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Suppression of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Mediated Aryl Hydrocarbon Receptor Transformation and CYP1A1 Induction by the Phosphatidylinositol 3-Kinase Inhibitor 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002)

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ABSTRACT. Numerous flavonoids are ligands of the aryl hydrocarbon receptor (AHR) and function as AHR antagonists and/or agonists. LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] is a widely used inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), and is structurally related to members of the flavonoid family. Concentrations of LY294002 ≥ 10 μM were cytostatic, but not cytotoxic, to cultures of the immortalized human breast epithelial cell line MCF10A-Neo. Treatment of MCF10A-Neo cultures with the AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) stimulated the transcriptional activation of *CYP1A1*, as monitored by measurements of steady-state CYP1A1 mRNA. Pretreatment of cultures with ≥ 10 μM LY294002 suppressed the TCDD activation of *CYP1A1* (IC_{50} ~10 μM). Electrophoretic mobility shift assays employing rat liver cytosol demonstrated that concentrations of LY294002 ≤ 400 μM did not transform the AHR into a DNA-binding species. However, the addition of LY294002 to cytosol just prior to TCDD addition completely suppressed AHR transformation by TCDD (IC_{50} ~35 μM). The PI 3-kinase inhibitor Wortmannin was weakly cytostatic, but not cytotoxic to MCF10A-Neo cultures at concentrations ≤ 500 nM. Exposure of cultures to Wortmannin (10–500 nM) did not suppress TCDD activation of *CYP1A1*. Analyses of the phosphorylation status of Akt-1, an *in vivo* substrate of PI 3-kinase, demonstrated that concentrations of LY294002 ≥ 50 μM and Wortmannin ≥ 10 nM completely suppressed PI 3-kinase activity. Hence, the ability of LY294002 to suppress TCDD-dependent activation of *CYP1A1* is unrelated to PI 3-kinase inhibition. Instead, this activity reflects LY294002 functioning as an AHR antagonist. Furthermore, most of the cytostatic activity of LY294002 towards MCF10A-Neo cells is unrelated to the inhibition of PI 3-kinase. BIOCHEM PHARMACOL **60**;5:635–642, 2000.

KEY WORDS. aryl hydrocarbon receptor; CYP1A1; LY294002; phosphatidylinositol 3-kinase; TCDD

The AHR† is a ligand-activated transcription factor [1, 2]. In its non-liganded form it appears to be a cytosolic protein in many tissues and cell lines. Upon binding ligand, the AHR translocates to the nucleus, where it complexes with the ARNT protein. AHR/ARNT heterodimers subsequently interact with enhancer sequences in target genes designated DREs, and stimulate the transcription of such genes [1, 2]. A variety of genes contain DRE sequences in their 5′-flanking regions and are transcriptionally activated by agonists of the AHR. Among these are several genes involved in the phase I metabolism (e.g. *CYP1A1*, *CYP1A2*, *CYP1B1*) and phase II metabolism (e.g. *ALDH4* and *NQO1*) of xenobiotics [1–3].

Mechanistic analyses of AHR activation commonly employ TCDD as the prototypical AHR agonist. However, a variety of planar, aromatic molecules are ligands of the AHR. Indeed, many flavonoids are AHR ligands [4–7]. The consequences of flavonoid binding to the AHR are often concentration-dependent. At the lower end of the concentration range at which they bind to the AHR, many flavonoids function as antagonists. As antagonists they can compete with TCDD for binding, but do not transform the AHR into a functional transcription factor. At higher concentrations, the same flavonoids function as AHR agonists and duplicate many of the actions of TCDD [4–6].

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† *Abbreviations:* AHR, aryl hydrocarbon receptor; ALDH4, aldehyde dehydrogenase class 4; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP, cytochrome P450; DRE, dioxin response element; EMSA, electrophoretic mobility shift assay; MEK, mitogen-activated protein kinase kinase; NQO1, NADP(H):quinone oxidoreductase; PI 3-kinase, phosphatidylinositol 3-kinase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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Although most flavonoids appear to have these concentration-dependent dual activities, a limited number of flavonoids appear to be exclusively AHR antagonists [6, 7].

