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Lack of the Aryl Hydrocarbon Receptor Leads to Impaired Activation of AKT/Protein Kinase B and Enhanced Sensitivity to Apoptosis Induced via the Intrinsic Pathway

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is best known for its role in mediating the toxicity of many environmental contaminants such as 2,3,7,8 tetrachlorodibenzo-*p*-dioxin. However, the endogenous role of AHR, especially with respect to the apoptotic process, is largely unknown and contradictory. In this report, we have used a mouse hepatoma cell line (Hepa1c1c7) and its AHR-deficient derivative (LA1) to examine the effect of differing AHR levels on apoptosis susceptibility, in particular, apoptosis regulated by the intrinsic pathway. Toward this end, the cells were subjected to UV irradiation, hydrogen peroxide, and serum starvation. Analyses of a number of different endpoints of apoptosis revealed that the LA1 cells were more sensitive to these stresses than the wild-type cells, indicating that the AHR plays a cytoprotective role in the face of stimuli that initiate the intrinsic apoptotic pathway. A direct role of the AHR in mediating this effect was confirmed using both pharmacological and molecular approaches. Further analyses imply that lack of the AHR leads to an impaired survival response mediated by phosphatidylinositol 3′-OH kinase-Akt/protein kinase B and, to a lesser degree, epidermal growth factor receptor activation. These findings indicate that exploring the use of the AHR antagonist as agents that enhance the proapoptotic actions of cancer therapies may be a valid approach.

The aryl hydrocarbon receptor (AHR) is a cytosolic protein that is activated by ligand binding and is a member of the basic helix-loop-helix/PAS (Per-Arnt-Sim) family (Kewley et al., 2004). The AHR has been best characterized as a mediator of the toxic and carcinogenic actions of compounds such as the polycyclic aromatic hydrocarbons and a regulator of xenobiotic metabolism (Nebert et al., 2004). At the biochemical level, activation of the AHR by its exogenous ligands, such as dioxin [2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD)], has been relatively well characterized (Petrulis and Perdew, 2002). Exposure to agonist is known to stimulate translocation of the AHR from the cytosol to the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and regulates gene expression by binding to dioxin response elements and interacting with a number of coactivators that assist in recruiting the RNA polymerase complex (Rochette-Egly, 2005).

Although the identity and function of the endogenous ligand of the AHR is poorly understood (Denison and Nagy, 2003; Marlowe and Puga, 2005), a number of clues have arisen using the AHR null mice and cultured cell lines that exhibit a lack of, or compromised, AHR signaling. At the cellular level, the absence of the AHR has been associated with an increase in senescence and apoptosis, altered cellular morphology, and altered cell cycle control (Ma and Whitlock, 1996; Elizondo et al., 2000; Fong et al., 2005). At the molecular level, the AHR signaling pathway has been shown to modulate a number of signaling pathways, such as those associated with EGFR signaling (i.e., tyrosine kinases, extracellular signal-regulated kinase, Src, and Akt/PKB) and cell cycle control (i.e., retinoblastoma protein) (Marlowe and Puga, 2005). These studies indicate that the AHR may impinge on central signaling pathways that regulate major

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; EGFR, epidermal growth factor receptor; PKB, protein kinase B; PI3K, phosphatidylinositol 3′-OH kinase; TGF, transforming growth factor; PBS, phosphate-buffered saline; PI, phosphatidylinositol; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein; RT, reverse transcriptase; QPCR, quantitative PCR; MNF, 3′-methoxy-4′-nitroflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; DMSO, dimethyl sulfoxide; siRNA, small interference; 7-AAD, 7-amino actinomycin; PD153035, 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline.

cellular processes in a manner that has important physiological consequences.

A critical process required for appropriate development and the maintenance of normal cellular homeostasis is apoptosis (Danial and Korsmeyer, 2004; Beere, 2005). At the cellular level, the apoptotic decision is mediated by an integration of competing death and survival signaling inputs. In large part, the survival signals such as those triggered by growth factors converge upon the PI3K-Akt/PKB axis. On the death side, there are two major pathways, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway (Hengartner, 2000). Activation of the intrinsic pathway by stimuli such as UV light, nutrient withdrawal, or H_2O_2 results in permeabilization of the mitochondrial outer membrane, release of cytochrome *c*, and activation of procaspase-9. Activation of the extrinsic pathway by ligands such as FasL that bind to the Fas death receptor results in cleavage and activation of caspase-8. Regardless of the mode of activation, both pathways converge to activate the "executioner" caspases, caspase-3, -6, and -7.

Although the role of AHR in determining apoptosis susceptibility is yet emerging (Gonzalez and Fernandez-Salguero, 1998; Reiners and Clift, 1999; Camacho et al., 2002; Park et al., 2005), much of the work in this area has been performed using conditions in which the AHR has been activated exogenously by TCDD, rather than by those that may mimic its endogenous role (Davis et al., 2000, 2001; Schrenk et al., 2004; Park et al., 2005). As reviewed recently by Marlowe and Puga (2005), the mechanisms by which the TCDD-activated AHR has thus far been proposed to alter apoptosis seems to involve the p53, EGFR, and/or TGFβ signaling pathways. In addition to the uncertainty surrounding the mechanisms by which the AHR alters the apoptotic response are the observations that lack of the AHR may result in either an increase or decrease in the number of apoptotic cells. For example, lack of the AHR has been found to correspond to a decrease in apoptosis stimulated by either ceramide (Reiners and Clift, 1999) or FasL (Park et al., 2005). However, mouse embryonic fibroblasts obtained from the AHR null mice exhibited increased apoptosis during culture conditions compared with the wild-type counterparts (Elizondo et al., 2000). One possibility that may explain these apparent discrepancies is that the impact of the AHR on apoptosis susceptibility may be dependent on whether apoptosis is activated by the intrinsic or extrinsic pathways. With these ideas in mind, we tested the hypothesis that lack of the AHR increases a cell's sensitivity toward apoptosis induced by the intrinsic pathway and set out to determine the mechanisms that underlie this action. Here, we report that the AHR plays a protective role in apoptosis induced by the intrinsic pathway and that this role seems to be mediated via its regulation of the PI3K-Akt/PKB and, to a lesser extent, the EGFR survival pathway. These findings indicate that exploring the use of AHR antagonist as agents that enhance the proapoptotic actions of cancer therapies may be a valid approach.

Materials and Methods

Reagents. Hepa1c1c7 and the AHR-deficient Hepa1c1c7 cell lines, LA1, were obtained from Dr. James P. Whitlock, Jr. (Stanford University, Stanford, CA) and were generated as described previously (Miller et al., 1983). The H_2O_2 was obtained from Sigma-Aldrich (St. Louis, MO). The FasL ligand and activated AKT cDNA expression kit were obtained from Upstate Biotechnology (Lake Placid, NY). The antibodies recognizing phosphorylated Akt/PKB (serine 473), total Akt/PKB, and EGFR were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture. The mouse hepatoma cells were maintained in Dulbecco's modified Eagle's medium with glucose and glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2.

Induction of Apoptosis. Approximately 16 h before exposure to the apoptotic stimuli, 1×10^6 cells were seeded onto 100-mm^2 or 1×10^5 onto 12-well plates (FasL treatment). Apoptosis was induced as follows.

UV Radiation. The cells were washed twice with warm PBS and were exposed to 20 J/m^2 ultraviolet light (254 nm) using a FLX-20M ultraviolet light source (Enprotech Corp., Cleveland, OH). Immediately after exposure, complete medium was added, and the cells were incubated for the indicated time periods.

H_2O_2 Treatment. Increasing concentrations (0, 100, 150, 200, or 300 μM) of H_2O_2 were added, and the cells were incubated for 24 h before harvesting.

Serum Starvation. The cells were incubated in media that lacked serum for the indicated time points.

FasL Treatment. Increasing concentrations (0, 5, 10, and 50 ng/ml) of the FasL ligand were added, and the cells were incubated for 4 h before harvesting.

EGFR Phosphorylation Inhibitor. The cells were serum-starved overnight and pretreated with 1 μM PD153035 for 1 h before exposure to UV light.

