Sulindac regulates the aryl hydrocarbon receptor-mediated expression of Phase 1 metabolic enzymes in vivo and in vitro

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Introduction

The non-steroidal anti-inflammatory drugs (NSAIDs) as a class are defined by their ability to inhibit cyclo-oxygenase enzymes, thereby blocking the synthesis of prostaglandins. Numerous epidemiological studies have demonstrated that long-term usage of NSAIDs is associated with a reduced incidence of several types of cancers (1,2). Studies in rodents have found that NSAIDs can inhibit the development of chemically induced cancers (3–7). Although effective as a chemopreventive agent when administered after carcinogen exposure, NSAIDs generally are most effective in chemoprevention in rodent models of carcinogenesis when administered before or concurrent with exposure to the carcinogen, rather than when treatment is delayed until the promotion/progression phase (8). This suggests that NSAIDs may affect the initiation phase of carcinogenesis.

Sulindac is a widely prescribed NSAID. It has been demonstrated that sulindac use is associated with a decreased incidence of several types of cancers (9–11). In rodent models, sulindac has been shown to inhibit dimethylbenzanthracene-induced mammary cancer (3), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane-induced lung cancer (4,5) and azoxymethane-induced colon cancer (6,7). These chemicals are all procarcinogens that are activated and detoxified by a pathway that begins with an oxidation step mediated by members of the cytochrome P450 (CYP) family of mono-oxygenases. CYPs involved in procarcinogen metabolism include the 1A1, 1A2 and 1B1 isoforms. Given the previously demonstrated chemopreventive activity of sulindac against chemically induced carcinogenesis in rodents, we hypothesized that sulindac exerts its chemopreventive effects, in part, by modulating the activity and expression of Phase 1 metabolic enzymes. To test this, we used Sprague–Dawley rats as an in vivo model, and, to determine the mechanisms of expression, we employed HepG2 human liver cancer cells, a well-differentiated cell line that has been extensively used to model CYP expression (12–14). The doses of sulindac used in our in vivo experiments, 200 or 400 mg/kg diet, are similar to those used in previously published rodent studies, which used up to 600 mg/kg diet (3–7). This results in a total dose per rat of ~10 or 20 mg/kg body wt/day of sulindac. This dosage is very similar to that used in human chemoprevention trials, which have used doses up to 22 mg/kg body wt/day (10,11,15,16).

Materials and methods

Materials

Male Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Human hepatoma HepG2 and MCF-7 human breast cancer cells were from American Type Culture Collection (Manassas, VA). RPMI 1640, TRIzol and LipofectAMINE were from Invitrogen (Carlsbad, CA). Glutamine and fetal bovine serum were from BioFluids (Rockville, MD).

Abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; DMSO, dimethylsulfoxide; EROD, ethoxyresorufin-O-deethylase; hnRNA, heterogeneous nuclear RNA; NSAID, non-steroidal anti-inflammatory drug; TCDD, 2,3,5,7-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic-responsive element.
Polydeoxyinosinic-deoxyycytidilic acid, protease inhibitors, ethoxyresorufin and sulindac were from Sigma (St Louis, MO). \[^{3}P\]dATP and \[^{32}P\]dCTP were from Dupont-NEN (Boston, MA). 2,3,5,7-Tetrachlorodibenzo-p-dioxin (TCDD) was from the Midwest Research Institute (Kansas City, MO). [\(^{3}H\)]2,3,5,7-TCDD was from Chemsyn 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).

**Animal treatment protocol**

Male Sprague–Dawley rats were received at 4 weeks of age and maintained for a 12 h light/dark cycle. Animals were provided with an AIN76A semi-purified powder diet and water ad libitum. Beginning at 5 weeks of age, their diet was supplemented with vehicle (olive oil), or sulindac at 200 or 400 mg/kg of diet. Each group consisted of eight animals. After 4 weeks, the animals were euthanized, and the liver was excised, quick frozen and stored at −80°C until analyzed.