The enzyme PI 3-kinase participates in processes affecting cell proliferation, cytoskeletal architecture, and susceptibility to apoptosis [8–14]. Wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] are used widely as specific inhibitors of PI 3-kinase, and have been used extensively to identify the functions of PI 3-kinase in biological processes [8–15]. However, pharmacological approaches to the study of enzyme function are dependent upon the specificity of the agent, and often an agent has activities in addition to its desired property. For example, the flavonoid PD98059 (2′-amino-3′-methoxyflavone) is widely used as a specific inhibitor of MEK and the kinases downstream of it [16]. Recently, we demonstrated that PD98059 suppresses the TCDD-dependent activation of AHR-responsive genes in the immortalized human breast epithelial cell line MCF10A-Neo [17]. The concentration–response curves defining this activity of PD98059 correlated perfectly with its ability to inhibit MEK [17]. However, the ability of PD98059 to inhibit the effects of TCDD was unrelated to its MEK inhibitory activity. Rather, it reflected the functioning of PD98059 as an AHR antagonist [17].

Exposure of MCF10A-Neo cultures to TPA strongly suppresses the TCDD-dependent transcriptional activation of *CYP1A1* [18]. TPA has been reported to be an activator of PI 3-kinase [19]. As a consequence of preliminary studies designed to determine whether PI 3-kinase mediates the effects of TPA, we found that LY294002 suppressed *CYP1A1* activation by TCDD, at concentrations that should have inhibited PI 3-kinase activity. However, since LY294002 is structurally similar to flavone (Fig. 1), and several flavonoids are known AHR antagonists [5], there existed the possibility that the suppressive effects of LY294002 on *CYP1A1* activation may be unrelated to its effects on PI 3-kinase activity. The current study was designed to determine whether LY294002 is an AHR antagonist, and if PI 3-kinase activity is required for the TCDD-dependent activation of *CYP1A1*.

MATERIALS AND METHODS

Materials

LY294002 was purchased from New England Biolabs. TCDD was the gift of Dr. S. Safe. Wortmannin was obtained from the Calbiochem-Novabiochem Corp. Trypsin, epidermal growth factor, penicillin/streptomycin solution, and horse serum were purchased from GIBCO BRL. [γ-^{32}P]dATP and [α-^{32}P]dCTP were purchased from DuPont–New England Nuclear. Antibodies raised in sheep to polypeptides corresponding to residues 466–480 and 467–477 of rat Akt-1 were purchased from Upstate Biotechnology and used to detect total and phospho-Akt-1, respectively. Protease inhibitor cocktail (product P2714) was purchased from the Sigma Chemical Co.

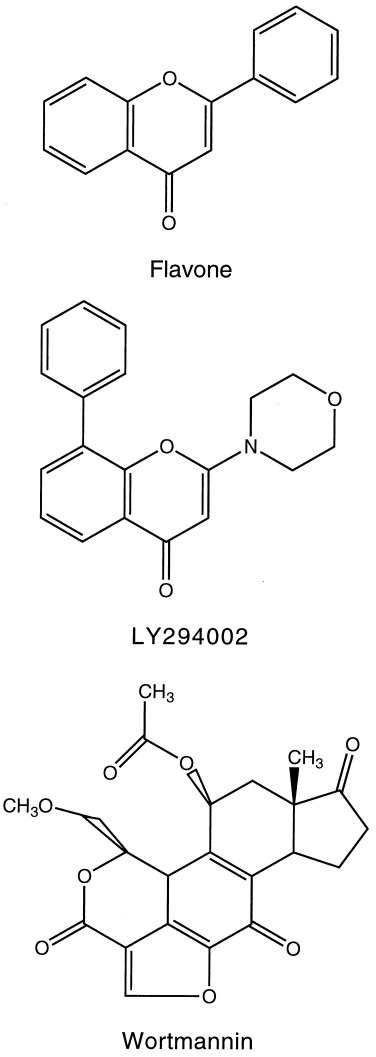

FIG. 1. Structures of flavone, LY294002, and Wortmannin.