Detection of Apoptosis by Flow Cytometry. Apoptosis was evaluated using the annexin V apoptosis detection kit (BD Pharmingen, San Diego, CA) according to the manufacturer's protocol. After washing twice with cold PBS, the cells were resuspended in 1× binding buffer and incubated with annexin V-fluorescein isothiocyanate and PI or annexin V-phosphatidylethanolamine and 7-AAD in the dark. After 15 min, flow cytometric analyses were performed using FACSCalibur (BD Biosciences, San Jose, CA) in the Flow Cytometry Core Facility (University of Kentucky). Flow cytometry used to analyze the GFP-labeled AHR was performed essentially as described previously (Kolluri et al., 1999).

Detection of Apoptosis by Enzyme-Linked Immunosorbent Assay and Cell Viability by Trypan Blue Staining. Apoptosis was measured by analysis of DNA fragmentation using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. For the trypan blue assay, the cells were collected by trypsinization and resuspended in PBS. The cells were then stained with trypan blue for 5 to 10 min and were counted using a hemocytometer. The unstained cells were regarded as viable cells.

Caspase-3 Assays. Caspase-3 activity was analyzed using the Caspase 3 Assay Kit (Sigma-Aldrich). The cells were removed from the plates by scraping and collected into 15-ml tubes. After centrifugation at 600*g* for 5 min at 4°C, the pellets were resuspended in 50 μl of 1× lysis buffer and incubated for 20 min. The lysed cells were centrifuged at 14,000*g* for 15 min at 4°C. The supernatants were collected and analyzed according to the manufacturer's instructions.

PI3K Activity Assays. The wild-type Hepa1 or LA1 cells (60–80% confluent) were serum-starved overnight followed by exposure to UV light. Cell extracts were prepared, and the PI3K enzyme was immunoprecipitated using 5 μl of the PI3K antibody (Upstate Biotechnology) and 60 μl of protein A-agarose (Sigma-Aldrich). The PI3K activity in the immunoprecipitates was analyzed with PI3K ELISA (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's instructions. The results depict the amount of phosphatidylinositol 3,4,5-trisphosphate generated.

Western Blot Analysis. The Western blot assays were performed as described previously (Ray and Swanson, 2004). In brief, total cellular extracts were prepared from cells following homogenization in F buffer (10 mM Tris, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 U/ml α2-macroglobulin, 2.5 U/ml pepstatin A, 2.5 U/ml leupeptin, 150 μM benzamidine, and 2.8 μg/ml aprotinin, pH 7.05) and were incubated on ice for 30 min. The homogenates were centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatants were collected. Aliquots (30–100-μg protein) were applied to a 10% SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose membrane (Pierce Chemical, Rockford, IL) and were incubated with 5% nonfat milk for 1 h at room temperature. The membranes were then probed with polyclonal antibodies that recognized phosphorylated Akt/PKB (serine 473), p85, p110, phosphorylated EGFR, total Akt/PKB, or total EGFR overnight at 4°C in 5% bovine serum albumin solution.

Plasmid Construction. The oligonucleotides were synthesized from Integrated DNA Technologies (Coralville, IA) and were: forward primer, 5′-GCCTGGAGACCATGAGCAGCGGCGCCAACATC-ACC-3′; and reverse primer, 5′-GCCTCGAGTCAGAAGTCTAGCTT-GTGTTT-3′. The AHR constructs were generated by PCR using the described previously constructs as templates (Swanson and Yang, 1996) and the forward and reverse primers. The PCR fragments were subcloned into pIRES2-EGFP (Clontech Laboratories, Inc., Palo Alto, CA) to generate AHR-GFR.

Transient Transfections. Transient transfections were performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. Cells were plated in antibiotic-free medium with serum overnight to reach 50 to 80% density. FuGENE 6 and the DNA mixture were directly added to the medium. Cells were incubated for an additional 48 h before UV irradiation.

siRNA Treatment. siRNA *SMART*pool that targeted the mouse AHR, mouse EGFR, and mouse p110 were designed by Dharmacon, Inc. (Lafayette, CO). As an siAHR control, we used si*CONTROL* Nontargeting siRNA#1. Transfections of siRNAs were performed using TransIT-TKO transfection reagent (Mirus Bio Corporation, Madison, WI) according to the manufacturer's instructions using a 25 or 50 nM concentration of the siRNA molecules. The cells were transfected when 50% confluent (six-well plates) and were incubated for 50 to 72 h before further manipulations.

Quantitative RT-PCR. The cells were harvested using Trypsin-EDTA-4Na (Invitrogen) and washed with phosphate-buffered saline. The total cellular RNA was extracted using TRIzol Reagent (Invitrogen). cDNA was synthesized from 1 μg of RNA using random primers and Omniscript reverse transcriptase (QIAGEN, Valencia, CA) according to the supplier's protocol. The mRNA expression levels were measured using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and the MX3000P instrument (Stratagene) according to the manufacturer's protocol. Sample loading was controlled by normalizing all values to glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analyses. Statistical analyses were performed using the Prism software (GraphPad Software Inc., San Diego, CA) and consisted of either the Student's t test or one-way analysis of variance and the Student's t test for differences among several means.

Results

Cells Defective in AHR Signaling Exhibit a Greater Sensitivity to UV-Induced Apoptosis. Our overall goal was to determine whether the AHR, in the absence of exogenous ligands, may play a role in protecting a cell from environmental apoptotic stresses. Toward this end, we have employed the mouse Hepa1c1c7 cell line. The Hepa1c1c7 cells have been a prototypical model that has been used to mechanistically examine the biochemistry and function of the AHR given its responsiveness to AHR agonists (Hankinson, 1979) and high AHR expression levels (Holmes and Pollenz, 1997). A further advantage of this cell line is the availability of variants that have been generated based on their resistance to benzo[a]pyrene toxicity and their alterations in the AHR signaling pathway (Miller et al., 1983; Hankinson et al., 1985). For example, the LA1 variant expresses approximately 10% of the wild-type AHR expression levels.

We chose to test our hypothesis using stimuli such as UV irradiation, H_2O_2, and serum starvation because use of pharmacological agents is probably complicated by the fact that the AHR plays a major role in the metabolism of many xenobiotics and drugs. Toward this end, we exposed AHR-deficient (LA1) and wild-type Hepa1c1c7 cells to UV light and first questioned whether the absence or presence of the AHR could alter a cell's viability following exposure to a stressor such as UV light. The conditions used in these experiments were based on those described previously (Li et al., 2006). As shown in Fig. 1A, exposure to UV light resulted in a greater loss of viability in the LA1 cells compared with that in the wild-type cells. Given these results, we then performed additional analyses to determine whether the loss of viability was as a result of an increase in apoptosis or necrosis by staining the cells with annexin V and PI and performing flow cytometry analyses. As shown in Fig. 1B, the number of cells that stained positive for PI (i.e., necrotic cells) was similar in the two cells lines. However, the number of cells that displayed positive annexin V staining and hence seemed to be apoptotic (Fig. 1B, lower right quadrant) was greater in the LA1 compared with that in the wild-type cells (i.e., after 24 or 48 h). Quantitation of the flow cytometry results is shown in Fig. 1C. Additional measures of apoptosis were then performed by analyzing for either caspase-3 activity (Fig. 1D) or DNA fragmentation (Fig. 1E). As shown, treatment with UV light resulted in an increase in caspase-3 activity (Fig. 1D) and an increase in DNA fragmentation (Fig. 1E) in the LA1 cells compared with that of the wild-type cells. Taken together, these results indicate that the UV-induced apoptotic response is greater in the LA1 cells that lack expression of the AHR.

