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Dioxin Causes a Sustained Oxidative Stress Response in the Mouse

Howard G. Shertzer,[1] Daniel W. Nebert, Alvaro Puga, Michelle Ary, David Sonntag, Kathleen Dixon, Lindsey J. Robinson, Eli Cianciolo, and Timothy P. Dalton

Department of Environmental Health and Center for Environmental Genetics, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, Ohio 45267-0056

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Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) is the prototype for environmental agonists of the aromatic hydrocarbon receptor (AHR) that are known to produce multiple adverse effects in laboratory animals as well as humans. Although not directly genotoxic, dioxin is known to increase transformation and mutations in mammalian cell culture and to cause an exaggerated oxidative stress response in the female rat. In humans and mice, however, dioxin-mediated oxidative stress appears to be more subtle, causing a response that has been poorly characterized. Using the female C57BL/6J inbred mouse, we show here that intraperitoneal treatment of 5 μg TCDD per kilogram on 3 consecutive days produces a striking, prolonged oxidative stress response: hepatic oxidized glutathione levels increase 2-fold within 1 week, and these effects persist for at least 8 weeks despite no further dioxin treatment. Urinary levels of 8-hydroxydeoxyguanosine—a product of DNA base oxidation and subsequent excision repair—remain elevated about 20-fold at 8 weeks after dioxin treatment, consistent with chronic and potentially promutagenic DNA base damage. These results demonstrate that dioxin exposure does produce a sustained oxidative stress response in the mouse.

Certain polycyclic aromatic hydrocarbons—including polyhalogenated dibenzodioxins, dibenzofurans and biphenyls—are known or suspected environmental carcinogens, toxicants and teratogens in the laboratory animal as well as the human (1–7). Having long biological half-lives, these compounds bind to and activate the cytosolic aromatic hydrocarbon receptor (AHR) which, after heterodimerization with the Ah receptor nuclear translocator (ARNT), transcriptionally activate genes containing aromatic hydrocarbon response elements (AHREs) in their regulatory regions (3, 5, 8). Such genes include at least three mammalian cytochromes P450—*CYP1A1, CYP1A2,* and *CYP1B1*—and several Phase II antioxidant enzymes (3, 9). In addition, the AHR appears to participate in cell cycle regulation (10, 11) and apoptosis (12), which might contribute to the capacity of AHR agonists to be tumor promoters.

The prototypic polyhalogenated aromatic hydrocarbon, dioxin, is a potent hepatic tumor promoter (13), as well as a complete liver carcinogen in both male and female mice (14–16). Epidemiologic studies also suggest that dioxin is a human liver carcinogen in both men and women (17). In contrast to the mouse and human, the female rat appears to be much more susceptible than the male to dioxin-induced hepatocarcinogenesis (14, 16). In the female rat, dioxin has also been shown to induce an oxidative stress response (reviewed in Ref. 1) characterized by lipid peroxidation, peritoneal macrophage activation, and decreased hepatic antioxidant levels. The concept that the female rat model might represent an exaggerated toxic response to dioxin led us to search for more subtle oxidative stress responses that might occur in other species, such as the mouse. We postulated that the molecular mechanisms underlying dioxin toxicity might be common to all mammalian species. Here we examine this hypothesis by measuring several parameters of oxidative stress during an 8-week period following TCDD exposure in the mouse.

MATERIALS AND METHODS

Animals. Sexually immature female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), 5 weeks of age, were treated intraperitoneally on Days 0, 1, and 2—each day with 5 μg TCDD/kg body weight in dimethyl sulfoxide (DMSO; 10 μl/mouse); controls received DMSO alone. At the indicated times (24 h, or 1, 5, or 8 weeks), livers were excised and placed in ice-cold 0.9% NaCl. Thus, animals representing the 24-h time point had received a single dose of TCDD whereas all other animals had received the three doses of TCDD. Experimental and control mice (N = 3 or 4) were assayed at each

[1] To whom correspondence should be addressed. Fax: (513) 558-4397. E-mail: shertzhg@ucmail.uc.edu.

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time point. Because the mean values and variances for each data set in the control groups were not different as determined by ANOVA, these data were pooled.

CYP1A1 and CYP1A2 enzyme activities. Liver was homogenized and fractionated as described (18). CYP1A1 enzyme activity was assayed as the fluorescent hydroxylation products of benzo[*a*]pyrene (19), whereas CYP1A2 activity was assayed by HPLC as acetanilide 4-hydroxylase (20).