Cell Culture and Treatment

The MCF10A-Neo cell line was obtained from the Cell Lines Resource, Karmanos Cancer Institute. The MCF10A-Neo line was derived by transfection of the spontaneously immortalized, normal human breast epithelial cell line MCF10A with the pHo6 plasmid and subsequent selection for resistance to G418. The derivations and characterizations of the parental and MCF10A-Neo cell lines have been described elsewhere [20, 21]. The MCF10A and MCF10A-Neo cell lines are very similar in their growth properties and responses to TCDD [22]. However, MCF10A-Neo cells accumulate more CYP1A1 RNA following TCDD exposure than do MCF10A cells [22].

MCF10A-Neo cells were cultured in supplemented Dulbecco's modified Eagle's medium/Ham's F-12 medium as described by Basolo *et al.* [23] in a humidified atmosphere of 95% air/5% CO_2 at 37°. The supplements consisted of several growth factors and 10% horse serum. Subconfluent cultures were treated with various concentrations of chemicals dissolved in DMSO (absolute volume of solvent $\leq$

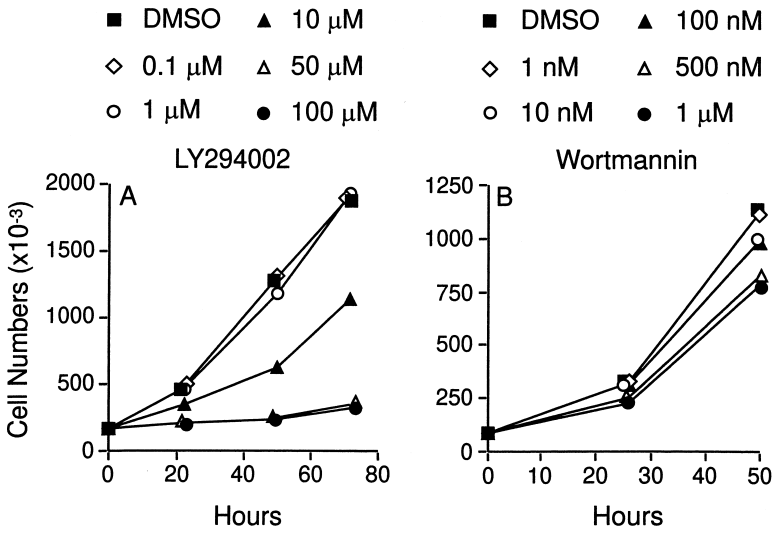

FIG. 2. Cytostatic effects of LY294002 and Wortmannin. Exponentially growing MCF10A-Neo cultures were harvested for cell counts and estimates of viability at various times after treatment with DMSO, LY294002, or Wortmannin (concentrations of treatment are provided in the figure). Data represent means ± SD of three plates.

0.1% of medium volume). Details of treatment are provided in the text. Viability was assessed by measurement of trypan blue exclusion. Cultures earmarked for RNA isolation were washed twice with calcium- and magnesium-free PBS at the time of harvesting and stored at -80°.

RNA Preparation and Northern Blot Analyses

Total cellular RNA was isolated using the acidic phenol extraction method of Chomczynski and Sacchi [24]. RNA was resolved on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes as described previously [22]. The probes used for the detection of CYP1A1 and 7S RNAs and the conditions used for hybridization have been described in detail in a previous publication [22].

EMSA

The conditions reported by Shen *et al.* [25] were used for the EMSA. Complementary oligonucleotides 5′-GATC CGGCTCTTCTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCG-3′ (the single-core DRE recognition sequence is underlined) were annealed and used to detect activated AHR/ARNT complexes.

