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Activation of mitogen-activated protein kinases (MAPKs) by aromatic hydrocarbons: role in the regulation of aryl hydrocarbon receptor (AHR) function

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Abstract

The aromatic hydrocarbon (Ah) receptor (AHR) is the only known cellular receptor of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and of many other widespread environmental contaminants that cause diverse toxic effects in animals and humans. Most, if not all, the biological effects of TCDD are mediated by the activation of AHR, which is a ligand-activated transcription factor required for ligand-induced expression of several detoxification genes, including those encoding for cytochrome P450 enzymes CYP1A1, CYP1A2, and CYP1B1. Environmental agents also activate several mitogen-activated protein kinase (MAPK) pathways, believed to modulate transcription factor function and to regulate gene expression. However, the contribution to TCDD toxicity resulting from cross-talk between AHR and MAPK pathways has yet to be determined. In this study, we show that TCDD and other AHR ligands induced the immediate activation of the extracellular signal-regulated kinases and the Jun N-terminal kinases, but not the p38 MAPKs. MAPK activation by TCDD did not require the AHR, since it occurred equally well in AHR-negative CV-1 cells and in *Ahr* (−/−) mouse embryonic fibroblasts as in AHR-positive cells. Distinct from serum factors and the tumor promoter TPA-induced MAPKs, which resulted in transcriptional activation of ELK or c-JUN, TCDD-stimulated MAPKs were critical for the induction of AHR-dependent gene transcription and CYP1A1 expression. These data indicate that AHR ligands elicit AHR-independent non-genomic events that are essential for AHR activation and function.

Keywords: Dioxin; Ah receptor; MAP kinase activation; Non-genomic effect; AHR-dependent gene transcription; CYP1A1 induction

1. Introduction

TCDD is the most potent congener of the dioxin-like compounds present as ubiquitous environmental agents in the atmosphere, soil, water, and food [1]. TCDD exerts diverse species-specific toxic effects in animals and humans, including chloracne, immune, reproductive, and developmental toxicity, carcinogenicity, wasting syndrome, and death. It is widely accepted that the majority of biological effects of TCDD in higher organisms are mediated by the activation of the cytosolic AHR [2,3]. The AHR is a ligand-activated transcription factor that, as a heterodimer with the ARNT protein, is required for TCDD-induced expression of several cytochrome P450 enzymes, including CYP1A1, CYP1A2, and CYP1B1, and of many phase II detoxification genes. *Ahr*-null mice are resistant to the toxic and pathologic effects of TCDD [4].

In both experimental animals and cell culture systems, it has been demonstrated that TCDD triggers the immediate activation of various intracellular signal transduction pathways [5,6]. For instance, TCDD induces a rapid activation of c-Src, which is mediated by the AHR, since AHR antagonists block the activation [7]. Recent studies have shown that TCDD stimulates the activity of extracellular signal-regulated kinases (ERKs) in experimental animals [8] and that it activates Jun-N-terminal kinases (JNKs) in

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Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, Ah receptor nuclear translocator; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B[a]P, benzo[a]pyrene; BP-dione, benzo[a]pyrene-6,12-dione; BP-7,8-diol, benzo[a]pyrene-*trans*-7,8-dihydrodiol; BPDE, benzo[a]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide; MEF, mouse embryo fibroblast; AP-1, activation protein-1; EGF, epidermal growth factor.

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leukemic T cells [9]. Whether ERK and JNK activation by TCDD, like activation of Src, depends on the AHR was not addressed in those studies.

The ERKs and JNKs, along with the p38s, are serine/threonine protein kinases that comprise the family of mitogen-activated protein kinases (MAPKs). MAPKs are important intracellular signaling mediators, controlling gene expression through phosphorylation of transcription factors and modulation of the function of these factors [10]. For instance, both serum factor and tumor promoter TPA activates ERK, leading to the phosphorylation of transcription factor ELK. Phosphorylation results in the increase of ELK transcription activity and the consequent induction of ELK target genes, including immediate-early proto-oncogenes, such as *c-Fos*. Likewise, serum factors or environmental stress-induced JNK phosphorylates JUN family proteins and enhances their transcriptional activity. JUN homodimers or JUN/FOS heterodimers constitute the AP-1 transcriptional complex, critically involved in the transcription regulation of immediate-early gene expression, and the MAPKs, through regulation of AP-1 activity, are implicated in a wide array of cellular activities, including proliferation, transformation, and apoptosis [11]. Induction of immediate-early genes by TCDD has been observed in mouse hepatoma cells and in guinea pig and macaque liver cells, and it has been postulated that it may contribute to TCDD carcinogenicity [12–14]. Whether this effect of TCDD is the consequence of MAPKs-induced activation of cJUN and ELK has never been determined.

In addition to ELK and JUN, an increasing numbers of transcription factors can be modulated by the MAPKs [10,15]. The AHR, as a transcription factor, is primarily activated by ligand-binding, but its optimal activity requires the participation of intracellular signaling pathways [16–18]. In this article, we have tested the hypothesis that TCDD, and other AHR ligands, cause the immediate activation of the ERK and the JNK MAPK pathways and that activation of these kinases plays a critical role in AHR function. Our findings indicate that these AHR ligands induce the activation of MAPK functions independently of the presence of the AHR and that the ensuing MAPK activity is required for AHR-dependent gene expression, strongly supporting the view that induction of MAPK activities is an essential regulatory component of the ability of the AHR to function as a transcription factor.

2. Materials and methods

2.1. Chemicals

TCDD was purchased from Acustandard. Benzo[a]pyrene (B[a]P), benzo[a]pyrene-6,12-dione (BP-dione), benzo[a]-pyrene-*trans*-7,8-dihydrodiol (BP-7,8-diol) and benzo[a]-pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE) were purchased from the NCI Chemical Carcinogen Repository operated by the Midwest Research Institute. The laboratories where this work was performed, are certified for use of carcinogenic and toxic chemicals by the University of Cincinnati Biohazardous Chemicals Committee.

