

Epigallocatechin Gallate Inhibits Aryl Hydrocarbon Receptor Gene Transcription through an Indirect Mechanism Involving Binding to a 90 kDa Heat Shock Protein[†]

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ABSTRACT: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor known to mediate the toxic effects of numerous environmental contaminants, including the polycyclic aromatic hydrocarbons (PAHs). Historically, binding of PAHs to the AhR and the events leading to the generation of DNA adducts have been associated with chemical carcinogenesis. Previous investigations have implicated green tea (GT) as affording protection against PAH-induced cancers in animal models. Investigations in our laboratory have demonstrated that the GT polyphenol epigallocatechin gallate (EGCG) is capable of antagonizing AhR-mediated gene transcription, implicating inhibition of AhR signaling as a potential chemopreventive mechanism. This line of investigation was directed at elucidating the molecular mechanism of this antagonism. Competitive binding assays strongly suggest that EGCG does not bind to the AhR ligand binding site, indicating this compound functions through a mechanism unlike that of typical AhR antagonists. Affinity chromatography experiments implicate an indirect mechanism of action involving direct binding of EGCG to the AhR chaperone protein, hsp90. This induces an AhR conformation capable of nuclear localization but incapable of binding DNA. These altered signaling events correlate with the formation of a complex with sedimentation characteristics different from those of the latent or ligand-activated AhR. These data implicate a model in which EGCG inhibits release of hsp90 from the AhR, stabilizing the complex in an intermediary state associated with XAP2. This is the first time EGCG has been demonstrated to directly bind hsp90 and the first indication that GT may exert its chemopreventive effects through an interaction with the common chaperone hsp90.

The AhR¹ is a ligand-dependent transcription factor that can be activated by numerous structurally diverse synthetic and naturally occurring compounds such as polycyclic aromatic hydrocarbons, indoles, and flavonoids. In an unliganded state, the AhR is present in a latent conformation in the cytoplasmic compartment of the cell associated with two molecules of molecular chaperone hsp90 (1, 2), an immunophilin-like protein, XAP2 (3–5), and the hsp90 interacting protein, p23 (6). Ligand binding initiates a cascade of poorly characterized events involving translocation to the nucleus, release of hsp90, and heterodimerization with ARNT (7, 8). The ligand-bound AhR–ARNT complex is capable of recognizing consensus sequences termed dioxin-response elements (DREs) located in the promoter region of CYP1A1 and other responsive genes, thereby modulating transcription (7, 8).

Hsp90 has been shown to be an essential component of the AhR signaling pathway. Its presence has been demonstrated to be necessary in both the proper folding and stability of the AhR complex (9, 10). Additionally, the hsp90–AhR interaction represses AhR activation either through potential steric interference with ARNT dimerization (11, 12) or by interfering with the interaction between the C-terminal transactivation domains or other putative cofactors (13). However, the role this protein serves in nuclear translocation remains unclear. For example, the detection of an hsp90–AhR complex in the nuclei of TCDD-exposed cells (2, 14) strongly implies that hsp90 dissociation may not be required for nuclear import. Conversely, deletion of the PAS domain of the AhR has been shown to result in ligand-independent nuclear translocation of the AhR (15), suggesting the association of hsp90 with the PAS domain prevents the unliganded AhR from entering the nucleus. On the basis of these and other data, it remains unclear whether dissociation of hsp90 is necessary for nuclear import of the AhR or whether its dissociation regulates dimerization with ARNT within the nuclear compartment of the cell. There also remains ambiguity concerning how and when the many other identified AhR-associated proteins, such as p23, XAP2, p60, hsp70, and p48, affect the AhR signaling pathway.

One approach to understanding events required for AhR activation is delineating mechanisms involved in turning this signaling pathway off. Currently, very little is known

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¹ Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DS, degradation signal; DRE, dioxin response element; EGCG, epigallocatechin gallate; GA, geldanamycin; GT, green tea; hsp90, heat shock protein 90; 3M4NF, 3'-methoxy-4'-nitroflavone; NLS, nuclear localization signal; SD, standard deviation.

regarding the mechanism of action of AhR antagonists. Two of the most potent and well-characterized AhR antagonists include the synthetic flavonoid, 3'-methoxy-4'-nitroflavone (3M4NF), and the indole derivative 3,3'-diindolylmethane (DIM). These compounds have been shown to function through direct competition for binding to the AhR ligand-binding site (16, 17). Interestingly, the fate of the AhR upon binding of these structurally distinct antagonists is very different. Binding of 3M4NF to the AhR inhibits TCDD-mediated nuclear localization, ARNT dimerization, and DNA binding (16). 3M4NF is believed to inhibit a conformational change within the AhR complex necessary for exposure of the nuclear localization sequence, resulting in retention of the AhR in the cytoplasmic compartment of the cell. Conversely, binding of DIM to the AhR allows nuclear localization, ARNT dimerization, and subsequent DNA binding. However, unlike the TCDD-bound AhR-ARNT dimer, this DIM-bound complex is incapable of recruiting the necessary cofactors responsible for initiating transcription (17). These findings strongly support the hypothesis that antagonists affect AhR conformation differently than agonists, and provide evidence that structurally diverse antagonists are capable of altering the activation process very differently.

On the basis of the observations described above and what is known about the AhR signal transduction pathway, it is conceivable that an antagonist could interfere with the AhR at numerous stages. These include (1) prevention of release of associated proteins such as hsp90 from the complex, (2) prevention of the association of the ligand-bound AhR with ARNT, and (3) formation of a complex which includes ARNT, but lacks DRE binding ability. In addition, a compound could potentially antagonize AhR activation through indirect processes that do not involve direct binding to the AhR (i.e., ligand independent), including (1) direct inhibition of the proteins involved in nuclear import, (2) direct binding to an associated AhR chaperone protein, (3) inhibition of kinases involved in phosphorylation events, and (4) stimulating protein degradation.

Previous studies from our lab and others have shown the green tea (GT) compound epigallocatechin gallate (EGCG) to have AhR antagonist activity (18-20). The goal of the studies described here was to elucidate the molecular mechanism and consequence of this inhibition. If EGCG were functioning as a competitive antagonist, it would be important to determine how this was altering the AhR-protein complex. Conversely, if EGCG were functioning through a ligand-independent mechanism, it would be important to identify the protein target. On the basis of the structural similarity between EGCG and the known AhR antagonist 3M4NF, it was hypothesized that EGCG functions through a similar mechanism involving competition for binding to the AhR ligand binding site. Interestingly, this was not the case. Competitive binding assays under numerous conditions optimal for low-affinity ligands strongly suggest that EGCG does not bind directly to the AhR. In fact, these studies suggest a ligand-independent mechanism of antagonist action involving direct binding to the chaperone protein hsp90. This binding of EGCG to hsp90 results in nuclear localization of an AhR form incapable of binding to DNA, supporting a model in which the AhR is translocated to the nucleus in the presence of hsp90. These data may also

implicate a novel mechanism responsible for the reported chemopreventive activity of green tea and EGCG.

EXPERIMENTAL PROCEDURES

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Cambridge Isotope Laboratories (Cambridge, MA). [³H]TCDD (specific activity of 34.7 Ci/mM) was purchased from Chemsyn Science Laboratories (Lenexa, KS). EGCG was purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was a kind gift from S. Safe (Texas A&M University, College Station, TX). [*methyl*-¹⁴C]Bovine serum albumin was purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA). 3M4NF and 3'-nitroflavone were synthesized in the laboratory of A. Kende (Department of Chemistry, University of Rochester) as previously described (16). [³H]-β-Naphthoflavone ([³H]BNF) was a kind gift from M. Hahn (Woods Hole, MA).

