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Activation of the Ah Receptor by Extracts of Dietary Herbal Supplements, Vegetables, and Fruits

ANOEK JEUKEN,† BART J. G. KESER,† ELAINE KHAN,§ ABRAHAM BROUWER,# JAN KOEMAN,† AND MICHAEL S. DENISON*,§

Department of Environmental Toxicology, University of California, Davis, California 95616-8588; Department of Toxicology, Agriculture University of Wageningen, The Netherlands; and Department of Toxicology and Ecology, Free University of Amsterdam, Amsterdam, The Netherlands

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that can be activated by a structurally diverse range of synthetic and natural chemicals, and it mediates the toxic and biological effects of environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The spectrum of chemicals that bind to and activate the AhR signal transduction pathway and the identity of materials containing AhR active chemicals is only now being defined. Utilizing AhR-dependent gel retardation and reporter gene bioassays, the screening of extracts of 22 dietary herbal supplements and 21 food products (vegetables and fruits) was performed to identify those containing AhR agonists. Several herbal extracts (ginseng, Fo-Ti, white oak bark, licorice, ginkgo biloba, and black cohosh) stimulated AhR DNA binding and gene expression to levels between 20 and 60% of that produced by TCDD. Although some food extracts (corn, jalapeño pepper, green bell pepper, apple, Brussels sprout, and potato) were relatively potent activators of AhR DNA binding (30−50% of TCDD), only corn and jalapeño pepper extracts induced AhR-dependent luciferase reporter gene expression. However, dilution of corn, jalapeño pepper, bell pepper, and potato extracts dramatically increased their ability to induce luciferase activity, suggesting that these extracts contained AhR antagonists whose effectiveness was overcome by dilution. Overall, these results demonstrate that dietary products can be a major source of naturally occurring AhR ligands to which animals and humans are chronically exposed.

KEYWORDS: Ah receptor; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD; natural ligands; herbs; vegetables; fruits; natural products

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a soluble, inducible ligand-dependent transcription factor that mediates the biological and toxic effects of a structurally diverse range of chemicals (*1*, *2*). Mechanistically, binding of these chemical ligands to the cytosolic AhR complex is suggested to stimulate a conformation change in the AhR, exposing a nuclear localization sequence, which leads to nuclear accumulation of the ligand:AhR complex (*1*, *2*). Once in the nucleus, the AhR dissociates from its associated protein subunits [two heat shock proteins of 90 kDa (hsp90), the hepatitis B virus X-associated protein and the co-chaperone p23], and dimerization with its nuclear protein partner Arnt (Ah receptor nuclear translocator) converts the AhR complex to its DNA-binding form (*3*−*6*). Binding of the ligand:AhR:Arnt complex to its specific DNA recognition site, the dioxin responsive element (DRE), adjacent to a responsive gene results in activation of the promoter and transcription of the gene (*7*). Although numerous AhR-responsive genes have been identified (*7*, *8*), the most responsive genes are those involved in xenobiotic metabolism [e.g., cytochrome P450s (CYP1A1, CYP1A2, and CYP1B1), glutathione-*S*-transferase Ya, UDP-glucuronosyl transferase, and NADPH quinone reductase]. Whereas persistent activation of the AhR signal transduction pathway has been proposed to be responsible for the spectrum of adverse effects produced by metabolically stable AhR ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), activation of the AhR signaling pathway by TCDD at nontoxic concentrations or by less persistent AhR agonists has also been shown to produce some beneficial antitumorigenic/antiestrogenic activities (*9*, *10*).

The best studied and highest affinity ligands for the AhR are primarily widespread synthetic environmental contaminants, which include both halogenated aromatic hydrocarbons (HAHs) such as the polychlorinated dibenzo-*p*-dioxins (including TCDD), dibenzofurans, and biphenyls and polycyclic aromatic hydro-

* Address correspondence to this author at the Department of Environmental Toxicology, Meyer Hall, One Shields Ave., University of California, Davis, CA 95616-8588 [telephone (530) 752-3879; fax (530) 752-3394; e-mail msdenison@ucdavis.edu].
† Agriculture University of Wageningen.
§ University of California.
# Free University of Amsterdam.

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carbons (PAHs) such as benzo[*a*]pyrene and dibenz[*a*,*h*]-anthracene (*11*, *12*). More recently, numerous other classes of synthetic AhR ligands have been described, and these ligands differ dramatically from the strict structural requirements previously defined for HAH and PAH ligands (*1*, *2*, *9*). Although the majority of these other classes of ligands are relatively weak agonists when compared to the more potent HAHs, such as TCDD, their diversity in structure demonstrates the promiscuous ligand-binding activity of the AhR. The documented promiscuity of substrate binding by numerous AhR-induced xenobiotic metabolizing enzymes (such as CYP1A1) and the established ability of these enzymes to metabolize a large number of AhR ligands is consistent with a major role of this receptor pathway as a sensory system for detoxification whereby the inducing chemical stimulates its own degradation (*1*, *9*, *13*). In addition, developmental defects in AhR knockout animals and other biochemical and genetic studies also suggest a role for the AhR in normal endogenous activities (*7*, *9*, *13*−*16*). Thus, the AhR appears to have multiple functions both as a regulator of endogenous activities (endogenous pathway) and in xenobiotic/endobiotic chemical metabolism/detoxification (adaptive response pathway) (*14*). The majority of ligands identified to date represent synthetic and xenobiotic compounds (*9*), but numerous naturally occurring and endogenous AhR ligands have been identified and characterized as well (reviewed in refs *1* and *2*).

Certainly, the greatest source of exposure of animals and humans to AhR ligands is through their consumption of foods containing these chemicals. Several studies have described a variety of naturally occurring dietary compounds that can directly activate and/or inhibit the AhR signaling pathway (*1*, *2*). Consumption of indole-3-carbinol (I3C), a naturally occurring compound found in members of the *Brassica* family (e.g., Brussels sprout, broccoli, and cauliflower), has been shown to induce AhR-dependent gene expression (i.e., CYP1A1) in rodents and humans (1Z7). In the stomach, I3C undergoes acid condensation into indolo[3,2-*b*]carbazole (ICZ), a compound that binds the AhR with an affinity similar to that of TCDD and is a potent activator of AhR-dependent gene expression (1Z7). More recently, a variety of other naturally occurring indoles (e.g., tryptophan and its metabolites), flavonoids, polyphenolics, and oxidized carotinoids have been identified as AhR ligands/agonists (*1*, *2*, *18*−*22*). Whereas some specific flavonoids (e.g., diosmin, tangeritin, and tamarixetin) are AhR agonists and activate the AhR signal transduction pathway (*23*−*26*), others exert antagonist activity and repress AhR-dependent gene expression (*25*−*28*). Flavonoids are widely distributed in dietary vegetables, fruits, and teas (*28*−*31*), and they have been found in human blood in the low micromolar range (*32*−*34*), a concentration sufficient for many of these ligands to activate or inhibit the AhR. Thus, naturally occurring chemicals appear to represent a major source of xenobiotic AhR ligands to which animals and humans are exposed.

