Inflammatory Signaling and Aryl Hydrocarbon Receptor Mediate Synergistic Induction of Interleukin 6 in MCF-7 Cells

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Abstract

The pleiotropic cytokine interleukin 6 (IL-6) is involved in immune cell homeostasis. Additionally, IL-6 expression and signaling in tumor cells have been shown to elicit both protumor and antitumor properties. There is a plethora of mechanistic knowledge regarding how IL-6 signal transduction translates to biological responses. However, there is little understanding as to what factors control IL-6 expression within a tumor cell environment. The studies presented herein show that, in MCF-7 breast and ECC-1 endocervical cancer cells, the stimulation of aryl hydrocarbon receptor (AHR) activity, in combination with IL-1β or phorbol 12-myristate 13-acetate (PMA) treatment, results in a marked synergistic induction of IL-6 levels over what is seen without AHR activation. Chromatin immunoprecipitation experiments suggest that the regulation of IL-6 mRNA expression occurs at the chromatin level, as AHR presence on the IL-6 promoter was observed in response to treatment with AHR ligand. Synergistic induction of IL-6 expression was sustained for 72 hours, with accumulation of IL-6 protein reaching levels 4.8-fold above IL-1β treatment alone. In addition, transcriptional regulation of the prototypic AHR responsive gene Cyp1a1 was negatively regulated by PMA and IL-1β treatment. Silencing of RELA expression alleviated IL-1β-mediated repression of AHR transcriptional activity, whereas PMA-mediated repression was maintained. Additionally, small interfering RNA studies reveal that AHR and RELA are necessary for synergistic induction of IL-6. The findings presented here reveal the AHR as a potential therapeutic target for selective modulation of IL-6 expression in some tumor cell types. The data also suggest a possible previously unrecognized mechanism of AHR-mediated tumor promotion. [Cancer Res 2008;68(10):3609–17]

Introduction

During the late 1960s, an autosomal dominant genetic factor was found to be responsible for mediating the onset of a plethora of deleterious health effects associated with exposure to environmental pollutants from the polycyclic and halogenated aromatic hydrocarbon families (1). This genetic factor was called the *aryl hydrocarbon (ah)* locus, which later was cloned and identified as a soluble ligand-activated transcription factor, termed the *ah* receptor (AHR; ref. 2). The AHR has often been studied through its role in mediating the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This persistent environmental contaminant bioaccumulates in the food chain and is essentially not metabolized by mammals. Its adverse biological effects in vertebrates include teratogenesis, reproductive and liver toxicity, immune dysfunction, and tumor promotion. These deleterious effects observed are both species-specific and organ-specific, with the hallmark of acute human exposure being chloracne. TCDD has been shown to be a potent promoter of ovarian, liver, and skin tumor formation in two-stage carcinogenesis rodent models (3–5). Interestingly, formation of skin tumors after sequential treatment with a mutagen and TCDD in mice was linked to the hairless locus. Transgenic mice expressing a constitutively active mutant AHR form liver tumors after exposure to *N*-nitrosodiethylamine as an initiator (6). This study further supports the concept that TCDD mediates its tumor promotional effects through the activation of the AHR. The precise mechanism(s) of AHR-mediated tumor promotion are largely unknown, although several theories have been proposed (7).

Currently, it is believed that the AHR functions through its ability to directly modulate transcription. The AHR is also known to functionally interact with numerous transcription factor pathways, and various reports have shown cross-talk between the immunologically relevant estrogen receptor (ER) or nuclear factor-κB (NF-κB) transcription factors and the AHR (for reviews, see refs. 8, 9). For example, influence of AHR on NF-κB signaling has been shown in immune and nonimmune cells (10, 11). Through these interactions, the AHR can function cooperatively to regulate gene expression in a range of cell types apparently without binding to its cognate response element. Whether the AHR can modulate expression of key NF-κB target genes, such as interleukin 6 (IL-6), is poorly understood. However, TCDD has been shown to repress IL-6 production in BMS2 cells, a bone marrow stromal cell line (12).

IL-6 is a pleiotropic cytokine classically involved in the acute phase response, as well as lymphocyte differentiation and proliferation after inflammatory stimuli. Similar to transcription factors, like NF-κB, IL-6 expression and function are also detected in various types of nonimmune tissues, such as skin (13, 14) and the nervous system (15). Additionally, IL-6 expression in tumor cells is known to elicit both protumorigenic and antitumorigenic properties. High expression of IL-6 in prostate cancer cells has been shown to result in enhanced androgen receptor–dependent growth rate (16), decreased sensitivity to chemotherapeutic drugs (17), and resistance to apoptosis (18), whereas numerous clinical studies have shown that cervical cancer leads to elevated IL-6 levels and elevated IL-6 gene expression has been correlated with the presence of invasive cervical carcinomas (19–21). However, IL-6...
expression in breast cancer has been implicated to correlate both positively and negatively with overall prognosis (as reviewed in ref. 22). Interestingly, whereas IL-6 expression has been detected in normal mammary epithelial cells, there seems to be a lack of IL-6 expression in breast ductal carcinoma tumors (23). Low IL-6 levels have been observed in certain established breast cancer cell lines, such as MCF-7 cells (24). Whether the lack of IL-6 expression enhances or represses the tumorigenic potential or ability to metastasize in vivo is poorly understood and is dependent on the tumor type, as well as the level of tumor progression.

