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Suppression of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-mediated *CYP1A1* and *CYP1B1* induction by 12-*O*-tetradecanoylphorbol-13-acetate: role of transforming growth factor β and mitogen-activated protein kinases

Meng Guo[a], Aby Joiakim[a], David T. Dudley[b], John J. Reiners, Jr.[a,*]

[a]*Institute of Environmental Health Sciences, Wayne State University, 2727 Second Ave., Rm. 4000, Detroit, MI 48201, USA*
[b]*Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI 48105, USA*

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Abstract

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) enhances or suppresses the transcriptional activation of *CYP1A1* by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in a cell/tissue-specific manner. The basis for these effects is not known. Exposure of the immortalized human breast epithelial cell line MCF10A-Neo to TPA at the time of, or up to 12 hr prior to, the addition of TCDD strongly suppressed the transcriptional activation of *CYP1A1* and *CYP1B1* (IC_{50} ~0.5 nM). A recent study (Carcinogenesis 2000;21:1303–12) demonstrated that TPA-treated MCF10A-Neo cells rapidly activate the latent transforming growth factor β (TGFβ) in the serum used to supplement the culture medium. The suppressive effects of TPA on *CYP1A1* induction by TCDD in MCF10A-Neo cultures could be partially suppressed by: (a) co-incubation of TCDD + TPA-treated cultures with a neutralizing TGFβ pan antibody; (b) prior removal of latent TGFβ from the culture medium; or (c) switching cultures to serum- and growth factor-free medium immediately before the addition of TPA and TCDD. Exposure of cultures to TPA 24–48 hr prior to subsequent TPA + TCDD treatment not only inhibited the suppressive effects of TPA, but markedly enhanced CYP1A1 mRNA accumulation. TPA caused a rapid and protracted activation of extracellular signal-regulated kinases (ERKs). Pretreatment of cultures with the mitogen-activated protein kinase kinase (MEK) inhibitor PD184352 [2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropyl-methoxy-3,4-difluoro-benzamide] completely inhibited ERK activation by TPA. However, PD184352 did not prevent the suppressive effects of TPA on *CYP1A1* activation by TCDD. These studies demonstrate that TPA initiates protein kinase C-dependent, ERK-independent processes that suppress *CYP1A1* activation by TCDD in MCF10A-Neo cells. Furthermore, TGFβ mediates a small portion of this suppressive activity.

Keywords: CYP1A1; MAP kinase; TCDD; TGFβ; TPA

1. Introduction

The AHR is a ligand-activated transcription factor [1,2]. In the absence of ligand, it resides in the cytoplasm of many types of cells. Upon binding ligand, the AHR translocates to the nucleus where it complexes with the ARNT protein. Heterodimers of AHR and ARNT, in conjunction with possibly other co-activating proteins, interact with enhancer sequences (designated DREs) in target genes, and stimulate the transcription of such genes [1,2]. Several genes involved in phase I metabolism (e.g. *CYP1A1, CYP1A2, CYP1B1*) and phase II metabolism (e.g. *ALDH4* and *NQO1*) of xenobiotics contain DRE sequences in their 5′-flanking regions, and are transcriptionally activated by AHR agonists [1–3]. Among the many planar aromatic molecules identified as AHR agonists, TCDD is probably the most potent.

A variety of agents affect the transcriptional activation of *CYP1A1* and *CYP1B1* by AHR agonists. The cytokine TGFβ suppresses the transcriptional activation of these two P450s in some [4–7], but not all, cell types [8,9]. The basis for the suppression is not well characterized, but presum-

* Corresponding author. Tel.: +1-313-963-7661; fax +1-313-577-0082.
E-mail address: john.reiners.jr@wayne.edu (J.J. Reiners, Jr.).

Abbreviations: AHR, aryl hydrocarbon receptor; ALDH4, aldehyde dehydrogenase class 4; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP, cytochrome P450; DRE, dioxin response element; ERKs, extracellular signal-regulated kinases; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NQO1, NADP(H):quinone oxidoreductase; PKC, protein kinase C; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGFβ, transforming growth factor β; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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ably requires protein synthesis since it can be blocked by pretreatment with cycloheximide [6]. A second widely studied agent is the phorbol ester TPA. Its effects on the transcriptional activation of *CYP1A1* by AHR agonists vary markedly among cell types. For example, *in vivo* exposure of rodents to TPA prior to AHR agonist treatment suppresses *CYP1A1* induction in liver [10], skin [11], and thymus [12]. Pretreatment with TPA also suppresses TCDD-dependent induction of *CYP1A1* in cultures of MCF-7 cells [13] and human primary keratinocytes [14]. In contrast, TPA pretreatment potentiates the effects of TCDD 2- to 3-fold in HepG2, HeLa, and COS-1 cells transfected with constructs designed to assess the transactivating activity of AHR/ARNT complexes bound to DREs [15–17]. Inhibitor studies suggest that this potentiation is mediated by PKCs [15–17]. The basis for the differential effects of TPA in different cell types is unclear.