The majority of studies examining the AhR activity of dietary products have focused predominantly on the effect of individual chemicals rather than assessing the overall activity of a crude extract of a product that would contain a complex mixture of chemicals. Analysis of individual chemicals isolated from dietary products provides information as to their potential to activate or inhibit the AhR pathway, but this analysis does not provide any insight into whether these chemicals can actually exert any AhR-dependent biological activity when present in an extract of the product or in the product itself. Thus, assessment of the overall AhR activity of a crude extract of a dietary product would provide more biologically and physiologically relevant information. While we were conducting studies examining the AhR agonist activity of crude extracts of a variety of dietary products, Amakura and co-workers (*35*) reported the ability of crude extracts of several vegetables, teas, fruits, and natural herbal products to antagonize the AhR signaling pathway. However, the AhR agonist activity present in these extracts was not assessed. Here we describe results of studies designed to assess the presence of AhR agonists in crude extracts of various fruits, vegetables, and dietary herbal products.

MATERIALS AND METHODS

Materials. TCDD was obtained from Dr. Steve Safe (Texas A&M University). [γ^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham (Piscataway, NJ), dimethyl sulfoxide (DMSO) and hexane were from Fisher Scientific (Pittsburgh, PA), poly dI·dC was from Roche Molecular Biochemicals (Indianapolis, IN), and the Luciferase Assay System and lysis reagent were from Promega (Madison, WI). Lyophilized dietary herbal supplements were obtained from local health food stores and were all manufactured by Natures Way products (Springsville, UT), except for valerian root (from Solaray, Ogden, UT) and *Ginkgo biloba* (from the Davis Food Co-op Market, Davis, CA). The indicated vegetables and fruit were obtained from local supermarkets and were stored frozen at −80 °C until use.

Preparation of Cytosol. Male Hartley guinea pigs (250−400 g) were purchased from Charles River Breeding Laboratories (Wilmington, DE). The animals were exposed to 12 h of light and 12 h of dark daily and allowed free access to food and water. Liver cytosol was prepared in HEDG buffer (25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 10% v/v glycerol) as previously described (*36*) and stored at −80 °C until use. Protein concentrations were determined according to the method of Bradford (*37*) using bovine serum albumin as the standard.

Preparation of Extracts of Dietary Herbal Supplements, Vegetables, and Fruits. Lyophilized dietary herbal supplements were removed from their gelatin capsules and extracted by vortexing for 2 min in the presence of hexane (1 g of herbal product/10 mL). A 1 mL aliquot of the mixture was transferred into a microcentrifuge tube and centrifuged for 15 min at 1500 rpm to remove the particulate material. The supernatant was transferred to a fresh microfuge tube and recentrifuged for 15 min at 1500 rpm. The resulting hexane extract was dried under a stream of nitrogen and the residue redissolved in 1 mL of DMSO for bioassay analysis. Vegetables and fruits were homogenized in 15 mM Tris buffer (pH 8) using a PowerGen 700 homogenizer (Fisher Technologies) at a ratio of 1 g of vegetable/10 mL of buffer. Because of the waxy cuticle present in most vegetable and fruit samples, we were unable to directly extract them with hexane as was done with the lyophilized herbal samples. The resulting Tris homogenate was centrifuged twice at 13500 rpm for 10 min, and the supernatant was collected and extracted with an equal volume of hexane. The hexane extracts were dried under a stream of nitrogen, and the residue was dissolved in 1 mL of DMSO for bioassay analysis. Appropriate extraction control samples were prepared to eliminate the possibility that AhR ligands were obtained from the solvents and/or the extraction procedures as we have previously observed (data not shown).

Gel Retardation Analysis. A complementary pair of synthetic oligonucleotides containing the DRE3 binding site for the transformed AhR:Arnt complex (5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′) were synthesized, purified, annealed, and radiolabeled with [γ^{32}P]ATP as we have described (*36*). For gel retardation analysis, an aliquot (125 μL) of liver cytosol (8 mg of protein/mL) was incubated with 2.5 μL of DMSO, TCDD (20 nM final concentration), ICZ (100 nM), or 10 μL of the indicated herbal/vegetable/fruit extract in DMSO (equivalent to 1 mg of original herbal/vegetable/fruit sample) for 2 h at room temperature. A 10 μL aliquot of each incubation was analyzed by gel retardation analysis (*36*), and protein−DNA complexes were resolved by polyacrylamide gel electrophoresis and autoradiography of the dried gel. The amount of γ^{32}P-labeled DRE present in the induced protein−DNA

complex was determined using a Molecular Dynamics phosphorimager. The amount of radioactivity present in the induced (TCDD or extract) protein−DNA complex minus that present in the same position in the DMSO- or blank-treated sample lane represents the amount of specific inducible binding of transformed AhR to the [γ^{32}P]DRE oligonucleotide. The amount of extract-induced protein−DNA complex was expressed relative to that induced by a maximal inducing concentration of TCDD after subtraction of the DMSO background signal.

Cell Culture and Induction of Luciferase Activity. Recombinant mouse hepatoma (H1L1.1c2) cells were grown and maintained in α-MEM (Gibco/Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals) at 5% CO_2 and 37 °C as described (*38*). These cells contain a stably integrated DRE-driven firefly luciferase reporter gene plasmid whose transcriptional activation occurs in an AhR-dependent manner (*38, 39*). H1L1.1c2 cells were grown to confluence in a sterile 96-well white CulturPlate (Packard Instruments) for 24 h, washed twice with phosphate-buffered saline (PBS), and incubated with 2 μL of DMSO (2% final volume), TCDD (1 nM final concentration) in DMSO, or 2.5 μL of the indicated sample extract (equivalent to 0.25 mg of the original herbal/vegetable/fruit sample) for 4 h at 37 °C. After the incubation, cells were washed twice with PBS, 25 μL of 1× lysis buffer (Promega) was added to each well, and the plate was placed on a plate shaker until cells were lysed (∼20 min). Luciferase activity was measured using an automated microplate luminometer (Dynatech ML3000, Chantilly, VA) in enhanced flash mode with the automatic injection of 50 μL of Promega stabilized luciferase reagent. Luciferase activity measured as relative light units (RLUs) was normalized to sample protein concentration using the fluorescamine protein assay (*39*), with bovine serum albumin as the standard. Final values are expressed as a percentage of maximal TCDD induction (after subtraction of the DMSO background activity).