2.2. Cell lines, growth conditions, transfections, and infections

The mouse Hepa-1 hepatoma cell line, Hepa-AhRDTK-Luc3, a Hepa-1 derivative with a stably integrated luciferase reporter driven by the AHR/ARNT responsive domain in the mouse CYP1A1 gene [19], the human A549 lung carcinoma cell line (obtained from ATCC) and the African Green Monkey Adult Kidney CV-1 cells, were cultured in α-MEM (Life Technologies) supplemented with 10% fetal bovine serum and 1% anti-mycotic/antibiotics (Life Technologies) in a humidified 5% CO_2 atmosphere. To prepare mouse embryo fibroblasts (MEF) from *Ahr* (+/+) and *Ahr* (−/−) mice, the knock-out mice were first back-crossed for seven generations into the C57BL/6J background of the *wild-type* mice to obtain congenic mouse lines differing at the *Ahr* locus (in fact, after seven back-cross generations the two mouse lines differ by a theoretical 0.78% of the genome, which corresponds to a few hundred genes, including *Ahr*). Thereafter, MEFs were prepared by standard techniques from 14.5-day-old embryos and grown in α-MEM medium. All cells were starved in serum-free medium for 24–48 hr prior to being used for the experiments. Cells were transfected using Lipofectamine (Life Technologies) following the protocols recommended by the manufacturer.

Twelve hours after transfection, the cells were starved for 24 hr and treated in serum-free medium with the chemicals to be tested for the length of time indicated in the figures. Cell lysates were prepared in Reporter Lysis Buffer™ (Promega) to determine luciferase and β-galactosidase activities in a Wallac plate reader luminometer. Relative luciferase activities were obtained after normalization to β-galactosidase activity. For infection experiments, Hepa-AhRDTKLuc3 cells were infected with an adenovirus vector expressing either a dominant negative MEK1 or a β-galactosidase gene, starved for 24 hr and treated in serum-free medium. Luciferase activities were normalized to protein concentration of the lysates. For experiments where MAPK inhibitors were used, cells were starved and pre-treated with U0126, PD98059, or SB202190 (CalBiochem) for 30–60 min. Thereafter, cells were treated with TCDD or vehicle control in the presence of the inhibitors for various lengths of time. Cell lysates were prepared and used for luciferase determinations or Western blot as needed.

2.3. Plasmid constructs

The luciferase reporter pAhRDTKLuc3, contains the mouse *Cyp1a1* AhRD enhancer fused to the herpes simplex virus type 1 thymidine kinase minimum promoter and has

been described previously [19]. Adenoviruses expressing dominant-negative MEK1 and β-galactosidase were a gift from J. Molkentin, Children's Hospital Medical Center [20]. ELK1 and c-JUN *trans*-reporter systems (PathDetect™, Stratagene) use fusions of ELK1 and c-JUN transactivation domains, respectively, to the DNA-binding domain of the yeast GAL4 protein to measure the expression of a luciferase reporter driven by a GAL4 promoter. These expression systems were used as recommended by the manufacturer.

2.4. Western blot analyses and JUN pull-down kinase assays

Cells were washed with ice-cold phosphate buffered saline (PBS) and extracted on ice for 30 min with lysis buffer (0.3 M NaCl, 3 mM EDTA, 1% Nonidet P-40, 10% glycerol, 50 mM Tris, pH 7.6) including protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (0.1 mM sodium orthovanadate, 10 mM tetrasodium pyrophosphate, 10 mM sodium fluoride). The cell lysates were clarified by centrifugation at 15,000 *g* for 5 min. Proteins were resolved on 10% SDS–polyacrylamide gels (SDS–PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in PBS containing 0.1% Tween 20 and sequentially incubated with primary and secondary antibodies. Protein visualization was by chemiluminescent detection using a SuperSignal™ kit (Pierce Chemical Company). Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were purchased from New England BioLabs; anti-phospho-JNK and anti-JNK, were from Promega and Pharmingen, respectively; anti-phospho-p38 and anti-p38 from Promega and Cell Signaling, respectively; anti-AHR from BioMol; and anti-CYP1A1 from GenTest. Anti-β-actin was a gift from J. Lessard, Children's Hospital Medical Center. A JUN pull-down kinase assay was used for JNK kinase activity determinations as described previously [21]. Briefly, whole cell extracts, prepared as described above, were incubated with GST-c-JUN (1–79) bound to agarose beads at 4° for 3 hr in binding buffer (0.15 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton-X100, 25 mM Hepes, pH 7.2, and the same protease and phosphatase inhibitors indicated above). The beads were washed five times with binding buffer and once with kinase buffer (20 mM Hepes, pH 7.6, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 50 μM sodium orthovanadate, 20 mM β-glycerolphosphate, 1 mM *p*-nitrophenyl phosphate). Kinase reaction was performed at 30° for 30 min in kinase buffer containing 2 μCi of [γ-^{32}P]ATP (Dupont-NEN). Phosphorylation products were resolved on 10% SDS–PAGE and autoradiographed after drying the gel.

