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Ultraviolet Photoproducts of Tryptophan Can Act as Dioxin Agonists

WILLIAM G. HELFERICH and MICHAEL S. DENISON

Department of Food Science and Human Nutrition (W.G.H.) and Department of Biochemistry (M.S.D.), Michigan State University, East Lansing, Michigan 48824-1224

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SUMMARY

Tryptophan (TRP) has been implicated in the mechanism of induction of microsomal aryl hydrocarbon hydroxylase (AHH) activity by UV light. UV irradiation of TRP resulted in formation of chloroform-soluble photoproducts that can specifically bind to the Ah receptor (AhR), an intracellular protein that mediates the induction of AHH activity by xenobiotics. Gel retardation analysis revealed that the TRP photoproducts, like other AHH inducers, can induce both AhR transformation and binding of the ligand-AhR complex to its specific DNA recognition site. Transient transfection experiments also demonstrated that the photoproducts could induce gene expression in an AhR-dependent manner. Thus, induction of AHH activity by UV light could be attributed, at least in part, to the formation of TRP photoproducts, which appear to act as AhR ligands.

Dietary intake of high concentrations of TRP produces a variety of toxic and biological endpoints in animals and humans. Increases in the number of histochemically altered hepatic foci, as well as aromatic amine-induced urinary bladder tumors, have been reported in animals on high TRP-containing diets, suggesting that TRP and/or some of its derivatives may act as tumor promoters (1–3). More recently, dietary intake of high levels of TRP-containing supplements in humans has been associated with an outbreak of eosinophilia-myalgia syndrome (4). The exact role of TRP in these responses and whether the toxicity associated with TRP is due to the parent compound itself, a TRP metabolite, or a contaminant present in the TRP preparation is currently unknown.

TRP also appears to be involved in the mechanism of induction of microsomal AHH activity by UV light (5–7). The addition of UV-irradiated tissue culture media to rat hepatoma (H4IIe) cells resulted in the specific induction of AHH activity, whereas UV-irradiated media lacking TRP and histidine failed to induce AHH, suggesting a role for these amino acids in the induction response (5–7). Increases in AHH activity in skin and liver of UV-irradiated rats and mice have also been reported (8–10) and suggest that a similar event(s) might occur *in vivo*, although this remains to be determined.

Induction of AHH, a microsomal monooxygenase activity that is primarily associated with a specific member of the cytochrome P450 superfamily, P450IA1 (11, 12), is mediated by a soluble receptor protein, the AhR, to which the inducing chemical binds with high affinity (13, 14). After ligand binding, the AhR undergoes transformation to its DNA-binding form (15–17), with the subsequent accumulation of liganded-AhR complexes within the nucleus (13, 15, 17). The binding of these nuclear AhR complexes to specific DNA transcriptional enhancer sequences (DREs) present in the 5′-flanking region of the *CYPIA1* gene induces transcription (13, 17–19). The most widely studied AhR ligands (inducers of cytochrome P450IA1) include a variety of HAHs, such as polychlorinated and polybrominated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (20, 21). Exposure to TCDD (dioxin), the prototypical and most potent inducer of cytochrome P450IA1, results in a wide variety of species- and tissue-specific toxic and biological effects, including tumor promotion, thymic involution, immune suppression, teratogenesis, hepatoxicity, and induction of numerous enzymes (20, 21). Structure-activity relationship studies using a series of TCDD and HAH congeners have implied that the AhR, in addition to mediating the induction of cytochrome P450IA1, is involved in mediating many, if not all, of the other biological and toxic effects of these compounds (20, 21).

A recent study by Rannug *et al.* (22) indicated that UV irradiation of TRP and histidine produced at least two distinct photooxidation products that could specifically bind to the AhR. One of these TRP photoproducts appears to induce AHH

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ABBREVIATIONS: TRP, tryptophan; AHH, aryl hydrocarbon hydroxylase; AhR, aromatic hydrocarbon receptor; DMSO, dimethyl sulfoxide; DRE, dioxin-responsive element; HAH, halogenated aromatic hydrocarbon; HPLC, high performance liquid chromatography; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; TLC, thin layer chromatography; PAP, placental alkaline phosphatase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

activity in rat hepatoma (H4IIe) cells (23). In the present study, we have prepared and examined partially purified TRP photooxidation products and demonstrate, using ligand binding, DNA binding, and transient expression assays, that these products interact with the AhR and act as TCDD agonists.