Western Blot Analyses of Akt-1

Culture dishes were washed twice with cold PBS (containing 1 mM NaVO) before the addition of lysis buffer [10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, 10 mM $NaPP_i$, 1 mM $NaVO_4$, and 1/10 (v/v) of a 10x solution of protease inhibitor cocktail]. Extracts were sonicated for 5 sec. Insoluble material was removed by centrifugation, and the supernatant was boiled in Laemmli's sample buffer. Equal amounts of protein were resolved on SDS–7.5% polyacrylamide gels and then transferred to nitrocellulose membrane. The resulting protein blot was blocked for 3 hr at room temperature in PBST (PBS containing 5% nonfat dry milk and 0.05% Tween 20). Blocked blots were incubated overnight at room temperature with 1 μg/mL of anti-Akt-1 or anti-phospho-Akt-1 (diluted in PBS containing 2% nonfat dry milk). Blots were subsequently washed for 3 × 10 min with PBST, and then incubated with rabbit anti-sheep Ig conjugated with horseradish peroxidase (diluted in PBS containing 2% nonfat dry milk) for 2 hr at room temperature. Blots then were washed and developed using Amersham enhanced chemiluminescence reagents. Akt-1-immunoglobulin conjugates were recorded on x-ray film. Enhanced chemiluminescence development was conducted according to the manufacturer's specifications.

^{32}P Detection and Quantitation

^{32}P-Labeled nucleic acids were detected either by autoradiography with x-ray film or with a BioRad GS-525 Molecular Imager. Detection by the latter technique allowed quantitation with Molecular Dynamics software.

RESULTS

Cytostatic and Cytotoxic Effects of LY294002

Exposure of MCF10A-Neo cultures to LY294002 resulted in a concentration-dependent inhibition of cell proliferation (Fig. 2A). Concentrations of LY294002 ≥ 50 μM completely suppressed MCF10A-Neo proliferation for at least 3 days. The antiproliferative effects of LY294002 occurred in the absence of any cytotoxicity (Guo M, unpublished studies).

LY294002 Suppression of Transcriptional Activation of CYP1A1

CYP1A1 mRNA was not detected in asynchronous, DMSO-treated MCF10A-Neo cultures, or in cultures ex-

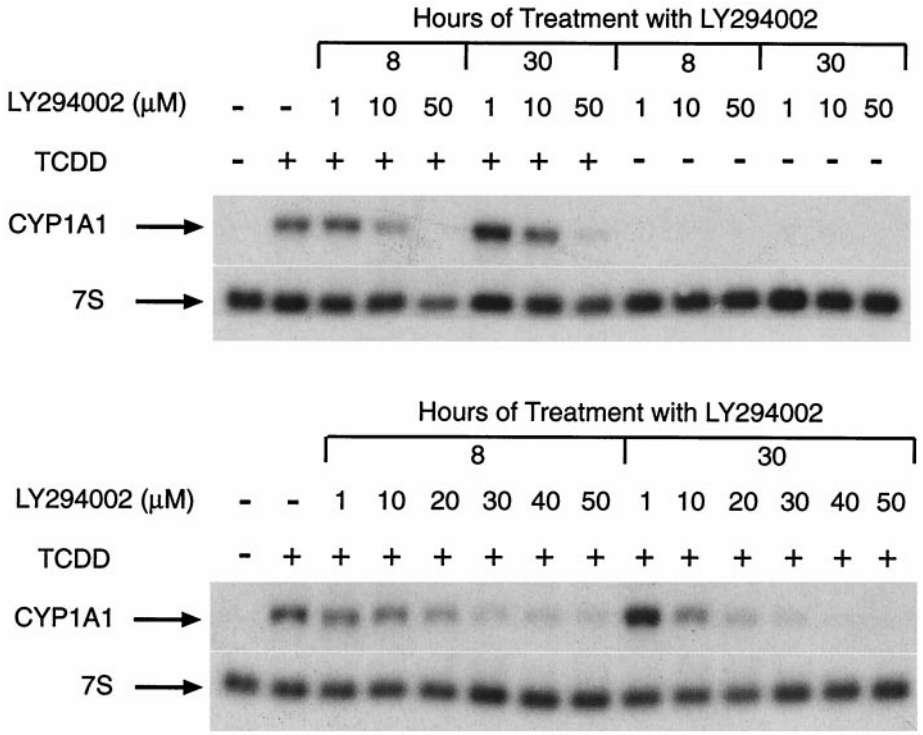

FIG. 3. LY294002 effects on *CYP1A1* activation. Exponentially growing MCF10A-Neo cultures were treated with 10 nM TCDD for 6 hr, or with various concentrations of LY294002 for 8 or 30 hr, prior to harvesting and isolation of RNA for analyses of CYP1A1 and 7S RNAs. Some cultures were treated with LY294002 for either 2 or 24 hr prior to the addition of 10 nM TCDD. These cultures subsequently were harvested 6 hr after TCDD addition.

