The Drug Salicylamide Is an Antagonist of the Aryl Hydrocarbon Receptor That Inhibits Signal Transduction Induced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a widespread environmental contaminant, that has been linked with a variety of deleterious effects on human health, including increased cancer rates and reproductive anomalies. The detrimental effects of TCDD are mediated via the aryl hydrocarbon receptor (AhR), a transcription factor that regulates the expression of the xenocarcinogen-activating enzymes cytochromes P-450 (CYP) 1A1, 1A2, and 1B1. In the present study, we examined the ability of synthetic derivatives of salicylic acid to affect TCDD-stimulated AhR-mediated signal transduction in human hepatoma HepG2 cells. Salicylamide (SAL), an analgesic drug, caused a potent and long-lasting inhibition of TCDD-induced CYP enzyme activity. Acetylsalicylic acid (aspirin) and the naturally occurring phytochemical salicylic acid had no effect on CYP activity. SAL inhibited the increase in CYP1A1, -1A2, and -1B1 mRNA levels that occurs on exposure to TCDD. TCDD-induced transcription of these genes was also inhibited by SAL, but not by aspirin or salicylic acid, as demonstrated by luciferase reporter assays. The transcription of the CYP1 family of genes is regulated by the interaction of TCDD-activated AhR with the xenobiotic-responsive element present in the promoter regions of these genes. As shown by electrophoretic mobility shift assays, SAL completely blocked the binding of TCDD-activated AhR to the xenobiotic responsive element. Also, SAL substantially blocked the binding of TCDD to the cytosolic AhR. These results demonstrate that SAL, a commonly used analgesic, is a potent inhibitor of AhR-mediated signal transduction, and may be an effective agent in the prevention of TCDD-associated disease.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ubiquitously expressed cytosolic protein that is a member of the basic helix-loop-helix Per-Arnt-Sim super family of transcriptional regulatory proteins (1, 2). On binding the ligand, the “activated” AhR translocates to the nucleus, in which it forms a heterodimer with its partner, the aryl hydrocarbon nuclear translocator. Together, they form a transcription factor that binds to the xenobiotic-responsive element (XRE) present in the promoter region of a number of genes. The best characterized molecular response to AhR activation is the transcriptional induction of CYP1A1, CYP1A2, and CYP1B1, the genes that encode the 1A1, 1A2, and 1B1 enzyme isoforms, respectively, of cytochrome P-450 (CYP). These enzymes catalyze the epoxidation of certain classes of xenobiotics, resulting in the generation of highly reactive electrophilic metabolites. These metabolites can covalently bind specific residues of DNA, causing DNA adduct formation that may result in mutation and subsequent cellular transformation. The AhR and the genes that it regulates are, therefore, central in mediating the genotoxicity of many environmental contaminants.

Ligands of the AhR include several classes of environmental carcinogens, including polyhalogenated biphenyls such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), aryl hydrocarbons such as benzo(a)pyrene, and heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. These classes of xenobiotics are all suspected human carcinogens. For example, TCDD, a widespread environmental contaminant generated by a variety of industrial processes, has been linked to an increase in many types of human cancers, including hepatocellular cancer (3, 4). In animal models, TCDD has been shown to cause acute toxicity (5), increased oxidative stress (6), impaired ovulation (7), and immunotoxicity (8). These effects in animal models are believed to closely reflect the deleterious effects of TCDD in humans (9). TCDD has the highest affinity for the AhR of any known compound, and is considered the prototypical AhR ligand. Its interaction with the AhR is essential to its toxicity, as demonstrated in AhR-knockout mice, which are impervious to TCDD exposure (10).

Because of the central role of the AhR in the toxicity of TCDD and genotoxicity of numerous xenobiotics, the development of inhibitors of AhR-mediated signal transduction has been an important goal of chemoprevention science. In the present study, we have examined the effects of the salicylates on the AhR and the metabolic pathway it regulates. Salicylic acid was first identified in willow bark extracts as an active anti-inflammatory compound more than 200 years ago, and its synthetic derivative, acetylsalicylic acid (aspirin), has been used to treat a number of human maladies for over a century. Recent studies have demonstrated that salicylate usage is associated with a reduction of incidence of several types of cancer (11–13), and animal studies have shown that salicylates are potent inhibitors of chemically induced carcinogenesis (14, 15). We have, therefore, examined the effects of salicylates on the AhR function in HepG2 cells, a well-differentiated human liver cancer cell line that has been extensively used as a model system to study the AhR (16–19). We demonstrated that the synthetic salicylate, salicylamide (SAL), is a potent and long-lasting inhibitor of TCDD-induced signal transduction mediated by the AhR.

