 20 November 1997.

involves modulation of growth factor receptors and components of their signal transduction pathways.

Many signal transduction pathways rely upon cellular protein kinase/phosphatase cascades to achieve activation and deactivation of *trans*-acting nuclear proteins in order to coordinate changes in gene expression [6, 7, 18]. Kinases play an important role in cell estrogenicity, and toxicants can block cellular responses to estrogens in many cell types by interference with protein kinase signaling [9–11]. Estrogens alone can stimulate cell growth and transformation. Growth factors and other molecules that are known to increase the activity of protein TKs have been shown to act in synergy with E_2 to modulate cell function [19, 20]. Mukku and Stancel [21] reported that treatment of immature female rats with E_2 resulted in a significant increase of EGFR binding activity, which appeared to be mediated through a transcriptional mechanism. There is increasing evidence that peptide growth factors can be mediators of estrogen action in both normal and neoplastic tissues [22, 23], and in the mouse uterus the ER is required for the E_2-like effects of EGF [24]. In MCF-7 human breast cancer cells, there is also a requirement for the ER in EGF-induced proliferation [25], as well as for induction of progesterone receptor levels by insulin-like growth factor I [20, 26]. These observations suggest that peptide growth factors may influence the transcriptional activity of the ER or other factors in the ER-transcriptional complex.

The ER is a phosphoprotein, and its activity is controlled primarily by its ligand, which releases it from heat-shock proteins [27] to induce the formation of active homodimers [28] and the phosphorylation of the receptor at specific tyrosine and serine residues [29]. ER binding to the ERE at the *trans*-activating site of DNA is strongly influenced by several other hormone receptors and nuclear transcription factors such as AP-1 [30]. The c-*jun* and c-*fos* genes are functionally linked in that the proteins they encode can associate to generate AP-1. Recently, we found that E_2 significantly increases the DNA binding activity of AP-1 in human luteinized granulosa cells (hLGCs) and that treatment of hLGCs with TCDD antagonizes this effect [10]. E_2 has been shown previously to induce the expression of c-*fos* in the adult rat uterus [31, 32]. A direct action of E_2 on the regulation of c-*fos* transcription is suggested by the finding that the E_2-induced expression of this gene is not prevented by protein synthesis inhibitors [32, 33]. In other studies, Safe and coworkers [34] found that E_2 produces an increase of rat uterine c-fos mRNA, and that following treatment of the animals with TCDD, there is a dose-dependent decrease in these levels. Regulation by E_2 of c-*jun* expression has been studied extensively in MCF-7 human breast cancer cells and in the organs of immature and adult animals [35–37]. A transient increase in c-*jun* transcription occurs very rapidly after E_2 administration and is independent of ongoing protein synthesis [36]. c-jun mRNA levels have been found to be inducible in both epithelial and stromal myometrial tissue fractions of the adult rat uterus [36].

Our working hypothesis is that TCDD shares cytosolic transduction pathways with E_2 and that interference with these common pathways is an important mechanism for disruption of E_2 action in cells. In the present study, we investigated the responses of non-reproductive tract cells from mature and immature female rats to TCDD and E_2, and we studied the interaction between the signal transduction pathways that mediate these responses. In addition, we addressed if there is a difference in age-specific susceptibility to TCDD and if this age-specific difference is mediated through signal transduction pathways. We utilized a cell-free system from which the nucleus had been removed to separate cytosolic and nuclear events. The results of these experiments demonstrated an interaction between TCDD and E_2 at the level of the cytosolic signaling pathways, as well as in the nucleus of target cells.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (3000 and 6000 Ci/mmol) was purchased from Amersham. [2,4,6,7-^{3}H]E_2 (95.3 Ci/mmol = 2.9 μg/mL) and ^{125}I-Labeled EGF (murine, sp. act. 176 μCi/μg) were purchased from NEN. Substrate peptides for TK (RR-SRC), MAP2K (APRTPGGRR), and PKA (Kemptide) were purchased from UBI. Double-stranded AP-1 response element DNA and double-stranded ERE DNA were purchased from Stratagene. TCDD was purchased from CIL (Woburn, MA.), with purity higher than 99.9%. All other biochemicals were purchased from Sigma.

Animals

Three- to four-month-old (mature) and three- to four-week-old (immature) female Sprague–Dawley rats were purchased from Simenson Laboratories, Inc. Animals were housed in suspended stainless steel cages and provided with food and water *ad lib*. All animals were maintained on a 12-hr light/12-hr dark cycle at constant temperature and humidity.

Experiments In Vivo

EFFECT OF E_2 AND TCDD ON KINASE ACTIVITIES IN FEMALE ADIPOSE CELLS. Mature and immature female rats were treated *in vivo* according to three experimental protocols. One set of animals was treated with a single i.p. injection of 115 μg of TCDD/kg in a corn oil/acetone vehicle (19:1); control animals received an equal volume of the vehicle only. A second set of animals was treated with a single i.p. injection of 15 μg of E_2/kg or with vehicle alone. The third set of animals was treated with 15 μg of E_2/kg 24 hr before a single dose of 115 μg of TCDD/kg. Five animals were used for each treatment or control group. Animals were killed 24 hr following the final treatment; their abdominal adipose tissues were removed rapidly, rinsed in cold normal saline-PBS, and chilled on ice. The effects of the various treat-

ments on TK, MAP2K, and PKA activities in the cytosolic fractions of adipose cells were investigated as described below.

DOSE–RESPONSE EFFECT OF TCDD ON TK ACTIVITY IN FEMALE ADIPOSE CELLS. Dose–response studies were performed with adipose tissues from mature and immature female rats to determine the threshold concentration of TCDD that induced changes in kinase activities in animals of different ages. Experimental and control animals were treated with TCDD or with vehicle alone as described above. Five treatment groups of three animals each were used and the single doses of TCDD were 0.1, 1.0, 2.0, 10.0, and 50.0 μg/kg. Animals were killed 7 days following TCDD injection because we found that by this time TCDD significantly decreased ^{125}I-labeled EGF binding to EGFR (see below). Adipose tissues were recovered, and TK activity in the cytosolic fractions of adipose cells was measured as described below.

TIME–COURSE EFFECT OF TCDD ON TK ACTIVITY IN FEMALE ADIPOSE CELLS. Time–course studies were performed with adipose tissues from mature and immature female rats to determine when the minimum effective doses of TCDD changed kinase activities in animals of different ages. Experimental and control animals were treated with TCDD or with vehicle alone as described above. The minimum effective dose of TCDD, as determined in the dose–response study, was used (10.0 μg/kg for mature females and 2.0 μg/kg for immature females). Seven treatment groups of three animals each were used, and the animals were killed at 1, 3, 5, 10, 14, 21, and 28 days following single doses of TCDD. Adipose tissues were recovered, and TK activity in the cytosolic fractions of adipose cells was measured as described below.

EFFECT OF TCDD ON FEMALE BODY WEIGHT AND ADIPOSE TISSUE WEIGHT. To investigate the relationship between TCDD-induced effects on kinase activities and the wasting syndrome, groups of immature and mature female rats were treated with a single i.p. dose of 2 μg of TCDD/kg or with vehicle alone. Body weight and daily food consumption were monitored through the 90-day time–course of the experiment. Ten animals per group were killed at 10 time points during the experiment, and adipose tissue weight was measured.

TIME–COURSE EFFECT OF TCDD ON EGFR BINDING ACTIVITY IN FEMALE ADIPOSE CELLS. Time–course studies were performed with adipose tissues from mature and immature female rats to determine when TCDD changed EGFR binding activity in animals of different ages. Experimental and control animals were treated with 2.0 μg of TCDD/kg or with vehicle alone as described above. Eight treatment groups of three animals each were used, and the animals were killed at 1, 3, 5, 7, 10, 14, 21, and 28 days following single doses of TCDD. Adipose tissues were recovered, and EGFR binding activity in the microsomal fractions of adipose cells was measured as described below.

EFFECT OF TCDD ON ER LEVELS AND DNA BINDING ACTIVITY OF ERE AND AP-1. Mature and immature female rats were treated *in vivo* according to three experimental protocols. One set of animals was treated with a single i.p. injection of 10 μg of TCDD/kg (for mature animals) and 2 μg of TCDD/kg (for immature animals) in a corn oil/acetone vehicle (19:1); control animals received an equal volume of the vehicle only. A second set of mature and immature animals were treated with a single i.p. injection of 15 μg of E_2/kg or with vehicle alone. The third set of animals was treated with 15 μg of E_2/kg 24 hr before a single dose of 10 μg of TCDD/kg (for mature animals) and 2 μg of TCDD/kg (for immature animals). Three animals were used for each treatment or control group. Animals were killed 24 hr following the final treatment, and adipose tissues were recovered. ER levels were measured in cytosolic and nuclear protein fractions of adipose cells, and the DNA binding activities of ERE and AP-1 were measured in the nuclear fractions as described below.