Materials and Methods

Chemicals. [^{3}H]TCDD (37 Ci/mmol), unlabeled TCDD, and TCDBF were obtained from Dr. S. Safe (Texas A&M University). These compounds are extremely toxic substances and were handled as described by Poland *et al.* (14). L-Tryptophan was purchased from Sigma Chemical Co. (St. Louis, MO); solvents were of chromatographic grade or better and were purchased from Mallinckrodt (Paris, KY).

Production and purification of photoproducts. Photoproducts were produced by exposure of a 10 mg/ml aqueous solution of TRP to an unfiltered high pressure mercury lamp (BLAK-RAY, San Gabriel, CA), at a distance of ~15 cm, for 4 hr. The resultant solution was used directly or was extracted with chloroform and dried under nitrogen. Gravimetric determination indicated that the rate of conversion of TRP to UV TRP photoproducts was approximately 0.3%. Dried chloroform extracts were resuspended in chloroform/hexane (1:1), spotted onto a silica gel G-25 TLC plate (Alltech, Deerfield, IL) that had been prewashed with chloroform, and separated using butanol/water/acetic acid (4:1:1) as the tank solvent. Fluorescent spots were scraped from the plate and eluted with chloroform. Eluted samples were further purified by HPLC using a C_{18} column, methanol/water (80:20) as the mobile phase, and dual-wavelength UV detection at 254 and 280 nm. The major peak was collected for further analysis.

Animals and cytosol preparation. Male Hartley guinea pigs (250–300 g) were obtained from the Michigan State Department of Health (Lansing, MI). Animals were exposed to 12 hr of light and 12 hr of dark and were allowed free access to food and water. Hepatic cytosol was prepared in ice-cold HEDG buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10%, v/v, glycerol), as previously described (24), and aliquots were stored at −80° until use. Protein concentrations were measured by the method of Bradford (25), using bovine serum albumin as the standard.

AhR binding assay. Specific binding of [^{3}H]TCDD to guinea pig hepatic cytosol was measured using the hydroxylapatite binding assay, as previously described (26). Aliquots (0.5 ml) of cytosol (2 mg/ml) were incubated with 2 nM [^{3}H]TCDD, in the absence or presence of the competitor TCDBF (200 nM), for 2 hr at 20°. After incubation, 0.2-ml aliquots of the incubation mixture were added to tubes containing hydroxylapatite (approximately 125 mg of hydroxylapatite in 250 μl of HEDG buffer). After a 30-min incubation period, hydroxylapatite pellets were washed three times with 1-ml aliquots of HEDG buffer containing 0.05% Tween 80. After the last wash, the pellet was resuspended in 1 ml of scintillation cocktail and the mixture was transferred into a scintillation vial containing 4 ml of scintillation cocktail. The incubation tube and pipette were rinsed with 1 ml of ethanol, and this rinse was added to the scintillation vial. Radioactivity was quantitated in an LKB Rackbeta scintillation counter, with sample quench corrected by external standardization. Specific binding of the [^{3}H]TCDD was computed by subtracting the amount of [^{3}H]TCDD bound in the absence of competitor. Competitors were added to the incubation in 5 μl of DMSO. The total cytosolic binding of [^{3}H]TCDD in the presence and absence of the various competitors was determined, and the level of competition was expressed as the percentage of displacement of [^{3}H]TCDD specific binding.

Synthetic oligonucleotides. A complementary pair of synthetic DNA oligonucleotides containing the sequences 5′-GATCTGGCTCT-TCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAA-GAGCCA-3′ [corresponding to the 21-base pair AhR binding site of DRE3 (18, 27) and designated here as the "DRE oligonucleotide"] were synthesized, purified, annealed, and radiolabeled with [^{32}P]ATP, as described previously (8). Annealing generates a double-stranded DNA fragment with a *Bam*HI cohesive end at its 3′ terminus and a *Bgl*II cohesive end at its 5′ terminus.

Gel retardation assay. TCDD-, DMSO-, TRP-, or TRP photoproduct-treated guinea pig hepatic cytosol (16 mg of protein/ml) was incubated with DMSO (20 μl/ml), TCDD (20 nM), TRP, or TRP photoproduct, in DMSO, for 2 hr at 20°, followed by gel retardation analysis as previously described (16, 20). Cytosol (80 μg of protein) was then incubated in binding buffer [25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10%, v/v, glycerol, 225 ng of poly(dI·dC), 80 mM KCl] for 15 min at 20°, followed by the addition of ^{32}P-DRE oligonucleotide (100,000 cpm, 0.5 ng). After an additional 15-min incubation at 20°, samples were loaded onto a 4% nondenaturing polyacrylamide gel, and protein-DNA complexes were resolved by autoradiography of the dried gels.