TGFβ and TPA are cytostatic to many cell types [18–28]. The antiproliferative effects of the latter agent may be partially mediated by TGFβ. TPA is cytostatic to MCF-7 cells [21–25], and induces the *de novo* synthesis and extracellular release of TGFβ [23,24]. We recently demonstrated that the immortalized human breast epithelial cell line MCF10A-Neo undergoes a G_1 arrest following exposure to concentrations of TPA as low as 1 nM [29]. This arrest occurred rapidly, but was transient. Cultures reentered the cell cycle within 24 hr of TPA treatment. A variety of approaches demonstrated that TGFβ was partially responsible for the cytostatic effects of TPA. An unusual aspect of the study was the source of TGFβ. MCF10A-Neo cultures did not synthesize TGFβ in response to TPA. Instead, TPA stimulated the activation of a proteolytic process leading to the processing of latent TGFβ supplied by the serum used to supplement the culture medium. This activation of serum-derived latent TGFβ occurred within 1 hr of TPA treatment.

In preliminary studies, we observed that TPA strongly inhibited the TCDD-mediated induction of *CYP1A1* in MCF10A-Neo cultures. Given the activation of latent TGFβ occurring in TPA-treated MCF10A-Neo cultures, and the ability of TGFβ to inhibit *CYP1A1* induction in some cell types, we hypothesized that the suppressive effects of TPA on the TCDD-dependent activation of *CYP1A1* in MCF10A-Neo cultures were mediated by TGFβ. The studies reported herein demonstrate that this hypothesis is partially correct, and are the first to demonstrate that TPA-mediated inhibition of *CYP1A1* activation by an AHR agonist reflects the consequences of PKC activation, as opposed to PKC inhibition or loss.

2. Materials and methods

2.1. Materials

TCDD was purchased from Midwest Research Institute. Trypsin/EDTA, epidermal growth factor (EGF), penicillin/streptomycin solution, horse serum, rabbit polyclonal IgG fractions made to amino acids 577–592 of PKC-ζ, amino acids 726–737 of PKC-ϵ, amino acids 661–671 of PKC-βI, and amino acids 660–673 of PKC-βII were purchased from Gibco BRL. [γ-^{32}P]dATP and [α-^{32}P]dCTP were purchased from DuPont-New England Nuclear. TPA, protease inhibitor cocktail (Product No. P2714), control rabbit IgG, and a neutralizing polyclonal pan rabbit antibody made to a mixture of TGFβ1 + TGFβ1.2 + TGFβ2 + TGFβ5 (IgG fraction, Product No. T929) were purchased from the Sigma Chemical Co. PD184352 [2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropyl-methoxy-3,4-difluoro-benzamide] was the gift of Pfizer Global Research and Development. Antibodies specific for the dually phosphorylated (active) forms of ERK1 and ERK2 were purchased from Promega. Antibodies recognizing both phosphorylated and non-phosphorylated forms of ERK1 and ERK2 were obtained from Santa Cruz Biotechnology. A murine monoclonal IgG to rabbit PKC-α and an affinity-purified rabbit polyclonal IgG to rat PKC-δ were purchased from Upstate Biotechnology. Alkaline phosphatase conjugated goat anti-mouse antibody and goat anti-rabbit antibody were purchased from TROPIX.

2.2. Cell culture and treatment

The MCF10A-Neo cell line was obtained from the Cell Lines Resource, Karmanos Cancer Institute. The MCF10A-Neo line was derived by transfection of the spontaneously immortalized, non-tumorigenic human breast epithelial cell line MCF10A with the pHo6 plasmid and subsequent selection for resistance to G418. The derivations and characterizations of the parental and MCF10A-Neo cell lines have been described elsewhere [30,31]. The MCF10A and MCF10A-Neo cell lines are very similar in their growth properties and responses to TCDD [32]. However, MCF10A-Neo cells accumulate more CYP1A1 RNA following TCDD exposure than do MCF10A cells [32].

MCF10A-Neo cells were cultured in supplemented Dulbecco's modified Eagle's medium/Ham's F-12 medium as described by Basolo *et al.* [33] in a humidified atmosphere of 95% air/5% CO_2 at 37°. The supplements consisted of human insulin (10 μg/mL), EGF (10 ng/mL), cholera toxin (100 ng/mL), hydrocortisone (0.5 μg/mL), 100 units/mL of penicillin-G, 100 μg/mL of streptomycin sulfate, and 5% horse serum. Subconfluent cultures were treated with various concentrations of chemicals dissolved in DMSO (absolute volume of solvent $\leq$ 0.1% of medium volume). Details of treatment are provided in the text. Viability was assessed by measurement of trypan blue exclusion. Cultures earmarked for RNA isolation were washed twice with calcium- and magnesium-free PBS at the time of harvesting and stored at −80°.

2.3. RNA preparation and northern blot analyses

Total cellular RNA was isolated using the acidic phenol extraction method of Chomczynski and Sacchi [34]. RNA was resolved on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes as described previously [32]. The probes used for the detection of CYP1A1, CYP1B1, and 7S RNAs, and the conditions used for hybridization have been described in detail [32]. CYP1A1/7S or CYP1B1/7S mRNA ratios were used to compare treatment groups. ^{32}P-Labeled nucleic acids were detected by either autoradiography with x-ray film or with a BioRad GS-525 Molecular Imager.