Preparation of Cytosol. Mouse hepatoma cells, Hepa1c1c7 (Hepa), and BP1C1 cells were maintained in modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, L-glutamine, sodium bicarbonate, and Gentamicin (MEM+), at 37 °C in a humid atmosphere with 5% CO₂. BP1C1 cells are derivatives of Hepa1c1c7 cells that lack ARNT protein expression and function (21). Upon reaching 90% confluency, cells were harvested and homogenized in HEDG buffer [25 mM HEPES, 1.5 mM Na₂EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol (pH 7.6)] containing protease inhibitors (Roche Applied Science complete mini cocktail tablet, Roche Applied Science, Indianapolis, IN). Both Hepa and BP1C1 cytosols were prepared by centrifugation of the homogenate at 100000g for 45 min.

SDS-PAGE and Western Blot Analysis. Hepa cells were plated onto six-well plates at a density of 5 × 10⁵ cells/well and incubated overnight at 37 °C. Cells were treated with vehicle (DMSO), TCDD (150 pM), EGCG, or EGCG in the presence of TCDD (150 pM) for 4 h. Cells were lysed (0.2% Triton X-100 and 5 mM EDTA in PBS), and the total protein was quantified using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Protein (30 μg) was separated by SDS-PAGE (7.5% acrylamide resolving gel) and transferred to a PVDF membrane (Millipore, Bedford, MA). Membranes were probed with antibodies recognizing CYP1A1 (Xenotech, Lenexa, KS), AhR (Biomol, Plymouth Meeting, PA), and actin (Sigma). The secondary IgG antibodies were coupled to horseradish peroxidase (Jackson Immuno Research, West Grove, PA). Both primary and secondary antibodies were used at a dilution of 1:5000 in TBST [50 mM Tris, 300 mM NaCl, and 0.5% Tween 20 (pH 7.5)] containing 5% milk. Proteins were visualized by chemiluminescence (KPL, Gaithersburg, MD).

Luciferase Reporter Gene Assay in Human Cells. Human hepatoma cell line HepG2 was stably transfected with a luciferase expression vector downstream from exon 1, a portion of intron 1, and 1612 bp of the 5'-flanking sequence of the human CYP1A gene to generate the HepG2.101L cell line, as previously described (22). Cells were grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and Gentamicin (DMEM) at 37 °C in a humid atmosphere with 5%

CO₂. Treatment and reporter gene activity were assessed as previously described (18) with the following changes. HepG2.101L cells were added to hydrated Cytodex microcarrier beads (Sigma) to create a density of 1.5×10^6 cells per 30 mg of beads per 10 mL of DMEM. Luminescence was detected using the Packard (Meriden, CT) Lumicount.

Sucrose Density Gradient Analysis. Competitive binding of EGCG was analyzed by velocity sedimentation on sucrose gradients in a vertical tube rotor (23, 24). Hepa cytosol (100 μ L of a 2.5 mg/mL solution) or BPC1 cytosol was incubated for 2 h at room temperature with either 3 nM [³H]TCDD, 0.5 nM [³H]TCDD, or 5 nM [³H]BNF, in the presence of DMSO, a 150-fold excess TCDF, 1 μ M 3M4NF, or EGCG as indicated in the figure legends. Cytosols were washed with charcoal dextran (1 mg of charcoal/mg of protein in HEDG), and applied to a 10 to 30% (w/v) sucrose gradient in Beckman Quick-Seal centrifuge tubes (Beckman, Palo Alto, CA). [*methyl*-¹⁴C]BSA was used as an internal sedimentation marker. The gradients were centrifuged for 2 h at 372000g in a Beckman VTI-80 rotor. Fractions were collected from the top at a rate of 1 mL/min, with 0.2 mL/fraction. For competitive binding assays, fractions were assayed for radioactivity with ScintiVerse (Fisher Scientific, Fair Lawn, NJ). The portion of bound TCDD that could compete with a 150-fold excess of TCDF, a known high-affinity AhR ligand and potent agonist, represents AhR specific TCDD binding. For AhR and hsp90 density analysis, protein contained within an aliquot (20 μ L) of each fraction was separated by SDS-PAGE (7.5% acrylamide resolving gel) and transferred to PVDF membranes. Membranes were probed with antibodies recognizing the AhR (Biomol) or hsp90 (Stressgen, Victoria, BC). A crude estimate of the sedimentation coefficient for the EGCG-shifted AhR complex was calculated by the method of Martin and Ames (25) relative to the [¹⁴C]BSA (4.4 S) standard.

Plasmid Constructs. Murine AhR and ARNT cDNA (obtained from J. Whitlock and O. Hankinson, respectively) were inserted into pcDNA3 (Invitrogen, Carlsbad, CA). Chicken hsp90 constructs (Figure 5), c90711hsp (wild type), c90G94D [wild-type Gly94 \rightarrow Asp point mutant (26)], c90D92A [wild-type Asp92 \rightarrow Ala point mutant (27)], c90N221 [wild-type truncation mutant encoding amino acids 221–728 (27)], c90N303 (amino acids 303–728), and c90N538 (amino acids 538–728), were kind gifts from D. Toft (Rochester, MN). C-Terminal truncation mutant c90C507 (amino acids 1–221) was obtained from L. Neckers (Rockville, MD) (26, 28). Human p23 cDNA was excised from pQE80-p23 (obtained from W. Chan, Stockton, CA) by sequential digests using the restriction enzymes SacI (3'-end) and BamHI (5'-end) and inserted into the similarly digested pET28a (Novagen, Madison, WI). The mouse histidine-tagged XAP2 construct, pET-AIP (29), was a kind gift from D. Bell (Nottingham, U.K.). All constructs are in the T7 orientation with the exception of c90C507, which is driven by an SP6 promoter.

In Vitro Transcription/Translation. AhR, ARNT, hsp90 (wild-type, point mutants, and truncation mutants), p23, and XAP2 were generated (separately) by coupled transcription/translation in rabbit reticulocyte lysate (RRL) using the TNT system according to the manufacturer's protocol (Promega, Madison, WI). In this system, [³⁵S]methionine was included

in the transcription/translation mix to generate ³⁵S-labeled protein.

Immobilized Metal Affinity Chromatography (IMAC) Purification of Histidine-Tagged XAP2. The TALON IMAC method was used for purification of XAP2 from RRL following a modified protocol (Clontech). A portion (500 μ L, 50% slurry) of cobalt resin was washed at room temperature three times with TALON buffer [50 mM Na₂PO₄ and 300 mM NaCl (pH 7.0)] according to the manufacturer's protocol. A 175 μ L aliquot of undiluted [³⁵S]XAP2 in RRL was added to a 15 mL conical tube containing the washed resin in TALON buffer for 20 min at room temperature end over end. The resin-bound XAP2 was pelleted and washed with TALON buffer for 15 min at room temperature. The pelleted resin-bound XAP2 was additionally washed with 7.5 mM imidazole in TALON buffer for 45 min at room temperature. The resin-bound XAP2 was then transferred and packed into a 2 mL disposable column (Pierce) and washed with 5 column volumes of 7.5 mM imidazole in TALON buffer. Ten 200 μ L aliquots of 150 mM imidazole in TALON buffer were used to elute the XAP2 from the column. Each aliquot was collected separately and analyzed for the presence of [³⁵S]XAP2 by SDS-PAGE followed by Phosphorimaging (PSI; Molecular Dynamics, Sunnyvale, CA).