2.5. AHR-binding assays

Two fragments of the mouse AHR cDNA encoding truncated peptides were cloned into the pQE3O vector (Qiagen) for expression of 6-His tagged proteins. Plasmid pΔ376–805 encodes the mouse AHR amino-proximal 375 amino acids, from the first residue to the end of the PAS region, including the bHLH and ligand-binding domains. Plasmid pΔl–425 encodes the carboxyl-terminal 379 amino acids, and lacks the ligand-binding domain. Construction of these plasmids has been described [22]. Twenty picomoles of bacterially expressed 6-His-tagged AHR truncated peptides were mixed in a total reaction volume of 100 ul with 20 pmol of ^{3}H-TCDD (S.A. 40 Ci/mmol, Cambridge Isotopes) in the presence or absence of a 50-fold excess of unlabeled TCDD or a 500-fold excess of each MAP kinase inhibitor. After 1 hr at 30°, receptor-bound of ^{3}H-TCDD was captured with nickel–agarose magnetic beads, the beads were washed, and the radioactivity remaining on the beads was determined by scintillation counting to calculate the percent inhibition of TCDD-binding to AHR by the various competitors. Correction for non-specific binding was done by subtracting the values obtained with the Δ1–425 peptide, lacking the ligand-binding domain, from the values found with the Δ376–805 peptide.

3. Results

3.1. The immediate activation of ERK and JNK MAPKs by TCDD

To evaluate the possible involvement of the MAP kinase pathways in cellular responses to xenobiotics, we asked whether TCDD could induce the activation of MAP kinases in serum-deprived mouse hepatoma Hepa-1 cells. To measure MAPK activation, we used antibodies that detect the active, phosphorylated state of ERK1/2 and p38, which has been demonstrated to be responsible for and correlated to the MAPK activities [23,24]. For JNK, we measured its activity, by determining its ability to phosphorylate a c-JUN substrate in a GST-c-JUN pull-down kinase assay. TCDD elicited the immediate phosphorylation of ERK1/2 (Fig. 1A) and the activation of the JNKs (Fig. 1C), as early as 10 min after exposure. Under the same stimulation conditions, TCDD did not activate the p38 pathway (Fig. 1B). Expression of total ERK, JNK, and p38, as determined with specific antibodies to each protein, was not affected by TCDD treatment (Fig. 1). These data strongly indicate that exposure to TCDD for a very short time was sufficient to induce the activation of two of the three MAPKs tested.

3.2. TCDD-induced MAPK activation independent of AHR

TCDD toxicity is mediated by its cytoplasmic receptor, the AHR, which plays a critical role in the activation of certain signaling pathways. For instance, c-SRC activation

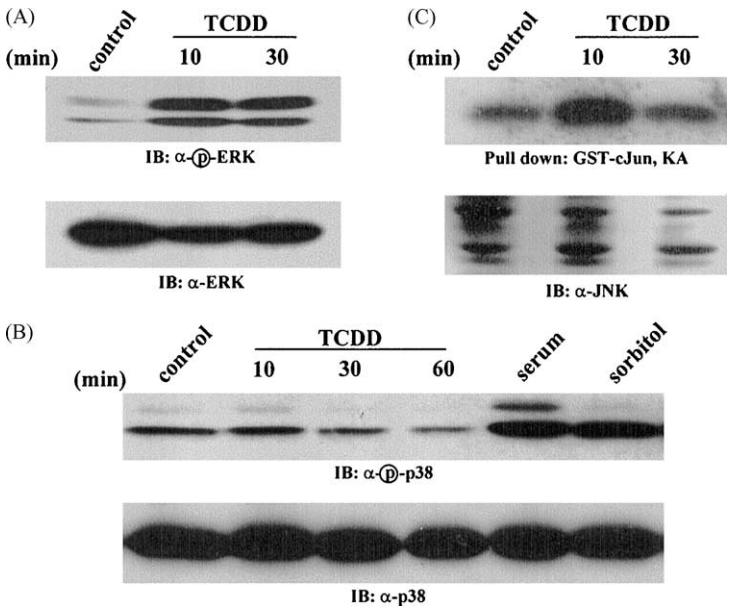

Fig. 1. TCDD activates ERK and JNK MAP kinases. Mouse Hepa-1 hepatoma cells were serum-starved and treated with 10 nM TCDD for the indicated times, or with 10% serum or 300 mM Sorbitol for 30 min as indicated. Cell lysates (50 μg) were subjected to SDS–PAGE and Western blot analysis were performed using (A) anti-phospho-ERK (top panel) and anti-ERK (bottom panel), or (B) anti-phospho p38 (top panel) and anti-p38 (bottom panel). (C) Lysates were analyzed for JNK activity using a GST-c-JUN pull-down kinase assay. The kinase reactions were resolved on SDS–PAGE and the phosphorylation of c-JUN was visualized by radioautography (top panel). The lysates were also analyzed for the expression of total JNK by immunoblotting with anti-JNK (bottom panel).

by TCDD requires the presence of AHR, as shown by the finding that AHR antagonists prevent TCDD-induction of SRC kinase activity [7]. The timing of the MAPKs induction determined in the previous set of experiments suggest a non-genomic effect of TCDD on MAPK activation. To address directly whether TCDD-induced MAPK activity required the AHR, we studied ERK and JNK activation in CV-1 cells, which lack AHR expression [19]. TCDD elicited significant ERK phosphorylation in these cells even at concentrations as low as 0.1 nM. Maximum induction of ERK in these cells took place at 1 nM TCDD after a 30-min treatment (Fig. 2A). Phosphorylation of JNK was also induced by TCDD in CV-1 cells (Fig. 2A). In quantitative terms, induction of both ERK and JNK by TCDD was slightly less than by 10% serum and the timing of maximum induction was delayed, being 5 min in serum-treated cells.

To confirm further the lack of a role for the AHR in TCDD-induced MAPK activity, we employed MEFs isolated from congenic *wild-type* and *Ahr*-null embryos. AHR was clearly expressed only in *wild-type*, but not in *Ahr* (−/−) MEFs and TCDD could induce CYP1A1 expression only in the *wild-type* but not in *Ahr* (−/−) MEFs (Fig. 2B), confirming that AHR plays an essential role in the induction of CYP1A1. In contrast, TCDD was able to induce ERK phosphorylation just as well in *Ahr* (−/−) as in *Ahr* (+/+) MEFs, indicating that the absence of AHR does not affect the induction of ERK phosphorylation by TCDD (Fig. 2C). Total ERK expression, as determined with anti-ERK antibodies, was also independent of AHR status (Fig. 2C). We conclude that MAPK activation by TCDD is not mediated through the AHR.

