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# INHIBITION OF Ah (DIOXIN) RECEPTOR TRANSFORMATION BY 9-HYDROXY ELLIPTICINE

## INVOLVEMENT OF PROTEIN KINASE C?

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**Abstract**—9-Hydroxy ellipticine (9-OHE), a metabolite of the anti-neoplastic agent ellipticine, is known to bind the aryl hydrocarbon (Ah) receptor in rat lung cytosol and to inhibit aryl hydrocarbon hydroxylase activity (AHH) in rat hepatic microsomes. In this study, the effects of 9-OHE on the transformation of the rat hepatic cytosolic Ah receptor to a form that binds the xenobiotic responsive enhancer element-3 (XRE-3) of the cytochrome P4501A1 gene was investigated. Sucrose density gradient analysis of [$^3$H]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) binding in rat hepatic cytosol indicated that 9-OHE inhibited binding of the radiolabeled ligand to the Ah receptor with an $IC_{50}$ of 90 $\mu$M. Gel retardation assays revealed that at low concentrations of 9-OHE, the Ah receptor bound to XRE-3, as was the case with the TCDD-liganded receptor. However, in the presence of high concentrations of 9-OHE, the Ah receptor failed to transform to a form that could bind to XRE-3. *In vitro* studies indicated that incubation of rat hepatic cytosol with TCDD resulted in concentration-dependent increases in levels of protein kinase C (PKC) mediated phosphorylation as compared to vehicle-treated extracts. Furthermore, 9-OHE concentrations that exhibited agonist activity with respect to Ah receptor transformation did not alter PKC phosphorylation in hepatic cytosol, whereas higher concentrations exhibited significant concentration-dependent decreases in PKC-mediated phosphorylation. These results demonstrate that the antagonistic effect of 9-OHE observed at high concentrations is due to inhibition of Ah receptor–XRE complex formation, a phenomenon that correlates with alterations in PKC activity.

The ellipticines constitute a family of aromatic, planar alkaloids that are structurally similar to the geometry of a purine-pyrimidine base pair [1]. Analogs of ellipticine are in clinical use in Europe for the treatment of breast cancer under the trade names Celiptium® and Detalliptinium®. The anti-neoplastic effects of the ellipticines are attributed to inhibition of topoisomerase II through sequence-specific intercalation with DNA, followed by entrapment of the DNA–topoisomerase II "cleavable complex" [2–4]. The end result of this process is double-stranded DNA breakage. 9-Hydroxy ellipticine (9-OHE§), shown in Fig. 1, is the major metabolite of ellipticine, and is more active than the parent compound in cytotoxicity and anti-tumorigenic effects [3, 4].

Ellipticine and 9-OHE are known to specifically bind to the aryl hydrocarbon (Ah) receptor in rat hepatic [5, 6] and lung [7] cytosol. In rat hepatic microsomes, both compounds are inhibitors of the dioxin-inducible enzyme, aryl hydrocarbon hydroxylase (AHH) [8], and 9-OHE has been reported to inhibit RNA synthesis and processing in rat hepatocytes [9]. Moreover, it has been reported that 7-hydroxy ellipticine antagonizes AHH induction by the Ah receptor ligand benzo(*a*)pyrene (B(a)P) [10]. The current model for biological effects such as induction of AHH by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, shown in Fig. 1) and its congeners

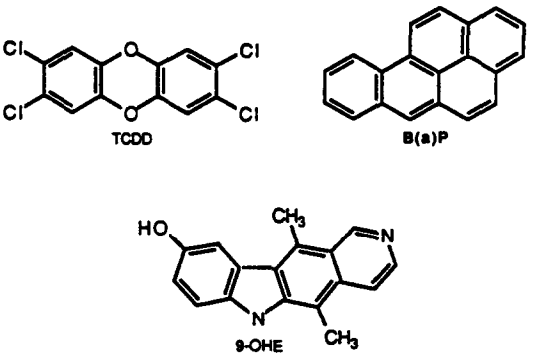


Fig. 1. Chemical structures of Ah receptor ligands utilized in this study. Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B(a)P, benzo(*a*)pyrene; and 9-OHE, 9-hydroxy ellipticine.

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§ Abbreviations: Ah, aryl hydrocarbon; AHH, aryl hydrocarbon hydroxylase; B(a)P, benzo(*a*)pyrene; CYP1A1, cytochrome P450IA1; $ME_2SO$, dimethyl sulfoxide; 9-OHE, 9-hydroxy ellipticine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and XRE, xenobiotic responsive element.

involves liganding of agonists to the cytoplasmic Ah receptor, followed by transformation of the ligand–receptor complex to a nuclear, heterodimeric state [11–13]. The "transformed" or activated Ah receptor binds enhancer sequences known as xenobiotic responsive elements (XRE) located upstream of the cytochrome P4501A1 (CYP1A1) gene to increase gene transcription [11, 13].