Lack of AHR Increases Susceptibility to Apoptosis Induced by either H_2O_2 or Serum Starvation but Not FasL. To verify that the results obtained in Fig. 1 were indicative of a response to stimuli of the intrinsic pathway that involves mitochondrial events and were not restricted to the UV-induced response (or DNA damage response), we performed similar experiments using either H_2O_2 or serum starvation to induce apoptosis. The concentrations and time points chosen for use of H_2O_2 and serum starvation were based on similar experiments that were described previously (Davis et al., 2000; Murakami et al., 2005). As shown in Fig. 2, using annexin V staining as a marker of apoptosis, lack of the AHR in the LA1 cells was found to result in a significant increase in the number of apoptotic cells detected following treatment with either H_2O_2 (Fig. 2A) or serum starvation (Fig. 2B). These results are consistent with those shown in Fig. 1. We then questioned whether the impact of the AHR on the apoptotic response may vary depending on the type of apoptotic pathway employed. For example, it has been reported recently that in response to apoptosis initiated by FasL, lack of the AHR was found to correspond to a decrease

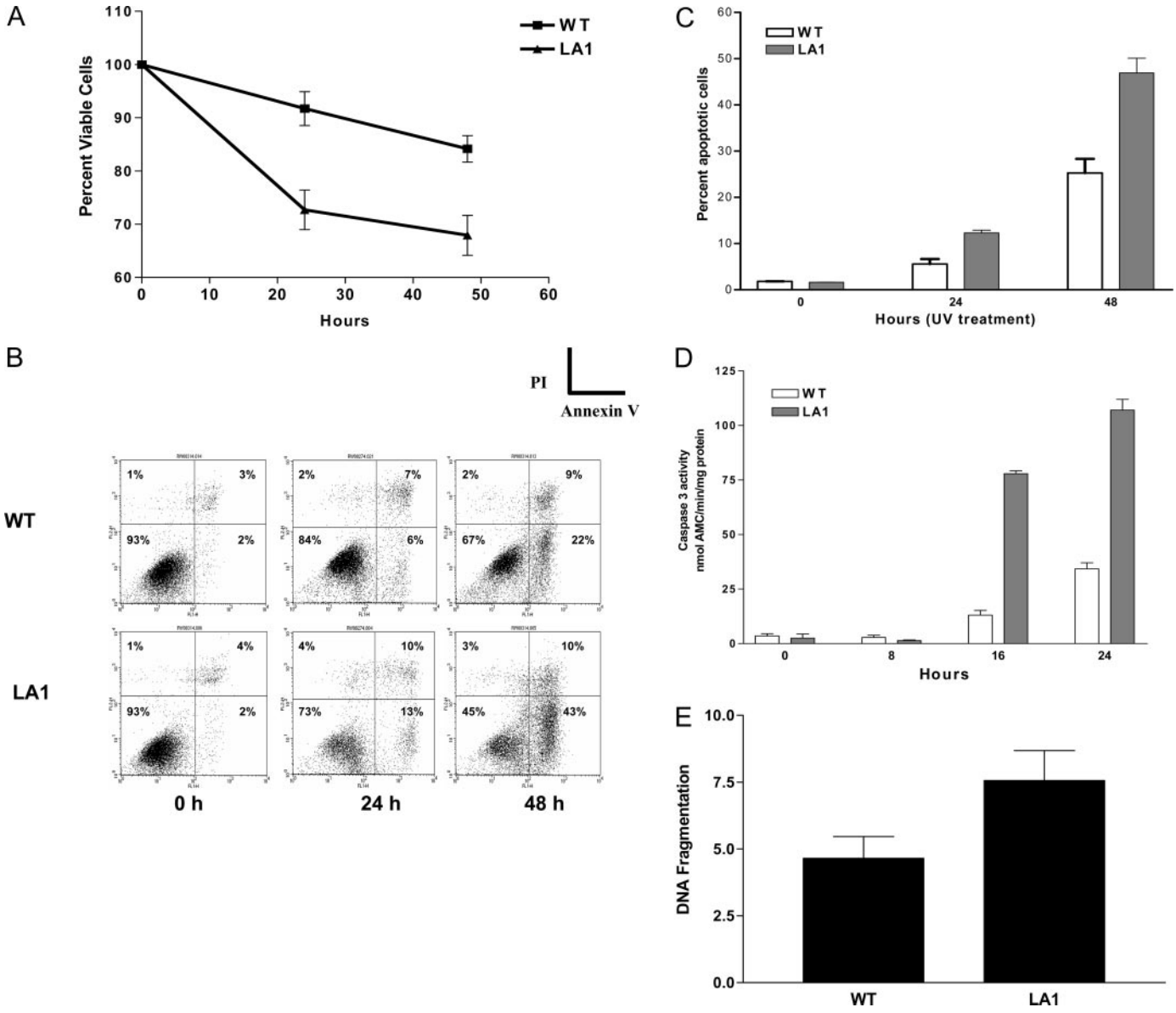

Fig. 1. Mouse hepatoma cells defective in the AHR signaling pathway exhibit a greater sensitivity to UV-induced apoptosis compared with their wild-type counterpart. Either LA1 (AHR−) or wild-type (WT) (Hepa1c1c7) mouse hepatoma cells were cultured to near confluence, washed with PBS, and exposed to UV light (20 J/m^2). After the indicated time periods, the cells were collected and analyzed for cell viability using trypan blue staining (A) and apoptosis using annexin V and PI staining and flow cytometry (B and C), analyzing for caspase-3 activity (D) and determining the extent DNA fragmentation by ELISA (E). The data shown in (C) represent quantitative analysis of the results obtained from the flow cytometry shown in (B). The results represent averages of at least three independent experiments.

in the apoptotic response (Park et al., 2005). Based on these observations, we then performed experiments to analyze the response to FasL-induced apoptosis in the wild-type and LA1 cells. As shown in Fig. 2C, the apoptotic response induced by FasL was the reverse of that resulting from apoptosis induced by UV light (Fig. 1), H_2O_2 (Fig. 2A), or serum starvation (Fig. 2B), where the LA1 cells exhibited a decreased, rather than increased, susceptibility to apoptosis. Thus, the impact of the AHR on apoptosis seems to be dependent on which apoptotic pathway is involved. With respect to the intrinsic pathway, lack of the AHR leads to increased apoptotic susceptibility, but in the case of the extrinsic pathway, lack of the AHR leads to a decrease in the apoptotic response.

The AHR Antagonist, MNF, Increases Sensitivity to Apoptosis. We then validated the results shown in Figs. 1 and 2 by using a pharmacological approach and the AHR antagonist, MNF. As shown in Fig. 3A, increasing concentrations of MNF resulted in an increase in caspase-3 activity that was induced in the wild-type Hepa1c1c7, but not LA1, cells following UV treatment. We verified that MNF was not overtly toxic by performing experiments that were similar to those shown in Fig. 3A but lacked UV irradiation and failed to detect caspase-3 activity at any concentration of MNF tested (data not shown). These results indicate that blocking the actions of the AHR through use of an antagonist is sufficient to alter sensitivity to UV-induced apoptosis.

Restoration of AHR to AHR-Deficient Cells Decreases the Apoptotic Response. To further confirm that the AHR is involved in determining a cell's apoptotic response to UV light, we questioned whether increasing the

