ACTIVATION OF Ah AND ESTROGEN RECEPTOR-BASED CELL BIOASSAY SYSTEMS BY EXTRACTS OF NATURAL DIETARY HERBAL SUPPLEMENTS

Jessica E. Bohonowych, Jane M. Rogers, Anoek Jeuken, and Michael S. Denison

Department of Environmental Toxicology, University of California, Davis, CA 95616

Introduction

Determination of the exposure of an organism to a given class(es) of environmental chemicals is crucial to evaluating the potential for adverse outcomes. Although chemical analysis methods allow accurate assessment of the amount of a given toxicant or closely related toxicants (i.e. PCDDs, PCDFs, etc...) in a sample, they do not provide any information as to the presence of other chemicals of classes of chemicals that can have similar or opposing effects. The application of mechanistically-based bioassay systems is an approach which has been successfully used for the detection and relative quantitation of chemicals which can exert their action through a common pathway. We and others have developed recombinant cell bioassay systems which take advantage of a ligand-dependent receptor transcription factor to detect chemicals which have dioxin or steroid hormone (endocrine) like effects[1,2]. These bioassays can detect chemicals that exert their action(s) via ligand-dependent and/or ligand independent mechanisms on the aryl hydrocarbon receptor (AhR) and hormone receptor (HR) signal transduction pathways. In addition, these systems can identify chemicals which can antagonize these signaling pathways irrespective of whether it is direct (i.e. via competitive inhibition at the ligand binding site) or by a mechanism which does not involve ligand binding (i.e. effects on other points of the pathway).

We and others have used these bioassays to detect the presence of dioxin-like and/or endocrine disrupting chemicals in extracts if a variety of materials including sediment, fly ash, and various biological samples (food, milk, tissue and blood) and to determine dioxin-like and/or endocrine disrupting activities of pure chemicals[1-4]. We have recently described modifications of our AhR-based chemically-activated luciferase gene expression (CALUX) cell bioassays to detect AhR ligands (i.e. polycyclic and halogenated aryl hydrocarbons (PAHs; HAHs) in whole serum samples[4]. Similar approaches are currently being undertaken for the detection of xenoestrogens in serum and other matrices using our ER-dependent luciferase gene expression (ER-Lite) bioassay. Given the cross-talk between AhR and ER signal transduction pathways and the ability of numerous AhR ligands to act as antiestrogens[5,6], we have begun to examine the ability of a variety of pure chemicals and sample extracts to activate and/or inhibit both of the signaling systems. The presence of these materials in biological (i.e. blood) or environmental samples and their ability to cross-talk (ie., to inhibit and/or stimulate responses) could modulate the level and duration of response in each system and thus, complicate accurate data analysis. Here we describe initial analysis of naturally occurring substances and an assessment of their ability to affect AhR- and ER-dependent signal transduction.

Materials and Methods

PCBs and Sample Preparation. PCBs were obtained from Dr. Isaac Pessah (UCDavis) and Accustandard. Hexane extracts of the indicated commonly available herbal dietary supplement were prepared by overnight incubation of the product in hexane (1g/10ml). An aliquot of extract (1 ml) was pelleted down, the supernatant was removed, evaporated to dryness under nitrogen and resuspended in DMSO (200µl for the ER assays, and 1 ml for the AhR assays.

Estrogen Receptor Binding and Gel Retardation Analysis. Bovine uterine cytosolic AhR was used in competitive ER ligand binding assays as we have described[7]. For gel retardation analysis,

guinea pig hepatic was incubated with 2.5 µl herbal extract, DMSO (20 µl/ml), or TCDD (20 nM) and AhR:Arnt:DRE complex formation determined as previously described[8].

ER-Lite and CALUX Bioassays. For analysis of activation of ER-signal transduction, recombinant human ovarian cells (BG1Luc4E_2) were grown in estrogen stripped media (ESM) (phenol red free MEM with 5% dextran-coated charcoal stripped fetal bovine serum) and incubated for 24 hr with 0.1% herbal extract in ESM for 24 hr. For analysis of AhR activation, recombinant mouse hepatoma cells (H1L1.1c2) treated with 2% herbal extract in MEM for 4 hr. Following incubation, all cells were lysed and luciferase activity measured as previously described[1,2]. BG1Luc4E_2 and H1L1.1c2 cells contain stably transfected reporter genes which respond to estrogen or 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), respectively, with the induction of firefly luciferase.

Results and Discussion

We have previously demonstrated that coplanar PCBs (i.e. 3,3',4,4'-TCB and 3,3',4,4',5-PCB) can bind to the AhR and activate gene expression in the CALUX bioassay[1,4], but not the ER-lite assay[2]. In contrast, non-coplanar PCBs, such as 2,3,4,4'-TCB and 2,2',3,5,6-PCB, were active in the ER-lite assay[2], while other non-coplanars were inactive in the CALUX bioassay. Not only are these results consistent with what is know about AhR ligand binding specificity, but it reveals two new PCBs which are potential endocrine disruptors.

