

Activation of Aryl Hydrocarbon Receptor Signaling by Cotton Balls Used for Environmental Enrichment

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Dioxins are nearly ubiquitous environmental contaminants that are produced as byproducts during industrial processes, including the bleaching of paper and textiles. Contamination of animal bedding material with dioxins has been a concern for both laboratory and farm animals. The objective of this study was to determine whether the presence of cotton balls, provided to mice for enrichment, caused induction of the cytochrome P450 1A1 gene (*Cyp1A1*), which typically is stimulated through activation of the aryl hydrocarbon receptor (AhR) by dioxins and dioxin-like compounds. *Cyp1A1* transcripts and protein in the liver were increased significantly by either exposure to cotton balls or treatment with a single dose of 2,3,7,8-tetrachlorodibenzo-*para*-dioxin. Unexposed controls displayed low levels of *Cyp1A1* transcript and undetectable levels of CYP1A1 protein. These results suggest that cotton balls are potentially contaminated with dioxins and/or dioxin-like compounds that act as potent inducers of *Cyp1a1* in laboratory animals if used as nesting material. This study underscores the necessity of considering dioxin content in products used for enrichment in animal facilities.

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear transporter; Cyp1A1, cytochrome P450 1A1.

The primary function of the cytochrome P450 1 (Cyp1) family of hemoproteins is the metabolism of foreign compounds including drugs, food additives, and environmental pollutants,⁴¹ although more recent evidence suggests a role in homeostatic functions. Induction of Cyp1A1/2 occurs in response to increased activity of the aryl hydrocarbon receptor (AhR). AhR is activated by numerous, structurally diverse chemical substances as well as a variety of endogenous ligands. Endogenous ligands include indoles, tetrapyroles, arachidonic acid metabolites, retinoids, and oxysterols, as well as heme and tryptophan metabolites.^{14,44} These weak AhR agonists likely cause transient induction of Cyp1A1/2, which leads rapidly to their degradation.¹⁴ A number of naturally occurring, dietary compounds are also AhR agonists; flavonoids and indoles, or gut-derived metabolites of these compounds, are the most common. Like the endogenous ligands, these substances are weak AhR agonists and substrates for degradation by Cyp1A enzymes.¹⁴ Anthropogenic chemicals, produced largely as byproducts of industrial processes, are the most well-studied ligands of AhR. Polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene and 3-methylcholanthrene, are biotransformed by Cyp1A enzymes into carcinogens.⁴⁴ Toxic effects linked to AhR activation occur after exposure to high levels of polychlorinated biphenyls, which are chemically stable constituents of many commercial products. However, the most potent of the known AhR agonists are the dioxins.

The term 'dioxins' refers to polyhalogenated aromatic hydrocarbons that are structurally similar and share a common mechanism of toxicity. The dioxin family includes several polychlorinated dibenzofurans, polychlorinated biphenyls, and polychlorinated dibenzo-dioxins. Dioxins are among the most toxic chemicals known to man.^{5,44} The detrimental human health effects of the most toxic of the dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were recently highlighted in the popular press after the deliberate poisoning of Ukrainian president Victor Yushchenko during his 2004 election campaign.^{8,18} Accidental human exposures at high concentrations in Italy,⁴⁸ Japan,¹ and Vietnam³⁶ have resulted in a plethora of health problems, including dermal and ocular lesions, altered immune responses, irregular menstrual cycles, and multisite carcinogenesis.