Cell culture, transfection, and PAP expression assays. Wild-type and variant (BPRc1) mouse hepatoma (hepa1c1c7) cells were transfected with 30 μg of either of the expression vectors pMpap1.1 or pSV_2Apap, using polybrene, as previously described (8). Transfected cells were allowed to grow for 48 hr, followed by treatment of pMpap1.1-transfected cells with DMSO, TCDD (1 nM in DMSO), or the UV TRP photoproducts. Cells were harvested 24 hr later and, after heating to 65° for 30 min, PAP activity was assayed as described by Henthorn *et al.* (29).

Results and Discussion

A previous study (22) demonstrated that UV photoproducts of TRP can bind with high affinity to the AhR and, although it was implied that these compounds act as Ah agonists, no induction studies were carried out. In a subsequent study, Rannug and Rannug (23) indicated that exposure of rat hepatoma cells to one of the UV TRP photoproducts for 3 days resulted in increased AHH activity. In this study, we have not only analyzed the ability of our UV TRP photoproducts to bind to the AhR and induce AhR transformation, but we have also examined the AhR-dependent nature of induction of gene expression by these compounds.

The TRP photoproducts, produced by UV exposure of an aqueous solution of TRP, were analyzed for their ability to bind to guinea pig hepatic cytosolic AhR (Table 1). Aqueous TRP and a chloroform extract of aqueous TRP decreased [^{3}H]TCDD specific binding slightly, but not significantly. In contrast, the addition of aqueous or chloroform-extracted TRP samples that had been UV-irradiated significantly decreased [^{3}H]TCDD specific binding (Table 1), indicating the presence of a UV photoproduct(s) that can bind to the AhR. Preliminary purification and characterization of the chloroform extracts separated by TLC revealed that the silica gel matrix of many commercial TLC plates contains a potent chloroform-extractable "factor(s)" that not only could bind to the AhR but also could cause AhR transformation and DNA binding (data not shown). This factor could be eliminated from the TLC plates by prerunning the plate twice in chloroform. Subsequent analysis and separations utilized these "clean" plates.

Separation of the chloroform-extracted UV TRP material by TLC in butanol/water/acetic acid (4:1:1) revealed at least six distinct UV-fluorescent spots, with R_F values ranging from 0.68 to 0.96 (Table 2). Each spot was scraped from the silica gel plate, eluted with chloroform, and further purified by reverse phase HPLC, and the major peak was collected and assayed for AhR binding. TLC spots with R_F values of 0.68 and 0.80 were not readily detected by HPLC and thus were not used for further analysis. Although all of the other HPLC-purified sam-

TABLE 1

Competitive displacement of [^{3}H]TCDD specific binding by TRP and UV-irradiated TRP photoproducts

TRP and TRP samples were prepared as described below and added to the binding incubation simultaneously with [^{3}H]TCDD. The TRP-AQ competitor represents the addition of 5 μl of aqueous TRP solution (1 g/100 ml) to the binding incubation. TRP extract (TRP-EXT) competitor represents aqueous TRP that had been extracted three times with chloroform (25 ml); the extract was dried over anhydrous sodium sulfate and evaporated to dryness. The dried crude extract was resuspended in 10 ml of DMSO, and 5 μl were added to the binding assay. TRP-UV competitor represents the addition of 5 μl of aqueous TRP solution that had been UV irradiated for 4 hr. The TRP-UV-EXT competitor was prepared as described for the TRP-EXT sample, except that before extraction the aqueous TRP sample was UV irradiated for 4 hr.

Competitor	Treatment		[^{3}H]TCDD specific binding	Displacement[a]
	UV irradiation	$CHCl_3$ extraction		
			fmol/mg of protein	%
None	−	−	73.9 ± 11.4[b]	0.0
TRP-AQ	−	−	66.5 ± 6.0	10.0
TRP-EXT	−	+	60.4 ± 14.3	18.2
TRP-UV	+	−	18.5 ± 9.2[c]	74.9[c]
TRP-UV-EXT	+	+	11.4 ± 10.2[c]	115.4[c]

[a] Displacement of [^{3}H]TCDD from guinea pig hepatic AhR, as measured by the hydroxylapatite binding assay. Binding is expressed as a percentage of the total [^{3}H]TCDD specific binding.