Affinity Chromatography. EGCG was conjugated to cyanogen bromide (CNBr)-activated Sepharose (Sigma). EGCG (2.5 mg) was dissolved in 500 μ L of coupling buffer [0.1 M NaHCO₃ and 0.5 M NaCl (pH 6.0)]. CNBr-activated Sepharose was swelled and washed in 1 mM HCl on a sintered glass filter followed by a wash with coupling buffer. CNBr-activated Sepharose beads were added to the EGCG in coupling buffer at a final concentration of 5 mg of EGCG/mL of wet gel. The coupling solution containing EGCG and Sepharose was mixed end over end at 4 °C overnight. Remaining active groups were blocked for 2 h at room temperature in Tris-HCl (0.1 M, pH 8). EGCG-conjugated Sepharose was washed with three cycles of alternating pH wash buffers [buffer 1, 0.1 M acetate and 0.5 M NaCl (pH 4.0); buffer 2, 0.1 M Tris-HCl and 0.5 M NaCl (pH 8.0)]. EGCG-conjugated beads were then equilibrated in binding buffer [0.05 M Tris-HCl and 0.15 M sodium chloride (pH 7.5)]. The control unconjugated CNBr-activated Sepharose beads were prepared as described above in the absence of EGCG.

Approximately 25 μ L of 1:4 diluted RRL containing ³⁵S-labeled in vitro transcribed protein was incubated with 40 μ L of either unconjugated or EGCG-conjugated Sepharose beads in binding buffer (50% slurry). For the smallest truncation hsp90 mutants (C507 and N538), 50 μ L of 1:4 diluted ³⁵S-labeled in vitro transcribed protein was incubated with 80 μ L of Sepharose beads to compensate for the decreased signal due to fewer methionines. The mixture was diluted with 500 μ L of binding buffer and incubated end over end for 1 h at room temperature. The protein-bound Sepharose beads were pelleted by microcentrifugation at maximum speed for 15 s. The beads were washed three times with binding buffer. The bound protein was eluted with SDS loading buffer [0.125 M Tris, 4% SDS (w/v), 20% glycerol (v/v), 200 mM dithiothreitol, and 0.01% bromophenol blue (w/v) (pH 6.8)]. The samples were boiled for 5 min, and the bound protein was separated by SDS-PAGE. The protein

was transferred to a PVDF membrane (Millipore). ^{35}S -labeled AhR, ARNT, p23, XAP2, and hsp90 truncated and point mutants were detected by Phosphorimaging. Hsp90 inherent to RRL was detected by immunoblotting using an anti-hsp90 monoclonal antibody (Stressgen) followed by a secondary antibody coupled to horseradish peroxidase (Jackson Immuno Research). Both primary and secondary antibodies were used at a dilution of 1:5000 in TBST.

For affinity chromatography using histidine-purified XAP2 and purified human hsp90 (Stressgen), 30 μL of unconjugated or EGCG-conjugated Sepharose was incubated for 1 h at room temperature with either 1:4 diluted [^{35}S]XAP2 in RRL, 30 μL of histidine-purified [^{35}S]XAP2, 0.6 μg of purified hsp90, or 20 μL of histidine-purified [^{35}S]XAP2 in the presence of 0.6 μg of purified hsp90. All incubations were adjusted to contain the same concentration of imidazole as the histidine-purified sample. Protein was bound, and beads were washed and eluted as described above.

Immunocytochemistry. Hepa cells were plated onto four-well chamber slides (Becton Dickinson, Bedford, MA) at a density of 2.0×10^4 and incubated overnight. Cells were treated with either vehicle (DMSO), TCDD (150 pM), EGCG alone (200 μM), or EGCG in the presence of TCDD (150 pM) for 1 h. Cells were fixed with 3.7% formalin at room temperature for 10 min, followed by incubation for 4 min in anhydrous methanol at 4 $^{\circ}\text{C}$. All antibodies were filtered through a 0.45 μm filter before staining. Cells were blocked for 1 h in phosphate-buffered saline containing 4% BSA and incubated with anti-AhR at a 1:2000 dilution for 2 h at room temperature, followed by a 1 h incubation with 1:1000 anti-rabbit Alexa-Fluor conjugated secondary antibody (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (0.5 $\mu\text{g}/\text{mL}$) (Molecular Probes) for 3 min at room temperature. Slides were mounted with 50% glycerol and coverslipped. AhR staining and nuclear staining were visualized using a Nikon Eclipse TS110 fluorescence microscope (40 \times magnification). Fluorescent images were captured using SPOT advanced software.

Electrophoretic Mobility Shift Assay. Hepa cytosol (2.5 mg of protein/mL) was incubated with a range of concentrations of EGCG (1–200 μM) or EGCG in the presence of 3 nM TCDD for 2 h at room temperature. Treated cytosols (21–25 μg) were mixed with nonspecific DNA (herring sperm), 0.08 M NaCl, and 25000–45000 cpm of ^{32}P -end-labeled oligonucleotide containing a single consensus DRE (30). Samples were subjected to nondenaturing electrophoresis (4% acrylamide) and visualized using a Phosphorimager.

RESULTS

EGCG Inhibits TCDD-Induced Gene Expression. Although we have previously demonstrated that EGCG blocks TCDD-induced transcription of a DRE-dependent reporter gene (18), it was important to assess the ability of EGCG to influence an endogenous AhR-regulated gene. To do this, the effect of EGCG on CYP1A1 expression in mouse hepatoma cells was determined. CYP1A1 is highly expressed in this cell type and is known to be transcriptionally induced upon ligand activation of the AhR (31). As shown in Figure 1A, treatment with EGCG alone produced no or only a slight increase in CYP1A1 protein levels. However, treatment of cells simultaneously with EGCG and TCDD showed a

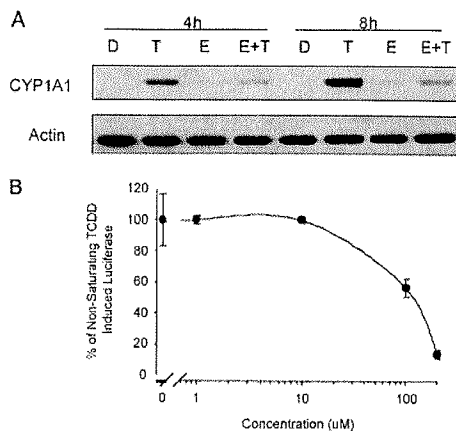


FIGURE 1: EGCG inhibits TCDD-mediated gene induction in both mouse and human hepatoma cells. (A) Hepa1c1c7 cells were treated for the indicated time with either DMSO (D), 150 pM TCDD (T), 200 μM EGCG alone (E), or 200 μM EGCG in the presence of 150 pM TCDD (E+T). Proteins were separated by SDS-PAGE and blotted for CYP1A1 and actin as a loading control. (B) HepG2.101L cells were treated with 500 pM TCDD and increasing concentrations of EGCG for 4 h ($n = 4$). Values are presented as the percentage of observed luciferase induction in the presence of 500 pM TCDD alone \pm SD. Representative data from one of at least three separate experiments are shown.

concentration-dependent inhibition of TCDD-mediated CYP1A1 gene induction. These data support the hypothesis that EGCG is an AhR antagonist capable of inhibiting AhR transcription of an endogenous gene.

To determine if this effect could be observed in other cell types, the antagonist activity of EGCG was assessed in the stably transfected human hepatoma cell line, HepG2. The reporter plasmid, described previously (22), contains the human CYP1A1 promoter and 5'-flanking sequence upstream of the luciferase gene. In this system, EGCG treatment alone did not induce luciferase activity significantly over background (data not shown). However, EGCG inhibited TCDD-induced luciferase activity in a concentration-dependent manner (Figure 1b). This result was similar to that observed previously with mouse hepatoma cells (18). EGCG also inhibited TCDD-mediated CYP1A1 gene induction in HepG2 cells (data not shown). These data suggest that the antagonist effect of EGCG is not specific for mouse hepatoma cells and that this compound is capable of modulating the activity of the human AhR. The remainder of these experiments focus on the inhibitory mechanism of EGCG on the mouse AhR.