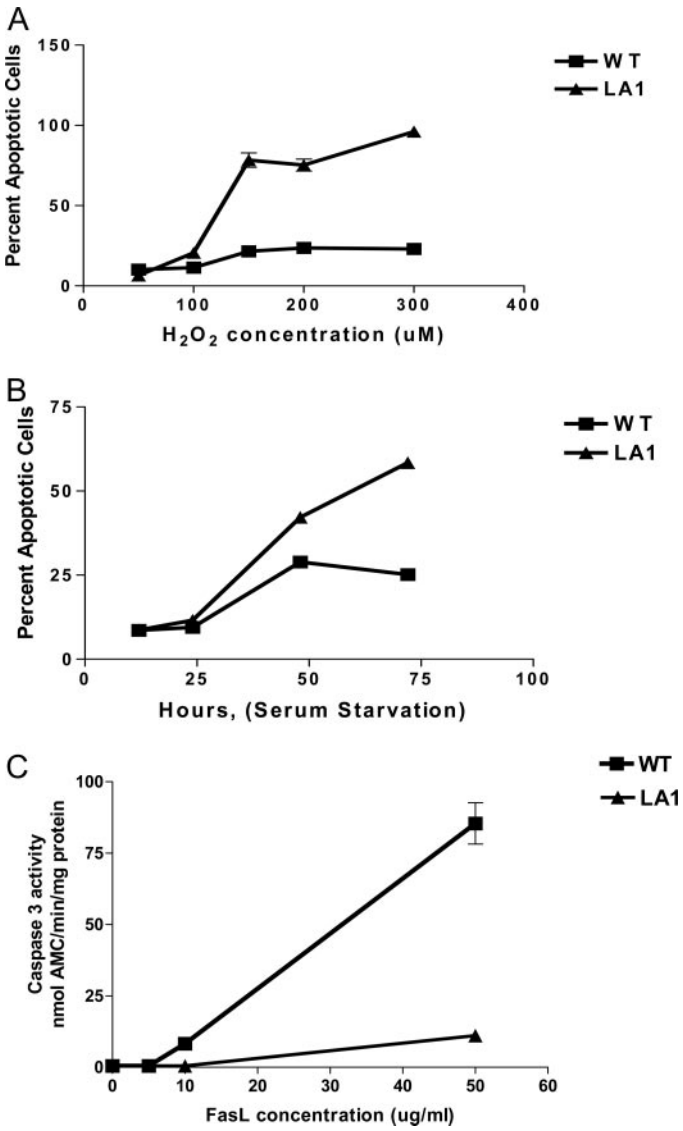

Fig. 2. Lack of AHR increases susceptibility to apoptosis induced by either H_2O_2 or serum starvation, but not FasL. The LA1 or wild-type cells were either cultured in the presence of increasing concentrations of H_2O_2 (A) or the absence of serum (B). After 24 (A) or 24, 48, and 72 (B) h, the cells were harvested, stained for annexin V and PI, and analyzed by flow cytometry. The results represent averages of at least three independent experiments. C, LA1 or wild-type cells were cultured in the presence of increasing concentration of FasL ligand. After 4 h, cells were harvested, and apoptosis was determined by caspase-3 analysis, $n = 6$.

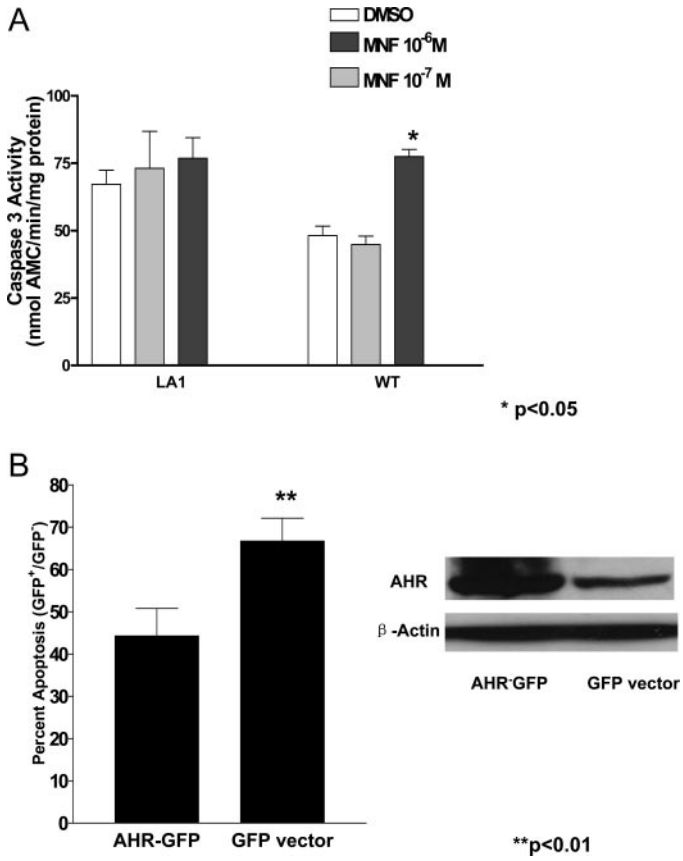

Fig. 3. Altered AHR signaling results in a corresponding change in response to UV-induced apoptosis. A, incubation with the AHR antagonist, MNF, increases the apoptotic response in the wild-type (WT), but not AHR− (LA1), Hepa 1 cells. The indicated cells were incubated with either DMSO or increasing concentrations of MNF for 24 h. After UV light exposure (20 J/m^2), the cells were incubated for an additional 24 h, and the apoptotic response was determined following caspase-3 analysis. The data represent the means of three independent experiments ± S.E. *, $p < 0.05$ versus DMSO treatment. B, increased expression of the AHR results in a decrease in the UV light-induced apoptotic response. The LA1 cells were transiently transfected with either the pIRES-EGFP bearing the murine AHR or the empty vector using FuGENE 6. After 48 h, cells were exposed to UV light (20 J/m^2), and the cells were harvested after an additional 24-h incubation. Apoptosis was determined by staining for annexin V-phosphatidylethanolamine (red) and flow cytometry. The cells that stained positive for 7-AAD were excluded from the analysis by gating. The apoptosis rate in the transfected green fluorescing cells (GFP^+) was divided by the apoptosis rate in the untransfected cells (GFP^-) from the same culture dish. The data represent the means ± S.E. **, $p < 0.01$ versus EGFP empty vector, $n = 8$. Protein expression levels of the AHR in the cells transfected with either the GFP control or AHR-GFP plasmid was determined using Western blot analysis.

expression levels of the AHR in the AHR-deficient cells would alter their response to UV-induced apoptosis. Toward this end, the cDNA encoding the murine AHR was subcloned into the pIRES-EGFP vector (AHR-GFP) and was transiently transfected into the LA1 cells. The cells were again subjected to UV-induced apoptosis as described in Fig. 1, and their apoptotic response was determined by annexin V staining. As shown in Fig. 3B, transfection with the AHR-GFP construct resulted in a significant lower number of apoptotic cells compared with those transfected with the empty vector (GFP vector). Western blot analyses confirmed that the cells transfected with the AHR-GFP construct expressed higher levels of AHR protein compared with those transfected with the GFP control vector (Fig. 3B, right). These results indicate that increasing the expression levels of the AHR in the LA1 cells results in a corresponding decrease in their susceptibility to UV light-induced apoptosis and further support the idea that the AHR plays a cytoprotective role.

Cells That Lack the AHR Exhibit Impaired Akt/PKB Activation. Two possible mechanisms by which lack of the AHR would result in an increase in UV light-induced apoptosis are enhanced proapoptotic pathway(s) or impaired anti-apoptotic pathway(s). We focused on the latter possibility and a putative role of the Akt/PKB pathway because of recent evidence that lack of the AHR is associated with impaired Akt/PKB signaling (Mulero-Navarro et al., 2005) and the critical role that the Akt/PKB pathway plays in determining apoptosis susceptibility (Kennedy et al., 1997; Song et al., 2005). Toward this end, we first questioned whether the

Akt/PKB signaling pathway may be impaired in the AHR-deficient cells. As shown in Fig. 4A, exposure to UV light resulted in an expected increase in the phosphorylated levels of Akt after 15 min. However, this UV light-induced increase in phosphorylation levels was compromised in the LA1 cells compared with the wild-type cells. Furthermore, blocking the AHR signaling pathway using the antagonist MNF resulted in a significant reduction in the phosphorylated levels of Akt (Fig. 4B). Finally, we questioned whether modulation of Akt expression would be sufficient for reducing apoptosis susceptibility in the LA1 cells using an overexpression approach. As shown in Fig. 4C, overexpression of Akt in the LAI cells before UV treatment resulted in a corresponding decrease in caspase-3 activity in the LAI cells. Similar results were obtained using a pharmacological inhibitor of Akt phosphorylation, LY294002 (data not shown).

Cells That Lack the AHR Exhibit Decreased PI3K Activity. We then questioned whether lack of the AHR may influence events that lie further upstream Akt by examining the expression levels and activity of PI3K. As shown in Fig. 5A, the expression levels of both the p85 and p110 subunits were similar in the Hepa1c1c7 and LA1 cells. However, induction of PI3K activity seems to be compromised in the LA1 compared with that in the wild-type cells (Fig. 5B). As shown, in the wild-type Hepa1c1c7 cells, treatment with UV light resulted in an increase in PI3K activity that was evident after 30 and 60 min of treatment. Similar treatment of the LA1 cells, however, failed to alter PI3K activity. To confirm that PI3K plays a role in dictating the response of the wild-type cells to UV-induced apoptosis, we used siRNA designed to inhibit expression of the p110 subunit of PI3K (Fig. 5C). As shown, treatment with the si-p110 significantly increased caspase-3 activity in the wild-type, but not LAI, cells.

