Biochemical Pharmacology, Vol. 52, pp. 1883–1893, 1996.

ISSN 0006-2952/96/$17.00 + 0.00
PII S0006-2952(96)00036-6

Induction of Cytochrome P4501A1 by Photooxidized Tryptophan in Hepa lclc7 Cells*

Ram K. Sindhu,† Suzanne Reisz-Porszasz,‡ Oliver Hankinson‡ and Yutaka Kikkawa†§

†Department of Pathology, College of Medicine, University of California at Irvine, Irvine, CA 92697; and ‡Department of Pathology and Laboratory Medicine, University of California at Los Angeles, Los Angeles, CA 90024, U.S.A.

ABSTRACT. Mouse hepatoma Hepa-lclc7 (Hepa-1) cells were cultivated in the presence of UV-irradiated amino acids. The results demonstrated that all of the amino acids tested, UV-oxidized tryptophan caused the highest induction of 7-ethoxyresorufin O-deethylase (EROD) activity compared with the controls ($P < 0.01$). The induction of EROD activity by oxidized tryptophan was dose dependent, and maximal induction was obtained at 12 hr after administration. Studies with various Hepa-1 mutants, which are defective in either the aryl hydrocarbon (Ah) receptor or Ah receptor nuclear translocator protein, indicated that the induction of EROD activity by oxidized tryptophan occurs through the Ah receptor. Gel mobility shift assays using nuclear extracts of Hepa-1 cells revealed that oxidized products of tryptophan can induce both Ah receptor transformation and binding of the liganded Ah receptor complex to its specific DNA recognition site. CYP1A1 mRNA, quantified by reverse transcription-polymerase chain reaction, and CYP1A1 protein were induced markedly in the oxidized tryptophan group compared with the controls. Injection of isolated oxidized tryptophan products into adult male rats caused significant induction of EROD activity in the pulmonary and hepatic microsomes compared with the controls ($P < 0.01$). These results demonstrated that oxidized tryptophan induces Ah receptor activation and binding of the liganded Ah receptor complex to its specific DNA recognition site, thereby initiating transcription and translation of the *CYP1A1* gene with concomitant increase of EROD activity in Hepa-1 cells. Induction of EROD activity in the liver and lungs after injection of isolated oxidized tryptophan products into rats suggests that a similar mechanism may be operative *in vivo*. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1883–1893, 1996.

KEY WORDS. oxidized tryptophan; cytochrome P4501A1; 7-ethoxyresorufin O-deethylase; Ah receptor; mRNA; reverse transcription -polymerase chain reaction

The cytochrome P450 monooxygenase system is involved in the oxidative metabolism of a wide variety of xenobiotics such as drugs, environmental pollutants, pesticides, and natural plant products, as well as endogenous substrates, including steroid hormones such as testosterone and androsterone. Of the 74 gene families of cytochrome P450 described so far, 14 families exist in all mammals examined to date [1]. In mammals, the CYP‖ family 1 has been reported to consist of three isozymes, CYP1A1, 1A2 [1] and 1B1 [2].

The present investigation was undertaken as a corollary to our earlier observations that exposure of adult male rats to hyperoxia ($O_2 > 95\%$) causes induction of mRNA, protein, and corresponding monooxygenase activity of CYP1A1 in the lung, and CYP1A1 and 1A2 in the liver [3]. This induction of CYP1A1/1A2 by hyperoxia is unusual because induction of these isozymes has been associated primarily with the administration of xenobiotic compounds such as polychlorinated or polybrominated biphenyls and polycyclic aromatic hydrocarbons, which are known to initiate transcription of CYP1A1 by binding to the Ah receptor [4, 5]. Thus, our working hypothesis to explain CYP1A1/1A2 induction by hyperoxia has been that endogenous ligand(s) of the Ah receptor is produced by hyperoxia, followed by Ah receptor-mediated transcriptional activation of the *CYP1A1/1A2* genes [3]. Since the lung is the primary target organ for hyperoxia, the endogenous ligand(s) for the Ah receptor may have been produced initially in the lung and must be sufficiently stable to be transported to the liver where secondary induction of CYP1A1/

* This work was presented at the annual meeting of the Federation of American Societies for Experimental Biology, Washington, DC, April 14–17, 1996.

§ Corresponding author: Yutaka Kikkawa, MD, Department of Pathology, College of Medicine, University of California at Irvine, Irvine, CA 92697. Tel. (714) 824-8031; FAX (714) 824-2160.

‖ *Abbreviations:* CYP, cytochrome P450; EROD, 7-ethyoxyresorufin O-deethylase; Ah, aryl hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHH, aryl hydrocarbon hydroxylase; RT, reverse transcription; PCR, polymerase chain reaction; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; ARNT, aryl hydrocarbon receptor nuclear translocator; and XRE, xenobiotic (dioxin) responsive element.

Received 15 February 1996; accepted 3 July 1996.

1A2 occurs. Hyperoxia is known to generate reactive oxygen species that have been shown to oxidize histidine residues in proteins and peptides [6]. Oxidized histidine has been reported to induce the activity of AHH in an inducible hepatoma cell line [7].

Previous studies have shown that apart from oxidized histidine, photooxidized tryptophan and proline may also cause an induction of AHH in hepatocyte culture [7–12]. Using hepatocyte culture, it was shown [7–9] that UV irradiation of cell culture medium resulted in the formation of an inducer of AHH, which was presumed to be a histidine-derived oxidation product [7]. Rannug *et al.* [13] reported that tryptophan and histidine gave products upon UV irradiation that competed with TCDD for its binding to the Ah receptor. One of these photoproducts of tryptophan appeared to induce AHH activity in rat hepatoma cells [14]. Helferich and Denison [15] showed that tryptophan photoproducts interact with guinea pig hepatic cytosolic Ah receptor and act as TCDD agonists. Nemoto *et al.* [16] reported an induction of CYP1A1 mRNA in primary culture of rat hepatocytes and implicated proline as the critical requirement for this induction. Kocarek *et al.* [17] showed that photooxidized tryptophan, but not histidine, caused increased CYP1A1 mRNA expression in primary cultures of adult rat hepatocytes. Furthermore, exposure of rats or mice to UV light has been reported to induce AHH activity in the skin and liver [10–12]. Taken together, these observations suggest that UV irradiation of animals produces oxidized products *in vivo* which can induce AHH directly in the irradiated skin cells and that these products may be transported to the liver where they induce hepatic CYP1A1. From the aforementioned studies it is clear, however, that the results on the induction of AHH activity are equivocal. For example, it is not clear which of the amino acids are actually involved in this induction. Furthermore, most of the studies mentioned above investigated the effect of oxidized amino acids on AHH activity, and none addressed the effects on CYP1A1 protein. Finally, no information is available as to whether these photoproducts would cause an induction of CYP1A1 when injected into intact animals.

The present study was undertaken with UV irradiation to examine all of the amino acids for their ability to induce CYP1A1 in a cell culture system by assaying EROD activity. The results obtained show that of all the amino acids tested, only photooxidized tryptophan caused a substantial induction of EROD activity in the mouse hepatoma cell line Hepa-1 in a dose-dependent manner. No induction of EROD activity by oxidized tryptophan was observed in several Hepa-1 mutants which are defective in either the Ah receptor or the ARNT protein, indicating the involvement of the Ah receptor. The photooxidized products of tryptophan can induce both Ah receptor transformation and binding of the liganded Ah receptor complex to its specific DNA recognition site, thereby activating the transcription of the *CYP1A1* gene and expression of the corresponding protein in Hepa-1 cells.