EFFECT OF GELDANAMYCIN TREATMENT ON THE ACTION OF TCDD. To investigate the role of Src kinase in the actions of TCDD, groups of 15 mature and 20 immature female rats were treated with four doses of 1 μg of geldanamycin/kg body weight. The first dose was administered i.p. at day −1 before a single i.p. dose (day 0) of 115 μg of TCDD/kg. The other three doses of geldanamycin were given i.p. on days +3, +7, and +10 after TCDD treatment. Two control groups were used. The first control group received solvent only, and the second control group received geldanamycin alone. Body weight and daily food intake were monitored every other day. Three animals were killed at day +14 because EGFR binding activity was decreased significantly by TCDD at this time point in both immature and mature female adipose tissues (see below). Adipose and reproductive tract tissues were collected and weighed. EGFR binding activity and TK activity in the cytosolic fraction of adipose cells were measured as described below.

Cell-Free Studies In Vitro

To determine whether the effects of TCDD on cytosolic kinase activities were independent of the cell nucleus, TCDD and/or E_2 were added directly to cytosolic fractions of adipose cells from untreated mature and immature female rats. Cytosolic fractions (100,000 *g* supernatant) were prepared as described below, and kinase activities were measured in this cell-free system from which the nucleus had been removed. In one set of experiments, 10 nM of TCDD was incubated with 20 μg of protein in 80 μL of each specific kinase buffer described below. In a second set of experiments, 10 nM of E_2 was added to the cell-free preparations. In the third set of experiments, 10 nM of E_2 was added 10 min before the addition of 10 nM of TCDD.

Incubations were carried out for 10 min at 30° before kinase activities were assayed as described below. Each experiment was replicated five times.

Preparation of Cell Fractions

Abdominal adipose tissues were homogenized in 3 vol. (w/v) of buffer A (10 mM of HEPES, pH 7.9, 1.5 mM of $MgCl_2$, 10 mM of KCl, and 0.05 mM of DTT). The homogenates were centrifuged at 7000 *g* for 10 min at 4° in a Sorvall SS34 rotor to pellet nuclei. The low-speed (7000 *g*) supernatant from this step was designated as the post-nuclear fraction, which was decanted carefully and centrifuged for 60 min at 100,000 *g* (Beckman type 50 rotor). The cytosol and pellet were obtained; the latter was resuspended in enough volume of buffer A to give a protein concentration of 5 mg/mL and was used as the microsomal fraction. The nuclear extract was prepared as described by Dignam *et al.* [38], with some changes. Briefly, the pellet obtained from the low-speed centrifugation of the homogenate was resuspended in 3 vol. of buffer A and was centrifuged a second time for 20 min at 25,000 *g*. The pellet (crude nuclei) was resuspended in 500 μL of buffer B, consisting of 20 mM of HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M of $NaCl_2$, 1.0 mM of EDTA, 0.5 mM of phenylmethylsulfonyl fluoride, 0.5 mM of DTT plus 10 μg/mL of the protease inhibitors leupeptin, aprotinin, and chymostatin. The protease inhibitors were added to the buffer just before the extraction of the pellet with a glass/glass homogenizer. The suspension was stirred gently with a magnetic stirring bar at 4° for 30 min and then centrifuged for 30 min at 25,000 *g*. The resulting clear supernatant was used as a nuclear extract.

Assays of Kinase Activities

Aliquots of 20 μg of cytosolic protein were incubated with assay buffer specific for each enzyme. The assay buffer for TK contained 50 mM of HEPES, pH 7.9, 10 mM of $MnCl_2$ and 10 μM of Na_3VO_4. For PKA, the assay buffer contained 100 μM of cAMP, 20 mM of $MgCl_2$, in 10 μL of 50 mM Tris, pH 7.5. For MAP2K, the assay buffer contained 12.5 mM of MOPS, pH 7.2, 7.5 mM of $MgCl_2$, 0.05 mM of Na_3VO_4, 0.5 mM of EGTA, 0.05 mM of NaF, and 2 mM of DTT. After 10 min, each enzymatic reaction was initiated by the addition of 5 μL of assay buffer containing 0.5 μCi [γ-^{32}P]ATP (1 μM final concentration) and a 200-μM concentration of specific substrate peptide for the corresponding enzyme (see above). The reaction was terminated after 5 min by the addition of 5 μL of 5% trichloroacetic acid and 1 mg/mL BSA. After 5 min, the samples were centrifuged at 5000 rpm for 5 min at 4° using an Eppendorf microcentrifuge. Three aliquots from each sample (20 μL each) were spotted onto phosphocellulose paper to measure the radioactivity [3]. Each assay was run in triplicate. The data are calculated as means ± SD.

^{125}I-labeled EGF and [^{3}H]E_2 Binding Assays

An aliquot of 200 μg of microsomal protein was used for the ^{125}I-labeled EGF binding assay, and 200 μg of cytosolic or nuclear protein was used for the [^{3}H]E_2 binding assay. The protein was mixed with 1 mL binding assay buffer (Dulbecco's Modified Eagle's Medium: PBS, pH 7.4, 1:1, containing 1% BSA). The buffer contained either 100,000 cpm ^{125}I-labeled EGF (0.25 ng) or [^{3}H]E_2 (0.20 ng). After 50 and 30 min respectively at 37°, the samples were centrifuged at maximum speed in an Eppendorf centrifuge, and the surfaces of the pellets were rinsed carefully one time with ice-cold PBS, then lysed in 1% SDS, and transferred into 4 mL of liquid scintillation fluid. The nonspecific binding was measured using 25 ng of unlabeled EGF with ^{125}I-labeled EGF or 20 ng of unlabeled E_2 with [^{3}H]E_2. The data were calculated as the difference between the values in the presence and absence of unlabeled ligand. Each assay was run in quadruplicate.

Gel-Retardation (Mobility Shift) Assay (EMSA)

To prepare the samples for gel-retardation assay, a 5-μL aliquot of 5 μg of isolated nuclear protein and ATP (final concentration 1 μM) was added along with 5 μL of incubation buffer (50 mM of Tris, pH 7.5, 500 mM of NaCl, 5 mM of DTT, 5 mM of EDTA, 20% glycerol, and 0.4 mg/mL of sonicated salmon sperm DNA) with or without 2 μg of the competitor oligonucleotide, unlabeled AP-1 response element or ERE DNA. The samples were preincubated at 4° for 15 min before the addition of 50,000 cpm of the ^{32}P-labeled ERE or AP-1 response element oligonucleotide and incubated at room temperature for 20 min. Two microliters of 0.1% bromophenol blue in 70% glycerol was added to each sample, which was analyzed on 6% polyacrylamide gels (29:1 acrylamide:bis) in 0.25x TBE (Tris/borate/EDTA) buffer.

RESULTS

Experiments In Vivo

EFFECT OF E_2 AND TCDD ON KINASE ACTIVITIES IN FEMALE ADIPOSE CELLS. At high dose (115 μg/kg), TCDD decreased the activity of TK, PKA, and MAP2K in adipose tissues of mature female rats 24 hr after treatment (Fig. 1A). This negative effect of TCDD on cytosolic phosphorylation contrasts with the positive effects of TCDD on these pathways, as reported frequently in studies of cells from male rodents [3, 4]. E_2 stimulated the activity of TK and PKA in adipose cells from mature females (Fig. 1A), which contrasted with the negative effects of TCDD on the activity of these kinases. When administered 24 hr after E_2, TCDD antagonized the stimulatory action of E_2 on TK and PKA activities (Fig. 1A). E_2 alone decreased the activity of MAP2K; this effect was antagonized by TCDD (Fig. 1A). In immature female rats, TCDD induced similar changes in TK, MAP2K, and PKA activities 24 hr after treatment,

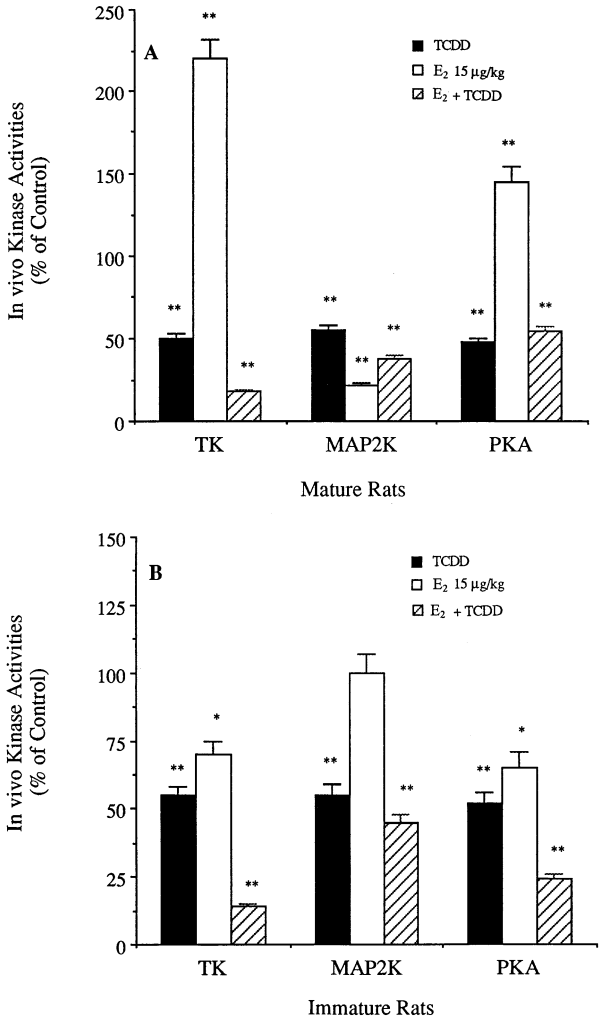

FIG. 1. *In vivo* effect of E_2 and TCDD on kinase activities of (A) mature and (B) immature female rats. The animals were treated with E_2 (15 μg/kg) for 24 hr before a single i.p. dose of 115 μg of TCDD/kg. Abdominal adipose tissue was removed, and 100,000 *g* cytosol was prepared and used to measure TK, MAP2K, and PKA activities. Absolute values for the mature controls (dpm ^{32}P/1 nmol substrate peptide/5 μg of protein ± SD) are: TK, 2550 ± 180; MAP2K, 1590 ± 110; PKA, 1970 ± 160. Absolute values for the immature controls (dpm ^{32}P/1 nmol substrate peptide/5 μg of protein ± SD) are: TK, 3470 ± 240; MAP2K, 2010 ± 160; PKA, 2490 ± 190. Data are the means ± SD of five independent experiments. Significantly different from control values at *$P \leq 0.05$ and **$P \leq 0.01$, respectively (Cochran *t*-test).

while E_2 significantly inhibited TK and PKA activities but had no effect on MAP2K (Fig. 1B).