EGCG Does Not Compete for Binding to the AhR Ligand Binding Domain. There are many possible mechanisms by which EGCG may function to inhibit TCDD-mediated gene induction. Previous findings from our laboratory suggest that flavonoid antagonists function through direct competition for binding to the TCDD ligand binding site on the AhR (16). This binding of antagonist is believed to result in an AhR conformation incapable of nuclear translocation, DRE binding, and transcriptional enhancement. It was therefore hypothesized that EGCG exerts its effects through an identical mechanism involving direct binding to the AhR ligand binding site.

Velocity sedimentation of the AhR on sucrose density gradients was used to determine if EGCG could inhibit the specific binding of TCDD to the mouse AhR. This methodology was chosen over other binding assays because it

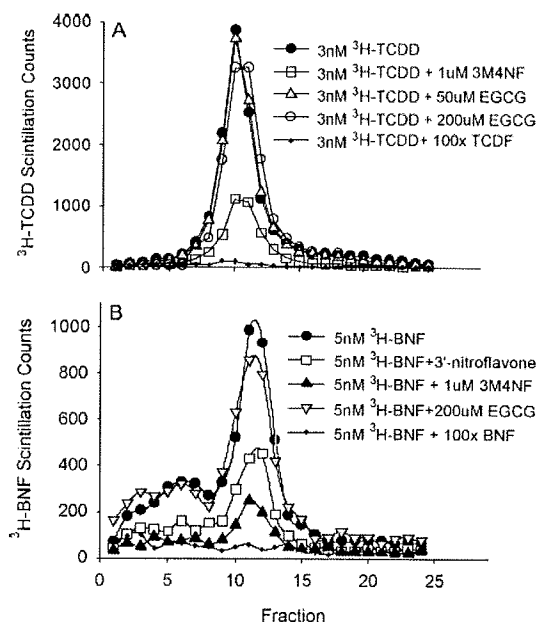


FIGURE 2: EGCG does not compete for binding to the AhR ligand binding site. Competitive binding was analyzed by velocity sedimentation on 10 to 30% sucrose gradients in a vertical tube rotor. Hepa cytosol was treated with 3 nM [^3H]TCDD (A) or 5 nM [^3H]BNF (B) in the presence of the indicated compound for 2 h at room temperature. Cytosols were loaded onto sucrose gradients, spun, and fractionated. Fractions were analyzed for the presence of ^3H , indicative of a ligand-bound AhR complex. The data are representative of at least three experiments.

provides a reliable measure of specific binding to the AhR (32), and has proven to be successful in detecting binding of many low-affinity ligands (33, 34). Incubation of Hepa cytosol with [^3H]TCDD led to the formation of specifically bound [^3H]TCDD–AhR protein complexes within the 9S region (~fractions 10–15) of the gradient (Figure 2A). Co-incubation with [^3H]TCDD and the known AhR antagonist 3M4NF inhibited the formation of this [^3H]TCDD peak, consistent with previous data suggesting that 3M4NF competes for binding to the AhR ligand binding site (16). Interestingly, co-incubation with EGCG failed to attenuate the [^3H]TCDD 9S signal. These data indicate that either EGCG does not bind to the TCDD ligand binding site or it does so with very low affinity.

Sucrose density gradient experiments were also performed using the lower-affinity AhR ligand, [^3H]BNF. Again, EGCG failed to inhibit binding of BNF to the AhR in Hepa cytosol (Figure 2b). However, 3'-nitroflavone, which has been demonstrated to weakly compete for binding with TCDD to the AhR and weakly inhibit TCDD-induced transcription (unpublished data), very efficiently competed with BNF binding in this experimental system. This demonstrates that under these conditions low-affinity ligands are capable of binding to the AhR ligand binding site. Additional experimental adjustments to both treatment time and exposure temperature also failed to alter this experimental outcome for EGCG (data not shown).

To further address the possibility that EGCG is a very low-affinity AhR ligand, additional alterations in the experimental system that could possibly enhance its ability to compete were made. Data from our laboratory (unpublished) and others (35, 36) suggest that binding of ARNT to the

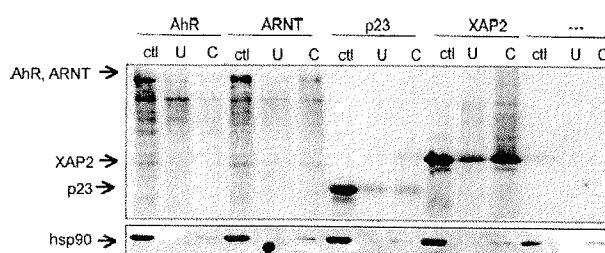


FIGURE 3: Hsp90 and XAP2 elute from EGCG-conjugated beads. [^{35}S]AhR, -ARNT, -p23, and -XAP2 were synthesized *in vitro* in RRL. Unconjugated CNBr-activated Sepharose (U) or EGCG-conjugated Sepharose (C) was incubated with either TnT-translated [^{35}S]AhR, -ARNT, -p23, or -XAP2 in rabbit reticulocyte lysate (RRL), or with RRL alone. The beads were washed, and the bound protein was eluted. The total eluted protein was separated by SDS-PAGE. Input lysate (5 μL) was loaded as a control (ctl). ^{35}S -labeled protein was detected by Phosphorimaging. The hsp90 inherent to RRL was detected by immunoblotting. The gels are representative of three experiments.

TCDD–AhR complex results in enhanced stabilization of the TCDD–AhR interaction into a nearly irreversible complex. If this is occurring, a ligand with low binding affinity may not be able to effectively compete with [^3H]TCDD under conditions that shift the equilibrium to favor a TCDD–AhR–ARNT complex. Therefore, ligand binding was re-assessed in ARNT deficient cytosol obtained from BP $^{\circ}$ C1 cells. In addition, lower concentrations of [^3H]TCDD were used to further favor competitive binding by a weak ligand. Despite these alterations, EGCG was incapable of displacing TCDD from the ligand binding site (data not shown). Pretreatment of BP $^{\circ}$ C1 cytosol for 30 min with EGCG also failed to inhibit TCDD binding (data not shown). Together, these data support a mechanism of action that does not involve direct binding of EGCG to the AhR ligand binding site.

Hsp90 and XAP2 Are Eluted from EGCG-Conjugated Sepharose Beads. On the basis of the competitive binding experiments described above, it is unlikely that EGCG is binding to the TCDD ligand binding site on the AhR. This suggests that EGCG either is binding another site on the AhR or is affecting AhR activity through an indirect mechanism, perhaps involving binding to another protein in the AhR complex such as hsp90, XAP2, p23, or ARNT. To address these possibilities, affinity chromatography was performed using EGCG-conjugated Sepharose. XAP2, ARNT, p23, and AhR proteins were separately transcribed *in vitro* in the presence of [^{35}S]methionine and incubated with either unconjugated Sepharose or EGCG–Sepharose. Binding of these proteins to the Sepharose beads was assessed by Phosphorimaging following SDS-PAGE of the eluted protein. Hsp90 is inherent to RRL; therefore, the ability of this protein to bind EGCG was assessed by immunoblotting. As shown in Figure 3, *in vitro*-translated AhR was not able to bind immobilized EGCG. This supports the competitive binding data suggesting that EGCG does not bind the TCDD ligand binding site and also suggests that EGCG is not binding another site on the AhR. ARNT and p23 were also incapable of binding immobilized EGCG. However, both hsp90 and XAP2 were eluted from EGCG–Sepharose, implicating these two proteins as direct targets of EGCG. Hsp90 and XAP2 were also eluted from EGCG–Sepharose incubated with Hepa cytosol (data not shown), demonstrating that this interaction is not specific to this *in vitro* system.