RESULTS

Dietary Herbal Supplement Extracts. *Dietary Herbal Supplement Extracts Stimulate AhR DNA Binding in Vitro.* We have previously demonstrated that the AhR can be transformed in vitro into its high-affinity DNA-binding form by TCDD and a variety of structurally diverse chemicals (*40*−*42*). In addition, numerous studies have demonstrated the ability of isolated plant products (e.g., indole carbinols, flavones, phenolics, and other chemicals) to bind to and stimulate AhR transformation and DNA binding (*24*, *25*, *43*, *44*). [In this paper, we have defined transformation as the process by which the liganded AhR complex is converted into a form(s) which binds to DNA with high affinity.] Although these studies demonstrate the existence of plant-derived AhR ligands, they do not provide any information as to the diversity of plant materials that contain AhR ligands. To determine the presence of AhR ligands (agonists) in a variety of plant products, we first examined the ability of chemicals present in hexane extracts of a variety of dietary herbal supplements to stimulate AhR transformation and DNA binding. Incubation of guinea pig hepatic cytosol with extracts of various herbal products and subsequent gel retardation analysis revealed that numerous extracts induce formation of a protein−DNA complex, which migrated to the same position as that produced by TCDD (**Figure 1**). This inducible protein−DNA complex has been previously demonstrated to represent the transformed AhR complex bound to the DRE (*36*, *45*). Although some herbal extracts were inactive (hawthorn, saw palmetto, nettle, damiana, and others), several extracts (ginseng, licorice, Fo-Ti, white oak bark, *Ginkgo biloba*, and black cohosh root) were relatively active (the amount of induced AhR:DRE complex was between 40 and 60% of that obtained with a maximally inducing concentration of TCDD). Quantitative determination of the amount of inducible AhR:DRE complexes obtained in triplicate experiments with each sample are shown in **Figure 2**, and the data are expressed relative to that of TCDD.

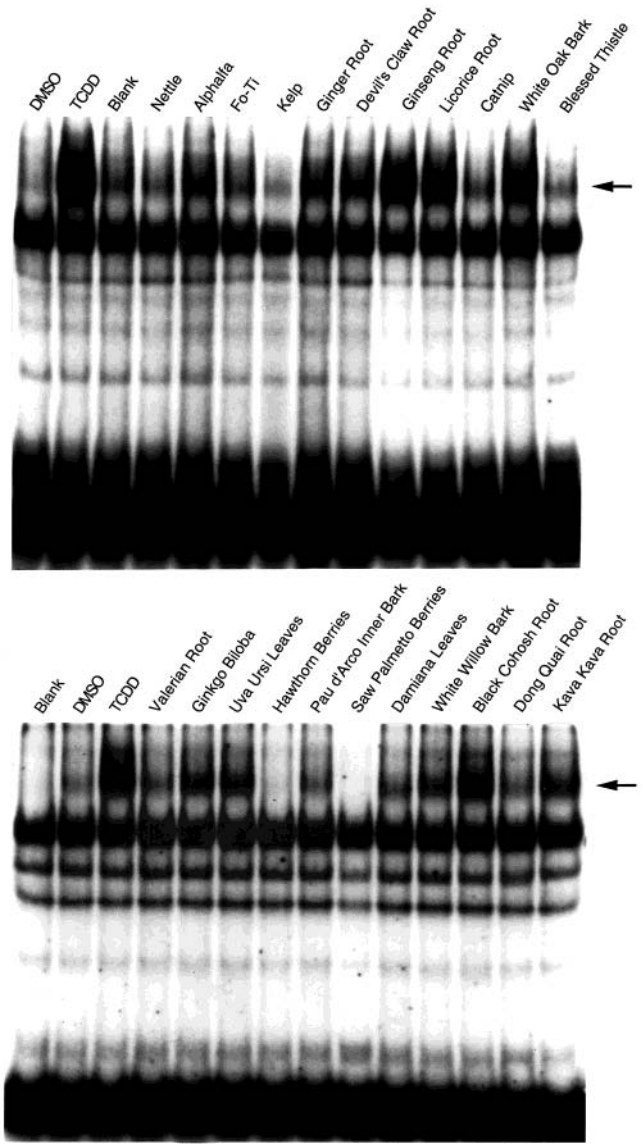

Figure 1. Extracts of dietary herbal supplements stimulate AhR transformation and DNA binding in vitro. Guinea pig hepatic cytosol was incubated with carrier solvent (2.5 μL of DMSO), 20 nM TCDD (in DMSO), or the indicated sample extract (equivalent to 1 mg of the original herbal supplement) for 2 h at 20 °C. Aliquots of each sample were further incubated with [^{32}P]DRE and protein–DNA complexes resolved by gel retardation analysis as described under Materials and Methods. The arrow indicates the position of the TCDD-inducible AhR:DRE complex.

In the studies described here, we focused our analysis on hexane extracts in order to determine whether AhR agonists are present in these products. We realize that these products likely contain AhR agonists that are refractory to extraction by hexane and thus we would underestimate the total activity present in these products. However, we have carried out limited extraction studies using other solvent systems. Incubation of chloroform extracts of selected herbal products or the pellet remaining from the hexane extraction of these products also resulted in formation of the AhR:DRE complex (data not shown), indicating the presence of additional more polar AhR active chemicals in these herbal products that were not hexane extractable. Overall, these results clearly demonstrate that the AhR can be transformed into its DNA-binding form by naturally occurring chemicals present in extracts of a wide variety of dietary herbal supplements.