DOSE–RESPONSE EFFECT OF TCDD ON TK ACTIVITY IN FEMALE ADIPOSE CELLS. The data shown in Fig. 2 demonstrate that TCDD decreased the activities of TK, MAP2K, and PKA in a dose-dependent manner. A significant decrease in TK, MAP2K, and PKA activities was observed in mature females at a dose of 10 μg of TCDD/kg (Fig. 2A) and in immature females at a dose of 2 μg/kg (Fig. 2B).

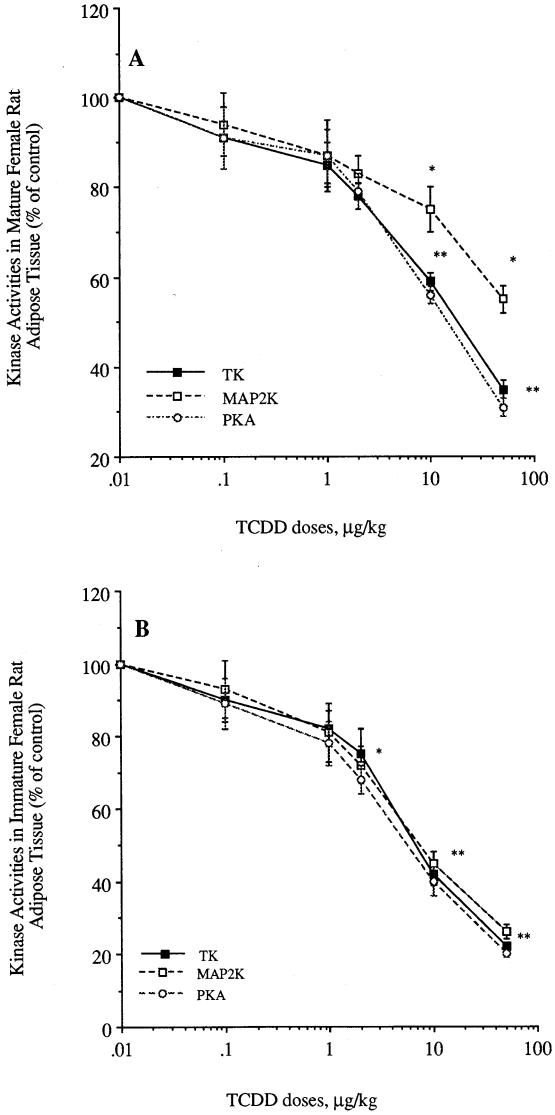

FIG. 2. *In vivo* dose–response study of the effect of TCDD on cytosolic TK, PKA, and MAP2K activities in adipose tissues from (A) mature and (B) immature female rats. Absolute values for TK, MAP2K, and PKA were similar to the values in Fig. 1. Data are the means ± SD of three independent experiments. *$P \leq 0.05$ and **$P \leq 0.01$ (Cochran *t*-test).

TIME–COURSE EFFECT OF TCDD ON TK ACTIVITY IN FEMALE ADIPOSE CELLS. When the minimum effective doses of TCDD were used to study the time–course of TCDD effects on TK activity, a significant decrease in TK activity was demonstrated in mature females 5 days after TCDD treatment, and the maximum decrease in TK activity was observed 14 days after treatment (Fig. 3A). In immature females, a significant decrease in TK activity was first detected 3 days after treatment, but by 5 days the decline had ended and TK activity reached a plateau by 10 days, which remained significantly lower than control values (Fig. 3B).

EFFECT OF TCDD ON FEMALE BODY WEIGHT AND ADIPOSE TISSUE WEIGHT. A significant decrease in the body weight and adipose tissue weight of immature female rats was

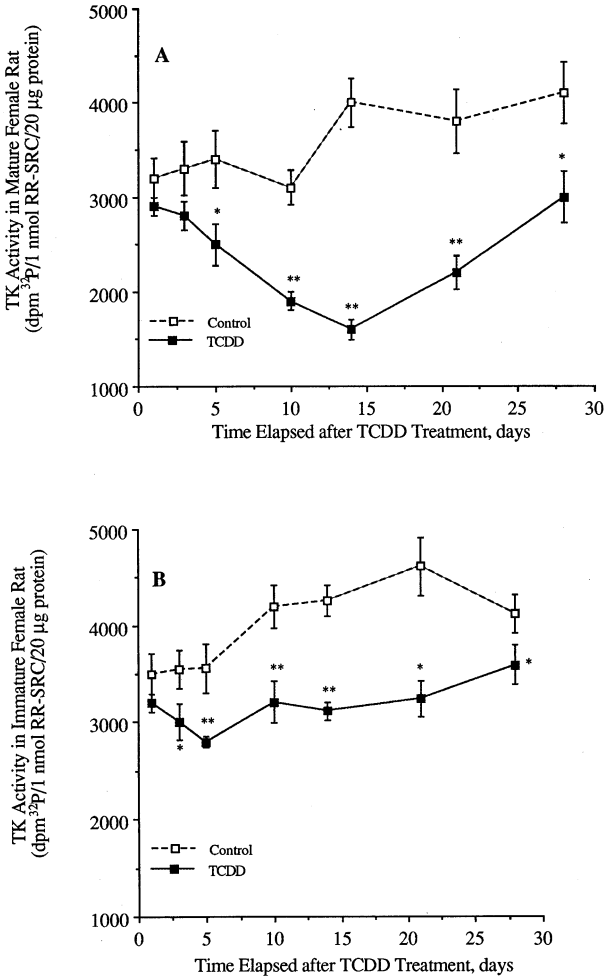

FIG. 3. Time–course effect of TCDD on cytosolic TK activity in (A) mature (10 μg of TCDD/kg) and (B) immature (2 μg of TCDD/kg) female rat adipose tissues. Values are means ± SD, N = 3. *P ≤ 0.05 and **P ≤ 0.01 (Cochran *t*-test).

observed 14 days following treatment with high doses of TCDD (10–115 μg/kg) (data not shown). At lower doses (less than 10 μg/kg), TCDD did not induce wasting in mature females. However, a decrease in the body and adipose tissue weight was observed in immature females 21 days after treatment (Fig. 4). No changes in daily food intake were recorded during the experiment, except for the first 48 hr after TCDD treatment of immature females.

Geldanamycin treatment provided partial protection against the TCDD-induced decrease in body weight, adipose tissue weight, and ovary weight in immature and mature females (Table 1). On the other hand, geldanamycin treatment did not antagonize the decrease in uterine weight that resulted from TCDD treatment. The TCDD-induced mortality at the 115 μg/kg dose was lower in geldanamycin-treated immature female rats (20 vs 60% mortality with TCDD alone). In mature females, no mortality was observed in geldanamycin-treated animals.

TIME–COURSE EFFECT OF TCDD ON EGFR BINDING ACTIVITY IN FEMALE ADIPOSE CELLS. TCDD increased EGFR binding activity in adipose tissue from immature and mature females within 24 hr of a single treatment (Fig. 5). However, EGFR binding activity then decreased in both immature and mature females, reaching significantly lower levels by 5 and 7 days following TCDD treatment, respectively (Fig. 5). The decrease in EGFR binding activity (Fig. 6A) and in TK activity (Fig. 6B) in adipose cells of TCDD-treated animals was partially antagonized by prior treatment with geldanamycin.

