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Evaluation of Polycyclic Aromatic Hydrocarbons in the Activation of Early Growth Response-1 and Peroxisome Proliferator Activated Receptors

Jeong-Ho Kim,* Kiyoshi Yamaguchi,* Seong-Ho Lee,* Patricia K. Tithof,* Gary S. Sayler,† Joo-Heon Yoon,‡ and Seung Joon Baek*,[1]

Department of Pathobiology, and †Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996, and ‡Department of Otorhinolaryngology, Yonsei University College of Medicine, Seoul, South Korea

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental and food contaminants with known or suspected carcinogenic properties. In this study, we have evaluated whether PAHs activate the early growth response (EGR-1) gene and bind to peroxisome proliferator-activated receptor alpha (PPARα) and delta (PPARβ/δ) in cell culture systems. Luciferase reporter systems were employed and several PAHs were evaluated for their ability to activate EGR-1 and PPARs. Some PAHs enhanced EGR-1 expression and activated PPARα and PPARβ. Among them, benz(a)anthracene was found to act as a relatively potent activator of PPARα and PPARβ/δ, and to significantly enhance EGR-1 transcription. These *in vitro* assays were confirmed by Western blot analysis, using cell lysates of tissue samples from mouse trapped at a highly contaminated Superfund site in the Chattanooga Creek floodplain in Chattanooga, Tennessee. We have found that a PPAR target gene, glycogen synthase kinase-3β (GSK-3β), was down-regulated and EGR-1 was up-regulated in the mouse samples of Chattanooga Creek. In addition, select PAHs repressed GSK-3β and induced CYP4A in FaO rat hepatoma cells. In conclusion, PAHs activate PPARα and PPARβ/δ, and up-regulate EGR-1 expression *in vitro* as well as *in vivo*. These data may provide a diversity of PAH activity in several biological pathways.

Key Words: **PAHs; PPARα; PPARδ; EGR-1; GSK-3β.**

Polycyclic aromatic hydrocarbons (PAHs) are toxic and ubiquitous environmental pollutants. They are generally formed and emitted into the environment as a result of incomplete combustion of fossil fuels, wood, and other organic materials and from industrial processes. Humans and animals are exposed to PAHs from air, water, dietary, and occupational sources, and also from cigarette smoke. Their very long biological half-lives in combination with biological effects at very low concentrations have caused health concerns about carcinogenicity (Rubin, 2001), teratogenicity (Couture *et al.*, 1990; Incardona *et al.*, 2004), and cardiovascular disease (Gustavsson *et al.*, 1996, 2001; Thirman *et al.*, 1994). Although many reports focus on elucidating the molecular mechanism of PAHs in several diseases, pathways other than aryl hydrocarbon receptor (AhR) activation have received little attention.

Many chronic diseases, including cancer and cardiovascular diseases have been linked to heredity and/or the environment, which can either enhance or inhibit the disease process. One such molecular link between disease and the environment is early growth response-1 (*EGR-1*, also known as *NGFI-A*, *Zif268*, *Krox24,* and *Tis8*). EGR-1 is the prototypical member of a family of zinc finger transcription factors that includes at least three other members, EGR-2, -3, and -4. EGR-1 is especially induced by a range of physiological and environmental stimuli including growth factors, cytokines, ultraviolet light, ionizing radiation, and mechanical injury (Gashler and Sukhatme, 1995; Khachigian and Collins, 1998). EGR-1 appears to be critically involved in several diseases including angiogenesis and tumor formation. Alteration in expression could contribute to the deleterious effects of PAH exposure. Recently, Martinez *et al.* reported that halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), induces EGR-1 expression in human lung carcinoma cells (Martinez *et al.*, 2004). However, expression of EGR-1 in nonneoplastic cells underlies diverse pathophysiological responses such as survival responses to damaging irradiation (Huang *et al.*, 1999), development of vascular occlusion in arteriosclerosis (Silverman and Collins, 1999), and formation of severe pulmonary emphysema (Zhang *et al.*, 2000). In addition to these diverse actions, expression of EGR-1 is commonly down-regulated in tumor cells in contrast with their normal tissue counterparts (Calogero *et al.*, 2004; Hao *et al.*, 2002; Huang *et al.*, 1997; Levin *et al.*, 1995; Shozu *et al.*, 2004). However, EGR-1 is expressed at a higher level and promotes cell growth in prostate cancer when compared with normal tissues (Eid *et al.*, 1998; Thigpen *et al.*, 1996). Thus,

[1] To whom correspondence should be addressed at Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996. Fax: (865) 974-5616. E-mail: sbaek2@utk.edu.

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EGR-1 has multiple functions in tumorigenesis, and the exact biological function of EGR-1 may be dependent on cell context as well as tissue types (Baek *et al.*, 2004). Nonetheless, the EGR-1's role in vascular disease has been firmly established by the fact that EGR-1 controls the expression of several genes implicated in the pathogenesis of atherosclerosis and restenosis (Breslow, 1996; Harja *et al.*, 2004; McCaffrey *et al.*, 2000; Silverman and Collins, 1999).

Peroxisome proliferator activated receptors (PPARs) are another molecular link between chronic disease and the environment. PPARs are members of the nuclear receptor superfamily and exist as three subtypes designated α, β (or δ), and γ. Among those, PPARα activation is responsible for the pleiotropic effects of peroxisome proliferator such as enzyme induction, peroxisome proliferation, liver enlargement, and tumors (Klaunig *et al.*, 2003). PPARα also plays a critical role in regulation of cellular uptake and β-oxidation of fatty acids (Berger and Moller, 2002; Marx *et al.*, 2004). In contrast, PPARδ (also known as PPARβ) is widely expressed with relatively higher levels in brain, colon, and skin. Although there have been extensive studies on PPARα, much less is known about the function of PPARδ. Nonetheless, recent studies suggest that PPARδ plays a role in colon cancer (Gupta *et al.*, 2004; He *et al.*, 1999; Wang *et al.*, 2004), and preadipocyte proliferation (Hansen *et al.*, 2001).

We hypothesized that transcription factors, EGR-1 and PPARs may link environmental toxic compounds to human diseases. The aim of this study is to determine whether PAHs affect EGR-1 and PPARs activity and to identify the different activity of PAHs in reporter system. In this study, fifteen PAHs, which are commonly found in the environment, were examined as potential activators of PPARα or PPARβ/δ, and inducer of *EGR-1* gene expression in A549 human lung adenocarcinoma cells and HCT-116 human colorectal adeno carcinoma cells. The luciferase reporter genes were used to measure the activity of PPARs and transactivation of the EGR-1 promoter. We have demonstrated that some PAHs may activate PPARα and PPARβ/δ, and transactivate EGR-1 promoter activity. Among those, benz(a)anthracene (BaA) induces EGR-1 and PPAR activation in culture systems. Furthermore feral mice (*Peramyscus gossypinus*) trapped along the flood-plain of a Superfund site with high levels of PAH contamination demonstrated significant up-regulation of EGR-1 and down-regulation of GSK-3β, a PPAR target gene. These data provide evidence for diverse effects of PAHs that may be important in diseases linked to environmental pollution such as cardiovascular disease and carcinogenesis.