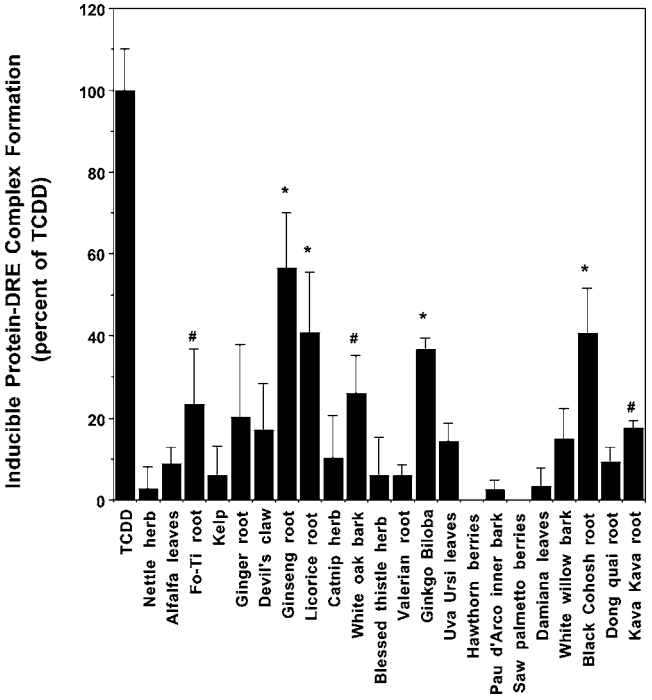

Figure 2. Quantitative determination of the amount of inducible AhR:DRE complexes. Phosphorimager quantitation of triplicate gel retardation analyses of the samples indicated in **Figure 1** are expressed relative to maximal protein–DRE complex formation induced by TCDD. Values represent the mean ± SD of at least triplicate samples, and those significantly greater than control at $P < 0.05$ (#) or $P < 0.01$ (∗) as determined by Student's *t* test are indicated.

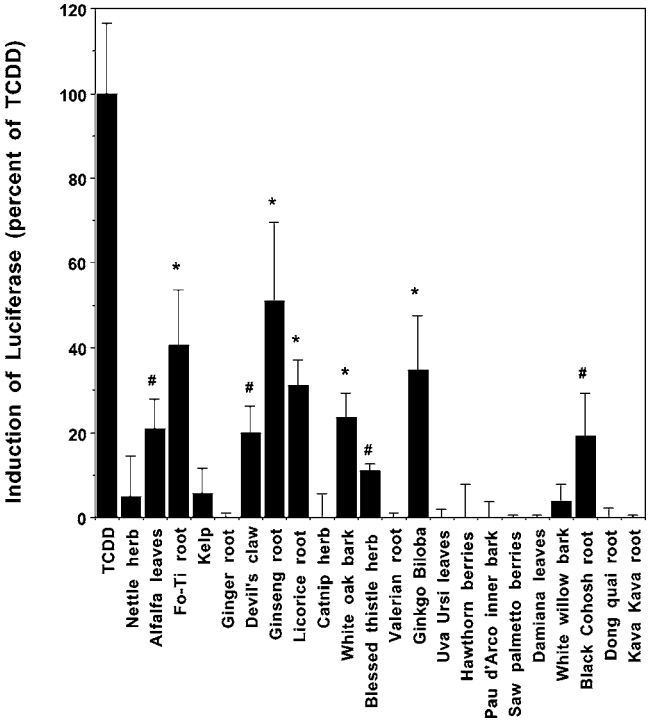

Figure 3. Induction of luciferase reporter gene activity in a stably transfected mouse hepatoma (H1L1.1c2) cell line by selected herbal extracts. Confluent 96 well plates of cells were incubated with carrier solvent (2 μL of DMSO), TCDD (1 nM final concentration) in DMSO or 2.5 μL of the indicated sample extract (equivalent to 0.25 mg of the original herbal supplement) for 4 h at 37 °C. Luciferase activity in cell lysates was determined as described under Materials and Methods. Values represent the mean ± SD of at least triplicate determinations and were expressed relative to the activity obtained with a maximal inducing concentration of TCDD. Values significantly greater than control at $P < 0.05$ (#) or $P < 0.01$ (∗) as determined by Student's *t* test are indicated.

Dietary Herbal Supplement Extracts Induce AhR-Dependent Reporter Gene Expression. Whereas the AhR mediates the induction of DRE-dependent gene expression, recent results in our laboratory demonstrated that the ability of a chemical to stimulate AhR DNA binding in vitro does not necessarily correlate with its ability to induce AhR-dependent gene expression (*40*). To examine the ability of each of the herbal extracts to induce AhR-dependent gene expression, we utilized a recombinant mouse hepatoma cell line (H1L1.1c2) that contains a stably integrated firefly luciferase gene whose expression is under the control of four DREs (*38*, *39*). We have previously demonstrated that treatment of these cells with AhR ligands (agonists) induces luciferase gene expression in a time-, dose-, and AhR-dependent manner (*38*). Induction of luciferase following incubation of H1L1.1c2 cells for 4 h with the indicated herbal extracts is shown in **Figure 3**. The results of these experiments are relatively consistent with those of the gel retardation/DNA binding studies in that extracts that stimulated AhR DNA binding also induced reporter gene expression (Fo-Ti, ginseng, licorice, white oak, *Ginkgo biloba*, and black cohosh). In these experiments, kava kava was the only herbal extract that significantly stimulated AhR DNA binding but was essentially inactive as an inducer of luciferase reporter activity. Unlike many of our previous studies (*40*), direct comparison of these results (**Figure 4**) revealed a relatively good correlation between the ability of a given herbal extract to stimulate AhR DNA binding and its ability to induce gene expression. The relatively good correlation between DNA binding and induction observed in these extracts also suggests that few interaction effects (i.e., antagonism or synergism) are occurring between chemicals present in the herbal extracts and the AhR signal transduction pathway.

Vegetable and Fruit Extracts. *Vegetable and Fruit Extracts Stimulate AhR DNA Binding in Vitro.* To determine the presence of AhR agonists in vegetables and fruit, we examined the ability of extracts of these materials to stimulate AhR transformation and DNA binding in vitro using gel retardation analysis. Similar to our results with the herbal extracts, numerous Tris/hexane extracts of vegetables and fruits stimulated AhR transformation and DNA binding (**Figure 5**). Phosphorimager quantitation of these complexes (**Figure 6**) demonstrated that chemicals present in extracts of corn, jalapeño pepper, green bell pepper, apple, Brussels sprout, coffee, and potato were relatively potent activators, stimulating AhR:DRE complex formation to between 30 and 50% of that produced by TCDD or ICZ. Extracts of broccoli, cauliflower, and lemon were less potent, inducing protein−DNA complex formation to between 20 and 30% of that of TCDD or ICZ. In preliminary studies we also observed that Tris/chloroform extracts of selected vegetables stimulated AhR:DRE complex formation, indicating that these products also contain more polar AhR active chemicals in these products in addition to those that were hexane extractable. Overall, these results demonstrate that many vegetable and fruit extracts contain chemicals that can stimulate AhR transformation and DNA binding in vitro.