Recently, it has been suggested that protein phosphorylation, particularly protein kinase C (PKC) activity, may play a role in regulating Ah receptor nuclear transformation and xenobiotic-mediated induction of CYP1A1 mRNA [14–18]. In this study we have investigated the effects of TCDD and 9-OHE on PKC-dependent protein phosphorylation in a cell-free rat hepatic cytosol system, and correlated these results with the process of Ah receptor transformation. We demonstrated that 9-OHE competes with [$^3$H]-TCDD for binding to the Ah receptor in rat hepatic cytosol and, at high concentrations, 9-OHE prevents transformation of the receptor to a form that binds to the XRE. We also provide evidence that TCDD and low concentrations of 9-OHE stimulated PKC activity in rat hepatic cytosol. Moreover, 9-OHE concentrations that diminished Ah receptor transformation inhibited PKC activity through a mechanism independent of intact cells or nuclei. These results further implicate PKC as a mediator of Ah receptor transformation, and they reveal novel pharmacological properties of 9-hydroxy ellipticine.

## MATERIALS AND METHODS

*Chemicals.* [1,6-$^3$H]-TCDD (40 Ci/mmol) and radioinert TCDD were obtained from Cambridge Isotope Laboratories (Woburn, MA). Benzo(a)pyrene was obtained from the Sigma Chemical Co. (St. Louis, MO). Ellipticine and 9-OHE were from the Drug Synthesis and Chemistry branch of the National Cancer Institute (Bethesda, MD). [$\gamma$-$^{32}$P]-ATP (7000 Ci/mmol) was from ICN (Irvine, CA). Poly [d(I-C)] and herring sperm DNA were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were of molecular biology grade.

*Preparation of hepatic cytosol.* Male Sprague–Dawley rats (70–100 g) from Taconic Farms (NY) were killed under anesthesia, and the livers were perfused *in situ* with phosphate-buffered saline. Hepatic tissue was finely minced and homogenized in buffer containing HEPES (25 mM, pH 7.6), EDTA (2 mM), 2-mercaptoethanol (2 mM), phenylmethylsulfonyl fluoride (PMSF, 1.0 mM) and 10% (v/v) glycerol. The homogenate was centrifuged at 105,000 *g* for 60 min, and the supernatant freed from the lipid layer was frozen at −80° in aliquots.

*Sucrose density gradient analysis.* Cytosol (6 mg/mL) was incubated for 2 hr at 22° with [$^3$H]-TCDD in the absence or presence of 9-OHE. The gradients [10–40% (w/v) sucrose in homogenization buffer] were centrifuged in a Beckman rotor (SW55) at 200,000 *g* for 18 hr at 4°. Ten-drop fractions were collected by piercing the bottom of the tubes, and radioactivity was measured using 5 mL of Cytoscint ES (ICN).

*Gel shift assays.* The complementary oligodeoxyribonucleotides corresponding to the XRE-3 region of the CYPIA1 (5′-CGACCTCGGAGTTGCGTGAGAACAGCC-3′) gene were synthesized, annealed, and $\gamma$-$^{32}$P-labeled at the 5′ ends using T-4 polynucleotide kinase. Cytosol was initially incubated for 2 hr at 22° with TCDD, 9-OHE, or vehicle. Thereafter, aliquots corresponding to 80 $\mu$g cytosolic protein were mixed with gel shift buffer [15 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 60 mM KCl, 5 mM $Zn_2SO_4$, 1 mM EDTA, 5 mM dithiothreitol, 12% (v/v) glycerol, 500 ng sonicated herring sperm DNA, and 200 ng poly d[I-C]) for 10 min at 22°. $^{32}$P-Labeled probe was added and incubated for 20 min at 22° and then analyzed by non-denaturing gel electrophoresis (4% acrylamide, 95 V/4 hr in buffer containing Tris–boric acid (90 mM) and EDTA (2 mM, pH 8.0). The gel was dried and exposed to XAR-5 film at −80°.

*Densitometric analysis.* Autoradiographs from gel retardation assay experiments were scanned with a Microtech 600ZS scanner and the Ah receptor–XRE 3 complexes on each film were analyzed and quantitated using NIH Image 1.41 (NIH, Bethesda, MD).

*Probe degradation assay.* [$\gamma$-$^{32}$P]-ATP-labeled XRE-3 was incubated exactly as described above for the gel shift assay with rat hepatic cytosol that had been preincubated with various concentrations of ligand for 2 hr at 22°. Thereafter, aliquots corresponding to 200,000 cpm were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was then exposed to Kodak XAR-5 film for several hours at −80°. Samples were run alongside [$\gamma$-$^{32}$P]-ATP to determine the mobility of unincorporated radiolabeled nucleotide.