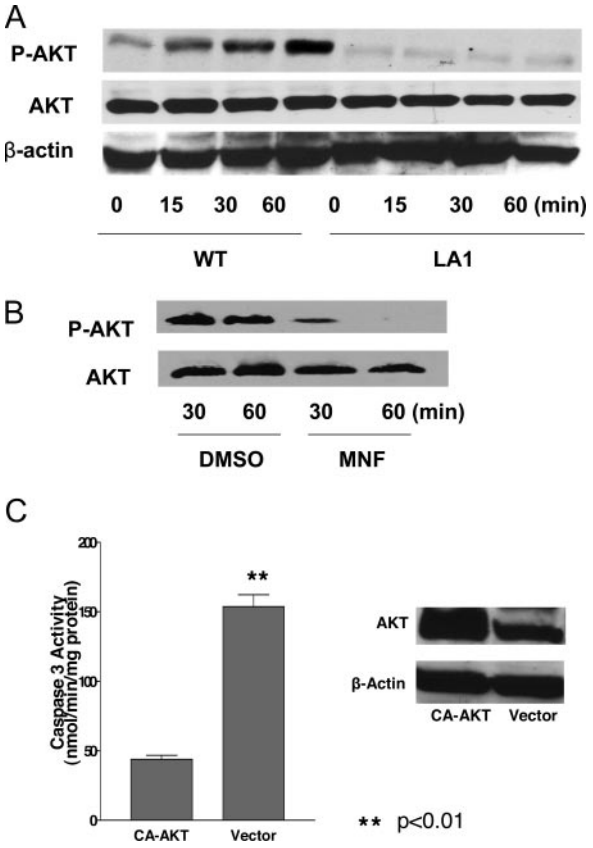

Fig. 4. Altered AHR signaling results in a corresponding change in response to UV-induced Akt/PKB activation. A, UV treatment results in phosphorylation of Akt/PKB in wild-type Hepa 1c1c7, but not the LA1 cell lines. Either wild-type Hepa 1c1c7 or the LA1 cell lines were exposed to UV light as described in Fig. 1. At the indicated time points, the cells were harvested, and whole-cell homogenates were prepared. Serine 473-phosphorylated and total Akt/PKB levels were determined following Western blot analysis. B, inhibition of Akt/PKB phosphorylation in wild-type Hepa1c1c7 cells by MNF. The Hepa1c1c7 cells were incubated with MNF (10^{-5} M) for 24 h, exposed to UV light, and the total and phosphorylation levels of Akt/PKB were determined following Western blot analyses. C, overexpression of Akt results in decreased caspase-3 activity in the LA1 cells. LA1 cells were transfected with either active Akt cDNA or empty vector for 48 h before UV light exposure. After 24-h incubation, caspase-3 activity was determined. The results are averages of three independent experiments. The protein levels of Akt in the LA1 cells were determined following Western blot analyses.

Cells That Lack the AHR Exhibit Decreased mRNA and Protein Levels of EGFR. It has been proposed previously that a mechanism by which the TCDD-activated AHR pathway inhibits apoptosis occurs via its ability to stimulate the EGFR pathway (Davis et al., 2000, 2001). Given this and the fact that EGFR activation leads to increased Akt and PI3 activity, we then examined the mRNA levels of EGFR and its ligand, TGFα, in the Hepa1c1c7 cells compared with that in the LA1 cells. As shown in Fig. 6A, the mRNA expression levels of both EGFR and TGFα were considerably lower in the LA1 cells. As expected, the mRNA levels of both the AHR and its prototypical target gene, CYP1A1, were also lower in the LA1 cells compared with that in the wild-type Hepa1c1c7 cells (i.e., approximately 6% that of the Hepa1c1c7 cells). To confirm that the differences in the mRNA levels of EGFR and TGFα were a result of decreased expression of the AHR and not a consequence of other genetic differences that may exist between the LA1 and Hepa1c1c7 cell lines, we used siRNA to decrease the mRNA levels of the AHR in the Hepa1c1c7 cells. As shown in Fig. 6B, the siRNA-induced decrease in expression of the AHR corresponded to a decrease in the mRNA levels of both EGFR and TGFα. Western blot analyses confirmed that the differences in EGFR mRNA levels observed between the LA1 and Hepa1c1c7 cell lines appropriately mirrored their EGFR protein levels (Fig. 6C).

We then determined whether these two cell lines also differed in the extent to which UV-light could induce EGFR phosphorylation. As shown in Fig. 6D, treatment with UV light resulted in a significant increase in phosphorylation in the wild-type cells that was clearly evident after 15 min. Although induction of EGFR phosphorylation was still evident in the LA1 cells, comparison with the wild-type cells revealed that the extent to which EGFR phosphorylation was induced in these cells was significantly diminished. Finally, we confirmed the role of the AHR in the extent to which the EGFR was induced following a 30-min treatment with UV light again using the AHR antagonist, MNF (Fig. 6E).

Impact of EGFR Signaling on Determining Susceptibility to UV Light-Induced Apoptosis. We then determined the extent to which impaired EGFR signaling in the LA1 cells accounted for their relatively high susceptibility to UV light-induced apoptosis. As shown in Fig. 7A, inhibiting EGFR phosphorylation using PD153035 significantly increased the UV light induction of caspase-3 activity in the Hepa1c1c7 cells. However, given that the extent of induction by PD153035 in the wild-type cells was substantially less than that observed in the untreated LA1, it seems that im-