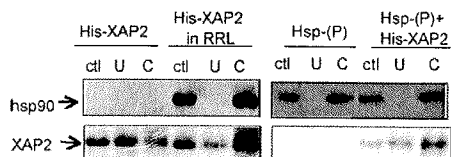


FIGURE 4: Elution of XAP2 from EGCG-conjugated Sepharose requires hsp90. Unconjugated CNBr-activated Sepharose (U) or EGCG-conjugated Sepharose (C) were incubated with either His-purified [35 S]-XAP2 (His-XAP2), His-XAP2 in RRL, purified hsp90 [Hsp-(P)], or His-XAP2 in the presence of Hsp-(P). The beads were washed, and the bound protein was eluted. The total eluted protein was separated by SDS-PAGE. Input lysate (5 μ L) was loaded as a control (ctl). XAP2 was visualized by Phosphorimaging and hsp90 visualized by immunoblotting. The gels are representative of three experiments.

EGCG Binds an Hsp90–XAP2 Associated Complex. It has been demonstrated that XAP2 binds hsp90–AhR complexes and is also capable of binding both proteins independently (37). Therefore, it is conceivable that hsp90 and XAP2 are being eluted as a complex. Considering this, the data in Figure 3 can be interpreted in two ways: (1) EGCG binds two different proteins in the AhR complex, or (2) EGCG binds one protein directly and the other indirectly through a protein–protein interaction. To delineate between these two possibilities, *in vitro*-transcribed histidine-tagged XAP2 was purified using metal affinity chromatography. Purified XAP2 and purified hsp90 (commercially available) were incubated with EGCG–Sepharose alone, or in combination. The beads were washed, and the bound protein was eluted. As shown in Figure 4, purified hsp90 was eluted specifically from EGCG–Sepharose, strongly implicating a direct interaction between this ligand and the hsp90 protein. Interestingly, purified XAP2 was not specifically eluted from immobilized EGCG. Note that this purified protein is devoid of detectable hsp90 (Figure 4, lane 1). However, reconstitution of His-purified XAP2 with purified hsp90 or RRL restores XAP2 elution, strongly suggesting that EGCG directly targets hsp90 and XAP2 is eluted as a result of its interaction with hsp90.

EGCG Binds the C-Terminus of Hsp90. Hsp90 is composed of well-conserved amino- and carboxyl-terminal regions both containing ATP binding domains (28, 38, 39). ATP binding and hydrolysis are essential for the activity of the protein (38), and inhibition of binding of ATP to either domain has been demonstrated to disrupt the chaperone activity of hsp90 and therefore the activity of the client protein (26, 40–42). However, inhibition of the N-terminal domain alters maturation of the hsp90 complex in a manner very different from that of inhibition of the C-terminal domain, suggesting these two domains serve different functions. Furthermore, these domains possess distinct nucleotide binding specificity (39), which provides a means for separating the functions of these domains with the use of site specific inhibitors (28). Therefore, identification of the EGCG binding site on hsp90 was considered to be important in understanding its effects on hsp90 complex association.

To determine which site was responsible for the interaction between EGCG and hsp90, several hsp90 mutants were tested for their ability to bind to immobilized EGCG. Geldanamycin (GA) is known to specifically bind the N-terminus of hsp90 (26). As shown in Figure 5, the amino-terminal hsp90 fragment containing the GA binding site (C507) failed to bind to immobilized EGCG. Furthermore, several N-terminal

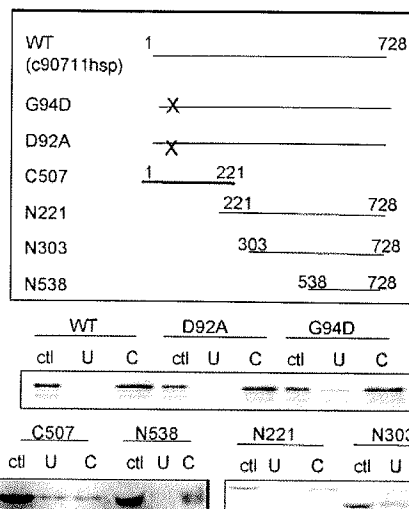


FIGURE 5: Map of chicken hsp90 constructs and their ability to bind EGCG-conjugated Sepharose. The top panel illustrates various mutant hsp90 constructs. Wild-type (wt) chicken hsp90 consists of 728 amino acids. Various mutated amino acids are marked with an X. In the bottom panels, RRL containing the indicated *in vitro*-transcribed [35 S]hsp90 construct was incubated with either unconjugated (U) or EGCG-conjugated (C) Sepharose. The beads were washed, and the bound protein was eluted. The total eluted protein was separated by SDS-PAGE. Input lysate (5 μ L) was loaded as a control (ctl). Hsp90 was visualized by Phosphorimaging. All truncation mutants were run on the same gel. The lower signal associated with the smaller fragments required additional gray scale image adjustments and therefore appears here as a separate image. The data are representative of three experiments.

point mutants (D92A and G94D) known to abrogate binding of GA to hsp90 (26) bound to EGCG–Sepharose as well as or better than wild-type hsp90. Interestingly, all C-terminal fragments bound EGCG. Analysis of the smallest truncation mutant containing amino acids 538–728 (N538) revealed that binding of EGCG occurs within this region of the protein, suggesting an interaction with the C-terminal ATP binding site (Figure 5).

EGCG-Induced Nuclear Localization of the AhR. To begin to understand the molecular consequences of the EGCG–hsp90 interaction on AhR function, it was important to determine which process within the AhR activation pathway EGCG is capable of inhibiting. After ligand binding, the next well-defined event required for AhR gene activation to occur is nuclear localization. To assess the effects of EGCG treatment on nuclear uptake of the AhR, Hepa cells were treated and the subcellular localization of the AhR was visualized by immunofluorescence microscopy. As expected, treatment of cells with TCDD for 1 h resulted in a redistribution of the AhR from the cytosol to the nucleus (Figure 6) (43). Treatment with EGCG did not attenuate this TCDD-induced nuclear localization of the AhR. Interestingly, when cells were exposed to EGCG alone, there was a remarkable redistribution of the AhR from the cytosol to the nucleus. In fact, EGCG treatment alone was just as good, if not better, at nuclear redistribution of the AhR than TCDD. These data indicate that binding of EGCG to hsp90 substantially alters the conformation or protein–protein interactions of the AhR complex, resulting in a redistribution of the AhR to the nuclear compartment of the cell. These observations suggest that EGCG affects the AhR within the cytoplasmic compartment of the cell. These data also

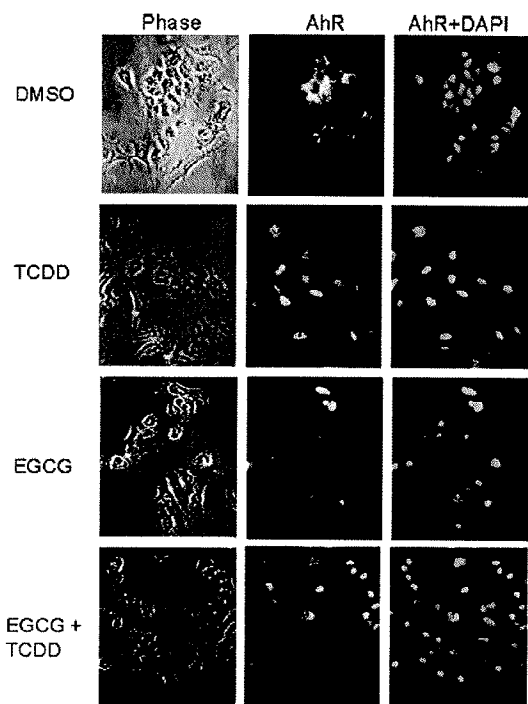


FIGURE 6: EGCG induces nuclear localization of the AhR. Hepa cells were treated for 1 h with DMSO, 150 pM TCDD, 200 μ M EGCG alone, or 200 μ M EGCG in the presence of 150 pM TCDD. Cells were stained with anti-AhR and visualized with Alexa Fluor-conjugated fluorescent secondary antibody (middle). To help visualize nuclear localization, nuclei were detected with DAPI (blue) and overlaid with the AhR (red) image (right). Nuclear localization is emphasized by the absence of blue staining and the presence of purple staining within the nuclear compartment of the cell. The images depicted in this figure are representative of three experiments.

demonstrate that nuclear localization of the AhR does not necessarily reflect the transcriptional activity of this protein.