**Cell culture**

HepG2 human hepatoma cells were grown with RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco, Grand Island, NY). Cells were grown in a humidified 5% CO2 atmosphere at 37°C. Cells were harvested by trypsinization, washed twice with cold PBS, and suspended in fresh medium at a concentration of 1 x 10⁶ cells/ml.[ECF01]

**Assay of CYP enzyme activity in rat liver**

CYP enzyme activity from rat liver was determined by ethoxyresorufin-O-deethylase (EROD) or ethoxyresorufin-O-demethylase (MROD) activity assay described previously (23). Total CYP protein was determined by Coomassie blue staining of the gel slices after separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (28).

**Reverse transcription–polymerase chain reaction (RT–PCR)**

Total RNA was isolated from a small piece of liver that had been subjected to Polytron homogenization, or from HepG2 cells, using TRIzol as directed. cDNA synthesis, semi-quantitative RT–PCR for rat or human CYP1A1, 1A2, 1B1, glyceraldehyde-3-phosphate dehydrogenase (GPDH) and analysis of results were performed as described previously (23). Primer sequences for human CYP1A1 and CYP1B1 were from Walker et al. (26) and sequences for rat CYP1A1 and CYP1B1 were from Chung and Bresnick (25). Sequences for rat CYP1A1, 1A2 and 1B1 were from Walker et al. (26) and sequences for rat and human GPDH were from Clontech (Palo Alto, CA), with an excitation of 530 nm and emission at 590 nm. The reaction was allowed to run for 30 min. A standard curve was constructed using resorufin.

**Transcription of CYP1A1**

The ability of sulindac to affect CYP enzyme activity was evaluated in intact cells by measurement of EROD activity. In 24-well plates, HepG2 or MCF-7 cells were incubated with sulindac at the concentrations and times indicated in the graph legends. Medium was decanted, and cells were washed once with PBS. Medium containing 5 µM ethoxyresorufin was added, and EROD activity was measured as described above.

**Electrophoretic mobility shift assay**

HepG2 cells were incubated with DMSO (0.1%), 10 nM TCDD alone or 250 µM sulindac for 2 h at 37°C. Nuclear protein was isolated, and EMSA was performed by the method of Denison et al. (29). Synthetic oligonucleotides containing the AhR-binding site of the xenobiotic-responsive element (XRE) (forward primer, 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′; reverse primer, 5′-CGGCTTCTTCTACCGAATCCCCAGTCG-3′) were labeled with \[^{3}P\]dCTP. Binding reactions were performed for 25 min (10 min pre-incubation without probe + 15 min incubation with probe) at room temperature and contained 10 µg of nuclear protein, 1 µg of poly-dIdC and labeled probe (≈50,000 c.p.m.) 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 non-denaturing conditions on a 6% (w/v) polyacrylamide gel with 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.

**Ligand-binding assay**

AhR ligand binding was measured according to the methods of Raha et al. (30). Cytosolic protein (1.5 mg/500 µl) was incubated with 20 nM \[^{32}P\]TCDD in the presence of DMSO (5 µl/500 µl), 2.5 mM sulindac 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 Beckman Quick-Seal rotor tubes at 65,000 rpm for 2 h at 4°C in a Beckman VT-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 analysis of variance (ANOVA), followed by Fisher’s protected least significant difference post-hoc analysis for pairwise comparison of means.

**Results**

**Effect of sulindac on CYP enzyme activity and expression in vivo**

Rats fed a diet containing 200 or 400 mg/kg sulindac for 4 weeks had a 3- and 7-fold increase, respectively, in CYP enzyme activity in microsomes isolated from liver compared with animals fed a control diet (Figure 1). The hepatic levels of mRNA of the three CYP isozymes responsible for EROD activity were measured by RT–PCR. There was a dose-dependent increase in CYP1A1 mRNA in livers of sulindac-treated rats compared with controls, a modest increase in CYP1A2 mRNA and no increase in CYP1B1 mRNA (Figure 2).