*Measurement of cytosolic protein kinase C activity.* A 200-$\mu$L aliquot of extraction buffer [20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM ethylene glycol-bis($\beta$-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.5% Triton X-100, 25 $\mu$g/mL aprotinin, 25 $\mu$g/mL leupeptin] was added to 200 $\mu$L of liver cytosol (protein concentration of 10 mg/mL) and vortexed for 15 sec at high speed. The sample was placed on ice for 20 min. Subsequently, the sample was diluted 1:5 with extraction buffer and 25 $\mu$L of the resulting solution was added to each tube for quantitation of PKC activity. For experiments where partially purified extract was required, the following DEAE cellulose column chromatography procedure was utilized. DEAE cellulose (0.25 g; Whatman DE-52) was suspended in column buffer [20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA] and the suspension was loaded into poly-prep (Bio-Rad, Richmond, CA) 2-mL columns. Subsequent steps were performed at 4°. The column was loaded with 400 $\mu$L of cytosol suspended in extraction buffer and washed with 5 mL of column buffer. The column was eluted with 2 mL elution buffer [20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM $\beta$-mercaptoethanol, 0.2 M NaCl]. Protein concentration of the eluate was approximately 1.1 mg/mL. Aliquots of 25 $\mu$L were used for PKC assays.

Materials for the PKC assay were obtained from a commercially available kit (GIBCO BRL, Gaithersburg, MD). PKC activity was determined

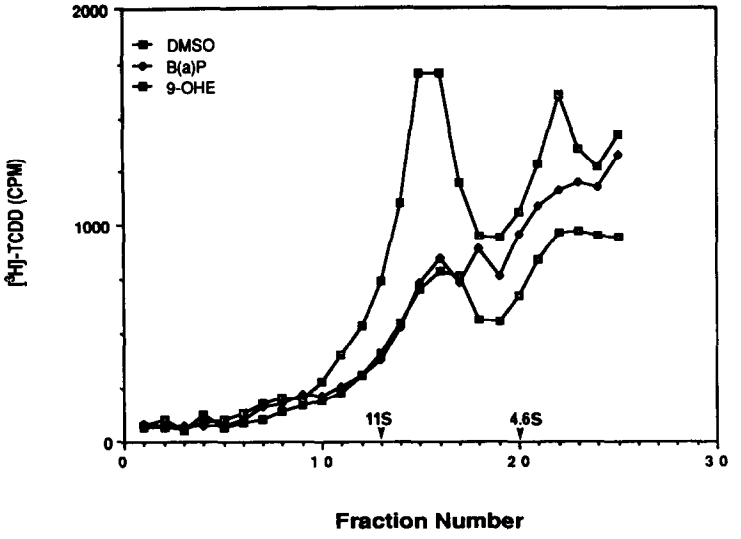


Fig. 2. Sucrose density gradient analysis of [$^3$H]-TCDD binding in rat hepatic cytosol. Cytosol was treated as described in Materials and Methods with 2 nM [$^3$H]-TCDD (sp. act. 40 Ci/mmol) in the presence or absence of 9-OHE (19 $\mu$M), and B(a)P (5.0 $\mu$M). Arrows indicate peak fractions of catalase (11S) and BSA (4.6S) markers determined spectrophotometrically.

by measuring the extent of incorporation of [$\gamma$-$^{32}$P]-ATP into a peptide substrate Ac-MBP (4–14) derived from a portion of the myelin basic protein [19]. All tubes contained a 25-$\mu$L aliquot of cytosol or DEAE column eluate. In addition, each set contained either 10 $\mu$M phorbol 12-myristate 13-acetate (PMA) and 0.28 mg/mL phosphatidylserine in Triton X-100 mixed micelles. The final volume was brought to 40 $\mu$L with sterile water. After incubation at 22° for 20 min, the reactions were initiated by the addition of 10 $\mu$L of substrate solution containing 50 $\mu$M Ac-MBP (4–14), 20 $\mu$M ATP, 1 mM $CaCl_2$, 0.1 $\mu$Ci [$\gamma$-$^{32}$P]-ATP, in 20 mM Tris (pH 7.5). This mixture was incubated for 5 min at 30°, and 25 $\mu$L of solution was spotted onto phosphocellulose discs, washed twice with 1% (v/v) $H_3PO_4$, washed twice with $H_2O$, and then counted in 5 mL Cytoscint ES (ICN). Specific PKC activity was determined by subtracting measured $\gamma$-$^{32}$P incorporation in the presence of 20 $\mu$M PKC (19–36), a peptide fragment of the PKC pseudosubstrate region which is known to potently inhibit PKC $\alpha$, $\beta$, and $\gamma$ subtypes [19].

*Protein concentration determination.* Protein concentrations for all experiments were measured by a kit from Bio-Rad, according to the manufacturer's instructions.