2.4. Western blot analyses

The conditions used for the preparation of extracts, separation of proteins on SDS–10% polyacrylamide gels, transfer to nylon or nitrocellulose membranes, and detection of phosphorylated and non-phosphorylated ERK1 and ERK2 have been described in detail [35]. The conditions used for western blot analyses of PKCs were similar to those used for ERK analyses through the transfer to nitrocellulose membranes except that lysis buffer consisted of 10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, 10 mM $NaPP_i$, 1 mM $NaVO_4$, and 1/10 (v/v) of a 10× solution of protease inhibitor cocktail (Sigma, Product No. P2714). Transferred blots were blocked overnight by incubation in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) containing 2% nonfat dry milk. After blocking, blots were washed three times for 5 min each with TBST and then incubated with primary antibodies (1 μg/mL in TBST with 2% nonfat dry milk) at room temperature for 4 hr. Thereafter, the blots were washed three times with TBST and incubated with secondary antibody (1:10,000 in TBST with 2% nonfat dry milk) at room temperature for 1 hr. After three 5-min washings with TBST, the immune-antigen complexes were detected with an ECL kit marketed by TROPIX and recorded on x-ray film. Enhanced chemiluminescence development was conducted according to the specifications of the manufacturer.

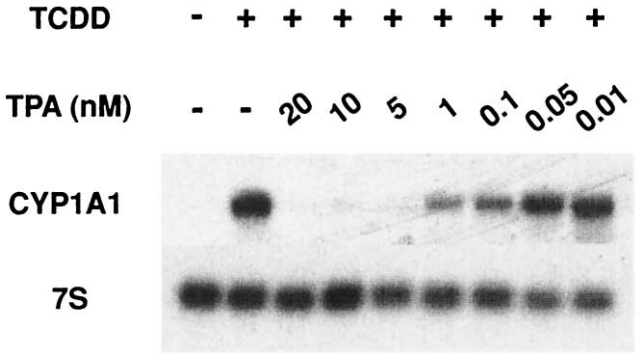

Fig. 1. Effects of TPA on CYP1A1 mRNA content in TCDD-treated cultures. Exponentially growing MCF10A-Neo cultures were treated with solvent or various concentrations of TPA for 1 hr prior to the addition of 10 nM TCDD. Cultures were harvested 6 hr after TCDD addition for isolation of RNA and analyses of CYP1A1 and 7S RNAs. Similar results were obtained in two additional experiments.

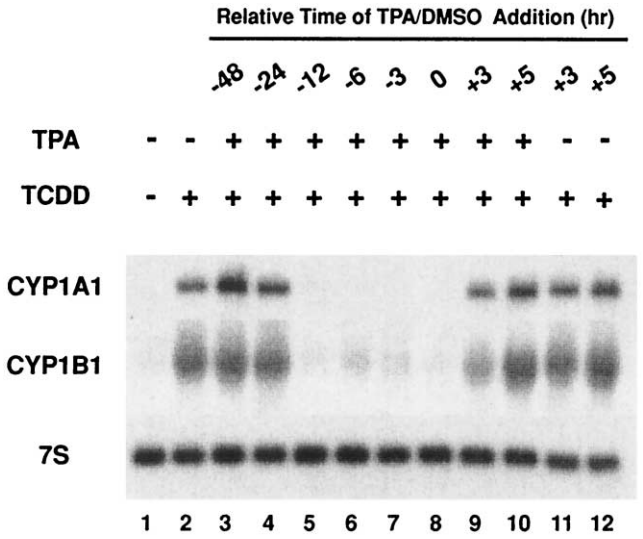

Fig. 2. Duration of suppressive effects of TPA on TCDD induction of *CYP1A1* and *CYP1B1*. Some MCF10A-Neo cultures were treated with 10 nM TPA before (- times), after (+ times), or at the time of addition of 10 nM TCDD (lanes 3–10). Other cultures were treated with DMSO 3 and 5 hr after TCDD addition (lanes 11 and 12). Cultures were harvested 6 hr after the addition of TCDD for isolation of RNA and analyses of CYP1A1, CYP1B1, and 7S RNAs. Similar results were obtained in two additional experiments.

2.5. Protein G column chromatography of serum

The procedure using polyclonal rabbit pan TGFβ IgG antibody and chromatography on columns of protein G-agarose to remove latent TGFβ from culture medium supplemented with horse serum has been described in detail [29].

3. Results

3.1. TPA suppression of transcriptional activation of CYP1A1 and CYP1B1

CYP1A1 mRNA was not detected in asynchronous, DMSO-treated MCF10A-Neo cultures (Fig. 1). In contrast, steady-state CYP1A1 mRNA content was elevated dramatically within 6 hr of exposure to the potent AHR agonist TCDD (Fig. 1). Treatment of cultures with TPA 1 hr prior to the addition of TCDD resulted in a concentration-dependent suppression of the accumulation of CYP1A1 mRNA (IC_{50} ~0.5 nM, based upon the data presented in Fig. 1 and two additional experiments). Co-treatment, or treatment with TPA for as long as 12 hr prior to TCDD addition, strongly suppressed CYP1A1 mRNA accumulation (Fig. 2).