A number of the herbal extracts contain ligands for the ER as indicated by their ability to competitively inhibit ^{3}[H] estradiol binding (Table 1). The supplements with the greatest binding activity included ginger, hawthorn, licorice, saw palmetto, and St. John's wort. Ginger, hawthorn, and licorice also induced the ER-Lite bioassay, suggesting that these extracts contain agonists. Damiana and dong quai extracts also induced expression in the ER-Lite bioassay and yet bound the ER weakly (dong quai) or not at all (damiana). These extracts may contain compounds that are metabolized to ER agonists *in vivo*, or may affect ER activity indirectly (i.e. by phosphorylation). In contrast, a previous study did not find that these herbal extracts (with the exception of licorice) contained ER ligands, nor did they induce cell proliferation in T47D breast cancer cells[11]. However, that study used extracts of whole herbs in ethanol and measured binding using intact human breast cancer cells while our studies examined ER binding in vitro and induction in human ovarian cells. These issues may account for some of these differences. Those extracts which were able to competitively inhibit binding of ^{3}[H] estradiol to the ER were also assayed for anti-estrogenic activity (Table 1). St. John's wort inhibited estrogen dependent induction in the bioassay by 78.55 ±8.89% (data not shown). Unlike the rest of the supplements, the brand of St. John's wort used also included a number of added ingredients, thus it is unknown whether a plant derived compound and/or an additive is responsible for the activity.

Extracts of ginseng, licorice, ginkgo biloba, and black cohosh were the greatest activators of AhR:DNA binding *in vitro*, indicating that they may contain AhR ligands (Fig. 1). These four extracts also induced gene expression in the CALUX bioassay (Fig. 2). However, the most active extract in the bioassay was devil's claw which was only weakly positive in the GRA. The lack of correlation between the ability of an extract to activate the AhR *in vitro* and to induce gene expression in whole cells may be explained by metabolism of the compound to a more, or less, potent agonist, bioavailability of the compound to its target site, or activation or recruitment of secondary proteins. These results clearly demonstrate the presence of AhR and ER ligands in a number of these herbal supplements with some extracts (ginger and licorice) containing ligands for both receptor systems. Further work needs to be conducted to determine the potency and pharmacological effects of these compounds *in vivo* and whether the levels that can be found in blood samples can interfere with analysis by the CALUX or ER-Lite assays.

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Table 1: ER binding and luciferase induction analysis of herbal supplement extracts.

Herbal Supplement	Percent Inhibition of Specific ^{3}[H] Estradiol Binding	Percent of ER Mediated Luciferase Induction
1nM Estradiol		100
Alfalfa	-2.63 ±1.79	-2.58 ±0.68
Black Cohosh	-1.11 ±1.93	-2.90 ±0.36
Blessed Thistle	8.49 ±1.03	-2.66 ±0.85
Catnip	-2.52 ±2.48	-2.11 ±0.96
Damiana	-11.65 ±2.31	17.48 ±2.21
Devil's Claw	-7.14 ±3.45	0.62 ±2.48
Dong Quai	17.31 ±1.58	24.67 ±6.16
Fo-Ti	6.98 ±2.56	0.60 ±1.27
Ginger	54.36 ±4.61	11.69 ±3.30
Ginkgo Biloba	2.37 ±1.11	-1.00 ±0.28
Ginseng	3.77 ±1.06	-1.23 ±0.22
Hawthorn	51.09 ±2.89	-1.30 ±0.30
Kava Kava	-5.87 ±5.49	-0.47 ±2.06
Kelp	7.65 ±0.93	1.87 ±1.17
Licorice	35.81 ±2.45	12.85 ±6.06
Nettle	5.23 ±0.41	-1.44 ±0.27
Pau d'Arco	6.78 ±0.77	-1.35 ±0.21
Saw Palmetto	72.66 ±4.32	1.47 ±0.70
St. John's Wort	24.37 ±1.33	-1.26 ±0.28
Uva Ursi	-0.07 ±2.93	-1.15 ±0.37
Valerian Root	0.32 ±1.27	3.12 ±5.45
White Oak	-0.57 ±3.52	-0.85 ±0.29
White Willow	-3.60 ±3.49	-1.54 ±0.10