3.3. MAPKs-activated by other AHR ligands

The effects of TCDD on MAPK pathways prompted us to test whether other AHR ligands could also activate ERK1/2 and the JNKs. B[a]P is a polycyclic aromatic hydrocarbon, also persistent as an environmental contaminant and a procarcinogen. B[a]P and many of its metabolic derivatives bind the AHR and activate AHR-directed gene transcription [25,26]. We examined the ability of several such polycyclic aromatic hydrocarbon compounds to activate MAPK pathways. In the human lung carcinoma cell line, A549, B[a]P and BP-7,8-diol, one of its metabolic products, induced ERK1/2 and JNK phosphorylation, which, by comparison with induction by TCDD, was more prolonged when B[a]P was the inducer and more transient when the inducer was BP-7,8-diol (Fig. 3A). ERK activation by B[a]P and its derivatives BP-7,8-diol, BP-dione, and BPDE, was also observed in *Ahr* (+/+) and *Ahr* (−/−) MEFs (Fig. 3B). MAPK activation appears to be a novel consequence of exposure to multiple AHR ligands and B[a]P metabolites, possibly contribute to the plethora of xenobiotic-elicited cellular responses.

3.4. TCDD-induced MAPK activity does not converge on ELK and c-JUN

The end point of the activation of ERK and JNK by TCDD may be the phosphorylation of transcription factors

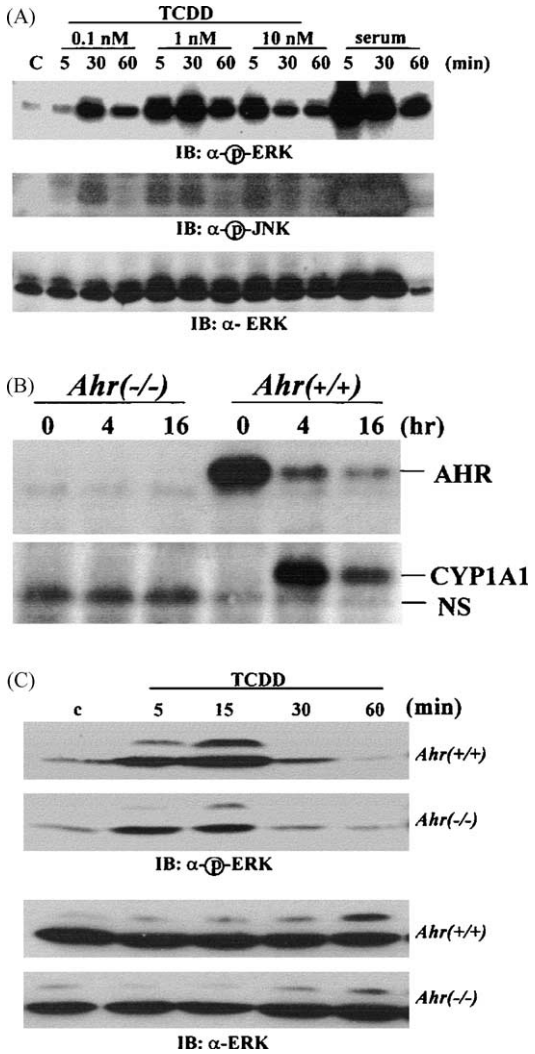

Fig. 2. TCDD-induced MAPK activation does not require AHR expression. (A) CV-1 cells were treated with various concentrations of TCDD or with 10% serum for the indicated length of time. Cell lysates were subjected to Western blot analysis for the detection of phosphorylated ERK (pERK), phosphorylated JNK (pJNK), and total ERK protein (ERK). (B) MEFs from *Ahr* (+/+) and *Ahr* (−/−) mice were treated with 10 nM TCDD for the indicated length of time. The cells were lysed and the lysates were subjected to 10% SDS–PAGE and transferred onto nitrocellulose membranes. Western blotting was performed using anti-AHR (top panel) and anti-CYP1A1 (bottom panel). NS: non-specific reaction. (C) *Ahr* (+/+) and *Ahr* (−/−) MEFs were serum-starved for 24 hr, followed by treatment with 2 nM TCDD for the indicated times. Anti-phospho-ERK and anti-ERK were used for Western blot analysis as indicated.

such as ELK and c-JUN. Although TCDD has been implicated in the induction of AP-1 DNA-binding activity, its role in the transcriptional activation and phosphorylation of ELK and c-JUN has never been elucidated. To test whether the MAPK cascade induced by TCDD concluded in the phosphorylation of ELK and c-JUN and the potentiation of their transcriptional activities, we used the PathDetect™ system, designed to measure activation of ELK and c-JUN using a luciferase expression vector as a reporter. TCDD did not induce ELK or c-JUN phosphorylation-dependent luciferase expression in Hepa-1 cells, although under the same experimental conditions, TPA and serum induced ELK-dependent and c-JUN-dependent luciferase expression, respectively (Fig. 4A and B). The fate of TCDD-stimulated MAPK activities appear to be distinct from that induced by serum factors and tumor promoters since they do not converge on the transcriptional activation of ELK and c-JUN.