Dioxins and other AhR agonists are responsible for a variety of health related effects in biologic systems.⁵⁵ Very small doses of dioxins can cause wasting syndrome and ultimately death in some laboratory animals.⁶ Dioxins cause developmental defects and immune deficiency³⁴ and are linked to pathologies of the central and peripheral nervous system.¹² Dioxins are well-known endocrine disruptors, interfering with reproductive^{19,20} and thyroid hormone homeostasis⁵⁹ and are associated with the development of diabetes.⁶² TCDD and other AhR agonists, benzo[*a*]pyrene, and 3-methylcholanthrene are carcinogenic.⁴⁴ Mechanistically, dioxins exert their biologic effects through the AhR, a ligand-activated basic helix-loop-helix transcription factor that belongs to the Per-Arnt-Sim domain family of chemosensors. Ligand binding promotes translocation of the AhR-ligand complex to the nucleus, where it associates with the aryl hydrocarbon nuclear transporter (ARNT), which directs the complex to dioxin response elements on target genes. Important target genes regulated by AhR-ARNT include the xenobiotic metabolizing enzymes, such as cytochrome P450 1A1 (Cyp1A1). Induction of Cyp1A1 is commonly used as an indicator

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of AhR activation.²⁵ Recent studies indicate that activated AhR also may interact directly with proteins involved in cell cycle control,²⁸ elements of the apoptotic machinery,¹² and other cellular kinases.⁵¹ Therefore, the vast array of effects attributable to AhR agonists should raise concern about their presence to a broad spectrum of researchers. Clearly, the presence of dioxins or dioxin-like compounds has the potential to create havoc in numerous research studies.

Human and animal exposure to dioxins typically occurs subsequent to their incorporation in food, water, soil, and air. Dioxins are produced as unwanted byproducts of industrial processes; they are formed during the combustion of organic substances in the presence of chlorine, such as during the process of paper and cotton bleaching, or from industrial chemical synthesis, such as the manufacture of certain pesticides, herbicides, and fungicides. Food of animal origin is a leading source of dioxin exposure in animals and humans.³¹ Previous investigations have demonstrated dioxin contamination of commercially available rodent chow.⁵⁶ Various types of laboratory animal and farm animal bedding have been found also to contain dioxins.^{26,47} Relevant to the present study, dioxin contamination occurs in pulp-based products and textiles, including tampons, diapers, cotton balls, and textiles, as a result of bleaching in the presence of chlorine.^{2,16,22} Dioxins are highly lipophilic compounds that demonstrate remarkable resistance to metabolism in vertebrate species: the half life of the most potent dioxin, TCDD, is estimated to be 2 to 4 wk in rodents⁵³ but can be as long as 11 y in humans.⁴⁹ These properties promote bioaccumulation throughout the food chain.

The *Guide for the Care and Use of Laboratory Animals*²⁴ states that the availability of cage enrichments should be considered as 1 of several factors to provide an adequate environment for the appropriate care of rodent species. Enrichment materials often are provided in the form of a rigid shelter or some sort of nesting material. Nesting materials, which allow animals to exert some degree of control over their environment and permit expression of natural 'nesting' behaviors, are preferred by mice over rigid shelters.⁶¹ Paper-derived nesting materials are preferred over wood-derived materials.⁶⁰ Therefore, the use of paper products, including paper towels, and cotton balls has become a common, inexpensive source for enrichment provided in animal care facilities. However, the potential effect of dioxin contamination in these products used for enrichment has not been explored. Because the bleaching process often generates dioxins as byproducts, potential contamination of enrichment materials is of great concern. After the Division of Animal Resources at our university instituted a policy of providing cotton balls to rodents for enrichment, we conducted the present study to determine whether the presence of cotton balls can sufficiently activate aryl hydrocarbon receptor signaling. We found that transcripts of the AhR target gene *Cyp1A1* were increased in the livers of mice provided cotton balls for enrichment.