MATERIALS AND METHODS

Materials

L-Tryptophan was purchased from the Aldrich Chemical Co. (Milwaukee, WI). 7-Ethoxyresorufin and resorufin were purchased from Pierce (Rockford, IL). Monoclonal CYP1A1/1A2 antibody (1-7-1) was a gift from Dr. S. S. Park of the N.C.I. (Frederick, MD). NADPH and all the amino acids (except tryptophan) were obtained from the Sigma Chemical Co. (St. Louis, MO). TCDD, shipped by the supplier (Cambridge Isotope Laboratories, Woburn, MA) in nonane solution, was dried under a gentle stream of nitrogen, solubilized in dimethyl sulfoxide, and used without any further purification. All other chemicals were of the highest purity commercially available.

Oxidation of Amino Acids

Aqueous solutions of amino acids (2 mM) were irradiated with a germicidal UV light for 6 hr at a distance of approximately 15 cm above the solutions. The irradiated solutions were protected from light and stored in the refrigerator. Distilled water, used routinely for the experiments, was also irradiated along with the amino acids and used as one of the controls.

Analysis of Tryptophan by HPLC

Unoxidized and UV-oxidized tryptophan was taken up in 50% methanol and analyzed by reversed-phase HPLC (Perkin–Elmer Series 410) equipped with a Zorbax C_{18} analytical column (4.6 × 250 mm, 5 μm) and an LC 235 Diode Array Detector. Elution of tryptophan off the column was carried out at a flow rate of 0.5 mL/min using a linear gradient of 0 to 100% methanol over a period of 60 min and held there for an additional 30 min. The absorbance of column eluates was followed at 254 and 280 nm.

Cells and Growth Conditions

Hepa-1 cells and the B^- (c12), C^- (c4), D^- (c35), and dominant (c31) mutants were propagated in ribonucleoside-free-minimum essential medium (Gibco BRL, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA) and 1% antibiotic-antimycotic (Gibco BRL) using 25 cm^2 canted neck tissue culture flasks (Becton Dickinson, San Jose, CA). When the cells were over 95% confluent, these were incubated with the test materials for varying periods of time at 37° as described in the text. At the end of the incubation period, the cells were scrapped, collected in sterile centrifuge tubes, and centrifuged at 1000 *g* for 5 min at 4°. The cell pellet was washed once with 5 mL of 0.15 M Tris–HCl, pH 8.0, containing 7.5% sucrose and finally resuspended in 0.25 mL of 0.15 M Tris–HCl, pH 8.0, containing 7.5% sucrose. The cells were sonicated with a Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY) using two 10-sec bursts at 50% power and an output of 3.

Biochemical Measurements

EROD activity was determined according to the method of Prough *et al.* [18]. Protein was measured by the method described by Bradford [19].

SDS–PAGE and Western Blotting

The proteins in the disrupted cell extracts were resolved by denaturing SDS–PAGE on discontinuous polyacrylamide (10%) slab gels using a Bio-Rad (Richmond, CA) Mini Protean II apparatus. The proteins were then electrophoretically transferred to nitrocellulose membranes (Bio-Rad) as described [20]. The filters were blocked with SuperBlock blocking buffer (Pierce) and probed with monoclonal anti-CYP1A1/1A2 (1-7-1). The antigen–antibody complex was incubated with horseradish peroxidase-conjugated second antibody (anti-mouse IgG; Amersham, Arlington Heights, IL) and visualized using chemiluminescent substrate (ECL kit, Amersham) according to the manufacturer's directions.

Isolation of mRNA

Control and oxidized tryptophan-administered Hepa-1 cells (1.8×10^6) were lysed with 250 μL of 10 mM Tris, pH 7.6, containing 1 mM EDTA, 0.5 M NaCl, 1% NP-40 (Sigma), and 10 mM vanadyl ribosyl complex (Gibco BRL) by vortexing for 2 min. mRNAs were isolated in 30 μL of nuclease-free H_2O using the PolyATract mRNA Isolation Systems kit from Promega (Madison, WI), according to the manufacturer's directions, and stored frozen at −80° until used.

cDNA Synthesis

The isolated mRNAs solution (1 μL) was diluted with 10.15 μL of nuclease-free H_2O, and 1 μL (5 pmol) of poly $(dT)_{17}$ was then added. The mixture was kept in boiling water for 5 min and then cooled on ice for an additional 5 min. After adding 4 μL of reverse transcriptase buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$; Gibco BRL), 1 μL of 10 mM dNTP mixture (Promega), 2 μL of 0.1 M DTT (Gibco BRL), 0.25 μL (10 U) of RNAsin (Promega), and 0.6 μL of M-MLV reverse transcriptase (120 U), the mixture was incubated at 37° for 1 hr. After the cDNA synthesis, reverse transcriptase was inactivated by incubation at 75° for 10 min. Ribonuclease H (1 μL, 1.5 U, Promega) was then added and incubated for 20 min at 37°. The single-stranded cDNA thus obtained was used for PCR amplification.

PCR

The primers for PCR amplification were determined by using the computer program (OligoProbe DesignStation) as described [21, 22] and were the most specific ones with minimum chance of cross-hybridization against other known P450 genes. The sequence of the sense and the antisense primers for CYP1A1 were as follows: 5′-CAATGAGGCTGTCTGTGATGTC-3′, and 5′-CCCTTTCAAGTATTTGGTGGTG-3′. β-Actin was amplified as a control. The sense and the antisense primers for β-actin were 5′-CTTCGCGGGCGACGATGC-3′, and 5′-CGTACATGGCTGGGGTGTTG-3′. cDNA (6.6 μL) was mixed with 1 μL of 10× PCR buffer (Promega), 0.4 μL of 25 mM $MgCl_2$ (Promega), 0.5 μL each sense and antisense primers (0.1 mg/mL), 0.8 μL of 10 mM dNTP mixture (Promega), and 0.2 μL of Taq polymerase (Promega) in a final volume of 10 μL and was amplified in a programmable thermal controller (M.J. Research, Inc., Watertown, MA) with 30 cycles by denaturation at 94° for 1.5 min, annealing at 55° for 1.5 min, and extension at 72° for 4 min.

Gel Mobility Shift Assays

The cells were grown in large tissue culture flasks (75 cm^2) as described earlier. When the cells were more than 95% confluent, these were incubated in the presence or absence of 0.1 mM UV-oxidized tryptophan for 3 hr in fresh medium. At the end of the incubation period, the cell monolayers were washed twice with 5 mL of ice-cold PBS. The cells were then scraped into a small volume of PBS (2 mL/dish), pooled in 50-mL centrifuge tubes on ice, and centrifuged at 400 *g* for 10 min at 4°. The cells were then suspended in 5 pellet volumes of PBS, recentrifuged, resuspended in 3 pellet volumes of HEPEs, pH 7.5, and kept on ice for 10 min. After recentrifugation, the cells were resuspended in 1 pellet volume HED buffer (10 mL of 250 mM HEPES, pH 7.5; 0.4 mL of 0.5 M EDTA, pH 8.0; 0.1 mL of 1 M DTT mixed just before use; volume was made up to 100 mL). The cells were transferred to a B-type Dounce homogenizer and broken with 10–20 strokes. One drop was analyzed under a microscope to ensure a high degree of cell disruption. The broken cells were centrifuged in a microfuge in Eppendorf tubes at maximum speed for 5 sec. The pellet nuclei were resuspended in 1 nuclei pellet volume of HED buffer supplemented with protease inhibitors (40 U/mL aprotinin, 200 μM PMSF, 100 μM leupeptin). KCl (2 M) was then added to a final concentration of 0.4 M. The nuclear suspension was agitated for 30 min at 4° with HAD buffer supplemented with protease inhibitors, and glycerol was then added to a final concentration of 20% and centrifugation was performed at 45,000 rpm for 30 min at 4° using a 75 Ti rotor. The supernatant was removed on dry ice–ethanol and stored at −80°. The nuclear extracts thus obtained were utilized for gel mobility shift assays that were carried out as described by Saatcioglu and coworkers [23] with minor modifications as described elsewhere [24]. The double-stranded 38-bp oligonucleotide as described was used as the wild-type probe. The mutant oligonucleotide used in competition experiments was the same except for the substitution of TA for the indicated CG nucleotides in the core region, CACGCNA of the XRE. This mutant oligonucleotide has been shown not to bind the liganded Ah receptor *in vitro* [25].