*Statistical analysis.* Data consisting of PKC activity measurements under various experimental conditions were transformed to percentage values according to the equation $P = [(V_e - V_c)/V_c](100)$ where $V_e$ represents the value obtained under the experimental condition, $V_c$ represents the value obtained for a concomitantly measured control, and $P$ represents the percentage value. Proportional values were compared for significant differences by use of the Mann–Whitney U-test.

## RESULTS

*Sucrose density gradient analysis of* [$^3$H]-*TCDD binding to rat hepatic cytosol in the absence or presence of competitors.* Initial experiments were performed to quantify displacement of [$^3$H]-TCDD from the Ah receptor in preparations of hepatic cytosol by 9-OHE. As shown in Fig. 2, and in agreement with previously published reports [11], [$^3$H]-TCDD bound to protein complexes that exhibited sedimentation coefficients of approximately 4S and 8-9S. The binding of [$^3$H]-TCDD was shown to be specific as it could be competed for by an excess of B(a)P, a congener of TCDD diagrammed in Fig. 1. 9-OHE also competed with [$^3$H]-TCDD for binding to the Ah receptor in hepatic cytosolic extracts (Fig. 2).

Displacement of specific [$^3$H]-TCDD binding in hepatic cytosol by 9-OHE was investigated further using a range of competitor concentrations. As shown in Fig. 3A, the ability of 9-OHE to compete for binding was concentration dependent with maximum displacement of 8-9S binding occurring at 190 $\mu$M competitor. In an effort to establish the concentration of 9-OHE required to inhibit 50% binding ($IC_{50}$) of [$^3$H]-TCDD, the area under the peak (8-9S) in the absence (i.e. [$^3$H]-TCDD only) or presence of increasing concentrations of 9-OHE was calculated (Fig. 3B). The $IC_{50}$ of 9-OHE was estimated to be 90 $\mu$M in rat hepatic cytosol (Fig. 3B).

*Gel shift analysis.* To examine the effects of 9-OHE binding on the transformation of the Ah receptor to the DNA-binding form, gel shift assays were performed using $^{32}$P-labeled XRE-3 probe and rat hepatic cytosol treated with various concentrations of ligand. As shown in Fig. 4A, gel retardation

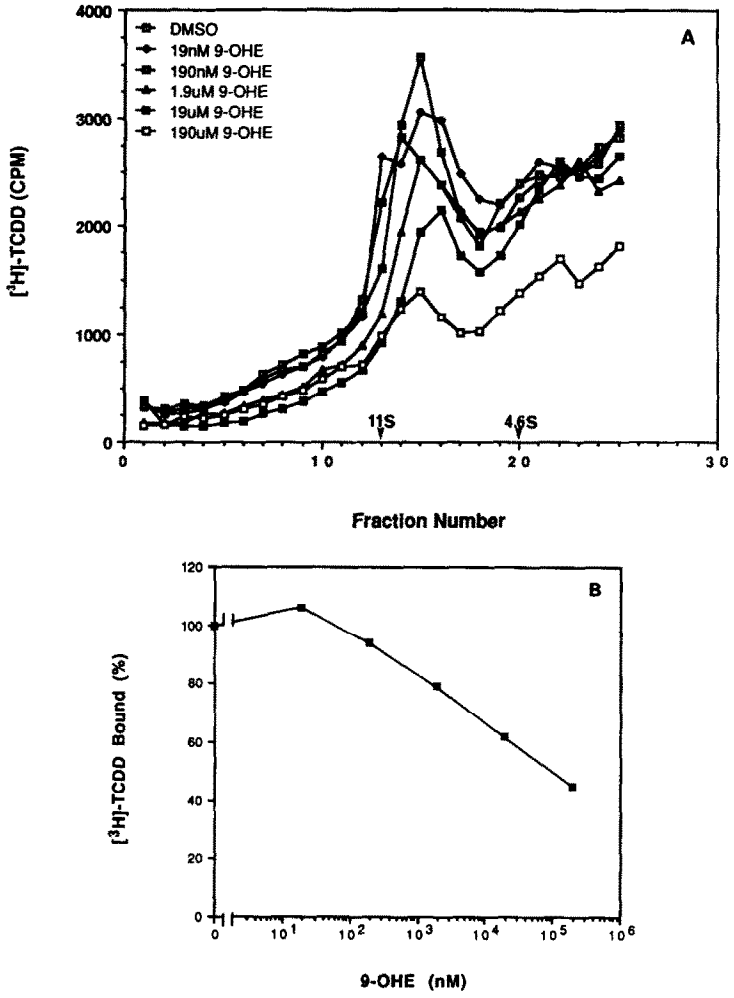


Fig. 3. Concentration-dependent competition of [$^3$H]-TCDD binding in rat hepatic cytosol. Cytosol was treated as described in Materials and Methods with 3.8 nM [$^3$H]-TCDD (sp. act. 40 Ci/mmol) in the presence or absence of the indicated concentrations of 9-hydroxy ellipticine. (A) Arrows indicate peak fractions of catalase (11S) and BSA (4.6S) standards determined spectrophotometrically. (B) Specific [$^3$H]-TCDD binding was calculated by subtracting the area under the 8-9S peak in the presence or absence of competitor. The $IC_{50}$ was determined to be 90 $\mu$M 9-OHE.