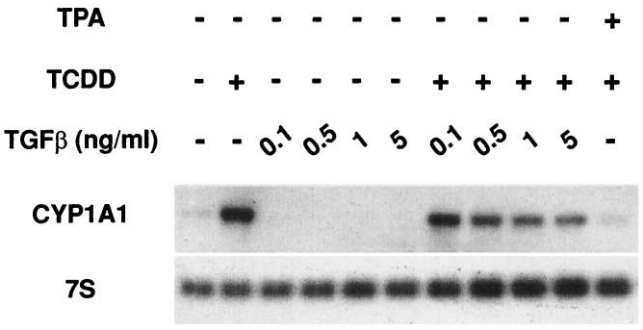

Fig. 3. Effects of TGFβ1 on CYP1A1 mRNA contents in TCDD-treated cultures. MCF10A-Neo cultures were treated with DMSO, 10 nM TPA, or various amounts of TGFβ1 1 hr prior to the addition of 10 nM TCDD. These cultures were harvested 6 hr after the addition of TCDD for isolation of RNA and analyses of CYP1A1 and 7S RNAs. Other cultures were treated with just TGFβ1 and were harvested 7 hr later for RNA isolation. Two additional studies were performed with slightly different concentrations of TGFβ1 and yielded similar results.

Treatment outside this window diminished the suppressive effects of TPA.

Like *CYP1A1*, *CYP1B1* is transcriptionally activated by TCDD via an AHR-mediated process [2]. Exposure of MCF10A-Neo cultures to TCDD dramatically increased CYP1B1 mRNA contents (Fig. 2). This accumulation of CYP1B1 mRNA was suppressed markedly by co-treating TCDD-exposed cultures with 10 nM TPA (Fig. 2). The kinetics defining the loss and recovery of TCDD induction of *CYP1A1* and *CYP1B1* in TPA-treated cultures were very similar (Fig. 2).

MCF10A-Neo cells were derived from the MCF10A cell line [31]. Studies similar to those reported in Figs. 1 and 2 were also performed with MCF10A cells. The responses of the MCF10A line to TPA were identical to those of MCF10A-Neo cultures (Guo M and Reiners JJ Jr, unpublished data).

3.2. TGFβ suppression of TCDD-mediated CYP1A1 induction

The kinetics defining the loss and recovery of responsiveness to TCDD noted in Fig. 2 mimic the kinetics of cell cycle arrest and reentry in TPA-treated MCF10A-Neo cultures [29]. These latter effects have been partially attributed to the activation of proteolytic processes in MCF10A-Neo cultures that convert the latent TGFβ found in culture medium to active TGFβ [29]. Previous studies [29] have shown that MCF10A-Neo proliferation is not affected by supplementing medium with 0.1 ng/mL of TGFβ1, but is maximally suppressed by 5 ng/mL (IC_{50} ~0.5 ng/mL). Supplementing MCF10A-Neo cultures with cytostatic and non-cytostatic amounts of active TGFβ had no detectable effects on constitutive CYP1A1 mRNA contents (Fig. 3). However, pretreatment with TGFβ1 diminished CYP1A1 steady-state mRNA contents following TCDD treatment.

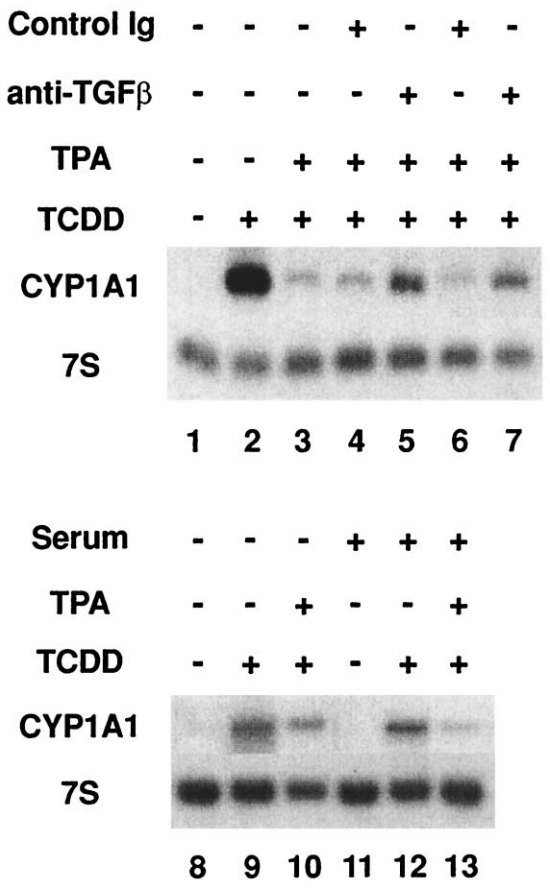

Fig. 4. Role of TGFβ in mediating the suppressive effects of TPA on *CYP1A1* induction by TCDD. In the top panel some MCF10A-Neo cultures were treated with 24 μg/mL of either control Ig (lane 4) or a neutralizing TGFβ pan antibody (lane 5) immediately prior to the addition of 10 nM TPA. Other cultures, just prior to the addition of TPA, were switched to a medium that had been incubated previously with either control Ig (lane 6) or the TGFβ pan antibody (lane 7, to remove latent TGFβ) and passed over a protein-G agarose column. Results similar to those reported in the top panel were obtained in two additional experiments. In the bottom panel, some cultures (lanes 11–13) were washed and refed with complete medium immediately before the addition of TPA, whereas other cultures (lanes 8–10) were washed and refed with medium lacking serum and growth factors. In all cases, TCDD (10 nM) was added 1 hr after the addition of TPA, and cultures were harvested 6 hr later for the isolation of RNA and analyses of CYP1A1 and 7S RNAs. Results similar to those reported in the bottom panel were obtained in a second independent study.