EFFECT OF TCDD ON ER LEVELS AND DNA BINDING ACTIVITY OF ERE AND AP-1. TCDD significantly reduced the ER level in the cytosolic and nuclear fractions of adipose tissue from mature female rats and in the nuclear fraction of immature female rats, while E_2 significantly increased ER levels in

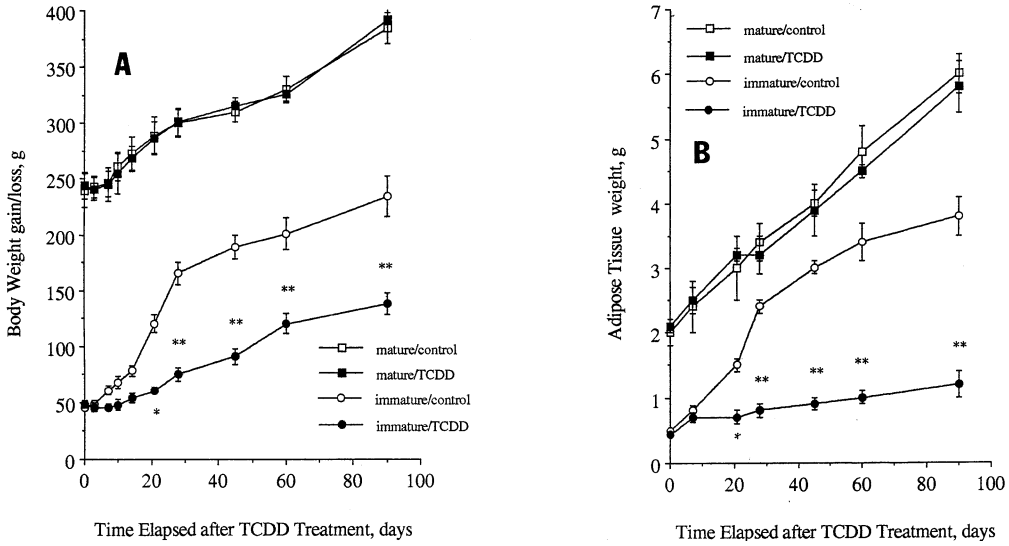

FIG. 4. Effect of 2 μg of TCDD/kg on the (A) body weight and (B) adipose tissue weight of mature and immature female rats. Values are means ± SD, N = 10. *P ≤ 0.05 and **P ≤ 0.01 (Cochran *t*-test).

TABLE 1. Effects of TCDD and the Src-kinase inhibitor geldanamycin on immature and mature female rats

	Weight (g)			
	Whole body	Adipose tissue	Ovary	Uterus
Immature				
Control	90 ± 4	1.6 ± 0.11	0.103 ± 0.001	0.153 ± 0.010
TCDD (115 μg/kg)	61 ± 2*	0.8 ± 0.12*	0.040 ± 0.002*	0.081 ± 0.001*
Geldanamycin‡ plus TCDD	78 ± 5†	1.3 ± 0.10†	0.077 ± 0.001†	0.084 ± 0.002*
Mature				
Control	300 ± 10	2.95 ± 0.61	0.163 ± 0.010	0.254 ± 0.013
TCDD (115 μg/kg)	271 ± 5†	2.00 ± 0.14†	0.071 ± 0.004*	0.192 ± 0.002*
Geldanamycin plus TCDD	284 ± 6	2.5 ± 0.31	0.101 ± 0.009†	0.210 ± 0.004*

Data are the means ± SD of five animals/group. All animals were killed 14 days after TCDD treatment.

*†Significantly different from control values at: $P \leq 0.01$ or $\dagger P \leq 0.05$, respectively (Cochran *t*-test).

‡There were no effects on body weight or organ weights in mature and immature female rats treated with only geldanamycin (data not shown).

both fractions (Fig. 7). In rats co-treated with E_2 plus TCDD, TCDD significantly inhibited the estrogen-induced E_2-ER binding in adipose tissue of mature and immature female rats. E_2 treatment significantly increased the ERE binding activity of adipose tissue from mature and immature females, and TCDD decreased this activity only in mature female rats when injected 24 hr after E_2 (Fig. 8). Similarly, TCDD decreased the DNA binding activity of AP-1 and antagonized the increase in AP-1 binding activity that was induced by E_2 (Fig. 9A). Interestingly, when 25 ng double-stranded ERE DNA was added to the AP-1 DNA binding assay, there was competitive binding of AP-1 as judged by the inhibition in AP-1 DNA binding activity in the presence of unlabeled double-stranded ERE DNA (Fig. 9B).

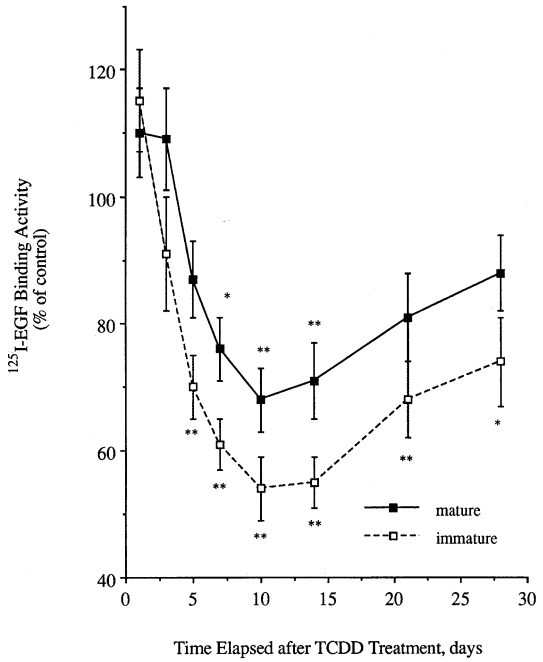

FIG. 5. Time–course effect of 2 μg of TCDD/kg on ^{125}I-labeled EGF binding to microsomal EGFR of adipose tissue from mature and immature female rats. Absolute values for the mature and immature controls were 155 ± 14 and 110 ± 9 fmol/200 μg of protein, respectively. Data are the means ± SD of specific binding of samples from three animals for each time point. Values were significantly different from control at: *$P \leq 0.05$ and **$P \leq 0.01$ (Cochran *t*-test).

Cell Free Studies In Vitro

In the absence of the nucleus, TCDD still decreased TK activity in cytosolic preparations of adipose tissue from mature and immature female rats, but the negative effects on MAP2K activity that were observed in adipose tissue preparations from animals treated with TCDD *in vivo* (Fig. 1) were no longer apparent (Fig. 10). The negative effect of TCDD on PKA activity that was observed in adipose tissues from mature and immature rats that were treated *in vivo* (Fig. 1) was observed only in cell-free preparations obtained from immature animals (Fig. 10). In adipose tissue preparations from mature females, E_2 stimulated TK activity in the absence of the nucleus, and this effect was antagonized by TCDD (Fig. 10), as in the *in vivo* experiments (Fig. 1). In contrast to the results of the *in vivo* experiments, E_2 also stimulated MAP2K activity in the cell-free system, and there was no antagonism of this E_2 action by TCDD (Fig. 10). As in the *in vivo* experiments, E_2 stimulated PKA activity, but the ability of TCDD to block this positive action was lost when the nucleus was absent (Fig. 10). In cell-free preparations from immature females, TCDD did not antagonize the stimulatory effect of E_2 on TK and MAP2K activities. However, the significant increase in PKA activity that was induced by E_2 was abolished by TCDD (Fig. 10).

DISCUSSION

Our previous observations have suggested that there are gender-specific differences in the mechanism of TCDD toxicity in adipose tissues, and that these differences may be mediated, in part, by sex steroid hormones [9]. As a

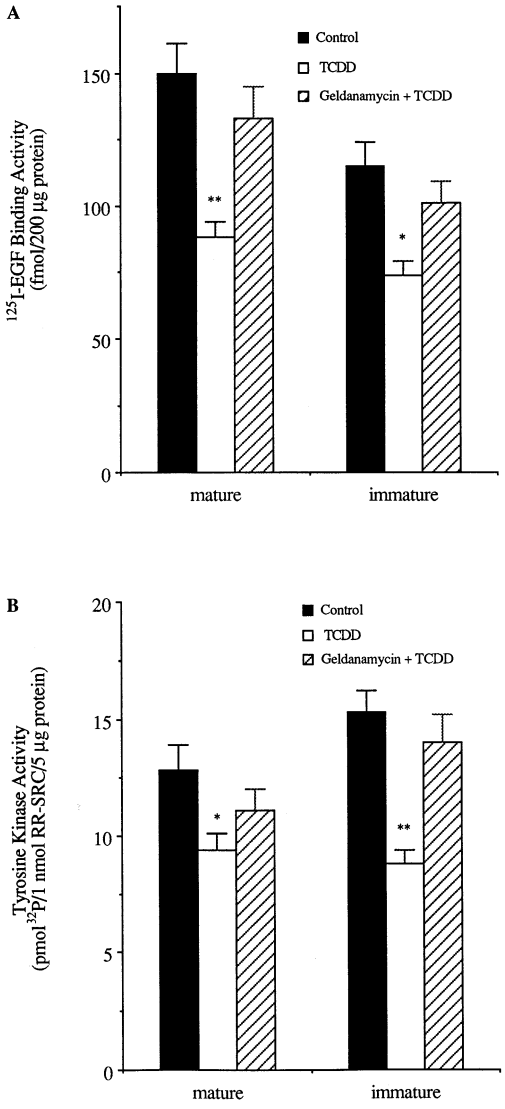

FIG. 6. Effect of geldanamycin on TK activity and EGFR binding activity in TCDD-treated and untreated mature and immature female rats. Four doses of 1 µg of geldanamycin/kg were given to female rats according to the treatment protocol, as described in Materials and Methods. All animals were killed 14 days after TCDD treatment. Adipose tissue was removed and rinsed in ice-cold PBS. Microsomal and cytosolic fractions were prepared and used to measure (A) microsomal EGFR binding using ^{125}I-labeled EGF and (B) cytosolic TK activity using RR-SRC (substrate peptide to tyrosine kinase), [γ-^{32}P]ATP and the phosphocellulose paper method, as described in Materials and Methods. Data are the means ± SD of three animals. These assays were repeated three times. Values were significantly different from control at: *$P \leq 0.05$ and **$P \leq 0.01$ (Cochran *t*-test).