Materials and Methods

Animals. Male C57BL/6J mice (age, 6 wk) from Jackson laboratory (Bar Harbor, ME). This strain is highly sensitive to dioxin, is the background strain for the AhR null mouse, and is frequently used for dioxin studies.^{27,30,32,37,39,45} Mice were entrained for at least 2 wk under controlled lighting (12:12-h light:dark cycle), temperature (22 °C), and humidity (39%) in light-tight chambers before initiation of experimentation. Rodent chow (8604, Harlan, Indianapolis, IN) and water were provided ad libitum. Animals were group-housed (2 to 4 animals per cage) in standard polycarbonate rodent cages (18 × 28 × 13 cm; Al-

lertown Caging Equipment, Allentown, NJ) on 1/8-in. corncob bedding (Mt Pulaski Products, Mt Pulaski, IL). Bedding was not tested for dioxin content. Mice were divided into control ($n = 6$), TCDD exposure ($n = 6$), and cotton ball exposure ($n = 6$) groups. The TCDD exposure group was given a single dose of TCDD (1 µg/kg body weight, 98% pure, Cambridge Isotope Laboratories, Woburn, MA) by gavage. The cotton ball exposure group was provided with a new cotton ball at each cage change, which occurred once weekly. All animals were euthanized by decapitation 4 wk after cotton ball exposure and 1 wk after TCDD treatment. These exposure times were chosen to reflect the typical amount of time animals might be exposed to cotton balls or TCDD in our laboratory. A liver sample was placed in RNAlater (Ambion, Foster City, CA) for preservation prior to RNA isolation and transcript analysis. Another liver sample was fixed in neutral buffered formalin prior to paraffin embedding and immunohistochemical analysis. All animal use was approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign, and experiments were conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.²⁴

Quantitative real-time PCR. Total RNA was isolated by using TRIzol (Gibco BRL, Carlsbad, CA), and quantitative PCR was performed as previously described.³⁹ Primers used for *Cyp1a1* are previously published.³⁹ The threshold cycle (C_t) value was obtained, and relative RNA amount was calculated by using the relative standard curve method according to the manufacturer's instructions. Serial dilutions of pooled liver RNA, representative of all treatment groups, were used to generate standard curves. Linear regression analysis produced an equation for determining doubling efficiency and expression of each transcript.

Immunohistochemistry. Paraffin-embedded liver samples were sectioned (7 mm) and mounted on slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). After deparaffination and hydration of slides, endogenous peroxidase was quenched by incubating in 1% hydrogen peroxide for 30 min. Sections were blocked by using 1% normal goat serum and then incubated at 4 °C overnight with rabbit antihuman CYP1A1 primary antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody staining was visualized by using 3-amino-9-ethylcarbazole (AEC, Sigma, St Louis, MO) as a chromagen after amplification of the signal with the ABC kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin, dehydrated, and cleared with xylene, and cover slips were secured (Permount, Fisher Scientific).

Statistical analysis. PCR results were analyzed by 1-way ANOVA using SYSTAT (SSI, Richmond, CA). Tukey posthoc analysis was applied when ANOVA returned a P value less than 0.05.

Results

Cotton balls and TCDD increase *Cyp1A1* transcripts. Transcript levels of the AhR target gene, *Cyp1a1*, in mice provided cotton balls for enrichment and animals treated with TCDD were compared with those of control mice that had no cotton balls or TCDD treatment. *Cyp1a1* transcripts in liver were increased ($P < 0.001$, ANOVA) approximately 1000-fold 7 d after TCDD treatment (Figure 1) compared with levels in mice that were not exposed to cotton balls. *Cyp1a1* transcripts in liver were increased ($P < 0.01$, ANOVA) approximately 4.5-fold in mice exposed to cotton balls for enrichment. Similar to those in control mice, *Cyp1a1* transcripts were also very low in liver from mice provided pads of dioxin-free nesting material for enrich-