**Effect of sulindac on CYP enzyme activity in HepG2 cells**

HepG2 cells treated with sulindac for 24 h demonstrated a dose-dependent increase in EROD activity (Figure 3A).

![Fig. 1. Effect of sulindac on hepatic CYP enzyme activity. Female Sprague–Dawley rats were fed for 4 weeks with a diet supplemented with vehicle control, or sulindac at 200 or 400 mg/kg of diet. Microsomes were isolated from liver and CYP enzyme activity was determined by EROD assay. Mean ± SE, n = 8. Asterisks indicate significant difference from control (*P < 0.05).](https://academic.oup.com/carcin/article-abstract/27/8/1586/2476185)
When cells were treated with a single concentration of sulindac, there was a sustained increase in CYP activity that reached a peak after 12 h and remained at maximal stimulation for 6 days (Figure 3B).

Effect of sulindac on CYP mRNA levels in HepG2 cells
There was a dose-dependent increase in CYP1A1 mRNA in HepG2 cells treated with sulindac, reaching a maximal stimulation of 7-fold greater than controls in cells treated with 250 \( \mu \)M sulindac (Figure 4A). There was a modest 2-fold increase in CYP1A2 mRNA (Figure 4A). There was no detectable increase in CYP1B1 mRNA (data not shown). Similar to enzyme activity, sulindac caused a sustained increase in CYP1A1 mRNA, which remained elevated compared with controls for 4 days after treatment commenced (Figure 4B).

Effect of sulindac on CYP1A1 transcription
Pretreatment of HepG2 cells with actinomycin D blocked the increase in CYP1A1 mRNA that occurred in cells treated with sulindac (Figure 5A). We also measured the level of heterogeneous nuclear RNA (hnRNA) of CYP1A1. As shown in Figure 5B, sulindac caused a dose-dependent increase in CYP1A1 hnRNA, which remained elevated compared with controls for 4 days after treatment commenced (Figure 4B).

Effect of sulindac on AhR activity
As shown in Figure 6, sulindac induced EROD activity in wild-type MCF-7 cells, but not in an AhR-deficient MCF-7 subclone. We then performed gel-shift analysis for the binding of ligand-activated AhR to oligonucleotides representing the XRE sequences that upregulate CYP1A1 gene expression. As shown in Figure 7, sulindac caused a marked band shift, indicating that it activated the DNA-binding capacity of the AhR. TCDD, the prototypical activator of the AhR, was included as a positive control. Finally, we examined whether sulindac could bind to the AhR using ligand-binding experiments. Sulindac was able to partially displace the potent AhR ligand TCDD (Figure 8).

Discussion
Although the chemopreventive effects of NSAIDs, such as sulindac, are generally attributed to their ability to inhibit cyclo-oxygenase activity, other biochemical mechanisms have been identified. It has been shown that sulindac induces apoptosis in tumor cells (10,18–21) and inhibits angiogenesis (31). Interestingly, sulindac has been shown to induce the activity and expression of Phase 2 carcinogen detoxifying enzymes, including QR in vitro (32) and glutathione

![Graph showing effect of sulindac on CYP mRNA levels in HepG2 cells.](image)

![Graph showing effect of sulindac on AhR activity in MCF-7 cells.](image)
S-transferase in vivo (33,34). These data suggest that an alternative mechanism may contribute to the chemopreventive effect of sulindac, the ability to modulate carcinogen detoxification pathways. However, the regulation of Phase 1 enzymes, which catalyze the initial step of xenobiotic detoxification, and the molecular mechanism that controls their expression by sulindac have not been examined. Because sulindac has been shown to inhibit carcinogenesis induced by tobacco carcinogens that are substrates of CYP enzymes, we hypothesized that the protective effect of sulindac may result from the modulation of the activity and expression of Phase 1 enzymes. The doses of sulindac used in our in vivo model were selected on the basis of previously published reports (3–7) and reflect the approximate doses used in Phase 1 clinical trials (10,11,15,16). For in vitro experiments, the doses were
for 6 days after treatment commenced (Figure 3B), and CYP1A1 mRNA levels were elevated above controls for 4 days (Figure 4B). Such a sustained elevation in CYP1A1 expression following exposure to sulindac would increase its chemopreventive efficacy.