Determination of reduced (GSH) and oxidized (GSSG) glutathione. Aliquots of liver tissue were homogenized in 5 mM diethylenetriaminepentaacetic acid (DTPA) and 10 mM ascorbic acid (in 20 mM HCl). GSH levels were assayed as previously described (9, 21), with certain modifications. The sample was divided, and one aliquot was derivatized directly (reduced thiols); the second was treated first with 1 mM *N*-ethylmaleimide to bind reduced thiols, then with 1 mM dithiothreitol to bind excess *N*-ethylmaleimide and to reduce the disulfides. Both aliquots were derivatized with monobromobimane in *N*-ethylmorpholine (pH 9.0). Authentic GSH and GSSH standards were run in parallel with the cell extracts. After 15 min at 37°C, 10% HCl was added, the tubes were mixed and microfuged, and the supernatant solution assayed for thiol-bimane fluorescence by reverse-phase HPLC with fluorescence detection at $Ex_{370\ nm}/Em_{485\ nm}$.

Urinary 8-hydroxydeoxyguanosine (8-OhdG). Urinary samples were collected from TCDD-treated and control mice housed in metabolic cages; the urine was collected in a redox-inactive solution containing 10% sulfosalicylic acid and 5 mM DTPA. Urine samples were microfuged at 10,000*g* for 5 min and stored at −80°C until analysis. Urinary 8-OHdG was assayed with an ELISA kit (Genox Corp., Baltimore MD).

Biohazard precaution. Because TCDD is a highly toxic compound, all personnel were instructed in safe handling procedures. Lab coats, gloves and masks were worn; contaminated materials were collected separately for disposal by the Hazardous Chemical Waste Unit. Dioxin-treated mice were housed separately, and carcasses were treated as contaminated biological material.

RESULTS AND DISCUSSION

Induction of CYP1A1 and CYP1A2 activity. To assess the efficacy and duration of dioxin effects in these experiments, we assayed CYP1A1 and CYP1A2 enzyme activities. Both were significantly elevated in dioxin-treated mice, compared with that in controls (Fig. 1). CYP1A2 activity was maximally induced 24 h following the first TCDD dose, and remained statistically unaltered for the rest of the 8-week experiment. In contrast, CYP1A1 activity was highest at 1 week and fell to 25% and 15% of maximum by 5 and 8 weeks, respectively.

The decline in CYP1A1 activity, while CYP1A2 levels remained elevated, has been observed previously (22), and is consistent with the finding that the mammalian *CYP1A2* promoter appears to be more sensitive to AHR-mediated induction than the *CYP1A1* promoter (23, 24). Moreover, the dioxin-activated AHR has been shown to bind to the AHR-binding response element (AHRE) of the *CYP1A2* promoter with a higher affinity than that of the *CYP1A1* promoter (25); thus, if the hepatic TCDD concentrations were declining by week 5 of the present study, inducible CYP1A1 activity would be expected to decrease at a faster rate than CYP1A2 activity. Other contributing factors might include the relative stabilities of CYP1A1 and CYP1A2 mRNA, which are known to differ and may change independently under variable inducing conditions (26). The CYP1A2 protein has also been shown to be a dioxin-binding "sink" that appears to be stabilized by the dioxin (25, 27–32).