MATERIALS AND METHODS

Cell culture and chemicals. A549 human lung adenocarcinoma cell lines and HCT-116 human colorectal carcinoma cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 10 μg/ml gentamycin. HCT-116 cells were cultured in Modified McCoy 5A medium supplemented with 10% FBS and 10 μg/ml gentamycin. FaO rat hepatoma cell lines were generously provided by Dr. Seong-Jin Kim (National Cancer Institute, Bethesda, MD) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 10 μg/ml gentamycin. All the PAHs were purchased from Sigma-Aldrich (St. Louis, MO) and completely dissolved in DMSO.

Plasmids. EGR-1 promoter (−1260 to +35) linked to the luciferase gene (pEGR1260-Luc) was described previously (Baek *et al.*, 2003). The plasmids used for studying PPARα and PPARβ/δ activators were a reporter gene containing four copies of a Gal4 binding site (MH100×4-TK-Luc) and chimeric receptors (pCMX-Gal-mPPARα-LBD for PPARα and pCMX-Gal-mPPARδ-LBD for PPARδ). In this system, when a compound binds to the ligand binding domain (LBD) from PPARα or PPARδ of the chimeric receptor (pCMX-Gal4-mPPARα-LBD or pCMX-Gal-mPPARδ-LBD), then the DNA binding domain of the yeast Gal4 (denoted as Gal) binds to co-transfected Gal4 binding site and initiates transcription of the firefly luciferase (Luc). A reporter plasmid containing three copies of the PPAR response element (PPRE×3-TK-Luc) and a mouse PPARα cDNA (pCDNA3-mPPARα) were previously described (Nixon *et al.*, 2003). This system directly measures activation of PPARα via transcriptional activation of the luciferase reporter gene as a result of PPARα binding to the PPAR response element (PPRE). All the PPAR and PPAR reporter plasmids were generously provided by Dr. Ronald M. Evans (Howard Hughes Medical Institute, CA).

Transient transfections and luciferase reporter assays. Cells (1×10^5 cells/well) were cultured in twelve-well plates in culture medium containing 10% FBS. After growth for 16 h, the internal control, 0.05 μg pRL-null (Promega, WI) and 0.5 μg of the other plasmids were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocol. After 24 h, the media were changed and the cells were treated with the various PAHs dissolved in DMSO. The final concentration of DMSO did not exceed 0.1% (v/v) in any of the samples. Treatments with PAHs were performed under serum-free conditions. After 24 h treatment, the cells were washed with PBS and harvested in 1X luciferase lysis buffer. The luciferase activity was measured by a dual luciferase assay kit (Promega, WI), and normalized to the internal control, pRL-null (renilla luciferase) activity.

Animal studies. Feral mice (*Peramyscus gossypinus*) were trapped along the floodplain of the Chattanooga Superfund site and in a control site two miles upstream from the contaminated section of creek. All animal procedures were in compliance with the National Institute of Health guidelines on animal use and were approved by the University of Tennessee Institutional Animal Care and Use Committee. Traps were set each evening and checked each morning. Trapped animals were transported to the laboratory and anesthetized with CO_2. After the chest cavities were opened, the mice were exsanguinated by cardiac puncture and the distal aorta, heart and lungs perfused with phosphate buffered saline (PBS) to remove clotted blood. Sections of heart, lung, and colon were removed and snap-frozen in liquid nitrogen for Western blot analysis.

Western blot analysis. The level of protein expression was evaluated by Western blot analysis. FaO cells were grown to 60–80% confluency in 6 cm plates, followed by 24 h treatment of selected PAHs in the absence of serum. Total cell lysates were isolated using RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing proteinase inhibitor, and the soluble protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL). For feral mouse tissues, frozen samples were lysed in ice-cold RIPA buffer as described above. All lysate proteins were separated by SDS-PAGE and transferred for 1 h onto nitrocellulose membrane (Osmonics Inc., MN). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline and Tween 0.05% and probed with GSK-3β (Cell Signaling, MA), EGR-1 (Santa Cruz Biotech., CA), CYP4A (Affinity Bioreagent, Golden, CO), or Actin (Santa Cruz Biotech., CA) antibody at 4°C overnight. After washing with Tris-buffered saline and Tween

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0.05%, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. The signals were detected by the enhanced chemiluminescence system (Amersham Biosciences, Arlington Height, IL). The signal intensities were measured by NIH Image program (Scion Corp., MD).

Statistical analysis. For luciferase activities of transient transfection experiments, data were expressed as mean ± SD for at least three independent repeats. For quantitative analyses, analysis of variance (ANOVA) with Tukey's multiple comparison test or *t*-test was used to compare mean values. SAS for Windows (9.1) (SAS Institute Inc., Cary, NC) statistical analysis software was used. A *p*-value of less than 0.05 was considered significant.

RESULTS

We have selected fifteen PAHs, containing two, three, four, or five aromatic rings. These PAHs were found to be in the highest concentrations in the Superfund site in Chattanooga, Tennessee (Elgayyar *et al.*, manuscript in preparation). Among these, benz(a)anthracene (BaA), benzo(a)pyrene (BaP), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), and chrysene (CHR) are considered to be carcinogenic, whereas acenaphthene (ANY), anthracene (ANT), naphthalene (NAP), pyrene (PYR), phenanthrene (PA), and triphenylene (TP) are not considered to be carcinogenic according to WHO (Table 1). A limited number of recent studies have shown that fluoranthene (FA) is an experimental carcinogen (Hecht *et al.*, 1995).

Effects of PAHs on EGR-1 Activation

Several PAHs were examined for the induction of *EGR-1* gene in A549 human lung carcinoma cell lines, which have been shown to induce EGR-1 expression (Martinez *et al.*, 2004). The pEGR1260-Luc construct was transiently transfected into A549 cells and the luciferase activity was measured in the presence of PAHs. The pEGR1260-Luc construct (Fig. 1A) contains 1.2 kb of the EGR-1 promoter (Baek *et al.*, 2005). All the PAHs tested here showed a significant increase in luciferase activity except BaP and TP (Fig. 1B). PA, BaA, and PA demonstrated higher luciferase activity, and PA also increased EGR-1 activity in 1 μM concentration (Fig. 1C). As a positive control, sulindac sulfide (30 μM) was used in the promoter assay (Baek *et al.*, 2005), showing a 4-fold induction of luciferase activity compared to vehicle treated sample.