3.5. AHR-dependent transcription attenuated by MAPK inhibitors

AHR activity requires posttranslational phosphorylation events involving the AHR itself or one or more of the accessory proteins constituting the AHR complex. Potential candidates for the modification of the AHR complex are the MAPK pathways induced by TCDD. If this were the case, inhibition of MAPK activity would interfere with AHR-dependent gene expression. To test the role of TCDD-induced MAPK activation in AHR function we used specific MAP kinase inhibitors. Two inhibitors, PD98059 and U0126 are specific for the MEKs, the upstream protein kinases that phosphorylate ERK1/2. A third inhibitor, SB202190, specifically inhibits p38, which is not activated by TCDD, at concentrations below 5 μM. At concentrations above 20 μM, SB202190 inhibits both JNK and p38. Treatment of Hepa-1 cells with U0126 and SB202190 blocked TCDD-stimulated phosphorylation of ERK1/2 and activation of JNK, respectively (Fig. 5A and B). PD98059, U0126, and SB202190, in a dose dependent manner, also affected the induction of AHR-dependent luciferase activity by TCDD in Hepa-AhPDTKLuc3 cells, with a significant suppression by 5 μM or higher of PD98059, 2.5 μM of U0126, and 25 μM of SB202190. Our results suggest that both the ERK and JNK pathways contribute to TCDD-induced AHR activity. Consistent with the inhibitory effect of U0126, TCDD-induced activation of AHR was also significantly suppressed by infection of Hepa-AhRDTKLuc3 cells with a replication deficient adenovirus carrying a dominant negative MEK1, but not with a control adenovirus that expresses β-galactosidase (Fig. 5D).

For inhibition of TCDD-induced AhRD-luciferase expression, the calculated IC_{50}s of U0126 and SB202190, being 1.2 and 10.8 μM, respectively (Fig. 5C), were similar to those effective in blocking other MAPK-mediated responses, such as AP-1 activation and nitric oxide production [27,28]. The PD98059 (IC_{50} = 2.3 μM) on AHRD-luc expression, however, was far more effective than its inhibitory role on nitric oxide production [28], possibly owed to the nature of this compound as a flavonoid antagonist of the AHR that directly interferes with AHR activation [29] (Table 1). Consequently, a complete block of AHR activity by this compound may be attributed to its role as an AHR antagonist rather than to the inhibition of MEK activity. In contrast, both U0126 and SB202190 are structurally distinct

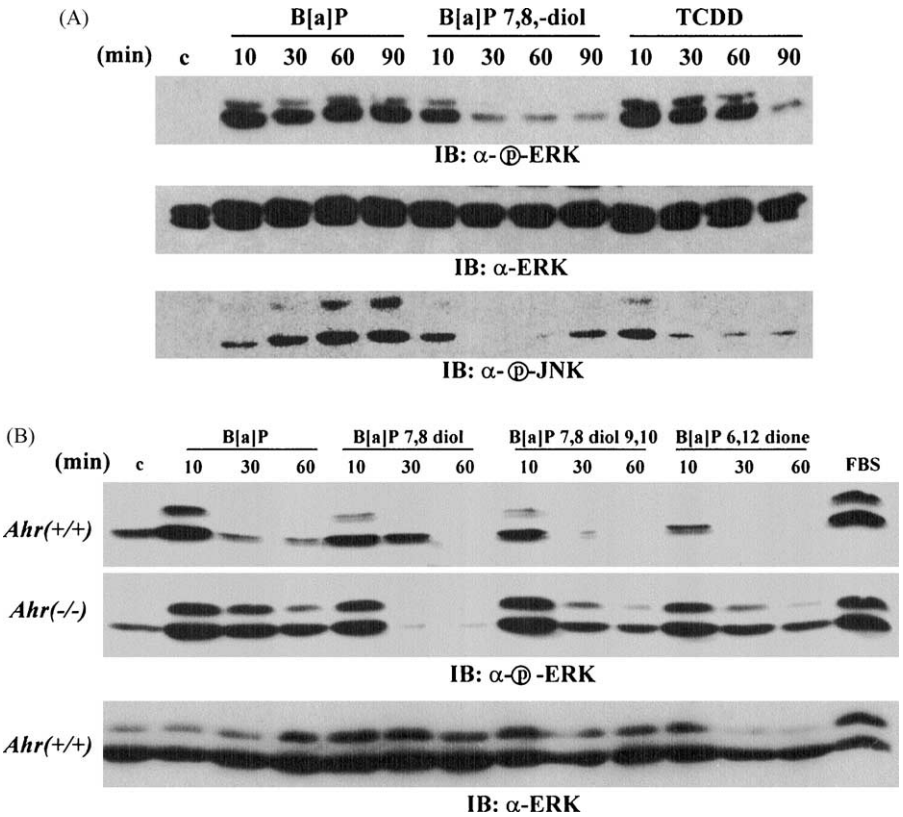

Fig. 3. MAPKs are activated by B[a]P and several of its metabolites. (A) A549 human lung carcinoma cells were deprived of serum and treated with 10 μM B[a]P, 10 μM B[a]P 7,8-diol and 10 nM TCDD for various times as indicated. The cell lysates (50 μg) were subjected to SDS–PAGE, followed by Western blotting using anti-phospho-ERK, anti-ERK, and anti-phospho JNK as specified. (B) MEFs from *Ahr* (+/+) and *Ahr* (−/−) mice were serum-starved, followed by stimulation with 10% fetal calf serum for 10 min, or with 10 μM each of B[a]P, BP-dione, BP-7,8-diol, or BPDE, as indicated. Cell lysates were analyzed by Western blot using anti-phospho-ERK and anti-ERK.