Isolation of tryptophan Photoproducts

Tryptophan was exposed (125 mL of a freshly prepared 40 mM solution) to an unfiltered germicidal UV light at a distance of 15 cm for 5 hr. The photoproducts were isolated with Sep-Pad C_{18} cartridges (Water, Milford, MA) that had been washed with 5 mL of water. The irradiated solution of tryptophan was slowly pushed through the Sep-Pak C_{18} cartridges. The cartridges were washed with 20 mL of water. The oxidized products were then eluted with 20 mL of methylene chloride and dried under nitrogen. The photoproducts of tryptophan were suspended in 3.125 mL of corn oil and stirred overnight in the cold room.

Treatment of the Animals with Photooxidized Products of Tryptophan

Adult male Sprague–Dawley rats of the CD strain, viral antigen and pathogen free, were obtained from Harlan Sprague–Dawley (San Diego, CA). Upon arrival, the animals were kept on a 12-hr light–dark cycle in the University of California, Irvine animal facility and allowed food (rodent laboratory chow) and water *ad lib*. The animals were divided into three groups, and each group had three animals. The control group received no treatment. The animals in the second group were injected i.p. for 3 consecutive days with 0.25 mL of corn oil containing oxidized tryptophan products as described above. The vehicle control group was injected i.p. for 3 consecutive days with 0.25 mL of corn oil containing the same amount of methylene chloride that was used to elute the photooxidized tryptophan off Sep-Pak columns and had been dried under nitrogen and suspended in corn oil. The rats were killed 24 hr after the last injection.

Tissue Preparation

The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the livers and the lungs were perfused with cold 0.9% NaCl containing 10 mM EDTA. The livers were weighed, homogenized in cold 1.15% KCl buffered with 50 mM Tris–HCl, pH 7.4 (4 mL buffer/g liver), by 10 strokes in a Potter–Elvehjem Teflon pestle glass homogenizer. The volumes of the whole homogenates were measured.

After recording the fresh weights, the lungs were suspended in cold 1.15% KCl buffered with 50 mM Tris–HCl, pH 7.4 (7 mL buffer/g lung), and subjected to three Polytron strokes of 15 sec each with 70% output using a tissumizer (Tekmar Co., Cincinnati, OH). These were then homogenized by 10 strokes in a Potter–Elvehjem Teflon pestle glass homogenizer, and the volumes were recorded.

Preparation of Microsomes

Pulmonary and hepatic microsomes were prepared as described earlier [26]. The microsomal pellet was suspended in 1.15% KCl buffered with 50 mM Tris–HCl, pH 7.4, and stored frozen at −80° until used.

Statistical Analysis

Statistical analysis of data was performed on a Macintosh II using StatView software. Results are expressed as means ± SD. Statistical significance of the difference between control and the experimental groups was determined by one-way ANOVA and Dunnett's *t*-test.

RESULTS

Effect of UV-Irradiated Amino Acids on EROD Activity

Wild-type Hepa-1 cells were incubated with aqueous solutions of UV-exposed amino acids at a final concentration of 0.2 mM for a total of 6 hr. Of all the amino acids tested, only oxidized tryptophan caused a significant ($P < 0.01$) induction of EROD activity compared with the controls (data not shown). Therefore, the induction of EROD by oxidized tryptophan was followed in greater detail.

Oxidation of Tryptophan by UV Light

Unoxidized and oxidized tryptophan was analyzed by reversed-phase HPLC as described in Materials and Methods. The column eluates were monitored for their absorbance at 254 nm to compare our results with those of Rannug *et al.* [13]. Figure 1A shows that unoxidized tryptophan was eluted as a single peak off the HPLC column, whereas there were several new peaks in the UV-oxidized tryptophan solution (Fig. 1B). Similar results were obtained when A_{280} of column eluates was monitored (data not shown).

HPLC analysis revealed that a total of 23 ± 3% of tryptophan was oxidized under the conditions described in Materials and Methods. However, we do not know what percent of this oxidized tryptophan is responsible for causing the induction of CYP1A1. Therefore, the concentrations of oxidized tryptophan presented in this paper are the concentrations of the parent compound used for oxidation.

Effect of the Period of UV Irradiation of Tryptophan on the Induction of EROD Activity

Tryptophan was oxidized by exposure to UV irradiation for various periods of times and was administered to growing Hepa-1 cells for 6 hr. The results show that photoproducts, with EROD-inducing capacity, were produced as early as 15 min after the exposure of tryptophan to UV light (Fig. 2). However, at 6 hr after UV exposure, the induction of EROD was maximal, and this time-point was used for the oxidation of tryptophan for further experiments.

Effect of Unoxidized and Oxidized Tryptophan on EROD Activity

The results reported in Table 1 show that when a sterile solution of unoxidized tryptophan was administered to

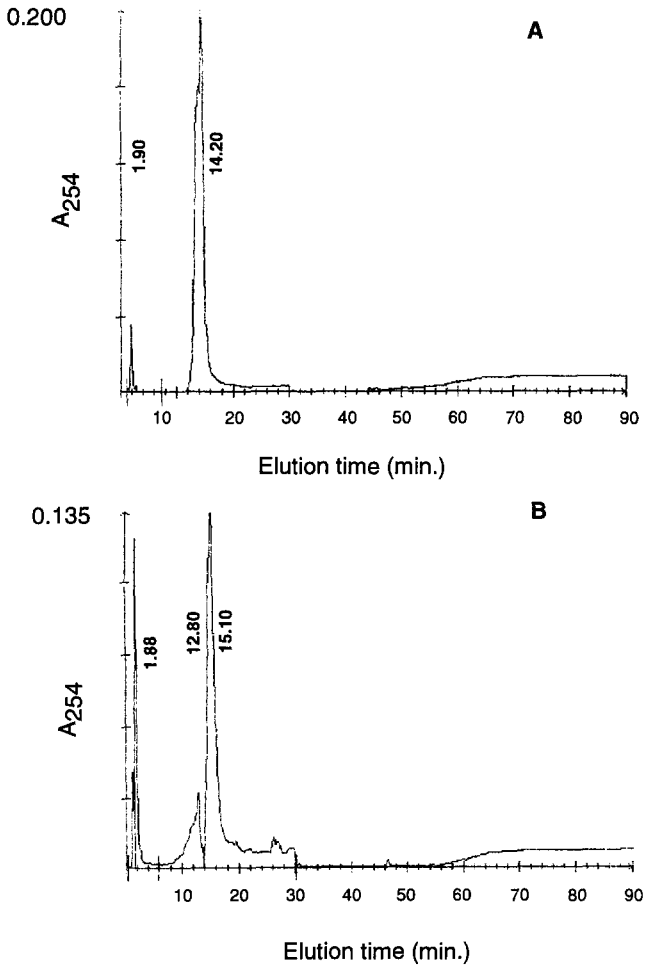

FIG. 1. Reversed-phase HPLC separation of unoxidized (A) and UV-oxidized (B) tryptophan using a C_{18} analytical column, as described in the text. The column eluant was monitored at 254 nm using an LC Diode Array Detector.