EGCG Inhibits TCDD-Induced Binding of the AhR to Dioxin Responsive Elements. Considering the EGCG-bound AhR localized to the nucleus, it was important to determine if EGCG was inhibiting TCDD-mediated gene induction through a mechanism involving inhibition of the AhR–DNA interaction. To address this question, Hepa cytosol was incubated with EGCG in the presence and absence of TCDD and DNA binding forms analyzed by EMSA. Cytosol treated with EGCG exhibited a concentration-dependent decrease in the TCDD–AhR–DRE shifted band (Figure 7). This compound was able to inhibit binding by nearly 100% at 100 μ M, providing evidence that EGCG alters the ability of a TCDD-bound AhR to transform into a DNA-binding form. EGCG had the same effect on TCDD-induced binding when extracts from treated whole cells were isolated (data not shown). In both systems, DRE binding was not observed with EGCG treatment alone.

EGCG Does Not Affect AhR Degradation. It is well established that the AhR protein is downregulated under many experimental conditions following agonist exposure both in vivo and in whole cells (44). Specifically, in Hepa cells, the concentration of the AhR rapidly declines after being exposed to TCDD for 2 h (43, 45), resulting in a dramatic decrease in the half-life of the AhR (46). Previous reports have demonstrated that inhibition of this ligand-

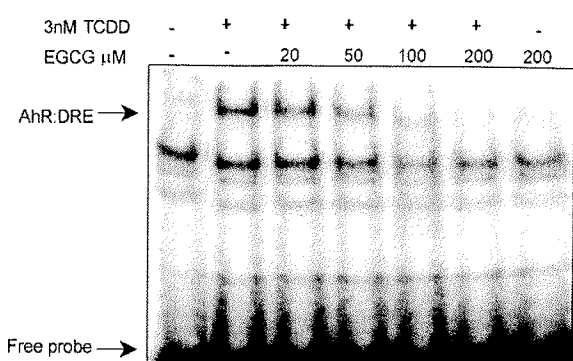


FIGURE 7: EGCG inhibits TCDD-induced DRE binding. Hepa cytosol was treated with the indicated concentrations of EGCG in the absence or presence of 3 nM TCDD for 2 h. Treated cytosols were incubated with [³²P]DRE and levels of transformed DRE–AhR complex determined as described in Materials and Methods. The autoradiograms illustrated in this figure are representative of three experiments.

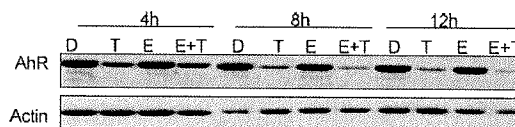


FIGURE 8: EGCG does not affect AhR degradation. Hepa cells were treated for the indicated time with either DMSO (D), 150 pM TCDD (T), 200 μ M EGCG (E), or 200 μ M EGCG in the presence of 150 pM TCDD (E+T). Proteins were separated by SDS–PAGE and blotted for AhR (top) and actin (bottom) as a loading control. The Western blots are representative of three experiments.

induced degradation results in an increase in the magnitude and duration of the induction of AhR-responsive genes (46, 47). Conversely, it has been demonstrated that TCDD-induced gene induction can be inhibited as a result of AhR degradation (48). These data emphasize the importance of AhR protein concentration in the response of cells to ligands, indicating that downregulation of the AhR serves a role in the attenuation of the gene regulatory response.

The importance of AhR stability and its effect on gene induction have been further emphasized in studies utilizing the hsp90 inhibitor GA. Exposure to GA inhibits TCDD-induced gene transcription through a mechanism involving destabilization of the hsp90–AhR complex, resulting in rapid proteolysis of the AhR (48, 49). On the basis of this knowledge, it is possible that EGCG could be inhibiting AhR gene induction through a mechanism involving enhanced protein degradation. To assess this possibility, cells were treated with DMSO, TCDD, EGCG, or EGCG in the presence of TCDD over a time period of 12 h and the levels of AhR protein determined by Western blotting. As expected, AhR levels decline rapidly upon exposure to TCDD (Figure 8). This decrease was not exacerbated or prevented by simultaneous treatment with EGCG. Furthermore, treatment with EGCG alone was not capable of inducing AhR degradation, supporting the hypothesis that EGCG does not destabilize the AhR–hsp90 interaction.

EGCG Affects AhR Complex Association Differently than TCDD. Sucrose density gradient centrifugation has been used extensively in the determination of molecular masses for individual and multiprotein complexes and to assess alterations in protein–protein interactions based on sedimentation coefficients within the gradient. The AhR in cytosolic

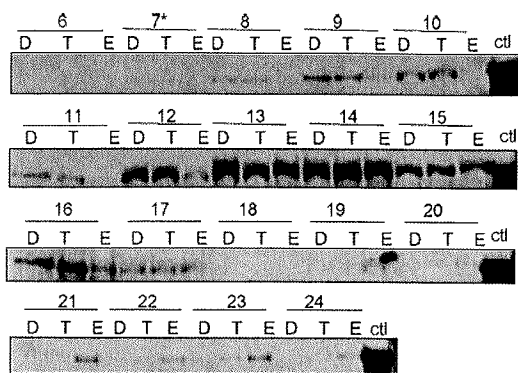


FIGURE 9: EGCG treatment results in an AhR complex that differs from both the latent and TCDD-activated complex. Hepa cytosol was treated for 2 h with DMSO (D), 10 nM TCDD (T), or 200 μ M EGCG (E) and loaded onto a 10 to 30% sucrose density gradient for analysis by velocity sedimentation in a vertical tube rotor. The gradients were fractionated, and the presence of the AhR within each fraction was assessed by Western blotting. [14 C]BSA (4.4S) was used as a sedimentation standard and was detected in fraction 7 as indicated by the asterisk. The AhR was not detected in fractions 1–5 under any treatment conditions. The Western blots are representative of three experiments.

samples sediments under conditions of low ionic strength as a specific peak in the \sim 9.8S region of the sucrose density gradient (32, 36). Previous studies have established that this peak represents an AhR complex associated with two molecules of hsp90 (2, 21). Interestingly, the sedimentation properties of the cytosolic AhR that has been transformed to a DNA-binding form *in vitro* have been reported to be the same as those of the native cytosolic AhR (50). This is observed in our sucrose density gradient experimental system and is demonstrated in Figure 2 where incubation of Hepa cytosol with [3 H]TCDD results in a specific peak of radioactivity approximately spanning fractions 8–14 (\sim 9S fraction).

Sucrose density gradients were used to determine the effects of EGCG on the sedimentation properties of the AhR. These experiments demonstrate that upon exposure of Hepa cytosol to TCDD, the AhR sediments in fractions 8–16 (Figure 9). This sedimentation profile corresponds to the \sim 9S region and correlates with the radioactive peak observed in Figure 2 upon detection of bound [3 H]TCDD. This sedimentation pattern is similar to the sedimentation pattern in the absence of ligand with the exception of a slightly higher density shift associated with TCDD treatment. This higher level of AhR in fraction 12 upon exposure to TCDD was consistently observed in repeat experiments. Interestingly, EGCG exposure consistently resulted in a different sedimentation pattern. Within the 9S region of the gradient, the EGCG-treated AhR was found predominantly in fractions 13–15. Furthermore, the AhR was detected within fractions 21–23 (\sim 13–14S) following EGCG treatment. These data suggest that EGCG affects the AhR complex very differently than TCDD, strongly implicating altered conformation or protein–protein interactions in mediating the effects of EGCG on the AhR.