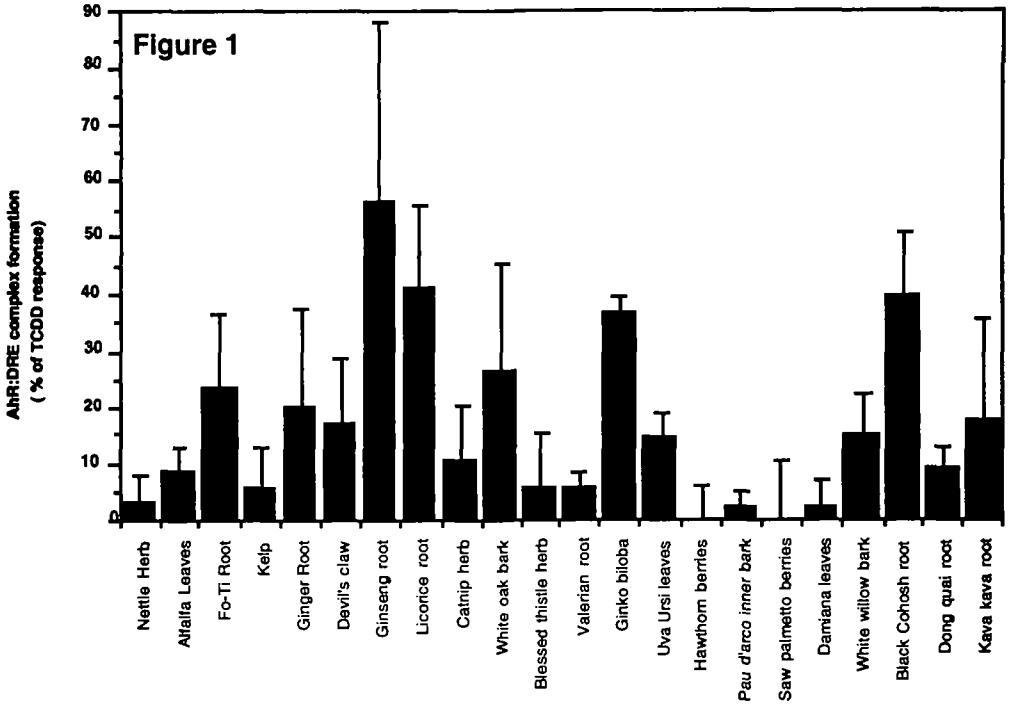
Figure 1
AhR:DRE complex formation
(% of TCDD response)
90
80
70
60
50
40
30
20
10
0
Nettle Herb
Alfalfa Leaves
Fo-Ti Root
Kelp
Ginger Root
Devil's claw
Ginseng root
Licorice root
Catnip herb
White oak bark
Blessed thistle herb
Valerian root
Ginko biloba
Uva Ursi leaves
Hawthorn berries
Pau d'arco inner bark
Saw palmetto berries
Damiana leaves
White willow bark
Black Cohosh root
Dong quai root
Kava kava root

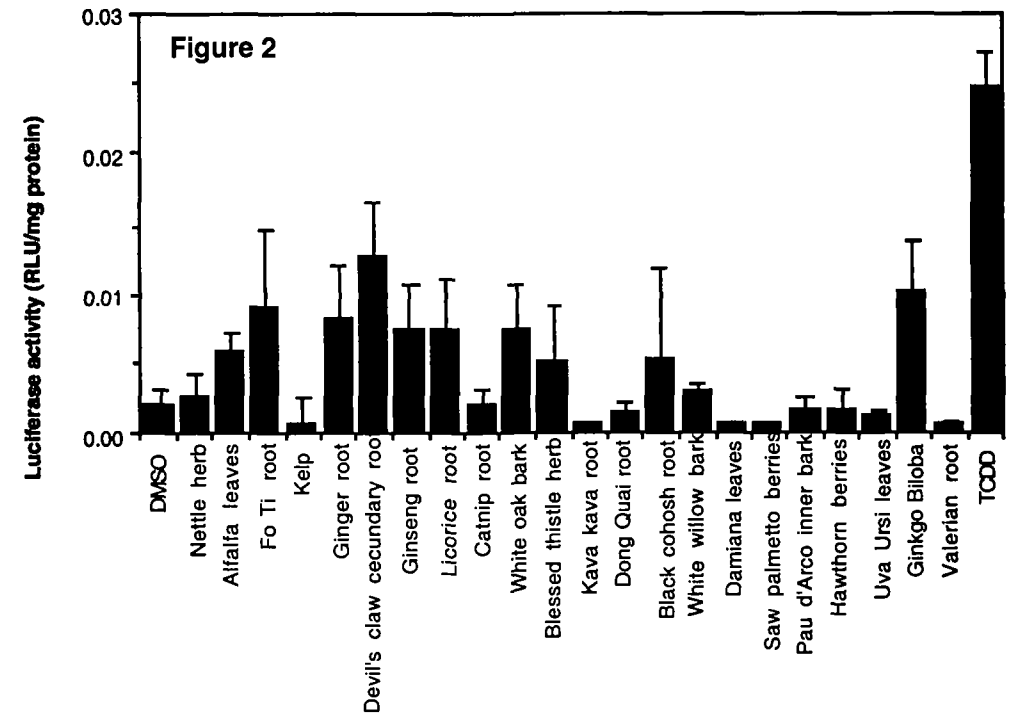
Figure 2
Luciferase activity (RLU/mg protein)
0.03
0.02
0.01
0.00
DMSO
Nettle herb
Alfalfa leaves
Fo Ti root
Kelp
Ginger root
Devil's claw cecundary roo
Ginseng root
Licorice root
Catnip root
White oak bark
Blessed thistle herb
Kava kava root
Dong Quai root
Black cohosh root
White willow bark
Damiana leaves
Saw palmetto berries
Pau d'Arco inner bark
Hawthorn berries
Uva Ursi leaves
Ginkgo Biloba
Valerian root
TCDD