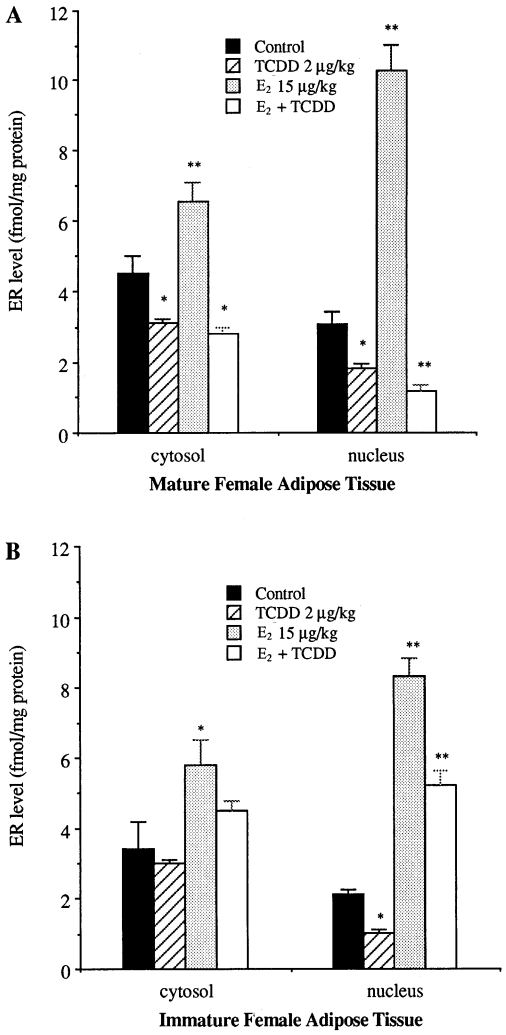

FIG. 7. Cytosolic and nuclear ER level in adipose tissue of mature and immature female rats. A single i.p. dose of 2 µg of TCDD/kg (for immature) and 10 µg of TCDD/kg (for mature) or 15 µg of E_2/kg was administered to animals. In a co-treatment experiment, TCDD was given 24 hr after E_2 (15 µg/kg) injection. Control animals received the same volume of vehicle only. All animals were killed 24 hr after the final treatment. Data are the means ± SD of [^{3}H]E_2 specific binding of three animals for each treatment. ER levels in adipose tissues from (A) mature animals and (B) immature animals are shown. Values were significantly different from control at: *$P \leq 0.05$ and **$P \leq 0.01$ (Cochran *t*-test).

continuation of these studies, we investigated the effects of E_2 and TCDD on adipose cells from immature female rats that had not been exposed to the adult levels of endogenous E_2 experienced by mature females. A series of experiments was performed to study the interactions of E_2 and TCDD on signal transduction pathways. First, a high dose of TCDD (115 µg of TCDD/kg) was used to assure that any lack of TCDD action on signal transduction pathways was due to the interaction between TCDD and E_2 and not to an inadequate dose of TCDD. Then a dose–response study was carried out to determine the lowest dose of TCDD required to induce significant changes in the activity of protein kinases and to address if there is a difference in age-specific susceptibility to TCDD. From these experiments, we found that 2 and 10 µg of TCDD/kg were the lowest doses that induced significant effects on kinases in immature and mature rats, respectively. In the following experiments, a dose of 2 µg of TCDD/kg was used in mature rats to demonstrate that the TCDD dose that did not induce changes in the kinases cascade also did not decrease body weight gain and adipose tissue weight. At this dose, TCDD

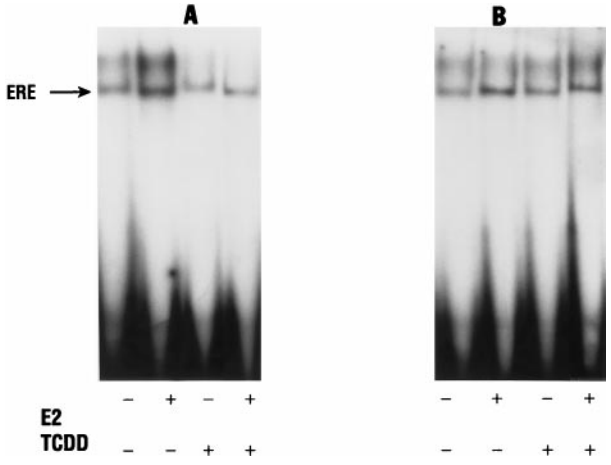

FIG. 8. ERE DNA binding activity in adipose tissue from immature and mature female rats. A single i.p. dose of 2 μg of TCDD/kg (for immature) and 10 μg of TCDD/kg (for mature) or 15 μg of E_2/kg was administered to animals. In a co-treatment experiment, TCDD was given 24 hr after E_2 (15 μg/kg) injection. Control animals received the same volume of vehicle only. All animals were killed 24 hr after the final treatment. Nuclear protein (5 μg) was incubated with 100,000 cpm ^{32}P-labeled double-stranded ERE DNA, and the complex was analyzed using EMSA, as described in Materials and Methods. In a competitive assay with 100-fold excess of ERE, no binding was detected (data not shown). The quantitative radio-scanning values of three gels for ERE DNA binding were as follows: (A) Mature females, control (1500 ± 170 cpm), E_2 (3200 ± 350 cpm), TCDD (800 ± 50 cpm), and TCDD plus E_2 (1100 ± 100 cpm). (B) Immature females, control (1320 ± 110 cpm), E_2 (3000 ± 140 cpm), TCDD (1440 ± 170 cpm), and TCDD plus E_2 (2350 ± 200 cpm).

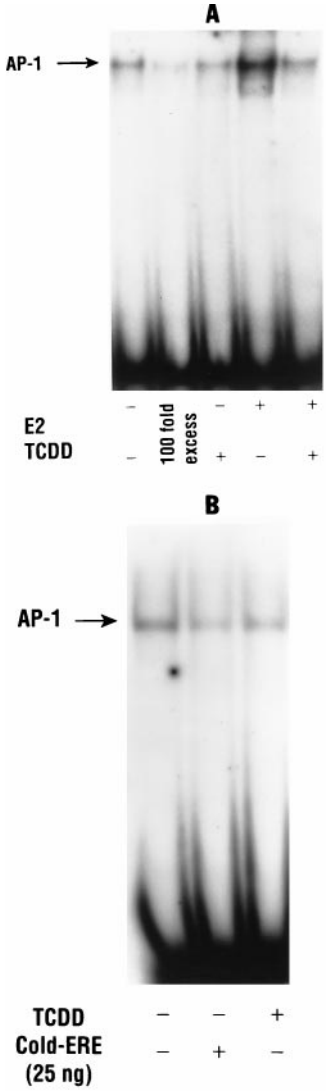

FIG. 9. AP-1 DNA binding activity in adipose tissue from mature female rats. A single i.p. dose of 10 μg of TCDD/kg or 15 μg of E_2/kg was administered to animals. In a co-treatment experiment, TCDD (10 μg/kg) was given 24 hr after E_2 (15 μg/kg) injection. Control animals received the same volume of vehicle only. All animals were killed 24 hr after the final treatment. In panel A, 5 μg of nuclear protein was incubated with 100,000 cpm ^{32}P-labeled double-stranded AP-1 DNA, and the complex was analyzed using EMSA as described in Materials and Methods. In panel B, the DNA binding activity of AP-1 was performed exactly as described in panel A, except that 25 ng of unlabeled ERE was incubated with the nuclear protein 20 min prior to the addition of ^{32}P-labeled double-stranded AP-1 DNA.

decreased body weight gain and adipose tissue weight in immature rats.

Our data demonstrated that TCDD inhibits several basal and E_2-induced responses in adipose tissues from both immature and mature female rats, and the data suggest that susceptibility to TCDD-induced toxicity in females is modified during the course of sexual development. These findings underscore the potential importance of E_2–TCDD interactions in the expression of TCDD-induced toxicity. Treatment of mature females with 2 μg of TCDD/kg did not decrease body weight or TK activity in adipose cells, but the same dose decreased both body weight and TK activity in adipose tissues of immature females. The results of experiments with geldanamycin support the idea that the TCDD-induced wasting syndrome is mediated through the kinases signaling cascade. Pretreatment with this Src kinase inhibitor blocked the TCDD-induced effects on body weight (Table 1) and inhibited the TCDD-induced down-regulation of EGFR binding activity (Fig. 6). Geldanamycin has been shown to block the mitotic signal by specifically inhibiting pp60^{src} [39], and we have shown recently that c-Src kinase is a component of the cytosolic AhR complex [6, 8]. In the current study, however, geldanamycin did not block the TCDD-induced decrease in uterine and ovarian weights, suggesting that the effect of TCDD on reproductive tract cells is, in part, Src independent. On the other hand, previous studies have also employed various tyrosine kinase inhibitors (genistein and herbimycin A) to block the proliferation of breast cancer cells [40–42]. Together, these data might explain some of the tissue specificity to TCDD exposure.