MATERIALS AND METHODS

Materials. Human hepatoma HepG2 cells were from American Type Culture Collection (Manassas, VA). RPMI 1640, TRIzol, and LipofectAMINE were from Life Technologies, Inc. (Grand Island, NY). Glutamine and fetal bovine serum were from BioFluids (Rockville, MD). Polydeoxyinosinic-deoxyctydilic acid, protease inhibitors, ethoxyresorufin, acetylsalicylic acid (aspirin), salicylic acid and SAL were from Sigma (St. Louis, MO). [3H]TCDD and [32P]dCTP were from DuPont-NEN (Boston, MA). TCDD was from the Midwest Research Institute (Kansas City, MO). [3H]TCDD was from Chem-syn Science Labs, Lanexa, KS. The Omniscript kit was from Qiagen (Valencia, CA). Tris-borate gels, Tris-borate running buffer, and high-density sample buffer were from Invitrogen (Carlsbad, CA).

Cell Culture. HepG2 human hepatoma cells were grown with RPMI 1640 supplemented with 2 mM glutamine and 10% fetal bovine serum in a 5% CO2 humidified incubator at 37°C. For all of the treatment compounds, DMSO was used as the vehicle and did not exceed 0.1%.

Carcinogen Activation Capacity in HepG2 Cells. The ability of SAL to affect CYP enzyme activity was evaluated in intact cells by measurement of ethoxyresorufin-O-deethylase (EROD) activity. In 24-well plates, HepG2 cells were incubated with increasing concentrations of salicylates in the presence of 1 nM TCDD for 24 h, for dose-response experiments. For time course experiments, cells were incubated with increasing concentrations of SAL, or with
Ligand-Binding Assay. AhR ligand binding was measured according to the methods of Rahm et al. (26). Cytosolic protein (1.5 mg/500 μl) was incubated with 20 nm [3H]TCDD in the presence of DMSO (5 μl/500 μl), 5 mM SAL, or 10 μM TCDD (unlabeled) for 2 h at 4°C. Samples were centrifuged in linear gradients of 5–30% sucrose (v/v) in 12 ml of Beckman Quick-Seal rotor tubes at 65,000 rpm for 2 h at 4°C in a Beckman VTI-65–1 rotor. Fractions (~500 μl each) of the gradients were collected, and radioactivity was determined by scintillation counting.

Statistical Analysis. Statistical analyses were performed with STATVIEW Statistical Analysis Software (SAS Institute, San Francisco, CA). Differences between group mean values were determined by a one-factor ANOVA, followed by Fisher’s protected least significant difference post hoc analysis for pairwise comparison of means. A two-sided P value of <0.05 was considered significant. CYP mRNA levels in Figs. 3 and 4 are shown on a linear scale.

RESULTS

Effect of Salicylates on TCDD-Induced CYP Enzyme Activity. In this study, we examined three salicylates the structures of which are illustrated in Fig. 1. The cellular capacity for enzymatic activation of procarcinogens was measured by EROD assay. Treatment of HepG2 cells with 1 nM TCDD resulted in an increase in EROD-specific activity from nondetectable levels to 17 pmol/min/well in controls (Fig. 2A). The addition of SAL caused a concen-

other test compounds at concentrations that inhibit 50% (IC50), in the presence of 1 nM TCDD for various times from 6 to 144 h. Medium was decanted, and cells were washed once with PBS. Medium containing 5 μM ethoxyresorufin was added. Increasing fluorescence as a result of the conversion of ethoxyresorufin to resorufin by CYP enzymes was measured using a CytoFluor multwell plate reader (Applied Biosystems, Foster City, CA), with an excitation of 530 nm and emission at 590 nm. The reaction was allowed to run for 30 min.