Vegetable and Fruit Extracts Induce AhR-Dependent Reporter Gene Expression. Vegetable and fruit extracts that stimulated AhR transformation and DNA binding (broccoli, corn, jalapeño, green bell pepper, apple, cauliflower, Brussels sprout, lemon,

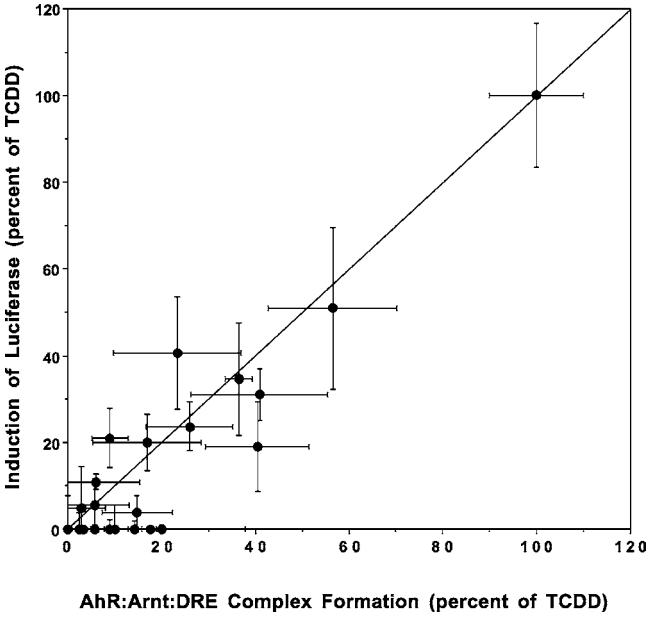

Figure 4. Direct comparison of the ability of a given herbal extract to stimulate AhR DNA binding and its ability to induce AhR-dependent gene expression. The diagonal line in the figure represents data that would fit a perfect correlation between AhR:DNA complex formation and AhR-dependent gene expression.

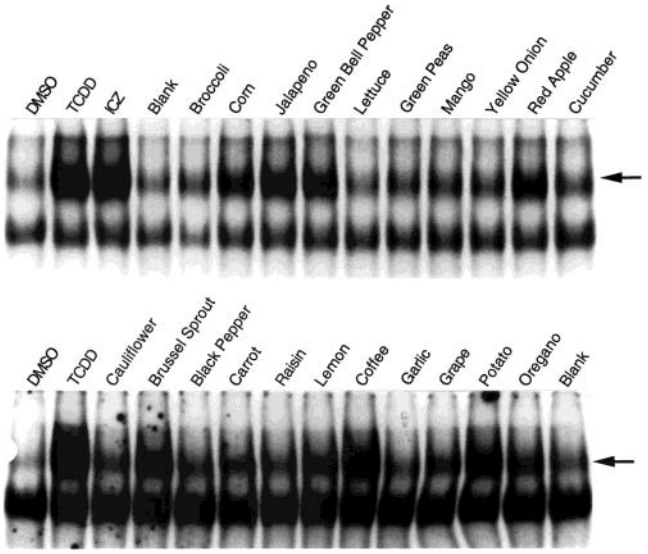

Figure 5. Extracts of fruits and vegetables stimulate AhR transformation and DNA binding in vitro. Guinea pig hepatic cytosol was incubated with carrier solvent (DMSO), 20 nM TCDD, ICZ (100 nM), or 10 μL of the indicated extract (equivalent to 1 mg of the original vegetable or fruit) for 2 h at 20 °C. Aliquots of each sample were further incubated with [^{32}P]-DRE and protein–DNA complexes resolved by gel retardation analysis as described under Materials and Methods. The arrow indicates the position of the inducible TCDD:AhR:DRE complex.

and potato) and two relatively weak or inactive extracts (pea and mango) were further analyzed for their ability to induce AhR-dependent gene expression (**Figure 7**). In contrast to the herbal extract results, incubation of H1L1.1c2 cells with vegetable and fruit extracts for 4 h resulted in significant induction of luciferase activity by only two samples (jalapeño pepper and corn extracts). A plot of the relative ability of these extracts to stimulate AhR DNA binding and gene expression (**Figure 8**) revealed that these vegetable and fruit extracts were

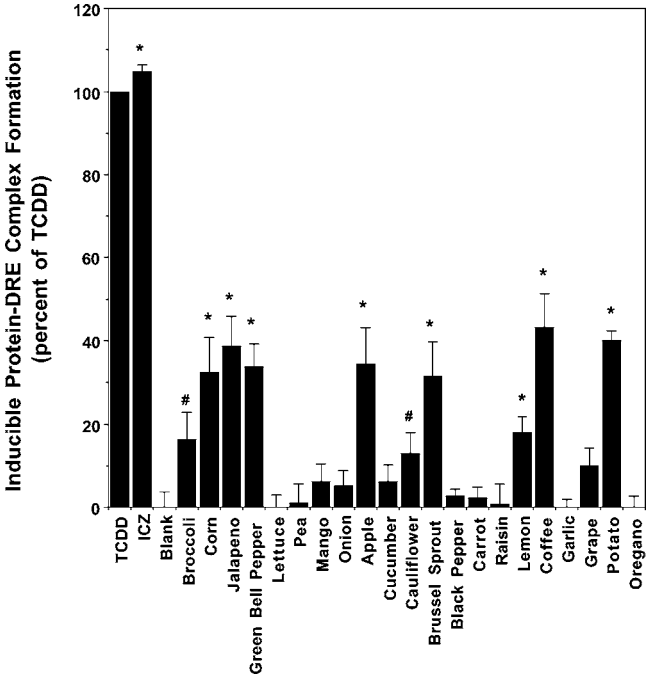

Figure 6. Quantitative determination of the amount of inducible AhR:DRE complexes. Phosphorimager quantitation of triplicate gel retardation analyses of the samples indicated in **Figure 5** are expressed relative to the maximal amount of protein–DRE complex formation induced by TCDD. Values represent the mean ± SD of at least triplicate samples, and those significantly greater than control at $P < 0.05$ (#) or $P < 0.01$ (∗) as determined by Student's t test are indicated.