The present data show that the responsiveness of the EGFR/kinase cascade is modulated by female maturation and that some kinase activities are inhibited by TCDD in the presence of the nucleus. It has been reported that the initial activation of EGFR by its ligands requires the

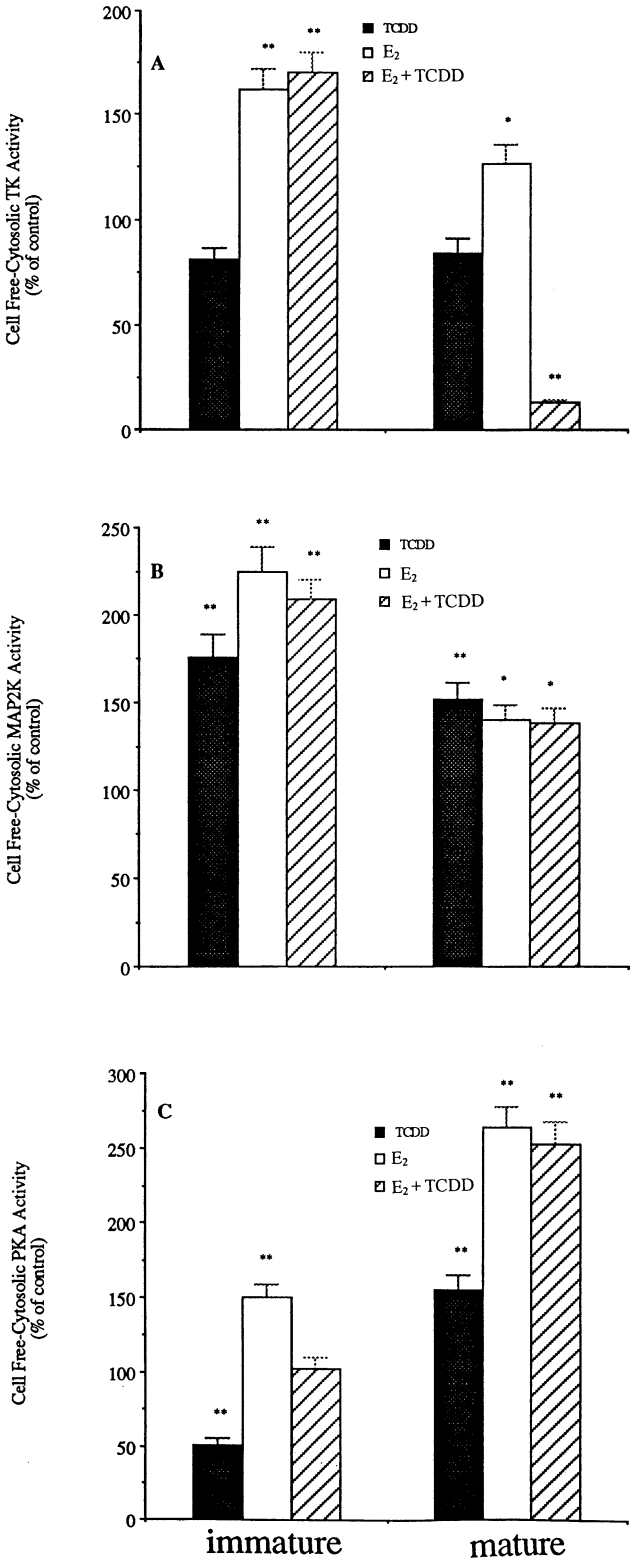

FIG. 10. Effect of E_2 and TCDD on kinase activities in the cytosolic fraction of adipose cells from untreated mature and immature female rats. TCDD (10 nM) and E_2 (10 nM) were incubated with 20 μg of protein and 10 μL of specific kinase buffer in a total volume of 80 μL for 10 min at 30° before kinase assay. In the co-treatment experiment, E_2 was added 10 min before TCDD. (A) Absolute values for TK activity in mature and immature rats were 980 ± 70 and 1110 ± 90 dpm ^{32}P/1 nmol substrate peptide/5 μg of protein, respectively; (B) absolute values for MAP2K activity in mature and immature rats were 660 ± 30 and 480 ± 20 dpm ^{32}P/1 nmol substrate peptide/5 μg of protein, respectively; (C) absolute values for PKA activity in mature and immature rats were 720 ± 60 and 690 ± 60 dpm ^{32}P/1 nmol substrate peptide/5 μg of protein, respectively. Data are the means ± SD of five independent experiments. Values were significantly different from control at *P ≤ 0.05 and **P ≤ 0.01 (Cochran *t*-test).

activation of its own protein TK [43]. Down-regulation in EGFR occurs as a subsequent step following receptor activation. Therefore, we have focused on TK activity as a way to study the onset of TCDD-triggered changes in signal transduction pathways.

In agreement with the differences observed between males and females, there was evidence for a different effect on the kinases signaling cascade when adipose cells of immature females were exposed to TCDD in comparison with the response of cells from adult females. While TCDD consistently decreased the activities of all three kinases in both mature and immature animals, only in mature animals did TCDD antagonize the actions of E_2 on TK and PKA activities. These data demonstrate that the interactions between E_2 and TCDD are different in immature and mature animals and suggest that it is the exposure to mature levels of E_2 that determines the subsequent cellular responses of adipose tissue to E_2 and TCDD, as well as the interactions between them.

The cell-free system provides additional information regarding the mechanism by which the maturation of females changes the actions of TCDD. The effect of TCDD on TK activity was the same in both immature and mature females, regardless of the presence or absence of the nucleus. In all cases, TK activity was inhibited by TCDD treatment. In contrast, the effect of E_2 treatment on TK activity was different in immature and mature females, with TK activity being inhibited in the immature animal and stimulated in the mature animal. In the absence of the nucleus, this difference was no longer apparent, and TK activity was stimulated under cell-free conditions in both immature and mature females. In addition, the combination of E_2 and TCDD treatments, which resulted in a negative effect on TK activity in cells of immature animals (Fig. 1), also resulted in a positive effect when the nucleus was absent (Fig. 10). In the presence of the nucleus, TCDD potentiated the inhibitory action of E_2 on TK in immature rat adipose tissue (Fig. 1), an effect that disappeared in the absence of the nucleus (Fig. 10). In mature rats, the absence of nucleus did not change the effect of E_2–TCDD interaction on TK activity. These data suggest that maturation of the female leads to a change in the response pattern of TK (or factors upstream of TK) to estrogen but not to TCDD. In addition, these data demonstrate that the cytosolic factors that respond to estrogen also interact with nuclear elements, since removal of the nucleus resulted in additional changes in response patterns. For example, the effect of TCDD on MAP2K involves interactions with the

nucleus, since an opposite response of MAP2K activity to TCDD was observed in the cell-free system compared with the response of the intact cell. Although PKA responded differently to E_2 following maturation of the animal, the effect of TCDD on this kinase in intact cells was always inhibitory. In cell-free preparations, the effect of TCDD on PKA activity again suggested involvement of interactions with the nucleus. The response patterns of PKA are inconsistent with those of TK and MAP2K and suggest that both E_2 and TCDD may act on PKA through mechanisms that are independent of the TK/MAP2K cascade. These observations suggest that interactions between TCDD and E_2 at several loci in the cytoplasm are determined or modified by previous exposure to sex steroids. Taken together, these data demonstrate that after female animals reach maturity, the response of nonreproductive tract cells to TCDD is changed and that the capacity of TCDD for disruption of E_2 action is increased.

We hypothesize that some xenobiotics are specific in utilizing pathways that are regulated by sex steroids. According to this model, the TK/MAP2K pathway, which is used by TCDD to transduce its toxic signal, is regulated by E_2 and/or androgen. The time–course of the cytosolic responses to E_2 and TCDD provides strong evidence that some of the interactions between these two molecules are independent of the nucleus and involve the triggering of cytosolic phosphorylation pathways. The concept of nuclear independent action is further supported by the consistent antagonism between E_2 and TCDD in regulating TK activity in whole cells and cell-free preparations. The studies of immature and mature rats treated with E_2 indicate that these mechanisms are age-specific and are probably E_2-dependent in the adult animal. Thus, the primary interaction and antagonism between E_2 and TCDD appear to involve TK, EGFR, and MAP2K through an upstream mechanism(s), and this model is consistent with the attenuation of the adverse affects of TCDD by geldanamycin, which blocks the Src-mediated phosphorylation cascade resulting from interaction between TCDD and AhR.