Effects of PAHs on PPARα activation

In addition to cyctochrome p450 based biomarkers, peroxisome proliferators have been used for a pollution biomarker in aquatic organism (Cajaraville *et al.*, 2003). Among those, PPARα has received much attention from many researchers since PPARα expression is tightly regulated by various hormones. In addition, PPARα plays a pivotal role in tumorigenesis and atherogenesis (Klaunig *et al.*, 2003; Marx *et al.*, 2004). Therefore, we have examined whether PAH compounds activate PPARα. The luciferase reporter construct and expression vector (Fig. 2A) were co-transfected into HCT-116 cells that have been shown a high transfection efficiency (Baek *et al.*, 2001). The cells were treated with 10 μM of various PAHs for 24 h and luciferase activity was examined. The

TABLE 1
Characteristics of Polycyclic Aromatic Hydrocarbons Used in This Study

Compound	Structure	Molecular weight	Genotoxicity	Carcinogenicity	AhR activity
Acenaphthene		154	(?)	(?)	
Acenaphthylene		152	(?)		
Anthracene		178	—	—	—
Benz(a)anthracene		228	+	+	++
Benzo(a)pyrene		252	+	+	++
Benzo(b)fluoranthene		252	+	+	+++
Benzo(k)fluoranthene		252	+	+	+++
Biphenylene		152			
Chrysene		228	+	+	+++
Fluoranthene		202	+	(+)	+
1-Methylanthracene	CH_3	192			
Naphthalene		128	—	(?)	
Phenanthrene		178	(?)	(?)	
Pyrene		202	(?)	(?)	+
Triphenylene		228	+	(−)	

Note. Genotoxicity and carcinigenecity according to WHO, 1998: +, positive; −, negative; ?, questionable; parentheses, result derived from small database (WHO, 1998). AhR-mediated activity (Machala *et al.*, 2001) value for benzo(*a*)pyrene (and PAHs with similar effects) was arbitrarily set as ++; values +++ and + indicate PAHs with more or less significant effects than benzo(*a*)pyrene; value of − indicates no effect.

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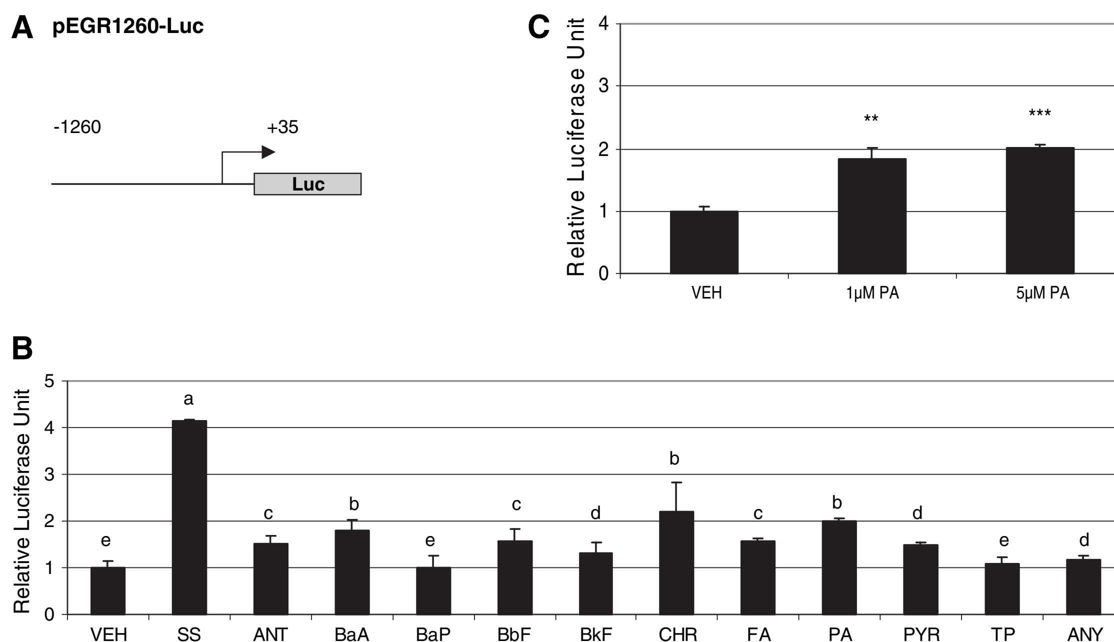

FIG. 1. PAH effects on EGR-1 expression. (A) Schematic diagram of pEGR1260-Luc construct. (B) A549 cells were exposed to PAHs after transfection with a pERG1260-Luc construct. Cells were treated with 5 μM PA and 10 μM of all other PAHs for 24 h. Sulindac sulfide was used as a positive control for EGR-1 activator (Baek *et al.*, 2004, 2005). Transfection efficiency for luciferase activity was normalized to the Renilla luciferase (pRL-null vector) activity. The *y*-axis shows relative luciferase unit (firefly luciferase unit/Renilla luciferase unit) by the fold increase in PAH treatment over vehicle treatment. The results are expressed as mean ± SD of three independent transfections and different letters indicate significant difference (Tukey's multiple comparision test, $p < 0.05$). VEH, vehicle; SS, sulindac sulfide; ANT, anthracene; BaA, benz(a)anthracene; BaP, benzo(a)pyrene; BbF, benzo(b)fluoranthene; BkF, benzo(k)fluoranthene; CHR, chrysene; FA, fluoranthene; PA, phenanthrene; PYR, pyrene TP, triphenylene; ANY, acenaphthylene. (C) A549 cells were transfected and exposed to 1 μM, 5 μM of PA. The luciferase activity was measured, and relative luciferase unit was shown at *y*-axis. *t*-test, $^{**}p < 0.01$; $^{***}p < 0.001$, compared to vehicle.

luciferase activity to PAH treatment was compared to that of vehicle treatment. BaA was estimated as a strong activator among PAHs tested here. Other PAHs showed slight increases or little changes in luciferase activity (Fig. 2B). As a positive control, Wy14643 was used in this system and we found around 55-fold induction of luciferase activity, compared to vehicle treated samples (data not shown). Transfected cells were also exposed to BaA and luciferase activity was significantly increased in a dose dependent manner (Fig. 2C). It is important to note that the results shown in Figure 2 reflect solely activator activity with exogenous transfectants. Therefore, to examine the transactivation of PPARα, A549 cells were transiently co-transfected with a pPPRE×3-TK-Luc and pCDNA3-mPPARα expression vector (Fig. 3A), and then treated with vehicle, and 1 or 10 μM of BaA. As shown in Figure 3B, we observed a significant induction of luciferase activity in a dose dependent manner, indicating that BaA binds to PPARα and transactivates the gene containing PPRE sites in the promoter.