significantly less potent as inducers of gene expression than would be expected. We have previously identified chemicals that can stimulate AhR transformation and DNA binding in vitro but not induce AhR-dependent gene expression (*40*, *41*), and there are several possibilities for this type of response. The fact that we were analyzing a complex mixture of chemicals in the extracts combined with the documented presence of naturally occurring AhR antagonists in vegetables and fruits (*26*, *35*) suggests that the decreased induction response from vegetable and fruit extracts results from AhR antagonists present in the extracts. To test this possibility, we examined the effect of dilution on the ability of selected vegetable and fruit extracts to induce luciferase in the H1L1.1c2 cells (**Figure 9**). These analyses revealed that treatment of cells with a 5-fold dilution of several extracts (notably, corn, jalapeño pepper, bell pepper, and potato) resulted in a dramatic induction of luciferase activity to levels equal to or greater than that induced by TCDD, as compared to extracts that had not been diluted. Significant increases in induction were also observed using 5-fold diluted apple and Brussels sprout extracts. These dilution results suggest the presence of AhR antagonists in these extracts whose inhibitory potency was reduced with dilution. It should also be noted that the lack of induction with the original sample extracts was not due to cell toxicity as observed by visual examination of the cells after treatment. A better correlation between the ability of the diluted extract to stimulate AhR DNA binding and to induce AhR-dependent gene expression was observed. The reduction in the magnitude of luciferase induction observed with the 25-fold diluted extracts, compared to the 5-fold diluted samples, likely results from a decrease in the concentration of the inducing chemical(s). Interestingly, the 5- and 25-fold diluted bell pepper and potato extracts induced luciferase maximally (i.e., equivalent to that induced by TCDD), suggesting that these

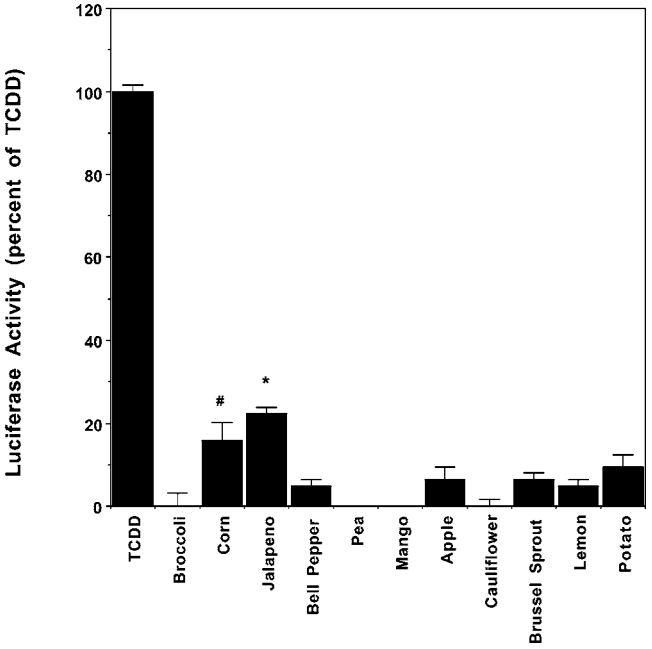

Figure 7. Induction of luciferase reporter gene activity by selected fruit and vegetable extracts. Confluent 96 well plates of H1L1.1c2 cells were incubated with carrier solvent (DMSO), TCDD (1 nM final concentration), ICZ (100 nM), or 2.5 μL of the indicated sample extract (equivalent to 0.25 mg of the original vegetable or fruit) for 4 h at 37 °C. Luciferase activity in cell lysates was determined as described under Materials and Methods. Values represent the mean ± SD of at least triplicate determinations and were expressed relative to the activity obtained with a maximally inducing concentration of TCDD. Values significantly greater than control at $P < 0.05$ (#) or $P < 0.01$ (∗) as determined by Student's *t* test are indicated.

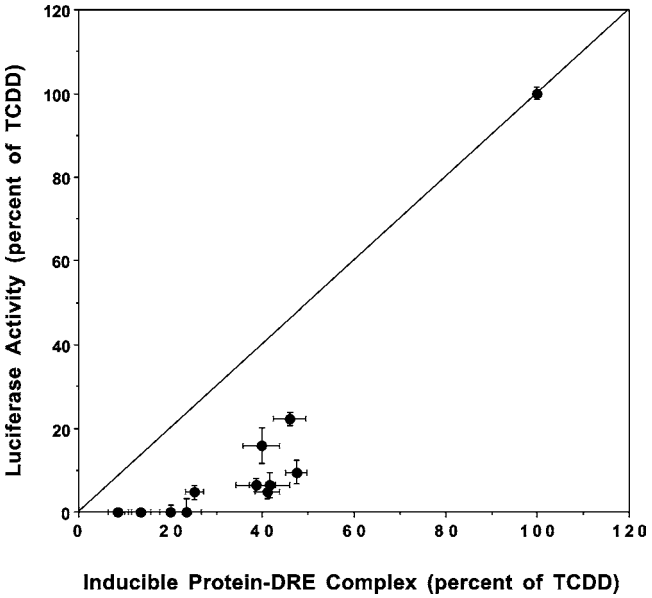

Figure 8. Direct comparison of the ability of a given herbal extract to stimulate AhR DNA binding and its ability to induce AhR-dependent gene expression. The diagonal line in the figure represents data that would fit a perfect correlation between AhR:DNA complex formation and AhR-dependent gene expression.

extracts contain a relatively high concentration of potent AhR agonists. Overall, these results demonstrate the presence of both AhR agonists and antagonists in extracts of a variety vegetables and fruits.

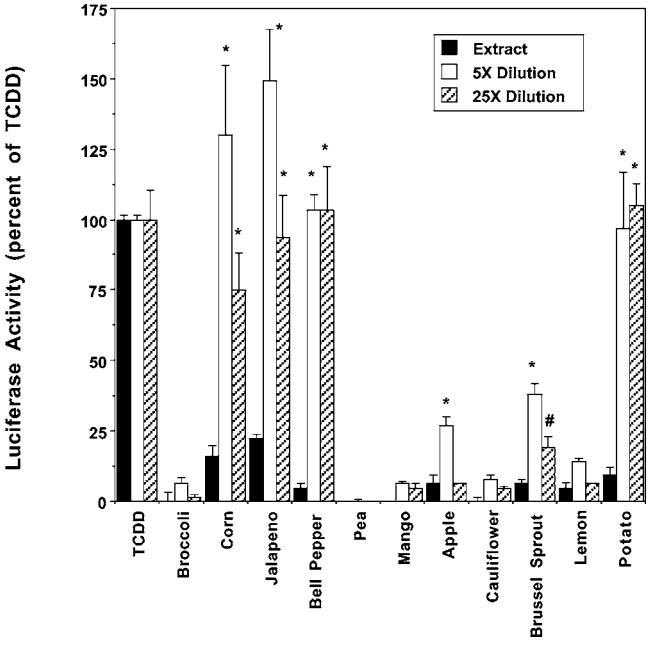

Figure 9. Effect of dilution on the ability of fruit and vegetable extracts to induce AhR-dependent luciferase reporter gene expression. Induction of luciferase reporter gene activity in stably transfected mouse hepatoma (H1L1.1c2) cells was facilitated by diluted fruit and vegetable extracts. Confluent 96 plates of cells were incubated with carrier solvent (DMSO), TCDD (1 nM final concentration) in DMSO, or 2.5 μL of undiluted or 5- or 25-fold diluted extract of the sample (equivalent to 0.25, 0.05, or 0.01 mg of the original vegetable or fruit, respectively) for 4 h at 37 °C. Luciferase activity in cell lysates was determined as described under Materials and Methods. Values represent the mean ± SD of at least triplicate determinations and were expressed relative to the activity obtained with a maximal inducing concentration of TCDD. Values significantly greater than control at $P < 0.05$ (#) or $P < 0.01$ (∗) as determined by Student's *t* test are indicated.

