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 (PPARδ) 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 PPARδ. Among them, benz(a)anthracene was found to act as a relatively potent activator of PPARα and PPARδ, 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 the early growth response (EGR-1) gene and bind to peroxisome proliferator-activated receptor alpha (PPARα) and delta (PPARδ) in cell culture systems. These data may provide a diversity of PAH activity in several biological pathways.

Key Words: PAHs; PPARα; PPARδ; EGR-1; GSK-3β.
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 (Burger 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 adenocarcinoma 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 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 CO2. 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.

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 CO2. 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% NP-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. The level of protein expression was evaluated by Western blot analysis. The level of protein expression was evaluated by Western blot analysis. The level of protein expression was evaluated by Western blot analysis. 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.

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 SA 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-Gal-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 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 CO2. 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.
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 cytochrome 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
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 PPAR\(\alpha\), A549 cells were transiently co-transfected with a pPPRE\(\times\)3-TK-Luc and pCDNA3-mPPAR\(\alpha\)-LBD constructs (Fig. 4A). The PPAR\(\beta/\delta\) 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-3\(\beta\) and EGR-1 Expression in the Presence of PAHs**

CYP4A is induced by PPAR\(\alpha\) activation in liver tissue (Johnson et al., 1996). GSK-3\(\beta\) is repressed when FaO rat hepatoma cells are exposed to PPAR\(\alpha\) and PPAR\(\beta/\delta\) ligands (Vanden Heuvel et al., 2003). We have shown that some PAHs are able to bind the PPAR\(\alpha\) and PPAR\(\beta/\delta\) as an activator. To determine whether PAHs affects PPAR\(\alpha\) 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-3\(\beta\) expression relative to vehicle-treated cells. BaA was the relatively strong suppressor of GSK-3\(\beta\) expression, whereas BaP or PA was the weak suppressor. These data are consistent with previous data showing that BaA is the relatively strongest PPAR\(\alpha\) and PPAR\(\beta/\delta\) activator, whereas BaP and PA are relatively weak (Figs. 2 and 4). Next, we examined GSK-3\(\beta\) expression in vivo...

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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 \(\mu\)M PA and 10 \(\mu\)M 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 comparison 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 \(\mu\)M, 5 \(\mu\)M 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.
using feral mice (*Peromyscus 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 downregulated 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
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.

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 comparison 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.
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 conclusion, our data suggest that some PAHs, particularly 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.

ACKNOWLEDGMENTS

We thank Dr. Seong-Jin Kim (National Cancer Institute, Bethesda, MD) for providing FaO cells, Wei Guan for technical assistance, and Jada Huskey for her critical reading of the manuscript. This work was in part supported by
grant from the National Institutes of Health (ES011657) and by start-up funds from the University of Tennessee. Financial support for J.H.K. was provided by Department of Occupational and Environmental Medicine, Inje University School of Medicine, Pusan, Korea.

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