Based upon a normalization of the CYP1A1 and 7S mRNA signal intensities presented in Fig. 3 and two additional experiments, cultures co-treated with 5 ng/mL of TGFβ1 and TCDD had 42 $\pm$ 16% of the CYP1A1 mRNA content of cultures exposed to only TCDD.

We recently demonstrated that the cytostatic effects of TPA on MCF10A-Neo proliferation could be partially suppressed by the inclusion of a neutralizing TGFβ pan antibody [29]. Inclusion of such an antibody partially inhibited the suppressive effects of TPA on *CYP1A1* induction, as monitored by measurements of steady-state CYP1A1 mRNA contents (Fig. 4). CYP1A1 mRNA contents in cultures co-treated with TCDD + TPA or pan antibody +

TCDD + TPA were 7 ± 4 and 30 ± 5%, respectively, of the CYP1A1 mRNA contents of TCDD-treated cultures (compare lanes 2, 3, and 5 in Fig. 4; mean values are from Fig. 4 and two additional experiments). Co-incubation with preimmune Ig afforded no protection (Fig. 4, compare lanes 3 and 4). We also tested the effects of TPA on MCF10A-Neo cells grown in complete culture medium depleted of latent TGFβ prior to phorbol ester addition. A comparison of lanes 2, 3, and 6 in Fig. 4 shows that pretreatment of complete medium with control Ig, followed by passage over protein G-agarose columns to remove Ig and immune complexes, did not inhibit the suppressive effects of TPA on *CYP1A1* activation. However, removal of latent TGFβ from culture medium prior to its addition to MCF10A-Neo monolayers reduced the suppressive effects of TPA on *CYP1A1* activation (Fig. 4, compare lanes 2, 3, and 7).

As a complement to the experiments presented in the top panel of Fig. 4 we also analyzed *CYP1A1* induction in MCF10A-Neo cultures refed with serum-free, growth factor-free medium immediately before the addition of TPA (Fig. 4, bottom panel). The CYP1A1 mRNA contents of cultures refed with serum- and growth factor-free medium (compare lanes 9 and 10) or complete medium (compare lanes 12 and 13) before TPA + TCDD addition were 34 and 17% of the contents measured in the corresponding control TCDD-treated cultures. Similar results were obtained in a second experiment (Guo M and Reiners JJ Jr, unpublished study).

3.3. Role of PKCS in mediating TPA effects

TPA is a well characterized activator of several PKC isoforms [36,37]. This activation is often accompanied by PKC association with membranes and subsequent proteolysis. A western blot analysis of PKC isoforms in MCF10A-Neo cells is presented in Fig. 5. MCF10A-Neo cells expressed PKC-α, -βII, -δ, -ϵ, and -ζ. We were unable to detect PKC-βI or -γ. PKC-α in non-treated and solvent-treated cultures existed in both cytosolic and particulate forms. Exposure to TPA caused the translocation of cytosolic PKC-α to the particulate fraction. TPA treatment had little effect on PKC-βII, which associated primarily with the particulate fraction. In contrast, exposure to TPA caused the rapid and sustained loss of cytosolic PKC-δ and -ϵ, and minor reductions in particulate PKC-ϵ and -δ contents (Fig. 5). Antibodies to PKC-ζ detected multiple bands in both cytosolic and particulate protein preparations from control and solvent-treated cultures. Exposure to TPA caused the loss of a high molecular weight PKC-ζ cytosolic band, and the corresponding gain of the band in the particulate fraction.

Pretreatment with TPA to down-regulate PKC contents is commonly used to implicate a role for PKCs in biological phenomena [22,38,39]. MCF10A-Neo cells treated with TPA 24 or 48 hr prior to the addition of TCDD remained responsive to TCDD, as monitored by analyses of steady-state CYP1A1 mRNA contents (Fig. 6, compare lanes 2, 4, and 5). Indeed, pretreatment with TPA modestly increased the relative amount of CYP1A1 mRNA detected after TCDD exposure. Treatment with TPA 24 or 48 hr prior to a second TPA exposure completely inhibited the suppressive effects of the second TPA exposure on TCDD-mediated induction of *CYP1A1* (Fig. 6, compare lanes 3, 6, and 7).