Reverse Transcription-PCR. In 6-well plates, cells were treated with various concentrations of SAL alone, or with 100 pm TCDD, for 8 h. Cells were washed twice with PBS, and total RNA was isolated with TRIzol reagent per manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA using an Omniscript kit according to the manufacturer’s instructions. Semiquantitative reverse transcription-PCR for cytochrome P4501A1 (CYP1A1), cytochrome P4501A2 (CYP1A2), and cytochrome P4501B1 (CYP1B1) expression was performed as described previously (20). Primer sequences and conditions for CYP1A1 and CYP1B1 were as described by Dohr et al. (21), and for cytochrome P4501A2 (CYP1A2) as described by Chung and Bresnick (22). Primers for glyceraldehyde-3-phosphate dehydrogenase were from Clontech (Palo Alto, CA). The optimum cycle number that fell within the exponential range of response for CYP1A1 (23 cycles), CYP1A2 (28 cycles), CYP1B1 (30 cycles) and glyceraldehyde-3-phosphate dehydrogenase (17 cycles) was used. The level of CYP mRNA was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Luciferase Assay. HepG2 cells were seeded onto 6-well plates at 400,000 cells/well. After 24 h, the cells were transiently transfected with a luciferase reporter vector (1.5 μg/well) containing three repeats of the XRE and a β-galactosidase vector (pCMV-β-gal). β-galactosidase activity was determined by the method of Rosenthal (23).

Electrophoretic Mobility Shift Assay. HepG2 cells were incubated with DMSO (0.1%), 10 nM TCDD alone, or 10 nM TCDD with 1 mM SAL for 2 h at 37°C. Nuclear protein was isolated, and electrophoretic mobility shift assay was performed by the method of Denison et al. (24). Synthetic oligonucleotides containing the AhR-binding site of the XRE (forward primer, 5'-GAT CTC AGC TCG GAG TTG CGT GAG AAG AGC CG-3'; reverse primer, 5'-CGG CTC TTC TCA CGC AAC TCC GAG CTC AG-3'; Ref. 25) were labeled with 11P [32P]dCTP. Binding reactions were performed for 25 min (10-min preincubation without probe + 15-min incubation with probe) at room temperature and contained 10 μg of nuclear protein, 2 μg of polydeoxyinosinic deoxycytidylic acid and labeled probe (~50,000 cpm) in a final volume of 20 μl of binding buffer [25 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM MgCl2, 1.5 mM EDTA, 0.5 mM DTT, 5% (v/v) glycerol]. To determine the specificity of binding to the oligonucleotide, excess unlabeled specific probe was incubated with the nuclear extract of TCDD-treated cells for 15 min. DNA–protein complexes were separated under nondenaturing conditions on a 6% (w/v) polyacrylamide gel with 0.5× Tris/borate/EDTA running buffer. The gels were dried, and the amount of labeled probe present in the DNA–protein complexes was determined with a Bio-Rad GS-363 molecular imaging system.

Fig. 1. Structures of salicylic acid, aspirin, and salicylamide (SAL).

Fig. 2. Effect of salicylates on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced cytochrome P-450 (CYP) activity in HepG2 cells. CYP activity was determined in intact cells by ethoxyresorufin-O-deethylase (EROD) assay. A, cells were treated with 1 nM TCDD and the indicated concentrations of salicylate for 24 h; B, cells were treated with TCDD and salicylamide (SAL) for the times indicated. n = 4 ± SE.
tration-dependent inhibition of EROD activity, with an interpo-
lated IC$_{50}$ of $\approx 170 \ \mu$M. SAL at 1 mM suppressed EROD levels to
10% of cells treated with TCDD alone. In contrast, both aspirin and
salicylic acid had no inhibitory effect at any concentration (Fig.
2A). TCDD caused a persistent increase in EROD activity in
HepG2 cells that remained at maximal levels for 6 days (Fig. 2B).
SAL, added along with TCDD, caused an inhibition of enzyme
activity that persisted throughout the 6-day time course, regardless
of the concentration used (Fig. 2B).

The inhibitory effect of SAL was further examined to test whether
this was due to a direct effect on CYP enzyme activity. The addition
of SAL (250 $\mu$M) to HepG2 cells that were first incubated with TCDD
(to induce CYP activity) did not have a significant effect on EROD
activity ($P = 0.16$), whereas 10 $\mu$M $\alpha$-naphthoflavone (a positive
control) inhibited EROD activity by 84%, compared with controls
(Table 1). SAL (250 $\mu$M) was also added to microsomes isolated from
TCDD-treated HepG2 cells, and EROD activity was assayed. There
was no significant effect on microsomal EROD activity ($P = 0.70$;
Table 1), whereas $\alpha$-naphthoflavone completely abolished EROD
activity.