growing Hepa-1 cells (>95% confluent) and included for 6 hr, it caused a 2-fold induction of EROD activity, whereas oxidized tryptophan caused a 10-fold induction of the EROD activity compared with the UV-treated H_2O controls ($P < 0.01$). In another group, when the cells were administered 2 nM TCDD, the induction of EROD activity was slightly over 8-fold compared with the controls ($P < 0.01$). When unoxidized tryptophan was recrystallized and administered to the cells, there was no difference in the induction of EROD activity compared with the group receiving unoxidized tryptophan used without recrystallization (data not shown).

Effect of the Concentration of Oxidized Tryptophan on EROD Activity

Different concentrations of oxidized tryptophan (0.001 to 0.2 mM) were administered to Hepa-1 cells for 6 hr, and EROD activity was measured as described in Materials and Methods. EROD activity increased as a function of tryptophan concentration up to 0.1 mM and leveled off after that

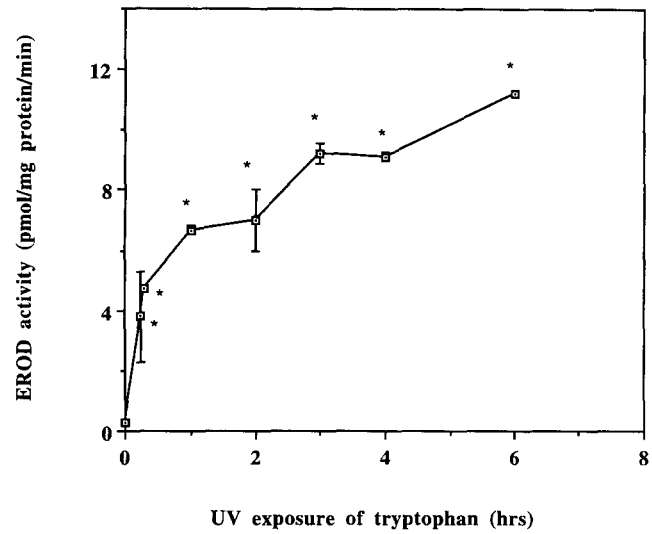

FIG. 2. Effect of period of UV irradiation of tryptophan on the induction of EROD activity. Tryptophan (2 mM) was oxidized by exposing it to UV irradiation for various periods of time and was administered to growing Hepa-1 cells for 6 hr at a final concentration of 0.1 mM. The cells were harvested, in groups of three dishes, and EROD activity was determined as described in the text. EROD activity is expressed as mean pmol resorufin formed/mg protein/min. Values are means ± SD, N = 3. For points where the SD is not shown, it was smaller than the symbol. Key: (*) significantly different from the control group, $P < 0.01$.

(Fig. 3). Therefore, in the following experiments 0.1 mM tryptophan was used. At concentrations of 0.01 mM and higher, oxidized tryptophan caused significant induction of EROD activity compared with the controls ($P < 0.01$).

Time Course of EROD Induction by Oxidized Tryptophan

The time course of the induction of EROD activity by oxidized tryptophan was investigated by administering 0.1 mM oxidized tryptophan to Hepa-1 cells. Oxidized tryptophan was also administered at 1 μM to investigate the kinetics of EROD induction at this low concentration.

TABLE 1. Effect of oxidized tryptophan on EROD activity

Treatment	EROD activity (pmol resorufin formed/mg protein/min)
UV-treated water	0.30 ± 0.04
Unoxidized tryptophan	0.66 ± 0.06
Oxidized tryptophan	3.0 ± 0.75*
TCDD	2.5 ± 0.01*

Hepa-1 cell cultures were treated with tryptophan for 6 hr at a final concentration of 0.1 mM. The cells were harvested, in groups of three dishes, and EROD activity was determined as described in the text. TCDD was administered at a final concentration of 2 nM for 6 hr in DMSO. The final concentration of DMSO was 0.2%. Values are means ± SD, N = 3.

* Significantly different from the UV-treated H_2O or unoxidized tryptophan-administered group, $P < 0.01$.