analysis of cytosol incubated with 15 nM TCDD revealed the presence of a TCDD-dependent protein–XRE complex, indicated by the arrow (Fig. 4A, lane 3), migrating near the top of the gel. The specificity of this complex was indicated by a competition experiment in which the band was diminished in the presence of a 50× molar excess of unlabeled XRE-3 (Fig. 4A, lane 4). Low concentrations of 9-OHE (19 and 190 nM) induced formation of a high molecular weight receptor–XRE complex (Fig. 4A, lanes 5 and 6) which migrated with the TCDD-Ah receptor–XRE-3 complex, indicating that 9-OHE acts as an Ah receptor agonist at these concentrations. However, in the presence of increasing concentrations of 9-OHE the Ah receptor–XRE-3 complex was increasingly diminished, suggesting that 9-OHE acts as an antagonist of the Ah receptor at higher concentrations. Co-incubation of the extracts with TCDD and 1.9 to 190 $\mu$M 9-OHE resulted in equal antagonism of Ah receptor of XRE-3 complex formation (data not shown). The possibility that 9-OHE induces proteolysis of proteins within the hepatic extract is argued against by the observation that the faster migrating constitutive XRE-3–protein complexes (lower arrowhead) were not diminished in the presence of even the highest 9-OHE concentration. Furthermore, equivalent concentrations of the parent compound ellipticine also behaved as an Ah receptor agonist/antagonist but with slightly reduced antagonist properties (data not shown), suggesting that the effects were not due to any contaminants in the 9-OHE preparation.

To quantitate the agonist/antagonist effects of 9-OHE on Ah receptor–XRE 3 complex formation, autoradiographs from three independent gel retar-