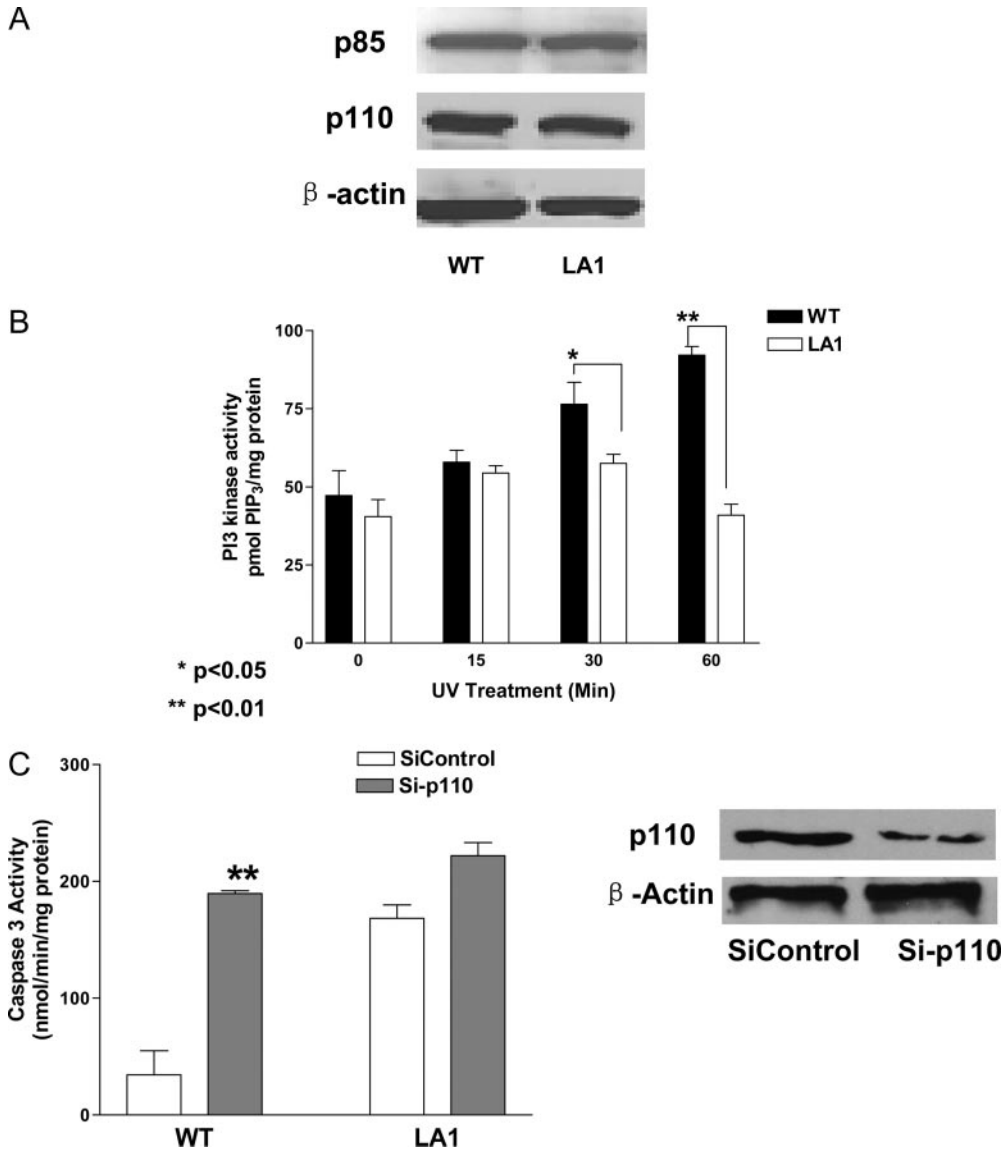

Fig. 5. Cells that lack the AHR exhibit decreased PI3K activity. A, expression levels of p85 and p110 are similar in the wild-type Hepa1c1c7 and LA1 cells. Lysates from the Hepa1c1c7 cells and LA1 cells were isolated, and the expression levels of the PI3K subunits, p85 and p110, were determined by Western blot analysis. B, UV light exposure increases PI3K activity in the wild-type Hepa1c1c7, but not LA1, cells. The cells were exposed to UV light as described in Fig. 1. After the indicated time points, the cells were harvested, PI3K was immunoprecipitated, and its activity was analyzed as described under *Materials and Methods*. The results depict total phosphatidylinositol 3,4,5-trisphosphate generated per milligram of protein lysate. C, overexpression of p110 increases UV light-induced caspase-3 activity in wild-type Hepa1c1c7, but not LA1, cells. The Hepa1c1c7 cells and LA1 cells were transfected with either si-Control or si-p110 (25 nM) for 50 h before UV light exposure. Protein expression levels of p110 were determined using Western blot analysis. The data represent at least three independent experiments.

paired EGFR phosphorylation in the LA1 cells may play only a partial role in determining their susceptibility to apoptotic stimuli. Use of siRNA targeted to inhibit expression of the EGFR yielded results similar to those shown in Fig. 7A, where transfection with siEGFR resulted in a small, but significant, increase in caspase-3 activity in the wild-type, but not LA1, cells (Fig. 7B). Taken together, these results indicate that the primary impact of endogenous signaling by the AHR on the intrinsic pathway most probably lies within events bordered by EGFR and PI3K activation.

Discussion

In this study, we have focused on a possible endogenous role of the AHR as a cytoprotective agent such that in its absence, a cell may become more susceptible to apoptosis mediated by intrinsic stimuli. Toward this end, we have found that murine hepatoma cells that are AHR-deficient (LA1) are more susceptible to apoptosis induced by UV light, H_2O_2, or serum starvation compared with that observed in their wild-type counterparts. Furthermore, our data also indicate that the AHR-dependent susceptibility is due, in part, to an impaired survival mechanism that involves loss of appropriate activation of the PI3K-Akt/PKB signaling pathway. Our ongoing studies performed in other cell types (i.e., HaCaT and normal human oral keratinocytes) indicate that these observations are not restricted to a single cell type (R. Wu, L. Zhang, M. S. Hoagland, and H. I. Swanson unpublished data).

The findings reported herein are consistent with those reported using primary mouse embryonic fibroblasts where cells obtained from the $AHR^{-/-}$ mice exhibited greater apoptosis than those from the $AHR^{+/+}$ mice that was presumably induced by the conditions associated with their in vitro culturing (e.g., exposure to 20% oxygen) (Elizondo et al., 2000). Furthermore, the role of the AHR in Akt/PKB signaling implied by Fig. 4 is supported by the observations obtained from immortalized mouse mammary fibroblasts that indicated the phosphorylation of Akt/PKB corresponds to the absence or presence of the AHR (Mulero-Navarro et al., 2005) and in studies performed in MCF10A cells in which exposure to the AHR agonist, TCDD, resulted in increased Akt/PKB phosphorylation (Davis et al., 2001).

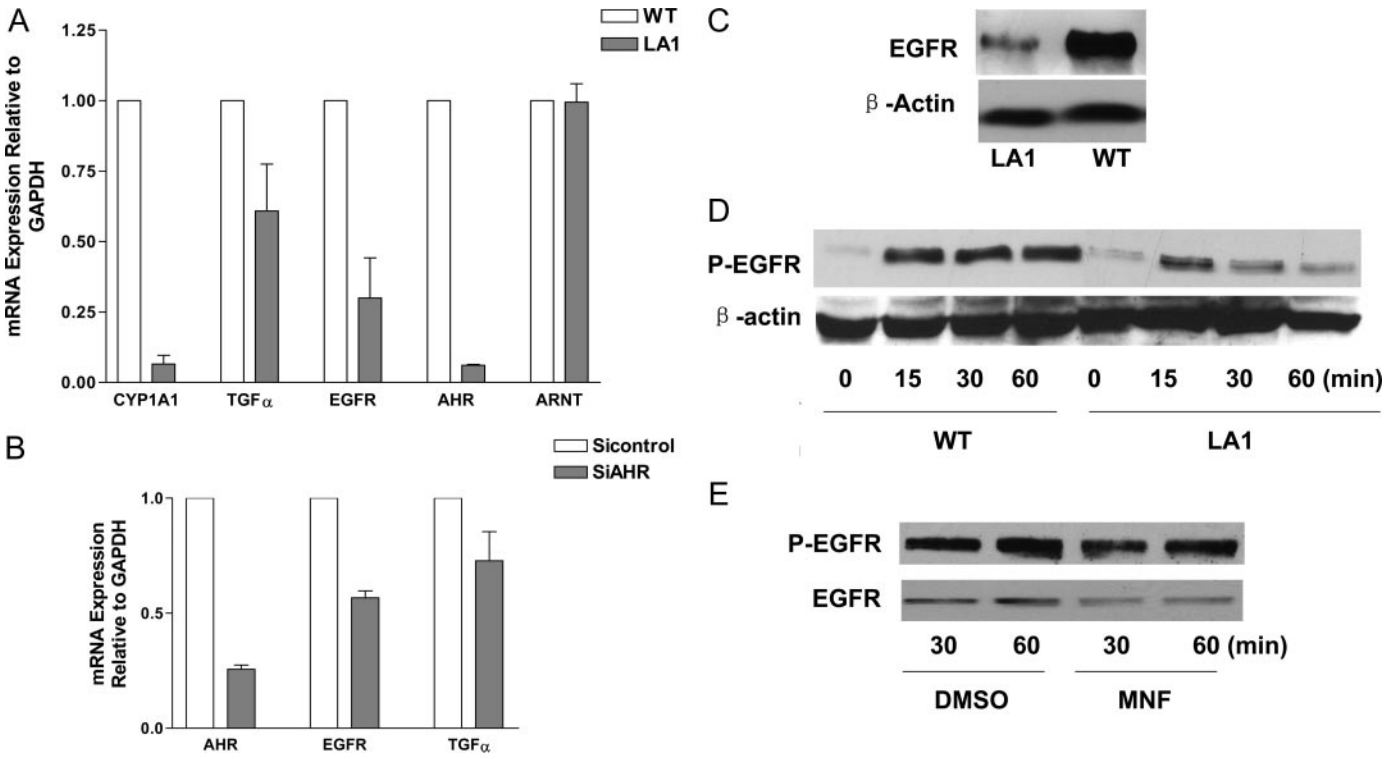

Fig. 6. Decreased expression of AHR is associated with decreased EGFR mRNA levels and EGFR phosphorylation. A, mRNA levels of CYP1A1, TGFα, EGFR, ARNT, and AHR in the wild-type Hepa1c1c7 and LA1 cell lines. Total mRNA was isolated from LA1 and wild-type Hepa1c1c7 cells, and the mRNA expression levels of CYP1A1, TGFα, EGFR, AHR, and ARNT were determined using RT-QPCR. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase. The experimental values are the average of three independent experiments. B, siRNA inhibition of AHR results in a decrease in the mRNA expression levels of EGFR and TGFα expression. Wild-type Hepa1c1c7 cells were transfected with siRNA targeted to the AHR or that of the control (50 nM). After a 50-h incubation period, the mRNA levels of EGFR and TGFα were determined using RT-QPCR. C, EGFR protein levels are decreased in the LA1 compared with that in wild-type Hepa1c1c7 cells. Lysates were prepared from the indicated cell lines and were subjected to Western blot analysis. D, UV light-induced phosphorylation of EGFR is decreased in the LA1 compared with that in wild-type Hepa1c1c7 cells. Either wild-type Hepa 1c1c7 or the LA1 cell lines were exposed to UV light as described in Fig. 1. At the indicated time points, the cells were harvested, and whole-cell homogenates were prepared. Phosphorylated EGFR was detected using Western blot analysis. E, treatment with MNF results in a decrease in P-EGFR and EGFR. The wild-type Hepa 1c1c7 was treated with either DMSO (0.1%) or MNF (10 μM) for 24 h. The cells were then exposed to UV light, harvested after either 30 or 60 min, and the homogenates were subjected to Western blot analyses. The results represent at least three independent experiments.