When the TK/MAP2K pathway has been modulated by previous exposure to E_2, both the ER and the nucleus must interact to transduce signals initiated either by ER-ligands or by AhR-ligands. Such ligands may activate steroid hormone receptors through an alteration in the protein phosphorylation state of the receptor protein itself. Steroid hormones have been shown to exist within cells as phosphoproteins [44], and binding of ligand has been shown to increase the overall phosphorylation state of several of these receptors [45, 46]. Since potential target sites for a variety of protein kinases are present on the ER [47], it is possible that a change in the phosphorylation state of the ER may alter its ability to activate gene transcription [27]. The recent finding by Kato *et al.* [48] is consistent with this concept and underscores the importance of MAPK family members and related signaling proteins such as Grb2, Ras, and PI3K in the activation of the ER. The present data also show that TCDD changed the activity of protein kinases in immature and mature females and that the prior treatment with E_2 induced age-specific effects of TCDD on the activity of TK, MAP2K, and PKA. This E_2–TCDD interaction suggests the possibility of ER-AhR overlapping through EGFR and signal transduction pathways.

Among the known transcription enhancer factors, the ER is paradoxical in that its activity is controlled primarily by its ligand, which causes its release from heat-shock proteins [28] to induce the formation of active homodimers [29] and the phosphorylation of the receptor at specific tyrosine and serine residues [30]. Upon ligand binding, the affinity of nuclear ER for a particular ERE is thought to increase and allow the receptor protein to serve as an enhancer or activator of gene transcription [49]. In addition, ER binding to ERE at the *trans*-activating site of DNA is strongly influenced by several other hormone receptors and nuclear transcription factors such as AP-1. The latter mechanisms, sometimes referred to as "cross-talk," recently have received particular attention, as they reveal an entirely new way to explain many aspects of regulatory cross-coordination between different signal transduction pathways [50–52]. Consistent with this mechanism, our data showed that EREs interacted with AP-1 and decreased its DNA binding activity, as judged by EMSA (Fig. 9B).

The present data illustrate that TCDD acts as an antiestrogen, since it significantly reduced the ER level in the cytosol and nucleus, while E_2 significantly increased ER levels in both fractions (Fig. 7). These data can be explained by the DNA binding activity of ERE, since E_2 significantly increased ERE binding activity and TCDD decreased this activity (Fig. 8). These results are consistent with a previous report by Safe and co-workers [53], who found that TCDD significantly decreases hepatic and uterine ER levels in female rats, while E_2 increases hepatic and uterine ER. A combination of TCDD and E_2 resulted in uterine and hepatic ER levels that were similar or lower than those observed after treatment with only TCDD [53].

Following initial reports that TCDD inhibited the development of spontaneous mammary and uterine tumors in rats [54], numerous studies have confirmed that TCDD inhibits a wide range of E_2-induced responses in rodents and in MCF-7 cells [55–57]. This effect was also observed in wild-type Ah-responsive Hepa 1c1c7 cells but not in the Ah-nonresponsive mutants [58]. Mechanistic studies have shown that the TCDD-mediated down-regulation of the nuclear ER in wild-type mouse Hepa 1c1c7 cells is inhibited by cycloheximide and actinomycin D and that the structure-dependent potencies of TCDD and related compounds are comparable to their competitive binding affinities for the AhR [58, 59]. It has been suggested that TCDD-mediated anti-estrogenicity could be due to either CYPIA1, increased metabolism of E_2 [60, 61] or induction of gene products other than CYPIA1, which modulate estrogen-induced responses [59, 62]. Our present results suggest that the antiestrogenic responses of TCDD in female adipose tissue are due to the down-regulation in

DNA binding activity of AP-1 and to AP-1–ERE cross-talk. This possibility is supported by the following observations: (1) treatment of female rats with TCDD results in a dose-dependent decrease in constitutive uterine c-fos mRNA [34], (2) the E_2-induced stimulation of c-fos mRNA is antagonized by TCDD [34], (3) an ERE that binds the ER and the AP-1 transcription factor has been identified upstream of the human c-*fos* gene [30], and (4) our present data that double-stranded ERE antagonized the DNA binding activity of AP-1 (Fig. 9B).

The results of the current study demonstrate that TCDD consistently opposed or antagonized the action of E_2 on the activities of TK, MAP2K, and PKA in adipose cells. Since MAP2K is essential for transducing mitogenic signals from growth factors through EGFR and for the nuclear expression of E_2 action through its classical transcription pathway, the present data suggest a previously unrecognized mechanism by which estrogens support normal cell function in a wide range of cell types. If the continued presence and action of E_2 are important for this central (kinases) pathway to function, then the blocking of E_2 action by xenobiotics at the level of TK and downstream MAP2K could lead to abnormal cell function. While specific changes in the intracellular signal transduction cascades, which take place during female maturation, are not revealed by these studies, the enhanced response of these kinases to acute estrogen exposure would be expected as a result of E_2 induction of its own receptor and response mechanisms.

Our data provide evidence for the involvement of growth factors and the kinases cascade in the TCDD-induced wasting syndrome. However, the observation that geldanamycin provided only partial protection against TCDD-induced wasting, decreased TK activity, and decreased EGFR binding activity suggests that other signal transduction components, in addition to Src kinase, may be involved in the mechanism of TCDD-induced toxicity in females. The involvement of other kinases, such as PKC, in TCDD toxicity is the subject of current investigations in our laboratory.

In conclusion, our data demonstrated that TCDD-induced phosphorylation, most likely through the TK/MAPKs and PKA pathways, interferes with E_2 action, and these observations provide strong evidence that TCDD and other toxicants may share cytosolic/nuclear transduction pathways with sex steroids. In this way, some toxicants can be modulated by sex steroids and may, in turn, mimic or antagonize the physiologic effects of sex hormones. The recent report that transduction of the ER-ligand signal requires phosphorylation of Ser118 through the Ras-MAP kinase cascade [48] underscores the significance of our data for understanding the mechanism of TCDD-induced toxicity to hormone-sensitive cells. According to our model, TCDD could disrupt the action of ER–ligand complexes at the cytosolic/nuclear level by preventing activation of the complex prior to interaction with its response element in the nucleus. Toxicants, such as TCDD, may block transduction from specific membrane receptor signals such as those of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by alteration of the PKA pathway. Similarly, steroid hormone and growth factor signaling pathways could be altered by disruption of the TK/MAPKs pathway. Our hypothesis, if correct, predicts differences in the biological effects of specific classes of environmental estrogens and antiestrogens and explains long-term effects of xenobiotics through changes in mitotic signal transduction pathways via the kinase/phosphatase cascade and changes in the differentiation of developing cells. More importantly, our model predicts that xenobiotics such as TCDD may have permanent effects on endocrine and growth factor signal transduction pathways that may be more deleterious than their actions as immediate hormone disruptors.

This work was supported, in part, by National Institutes of Health Grants P01 ES06198 and P30 ES05707.

References

1. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22:** 517–524, 1982.
2. Safe S, Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu Rev Pharmacol Toxicol* **26:** 371–399, 1986.
3. Enan E and Matsumura F, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induced alterations in protein phosphorylation in guinea pig adipose tissue. *J Biochem Toxicol* **8:** 89–99, 1993.
4. Enan E and Matsumura F, Significance of TCDD-induced changes in protein phosphorylation in the adipocyte of male guinea pigs. *J Biochem Toxicol* **9:** 159–170, 1994.
5. Enan E and Matsumura F, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-induced changes in glucose transporting activity in guinea pigs, mice, and rats *in vivo* and *in vitro*. *J Biochem Toxicol* **9:** 97–106, 1994.
6. Enan E and Matsumura F, Evidence for a second pathway in the action mechanism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): Significance of Ah-receptor mediated activation of protein kinase under cell-free conditions. *Biochem Pharmacol* **49:** 249–261, 1995.
7. Enan E and Matsumura F, Regulation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) of the DNA binding activity of transcriptional factors via nuclear protein phosphorylation in guinea pig adipose tissue. *Biochem Pharmacol* **50:** 1199–1206, 1995.
8. Enan E and Matsumura F, Identification of c-Src as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) through the protein phosphorylation pathway. *Biochem Pharmacol* **52:** 1599–1612, 1996.
9. Enan E, Overstreet J, Matsumura F and Lasley B, Gender differences in the mechanism of dioxin toxicity in rodents and in nonhuman primates. *Reprod Toxicol* **10:** 401–411, 1996.
10. Enan E, Moran F, VandeVoort CA, Stewart DR, Overstreet JW and Lasley BL, Mechanism of toxic action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in cultured human luteinizing granulosa cells. *Reprod Toxicol* **10:** 497–508, 1996.
11. Enan E, Lasley B, Stewart D, Overstreet J and Vandevoort C, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) modulates function of human luteinizing granulosa cells via cAMP signaling and early reduction of glucose transporting activity. *Reprod Toxicol* **10:** 191–198, 1996.
12. Whitlock JP Jr, Genetic and molecular aspects of 2,3,7,8-