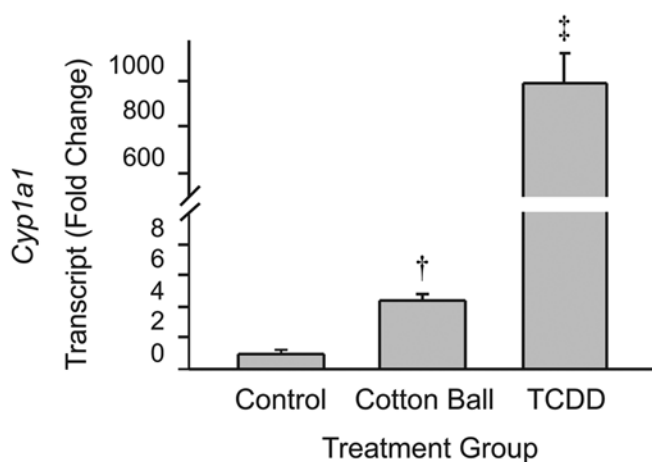


Figure 1. Quantitative real-time RT-PCR revealed that the quantity of *Cyp1a1* transcripts in liver is increased after exposure to cotton balls or TCDD. †, $P < 0.01$; ‡, $P < 0.001$ (ANOVA with Tukey posthoc analysis) compared with control value.

ment (data not shown). These data suggest that the presence of cotton balls led to activation of AhR signaling.

Cotton balls and TCDD increase CYP1A1 immunoreactivity. We used immunohistochemistry to determine whether cotton balls and TCDD altered CYP1A1 protein. Immunostaining of CYP1A1 was increased in the livers of animals treated with TCDD compared with control animals that were not exposed to cotton balls (Figure 2). CYP1A1 immunoreactivity in animals exposed to cotton balls was increased also. A characteristic centrilobular pattern of CYP1A1 expression was observed in the livers of mice that were either treated with TCDD or exposed to cotton balls. These data support the hypothesis that exposure to cotton balls led to activation of AhR signaling.

Discussion

The validity of scientific results derived from animal studies depends on the health and well-being of the animals used for investigation. Over that past few decades, standardization of housing in animal facilities has greatly enhanced experimental repeatability across laboratories. However, the lack of complexity in the environment of laboratory rodents raises questions regarding both animal welfare and the degree to which they represent 'normal' animals. Animals housed in complex environments are clearly different from their counterparts housed in barren surroundings (for review, see reference⁴). To enhance animal welfare, many facilities now provide enrichment for the animals. Sources of enrichment may include addition of (1) rigid structures that create microenvironments for hiding; (2) manipulada that allow chewing or engaging in fine motor movements; (3) novel foods with different tastes and textures; (4) increased social contact through group housing; and (5) olfactory or auditory stimuli.²³ Although few studies^{3,4,54} address the effects of enrichment on experimental outcomes, it is clear that rodents prefer enriched environments. Furthermore, rodents favor environments enriched with paper- or textile-based nesting material.⁴⁶ However, as the results of the present study indicate, care must be taken to provide nesting materials that will not compromise animal health or the validity of experimental measures.

During an ongoing project designed to investigate the role of AhR in regulation of circadian rhythms,^{37,39} the Division of Animal Resources at our university initiated the use of standard (not intended for laboratory animal use) cotton balls as envi-

ronmental enrichment for all mice in the animal facility. Shortly thereafter, we noticed increased Cyp1A1 levels in a control group that was part of a TCDD-treatment study, raising concerns regarding the presence of dioxins or dioxin-like compounds in the facility and thus prompting this investigation. Although the cotton balls we used were not tested for dioxins, the increased Cyp1A1 transcript and protein levels strongly suggest that dioxins or other dioxin-like compounds were present. Cyp1A1 is a sensitive indicator of AhR activation; Cyp1A1 levels in the liver are negligible in animals not exposed to AhR agonists but are increased in rat liver in response to a single dose of TCDD at 1 ng/kg body weight;⁶² the 1- μ g/kg dose used in the present study is a typical dose used in C57BL/6J mice.⁷ Induction of *Cyp1A1* in response to AhR agonists varies among species^{29,35,50} and even among strains of mice.⁴² Inbred mouse strains exhibit as much as 10-fold differences in sensitivity;⁶³ C57BL/6J mice are considered sensitive, whereas DBA/2 mice are resistant. Differences in sensitivity are attributable to specific structural differences in AhR.^{21,29,43} Increased *Cyp1A1* transcripts can be detected in mouse liver as early as 1 h after exposure to AhR agonists.³⁸ Maximal induction of *Cyp1A1* occurs by 24 h after a single dose of TCDD;⁵⁸ however, Cyp1A1 activity remains increased for as long as 35 d.¹⁷ Furthermore, Cyp1A1 is likely to remain increased with continued exposure to AhR agonists.