posed to non-cytostatic or cytostatic concentrations of LY294002 for 8 or 30 hr (Fig. 3). In contrast, steady-state CYP1A1 mRNA content was elevated dramatically within 6 hr of exposure to the potent AHR agonist TCDD (Fig. 3). Treatment of cultures with LY294002 for either 2 or 24 hr prior to the addition of TCDD resulted in a concentration-dependent suppression of the accumulation of CYP1A1 mRNA (Fig. 3; IC_{50} ~10 μM for a 2-hr preincubation, based upon the data presented in the figure and two additional experiments). The effects of LY294002 on the TCDD activation of *CYP1A1* were not altered markedly by extending the period of preincubation from 2 to 24 hr.

AHR Transformation and DNA Binding

The EMSA assay is commonly used to evaluate the ability of a chemical to (a) activate the AHR into a species capable of complexing with ARNT and binding to DNA (AHR agonist activity), or (b) inhibit the AHR agonist activity of TCDD (AHR antagonist activity). We used the EMSA assay to determine whether LY294002 could mediate or suppress AHR transformation in rat liver extracts. We used rat liver cytosol instead of MCF10A-Neo cytosol because the latter, in the absence of exogenous ligand, displays a high level of spontaneous AHR–DNA complex formation [22]. Incubation of rat liver cytosol with TCDD transformed the AHR into a species that bound to a labeled oligonucleotide containing a DRE sequence (Fig. 4). This binding could be prevented by co-incubation with a 20-fold excess of unlabeled (cold) oligonucleotide. In contrast to TCDD, no AHR–DNA complex was seen in extracts treated with concentrations of LY294002 as high as 400 μM (Fig. 4, left panel). A similar lack of AHR agonist activity was noted in studies employing concentrations of LY294002 as high as 1 mM (Reiners JJ Jr, unpublished data). However, co-incubation of liver extracts with TCDD and various amounts of LY294002 (Fig. 4, right panel) resulted in a concentration-dependent suppression of AHR–DRE complex formation (IC_{50} ~35 μM; as determined from Fig. 4 and three additional independent experiments).

Measurement of PI 3-Kinase Activity

The IC_{50} for the *in vitro* inhibition of PI 3-kinase activity by LY294002 is ~1.4 μM [15]. Hence, the concentrations at which LY294004 suppressed MCF10-Neo growth and the transcriptional activation of *CYP1A1* presumably also inhibited PI 3-kinase activity. This possibility was tested by assessing the phosphorylation status of Akt-1, an *in vivo* substrate of PI 3-kinase that is activated by phosphorylation [8]. Phospho-Akt-1 could be detected in exponentially growing cultures of MCF10A-Neo cultures (Fig. 5, lane 9). However, the signal was weak and variable from experiment to experiment. The amount of phospho-Akt-1 could be increased dramatically by maintaining established cultures for 24 hr in serum- and growth factor-depleted medium, and then refeeding them with complete medium.