**Effect of SAL on CYP1A1, -1A2, and -1B1 mRNA Levels.** TCDD caused a 13.3-, 7.5-, and 2.8-fold increase in CYP1A1, -1A2,
and -1B1 expression in HepG2 cells, respectively, compared with
DMSO controls. SAL cotreatment inhibited the TCDD-induced in-
crease of all three CYP isozymes in a concentration-dependent man-
er (Fig. 3A). SAL treatment alone had no effect on basal CYP
expression (Fig. 3B). Aspirin did not have an effect on TCDD-induced
CYP mRNA (data not shown).

**Effect of Salicylates on XRE-Controlled Luciferase Reporter
Activity.** HepG2 cells were transiently transfected with a lucifer-
ase reporter vector containing multiple copies of the XRE. TCDD
induced luciferase activity by $\approx 45$-fold above control levels. The
addition of SAL resulted in a concentration-dependent decrease of
luciferase activity (Fig. 4). Aspirin and salicylic acid had no effect
(Fig. 4).

**Effect of SAL on AhR Activation.** The effect of SAL on TCDD-
induced activation of the AhR was measured by electrophoretic
mobility shift assay. TCDD (10 nM) caused a significant increase
in AhR translocation to the nucleus and in binding to the XRE.
This was completely suppressed in cells cotreated with 1 mM SAL
(Fig. 5). The specificity of the bandshift was confirmed by treat-
ment of nuclear extracts with excess unlabeled XRE and by incu-
bation with an antibody to the AhR, which abolished AhR-XRE
binding (data not shown), as we have demonstrated previously
(27).

**Effect of SAL on AhR Ligand Binding.** Competitive binding
assays were performed using the cytosolic fraction from HepG2 cells.
Binding of [$^3$H]-TCDD to the AhR was competitively inhibited by
excess unlabeled TCDD or SAL (Fig. 6).

Table 1. The effect of salicylamide on cytochrome (CYP)-activity, after induction

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>20.16 ± 0.49</td>
</tr>
<tr>
<td>Control</td>
<td>2.05 ± 0.08</td>
</tr>
<tr>
<td>$\alpha$-Naphthoflavone</td>
<td>19.15 ± 0.22</td>
</tr>
<tr>
<td>Salicylamide</td>
<td>2.22 ± 0.06</td>
</tr>
<tr>
<td>Microsomes</td>
<td>2.22 ± 0.06</td>
</tr>
<tr>
<td>Control</td>
<td>Not detected</td>
</tr>
<tr>
<td>$\alpha$-Naphthoflavone</td>
<td>2.18 ± 0.16</td>
</tr>
<tr>
<td>Salicylamide</td>
<td>2.18 ± 0.16</td>
</tr>
</tbody>
</table>

$^a$ Activity for intact cells is in units of pmol/min/100 k cells; activity for microsomes
is in units of pmol/min/2.5 $\mu$g.
DISCUSSION

TCDD is a major environmental contaminant, generated by a variety of industrial processes. There are numerous thermal sources of TCDD, such as municipal, hazardous and medical waste incinerators, diesel engine emissions, fires, and residential heating. The manufacture of chlorinated chemicals, wood pulp, and paper also release TCDD (28). Major industrial accidents, including those in Nitro, West Virginia (1949) and Seveso, Italy (1976), resulted in the release of huge amounts of TCDD, causing acute exposure to both workers and residents in the surrounding areas. Because of the chemical stability of TCDD and other related dioxins, there is persistent contamination worldwide. The primary source of human exposure to TCDD is food, particularly dairy products, meat, and fish (29). TCDD is detectable in human serum and is known to accumulate in adipose tissue of humans and animals (30). It also reaches significant levels in human breast milk, providing a pathway for infant exposure (29).

The toxicity of TCDD and other dioxins have, as a result, been intensely studied. Animal experimentation has demonstrated that, based on the minimal effective carcinogenic dose, TCDD is the most potent animal carcinogen yet discovered (31). There is mounting experimental evidence suggesting that TCDD may affect human fertility, because it is associated with anovulation and dysfunctional spermatogenesis in primates and other animals (32–35). Developmental anomalies in infants may arise as a result of exposure via breast milk (36). Epidemiological studies have indicated that TCDD exposure is associated with an increase in many types of cancer, including lung, liver, and rectal cancers (3). TCDD has also been linked to increased ischemic heart disease (37, 38).