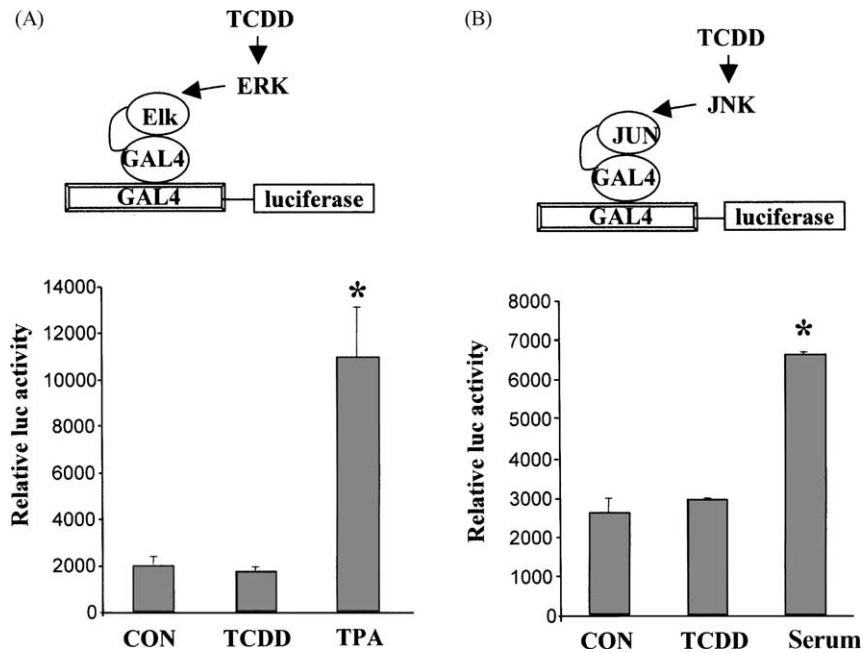

Fig. 4. The transcription activity of ELK and c-JUN is not induced by TCDD. Hepa-1 cells were transfected with a plasmid for β-galactosidase and with a GAL4-luciferase reporter, together with Gal4-ELK (A), or Gal4-JUN (B). Twelve hours after transfection, the cells were serum-starved for 24 hr, followed by treatment for 16 hr with 10 nM TCDD, 15 ng/mL TPA, or 10% serum, as indicated. The cells were harvested and luciferase and β-galactosidase activities were measured. Relative luciferase activities represent luciferase values normalized for transfection efficiency to the β-galactosidase activity in each extract. Group comparisons were made by one-way ANOVA on log-transformed data from 4–6 experiments. Significant differences to control at $P < 0.05$ are denoted by an asterisk (∗).

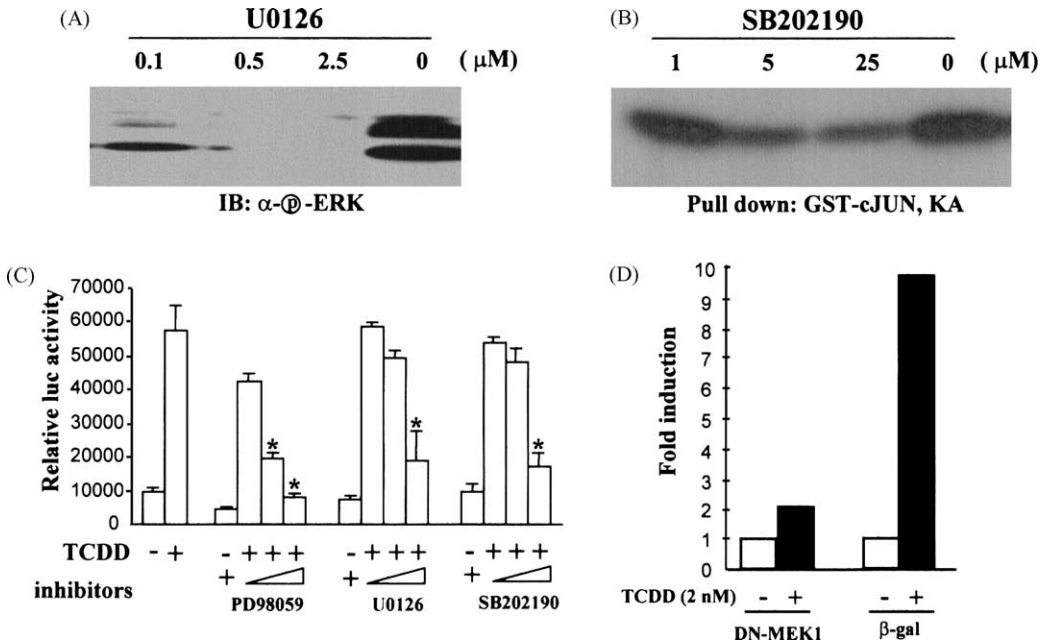

Fig. 5. ERK and JNK are needed for TCDD-induced AHR activation. Hepa-AHRDTKluc3 cells were deprived of serum for 24–48 hr and pretreated for 0.5 hr with U0126 (A) or SB202190 (B) at the indicated concentrations followed by stimulation with 2 nM TCDD for 10 min. Cell lysates were processed for Western blotting using anti-phospho-ERK for ERK phosphorylation (A) and GST-cJun pull-down kinase assay for JNK activity (B). (C) Hepa-AHRDTKluc3 cells were starved as described above and pretreated with increasing concentrations of MAPK inhibitors. PD98059 was used at 1, 5, and 25 μM; U0126 at 0.1, 0.5, and 2.5 μM; and SB202190 at 1, 5, and 25 μM, respectively. The cells were treated with 2 nM TCDD or with DMSO vehicle for 5 hr in the presence of the inhibitors and luciferase activities were measured and plotted relative to the protein concentration of the lysates. Group comparisons were made by one-way ANOVA on log-transformed data from 2–4 experiments. Significant differences to control at $P < 0.05$ are denoted by an asterisk (∗). Fifty percent inhibition was achieved with 2.3 μM of PD98059, 1.2 μM U0126, or 10.8 μM SB202190. (D) Hepa-AHRDTKluc3 cells were infected with adenovirus expressing a dominant negative MEK1 (DN-MEK1) or a β-galactosidase control (β-gal). After infection, the cells were serum-starved for 24 hr, followed by stimulation with 2 nM TCDD for 5 hr. Luciferase activities were measured and the effect of TCDD represented as fold-induction relative to activities in vehicle-treated cells and the results shown are a representative of three independent experiments.