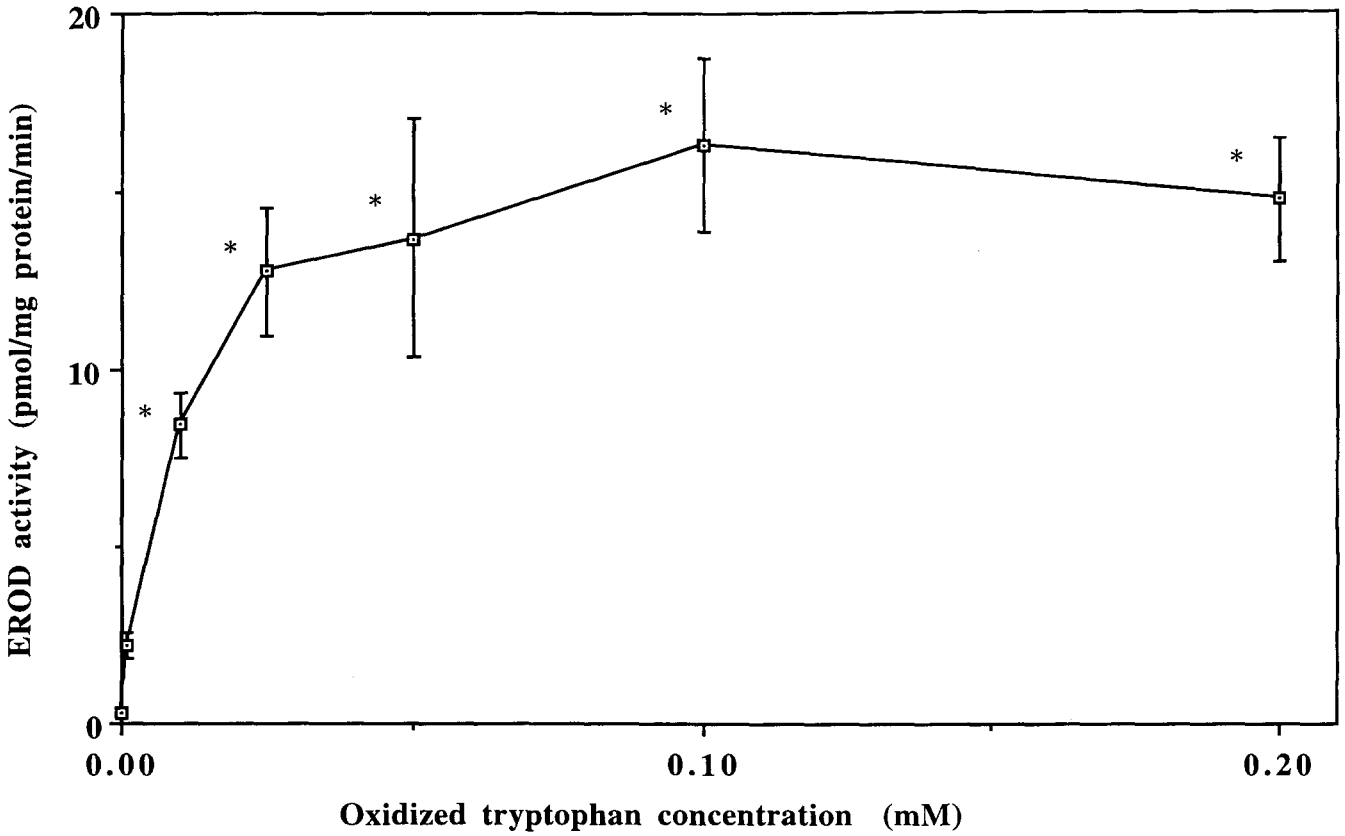

FIG. 3. Effect of oxidized tryptophan concentration on EROD activity. Various concentrations of oxidized tryptophan were administered to Hepa-1 cells in conditioned medium for 6 hr. The cells were harvested, in groups of three dishes, and EROD activity was determined as described in the text. EROD activity is expressed as mean pmol resorufin formed/mg protein/min. Values are means ± SD, N = 3. Key: (*) significantly different from the control group, $P < 0.01$.

When 0.1 mM oxidized tryptophan was administered to Hepa-1 cells, an induction of about 3-fold in EROD activity could be detected as early as 2 hr after the treatment (Fig. 4). The EROD activity at 3, 6, and 12 hr after the administration of 0.1 mM tryptophan was induced by 11-, 38-, and 47-fold, respectively, compared with the controls (Fig. 4). However, at 24 hr, there was a decrease in the induction response, although EROD activity still remained increased about 18-fold compared with the controls ($P < 0.01$). When 1 μM tryptophan was administered, there was no induction of EROD activity up to 3 hr. At 6 and 12 hr, EROD activity was induced by 5.6- and 3-fold, respectively, compared with the controls and returned to the control levels by 24 hr.

Effect of Oxidized Tryptophan on EROD Activity of Various Hepa-1 Mutants

To investigate the participation of the Ah receptor in the induction of EROD activity, wild-type and various mutants of Hepa-1 cells were incubated with oxidized tryptophan at a final concentration of 0.1 mM for 6 hr, and EROD activity was then assayed in the cell extracts as described earlier. The results presented in Table 2 show that in the wild-type cell extracts, oxidized tryptophan caused a significant induction of EROD activity ($P < 0.01$). There was a small amount of EROD activity in B^- mutants to which oxidized tryptophan had been administered (about 5% of that found in the oxidized tryptophan-administered wild type). In the H_2O-administered control B^- mutants, the EROD activity was below the limits of detection. The EROD activity in the control or oxidized tryptophan-administered extracts of the other Hepa-1 mutants, namely C^-, D^-, and dominant, were also below the limits of detection.

Ah Receptor Transformation by Oxidized Tryptophan In Vivo

Gel mobility shift assays were carried out to investigate whether the photooxidized products of tryptophan would induce transformation of the Ah receptor and DNA binding. Hepa-1 cells were incubated with TCDD or oxidized tryptophan, and the induced nuclear extracts were incubated with ^{32}P-labeled double-stranded XRE-containing oligonucleotide and then subjected to non-denaturing PAGE. The results presented in Fig. 5 show that a gel-shifted band (indicated by B) was observed when nuclear

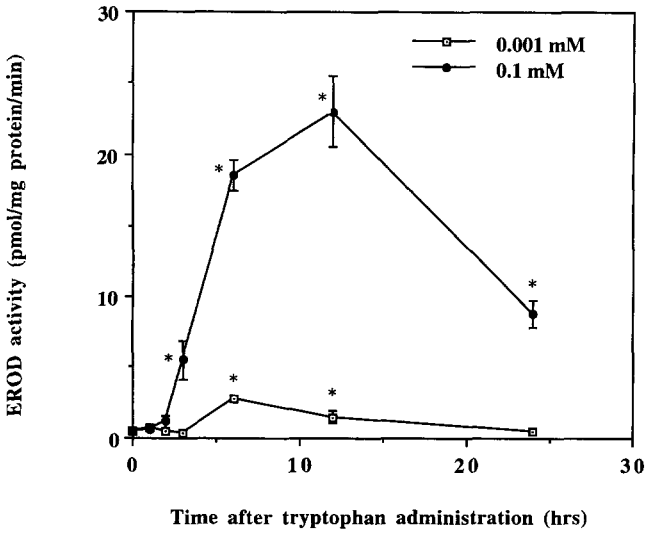

FIG. 4. Time course of EROD induction by oxidized tryptophan. Hepa-1 cells were grown as described in the text. Oxidized tryptophan was administered at a final concentration of 0.1 and 0.001 mM for various periods of time as indicated. The cells were harvested, in groups of three dishes for each group, and EROD activity was assayed as described in the text. EROD activity is expressed as mean pmol resorufin formed/mg protein/min. Values are means ± SD, N = 3. Key: (*) significantly different from the control group, $P < 0.01$.

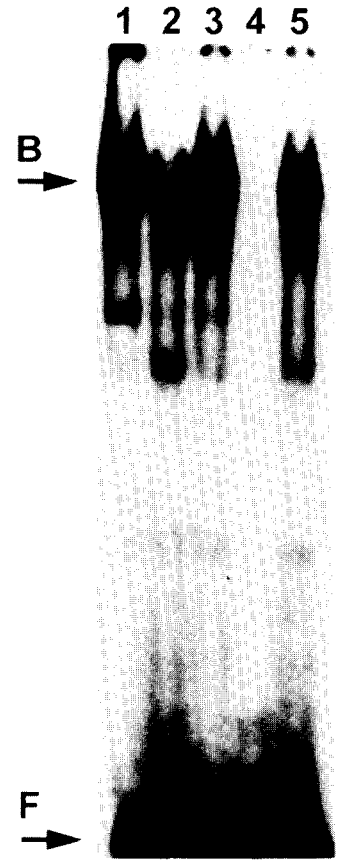

FIG. 5. Gel mobility shift assays using nuclear extracts of wild-type Hepa-1 cells incubated with ^{32}P-labeled XRE-containing double-stranded oligonucleotide. The cells were grown in the presence of 2 nM TCDD for 1 hr (lane 1), UV-treated H_2O (lane 2), or UV-oxidized tryptophan for 3 hr (lanes 3–5). Lanes 1–3 were incubated without competitor oligonucleotide. Lane 4 and 5 were preincubated with 100-fold excess of wild-type and mutant competitor, respectively. The locations of the Ah receptor-dependent DNA-protein complex (B) and the free probe (F) are indicated by arrows.

extracts prepared from Hepa-1 cells grown with TCDD were incubated with radiolabeled 38-bp oligonucleotide and subjected to non-denaturing PAGE (lane 1). The Ah receptor–XRE complex band was of almost equal intensity when Hepa-1 cells were treated with oxidized tryptophan (lane 3), suggesting that oxidized tryptophan was as effective as TCDD in causing the transformation of Ah receptor *in vivo*. However, this band was not detected when a 100-fold excess of unlabeled wild-type oligonucleotide was added to the incubation mixture (lane 4) but was readily detectable when a 100-fold excess of unlabeled mutant oligonucleotide was included in the assay (lane 5). There was a small signal for the Ah receptor-XRE band in the H_2O-administered control cell nuclear extracts (lane 2).