We determined that the increase in CYP1A1 mRNA in cells treated with sulindac was due to increased transcription of the CYP1A1 gene. This was demonstrated in two ways. Actinomycin D, an RNA polymerase inhibitor that prevents transcription, completely blocked the increase in CYP1A1 mRNA in cells treated with sulindac (Figure 5A). We also showed that the level of CYP1A1 hnRNA, an assay that has been previously characterized as a valid measure of CYP1A1 transcription (20,21), was increased in cells treated with sulindac (Figure 5B). The dose of sulindac at which a significant increase in CYP1A1 hnRNA was observed was higher than in EROD assay for enzyme activity or RT–PCR for mRNA level.

In our experience with other CYP1A1 transcription inducers, measuring hnRNA is less sensitive than those methods, accounting for the higher sulindac doses required.

CYP1A1 transcription is regulated by the AhR. The AhR is a cytosolic protein that, when activated by a ligand, translocates to the nucleus and binds to its protein partner, the aryl hydrocarbon nuclear translocator (36). Together, this heterodimer forms a transcription factor that binds to a specific sequence of nucleotides, the XRE, in the promoter region upstream of a number of genes involved in xenobiotic metabolism, including CYP1A1. Because sulindac induced an increase in CYP1A1 transcription, we investigated whether this activity was mediated by the AhR. First, we used a subclone of the MCF-7 human breast cancer cell line, which we have previously characterized as lacking in the AhR (37). Sulindac induced EROD activity in wild-type MCF-7 cells, but not in the AhR-deficient subclone (Figure 6), which suggests that sulindac’s effect is dependent on the AhR. To further test this, we examined whether sulindac could activate the DNA-binding capacity of the AhR to the XRE of CYP1A1 by gel-shift assay. As shown in Figure 7, sulindac treatment of cells caused a band shift similar to that caused by the prototypical AhR ligand, TCDD. We have previously characterized this band shift as AhR-specific, using unlabeled XRE or an antibody to the AhR, both of which eliminate the band shift (38). We also examined whether sulindac could disrupt [3H]TCDD binding to the AhR. This indirect method of ligand-binding determination is currently the best available assay to determine ligands of the AhR, as most ligands other than TCDD do not have sufficient affinity for the AhR to be distinguishable above non-specific binding. As shown in Figure 8, an excess of sulindac substantially reduced binding of [3H]TCDD from the receptor. Because of the relatively high affinity of TCDD compared with any competitor, a large excess of sulindac was required to compete with TCDD for the receptor. These data indicates that sulindac directly interacts with and is a ligand of the AhR. Sulindac is, therefore, a novel addition to the growing list of AhR ligands.

The data in the present work demonstrate, for the first time, that sulindac induces CYP1A1 expression in vivo and in vitro, and that this activity occurs in an AhR-dependent manner. This is the first demonstration of an NSAID that induces Phase 1 enzyme expression, and represents a novel biochemical mechanism that may contribute to the chemopreventive effect of sulindac in chemically induced tumorigenesis in rodent models. Several questions remain. Do other NSAIDs affect

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**Fig. 7.** Effect of sulindac on XRE-binding activity of the AhR. HepG2 cells were incubated with DMSO (vehicle control), 250 μM sulindac or 10 nM TCDD (positive control) for 2 h. Nuclear protein was isolated and activated AhR (indicated by the arrow) was measured by EMSA. Densitometry demonstrated a 3.23-fold increase in binding in TCDD-treated cells, and a 2.49-fold increase in sulindac-treated cells. Results are representative of two separate experiments.