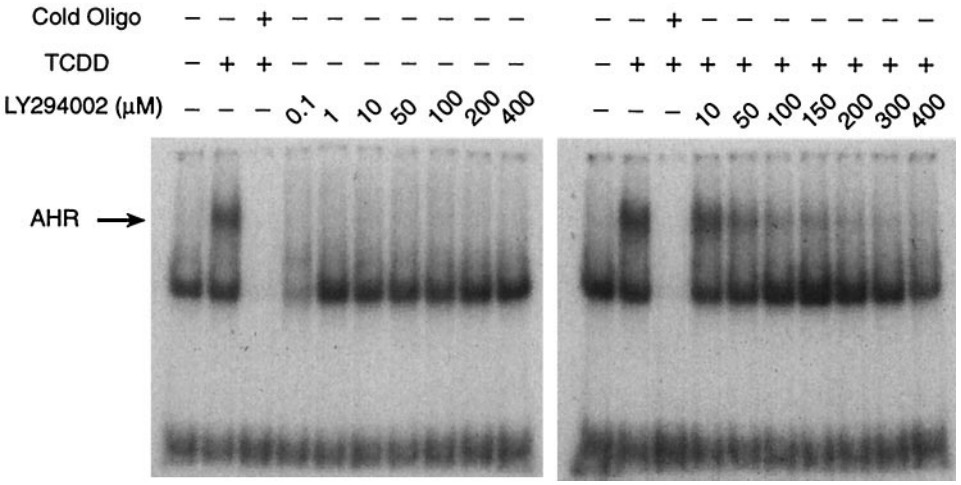

FIG. 4. EMSA of LY294002 as an AHR agonist and antagonist. Rat liver extract was transformed *in vitro* with various concentrations of LY294002 (left panel), or various concentrations of LY294002 and 2 μM TCDD (right panel), before being incubated with labeled DRE oligonucleotide and analyzed in a gel retardation assay. In lane 3 of each gel, a 20-fold excess of unlabeled (cold) DRE oligonucleotide was included in the EMSA reaction mixture.

Serum- and growth factor-depleted cultures arrested in G_o/G_1 (Reiners JJ Jr, unpublished data) and had undetectable levels of phospho-Akt-1 (Fig. 5, lane 1). However, phospho-Akt-1 was detected readily within 5 min of refeeding with complete medium, and remained phosphorylated for at least 4 hr (Fig. 5, lanes 2–8). PI 3-kinase is activated rapidly in growth factor-depleted cells following exposure to serum and growth factors [8]. Presumably the detected increases in phospho-Akt-1 reflected serum/growth factor activation of PI 3-kinase. Indeed, exposure of serum- and growth factor-starved cultures to LY294004 40 min prior to refeeding with complete medium resulted in a concentration-dependent suppression of Akt-1 phosphorylation (Fig. 6). Specifically, suppression required concentrations of LY294002 $>$ 10 μM. Complete suppression occurred with concentrations $\geq$ 50 μM.

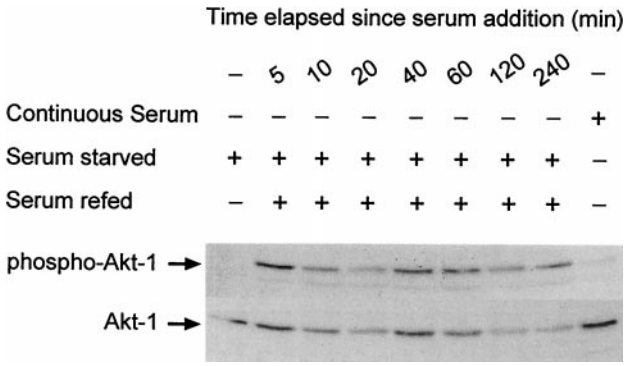

FIG. 5. Activation of Akt-1 by serum and growth factors. Exponentially growing MCF10A-Neo cultures were shifted to a medium lacking growth factors and serum for ~20 hr. Thereafter, most of the cultures were refed with complete growth medium. Cultures subsequently were harvested for the preparation of extracts for western blot analyses. Samples (25 μg protein) were separated on SDS–7.5% polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed as described in the text, using antibodies to Akt-1 and phospho-Akt-1.