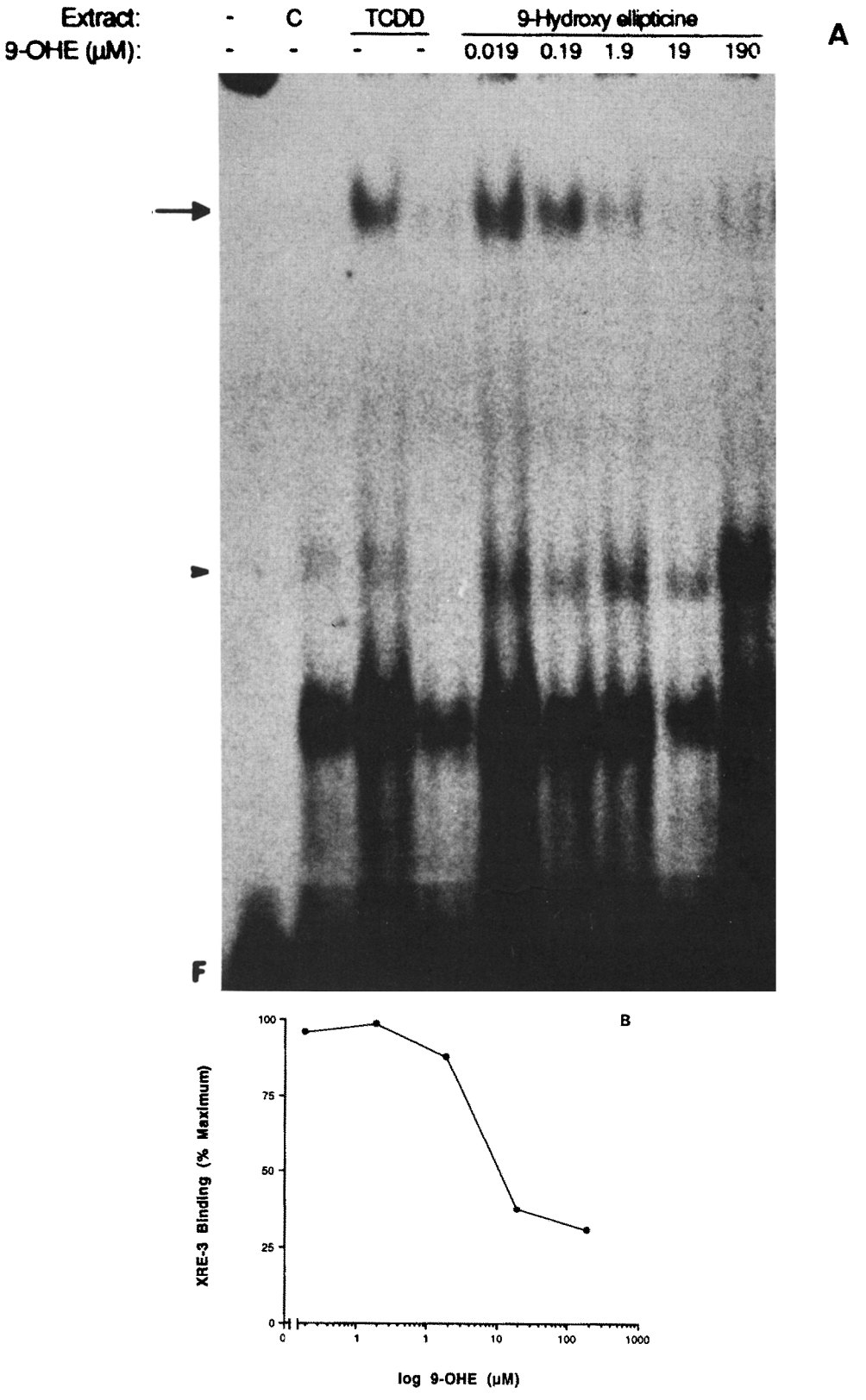


Fig. 4. (A) Agonist/antagonist effects of 9-hydroxy ellipticine on Ah receptor DNA binding. Cytosol was incubated *in vitro* as described in Materials and Methods with $ME_2SO$ (lane 2), 15 nM TCDD (lane 3), 15 nM TCDD + 50× molar excess of unlabeled XRE-3 (lane 4), 19 nM 9-OHE (lane 5), 190 nM 9-OHE (lane 6), 1.9 μM 9-OHE (lane 7), 19 μM 9-OHE (lane 8), or 190 μM 9-OHE (lane 9) and analyzed for DNA binding by gel retardation assay with a $^{32}P$-labeled XRE-3 probe. Lane 1 contains the probe (denoted by F) incubated without cytosol. The Ah receptor–XRE-3 complex is indicated by the arrow; the lower arrowhead indicates constitutive XRE-3 protein complexes. (B) Quantitation of 9-OHE induced agonism/antagonism of XRE-3 binding to the Ah receptor. Autoradiographic films from gel retardation experiments were analyzed and quantitated for Ah receptor–XRE 3 complex formation as described in Materials and Methods. Values are means from 3 independent experiments expressed as percentages of maximum autoradiographic density.

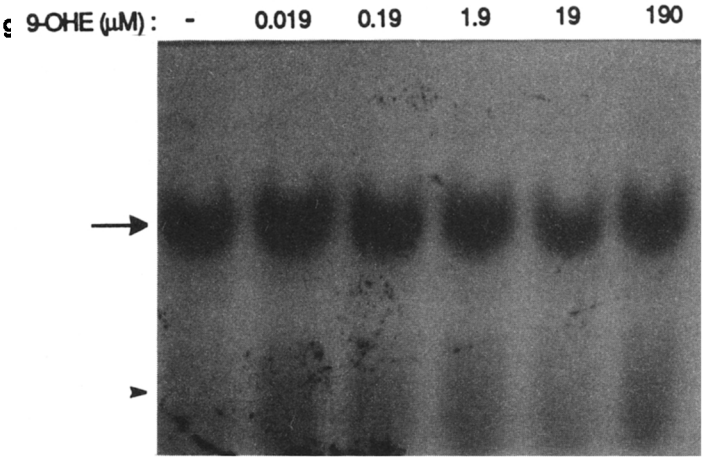


Fig. 5. Effects of 9-hydroxy ellipticine on XRE-3 degradation. $^{32}$P-Labeled XRE-3 was incubated with hepatic cytosol treated with vehicle (lane 1), 19 nM 9-OHE (lane 2), 190 nM 9-OHE (lane 3), 1.9 μM 9-OHE (lane 4), 19 μM 9-OHE (lane 5), or 190 μM 9-OHE (lane 6), and analyzed by SDS–PAGE and autoradiography. The arrow indicates oligonucleotide probe, and the arrowhead denotes unincorporated [$^{32}$P]-ATP.

dation assays were scanned and analyzed by densitometry. The autoradiographic densities of Ah receptor–XRE 3 bands (Fig. 4A, arrow) for each film were quantitated and expressed as percentages of the maximum density. As shown in Fig. 4B, maximal 9-OHE-induced Ah receptor–XRE 3 complex formation occurred at the two lowest concentrations, while in the presence of 190 μM 9-OHE there was approximately 30% XRE-3 binding.