TABLE 2. Effect of oxidized tryptophan on EROD activity of various Hepa-1 mutants

	EROD activity (pmol resorufin formed/mg protein/min)	
Type of strain	Control	Oxidized tryptophan-administered
Wild-type	0.57 ± 0.10	11 ± 0.98*
B^-	<0.09	0.56 ± 0.08
C^-	<0.09	<0.09
D^-	<0.09	<0.09
Dominant	<0.09	<0.09

Hepa-1 and various mutants were grown as described in the text. Oxidized tryptophan was administered at a final concentration of 0.1 mM for 6 hr. The cells were harvested, in groups of three dishes, and EROD activity was determined as described in the text. Values are means ± SD, N = 3.

* Significantly different from the controls, $P < 0.01$.

Effect of Oxidized Tryptophan on mRNA Expression

Results on the CYP1A1 quantification by RT-PCR (Fig. 6) showed that no CYP1A1 cDNA could be detected in H_2O-administered cells (lane 2). However, using the cells that had been incubated with UV-oxidized tryptophan for 3 hr at a final concentration of 0.2 mM, an expected 305 bp-long fragment of CYP1A1 cDNA was readily detected (lane 3). β-Actin-specific cDNA of 338 bp was detected in both the control (lane 4) and UV-oxidized tryptophan-administered cells (lane 5). RT-PCR amplifications were carried out at least twice to verify the reproducibility of results. Similar results have been reported by Kocarek *et al.* [17] who found that mild photoactivation of tryptophan

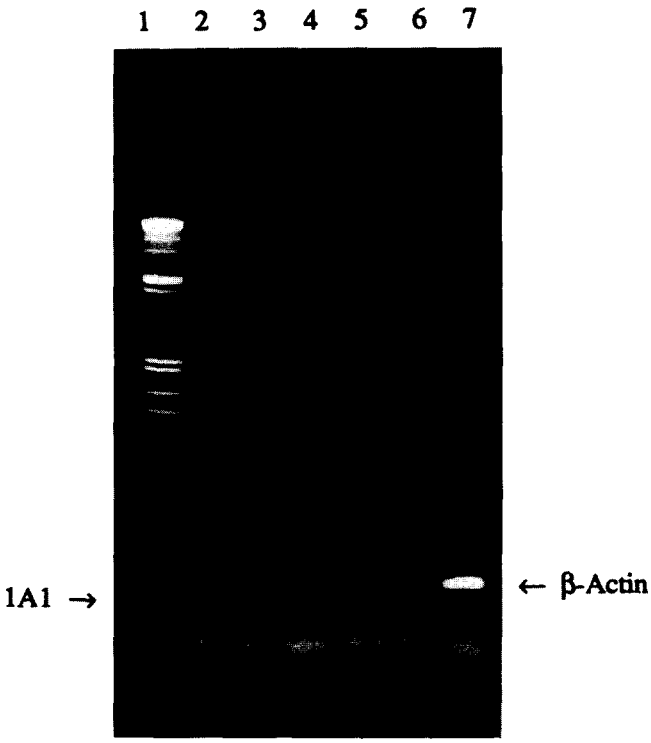

FIG. 6. RT-PCR amplification of CYP1A1 cDNA synthesized from mRNA as described in the text. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Lambda DNA/*Eco*RI + *Hind*III (0.75 μg) was used as the marker in lane 1. Lane 2 had RT-PCR amplified CYP1A1 cDNA using mRNA isolated from H_2O-administered Hepa-1 cells. No CYP1A1 cDNA band was observed. Lane 3 had RT-PCR amplified CYP1A1 cDNA using mRNA isolated from UV-oxidized tryptophan-administered Hepa-1 cells. Lanes 4 and 5 had RT-PCR amplified β-actin using mRNA from H_2O- and UV-oxidized tryptophan-administered Hepa-1 cells, respectively. Lane 6 was a negative control (nuclease-free H_2O was used instead of mRNA for RT-PCR). Lane 7 was a positive control where 1 pg of β-actin cDNA was used for amplification.

resulted in a substantially increased magnitude of CYP1A1 mRNA induction in primary cultures of adult rat hepatocytes.

Effect of Oxidized Tryptophan on the CYP1A1 Protein

Hepa-1 cells were grown as described in the text and used at >95% confluence. The conditioned medium was taken out and replaced with fresh medium and the test materials were administered for a total of 6 hr. Immunoblot analysis of the cell extracts (20 μg protein in each lane) with monoclonal anti-CYP1A1/1A2 presented in Fig. 7 show the presence of a CYP1A1 band in the control (lanes 1 and 2) and unoxidized tryptophan-administered group (lanes 3 and 4). The group to which oxidized tryptophan was administered (lanes 5–7) showed a marked induction of CYP1A1 protein. This induction of CYP1A1 protein was slightly higher than the group to which TCDD had been administered

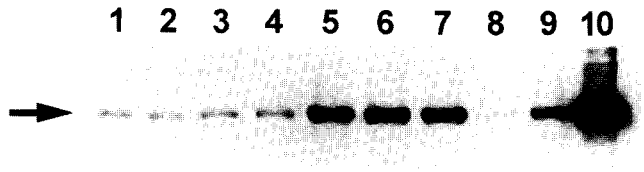

FIG. 7. Immunoblot analysis of CYP1A1 protein. Hepa-1 cell sonicates were subjected to SDS–PAGE followed by immunoblot analysis using monoclonal anti-CYP1A1 as described in the text. The CYP1A1 band is indicated by an arrow. Lanes 1 and 2 had 20 μg protein from Hepa-1 cell sonicates to which UV-treated H_2O had been administered for 6 hr in fresh medium; lanes 3 and 4 had 20 μg protein from Hepa-1 cell sonicates to which unoxidized tryptophan had been administered for 6 hr in fresh medium; lanes 5–7 had 20 μg protein from oxidized tryptophan-administered cell sonicates; lane 8 had 20 μg protein of cell sonicates to which UV-treated H_2O was added in conditioned medium; lane 9 had 20 μg protein from cells administered 2 nM TCDD; and lane 10 had 4 μg protein from 3-methylcholanthrene-induced mouse liver microsomes.

(lane 9). Lane 8 had 20 μg of protein to which UV-treated H_2O was added in the conditioned medium. Slight induction of CYP1A1 protein in the control group to which fresh medium had been added (lanes 1 and 2) is probably caused by exposure of medium to room light.

Effect of Oxidized Tryptophan on Rat Pulmonary and Hepatic Microsomal EROD Activity

After i.p. injection of adult male Sprague–Dawley rats with isolated tryptophan photoproducts suspended in corn oil, pulmonary microsomal EROD activity was increased 3.1- and 2.7-fold compared with the control ($P < 0.01$) and vehicle control groups ($P < 0.01$), respectively (Table 3). Hepatic microsomal EROD activity was induced 3.2- and 2.3-fold compared with control ($P < 0.01$) and vehicle control groups ($P < 0.01$), respectively.