Effects of PAHs on PPARβ/δ activation

It has been suggested that PPARβ/δ may play an important role in tumorigenesis and atherogenesis (Gupta *et al.*, 2004; He *et al.*, 1999; Marx *et al.*, 2004; Tong *et al.*, 2000). PAH compounds were tested for PPARβ/δ activity in HCT-116 cells transiently transfected with pMH100×4-TK-Luc and pCMX-Gal4-mPPARδ-LBD constructs (Fig. 4A). The PPARβ/δ activity of each PAH was compared with vehicle. Interestingly, treatment with BaA also induced strong luciferase activity among PAHs tested here. However, other PAHs also slightly increased luciferase activity, which is significant (Fig. 4B). BaA also increased luciferase activity in a dose dependent manner (Fig. 4C).

GSK-3β and EGR-1 Expression in the Presence of PAHs

CYP4A is induced by PPARα activation in liver tissue (Johnson *et al.*, 1996). GSK-3β is repressed when FaO rat hepatoma cells are exposed to PPARα and PPARβ/δ ligands (Vanden Heuvel *et al.*, 2003). We have shown that some PAHs are able to bind the PPARα and PPARβ/δ as an activator. To determine whether PAHs affects PPARα downstream in FaO cells, we performed Western blot analysis using cell lysates prepared from FaO cells treated with BaA, BaP, or PA. As shown in Figure 5A, all the PAHs tested increase CYP4A expression and suppressed GSK-3β expression relative to vehicle-treated cells. BaA was the relatively strong suppressor of GSK-3β expression, whereas BaP or PA was the weak suppressor. These data are consistent with previous data showing that BaA is the relatively strongest PPARα and PPARβ/δ activator, whereas BaP and PA are relatively weak (Figs. 2 and 4). Next, we examined GSK-3β expression *in vivo*

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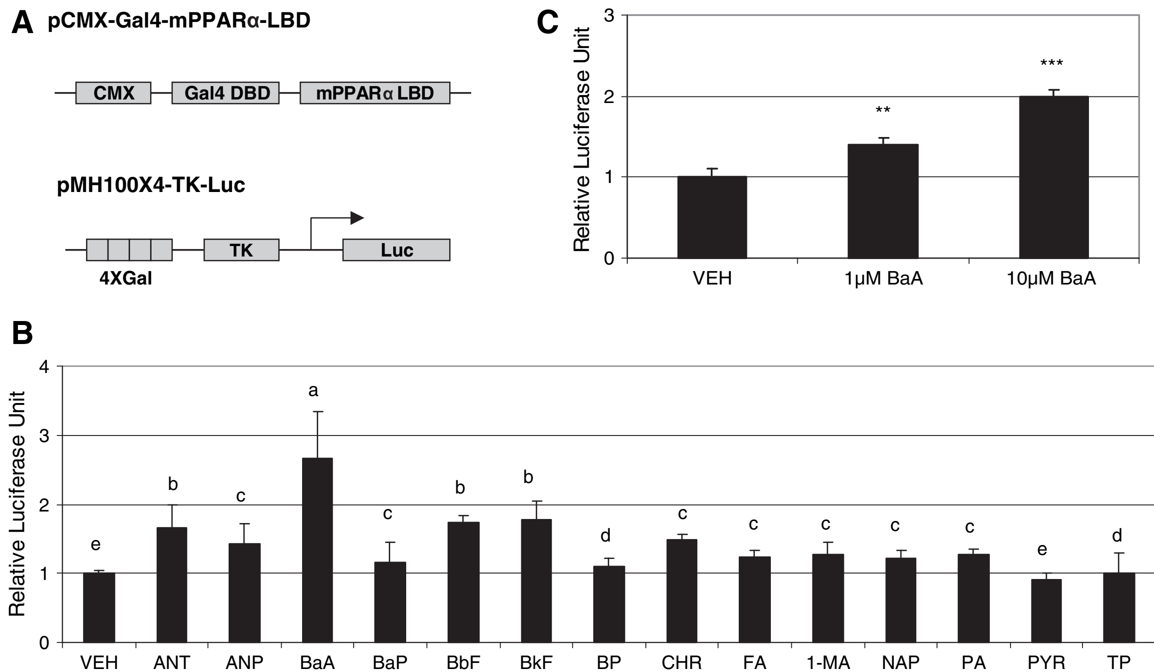

FIG. 2. PAH effects ozn PPARα binding affinity. (A) Schematic diagram of pCMX-Gal4-mPPARα-LBD and pMH100X4-TK-Luc vector. (B) HCT-116 cells were transfected with the vectors shown in Figure 2A, and the cells were treated with 10 μM of various PAHs indicated for 24 h. Transfection efficiency for luciferase activity was normalized to Renilla luciferase (pRL-null vector) activity. The *y*-axis shows relative luciferase unit (firefly luciferase unit/Renilla luciferase unit) by the fold increase in PAH treatment over vehicle treatment. The results are expressed as mean ± SD of four independent transfections and different letters indicate significant difference (Tukey's multiple comparison test, $p < 0.05$). ANP, acenaphthene; BP, biphenylene; 1-MA, 1-methylanthracene; NAP, naphthalene. Other abbreviations are listed in Figure 1B. (C) The transfected cells were treated with the indicated concentration of BaA for 24 h and luciferase activity was measured. *t*-test, $^{**}p < 0.01$; $^{***}p < 0.001$ compared to vehicle.

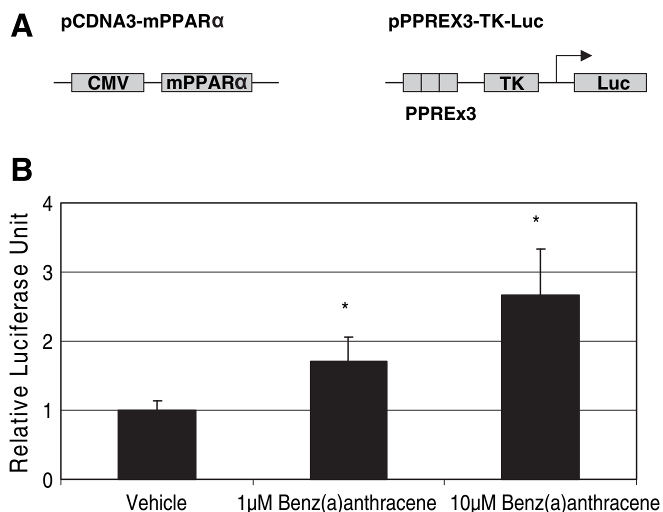

FIG. 3. PAH effects on PPARα transactivation in the promoter containing PPRE sites in A549 cells. (A) Schematic diagram of pCDNA3-mPPARα expression vector and pPPREX3-TK-Luc reporter vector. (B) A549 cells were transfected and the cells were treated with either vehicle or indicated BaA for 24 h. $^{*}p < 0.05$ compared to vehicle. Luciferase activity was measured as described above.