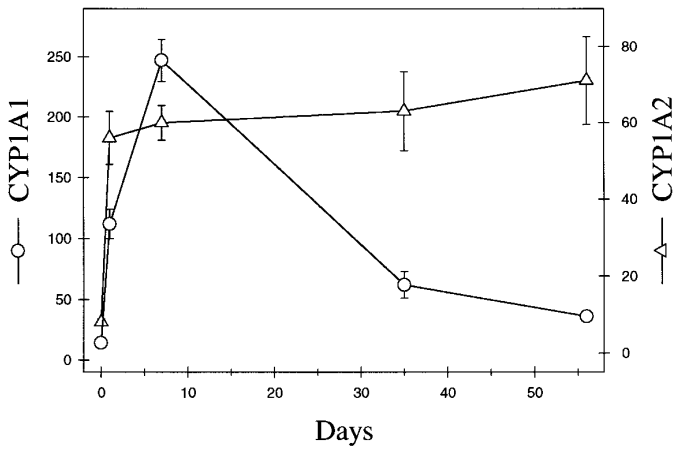

FIG. 1. Changes in CYP1A1 and CYP1A2 activity for 8 weeks following 3 consecutive days of intraperitoneal dioxin (5 μg/kg). N = 4 for control mice (DMSO vehicle alone) and N = 3 for each TCDD-treated time point; since the mean values for the data were the same in all control groups, these data were pooled. CYP1A1 (FU $\times$ 10^{-3}/min/g tissue) and CYP1A2 (AUC $\times$ 10^3/min/g tissue) activities are expressed as means ± SEM. Each mean value shown after TCDD treatment is greater than the mean value at Day 0 (prior to the first dose of TCDD), using a one-way ANOVA, followed by a Tukey pairwise multiple comparison procedure, at $P < 0.01$.

Alterations in GSH, GSSG and thiol pools. The oxidation of intracellular thiol pools is a hallmark of oxidative stress. Although thiol homeostasis was not altered after 1 day of dioxin exposure (Table 1), at 1 week we observed an oxidative stress response in the form of elevated GSSG levels. At the 8-week time point, both GSSG and protein-glutathione mixed disulfide levels were elevated. We found, at all time points, that the protein-cysteinyl mixed disulfides were below detection limits (<50 nmol/g tissue).

GSH is normally maintained in the reduced state due to the concerted action of glutathione reductase and NADPH (33). Oxidative stress, in the form of hydrogen peroxide and other organic peroxides, can be decreased at least in part by glutathione peroxidase in a reaction that generates GSSG and tends to increase the GSSG/GSH ratio (34). Increases in the GSSG/GSH ratio favor the formation of glutathione mixed-disulfides with reactive protein cysteinyl residues. Such residues are important in the regulation of many proteins involved in cellular proliferation and carcinogenesis—including Fos and Jun (35), protein kinase C (36), Ca^{2+}-ATPase (37, 38), collagenase (39), plasma membrane Ca^{2+} transporter (40, 41), and Src tyrosine kinases (42). Thus, the persistent oxidative shift in

TABLE 1

Dioxin-Induced Hepatic Changes in GSH, GSSG, and Thiol Pools in the Mouse[a]

	1 day		1 week		8 weeks	
Parameter[b]	Control	TCDD	Control	TCDD	Control	TCDD
(*N*)	(7)	(3)	(3)	(4)	(3)	(3)
GSH	9.3 ± 0.3	9.2 ± 0.4	9.5 ± 0.4	11 ± 0.3	9.8 ± 0.5	12 ± 0.3
GSSG	0.61 ± 0.03	0.59 ± 0.07	0.62 ± 0.03	1.4 ± 0.3*	0.65 ± 0.04	1.4 ± 0.02*
GSSG/GSH	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.13 ± 0.01*	0.06 ± 0.01	0.12 ± 0.01*
ProtS-SG	0.22 ± 0.3	0.18 ± 0.08	Not assayed		0.17 ± 0.04	0.51 ± 0.09*

Note. For comparison, values for liver of Fischer 344 female untreated rats were GSH = 6.9 ± 0.7 μmol/g tissue ($N = 4$); GSSG = 0.11 ± 0.01 ± μmol/g tissue ($N = 4$); and GSSG/GSH = 0.02 ± 0.01.

[a] Mice and data were treated as described in the legend to Fig. 1.

[b] GSH and GSSG (μmol/g tissue) and ProtS-SG (protein cysteinyl-glutathione mixed disulfide) (μmol/g tissue) are expressed as means ± SEM. GSSG/GSH ratios are expressed as means ± SEM of individual ratios.

* Significantly different from control animals at $P < 0.01$.

thiol homeostasis may play a role in the mechanism by which dioxin acts as a tumor promoter.

Urinary 8-hydroxydeoxyguanosine (8-OHdG). Reactive oxygen species have been demonstrated to react with DNA and cause mutations. 8-OHdG is the major oxidized base formed when reactive oxygen reacts with DNA (43). Oxidative damage to DNA is strongly associated with chemical carcinogenesis (44). *In vivo,* DNA that has been damaged by reactive oxygen is repaired by exonucleases and/or glycosylases, releasing deoxynucleosides and bases, respectively. These products of DNA repair are water-soluble and excreted into the urine without additional modification (45). Dietary DNA contributes to the excretion of oxidized bases, but does not contribute to the excretion of oxidized deoxynucleosides such as 8-OHdG (46). Thus, the urinary excretion of 8-OHdG is a valid measure of oxidative DNA damage in the intact animal.