Wortmannin Inhibition of PI 3-Kinase Activity

The concentrations at which LY294002 suppressed TCDD activation of *CYP1A1* and cell proliferation also inhibited PI 3-kinase (compare Figs. 2, 3, and 6). To determine whether the suppressive effects of LY294002 on *CYP1A1* activation and cell proliferation could be attributed to an inhibition of PI 3-kinase, we examined the effects of a

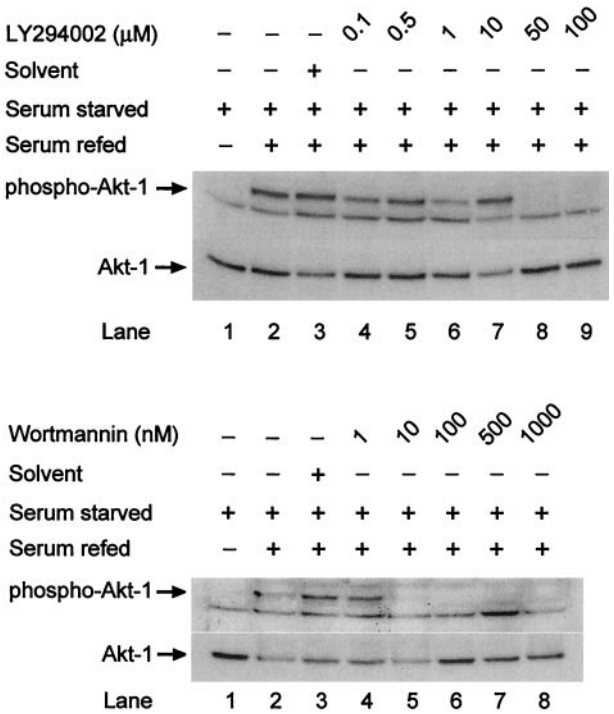

FIG. 6. Suppression of Akt-1 activation by LY294002 and Wortmannin. Exponentially growing MCF10A-Neo cultures were shifted to a medium lacking growth factors and serum for ~20 hr. LY294002 and Wortmannin were added to the cultures at the indicated concentrations ~40 min prior to refeeding the cultures with complete growth medium. Upon refeeding, the cultures were re-treated immediately with the PI 3-kinase inhibitors and harvested 10 min later for preparation of extracts for western blot analyses and subsequent Akt-1 detection.

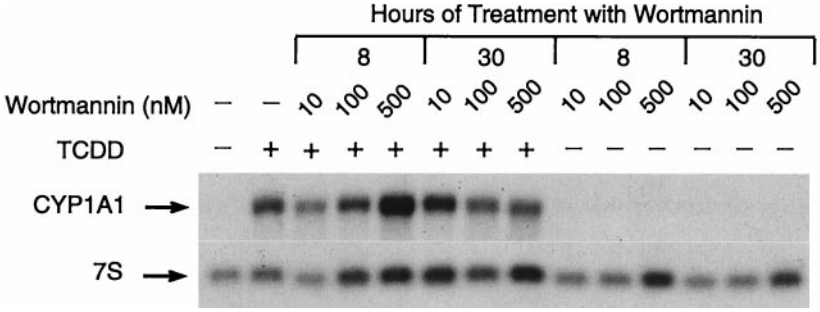

FIG. 7. Wortmannin effects on *CYP1A1* activation. Exponentially growing MCF10A-Neo cultures were treated with 10 nM TCDD for 6 hr, or various concentrations of Wortmannin for 8 or 30 hr, prior to harvesting and the isolation of RNA for analyses of CYP1A1 and 7S RNA. Some cultures were treated with Wortmannin for either 2 or 24 hr prior to the addition of 10 nM TCDD. These latter cultures subsequently were harvested 6 hr after TCDD addition.

second PI 3-kinase inhibitor. Wortmannin is an irreversible inhibitor of PI 3-kinase and is structurally unrelated to LY294002 (Fig. 1). It is used routinely in the 10–500 nM range to suppress PI 3-kinase activity in cultured cells [9–12]. Concentrations of Wortmannin ≥ 10 nM suppressed the phosphorylation of Akt-1 that occurred following serum/growth factor stimulation of serum- and growth factor-starved MCF10A-Neo cultures (Fig. 6).