[b] Values are expressed as the mean ± standard deviation of duplicates of three separate incubations.

[c] Values are significantly different from control at $p < 0.05$, as determined by the Student t test.

TABLE 2

Characteristics of UV photoproducts of TRP on chloroform-prewashed silica gel TLC plates, using butanol/water/acetic acid (4:1:1) as the mobile phase

Spot	R_F	Color
1	0.68	Blue
2	0.80	Faint pink
3	0.86	Blue
4	0.88	Blue violet
5	0.91	Yellow/green
6	0.96	Yellow/green

ple spots displaced [^{3}H]TCDD specific binding to varying degrees (Table 3), only that corresponding to the R_f 0.96 sample completely eliminated [^{3}H]TCDD specific binding (Table 3). This sample was further characterized by gel retardation and transient transfection experiments.

To determine whether the TRP photoproducts would, in addition to binding to the AhR, induce AhR transformation and DNA binding, we utilized a sensitive gel retardation assay (17, 30). We have previously demonstrated that TCDD-AhR complexes, transformed *in vitro*, can bind with high affinity to an oligonucleotide that contains a DRE consensus sequence (16). The ability of aqueous TRP and UV TRP photoproducts to induce protein-DRE complex formation is shown in Fig. 1. Aliquots of UV-irradiated TRP and chloroform-extracted UV-irradiated TRP samples, but not aqueous TRP, induced formation of a protein-DNA complex that migrated to a position comparable to that induced by TCDD. Additionally, samples of TLC spots 3 to 6 induced formation of a similar complex, although spots 5 and 6 (R_f 0.91 and 0.96, respectively) were the most effective (Fig. 2). Previous studies indicated that formation of the TCDD-inducible protein-DNA complex not only was AhR dependent but also demonstrated the presence of the AhR in the inducible protein-DNA complex (18). Thus, the

TABLE 3

Competitive displacement of [^{3}H]TCDD specific binding by partially purified, chloroform-extracted TRP UV photoproducts

UV TRP photoproducts were generated and extracted as described for Table 1, except that the sample extract was resuspended in 1 ml of chloroform/hexane (1:1) instead of DMSO. Aliquots (200 μl) were spotted on prewashed silica gel plates, and samples were resolved in TLC solvent. The spots of interest were scraped from the plates, eluted with chloroform, evaporated to dryness, and resuspended in 200 μl of methanol/water (80:20) for injection into the HPLC. The major 280-nm absorbance peak was detected at approximately 3.9 min (with a flow rate of 1 ml/min) and was collected and dried under vacuum. The resulting sample was resuspended in 100 μl of DMSO, and 5 μl were used in the binding assay.

Competitor	Treatment			[^{3}H]TCDD specific binding	Displacement[a]
	UV irradiation	$CHCl_3$ extraction	TLC/HPLC		
				fmol/mg of protein	%
None	−	−	−	73.9 ± 11.4[b]	0.0
TRP R_F 0.86	+	+	+	25.3 ± 22.2[c]	66.0[c]
TRP R_F 0.88	+	+	+	17.2 ± 15.8[c]	76.6[c]
TRP R_F 0.91	+	+	+	15.2 ± 15.2[c]	79.5[c]
TRP R_F 0.96	+	+	+	6.0 ± 7.4[c]	91.9[c]
TLC plate blank[d]	−	+	+	58.7 ± 6.3	20.6

[a] Displacement of [^{3}H]TCDD from guinea pig hepatic AhR, as measured by the hydroxylapatite binding assay. Binding is expressed as a percentage of the total [^{3}H]TCDD specific binding.

[b] Values are expressed as the mean ± standard deviation of duplicates of three separate incubations.

[c] Values are significantly different from control at $p < 0.05$, as determined by the Student *t* test.

[d] Chloroform extracts of a TLC plate were added as a negative control, because some commercial TLC plates contain a factor that acts as an Ah agonist (see text for details).

results described above suggest that the TRP photoproducts, in addition to specifically binding to the AhR, can also induce AhR transformation and DRE binding. Similar results were obtained with UV histidine photoproducts (data not shown). Although, the exact quantity of the specific AhR-binding UV TRP product in the partially purified samples is unknown, a conservative estimate, based on gravimetric measurements and preliminary mass spectral analysis, indicates that the concentration of the products in the incubation mixture is <25 nM. Preliminary mass spectral analysis indicates that our partially purified TRP product (R_F 0.96) contains at least two products, both of which have a mass ion size greater than those previously reported by Rannug *et al.* (22), indicating that our products may be different.