using feral mice (*Peramyscuc gossypinus*) trapped along the floodplain of a highly contaminated area of the Chattanooga Creek and also along a noncontaminated area. We evaluated GSK-3β expression in colon, heart, and lung tissues of mice from both areas. As shown in Figure 5B, GSK-3β was down-regulated in heart but not lung from mice trapped in the contaminated area of the Chattanooga Creek when compared to mice trapped in the control area. However, GSK-3β was not detected in colon tissue from both mice trapped from either site. We also measured EGR-1 expression and found that EGR-1 was induced in colon samples from mice trapped in the Superfund site when compared to control. However, no EGR-1 was detected in heart or lung samples from either site. EGR-1 induction in the *in vivo* study is consistent with *in vitro* data, showing that PAHs increase EGR-1 expression as assessed by a reporter system (Fig. 1). Taken together, these results suggest that some PAHs, particularly BaA activates PPARα and PPARβ/δ, and alters the PPAR target gene expression such as CYP4A and GSK-3β.

DISCUSSION

Humans and other living organisms are constantly exposed to a large number of potentially toxic environmental

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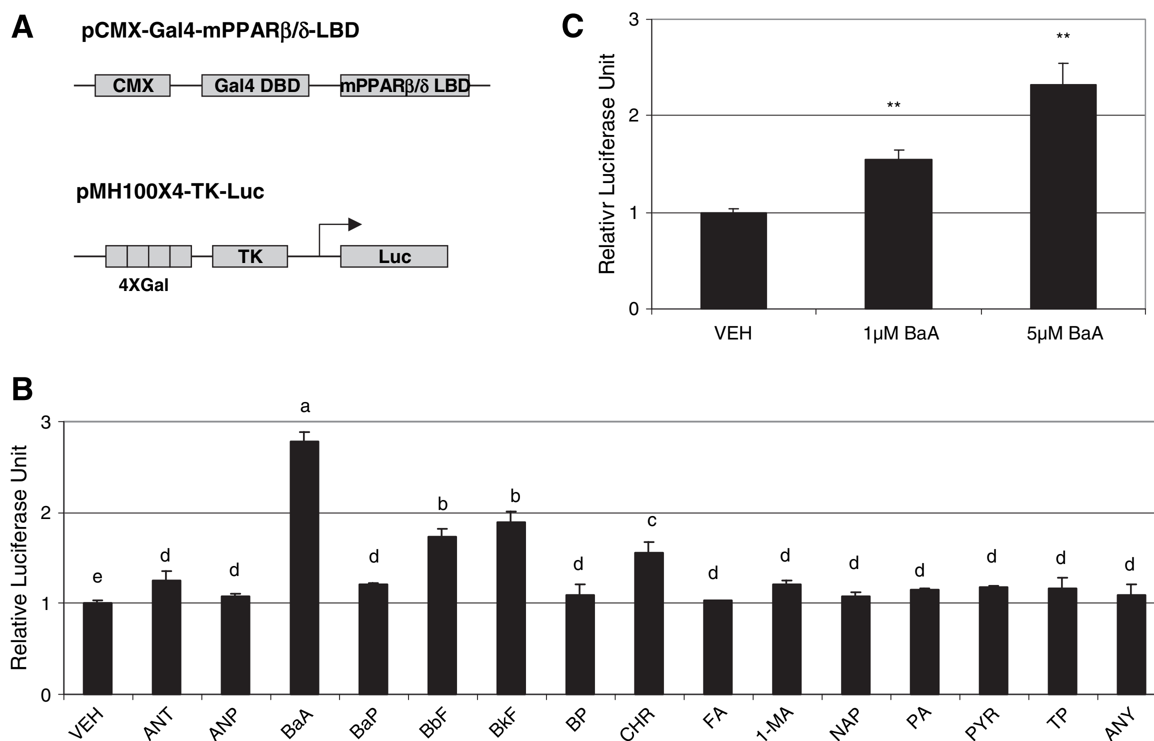

FIG. 4. PAHs effects on PPARβ/δ activation using luciferase assay. (A) A schematic diagram of the pCMX-Gal4-mPPARδ-LBD chimeric gene and the pMH100X4-TK-Luc reporter vector used in this study. (B) HCT-116 cells were transfected with vectors shown in A, and the cells were treated with 10 μM of various PAHs for 24 h. Transfection efficiency for luciferase activity was normalized to the Renilla luciferase (pRL-null vector) activity. The *y*-axis shows relative luciferase unit (firefly luciferase unit/Renilla luciferase unit). The results are expressed as mean ± SD of three independent transfections and different letters indicate significant difference (Tukey's multiple comparision test, $p < 0.05$). The abbreviations are described in Figure 2B. (C) Transfected cells were treated with indicated concentrations of BaA for 24 h and luciferase activity was measured. *t*-test,. $^{**}p < 0.01$; $^{***}p < 0.001$, compared to vehicle treatment.

chemicals, including the ubiquitous polycyclic aromatic hydrocarbons (PAHs). A number of PAHs have been known to affect several chronic diseases, including cancer and cardiovascular disease, in experimental animals following oral, pulmonary, dermal, or subcutaneous administration. Most of the biological effects of PAHs are considered to be mediated via aryl hydrocarbon receptor (AhR)-dependent gene expression (Nebert *et al.*, 2000). PAHs also cause oxidative DNA damage and DNA adduct formation, which is the most widely accepted mechanisms of PAH-induced tumorigenesis and cardiovascular disease (Lee and Blair, 2001; Toraason *et al.*, 2001; Wogan *et al.*, 2004). Compared to genotoxicity or AhR-mediated toxicities of PAHs, knowledge about the nongenotoxic effects of PAHs remains to be elucidated. The major goal of our investigation was to determine the cellular mechanisms that are induced by PAHs in AhR independent manner. EGR-1 and PPARs are the transcription factors that control many genes in the presence of stimuli, including chemical compounds. The ability of select PAHs to activate the *EGR-1* gene and to bind to PPARα and PPARβ/δ was examined in this study using several PAHs commonly found in a Superfund site in Chattanooga, Tennessee. We found that some PAHs activated *EGR-1* gene and enhanced PPARα and PPARβ/δ activity *in vitro*. The latter resulted in the suppression of GSK-3β expression, which may play an important role in tumorigenesis.