tetrachlorodibenzo-*p*-dioxin action. *Annu Rev Pharmacol Toxicol* **30:** 251–277, 1990.
13. Reyes H, Reisz-Porszasz S and Hankinson O, Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* **256:** 1193–1195, 1992.
14. Whitelaw M, Pongratz I, Wilhelmsson A, Gustafsson J-Å and Poellinger L, Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. *Mol Cell Biol* **13:** 2504–2514, 1993.
15. Denison MS, Fisher JM and Whitlock JP Jr, The DNA recognition site for the dioxin-Ah receptor complex: Nucleotide sequence and functional analysis. *J Biol Chem* **263:** 17221–17224, 1988.
16. Heuman DM, Gallagher EJ, Barwick JL, Elshourbagy NA and Guzelian PS, Immunochemical evidence for induction of a common form of hepatic cytochrome P-450 in rats treated with pregnenolone-16α-carbonitrile or other steroidal or non-steroidal agents. *Mol Pharmacol* **21:** 753–760, 1982.
17. Schuetz EG, Wrighton SA, Barwick JL and Guzelian PS, Induction of cytochrome P-450 by glucocorticoids in rat liver. I. Evidence that glucocorticoids and pregnenolone 16α-carbonitrile regulate *de novo* synthesis of a common form of cytochrome P-450 in cultures of adult rat hepatocytes and in the liver *in vivo*. *J Biol Chem* **259:** 1999–2006, 1984.
18. Meek DW and Street AJ, Nuclear protein phosphorylation and growth control. *Biochem J* **287:** 1–15, 1992.
19. O'Malley BW and Buller RE, Mechanism of steroid hormone action. *J Invest Dermatol* **68:** 1–4, 1977.
20. Katzenellenbogen BS and Norman MJ, Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: Interrelationships among insulin/insulin-like growth factor-1, serum and estrogen. *Endocrinology* **126:** 891–898, 1990.
21. Mukku VR and Stancel GM, Regulation of epidermal growth factor by estrogen. *J Biol Chem* **260:** 9820–9824, 1985.
22. Dickson RB and Lippman ME, Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr Rev* **8:** 29–43, 1987.
23. Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA and Korach KS, Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independent of protein kinase C or estradiol. *Endocrinology* **137:** 1735–1744, 1996.
24. Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA and Korach KS, Coupling of dual signaling pathways: EGF action involves the estrogen receptor. *Proc Natl Acad Sci USA* **89:** 4658–4662, 1992.
25. Vignon F, Bouton MM and Rochefort H, Antiestrogens inhibit the mitogenic effect of growth factors in breast cancer cells in the total absence of estrogens. *Biochem Biophys Res Commun* **146:** 1502–1508, 1987.
26. Aronica SM and Katzenellenbogen BS, Progesterone receptor regulation in uterine cells: Stimulation by estradiol, cyclic adenosine 3′,5′-monophosphate and insulin-like growth factor I and suppression by antiestrogens and protein kinase inhibitors. *Endocrinology* **128:** 2045–2052, 1991.
27. Smith DF and Toft DO, Steroid receptors and their associated proteins. *Mol Endocrinol* **7:** 4–11, 1993.
28. Denton RR, Koszewski NJ and Notides AC, Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. *J Biol Chem* **267:** 7263–7268, 1992.
29. Castoria G, Migliaccio A, Green S, Di Domenico M, Chambon P and Auricchio F, Properties of a purified estradiol-dependent calf uterus tyrosine kinase. *Biochemistry* **32:** 1740–1750, 1993.
30. Weisz A and Rosales R, Identification of an estrogen response element upstream of the human c-*fos* gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Res* **18:** 5097–5106, 1990.
31. Boettger-Tong HL, Murthy L and Stancel GM, Cellular pattern of c-*fos* induction by estradiol in the immature rat uterus. *Biol Reprod* **53:** 1398–1406, 1995.
32. Weisz A and Bresciani F, Estrogen induces expression of c-*fos* and c-*myc* protooncogenes in rat uterus. *Mol Endocrinol* **2:** 816–824, 1988.
33. Loose-Mitchell DS, Chiappetta C and Stancel G, Estrogen regulation of c-*fos* messenger ribonucleic acid. *Mol Endocrinol* **2:** 946–951, 1988.
34. Astroff B, Eldridge B and Safe S, Inhibition of the 17β-estradiol-induced and constitutive expression of the cellular proto-oncogene c-*fos* by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the female rat uterus. *Toxicol Lett* **56:** 305–315, 1991.
35. Wilding G, Lippman ME and Gelmann EP, Effects of steroid hormones and peptide growth factors on protooncogene c-*fos* expression in human breast cancer cells. *Cancer Res* **48:** 802–805, 1988.
36. Webb DK, Moulton BC and Khan SA, Estrogen induced expression of the c-*jun* proto-oncogene in the immature and mature rat uterus. *Biochem Biophys Res Commun* **168:** 721–726, 1990.
37. Weisz A, Cicatiello L, Persico E, Scalona M and Bresciani F, Estrogen stimulates transcription of c-*jun* protooncogene. *Mol Endocrinol* **4:** 1041–1050, 1990.
38. Dignam ID, Lebouitz RM and Roeder RG, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:** 1475–1489, 1983.
39. Yamaki H, Nakajima M, Seimiya H, Saya H, Sugita M, Tsuruo T, Inhibition of the association, with nuclear matrix of pRB, p70 and p40 proteins along with the specific suppression of c-MYC expression by geldanamycin, an inhibitor of Src tyrosine kinase. *J Antibiot (Tokyo)* **48:** 1021–1026, 1995.
40. Reddy KB, Mangold GL, Tandon AK, Mundy GR, Zilberstein A and Osborne CK, Inhibition of breast cancer cell growth *in vitro* by a tyrosine kinase inhibitor. *Cancer Res* **52:** 3636–3641, 1992.
41. Monti E and Sinha BK, Antiproliferative effect of genistein and adriamycin against estrogen-dependent and -independent human breast carcinoma cell lines. *Anticancer Res* **14:** 1221–1226, 1994.
42. Clark JW, Santos-Moore A, Stevenson LE and Frackelton AR Jr, Effects of tyrosine kinase inhibitors on the proliferation of human breast cancer cell lines and proteins important in the Ras signaling pathway. *Int J Cancer* **65:** 186–191, 1996.
43. Northwood IC and Davis RJ, Activation of the epidermal growth factor receptor tyrosine protein kinase in the absence of receptor oligomerization. *J Biol Chem* **263:** 7450–7453, 1988.
44. Moudgil VK, Phosphorylation of steroid hormone receptors. *Biochim Biophys Acta* **1055:** 243–258, 1990.
45. Hoeck W, Rusconi S and Groner B, Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. *J Biol Chem* **264:** 14396–14402, 1989.
46. Orti D, Bodwell JE and Munck A, Phosphorylation of steroid hormone receptors. *Endocr Rev* **13:** 105–128, 1992.
47. Green S, Walter P, Kumar V, Krust A, Bornet J-M, Argos P and Chambon P, Human oestrogen receptor cDNA: Sequence, expression and homology to v-*erb*-A. *Nature* **320:** 134–139, 1986.
48. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D and Chambon P, Activation of the estrogen

receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270:** 1491–1494, 1995.
49. Ham J and Parker MG, Regulation of gene expression by nuclear hormone receptors. *Curr Opin Cell Biol* **1:** 503–511, 1989.
50. Lamb P and McKnight SL, Diversity and specificity in transcriptional regulation: The benefits of heterotypic dimerization. *Trends Biochem Sci* **16:** 417–422, 1991.
51. Miner JN and Yamamoto KR, Regulatory crosstalk at composite response elements. *Trends Biochem Sci* **16:** 423–426, 1991.
52. Wagner S and Green MR, Retinoblastoma. A transcriptional tryst. *Nature* **352:** 189–190, 1991.
53. Romkes M, Piskorska-Pliszczynska J and Safe S, Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic and uterine estrogen receptor levels in rats. *Toxicol Appl Pharmacol* **87:** 306–314, 1987.
54. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA and Humiston CG, Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Toxicol Appl Pharmacol* **46:** 279–303, 1978.
55. Gallo MA, Hesse EJ, MacDonald GJ and Umbreit TH, Interactive effects of estradiol and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic cytochrome P450 and mouse uterus. *Toxicol Lett* **32:** 123–132, 1986.
56. Romkes M and Safe S, Comparative activities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and progesterone as antiestrogens in the female rat uterus. *Toxicol Appl Pharmacol* **92:** 368–380, 1988.
57. Astroff B and Safe S, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin as an antiestrogen: Effect on rat uterine peroxidase activity. *Biochem Pharmacol* **39:** 485–488, 1990.
58. Zacharewski T, Harris M and Safe S, Evidence for a possible mechanism of action of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated decrease of nuclear estrogen receptor levels in wild-type and mutant Hepa 1c1c7 cells. *Biochem Pharmacol* **41:** 1931–1939, 1991.
59. Harris M, Zacharewski T and Safe S, Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds on the occupied nuclear estrogen receptor in MCF-7 human breast cancer cells. *Cancer Res* **50:** 3579–3584, 1990.
60. Gierthy JF and Lincoln DW, Inhibition of postconfluent focus production in cultures of MCF-7 breast cancer cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Breast Cancer Res Treat* **12:** 227–233, 1988.
61. Spink DC, Lincoln DW II, Dickerman HW and Gierthy JF, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes an extensive alteration of 17β-estradiol metabolism in MCF-7 breast tumor cells. *Proc Natl Acad Sci USA* **87:** 6917–6921, 1990.
62. Biegel L and Safe S, Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on cell growth and the secretion of the estrogen-induced 34-, 52- and 160-kDa proteins in human breast cancer cells. *J Steroid Biochem Mol Biol* **37:** 725–732, 1990.