*XRE degradation assay.* It was reported recently by Fosse *et al.* [20] that an analog of 9-OHE preferentially induces DNA cleavage at the consensus sequence 5′-ANCNT(A/G)T. ∧NN(G/C)N(A/G)-3′. This cleavage consensus site is not present in the XRE-3 probe utilized in our studies, but it seemed advisable to investigate possible direct effects of 9-OHE on the probe. To examine if high concentrations of 9-OHE induce probe degradation through a direct interaction with 9-OHE and XRE-3, or through activation of nucleases that may have leaked into the cytosol, the experiment shown in Fig. 5 was performed. $^{32}$P-Labeled XRE-3 probe was incubated with cytosol treated with vehicle (Fig. 5, lane 1), or increasing concentrations of 9-OHE (Fig. 5, lanes 2–6) under the same conditions utilized in gel retardation assays and then analyzed by SDS–PAGE. Probe incubated with 9-OHE-treated cytosolic extracts ran at the same mobility as vehicle-treated extracts, indicating that integrity of the probe appears to be maintained throughout these conditions.

*Effects of TCDD and 9-OHE on protein kinase C activity.* We recently observed that rat thymocytes incubated with TCDD *in vitro* exhibited a rapid and transient increase in PKC activity [21], and it has been reported previously that *in vivo* administration of TCDD in rats and guinea pigs results in elevated levels of hepatic PKC activity [22]. To examine the changes in the phosphorylation state of proteins within rat hepatic cytosol upon incubation with TCDD, an *in vitro* PKC phosphorylation assay was performed. Cytosol was incubated for 2 hr in the presence or absence of increasing concentrations of TCDD, including the same concentration shown to induce Ah receptor–XRE-3 complex formation in the gel shift assay (Fig. 4A). Detergent-extractable PKC activity was then quantitated in these extracts. As shown in Table 1, hepatic extracts preincubated for 2 hr with 15 nM TCDD exhibited statistically significant increases in PKC activity relative to vehicle-incubated controls. When cytosol treated with 15 nM TCDD for 2 hr at 20° was chromatographed over a DEAE cellulose column, we observed significant increases in PKC activity over vehicle-treated cytosol (152.0 ± 18.4 pmol/mg/min vs 113.0 ± 17.1 pmol/mg/min, $P < 0.05$). These results demonstrate that TCDD elevated detergent

Table 1. Concentration-dependent effects of TCDD on PKC activity

| TCDD (nM) | PKC activity (% control) |
|---|---|
| 1.5 | 110 ± 3.7 |
| 15 | 123 ± 7.5* |
| 60 | 126 ± 9.5* |
| 120 | 137 ± 11.5* |

Rat hepatic cytosol was incubated for 2 hr with the indicated concentrations of TCDD or $ME_2SO$, and cytosol was assayed for PKC activity as described in Materials and Methods. Values are means ± SEM from at least 3 independent experiments determined in triplicate. The control (100%) value was 20.6 ± 6.6 pmol/mg/min.

* Statistically significant difference from control ($P < 0.05$) as analyzed by a Mann–Whitney U-test.

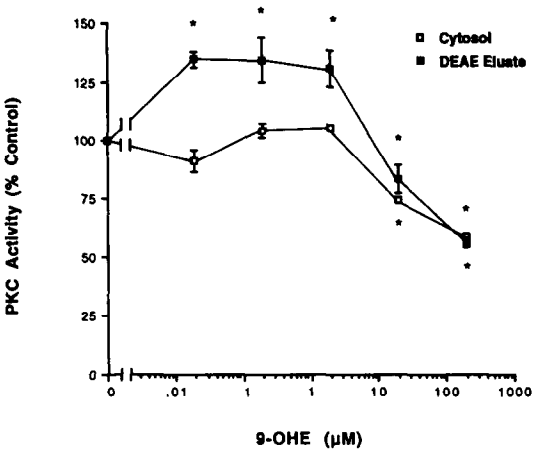


Fig. 6. Effects of 9-hydroxy ellipticine on PKC activity. Cytosol and DEAE eluate were incubated with $ME_2SO$ or the indicated concentrations of 9-OHE for 2 hr, and assayed for PKC as described in Materials and Methods. Values are means ± SEM from at least 3 independent experiments determined in triplicate. Key: (*) statistically significant difference from control ($P < 0.05$) as analyzed by a Mann–Whitney U-test. Control (100%) values of PKC activity were 22.8 ± 0.9 pmol/mg/min in cytosol, and 113.0 ± 17.1 pmol/mg/min in DEAE eluate.

extractable PKC activity in a concentration-dependent manner in a cell-free system devoid of *de novo* mRNA transcription and protein synthesis.