EGR-1 can induce expression of a set of vasculature genes, such as PDGF-A and B chain, bFGF, TGF-β, TNF-α, and intracellular adhesion molecule-1. Expression of the EGR-1 is elevated in prostate cancer and correlates with tumor progression. Thus, EGR-1 is the key mediator in orchestrating the functional characteristics of the vessel wall and tumorigenesis. However, EGR-1 can be related to anti-tumorigenesis and pro-tumorigenesis, depending on cell and tissue types. While EGR-1 induces anti-tumorigenic proteins including p53, PTEN, and NAG-1 (Baek *et al.*, 2005), EGR-1 is expressed at a higher level and promotes cell growth in prostate cancer (Eid *et al.*, 1998; Thigpen *et al.*, 1996). Thus, EGR-1 could play a role in both cell proliferation and growth arrest. In contrast to EGR-1 functions in tumorigenesis, experimental evidence is emerging to link EGR-1 to chronic vascular and inflammatory stress *in vivo*. The role of EGR-1 in atherosclerosis related to PAH exposure has not been examined. In this study, EGR-1 was not up-regulated in heart tissue from mice exposed naturally to environmental contaminants; however, vascular tissue was not examined. Further studies are warranted to evaluate the potential role of EGR-1 in atherosclerosis progression related to atherosclerosis and heart disease.

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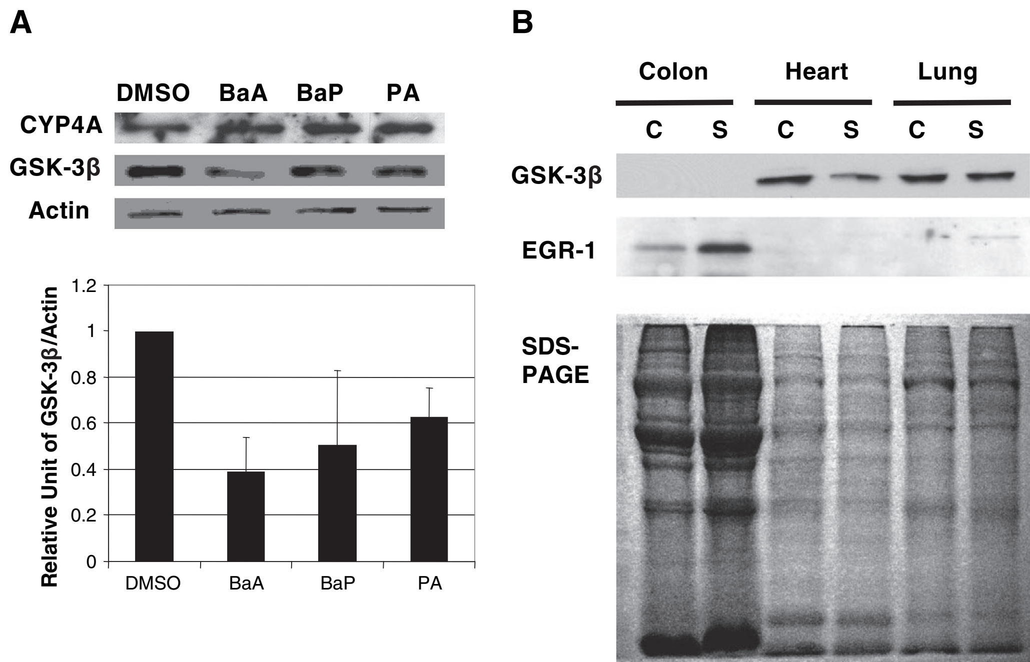

FIG. 5. Regulation of GSK-3β, CYP4A, and EGR-1 by PAHs. (A) FaO rat hepatoma cells were treated with 10 μM of various PAHs for 24 h (DMSO, vehicle control; BaA, benz(a)anthracene; BaP, benzo(a)pyrene; PA, phenanthrene). Western blotting was performed using a GSK-3β antibody (Cell Signaling, Beverly, MA) and CYP4A antibody (Affinity Bioreagent, Golden, CO). Equal loading (30 μg) was confirmed by determining actin immunoreactivity. The relative GSK-3β expression levels normalized by actin from three independent experiments are shown on the bottom. (B) Mouse tissue samples from colon, heart, and lung were isolated from mouse tissue collected from the control site (C) or from the Superfund site (S) in Chattanooga, Tennessee. Cell lysates (100 μg) were subjected to Western blot analysis for GSK-3β and EGR-1 protein expression. Equal loading was confirmed by Bio-Safe Coomassie staining (Bio-Rad, CA) of total proteins shown in bottom. The blot shown is a representative of two independent experiments.

PPARα is a key contributor in the processes of peroxisome proliferation, hypertrophy, cell proliferation, and hepatocarcinogenesis *in vivo*. Its over-expression is observed in advanced prostate cancer (Collett *et al.*, 2000), and activation of PPARα promotes cell proliferation in breast cancer cells (Klaunig *et al.*, 2003; Suchanek *et al.*, 2002). In addition, activation of PPARα has been demonstrated to modulate many aspects of lipoprotein metabolism and inflammation *in vitro,* as well as in animal and human studies (Israelian-Konaraki and Reaven, 2004). Thus, activation of PPARα may play a role in disease such as tumorigenesis and atherogenesis. On the other hand, the activation of PPARβ/δ plays an anti-apoptotic role in keratinocytes via transcriptional control of the AKT signaling pathway (Di-Poi *et al.*, 2002). Genetic disruption of PPARβ/δ also decreases the tumorigenicity of human colon cancer cells transplanted into mice (Park *et al.*, 2001). Our results support the contention that some PAHs with known carcinogenic activity are relatively strong PPARα and PPARβ/δ activators as assessed by reporter system. These results support that some PAHs may induce chronic disease through PPAR activation mechanism other than AhR activation. These results also suggest that minute differences in PAH structure result in the activation of two different PPARs.

In this report, we have shown that GSK-3β is suppressed in heart tissue from mice trapped in a highly contaminated Superfund site as well as in the FaO cells. GSK-3β is known to be a negative regulator of cardiac hypertrophy (Hardt and Sadoshima, 2002), and we have recently reported that AKT/GSK-3β plays an important role in apoptosis (Yamaguchi *et al.*, 2004). Therefore, the suppression of GSK-3β by environmental contaminants such as PAHs may be important in the processes by which environmental pollution accelerates cardiac disorders or tumorigenesis such as cardiomyopathy or cancer; however, further studies may be required to elucidate the exact molecular mechanism.

In conclusion, our data suggest that some PAHs, particulary BaA, are able to activate EGR-1 promoter and act as an activator of PPARα and PPARβ/δ *in vitro*. BaA can activate target genes of PPARα and PPARβ/δ, thereby repressing the GSK-3β expression *in vitro* and *in vivo* and inducing the CYP4A expression *in vitro*. The repression of GSK-3β and activation of EGR-1 by some PAHs may provide a novel approach to elucidating the various effects of PAHs on human chronic disease.