Given the ubiquity of TCDD contamination and its adverse impact on human health, the mechanisms of TCDD toxicity are of great interest. TCDD does not bind DNA, which indicates that it is not directly genotoxic. The primary mechanism of biological damage by TCDD has been reported to be the generation of reactive oxygen species (39–41). This occurs as a consequence of CYP enzyme activity. A postulated mechanism for this is the auto-oxidation of the cytochrome P-450 mono-oxygenase complex, which generates superoxide (40). Superoxide can spontaneously dismutate, liberating hydrogen peroxide as a byproduct, which, in the presence of transition metals, can produce hydroxy radicals, leading to indiscriminate damage to cellular biomolecules, including DNA. This hypothesis is consistent with the observation that the deleterious effects of TCDD are dependent on the AhR. The basal expression of CYP is very low. Only after ligands of the receptor, such as TCDD, activate the AhR, does the expression of CYP1A1 (as well as CYP1A2 and CYP1B1) increase. This AhR-dependent mechanism has been demonstrated in AhR-null mice, which do not up-regulate CYP expression when challenged with TCDD and are unaffected by TCDD, even at doses that are 10 times higher than that which induces severe toxic and pathological effects in wild-type mice (10).

Inhibition of AhR-mediated signal transduction would, therefore, prevent TCDD-induced biological damage. We, and others, have described natural and synthetic compounds that act in this regard, e.g., the synthetic flavonoid α-naphthoflavone acts antagonistically by competing with TCDD for binding of the AhR (42). However, by
inhibitory effects (at IC50). The inhibitory effect of SAL on CYP activity is, thus, long-lived. This is in contrast to the actions of other well-known inhibitors of CYP activity, such as resveratrol, α-naphthoflavone, difenzoylemethane, curcumin, and quercetin. The inhibitory effects (at IC50 concentrations) of these compounds rapidly decline, presumably because they are metabolized by the cells, and they are completely ineffective in abolishing TCDD-induced EROD activity after 48 h of incubation (data not shown). Therefore, SAL is the only compound identified thus far that produces a sustained inhibition of TCDD-induced CYP enzyme activity.

Two possible mechanisms may account for the inhibitory action by SAL. First, SAL could directly bind to the CYP enzymes and inhibit their activity. However, when cells were pretreated with TCDD to induce CYP activity, SAL treatment was ineffective (Table 1). Furthermore, SAL added to microsomes isolated from TCDD-treated cells also had no effect on CYP activity (Table 1). These results indicate that SAL does not directly affect CYP enzyme activity. We, therefore, hypothesized that the effect of SAL was at the level of CYP expression. The effect on mRNA levels was investigated by reverse transcription-PCR, which demonstrated that CYP1A1, CYP1A2, and CYP1B1 induction by TCDD was completely blocked in cells cotreated with SAL (Fig. 3A), but SAL treatment alone did not affect basal CYP expression (Fig. 3B). This is in contrast to some other inhibitors of induced CYP expression, such as difenzoylemethane (43), galangin (44), and curcumin (45), that act as agonists when administered alone. Because CYP expression is regulated by XREs, we evaluated the effect of SAL on XRE-driven luciferase activity as a measure of transcription. SAL suppressed the up-regulation of XRE-controlled transcription by TCDD in a concentration-dependent manner, whereas aspirin and salicylic acid, consistent with the lack of effect on EROD activity, had no effect (Fig. 4). The reason that salicylic acid and aspirin are ineffective, despite their structural similarity to SAL, is not known.

We used gel-shift assays to further determine whether SAL affected the binding of activated (ligand-bound) AhR to XREs present in the promoter regions of CYP genes. As shown in Fig. 5, SAL inhibited the binding of the TCDD-activated AhR to the XRE. We concluded from this that the decrease in TCDD-stimulated transcription and mRNA levels caused by SAL (Figs. 3 and 4) was caused by preventing the activation of the AhR to its DNA-binding form. This could occur as a result of inhibiting the binding of the ligand to the receptor, which was confirmed by ligand-binding assay. We demonstrated that SAL substantially reduced the binding of TCDD to the cytosolic AhR (Fig. 6). Although the inhibition was not completely blocked, this was the most likely cause of the high levels (20 nt) of [3H]-TCDD necessary to elicit detectable binding in this assay system (46).

This is the first demonstration of SAL as a potent inhibitor of the AhR that, as a result, can block signal transduction initiated by TCDD exposure. It is, furthermore, a pure antagonist of the AhR, unlike most other AhR inhibitors that have been characterized in this laboratory and others. Unlike several other compounds that directly bind and inhibit CYP enzyme activity, it is exceptionally long-lasting. Finally, SAL has already been extensively used in humans; therefore, its safety and pharmacokinetics are already well established. SAL may, therefore, serve as a promising chemopreventive compound that targets the AhR.

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