DISCUSSION

The presence of AhR agonists in food products has been previously reported by numerous investigators (*17*, *18*, *23*). However, these studies focused primarily on individual chemicals present in these products, and the overall AhR agonist activity in crude extracts of food has not been examined. Utilizing two sensitive AhR bioassay systems, we have demonstrated the presence of AhR agonists and antagonists in extracts from a variety of vegetables, fruits, and dietary herbs. Interestingly, herbal extracts appear to contain predominantly AhR agonists, whereas vegetable and fruit extracts appear to contain both AhR agonists and antagonists. Although the identity of the AhR-activating chemical(s) present in these extracts remains to be determined, it is apparent that AhR ligands are widely distributed in food products. Over the past several years, it has become increasingly apparent that the AhR (*1*, *2*, *9*), like numerous other ligand-activated nuclear receptors [i.e., estrogen, androgen, thyroid, pregnane X, and peroxisome proliferator activated receptors (*46*−*49*)], can be bound and activated (or inhibited) by a large number of structurally diverse chemicals and food extracts (*50*−*52*). These observations have not only raised questions regarding the actual spectrum of chemicals (both natural and synthetic) that can bind to these receptors but has also led to studies evaluating the biochemical and toxicological consequences of these interactions. One avenue we and others have followed to identify and characterize novel activators of the AhR has taken advantage of sensitive bioassay systems for high-throughput analysis of individual chemicals (*42*).

Whereas these approaches have identified new classes of AhR agonists, they have predominantly focused on the analysis of synthetic chemicals. Although the rather "sloppy" or promiscuous ligand-binding specificity of the AhR (*1*, *2*, *9*) might initially seem to be incompatible with its role as a selective ligand-dependent receptor, a case can be made that this characteristic may actually confer some adaptive advantage to the organism. The AhR is known to induce the expression of cytochrome P450s as well as other xenobiotic metabolizing enzymes (*7*, *12*). Because activation of this receptor system results in the expression of numerous detoxification enzymes, the majority of which exhibit broad substrate specificity, the promiscuous ligand-binding activity of the AhR would provide the organism with a greater dynamic range of "chemical detection" and metabolism. As a consequence of the promiscuous nature of AhR ligand binding, the spectrum of exogenous and endogenous chemicals that can activate the AhR may be significantly greater than what is currently known. We envision the existence of numerous endogenous physiological AhR ligands that have relatively weak affinity, compared to TCDD, and are rapidly degraded by the coordinately induced detoxification enzymes. Together, the broad spectrum of less persistent AhR ligands may provide a further advantage to the organism by decreasing its responsiveness to TCDD and related toxic AhR ligands as a result of antagonistic or agonistic occupancy of the receptor.

Several investigators have previously examined a variety of plant-derived compounds that interact with the AhR (*17*, *18*, *23*, *28*). However, these studies either utilized pure compounds or examined only the antagonist effects of plant extracts on the AhR (*23*−*27*). Here we have observed that extracts of a variety of fruits and vegetables contain AhR ligands, with both agonistic and antagonistic properties, whereas extracts of a variety of dietary herbal supplements exhibited predominantly agonist activity. Extracts of Brussels sprout, broccoli, and cauliflower, members of the *Brassica* family, are reported to be potent activators of the AhR (*17*, *44*) but were relatively inactive in our bioassays. However, chemicals in these plants (i.e., I3C) require acidic conditions to be converted into potent AhR agonists (i.e., ICZ). The extracts examined here were prepared at neutral pH. Extracts of ginseng root, licorice root, *Ginkgo biloba*, and black cohosh root were most potent in the gel retardation assay and appear to contain relatively potent AhR ligands. These plants contain a variety of natural chemicals (*53*, *54*). Ginseng root is known to contain steroidal glycosides, saponins, volatile oil, vitamin D, acetyleneic compounds, and sterols, whereas licorice root contains saponins, glycosides (including glycyrrhizin), estrogenic substances, coumarins, flavonoids, sterols, choline, asparagine, and volatile oil. Flavone glycosides, bioflavones, sitosterol, lactones, and anthocyanin are constituents of *Gingko biloba*. The main constituents of black cohosh root are triterpene glycosides (actein cimifugoside), isoflavones (formononetin), isoferulic acid, salicylic acid, tannins, and resins (*53*, *54*). Because the tested sample extracts contain complex mixtures of chemicals, it remains to be determined which chemical(s) is responsible for activation of the AhR and AhR-dependent gene expression. Ginseng, licorice, and black cohosh root extracts were less potent in the luciferase expression assay compared to their activity in the gel retardation assay. Some samples, like devil's claw secondary root and blessed thistle, were shown to be more potent in the luciferase expression assay than in the gel retardation assay. It should also be noted that our Tris/hexane vegetable/fruit extract results certainly underestimate the actual concentration of AhR ligands (agonists and antagonists) present in these products. This derives from the fact that the initial extraction was in aqueous buffer and the majority of the hydrophobic chemicals [which would include most AhR ligands identified to date (*1*, *2*, *9*)] would not be extracted. However, the fact that the isolated extracts we prepared are still relatively active strongly supports the existence of naturally occurring AhR ligands with water soluble characteristics. Bioassay-directed fractionation of extracts of these products provides us with an avenue to identify the constituent(s) responsible for AhR-dependent induction as well as to determine interactive effects (i.e., antagonist, additive, and/or synergistic) that occur between chemicals in a given extract.

Numerous studies have previously demonstrated a relatively good correlation between the ability of a variety of synthetic chemicals (mostly HAHs and PAHs) to bind to the AhR and stimulate AhR-dependent DNA binding and gene expression (*10*, *38*). However, recent detailed examination of a diverse range of other AhR ligands has revealed a relatively poor correlation between the ability of some chemicals to bind to the AhR and stimulate AhR DNA binding and their ability to stimulate AhR-dependent gene expression (*40*). Several extracts in this study were able to activate and transform the AhR into its DNA-binding form in vitro to a greater extent than they were able to induce AhR-dependent gene expression in intact cultured cells. This may be a consequence of the bioavailability of the chemical to its target site. In the in vitro assay, chemicals have direct access to the AhR and, as such, tend to appear to be more active than they are in the luciferase induction bioassay because the same chemical would now have to be able to pass through the cell membrane to bind the AhR. With the treatment of cells in culture, there is the added possibility of the test compound binding to and being sequestered by various serum proteins. Additionally, when the compound does enter the intact cell, it may be degraded by metabolic enzymes into a product that cannot interact with the AhR to induce gene expression and/or it may be ineffective in recruiting the proper cofactors necessary for transcriptional activation.