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REFERENCES

Baek, S. J., Horowitz, J. M., and Eling, T. E. (2001). Molecular cloning and characterization of human nonsteroidal anti-inflammatory drug-activated gene promoter. Basal transcription is mediated by Sp1 and Sp3. *J. Biol. Chem.* **276,** 33384–33392.

Baek, S. J., Kim, J. S., Moore, S. M., Lee, S. H., Martinez, J., and Eling, T. E. (2005). Cyclooxygenase inhibitors induce the expression of the tumor suppressor gene EGR-1, which results in the up-regulation of NAG-1, an antitumorigenic protein. *Mol. Pharmacol.* **67,** 356–364.

Baek, S. J., Kim, J. S., Nixon, J. B., DiAugustine, R. P., and Eling, T. E. (2004). Expression of NAG-1, a transforming growth factor-beta superfamily member, by troglitazone requires the early growth response gene EGR-1. *J. Biol. Chem.* **279,** 6883–6892.

Baek, S. J., Wilson, L. C., Hsi, L. C., and Eling, T. E. (2003). Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity. *J. Biol. Chem.* **278,** 5845–5853.

Berger, J., and Moller, D. E. (2002). The mechanisms of action of PPARs. *Annu. Rev. Med.* **53,** 409–435.

Breslow, J. L. (1996). Mouse models of atherosclerosis. *Science* **272,** 685–688.

Cajaraville, M. P., Cancio, I., Ibabe, A., and Orbea, A. (2003). Peroxisome proliferation as a biomarker in environmental pollution assessment. *Microsc. Res. Tech.* **61,** 191–202.

Calogero, A., Lombari, V., De Gregorio, G., Porcellini, A., Ucci, S., Arcella, A., Caruso, R., Gagliardi, F. M., Gulino, A., Lanzetta, G., Frati, L., Mercola, D., and Ragona, G. (2004). Inhibition of cell growth by EGR-1 in human primary cultures from malignant glioma. *Cancer Cell. Int.* **4,** 1.

Collett, G. P., Betts, A. M., Johnson, M. I., Pulimood, A. B., Cook, S., Neal, D. E., and Robson, C. N. (2000). Peroxisome proliferator-activated receptor alpha is an androgen-responsive gene in human prostate and is highly expressed in prostatic adenocarcinoma. *Clin. Cancer Res.* **6,** 3241–3248.

Couture, L. A., Harris, M. W., and Birnbaum, L. S. (1990). Characterization of the peak period of sensitivity for the induction of hydronephrosis in C57BL/6N mice following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Fundam. Appl. Toxicol.* **15,** 142–150.

Di-Poi, N., Tan, N. S., Michalik, L., Wahli, W., and Desvergne, B. (2002). Antiapoptotic role of PPARbeta in keratinocytes via transcriptional control of the Akt1 signaling pathway. *Mol. Cell.* **10,** 721–733.

Eid, M. A., Kumar, M. V., Iczkowski, K. A., Bostwick, D. G., and Tindall, D. J. (1998). Expression of early growth response genes in human prostate cancer. *Cancer Res.* **58,** 2461–2468.

Gashler, A., and Sukhatme, V. P. (1995). Early growth response protein 1 (Egr-1): Prototype of a zinc-finger family of transcription factors. *Prog. Nucleic Acid Res. Mol. Biol.* **50,** 191–224.

Gupta, R. A., Wang, D., Katkuri, S., Wang, H., Dey, S. K., and DuBois, R. N. (2004). Activation of nuclear hormone receptor peroxisome proliferator-activated receptor-delta accelerates intestinal adenoma growth. *Nat. Med.* **10,** 245–247.

Gustavsson, P., Alfredsson, L., Brunnberg, H., Hammar, N., Jakobsson, R., Reuterwall, C., and Ostlin, P. (1996). Myocardial infarction among male bus, taxi, and lorry drivers in middle Sweden. *Occup. Environ. Med.* **53,** 235–240.

Gustavsson, P., Plato, N., Hallqvist, J., Hogstedt, C., Lewne, M., Reuterwall, C., and Scheele, P. (2001). A population-based case-referent study of myocardial infarction and occupational exposure to motor exhaust, other combustion products, organic solvents, lead, and dynamite. Stockholm Heart Epidemiology Program (SHEEP) Study Group. *Epidemiology* **12,** 222–228.

Hansen, J. B., Zhang, H., Rasmussen, T. H., Petersen, R. K., Flindt, E. N., and Kristiansen, K. (2001). Peroxisome proliferator-activated receptor delta (PPARdelta)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J. Biol. Chem.* **276,** 3175–3182.

Hao, M. W., Liang, Y. R., Liu, Y. F., Liu, L., Wu, M. Y., and Yang, H. X. (2002). Transcription factor EGR-1 inhibits growth of hepatocellular carcinoma and esophageal carcinoma cell lines. *World J. Gastroenterol.* **8,** 203–207.

Hardt, S. E., and Sadoshima, J. (2002). Glycogen synthase kinase-3beta: A novel regulator of cardiac hypertrophy and development. *Circ. Res.* **90,** 1055–1063.

Harja, E., Bucciarelli, L. G., Lu, Y., Stern, D. M., Zou, Y. S., Schmidt, A. M., and Yan, S. F. (2004). Early growth response-1 promotes atherogenesis: mice deficient in early growth response-1 and apolipoprotein E display decreased atherosclerosis and vascular inflammation. *Circ. Res.* **94,** 333–339.

He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* **99,** 335–345.

Hecht, S. S., Amin, S., Lin, J. M., Rivenson, A., Kurtzke, C., and el-Bayoumy, K.(1995). Mammary carcinogenicity in female CD rats of a diol epoxide metabolite of fluoranthene, a commonly occurring environmental pollutant. *Carcinogenesis* **16,** 1433–1435.

Huang, R. P., Fan, Y., and Boynton, A. L. (1999). UV irradiation upregulates Egr-1 expression at transcription level. *J. Cell Biochem.* **73,** 227–236.

Huang, R. P., Fan, Y., de Belle, I., Niemeyer, C., Gottardis, M. M., Mercola, D., and Adamson, E. D. (1997). Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. *Int. J. Cancer* **72,** 102–109.

Incardona, J. P., Collier, T. K., and Scholz, N. L. (2004). Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **196,** 191–205.

Israelian-Konaraki, Z., and Reaven, P. D. (2004). Peroxisome proliferator-activated receptor-alpha and atherosclerosis: From basic mechanisms to clinical implications. *Cardiology* **103,** 1–9.

Johnson, E., Palmer, C., Griffin, K., and Hsu, M. (1996). Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *FASEB J.* **10,** 1241–1248.