DISCUSSION

The results obtained in the present investigation show that of all the amino acids tested, only oxidized tryptophan caused a marked induction of EROD activity and CYP1A1

TABLE 3. Effect of oxidized tryptophan on rat pulmonary and hepatic microsomal EROD activity

	EROD activity (pmol resorufin formed/mg protein/min)	
Group	**Lung**	**Liver**
Control	2.4 ± 0.69	2.6 ± 0.77
Vehicle control	2.8 ± 1.1	3.6 ± 1.4
Oxidized tryptophan	7.5 ± 1.9*	8.3 ± 1.2*

Values are means ± SD, N = 3 for each group.
* Significantly different from the control or the vehicle control group, $P < 0.01$.

protein in wild-type Hepa-1 cells. Our results are consistent with the report of Kocarek *et al.* [17], who demonstrated that photooxidized tryptophan but not histidine was responsible for the induction of CYP1A1 mRNA in primary cultures of adult rat hepatocytes. The results also show that the induction of CYP1A1 activity by oxidized tryptophan was dose-dependent and transient in nature. Kocarek *et al.* [17] reported that the induction of CYP1A1 mRNA by photooxidized tryptophan in primary cultures of adult rat hepatocytes was also transient in nature. They demonstrated that inactivation of the inducer (oxidized tryptophan) rather than down-regulation of the Ah receptor system is responsible for this transient nature of the CYP1A1 mRNA induction. The results obtained in the present investigation also indicate that oxidized tryptophan is being inactivated, suggesting that is being metabolized by CYP1A1 present in the Hepa-1 cells.

The unoccupied Ah receptor, found in the cytosol after conventional subcellular fractionation, is about 280 kDa in size and contains two molecules of the 90 kDa ligand-binding Ah receptor monomer [reviewed in Ref. 27]. Recently, Ah receptor has been suggested to play an important role in the expression of dioxin-inducible genes and in the normal development of the liver and the immune system [28]. Probst *et al.* [29] have shown that binding of the 9-S Ah receptor complex with TCDD results in the dissociation of 90 kDa heat shock protein and possibly other proteins and release of the Ah receptor monomer. The free Ah receptor monomer then heterodimerizes with the ARNT protein. In the nucleus, both ARNT and the Ah receptor bind directly to the core sequences of XRE located 5′ to the coding sequence of the *CYP1A1* gene, thereby activating the transcription of the *CYP1A1* gene. Helferich and Denison [15] previously showed that oxidized tryptophan can bind the Ah receptor, activate the Ah receptor to an XRE binding form *in vitro*, and activate transcription from an XRE-driven reporter gene in transfected Hepa-1 cells. Furthermore, transcriptional activation was shown not to occur in Hepa-1 mutants of the C class [15]. We have confirmed and extended the results of Helferich and Denison [15], demonstrating lack of induction of CYP1A1 by oxidized tryptophan in B^- and D^- mutants as well. The Ah receptor is present at much reduced levels in the B^- mutants, but is nevertheless able to translocate to the nucleus upon ligand binding [30]. C^- mutants are defective in the ARNT protein and are totally defective in nuclear translocation of the Ah receptor monomer [31]. The D^- mutant has a reduced level of Ah receptor that is diminished in its ability to translocate to the nucleus [32]. Hybrids between dominant mutants and the wild-type lack inducible CYP1A1 mRNA as well as AHH activity, and available evidence indicates that the dominant mutants synthesize a repressor protein of CYP1A1 transcription [24]. The lack of induction of EROD activity observed in the present investigation by oxidized tryptophan in C^-, D^-, and dominant mutants and the slight induction in the B^- mutant are consistent with the involvement of the Ah receptor.

The results of the gel mobility shift assays presented in the present study show that photooxidized tryptophan is as effective as TCDD in generating XRE binding in the nuclear extracts of Hepa-1 cells administered oxidized tryptophan. Helferich and Denison [15] have shown that when guinea pig hepatic cytosols are treated with UV-oxidized tryptophan *in vitro*, it induces the transformation of cytosolic Ah receptor and DNA binding. However, no attempt was made by these authors to investigate whether oxidized tryptophan would cause the transformation of the Ah receptor in the living cell. In the present investigation, the nuclei were isolated after the administration of oxidized tryptophan to Hepa-1 cells and the nuclear proteins were extracted and used for gel shift assays. Our results, therefore, suggest that oxidized tryptophan initiates the activation of Ah receptor, resulting in the binding of the ARNT protein and translocation of the liganded Ah receptor complex into the nucleus followed by binding of the activated Ah receptor directly to the core sequences of XRE of *CYP1A1* gene, thereby activating its transcription. The induction of CYP1A1 by traditional planar molecules such as polycyclic aromatic hydrocarbons and TCDD occurs exclusively through the interaction of these compounds with the Ah receptor. We are interested in identifying the structure(s) of the oxidized tryptophan product(s) which may fit in the planar model as the Ah receptor ligand.

It should be pointed out that the lower magnitude of EROD induction in rats after injection of isolated oxidized tryptophan products as compared with the results of cell culture may primarily be due to the dosage of oxidized tryptophan used since only about 23% of tryptophan was oxidized under the conditions described in the present investigation. Furthermore, we do not know what fraction of the oxidized tryptophan is actually responsible for causing the induction of CYP1A1. However, it is of interest to note that oxidized tryptophan-treated rats demonstrated a similar magnitude of EROD induction in both the lung and liver which is also similar to the pattern of EROD induction seen after hyperoxic exposure [3]. Induction of pulmonary EROD activity by prototypic inducers such as methylcholanthrene is about 10% of that seen in the liver [33].

Tryptophan is an essential amino acid that is normally ingested as a component of dietary protein. It is available as an over-the-counter nutritional supplement and has been used to treat insomnia and premenstrual syndrome. Since it is metabolized to serotonin, it has been prescribed for psychiatric disorders such as obsessive-compulsive behavior and depression [34]. It is commonly taken in doses that range from 1 to 15 g/day, which is considerably higher than its normal dietary requirement of a daily dose of 1 g or less. Dietary intake of high concentrations of tryptophan produces a variety of toxic and biological effects in animals and humans including eosinophilia myalgia syndrome [35, 36]. Although the exact role of photooxidized tryptophan in tumorigenesis remains to be elucidated, a tumor promoting effect of tryptophan and/or some of its derivatives has been observed in several studies [37–39].

In summary, the data presented in this paper demonstrate that UV oxidation of tryptophan produces products that can induce both Ah receptor transformation and binding of the liganded (activated) receptor to its specific DNA recognition site of the *CYP1A1* gene, thereby activating transcription of the CYP1A1 mRNA and expression of the corresponding protein, leading to a concomitant increase of EROD activity in Hepa-1 cells. Further, the results of *in vivo* experiments with oxidized tryptophan-treated rats showing increased EROD activity of the liver and lungs suggest that a similar mechanism is most probably also operative in the induction of CYP1A1 *in vivo*.

This work was supported by Grant HL-50450 from the National Heart, Lung and Blood Institute of the National Institutes of Health, and Grant CA 28868 from the National Cancer Institute, U.S. Public Health Service. We thank Dr. S. S. Park for his gift of the monoclonal CYP1A1/1A2 antibody, Vahag Vartanian and Artin Gevorkian for their help in some of the experiments, and Dr. M. Mitsuhashi for the gift of β-actin cDNA and helpful suggestions for RT-PCR.