**Fig. 8.** Competition of sulindac with [3H]TCDD for binding to the AhR. Cytosol isolated from HepG2 cells was incubated with [3H]TCDD in the presence of DMSO (control, filled circle), 10 μM unlabeled TCDD (open diamond) or 2.5 mM sulindac (open triangle) and subjected to sucrose density gradient centrifugation.

selected on the basis of previously published reports employing similar model systems (18–22).

In the present study, we demonstrated that sulindac induces hepatic CYP enzyme activity in vivo (Figure 1), as measured by EROD assay. Although three isozymes of CYP carry out EROD activity, the 1A1 form has a much higher specific EROD activity than 1A2 and 1B1 (35). This suggested that the majority of the increase in EROD activity in animals treated with sulindac would be due to increased 1A1 expression. This was demonstrated by RT–PCR, which showed that CYP1A1 mRNA is preferentially induced by sulindac treatment (Figure 2). In order to delineate the mechanism of this effect, we examined the effect of sulindac on CYP activity and expression in vitro.

As in the rat, sulindac caused a dose-dependent increase in EROD activity (Figure 3A) and CYP1A1 mRNA expression (Figure 4A) in HepG2 cells. Interestingly, the increase was particularly long-lasting, as enzyme activity remained elevated...
the AhR and CYP1A1 expression? Recently, we demonstrated that aspirin has no effect on the AhR-mediated pathway, but an aspirin derivative, salicylamide, inhibits AhR activity in vitro (39). Therefore, NSAIDs, as a class, do not necessarily affect AhR activity. Other NSAIDs, for example, the new generation of selective NSAIDs such as celecoxib, have not been examined with regard to the AhR. Secondly, do the metabolites of sulindac also affect AhR activity? Sulindac is a sulfone that is reduced in vivo to the pharmacologically active (in terms of cyclo-oxygenase inhibition) metabolite sulindac sulfide (40). This conversion is reversible, resulting in an equilibrium between the sulfone and sulfide forms of sulindac in vivo. Additionally, sulindac may be oxidized to a sulfone metabolite, which does not inhibit cyclo-oxygenase activity. The effect of these metabolites on Phase 1 enzyme expression is currently being examined. Finally, would an increase in CYP1A1 expression and activity induced by sulindac truly be chemopreventive? CYP1A1 enzyme activity is a two-edged sword. It catalyzes the mono-oxidation of carcinogens, creating reactive epoxides that may bind DNA and cause mutations. CYP1A1 activity may, therefore, be viewed as a key step in the carcinogenicity of environmental carcinogens, such as the tobacco carcinogen benzopyrene. Thus, hypothetically, inhibition of CYP1A1 activity and expression would protect against the activation of carcinogens. However, several lines of evidence call this hypothesis into question. The epoxides formed by CYP1A1 activity are better substrates for Phase 2 enzyme conjugations than the parent carcinogen, facilitating detoxification. Thus, a coordinate induction of Phase 1 and 2 enzymes would be protective against aryl hydrocarbons. The ability of sulindac to induce Phase 2 enzyme expression, including quinone reductase and glutathione-S-transferase, has previously been well characterized (32–34). Furthermore, chemicals that induce CYP1A1 expression via activation of the AhR, such as the dietary component indole 3-carboline (41), are protective against chemically induced carcinogenesis. Recent studies have shown that CYP1A1 knockout mice treated with benzopyrene have higher levels of benzopyrene-DNA adducts (42) and are more susceptible to benzopyrene-induced toxicity (43) than wild-type mice. Thus, the preponderance of evidence indicates that CYP1A1 induction should be viewed as the first step in carcinogen detoxification, and the induction of CYP1A1 activity and expression by sulindac demonstrated in the present manuscript would be chemopreventive.

Recent studies have indicated that the newer generation of NSAIDs, such as rofecoxib, are associated with an increased incidence of adverse cardiovascular events (44). Revisiting the chemopreventive potential of the non-specific NSAIDs such as sulindac is likely, and it is important to understand the basic biochemical mechanisms underlying their chemopreventive capacity.

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