A major finding of the current study is that in the presence of stimuli of the intrinsic pathway (i.e., exposure to UV light, H_2O_2, or growth factor deprivation), lack of the AHR increases a cell's susceptibility to apoptosis. In contrast, in the presence of stimuli of the extrinsic pathway (i.e., exposure to FasL ligand), lack of the AHR seems to be protective as apoptosis susceptibility is decreased (Fig. 2C) (Park et al., 2005). Likewise, lack of the AHR seems to play a protective role against apoptosis stimulated by ceramide (Reiners and Clift, 1999). The events underlying this apparent conundrum are currently unclear but most probably lie in the differences in the mechanisms by which these stimuli exert their apoptotic responses. One possibility is that the AHR enhances extracellular signaling. Under intrinsic conditions, this would aid external growth factor survival signals at the expense of internal signals (i.e., DNA damage, etc.), whereas under extrinsic conditions, it would aid the external death receptor signaling. The responses to apoptotic stimuli may also prove to be cell type-dependent because of the varying expression levels of the many mediators of these complex pathways (Beere, 2005).

We hypothesize here that the cytoprotective role of the AHR requires an endogenous ligand, rather than occurring in a ligand-independent manner. The evidence for this idea is supported by the experiments performed using the AHR antagonist, MNF, in the wild-type Hepa1c1c7 cells (Figs. 3A and 4B), where its presence resulted in both an increase in apoptosis and a decrease in activated Akt/PKB following UV light irradiation. As mentioned previously, the endogenous ligand of the AHR has not yet been conclusively demonstrated. Although it may arise from metabolism of arachidonic acid, it is also possible that it may be formed following UV irradiation of tryptophan (Denison and Nagy, 2003).

In considering the endogenous, cytoprotective role of the AHR, we propose that it acts primarily to bolster the survival pathway such that, in its absence, susceptibility to apoptosis is increased. This idea is supported by the data that show that elimination of PI3K in the wild-type cells results in an apoptotic index that is a complete match with that of the LA1 cells (Fig. 5C). Whether a deficiency of AHR modulates death signals was not addressed here, although there is evidence that this may be the case. For example, TCDD treatment has been shown to up-regulate the expression levels of p53 in some cases (Schrenk et al., 2004). However, our previous observations indicate that the endogenous levels of p53 do not differ significantly between the wild-type and LA1 cell lines (Hoagland et al., 2005). Thus, the ability of the AHR to affect p53 expression levels either requires exogenous rather than endogenous ligands or the events stimulated by exogenous ligands involving those associated with increased oxi-

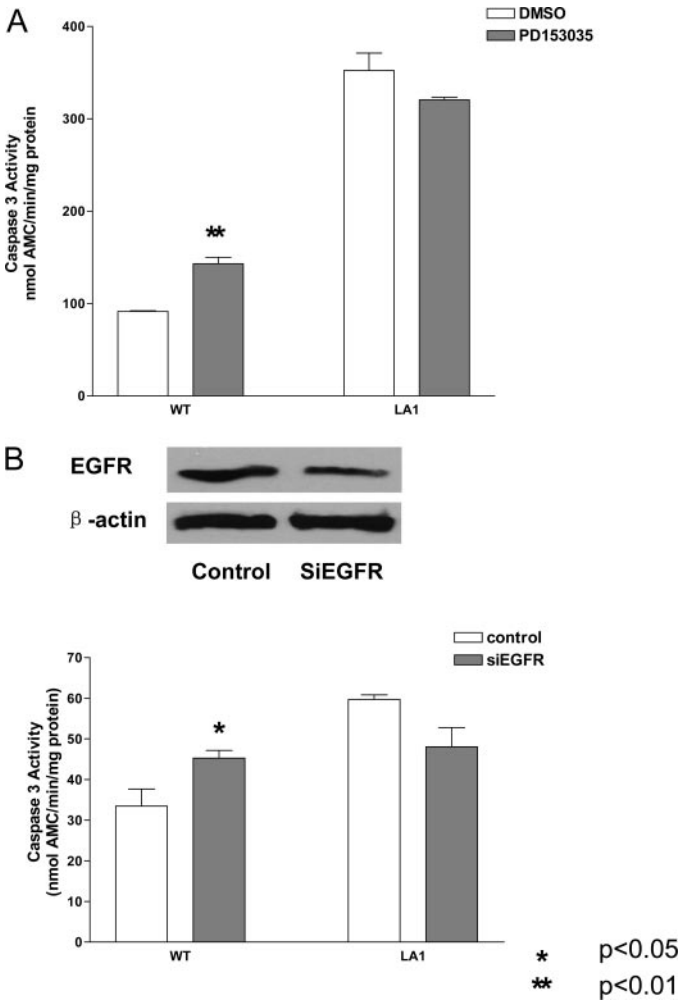

Fig. 7. Impact of EGFR signaling on determining susceptibility to UV light-induced apoptosis. A, treatment with PD153035 results in an increase in UV light-induced caspase-3 activity in the wild-type Hepa1c1c7, but not LA1, cells. The Hepa1c1c7 cells and LA1 cells were serum-starved overnight. One hour before UV light exposure, the cells were treated with either DMSO or PD153035 (1 μM). After 24-h incubation, caspase-3 activity was determined. B, decreased EGFR expression results in an increase in UV light-induced caspase-3 activity in the wild-type Hepa1c1c7, but not LA1, cells. Hepa1c1c7 cells and LA1 cells were transfected with either siEGFR or the siControl (25 nM) for 50 h before exposure to UV light. The protein levels of EGFR in the wild-type Hepa1c1c7 cells were determined using Western blot analyses. After 24-h incubation, caspase-3 activity was determined. The results are averages of three independent experiments.

dative stress (Senft et al., 2002), rather than solely involving the ability of the AHR to regulate gene transcription.

Our results also indicate that although EGFR signaling is also impaired in cells that lack functional AHR, in the experimental paradigm used in this study, its impact on determining apoptosis susceptibility does not completely explain the LA1 defect. The relationship observed between AHR and EGFR signaling reported in the current study is consistent with that reported previously. For example, the AHR has been shown to regulate the expression levels of not only TGFα (Choi et al., 1991; Davis et al., 2001) but also other EGFR ligands such as amphiregulin (Du et al., 2005) and epiregulin (Patel et al., 2006). Taken together, these studies imply that an endogenous role of the AHR may involve its ability to regulate the expression levels of EGFR ligands and in this manner, alter EGFR activity. Based on the observations that AHR/TCDD regulation of TGFα involves post-transcriptional mechanisms (Choi et al., 1991), that the promoter regions of both TGFα or EGFR lack dioxin response elements (Kel et al., 2004), but that AHR/TCDD regulation of epiregulin requires AHR/ARNT binding to its promoter, it seems that AHR/TCDD regulation of the EGFR pathway occurs via both direct and indirect mechanisms.