Khachigian, L. M., and Collins, T. (1998). Early growth response factor 1: A pleiotropic mediator of inducible gene expression. *J. Mol. Med.* **76,** 613–616.

Klaunig, J. E., Babich, M. A., Baetcke, K. P., Cook, J. C., Corton, J. C., David, R. M., DeLuca, J. G., Lai, D. Y., McKee, R. H., Peters, J. M., Roberts, R. A., and Fenner-Crisp, P. A. (2003). PPARalpha agonist-induced rodent tumors: Modes of action and human relevance. *Crit. Rev. Toxicol.* **33,** 655–780.

Lee, S. H., and Blair, I. A. (2001). Oxidative DNA damage and cardiovascular disease. *Trends Cardiovasc. Med.* **11,** 148–155.

Levin, W. J., Press, M. F., Gaynor, R. B., Sukhatme, V. P., Boone, T. C., Reissmann, P. T., Figlin, R. A., Holmes, E. C., Souza, L. M., and Slamon, D. J. (1995). Expression patterns of immediate early transcription factors in human non-small cell lung cancer. The Lung Cancer Study Group. *Oncogene* **11,** 1261–1269.

Machala, M., Vondracek, J., Blaha, L., Ciganek, M., and Neca, J. V. (2001). Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. *Mutat. Res.* **497,** 49–62.

Martinez, J. M., Baek, S. J., Mays, D. M., Tithof, P. K., Eling, T. E., and Walker, N. J. (2004). EGR1 is a novel target for AhR agonists in human lung epithelial cells. *Toxicol. Sci.* **82,** 429–435.

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Marx, N., Duez, H., Fruchart, J. C., and Staels, B. (2004). Peroxisome proliferator-activated receptors and atherogenesis: Regulators of gene expression in vascular cells. *Circ. Res.* **94,** 1168–1178.

McCaffrey, T. A., Fu, C., Du, B., Eksinar, S., Kent, K. C., Bush, H., Jr., Kreiger, K., Rosengart, T., Cybulsky, M. I., Silverman, E. S., and Collins, T. (2000). High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J. Clin. Invest.* **105,** 653–662.

Nebert, D. W., Roe, A. L., Dieter, M. Z., Solis, W. A., Yang, Y., and Dalton, T. P. (2000). Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem. Pharmacol.* **59,** 65–85.

Nixon, J. B., Kamitani, H., Baek, S. J., and Eling, T. E. (2003). Evaluation of eicosanoids and NSAIDs as PPARgamma ligands in colorectal carcinoma cells. *Prostaglandins Leukot. Essent. Fatty Acids* **68,** 323–330.

Park, B. H., Vogelstein, B., and Kinzler, K. W. (2001). Genetic disruption of PPARdelta decreases the tumorigenicity of human colon cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 2598–2603.

Rubin, H. (2001). Synergistic mechanisms in carcinogenesis by polycyclic aromatic hydrocarbons and by tobacco smoke: A bio-historical perspective with updates. *Carcinogenesis* **22,** 1903–1930.

Shozu, M., Murakami, K., Segawa, T., Kasai, T., Ishikawa, H., Shinohara, K., Okada, M., and Inoue, M. (2004). Decreased expression of early growth response-1 and its role in uterine leiomyoma growth. *Cancer Res.* **64,** 4677–4684.

Silverman, E. S., and Collins, T. (1999). Pathways of Egr-1-mediated gene transcription in vascular biology. *Am. J. Pathol.* **154,** 665–670.

Suchanek, K. M., May, F. J., Robinson, J. A., Lee, W. J., Holman, N. A., Monteith, G. R., and Roberts-Thomson, S. J. (2002). Peroxisome proliferator-activated receptor alpha in the human breast cancer cell lines MCF-7 and MDA-MB-231. *Mol. Carcinog.* **34,** 165–171.

Thigpen, A. E., Cala, K. M., Guileyardo, J. M., Molberg, K. H., McConnell, J. D., and Russell, D. W. (1996). Increased expression of early growth response-1 messenger ribonucleic acid in prostatic adenocarcinoma. *J. Urol.* **155,** 975–981.

Thirman, M. J., Albrecht, J. H., Krueger, M. A., Erickson, R. R., Cherwitz, D. L., Park, S. S., Gelboin, H. V., and Holtzman, J. L. (1994). Induction of cytochrome CYPIA1 and formation of toxic metabolites of benzo[a]pyrene by rat aorta: A possible role in atherogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 5397–5401.

Tong, B. J., Tan, J., Tajeda, L., Das, S. K., Chapman, J. A., DuBois, R. N., and Dey, S. K. (2000). Heightened expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor-delta in human endometrial adenocarcinoma. *Neoplasia* **2,** 483–490.

Toraason, M., Hayden, C., Marlow, D., Rinehart, R., Mathias, P., Werren, D., Olsen, L. D., Neumeister, C. E., Mathews, E. S., Cheever, K. L., Marlow, K. L., DeBord, D. G., and Reid, T. M. (2001). DNA strand breaks, oxidative damage, and 1-OH pyrene in roofers with coal-tar pitch dust and/or asphalt fume exposure. *Int. Arch. Occup. Environ. Health* **74,** 396–404.

Vanden Heuvel, J. P., Kreder, D., Belda, B., Hannon, D. B., Nugent, C. A., Burns, K. A., and Taylor, M. J. (2003). Comprehensive analysis of gene expression in rat and human hepatoma cells exposed to the peroxisome proliferator WY14,643. *Toxicol. Appl. Pharmacol.* **188,** 185–98.

Wang, D., Wang, H., Shi, Q., Katkuri, S., Walhi, W., Desvergne, B., Das, S. K., Dey, S. K., and DuBois, R. N. (2004). Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer Cell* **6,** 285–295.

WHO (1998). Environmental Health Criteria 202: Selected non-heterocyclic polycyclic aromatic hydrocarbons. In IPCS, International Programme on Chemical Safety. World Health Organisation, Geneva.

Wogan, G. N., Hecht, S. S., Felton, J. S., Conney, A. H., and Loeb, L. A. (2004). Environmental and chemical carcinogenesis. *Semin. Cancer Biol.* **14,** 473–486.

Yamaguchi, K., Lee, S. H., Eling, T. E., and Baek, S. J. (2004). Identification of nonsteroidal anti-inflammatory drug-activated gene (NAG-1) as a novel downstream target of phosphatidylinositol 3-kinase/AKT/GSK-3beta pathway. *J. Biol. Chem.* **279,** 49617–49623.

Zhang, W., Yan, S. D., Zhu, A., Zou, Y. S., Williams, M., Godman, G. C., Thomashow, B. M., Ginsburg, M. E., Stern, D. M., and Yan, S. F. (2000). Expression of Egr-1 in late stage emphysema. *Am. J. Pathol.* **157,** 1311–1320.

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