To determine whether these UV TRP photoproducts can also induce gene expression, we carried out transient transfection experiments using the recombinant expression vector pMpap1.1. This vector contains a heat-stable PAP gene and mouse mammary tumor virus promoter under TCDD-inducible control of four DREs.[1] Transfection of this vector into wild-type mouse hepatoma cells and measurement of PAP activity in extracts from TCDD-, aqueous TRP-, or UV TRP photoproduct-treated or untreated cells are shown in Table 4. All treatments, with the exception of aqueous TRP, induced PAP activity relative to control (DMSO-treated).

To demonstrate that the inducing action of the UV TRP photoproducts is AhR dependent, we transfected pMpap1.1 into AhR-defective class II variant mouse hepatoma cells. In these cells, TCDD-AhR complexes form normally, but they bind weakly to the DNA and thus fail to induce gene expression

[1] M. S. Denison and M. El-Fouly, unpublished observations.

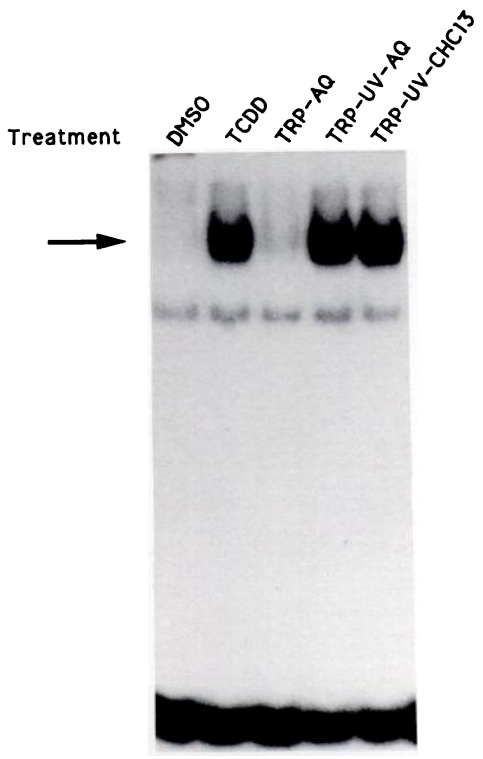

Fig. 1. Protein-DNA complexes induced by TCDD, TRP, and TRP UV photoproducts. Guinea pig hepatic cytosol (16 mg/ml) was incubated for 2 hr at 20° with DMSO, 20 nM TCDD, aqueous TRP (*TRP-AQ*), an aqueous UV-irradiated TRP solution (*TRP-UV-AQ*), or a chloroform extract of UV TRP solution (*TRP-UV-CHCl*$_3$). TRP samples were prepared as described for Table 1. Aliquots (5 μl) were analyzed for DNA binding by gel retardation analysis, as described in Materials and Methods. *Arrow*, position of the inducible protein-DNA complex.

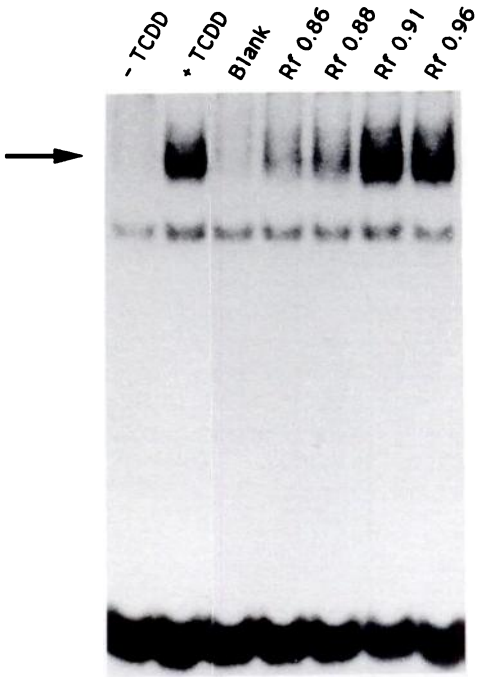

Fig. 2. Protein-DNA complexes induced by TCDD and partially purified chloroform extracts of TRP UV photoproducts. Guinea pig hepatic cytosol (16 mg/ml) was incubated for 2 hr at 20° with DMSO (*–TCDD*), 20 nM (*+TCDD*), control TLC plate sample (*Blank*), or TLC spots corresponding to R_F 0.86, 0.88, 0.91, or 0.96. TRP samples were prepared as described in Table 3. Aliquots (5 μl) were analyzed for DNA binding by gel retardation analysis, as described in Materials and Methods. *Arrow*, position of the inducible protein-DNA complex.