Recently, it was suggested that ligand-induced transformation of the Ah receptor to the DNA-binding state may be regulated by PKC-dependent phosphorylation [15, 16]. To investigate if the observed agonist/antagonist properties of 9-OHE on Ah receptor transformation may be mediated through protein phosphorylation, PKC assays were performed using rat hepatic cytosol incubated with increasing concentrations of 9-OHE. As shown in Fig. 6, low concentrations of 9-OHE (19 nM to 1.9 μM) had no effect or slightly increased PKC activity relative to control, whereas higher concentrations of 9-OHE (19–190 μM) significantly inhibited PKC-dependent phosphorylation in a concentration-dependent manner. The relative potency of 9-OHE as a PKC inhibitor at high concentrations was examined by performing parallel experiments with the known PKC inhibitor staurosporine [23], and estimating $IC_{50}$ values from concentration–response curves similar to the ones in Fig. 6. The resulting $IC_{50}$ values for staurosporine and 9-OHE in rat hepatic cytosol were 70 nM and 200 μM, respectively (data not shown). When cytosol was DEAE-cellulose purified, a profile of statistically significant PKC activation at low concentrations of 9-OHE and inhibition at high concentrations was observed (Fig. 6). Thus, the agonist/antagonist effects of 9-OHE on PKC-dependent protein phosphorylation correlated closely with the effects on Ah receptor–XRE 3 binding (Fig. 4B).

## DISCUSSION

It is currently believed that the stimulatory effects of TCDD and related compounds on gene transcription occur through binding of an agonist to a multimeric Ah receptor complex in the cell cytosol consisting of the Ah receptor nuclear translocator protein and a dimer of heat shock protein 90 kDa, followed by transformation of the receptor to a heterodimeric XRE-binding state [12, 13, 24]. Thus, an antagonist of the Ah receptor may exert its effects through multiple mechanisms that are not necessarily mutually exclusive. One model to explain the antagonistic effects of 9-OHE observed at high concentrations would be through a direct interaction at the putative 6.8 × 13.7 Å ligand binding pocket of the Ah receptor [25], as has been proposed for the aromatic hydrocarbon α-naphthoflavone [26]. A second possibility is that high concentrations of 9-OHE specifically induce formation of a transcriptionally inactive complex between XRE3 and a monomer of the Ah receptor, or an accessory protein such as the Ah receptor nuclear translocator protein [24]. This situation would be analogous to anti-estrogens such as tamoxifen and ICI 164,384, which alter the binding properties of the estrogen receptor to its cognate response element [27, 28]. Alternatively, the mechanism by which a given ligand inhibits Ah receptor binding or transformation to the XRE-binding state may be mediated through indirect means, such as changes in the phosphorylation state of the cytosolic receptor complex. In support of this latter possibility, several recent reports suggest that decreasing levels of PKC-dependent phosphorylation in cell extracts inhibit Ah receptor-mediated transcription [14–18]. Here we extend these findings by presenting evidence that TCDD elevates PKC activity in a concentration-dependent manner, and that agonism/antagonism of PKC activity correlates closely with agonism/antagonism of Ah receptor transformation. Furthermore, by utilizing a cell-free system, we demonstrated that *de novo* synthesis of RNA and protein are not required for these processes to occur.

In this study we determined that TCDD increased PKC activity in a concentration-dependent manner with significant increases occurring at 15–120 nM of the xenobiotic, while higher concentrations of 9-OHE inhibited PKC activity. It should be noted that the solubility of TCDD is limited, so that all of the drug may not be solubilized at 120 nM. The concentration of 9-OHE required to inhibit PKC activity was relatively high, and at variance with that required to inhibit transformation of the Ah receptor. This is likely due to the resolution of the assay systems utilized. The gel shift assay resolves specific binding of the Ah receptor, while the protein kinase C assay is a measure of total PKC activity within the cytosol. Thus, specific PKC-mediated phosphorylation of the Ah receptor would not be detected by this assay. An alternative hypothesis would be that 9-OHE inhibits PKC activity at its optimal concentration range through another mechanism(s), such as a direct interaction with the enzyme. The availability of sufficient quantities of purified Ah receptor and specific PKC isoforms may enable a more precise determination of the effect that 9-OHE has on Ah receptor phosphorylation, and the mechanism governing inhibition of PKC by 9-OHE.

*In vitro* studies demonstrate that the concentration of ellipticine required to induce maximal DNA cleavage is approximately 0.5 μg/mL (i.e. 2 μM) while DNA cleavage is inhibited at drug concentrations higher than 4 μM [29]. Therefore, the concentrations of ellipticine required to cleave DNA are in the same range as those that induce transformation of the Ah receptor. Recently it was reported that inhibiting PKC activity with staurosporine [30] or extended exposure to phorbol esters [31] results in an inhibition of topoisomerase II-mediated DNA cleavage. The results presented here suggest that interference of DNA cleavage at high concentrations of 9-OHE may also be mediated through inhibition of protein kinase C, a finding that may have clinical ramifications in terms of the pharmacodynamics of the ellipticines. Investigations are currently underway to examine the effects of 9-OHE on other specific kinase activities as well as additional factors governing the processes of Ah receptor transformation and TCDD-induced activation of PKC. Studies along these lines may aid in elucidating the mechanism(s) of Ah receptor agonist carcinogenesis and toxicity.

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