References

1. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyerelsen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Funsalus IC and Nebert DW, The P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6:** 1–42, 1996.
2. Bhattacharyya KK, Brake PB, Eltom SE, Otto SA and Jefcoate CR, Identification of a rate adrenal cytochrome P450 active in polycyclic hydrocarbon metabolism as rat CYP1B1. Demonstration of a unique tissue-specific pattern of hormonal and aryl hydrocarbon receptor-linked regulation. *J Biol Chem* **270:** 11595–11602, 1995.
3. Okamoto T, Mitsuhashi M, Fujita I, Sindhu RK and Kikkawa Y, Induction of cytochrome P450 1A1 and 1A2 by hperoxia. *Biochem Biophys Res Commun* **197:** 878–885, 1993.
4. Whitlock JP, Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu Rev Pharmacol Toxicol* **30:** 251–277, 1990.
5. Nebert DW and Gonzalez FJ, P450 genes: Structure, evolution, and regulation. *Annu Rev Biochem* **56:** 945–993, 1987.
6. Levine RL, Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *J Biol Chem* **258:** 11823–11827, 1983.
7. Paine AJ and Francis JE, The induction of benzo[*a*]pyrine-3-mono-oxygenase by singlet oxygen in liver cell culture is mediated by oxidation products of histidine. *Chem Biol Interact* **30:** 343–353, 1980.
8. Paine AJ, Induction of benzo[*a*]pyrine mono-oxygenase in liver cell culture by the photochemical generation of active oxygen species: Evidence for the involvement of singlet oxygen and the formation of a stable inducing intermediate. *Biochem J* **158:** 109–117, 1976.
9. Paine AJ, Comparison of the photochemical and enzymic generation of excited states of oxygen on the induction of benzo[*a*]pyrene mono-oxygenase in liver cell cultures. *Chem Biol Interact* **16:** 309–314, 1977.
10. Pohl RJ and Fouts JR, Xenobiotic metabolism in skin of hairless mice exposed to ultraviolet radiation (UV), Aroclor 1260, or chlordane. *Pharmacologist* **19:** 200, 1977.
11. Goerz G, Merk H, Bolsen K, Tsambaos D and Berger H, Influence of chronic UV-light exposure on hepatic and cutaneous monooxygenases. *Experientia* **39:** 385–386, 1983.
12. Mukhtar H, DelTito BJ, Jr, Matgouranis PM, Das M, Asokan P and Bickers DR, Additive effects of ultraviolet B and crude coal tar on cutaneous carcinogen metabolism: Possible relevance to the tumorigenicity of the Goeckerman regimen. *J Invest Dermatol* **87:** 348–353, 1986.
13. Rannug A, Rannug U, Rosenkranz HS, Winqvist L, Westerholm R, Agurell E and Grafstom AK, Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *J Biol Chem* **262:** 15422–15427, 1987.
14. Rannug A and Rannug U, UV-irradiation of tryptophan gives rise to AHH-inducing compounds with very high affinity for the Ah receptor. *Chemosphere* **18:** 1101–1106, 1989.
15. Helferich WG and Denison MS, Ultraviolet photoproducts of tryptophan can act as dioxin agonists. *Mol Pharmacol* **40:** 674–678, 1991.
16. Nemoto N, Sakurai J, Tazawa A and Ishikawa T, Proline-dependent expression of aryl hydrocarbon hydroxylase in C57BL/6 mouse hepatocytes in primary culture. *Cancer Res* **49:** 5863–5869, 1989.
17. Kocarek TA, Schuetz EG and Guzelian PS, Transient induction of cytochrome P450 1A1 mRNA by culture medium component in primary cultures of adult rat hepatocytes. *In Vitro Cell Dev Biol* **29A:** 62–66, 1993.
18. Prough RA, Burke MD and Mayer RT, Direct fluorometric methods for measuring mixed-function oxidase activity. *Methods Enzymol* **52:** 372–377, 1978.
19. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
20. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354, 1979.
21. Mitsuhashi M, Cooper A, Ogura M, Shinagawa T, Yano K and Hosokawa T, Oligonucleotide probe design—A new approach. *Nature* **367:** 759–761, 1994.
22. Mitsuhashi M and Hosokawa T, Novel computerized method for DNA probes and PCR primers. *Nippon Rinsho* **52:** 530–541, 1994.
23. Saatcioglu F, Perry DJ, Pasco DS and Fagan JB, Aryl hydrocarbon (Ah) receptor DNA-binding activity: Sequence specificity and Zn^{2+} requirement. *J Biol Chem* **265:** 9251–9258, 1990.
24. Watson AJ, Weir-Brown KI, Bannister RM, Chu F-F, Reisz-Porszasz S, Fujii-Kuriyama Y, Sogawa K and Hankinson O, Mechanism of action of a repressor of dioxin-dependent induction of *Cyp1a1* gene transcription. *Mol Cell Biol* **12:** 2115–2123, 1992.
25. Neuhold LA, Shirayoshi Y, Ozato K, Jones JE and Nebert DW, Regulation of mouse *CYP1A1* gene expression by dioxin: Requirement of two *cis*-acting elements during induction. *Mol Cell Biol* **9:** 2378–2386, 1989.
26. Ischiropoulos H, Nadziejko CE, Kumae T and Kikkawa Y, Oxygen tolerance in neonatal rats: Role of subcellular superoxide generation. *Am J Physiol* **257:** L411-L420, 1989.
27. Hankinson O, The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxiol* **35:** 307–340, 1995.
28. Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SST, Kimura S, Nebert DW, Rudikoff S, Ward JM and Gonzalez FJ, Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* **268:** 722–726, 1995.
29. Probst MR, Reisz-Porszasz S, Agbunag RV, Ong MS and Hankinson OH, Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. *Mol Pharmacol* **44:** 511–515, 1993.
30. Hankinson O, A genetic analysis of processes regulating cy-

tochrome P4501A1 expression. *Adv Enzyme Regul* **34:** 159–171, 1994.
31. Legraverend C, Hannah R, Eisen H, Owens O, Nerbert D and Hankinson O, Regulatory gene product of the Ah locus: Characterization of receptor mutants among mouse hepatoma clones. *J Biol Chem* **257:** 6502–6507, 1982.
32. Karenlampi SO, Legraverend C, Gudas JM, Carramanzana N and Hankinson OH, A third genetic locus affecting the Ah (dioxin) receptor. *J Biol Chem* **263:** 10111–10117, 1988.
33. Godden PMM, Kass G, Mayer RT and Burke MD, The effects of cigarette smoke compared to 3-methylcholanthrene and phenobarbitone on alkoxyresorufin metabolism by lung and liver microsomes from rats. *Biochem Pharmacol* **36:** 3393–3398, 1987.
34. Fuller RW, Pharmacologic properties of serotonergic and antidepressant drugs. *J Clin Psychiatry* **48**(Suppl): 5–11, 1987.
35. Centers for Disease Control, Eosinophilia-myalgia syndrome: New Mexico. *Morbidity Mortality Weekly Rep* **38:** 765–767, 1989.
36. Hertzman PA, Blevins WL, Mayer J, Greenfield B, Ting M and Gleich GJ, Association of the eosinophilia-myalgia syndrome with the ingestion of tryptophan. *N Engl J Med* **322:** 869–873, 1990.
37. Sidransky H, Verney E and Wang D, Effects of varying fat content of a high tryptophan diet on the induction of γ-glutamyltranspeptidase positive foci in liver of rats treated with hepatocarcinogen. *Cancer Lett* **31:** 235–242, 1986.
38. Sidransky H, Garrett CT, Murty CN, Verney E and Robinson ES, Influence of dietary tryptophan on the induction of β-glutamyltranspeptidase positive foci in the livers of rats treated with hepatocarcinogen. *Cancer Res* **45:** 4844–4847, 1985.
39. Cohen SM, Arai M, Jacobs JB and Friedell GH, Promoting effect of saccharin and DL-tryptophan in urinary bladder carcinogenesis. *Cancer Res* **39:** 1207–1212, 1979.