in response to TCDD (13). As expected, neither TCDD nor the TRP photoproducts induced PAP in these variant cells, indicating that PAP induction by these compounds was AhR dependent. The similarity in PAP activity in wild-type and variant cells transfected with the constitutive expression vector pSV$_2$Apap (29) indicated that the results described above are not simply due to differences in transfectability between these cells. Additionally, TCDD treatment of pSV$_2$Apap-transfected cells has no effect on the level of PAP enzyme activity (data not shown). Thus, these results demonstrate that UV TRP photoproducts can induce gene expression in an AhR-dependent manner, providing a plausible mechanism to explain, at least in part, the earlier observations of UV-inducible AHH activity in cells in culture (5–7).

The structural identity of the specific TRP products, in our partially purified preparation, that act as TCDD agonists is currently unknown. Because most of the AhR ligands identified to date are planar cyclic compounds (20, 21), we envision that the UV TR photoproduct(s) are similar in structure and are possibly produced by UV irradiation fusion of one or more molecules or fragments of TRP. Previous studies (31, 32) have indicated that various indole compounds can bind to the AhR and, because the structure of TRP is similar to these compounds, it is highly possible that our TRP photoproduct(s) may be structurally related.

The toxic and carcinogenic potential of these UV TRP photoproducts is currently not known. It is possible that these photoproducts can produce a myriad of toxic effects associated with TCDD and related HAHs. However, we think that this is probably not the case, because these toxic effects appear to be produced only by specific HAHs and not by their nonhalogenated derivatives (20, 21). More likely, these compounds behave similarly to numerous other polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene, dibenzanthracene, and 3-methylcholanthrene, which induce AHH activity via the AhR (20, 21) but do not produce the spectrum of HAH-inducible toxic responses. Additionally, although many of the latter compounds can be readily metabolized and transformed into active carcinogenic

TABLE 4

Expression of PAP activity in wild-type and variant mouse hepatoma cells exposed to TCDD, TRP, and TRP UV photoproducts

TRP and UV TRP products were prepared as described for Tables 1 and 3 and were added to the cultures as indicated in Materials and Methods.

Vector	Treatment	PAP activity[a]	
		Wild-type cells	Variant cells[b]
		units/ml	
pSV2Apap	DMSO	7582 ± 2[c]	8298 ± 442
pMpap1.1	DMSO	2960 ± 505	180 ± 118
	TCDD	9800 ± 50[d]	195 ± 190
	TRP-AQ	2057 ± 55	315 ± 12
	UV-TRP-EXT	8057 ± 865[d]	415 ± 72[d]
	TLC R_F 0.96	7510 ± 305[d]	595 ± 125[d]
	TLC plate blank	2685 ± 415	472 ± 45[d]

[a] One unit is defined as the amount of enzyme necessary to hydrolyze 1 nmol of substrate/min. Assays within each cell type contained similar numbers of cells.
[b] Mouse hepatoma variant (BPrc1) cells contain a defective AhR that fails to bind to DNA and activate gene expression (13).
[c] Values are expressed as the mean ± standard deviation of duplicates of three separate incubations.
[d] Values are significantly different ($p < 0.02$) from pMpap1.1 control (DMSO-treated) cells, as determined by the Student *t* test.

forms (33), the carcinogenic potential of these UV TRP photoproducts is unknown.

Thus, in conclusion, our results support a role for AhR-dependent induction of AHH activity by UV TRP photoproducts. Because UV irradiation of animals has been observed to induce AHH activity in skin and liver of rats and mice (8), our results suggest an explanation of such phenomena, i.e., that the UV irradiation of animals produces some UV photoproducts *in vivo* that can induce AHH activity directly in the irradiated skin cells and the products can apparently be transported to the liver, where they induce hepatic AHH. Because AHH activity has been associated with the metabolic activation of numerous secondary carcinogens, such as benzo[*a*]pyrene, the induction of this enzyme by UV light could result in some potentially serious effects, such as the enhancement of DNA-adduct formation. The exact role of these UV photoproducts in skin tumorigenesis, however, remains to be determined.

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Send reprint requests to: W. G. Helferich, Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224.