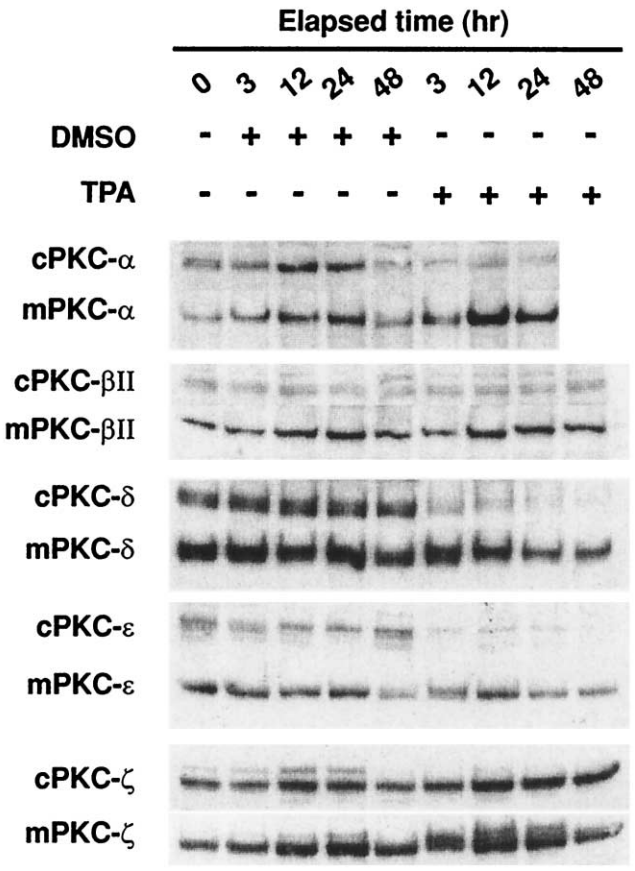

Fig. 5. Effects of DMSO and TPA on PKC distribution and content. Exponentially growing MCF10A-Neo cultures were treated with solvent or 10 nM TPA for various lengths of time prior to being harvested for western blot analyses (25 μg of protein per lane) of cytosolic (cPKC) and membrane/particulate (mPKC)-associated PKCs. Patterns similar to those reported in the figure were obtained in a second independent experiment.

3.4. TPA activation of ERKs and inhibition by PD184352

Exposure of MCF10A-Neo cultures to 10 nM TPA resulted in the rapid and sustained phosphorylation/activation of ERK1 and ERK2 (Fig. 7A). Phosphorylation was detected within 5 min of TPA treatment, and the ERKs remained phosphorylated for at least 6 hr. Although PD98059 (2′-amino-3′-methoxyflavone) is commonly used as a pharmacological inhibitor of MEK [40], the kinase responsible for the phosphorylation/activation of ERKs, we recently reported that it is an AHR ligand, and functions as an AHR antagonist at concentrations at which it inhibits MEK [35].

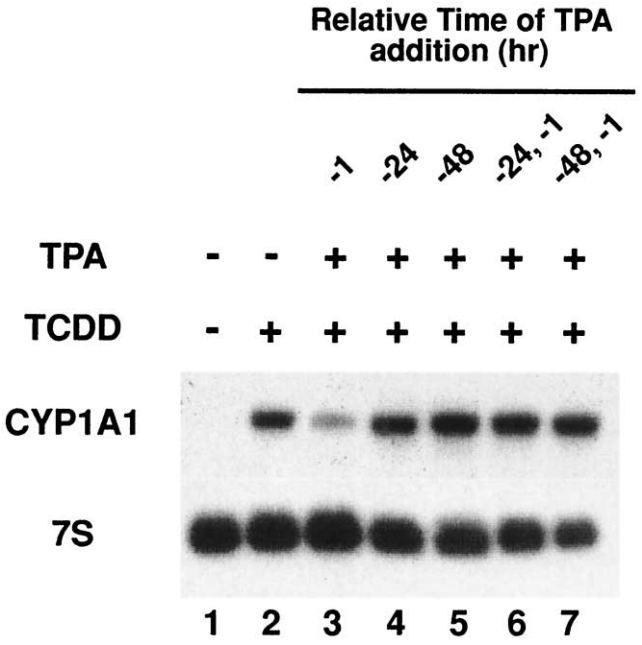

Fig. 6. Effects of PKC down-regulation on TPA suppression of *CYP1A1* induction by TCDD. MCF10A-Neo cultures were treated either once or twice with 10 nM TPA for varied lengths of time prior to the addition of 10 nM TCDD. Cultures were harvested for analyses of CYP1A1 and 7S RNAs 6 hr after the addition of TCDD. Results similar to those reported in the figure were obtained in an additional independent experiment.

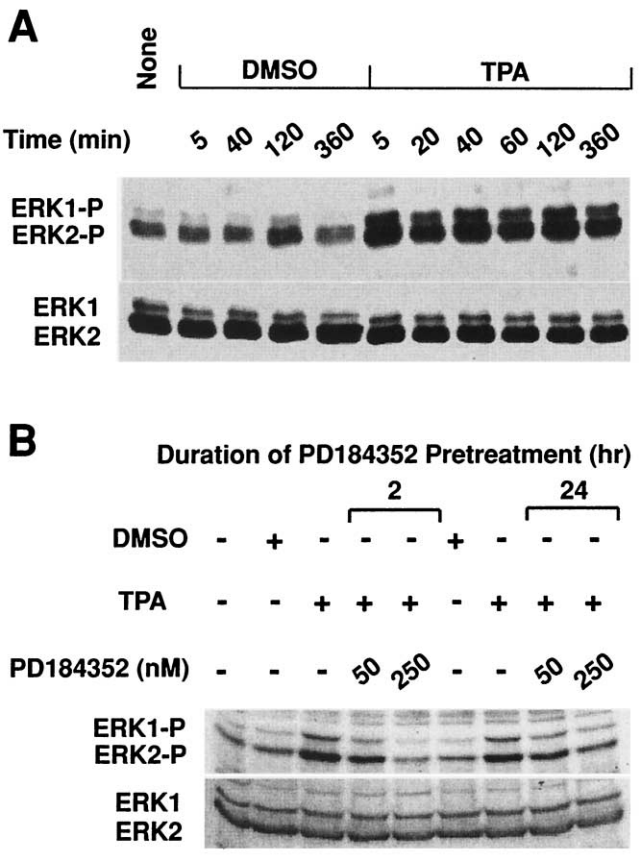

Fig. 7. PD184352 suppression of ERK activation by TPA. Panel A: MCF10A-Neo cultures were treated with DMSO or 10 nM TPA and harvested at various times thereafter for analyses of ERK1 and ERK2 phosphorylation. Panel B: MCF10A-Neo cultures were treated with 50 or 250 nM PD184352 either 2 or 24 hr prior to the addition of 10 nM TPA. Cultures were harvested 10 min after TPA addition for analyses of ERK1 and ERK2 phosphorylation. A second independent experiment yielded data almost identical to those reported in the figure.