Our results also show an increase in luciferase gene induction when diluted vegetable and fruit extracts were tested, suggesting that the undiluted extracts contain an AhR antagonist(s) or an inhibitor of the AhR signaling pathway. This inhibitory component(s), which may have been rendered ineffective by dilution, could adversely affect the cells themselves, the reporter gene, and/or decrease the inducing potency of an AhR agonist. The ability of compounds to induce AhR-dependent gene expression to levels disproportionately greater than their ability to bind the AhR in vitro, however, opens up new avenues for examining AhR signal transduction. Conversion of a chemical in the intact cell to a more potent ligand, either spontaneously or enzymatically, would be one avenue by which this could achieved. Alternatively, recent studies have shown that activation of other signaling pathways in the cell, such as that of protein kinase C (PKC), leads to augmentation of TCDD-induced, AhR-dependent gene expression (*55*, *56*), whereas down-regulation of PKC leads to inhibition of the AhR-dependent signaling pathway (*57*, *58*). This opens up the possibility that some dietary AhR ligands, in the absence of putative inhibitory factors in complex mixtures such as crude extracts, could also activate a signaling pathway(s), which results in augmentation or inhibition of induction of AhR-dependent gene expression. The specific mechanisms by which compounds are able to enhance or inhibit AhR signaling remain to be determined, but some proposed possibilities include the activation of kinase pathways and subsequent phosphorylation of the AhR, ARNT, and/or related cofactors, leading to alterations in the transcriptional machinery (*55*, *56*).

The results of our analysis demonstrate the presence of AhR agonists and antagonists in extracts from a variety of dietary herbal/vegetable/fruit products. Considering the relatively small amounts of each extract examined in our bioassays (1 and 0.25 mg equivalents of original materials in DNA-binding and luciferase induction assays, respectively), these dietary products must contain relatively high concentrations of AhR agonists. Estimates of the total amount of TCDD induction equivalents (IEQs) present in selected products by comparison to TCDD dose−response curves of gene induction (data not shown) indicate a single dietary serving of potato (150 g) would contain 96000 pg of TCDD IEQs, whereas bell pepper (125 g) contains 80000 pg of IEQs, corn (40 g) 26000 pg of IEQs, and Brussels sprout (85 g) 5500 pg of IEQs. The TCDD IEQs present in a single recommended dosage of Fo-Ti would be 1000−3000pg, ginseng would contain 2000−3000, *Ginkgo biloba* 70 pg, black chodosh 400 pg, white oak 400−400 pg, and licorice 500−1000 pg. When we consider that consumption of multiple servings of these vegetables and dosages of these hrebal supplements are recommended, combined with the fact that we have extracted only a fraction of the total AhR active chemicals present in these products, the actual human exposure levels would be predicted to be quite high. Differential fraction approaches combined with AhR bioassay analysis techniques will not only allow more accurate evaluation of the quantity and spectrum of AhR agonists/antagonists in these dietary products, but they will allow more accurate exposure assessment of humans to AhR-active chemicals.

In conclusion, numerous dietary plant products appear to contain relatively high concentrations of AhR ligands or products that can readily be converted into AhR ligands, and they may well contain the largest class of natural AhR ligands to which humans and animals are exposed. Although the full biological and toxicological impact of persistent exposure to these and other naturally occurring AhR ligands remains unknown, there have been documented biological effects of such compounds in humans and animals. For example, in humans, a decrease in serum bilirubin levels was reported with induction of cytochrome P4501A1 (which can metabolize bilirubin) by ICZ (*59*), whereas TCDD-like effects were observed in catfish chronically fed high levels of β-naphthoflavone, a PAH ligand for the AhR (*60*). The evolution of the AhR ~450 million years ago (*61*), prior to the anthropogenic introduction of HAHs and PAHs into the environment, along with the ability of the AhR-dependent enzymatic activities (i.e., CYP1A1 and others) to metabolize many AhR ligands, also supports the idea that there are endogenous and/or naturally occurring AhR ligands. Demonstration of the presence of relatively high concentrations of AhR agonists in extracts of fruits, vegetables, and dietary herbal supplements is consistent with the hypothesis that the AhR may have evolved, at least in part, as a ligand-inducible system to facilitate the metabolism of dietary and endogenous compounds. TCDD may mimic the binding of such compounds to the receptor, but its resistance to metabolism appears to be a major factor contributing to its toxic potency. As such, our results reinforce some of the proposed pitfalls of utilizing the toxic equivalency factor (TEF) method for overall risk assessment of dioxins and related chemicals (*62*). In this approach, the overall toxic potency or toxic equivalent (TEQ) of a solvent-extracted sample containing HAHs is determined by multiplication of the concentration of individual toxic HAHs (determined by instrumental analysis) by their respective TEF value and expressed relative to that of TCDD. However, this analysis ignores the contribution/effect of other non-HAH AhR ligands (exogenous and endogenous) that may be present in the original sample on the overall TEQ estimation. Although such chemicals would not contribute much to the overall TEQ, because they do not appear to produce TCDD-like toxic effects, depending on their concentration they could reduce the overall toxic potency of the HAHs in a mixture by effectively reducing the number of AhRs available to bind HAHs. This decrease in the toxic potency/TEQ of HAHs in the mixture would result from both metabolically labile, nontoxic AhR agonists and antagonists. Because it has also been documented that activation of the AhR can exert antitumorigenic/antiestrogenic activity (*9*, *10*), exposure to AhR active chemicals present in foods may also have beneficial therapeutic effects. However, further research is needed to examine the potential impact of acute and chronic exposure to naturally occurring AhR agonists and antagonists on the toxicological and biological effects and overall risk assessment of HAH and PAHs in humans and animals, as well as their potential utility as chemotherapeutic agents.

ABBREVIATIONS USED

AhR, aromatic hydrocarbon receptor; Arnt, Ah receptor nuclear translocator; DMSO, dimethyl sulfoxide; DRE, dioxin responsive element; HAH, halogenated aromatic hydrocarbon; I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; IEQ, induction equivalent; PAH, polycyclic aromatic hydrocarbon; RLU, relative light unit; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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