from AHR ligands. To rule out the possibility that the MAPK inhibitors functioned in our assays as receptor antagonists rather than as true MAPK inhibitors, we measured the ability of these compounds to compete with ^{3}H-TCDD for binding to the AHR. As shown by others [29], PD98059 inhibits TCDD-binding to AHR almost completely; on the other hand, a 500-fold molar excess of U0126 or SB202190 only blocked 8.3 and 17.8% of TCDD-binding, respectively (Table 1). The inhibitory effects of U0126 and SB202190 on AHR activation are likely to be the consequence of blocking the MAPK pathways, and not of AHR-binding, leading to the conclusion that TCDD-induced MAPK activation is critical for AHR activation.

Table 1
Competition of TCDD-binding to the AHR by MAPK inhibitors

Competitor	Percent inhibition
TCDD	87.5
PD98059	79.9
U0126	8.3
SB202190	17.8
SB202474	13.9
None	<1

3.6. MAPK activity needed for induction of CYP1A1 expression by TCDD

Expression of CYP1A1 is dependent on AHR activation by its ligands [30]. To assess the role of MAP kinases in the induction of an indigenous AHR-regulated gene, we determined the effect of MAPK inhibitors on the induction of CYP1A1 expression by TCDD. Basal expression of CYP1A1 was undetectable in untreated Hepa-1 cells and was considerably induced by TCDD treatment (Fig. 6, lanes 1 and 2). Induction was partially suppressed by pretreatment with U0126 or with 20 μM but not 5 μM SB202190 (Fig. 6, compare lanes 8, 10, and 12 to lane 2) and was blocked almost completely when the two inhibitors were used in combination (Fig. 6, lanes 14 and 16). The inhibitory effect was not caused by a general reduction of protein synthesis, since β-actin expression was not altered (Fig. 6, lower panels). When applied at the higher concentration (20 μM), U0126 induced CYP1A1 expression slightly (Fig. 6, lanes 7, 13, and 15). Since this compound is a poor, if at all, AHR antagonist (Table 1), its effect at high concentration on CYP1A1 expression is at present unexplained. Notwithstanding, our data indicate that TCDD-induced MAPK activity play an important role

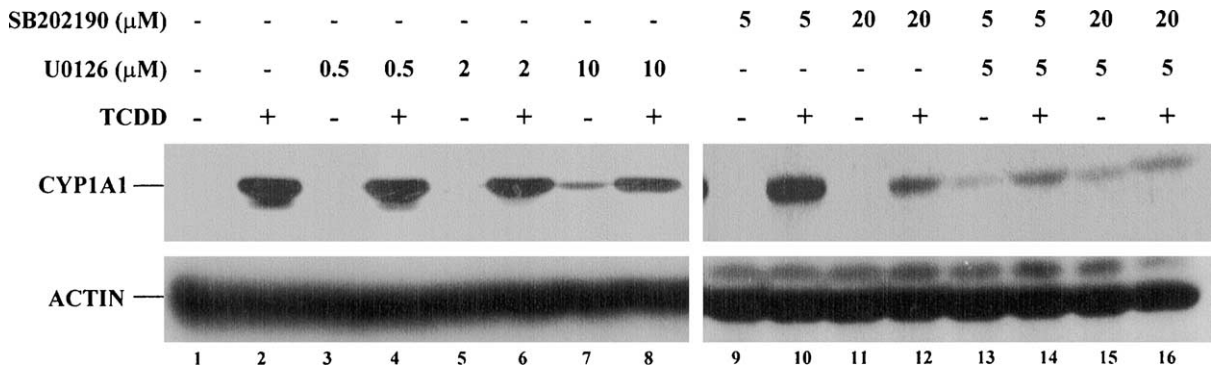

Fig. 6. ERK and JNK activities are needed for TCDD-dependent CYP1A1 induction. Hepa-1 cells were serum-starved for 24 hr, pretreated with DMSO or with various concentrations of U0126 or SB202190 as indicated, followed by stimulation with 10 nM TCDD for 12 hr. Cell lysates were analyzed by Western blot using anti-CYP1A1 and anti-β-actin antibodies.

in AHR activation and CYP1A1 induction. The ERK and JNK pathways, but not p38, appear to each make unique and separate contributions to induction.

4. Discussion

In this report, we show that ubiquitous environmental contaminants, including TCDD, B[a]P, and several B[a]P metabolites, induce an immediate activation of the ERK and the JNK MAP kinase pathways. Common to these MAPK inducers is that they are all ligands for the cytosolic AHR. However, MAPK activation is not dependent on ligand–receptor interactions, since it takes place equally efficiently in cells that have AHR as in cells that lack AHR expression. Hence, we conclude that these xenobiotics activate MAPK through a unique AHR-independent non-genomic mechanism.