This property confounds the use of this MEK inhibitor in the study of AHR function.

PD184352 is a newly described MEK inhibitor that is effective in cell culture in the nanomolar range [41]. Exposure of MCF10A-Neo cultures to PD184352 resulted in a concentration-dependent suppression of proliferation (IC_{50} ~80 nM). This effect reflected a cytostatic activity since no cytotoxicity was observed over the observation period (Guo M and Reiners JJ Jr, unpublished observation). The addition of PD184352 to cultures either 2 or 24 hr prior to treatment with TPA suppressed ERK phosphorylation (Fig. 7B). A complete suppression of ERK phosphorylation occurred in cultures pretreated for 2 hr with 250 nM PD184352. Partial suppression was obtained with 50 nM. Strong suppressions of ERK phosphorylation also were observed in cultures treated with PD184352 24 hr prior to TPA addition (Fig. 7B).

3.5. PD184352 effects on TPA suppression of CYP1A1 induction

Treatment of MCF10A-Neo cultures with concentrations of PD184352 sufficient to inhibit MEK activity affected neither basal CYP1A1 mRNA content nor the activation of *CYP1A1* by TCDD (Fig. 8A). Furthermore, pretreatment with PD184352 did not affect the ability of TPA to suppress CYP1A1 mRNA accumulation following TCDD exposure (Fig. 8B).

4. Discussion

Co-treatment of MCF10A-Neo cultures with TPA and TCDD, or pretreatment with TPA for up to 12 hr before TCDD addition, strongly suppressed the accumulation of CYP1A1 and CYP1B1 mRNAs. We previously demonstrated by run-on experiments that TCDD activates the transcription of both P450 genes in this cell line [32]. Studies in other systems suggest that TPA affects the transactivating activity of AHR/ARNT complexes [14–17]. Hence, although we have not determined the basis for the suppression noted in the current study, it most likely represents an inhibition of the AHR-dependent induction of the *CYP1A1* and *CYP1B1* genes.

We previously reported that TPA-treated MCF10A-Neo cultures initiate a proteolytic process that rapidly converts serum-derived latent TGFβ to active TGFβ [29]. Several findings in the current study suggest that the suppressive effects of TPA on *CYP1A1* induction by TCDD were mediated partially by TGFβ. First, we established that the

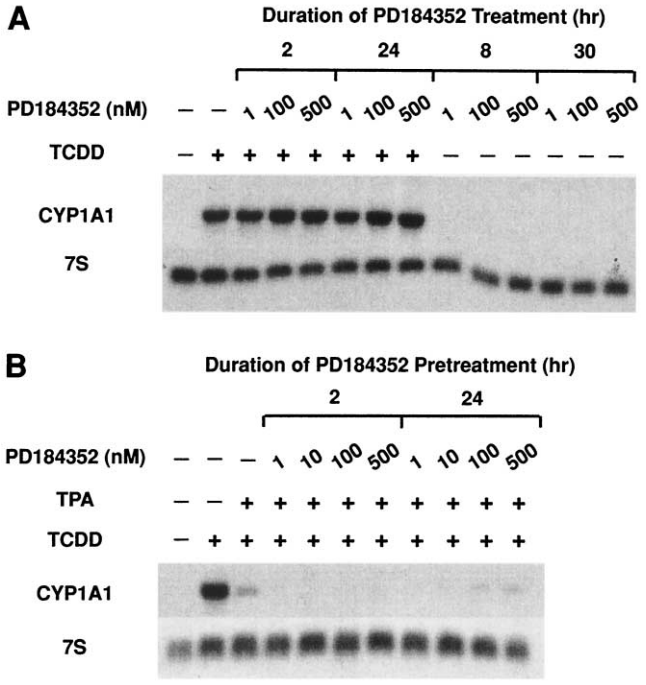

Fig. 8. Effects of PD184352 on TPA suppression of *CYP1A1* induction by TCDD. Panel A: Some MCF10A-Neo cultures were treated with various concentrations of PD184352 for either 2 or 24 hr prior to the addition of 10 nM TCDD. Cultures were harvested 6 hr later. Other cultures were treated with PD184352 for 8 or 30 hr prior to harvesting for analyses of CYP1A1 and 7S RNAs. Panel B: Cultures were treated with various concentrations of PD184352 for either 2 or 24 hr prior to the addition of 10 nM TPA. TCDD (10 nM) was added 1 hr after TPA treatment. Cultures were harvested 6 hr after TCDD addition for isolation of RNAs and analyses of CYP1A1 and 7S RNAs. Results similar to those reported in panels A and B were obtained in a second independent experiment.