Urinary 8-OHdG was elevated 2- to 3-fold at 5 weeks and, interestingly, 20-fold at 8 weeks following dioxin treatment (Fig. 2). Oxidized guanine bases in replicating DNA produce primarily G→T and A→C transversions (47–49). In addition, reactive oxygen-induced DNA damage activates error-prone polymerase DNA repair that may introduce mismatched bases (44). Although oxidative DNA damage tends to be repaired rapidly by exonucleases and glycosylases, the probability of mutation fixation increases with the duration of exposure to the mutagen, as well as with the mitotic rate. Dioxin is extremely lipophilic and metabolically stable, with biological half-lives of several weeks in rodents and 7–10 years in humans (50). The persistence of this chronic oxidative stress response, as opposed to acute severe effects, might be important in the action of TCDD as a complete liver carcinogen.

Speculation on mechanism. The mechanism by which dioxin causes a sustained oxidative stress is unclear at present. Previous experiments have demonstrated that an AHR- or CYP1A1-dependent increase in 8-OHdG released into the culture medium occurs in mouse hepatoma Hepa-1c1c7 cells treated with TCDD (51). The importance of CYP1A1 and CYP1A2 in the cellular oxidative stress response may be related to the formation of H_2O_2 by the peroxidase activity provided by enzymes such as dioxin-induced CYP1A1 and 1A2. Cytochromes P450 sequentially transfer two electrons to bound molecular oxygen from NADPH (52, 53). In the course of electron transfer, some of the activated oxygen can be released as superoxide and/or H_2O_2. The cytochromes P450 have clearly been shown to contribute significantly to the total cellular production of reactive oxygen in rat liver, even in the absence of enzyme induction (54). The importance of the metabolism of endogenous, as well as exogenous substrates by CYP1A1 and CYP1A2, however, cannot be overemphasized.

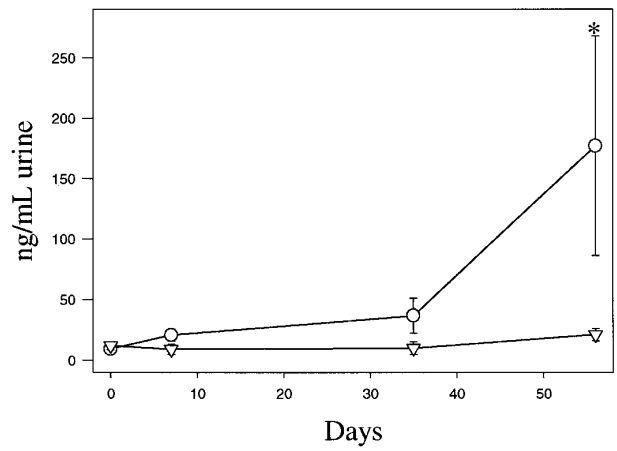

FIG. 2. Changes in urinary 8-OHdG for 8 weeks following 3 consecutive days of intraperitoneal dioxin (5 μg/kg). $N = 4$ for control mice (DMSO vehicle alone) and $N = 3$ for each TCDD-treated time point. 8-OHdG levels (ng/mL urine) are expressed as means ± SEM. * denotes significantly different from control animals, using a one-way ANOVA, followed by a Tukey pairwise multiple comparison procedure, at $P < 0.01$.

Dioxin is the prototype of a class of compounds, both natural and synthetic, that are agonists for the AHR. The oxidative stress response elicited by TCDD may therefore be common to a broad range of compounds. It is noteworthy that this oxidative stress response to dioxin may extend over long periods of time of exposure. Our experiments were conducted using dioxin doses that have previously been shown to result in persistent activation of the AHR in mice, which allowed us to examine oxidative stress responses over a time course of 8 weeks. We plan to study this phenomenon over longer periods of time, and to compare male with female C57BL/6J mice, as well as other inbred strains. In the context of a human life, 8 weeks in a mouse represents 5 or 6 human years. Given the cumulative nature of mutations on the initiation and progression of cancer, low-level chronic internal exposures to chemicals that activate the AHR—rather than high levels of acute environmental exposures—may be more appropriate to the mechanisms underlying the adverse impact that dioxins have on human health.

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