Although both TCDD and serum induce MAPK activity, the outcome of MAPK activation by TCDD, as demonstrated by transcription factor modulation, is rather different from that of activation by serum. Unlike the mitogenic MAPK inducers, such as serum factors and the tumor promoter TPA, TCDD-stimulated MAPK activity does not converge on the induction of ELK-1 or c-JUN transcriptional activity. Compared to serum, TCDD is a weak MAPK activator and induces the phosphorylation of ERK and JNK to a much lesser extent (Fig. 2), which may account for its inability to activate ELK and JUN (Fig. 4). MAPKs-induced by mitogenic stimuli modify cytoplasmic proteins; they also undergo nuclear translocation and phosphorylate nuclear transcription factors [31,32]. Induction of MAPK nuclear translocation by TCDD has never been demonstrated, and it is possible that the observed inability of TCDD to activate ELK and cJUN is caused by the lack of MAPK translocation to the nucleus. If this were the case it would be likely that these MAPKs would exert their effects on AHR activity in the cytoplasmic compartment. It is well accepted that the specificity of MAPKs in signal transduction is determined by the formation of MAPK modules [33] Possibly, the module activated by TCDD is distinct from the module induced by serum factors and tumor promoter TPA and does not lead to the transcription activation of ELK and JUN. This suggestion is further supported by the findings that TPA attenuates AHR-dependent gene expression [34], whereas MAPKs-induced by TCDD selectively activate AHR. It would be of great interest in future studies to identify the MAPK module specifically responsive to dioxin.

Using specific inhibitors of the ERK and the JNK/p38 pathways, we show for the first time the critical role of the MAPK pathways in AHR activation and CYP1A1 induction. U0126 completely blocked TCDD-induced ERK activation and partially suppressed AHR activity and CYP1A1 induction. Similarly, inhibition of the JNK pathway by SB202190 caused a remarkable reduction of AHR activation by TCDD (Figs. 5 and 6). Most significantly, dual inhibition of JNK and ERK pathways caused nearly complete suppression of CYP1A1 induction (Fig. 6). These results suggest that each pathway is only partially responsible for induction of AHR activity and that complete AHR activation is likely to involve multiple signaling MAP kinase cascades. Given that TCDD elicits complex intracellular signaling pathways [30], our data is of particular interest because it suggests that each pathway may be unique in AHR activity. Identifying TCDD-activated pathways and uncovering the interactions and cross-talks among them will be essential to understand the molecular mechanisms of TCDD toxicity.

Estrogen receptors (ERs), like AHR, are also members of the nuclear receptor superfamily and act as ligand-inducible transcription factors to activate transcription of a particular set of the target genes. Estrogens are the natural ligands for ER and they share remarkable similarity with the xenobiotics in terms of eliciting a rapid non-genomic effect at the plasma membrane, leading to an immediate activation of the MAPKs [35]. Estrogen-induced MAPKs, either phosphorylate directly ER at serine-118 [36] or phosphorylate a coactivator of ER [37], thus playing a critical role in ER activation and the induction of

ER-dependent gene transcription. Similarly, AHR transcriptional activity is dependent on the phosphorylation state of the AHR itself or proteins in the AHR complex [17,18]. Tyrosine phosphorylation is required for AHR DNA-binding and transcriptional activity [18] and phosphorylation on serine/threonine residues is equally important for AHR activation, because inhibition of serine/threonine phosphatases results in increased AHR activity [38]. It is attractive to speculate that MAP kinases may be responsible for the phosphorylation of the AHR or of a member of the AHR complex critical for AHR function.

The mechanisms by which xenobiotic agents activate MAPK appear to be distinct from the way that they activate Src, which is believed to be functionally associated with AHR/HSP90 complex in the cytosol and therefore activated as a consequence of ligand-induced and AHR-dependent complex disruption [7]. Emerging evidence indicates that TCDD elicits many cellular signaling events. For instance, TCDD activates epidermal growth factor receptor (EGFR)-associated tyrosine kinases and induces the molecular interactions of EGFR-associated adaptor proteins, including SHC, GRB2, and SOS, which may form signaling complexes in transmitting EGFR-originated signals [5,39]. Since TCDD itself is not an EGFR ligand, the nature of EGFR activation by TCDD has remained elusive [40]. Similar activation of EGFR-mediated pathways occurs by various physiological and environmental factors, such as epidermal growth factor (EGF) and UV light, which cross-link the EGFR on the plasma membrane and initiate receptor-mediated signaling pathways, leading to MAPK activation [41,42] Possibly, TCDD activates the MAPKs through signaling components and molecular pathways similar or identical to those employed by EGF. TCDD also trigger an immediate elevation of calcium influx rate and intracellular calcium mobilization, independent of AHR [43,44]. Free intracellular Ca^{2+} is a key second messenger in cell signaling and has been directly linked to the induction of MAPKs [45]. Hence, MAPK activation by dioxin may result from TCDD-induced increase in intracellular Ca^{2+} levels. In MCF10A human mammary epithelial cells, TCDD fail to elevate intracellular Ca^{2+}, which may be accountable for the inability of TCDD to activate the MAPKs in this cell type [46,47], further suggesting a role of Ca^{2+} in TCDD-induced MAPK activation.

Different xenobiotic stimuli cause variable duration of MAPK activity, a phenomenon particularly evident in A549 cells (Fig. 3A). Prior observations in PC12 neuroblastoma cells suggest that the duration of MAPK activation may determine cellular responses and cell fates. Sustained ERK activation results in PC12 cell differentiation, whereas transient activation leads to cell proliferation [48]. B[a]P, a strong tumor promoter and a complete carcinogen, may owe its properties to the combination of B[a]P-induced genotoxicity with the induction of prolonged MAPK activation.

Induction of MAPK takes place in all cells examined, including mouse hepatoma Hepa-1 cells, mouse embryo fibroblasts, African Green Monkey Kidney CV-1 cells, and human lung carcinoma A549 cells. Other xenobiotic agents tested, including B[a]P and several of its metabolic derivatives, also activate ERK and JNK, suggesting that MAPK activation is a novel and generalized cellular response to xenobiotics. Our data indicate that the MAPK pathways are important signaling mechanisms involved in the activation of AHR, in TCDD toxicity, and possibly in B[a]P carcinogenicity. Studies on inhibition of MAP kinases may be a productive direction to explore for the prevention and treatment of cellular damage and toxicity associated with exposure to environmental carcinogens and other toxic agents.

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