Cyp1A1/2 are critical mediators of the biotransformation of environmental toxicants, steroid hormones, pharmaceuticals, and carcinogens.^{10,11,33} Detoxification and maintenance of chemical homeostasis relies on the conversion of chemicals in the liver by Cyp1A1/2 into polar metabolites that can be excreted. Demethylation of caffeine and metabolism of theophylline occurs through Cyp1A1/2 in the liver.⁹ Endobiotics, including 17 β -estradiol, retinoids, and thyroxine, as well as pharmaceuticals such as clazapine, imipramine, and propranolol are high-affinity substrates for Cyp1A1/2 (for review, see reference⁵⁷). Anthropogenic contaminants classified as dioxins are, however, the most potent AhR agonists. Cyp1A1/2 act on polyaromatic hydrocarbons to cause formation of DNA and protein adducts that ultimately lead to tumor formation and toxicity. Therefore, persistent exposure to AhR agonists, leading to chronic Cyp1A1/2⁶³ activity, ultimately contributes to detrimental health effects. Because Cyp1A activity in liver is central to the conversion of chemicals into ultimate carcinogens and because our previous studies identified the liver as an important target site for the effects of AhR agonists on circadian rhythms,^{37,39} we examined Cyp1A1 levels in liver during the present study. We observed an approximately 4.5-fold increase in *Cyp1A1* transcripts in liver after exposure of mice to cotton balls (Figure 1). This level of induction of Cyp1A1 in the liver is sufficient to cause immunosuppression as measured by a decreased splenic antibody response to immunologic challenge.⁴⁰ In that study, a single dose of TCDD produced a sustained 6-fold increase in Cyp1A1 that was associated with significant accumulation of TCDD in multiple tissues and suggested that sensitivity to TCDD was higher in the immune system, kidney, and uterus as compared with liver.⁴⁰ Other studies^{15,52} also have indicated differences in sensitivity to AhR agonists among organs systems. In pigs, low level exposure to polycyclic aromatic hydrocarbons in soil leads to increased Cyp1A1, most prominently in the duodenum.⁵² Organ-specific responses are likely dependent on the route of exposure and the chemical nature of the AhR agonist.^{13,15}

The results presented here indicate that activation of AhR signaling can occur in the presence of contaminants found in products intended to provide enrichment for mice housed in