Cytostatic and Cytotoxic Effects of Wortmannin

Treatment of MCF10A-Neo cultures with Wortmannin resulted in a concentration-dependent suppression of cell proliferation (Fig. 2B). However, the anti-proliferative effects of Wortmannin were quite weak even at concentrations that suppressed PI 3-kinase activity totally (compare Figs. 2B and 6). The cytostatic effects of Wortmannin occurred in the absence of any cytotoxicity (Guo M, unpublished studies).

Wortmannin Effects on Transcriptional Activation of CYP1A1

Basal levels of CYP1A1 mRNA in MCF10A-Neo cultures remained undetectable following exposure to concentrations of Wortmannin sufficient to inhibit PI 3-kinase (Fig. 7). Similarly, treatment of cultures with Wortmannin either 2 or 24 hr prior to the addition of TCDD did not suppress the subsequent TCDD-stimulated accumulation of CYP1A1 mRNA (Fig. 7).

DISCUSSION

LY294002 is commonly used in studies designed to assess the contribution of PI 3-kinase to biological processes. An inherent assumption of such studies is that the effects seen in the presence of LY294002 reflect exclusively its ability to inhibit PI 3-kinase. The current study demonstrated that concentrations of LY294002 commonly used to inhibit PI 3-kinase in cultured cells also suppress the TCDD-dependent activation of *CYP1A1*, an AHR-dependent process. However, this latter effect was unrelated to changes in PI 3-kinase activity, since a similar suppression was not obtained with concentrations of Wortmannin that suppressed PI 3-kinase activity totally in our culture system. The Wortmannin results also suggested that PI 3-kinase activity is not required for an AHR-dependent activation of *CYP1A1*.

LY294002 is structurally similar to flavone, and numerous flavonoids are AHR ligands and function as AHR antagonists [4–7]. LY294002 displayed AHR antagonist activity, but no agonist activity, when analyzed by EMSA using an oligonucleotide containing a DRE. Indeed, the IC_{50} values estimated for LY294002 suppression of *CYP1A1* activation and AHR–DRE binding (as measured by EMSA) were very similar. Hence, the ability of LY294002 to inhibit the TCDD-dependent transcriptional activation of *CYP1A1* probably reflects its functioning as an AHR antagonist. Although the flavonoid literature and our data collectively suggest that LY294002 may be an AHR ligand, we have not performed the ligand competition assays needed to resolve the issue.

Concentrations of LY294002 (≥50 μM) sufficient to inhibit PI 3-kinase completely in MCF10A-Neo cultures were also very cytostatic. Several studies have implicated a role for PI 3-kinase in mitogenic signaling processes [8, 13]. However, concentrations of Wortmannin that totally suppressed PI 3-kinase activity were only weakly cytostatic to MCF10A-Neo cultures. Given the Wortmannin data, it is likely that only a portion of the cytostatic activity of LY294002 is mediated by its inhibition of PI 3-kinase.

Exposure to TCDD suppresses the proliferation of several, but not all, cultured cell types [26–30]. Studies employing rat 5L cells, and an AHR-deficient variant (BP8 cells) derived from them, have shown that the cytostatic effects of TCDD are dependent upon its interaction with the AHR [26, 27]. Numerous flavonoids are also cytostatic as a consequence of their induction of either a G_1 or G_2/M arrest [31–33]. Studies using 5L and BP8 cells have demonstrated that the cytostatic activities of flavonoids reflect both AHR-dependent and -independent processes [26]. In the case of the AHR-dependent processes, cytostatic effects were noted only at concentrations at which flavonoids function as AHR agonists [26]. Although LY294002 may be an AHR ligand, the EMSA results and its inability to activate *CYP1A1* strongly suggest that it functions as an AHR antagonist and has no agonist activity. Hence, it seems unlikely that the cytostatic effects of LY294002 in MCF10A-Neo cells are a consequence of its interaction with the AHR.

The extent to which the AHR antagonist activity of

LY294002 compromises its usefulness as a PI 3-kinase inhibitor will depend upon the nature of the study. If it involves an AHR agonist, and the process being investigated is dependent upon AHR activation, LY294002 is not the agent of choice for assessing the role of PI 3-kinase activity in the process.

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