TCDD-dependent induction of *CYP1A1* in MCF10A-Neo cultures could be suppressed partially by exogenous TGFβ1. Similar effects have been seen in other cell lines [4–7]. Second, we observed that the suppressive effects of TPA on *CYP1A1* induction could be inhibited partially by supplementing culture medium with a neutralizing TGFβ pan antibody just before the addition of TPA. Third, the suppressive effects of TPA on *CYP1A1* induction were also inhibited partially in cells cultured in serum-free medium or medium containing serum depleted of latent TGFβ. These findings collectively suggest that TGFβ mediates a portion ($\sim$17–23%) of the suppressive effect of TPA on *CYP1A1* induction in MCF10A-Neo cultures. This finding may be relevant to the mechanism by which TPA suppresses *CYP1A1* induction in MCF-7 cultures [13] and murine keratinocytes [11]. Specifically, TPA stimulates the synthesis/activation of TGFβ in both cell types [23,24,42,43].

Members of the 'conventional' and 'novel' PKC subfamilies bind TPA and are activated by it [36,37]. Activation of some PKCs by TPA is accompanied by their subsequent accelerated turnover [36,37]. Hence, PKC involvement in mediating biological processes is commonly assessed by pretreating cells with TPA to down-regulate PKC content/activity [22,38,39]. A 24- to 48-hr pretreatment of MCF10A-Neo cultures with TPA totally inhibited the suppressive effects of a subsequent TPA exposure on *CYP1A1* activation by TCDD. Western blot analyses demonstrated that the content/distribution of PKC-α, -δ, and -ϵ were affected markedly in TPA-treated MCF10A-Neo cultures. One or all of these PKC species may be responsible for the TGFβ component of the TPA response that inhibits TCDD-mediated *CYP1A1* induction. However, this latter speculation merits caution. Recent studies have identified several non-PKC, TPA-binding proteins that appear to participate in signaling processes [36].

The transcriptional activation of *CYP1A1* by TCDD is affected by a variety of agents and conditions. Addition of epidermal growth factor to serum/growth factor-depleted cultures of hepatocytes suppresses *CYP1A1* induction by AHR agonists [44], as does the introduction of the Ha-*ras* oncogene into MCF10A cells [35]. A signaling pathway activated by TPA, EGF, and p21-ras is the MAPK pathway (this study; [45–47]). In a previous study, we attempted to use the MEK inhibitor PD98059 to assess the role of the MAPK pathway in mediating the suppressive effects of oncogenic p21-ras on *CYP1A1* induction. However, the studies were confounded by the finding that PD98059 was an AHR antagonist at concentrations at which it inhibited MEK activity [35]. PD184352 is a newly described MEK inhibitor [41]. It neither activated *CYP1A1* transcription, nor inhibited the TCDD-dependent activation of *CYP1A1* in MCF10A-Neo cells. Electrophoretic mobility shift assays employing rat liver extract and an oligo containing a consensus DRE sequence also showed that PD184352 is neither an AHR antagonist nor an agonist (tested to 100 μM; Reiners JJ Jr, unpublished data). Hence, PD184352 circumvents the complications associated with the use of PD98059. Pretreatment of MCF10A-Neo cultures with concentrations of PD184352 sufficient to inhibit MEK and ERK activation did not inhibit the suppressive effects of TPA on *CYP1A1* induction by TCDD. Collectively, these findings suggest that MEK/ERK activities neither mediate the suppressive effects of TPA on *CYP1A1* induction, nor are required for AHR activation by TCDD.

A number of studies document either the suppression [10–14] or enhancement [15–17] of AHR-mediated transcriptional processes by TPA. In our studies, a strong suppression occurred even if 12 hr elapsed between the time of TPA and TCDD exposures. Thereafter, the suppressive effects of TPA were lost. However, we also observed that treatment of MCF10A-Neo cultures with TPA 24 or 48 hr prior to TCDD addition increased CYP1A1 mRNA contents above that obtained with just TCDD. Moore *et al.* [13] reported that *CYP1A1* induction was suppressed during the first 26 hr of co-treatment of MCF-7 cells with TPA and TCDD. However, by 72–96 hr of co-treatment, CYP1A1 mRNA levels were 2.8-fold higher than the contents measured in cultures treated with only TCDD [13]. Similar results have been observed in murine epidermis following

topical application of TPA and an AHR agonist [11]. Although the protocols used in the latter two studies and the current one are operationally different, they may be functionally comparable. The biphasic effects of TPA on CYP1A1 mRNA accumulations observed in MCF-7 and MCF10A-Neo cells and murine keratinocytes may represent distinct pathways that differentially affect AHR function. It is conceivable that the ability of TPA to enhance only the transactivating activity of AHR/ARNT complexes in some cell types may reflect the absence of, or resistance to, the suppressive pathway(s) in these cells. Indeed, our data suggest that the TPA-mediated suppression of *CYP1A1* induction occurring in MCF10A-Neo cells is mediated by at least two ERK-independent pathways, one of which involves TGFβ. TPA-mediated enhancement of AHR-dependent transactivating activity, in the absence of any suppressive activity, has been documented in HepG2 cells [15,16]. This line is non-responsive to TGFβ due to an absence of type II TGFβ receptor expression [48].

In summary, the current investigation demonstrated that TPA suppresses the TCDD-dependent induction of *CYP1A1* by two ERK-independent mechanisms, one of which involves TGFβ. This study, in conjunction with the published literature [10–17], also provides a plausible explanation for the differential effects of TPA on *CYP1A1* induction in different cell/tissue types.

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