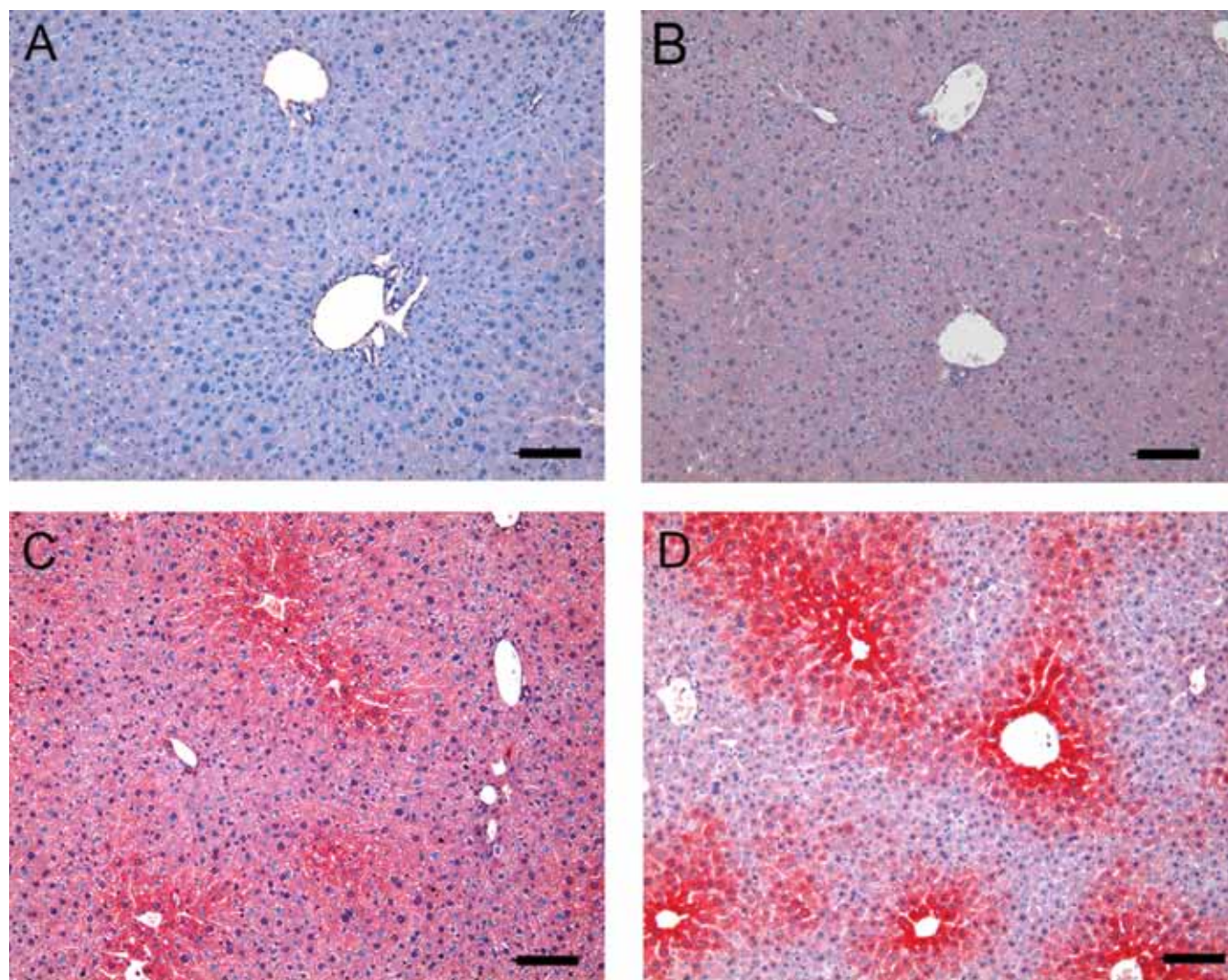


Figure 2. Representative images of immunohistochemistry for hepatic expression of CYP1A1 protein (red). (A) Negative control (omission of primary antibody). (B) Control animal (no exposure to TCDD or cotton balls). (C) Animal exposed to cotton balls. (D) Animal exposed to TCDD. Panels C and D both show a characteristic centrilobular pattern of CYP1A1 expression. Scale bar, 20 μ m.

laboratory animal facilities. The cotton balls used in our facility were not manufactured for use in laboratory animals and thus information regarding chemical residues was unavailable. A variety of paper-based products for enrichment are available from various laboratory animal suppliers, some of which are similar in consistency to cotton balls; these vendors routinely disclose information on residual pesticide and chemical concentrations in their enrichment and bedding products. Therefore, products that provide the desired enrichment of rodent environments yet do not compromise animal health are readily available.

This study underscores the necessity for awareness of potential sources of contamination in control groups of mice, especially those used for investigation of AhR activation. Given the critical role of Cyp1A enzymes in steroid and drug metabolism and chemical detoxification and their roles in chemical toxicity and carcinogenesis, the current study raises important questions regarding how the presence of low levels of AhR agonists might influence animal well-being and experimental outcomes. At the very least, the presence of dioxins or other AhR agonists potentially adds an additional scientific variable that is not accounted for in experimental results. This potential confound, however, can easily be avoided by the use of dioxin-free enrichment materials.

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