Considering EGCG targets hsp90 directly, it was of interest to assess its effects on hsp90 sedimentation as well. As shown in Figure 10, hsp90 sediments predominantly within fractions 8–10 in an untreated cell. TCDD treatment did not appear

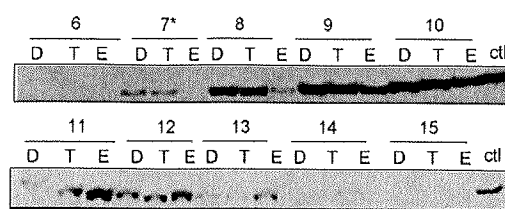


FIGURE 10: EGCG alters hsp90 complex association as assessed by density sedimentation. Hepa cytosol was treated for 2 h with DMSO (D), 10 nM TCDD (T), or 200 μ M EGCG (E) and loaded onto a 10 to 30% sucrose density gradient for analysis by velocity sedimentation in a vertical tube rotor. The gradients were fractionated, and the presence of hsp90 within each fraction was assessed by Western blotting. [14 C]BSA (4.4S) was used as a sedimentation standard and was detected in fraction 7 as indicated by the asterisk. Hsp90 was not detected in fractions 1–5 or 16–24 under any treatment conditions. The Western blots are representative of three experiments.

to influence this sedimentation pattern. However, EGCG treatment dramatically altered the sedimentation pattern of hsp90 as demonstrated by the loss of hsp90 detection in fractions 7 and 8 and an increase in the level of hsp90 detection in fractions 11–13. Although the limitations of this system prevent us from drawing any conclusions regarding hsp90–AhR association, these experiments strongly suggest that EGCG affects hsp90 and that these effects are not specific to the hsp90 bound to the AhR.

DISCUSSION

These experiments demonstrate that EGCG, a compound that is structurally similar to known AhR ligands, inhibits AhR activity through a mechanism that does not involve direct binding to the TCDD ligand binding site. To date, most, if not all, AhR antagonists have been found to bind to the AhR protein. Our data strongly emphasize that competing for binding to the AhR ligand binding site is not the only mechanism of action for AhR antagonists and that structural similarity to known AhR ligands does not necessarily imply competitive binding.

To conclusively determine that EGCG was not binding to the AhR, it was important to adjust our experimental system to favor low-affinity competition. Some of these modifications included (1) decreasing the concentration of TCDD, (2) increasing the concentration of EGCG, and (3) using the lower-affinity competing ligand, BNF. Successful competition by the low-affinity ligand 3'-nitroflavone indicated that these conditions were conducive for low-affinity binding (Figure 2B). However, EGCG was incapable of competing for binding to the AhR under any of these experimental conditions. Furthermore, *in vitro*-translated AhR failed to bind EGCG–Sepharose (Figure 3). Affinity chromatography using purified proteins further indicated that EGCG binds the C-terminus of hsp90 (Figure 5) and indirectly elutes XAP2 as a result of the hsp90–XAP2 interaction (Figure 4). Together, these data strongly imply that EGCG does not bind the ligand binding domain or any other domain on the AhR, and alters AhR activity through an interaction with hsp90.

The observation that EGCG treatment alone induces a rapid and profound redistribution of the AhR to the nuclear compartment of the cell (Figure 6) suggests that EGCG exerts its initial effect on the AhR within the cytoplasm. These data

emphasize that EGCG is not necessarily functioning only to block a TCDD-induced effect but is capable of modulating the receptor conformation/complex association on its own. Once in the nucleus, the EGCG-bound AhR complex is incapable of binding DREs (Figure 7). Notably, a recent publication observed that concentrations of EGCG as low as 1 μ M were effective at inhibiting binding of the rat AhR to DREs (20). Considering that the AhR cannot bind DNA in the absence of ARNT (21, 51), these data suggest that EGCG inhibits ARNT dimerization. The inability of EGCG treatment of Hepa cell cytosol to elicit DRE binding of the AhR (Figure 7) is also consistent with this.

In the cytoplasm, the AhR exists in a complex with two molecules of hsp90. These hsp90 molecules contact the AhR in two regions: the bHLH region located at the N-terminus of the protein and the PAS domain. Within the PAS domain, the AhR-hsp90 interaction overlaps with the ligand binding domain and the ARNT dimerization domain (11, 12, 52, 53), whereas within the basic region, it overlaps with both the DNA binding region (53, 54) and the nuclear localization sequence (55). These interactions result in an AhR conformation capable of binding a ligand with high affinity (9–11, 56) and incapable of ARNT dimerization due to steric interference (11, 12, 57). In response to the ligand, two possible pathways for the AhR have been proposed. (1) The AhR complex dissociates in the cytoplasm, and free AhR becomes associated with nuclear transport proteins to be translocated to the nucleus. (2) Ligand binding initiates nuclear translocation of the intact complex where hsp90 and XAP2 dissociate prior to, or in concert with, dimerization with ARNT. The data presented here are consistent with a model in which EGCG binding to hsp90 results in a conformational change responsible for a modification of the hsp90-AhR interaction with the bHLH region of the AhR and enhanced stabilization of the PAS-hsp90 interaction. This results in exposure of the nuclear localization signal, supporting a model for nuclear localization of an hsp90-associated AhR complex. In the nucleus, enhanced stabilization of the hsp90-AhR interaction within the PAS domain prevents further dissociation of this complex and possibly ARNT dimerization. Ligand-induced translocation of the AhR-hsp90 core complex prior to dissociation has been previously suggested, supporting the model proposed here (2, 58, 59).

We propose a model in which EGCG maintains AhR protein levels through stabilization of the AhR-hsp90 association. However, if this is occurring, then why upon simultaneous treatment with TCDD does the AhR still undergo TCDD-mediated degradation (Figure 8)? It is well established that the AhR-hsp90 interaction is an important determinant of AhR stability (48, 58). However, the processes responsible for mediating ligand-induced degradation of the receptor remain unclear. We propose that there are two distinct signals for AhR degradation: one mediated by the dissociation and/or altered binding of hsp90 and its associated proteins and the other by the agonist-elicited activation of the AhR. Currently, GA-mediated degradation of hsp90 client proteins, including the AhR (60), is thought to occur through a CHIP (C-terminal hsp70-interacting protein)-mediated mechanism. Although the details that control these pathways are poorly understood, CHIP initiates degradation through a process involving binding to the tetratricopeptide repeat motif

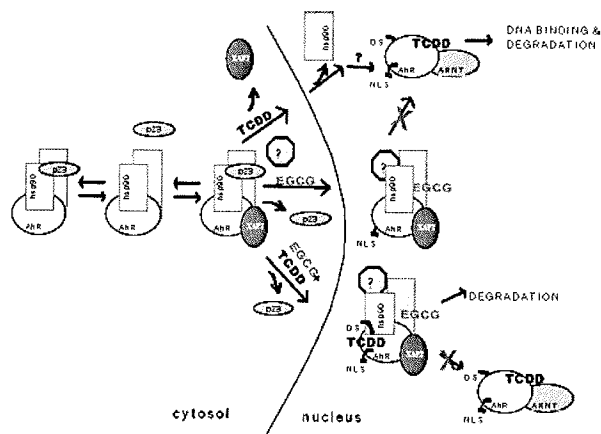


FIGURE 11: Proposed model for the dynamic complex association of the AhR. Following assembly of the AhR-hsp90 complex, the AhR exists in multiple forms within the cell determined by the absence or presence of XAP2 and/or p23. These receptor forms exist in a dynamic equilibrium with one another, and it is possible that they are functionally unique. TCDD binding to the AhR exposes the nuclear localization signal (NLS) and a proposed degradation signal (DS). However, this conformational change is not sufficient for DNA binding, and transformation to an AhR-ARNT conformation requires an additional currently undefined event (?). Binding of EGCG to hsp90 alters the AhR conformation to expose the NLS and shifts the equilibrium toward an AhR complex associated with XAP2 and devoid of p23. Unpublished observations also suggest the inhibitory effect of EGCG on AhR transformation involves recruitment of an unknown protein to the AhR complex (represented by the octagon). The additional presence of TCDD has its own influence on AhR conformation, resulting in exposure of the DS. However, the EGCG-induced conformation prevents TCDD-mediated AhR-ARNT association either through stabilization of the AhR-hsp90 interaction or through prevention of the currently undefined transformation event.