The results presented herein imply that an important relationship exists among the AHR, PI3K, and ultimately Akt/PKB signaling. Although Akt/PKB is a major mediator of cell survival that acts by directly inhibiting Bad and inhibiting cytochrome *c* release from mitochondria (Burgering and Coffer, 1995), it also plays important roles in processes such as the induction of neoplasias and appropriate glucose metabolism (Yang et al., 2004). Thus, it is possible that the ability of the AHR to regulate the PI3-Akt/PKB pathway is important to not only TCDD's tumor-promoting activities but also in its ability to induce other human disease states such as diabetes (Remillard and Bunce, 2002). Given the observation that lack of the AHR renders a cell more susceptible to apoptosis, future directions will be focused on determining whether AHR antagonists may be useful as adjunct therapies that may conquer a tumor cell's resistance to chemo- and radiation-based therapies.

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References

Beere HM (2005) Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Investig* **115:**2633–2639.
Burgering BM and Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature (Lond)* **376:**599–602.
Camacho IA, Nagarkatti M, and Nagarkatti PS (2002) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces Fas-dependent activation-induced cell death in superantigen-primed T cells. *Arch Toxicol* **76:**570–580.
Choi EJ, Toscano DG, Ryan JA, Riedel N, and Toscano WA Jr (1991) Dioxin induces transforming growth factor-alpha in human keratinocytes. *J Biol Chem* **266:**9591–9597.
Danial NN and Korsmeyer SJ (2004) Cell death: critical control points. *Cell* **116:** 205–219.
Davis JW 2nd, Lauer FT, Burdick AD, Hudson LG, and Burchiel SW (2001) Prevention of apoptosis by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the MCF-10A cell line: correlation with increased transforming growth factor alpha production. *Cancer Res* **61:**3314–3320.
Davis JW, 2nd, Melendez K, Salas VM, Lauer FT, and Burchiel SW (2000) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits growth factor withdrawal-induced apoptosis in the human mammary epithelial cell line, MCF-10A. *Carcinogenesis* **21:**881–886.
Denison MS and Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* **43:**309–334.
Du B, Altorki NK, Kopelovich L, Subbaramaiah K, and Dannenberg AJ (2005) Tobacco smoke stimulates the transcription of amphiregulin in human oral epithelial cells: evidence of a cyclic AMP-responsive element binding protein-dependent mechanism. *Cancer Res* **65:**5982–5988.
Elizondo G, Fernandez-Salguero P, Sheikh MS, Kim GY, Fornace AJ, Lee KS, and Gonzalez FJ (2000) Altered cell cycle control at the G(2)/M phases in aryl hydrocarbon receptor-null embryo fibroblast. *Mol Pharmacol* **57:**1056–1063.
Fong CJ, Burgoon LD, and Zacharewski TR (2005) Comparative microarray analysis of basal gene expression in mouse Hepa-1c1c7 wild-type and mutant cell lines. *Toxicol Sci* **86:**342–353.
Gonzalez FJ and Fernandez-Salguero P (1998) The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos* **26:**1194–1198.
Hankinson O (1979) Single-step selection of clones of a mouse hepatoma line deficient in aryl hydrocarbon hydroxylase. *Proc Natl Acad Sci USA* **76:**373–376.
Hankinson O, Andersen RD, Birren BW, Sander F, Negishi M, and Nebert DW (1985) Mutations affecting the regulation of transcription of the cytochrome P1–450 gene in the mouse Hepa-1 cell line. *J Biol Chem* **260:**1790–1795.
Hengartner MO (2000) The biochemistry of apoptosis. *Nature (Lond)* **407:**770–776.
Hoagland MS, Hoagland EM, and Swanson HI (2005) The p53 inhibitor pifithrin-alpha is a potent agonist of the aryl hydrocarbon receptor. *J Pharmacol Exp Ther* **314:**603–610.

Holmes JL and Pollenz RS (1997) Determination of aryl hydrocarbon receptor nuclear translocator protein concentration and subcellular localization in hepatic and nonhepatic cell culture lines: development of quantitative Western blotting protocols for calculation of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein in total cell lysates. *Mol Pharmacol* **52:**202–211.
Kel A, Reymann S, Matys V, Nettesheim P, Wingender E, and Borlak J (2004) A novel computational approach for the prediction of networked transcription factors of aryl hydrocarbon-receptor-regulated genes. *Mol Pharmacol* **66:**1557–1572.
Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, and Hay N (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev* **11:**701–713.
Kewley RJ, Whitelaw ML, and Chapman-Smith A (2004) The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int J Biochem Cell Biol* **36:**189–204.
Kolluri SK, Weiss C, Koff A, and Gottlicher M (1999) p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells. *Genes Dev* **13:**1742–1753.
Li L, Sampat K, Hu N, Zakari J, and Yuspa SH (2006) Protein kinase C negatively regulates Akt activity and modifies UVC-induced apoptosis in mouse keratinocytes. *J Biol Chem* **281:**3237–3243.
Ma Q and Whitlock JP Jr (1996) The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Mol Cell Biol* **16:**2144–2150.
Marlowe JL and Puga A (2005) Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *J Cell Biochem* **96:**1174–1184.
Miller AG, Israel D, and Whitlock JP Jr (1983) Biochemical and genetic analysis of variant mouse hepatoma cells defective in the induction of benzo(*a*)pyrene-metabolizing enzyme activity. *J Biol Chem* **258:**3523–3527.
Mulero-Navarro S, Pozo-Guisado E, Perez-Mancera PA, Alvarez-Barrientos A, Catalina-Fernandez I, Hernandez-Nieto E, Saenz-Santamaria J, Martinez N, Rojas JM, Sanchez-Garcia I, et al. (2005) Immortalized mouse mammary fibroblasts lacking dioxin receptor have impaired tumorigenicity in a subcutaneous mouse xenograft model. *J Biol Chem* **280:**28731–287341.
Murakami T, Takagi H, Suzuma K, Suzuma I, Ohashi H, Watanabe D, Ojima T, Suganami E, Kurimoto M, Kaneto H, et al. (2005) Angiopoietin-1 attenuates H_2O_2-induced SEK1/JNK phosphorylation through the phosphatidylinositol 3-kinase/Akt pathway in vascular endothelial cells. *J Biol Chem* **280:**31841–31849.
Nebert DW, Dalton TP, Okey AB, and Gonzalez FJ (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* **279:**23847–23850.
Park KT, Mitchell KA, Huang G, and Elferink CJ (2005) The aryl hydrocarbon receptor predisposes hepatocytes to Fas-mediated apoptosis. *Mol Pharmacol* **67:**612–622.
Patel RD, Kim DJ, Peters JM, and Perdew GH (2006) The aryl hydrocarbon receptor directly regulates expression of the potent mitogen epiregulin. *Toxicol Sci* **89:**75–82.
Petrulis JR and Perdew GH (2002) The role of chaperone proteins in the aryl hydrocarbon receptor core complex. *Chem Biol Interact* **141:**25–40.
Ray SS and Swanson HI (2004) Dioxin-induced immortalization of normal human keratinocytes and silencing of p53 and p16INK4a. *J Biol Chem* **279:**27187–27193.
Reiners JJ Jr and Clift RE (1999) Aryl hydrocarbon receptor regulation of ceramide-induced apoptosis in murine hepatoma 1c1c7 cells: a function independent of aryl hydrocarbon receptor nuclear translocator. *J Biol Chem* **274:**2502–2510.
Remillard RB and Bunce NJ (2002) Linking dioxins to diabetes: epidemiology and biologic plausibility. *Environ Health Perspect* **110:**853–858.
Rochette-Egly C (2005) Dynamic combinatorial networks in nuclear receptor-mediated transcription. *J Biol Chem* **280:**32565–32568.
Schrenk D, Schmitz HJ, Bohnenberger S, Wagner B, and Worner W (2004) Tumor promoters as inhibitors of apoptosis in rat hepatocytes. *Toxicol Lett* **149:**43–50.
Senft AP, Dalton TP, Nebert DW, Genter MB, Hutchinson RJ, and Shertzer HG (2002) Dioxin increases reactive oxygen production in mouse liver mitochondria. *Toxicol Appl Pharmacol* **178:**15–21.
Song G, Ouyang G, and Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* **9:**59–71.
Swanson HI and Yang J (1996) Mapping the protein/DNA contact sites of the Ah receptor and Ah receptor nuclear translocator. *J Biol Chem* **271:**31657–31665.
Yang ZZ, Tschopp O, Baudry A, Dummler B, Hynx D, and Hemmings BA (2004) Physiological functions of protein kinase B/Akt. *Biochem Soc Trans* **32:**350–354.

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