(TPR) on the hsp90-hsp70 chaperone complex, ubiquitination, and the 26S proteasome (61–65). Although GA induces degradation of the AhR to the same degree as TCDD (64), data suggest that these two degradation processes are distinct. Song and colleagues demonstrated that GA-mediated degradation occurs at a much faster rate than TCDD-mediated degradation (64). GA-mediated degradation is not altered by the nuclear export inhibitor leptomycin B, whereas TCDD-mediated degradation is (48). Furthermore, TCDD induces an AhR conformation void of its hsp90 chaperone complex. By releasing this complex, the TCDD-activated AhR loses its link to the CHIP-mediated degradation pathway, suggesting an alternative signal is responsible for TCDD-mediated degradation. In the model proposed here (Figure 11), stabilization of the AhR-hsp90 complex by EGCG may only affect the stronger interactions with the PAS domain of the AhR and may not interfere with the potential ligand-induced changes necessary for degradation. Alone, EGCG may maintain the hsp90 conformation in a state incapable of recruiting degradation cofactors such as CHIP. However, when TCDD binds in the presence of EGCG, the AhR conformation is altered, exposing the AhR degradation signal resulting in ligand-induced degradation following nuclear localization. Interestingly, a TPR half-site has been identified (65) in the N-terminal region of the AhR, and it may be that CHIP, or another unknown protein, binds directly to this site to mediate degradation. It has also been demonstrated that deletion of the transactivation domain of the AhR greatly inhibits TCDD-mediated degradation (46), suggesting the

degradation signal may be contained within the C-terminal domain of the receptor. In any case, we propose that TCDD binding to the AhR results in a conformational change responsible for exposing a proposed degradation signal on the AhR that is not dependent on its association with hsp90. These observations strongly implicate the involvement of distinct protein-protein interactions, and/or AhR domains in the degradation of the inactive versus the TCDD-activated AhR complex.

The data in this paper implicate a model in which EGCG inhibits the release of hsp90 from the AhR complex. This form of hsp90 appears to maintain its interaction with XAP2 (Figure 4). These data indicate that the hsp90-XAP2 complex remains bound to the AhR upon EGCG treatment, EGCG prevents ARNT dimerization, and EGCG stabilizes the receptor in a different complex upon additional treatment with TCDD. To date, attempts to definitively address the mechanisms for these events have been met with some difficulties. However, findings from these attempts add some useful insights. The mouse AhR used for these studies was transcribed *in vitro* in rabbit reticulocyte lysate (RRL) and immunoprecipitated using an antibody specific for amino acids 12-31 (data not shown). Although this antibody could precipitate the latent and TCDD-activated AhR complex from RRL, it was incapable of precipitating an EGCG-treated AhR (data not shown). Immunoprecipitation under denaturing conditions restored the ability to immunoprecipitate the AhR following EGCG treatment, suggesting that an EGCG-induced conformational change was responsible for the lack of antibody recognition. Interestingly, this conformational change did not appear to occur in Hepa cytosol because the AhR could be successfully immunoprecipitated from this system following EGCG treatment. Furthermore, titration of Hepa cytosol into the RRL system restored the ability to immunoprecipitate [³⁵S]methionine-labeled, *in vitro*-transcribed AhR. Similar discrepancies between systems were observed utilizing DNA binding as an end point. EGCG treatment of AhR and ARNT in RRL resulted in the formation of a strong DRE-AhR-ARNT shifted complex (data not shown). This is in direct contrast to the lack of AhR transformation by EGCG observed in Hepa cytosol (Figure 7). Furthermore, the EGCG-induced DNA binding complex had a mobility different from that of the TCDD-induced DNA binding complex, suggesting an altered conformation or protein association following AhR activation by EGCG as compared to TCDD in the RRL system. This EGCG-induced DNA binding complex could also be attenuated upon titration of Hepa cytosol into RRL. Together, these observations support the presence of an additional inhibitory factor within Hepa cytosol that is not present in RRL and that is pertinent to the antagonist effects of EGCG on the AhR complex.

The ability of hsp90 inhibitors to affect AhR function is not a novel concept. However, comparison of the effects of EGCG treatment on the AhR signaling pathway with those of two other characterized hsp90 inhibitors suggests a unique mechanism of inhibition. Binding of GA to the N-terminal ATP binding pocket on hsp90 functions to destabilize the hsp90-AhR interaction, resulting in the release of p23 and XAP2 (6, 40, 66, 67), signaling the AhR for degradation (48, 49). Conversely, the inhibitor molybdate stabilizes the hsp90-AhR interaction through an unknown mechanism,

resulting in enhanced association with p23 and stabilization of the AhR protein (6, 58). Interestingly, EGCG appears to alter AhR function in an intermediate manner. On the basis of the data in Figure 4 suggesting that EGCG targets an hsp90-XAP2 complex, we propose that EGCG retains the AhR in a conformation that remains bound to both hsp90 and XAP2. This retention of XAP2 directly reflects a particular conformational state of hsp90, and its presence within the AhR complex has important functional consequences. In the literature, XAP2 has been implicated in many processes, including enhancing AhR stability (37, 66), decreasing the extent of AhR ubiquitination (66), and enhancing nuclear targeting (4, 68). The actual end result of XAP2 association may also depend on the species of origin of the AhR (69). In the model proposed here for the mouse AhR, the simultaneous contact of XAP2 with the AhR and hsp90 in the presence of EGCG appears to enhance the AhR-hsp90 interaction, resulting in increased AhR stability and protein levels and enhanced nuclear uptake. More importantly, XAP2-mediated enhancement of the hsp90-AhR interaction may be pertinent in preventing the AhR from binding DNA.

Currently, there is extensive literature addressing the numerous biological effects of EGCG on cellular function, including inhibition of telomerase (70) and kinase (71-73) activities, as well as altering the normal function of numerous transcription factors (71, 74, 75). Interestingly, a large number of these affected proteins are also hsp90 client proteins. The observed shift in the sedimentation rate of hsp90 following EGCG treatment (Figure 10) suggests that EGCG may target any and all cellular hsp90. This overlap between hsp90 inhibition by EGCG and EGCG inhibition on numerous hsp90 client proteins provides a very desirable explanation for how one compound, and green tea, could have so many biological effects. Future studies aimed at assessing the general effect of EGCG on other hsp90 client proteins will help address this possibility.

In conclusion, EGCG inhibits AhR transcriptional activation through an indirect mechanism involving direct binding of EGCG to the C-terminus of the AhR chaperone protein hsp90. This is the first time EGCG has been demonstrated to bind hsp90 directly and therefore the first indication that this compound may function as an hsp90 inhibitor. EGCG appears to target a XAP2-bound hsp90 complex, suggesting this compound inhibits hsp90 function differently than currently reported hsp90 inhibitors. Elucidation of the exact mechanisms responsible for the effects of hsp90 inhibition by EGCG on AhR stability, DNA binding activity, cellular localization, and protein-protein interactions will help to refute or support a unique mechanism of hsp90 inhibition and provide further insight regarding how AhR-associated proteins are involved in receptor regulation.

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