Mounting evidence shows IL-6 expression and functionality in a broad range of tumor cells; however, there is less known about what factors regulate IL-6 expression in these cells. The results presented in this study show for the first time that activation of AHR signaling, coupled with inflammatory signaling, mediates the synergistic induction of IL-6 expression in some cancer cells that are normally largely refractory to cytokine-induced IL-6 transcription. Surprisingly, the signaling conditions leading to the greatest enhancement of IL-6 expression are repressive for AHR-responsive transcription of the prototypic AHR-regulated gene Cyp1a1. Furthermore, the results presented here identify a possible mechanism of AHR-mediated tumor promotion.

Materials and Methods

Cell culture. MCF-7 breast and ECC-1 endocervical tumor cells were maintained at 37°C, 5% CO2 in a high glucose DMEM (Sigma), supplemented with 7% fetal bovine serum (FBS; Hyclone Labs.), 1,000 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma).

Gene expression. MCF-7 cells were serum-starved, and ECC-1 cells were placed in media containing 1% dexamethasone- and charcoal-stripped serum (Hyclone) 18 h before treatment. Treatment of cells was performed by diluting compounds to the desired working concentration (see figure legends) in serum-free media supplemented with 3 mg/ml BSA, penicillin, streptomycin, and 100 ng/ml bovine sera albumin (BSA) or, in the case of ECC-1 cells, in 1% charcoal-stripped serum (Hyclone)-supplemented low-serum media containing 5 mg/mL BSA, penicillin, streptomycin, and 1% SDS, and crosslinks were reversed at 65°C overnight. Immunodabsorbed DNA was analyzed by PCR using the following primers: IL-6 promoter (5'-AGCATGTCGCAACAAAGGCAAC-3') and 5'-CAAGCGTGGTATTAGGAAAGG-3', Cyp1a1 enhancer (5'-TAAGGACCCCGCCACGTCTCCT-3') and 5'-TAGCTTGCGTGCAGCCGGCCGACAT-3'. Cyp1a1 and IL-6 ChIP primers are previously published (25, 26).

Gene silencing. RELA (Dharmacon, J-003333-06, J-003333-07), C/EBPβ (Ambion, AM16708), hIL-6 (Dharmacon, J004990-07), and c-JUN (Dharmacon, J-003268-10) mRNA levels were decreased using Dharmacon On-Target plus or Ambion silencer small interfering RNA (siRNA) oligos. Dharmacon transfections were performed essentially as recommended by the manufacturer using Dharmafect 1 transfection reagent. Oligos were transfected at 100 nmol/L onto cells in serum-free/antibiotic-free media. After 48 h, cells were treated and processed as described above for gene expression experiments. In some experiments, electroporation/nucleofection was performed using the Amaza nucleofection system essentially as described in manufacturer protocols. Briefly, cells were washed and suspended at a concentration of 3.0 × 10^6 per 100 μl of Amaza nucleofection solution from Nucleofection kit V. Control or target siRNA was added to the sample for a final concentration of 2 μmol/L per sample. Samples were electroporated using manufacturer's high efficiency program and plated into six-well dishes in complete media.

Conditioned media preparation. Undifferentiated THP-1 cells were plated at a density of 5 × 10^5 cells/mL in RPMI media supplemented with 7% FBS, 1,000 units/ml penicillin, and 0.1 mg/ml streptomycin. Phorbol 12-myristate 13-acetate (PMA) was added to the media for a final concentration of 81 nmol/L for 24 h to promote differentiation. THP-1 cells were washed with PBS followed by the addition of serum-free RPMI containing 5 mg/ml BSA, penicillin, streptomycin, and 100 ng/ml lipopolysaccharide (LPS) and incubated for 24 h.

Results

PMA-mediated repression of Cyp1a1 expression. Previous studies have shown that AHR activity is modulated after exposure to the tumor promoter PMA. However, whether the mechanism of PMA-mediated modulation of AHR target gene expression is similar to the type of repression seen with cytokines has not been...
explored. Furthermore, whether there is cross-talk between cytokine signaling and AHR that leads to AHR-mediated modulation of cytokine signaling has also not been significantly examined. The effects of PMA on AHR activity have been postulated to be due to the ability of PMA to alter protein kinase C (PKC) signaling. However, many of these effects have been measured through reporter studies (27, 28) and sometimes are in direct conflict with observations made in vivo (29). Therefore, we addressed PMA modulation of AHR activity with respect to endogenous gene regulation using quantitative real-time PCR. In contrast to previous reporter studies that described enhanced AHR activity resulting from PMA treatment, the cotreatment of MCF-7 cells with 10 nmol/L TCDD and 81 nmol/L PMA for 2 hours results in a marked decrease in the production of the AHR-regulated gene Cyp1a1 compared with TCDD alone (Fig. 1A). This decrease in Cyp1a1 mRNA production is presumably due to an overall decrease in AHR transcriptional activity, as evidenced by the reduction in histone-4 acetylation at the Cyp1a1 enhancer (Supplementary Fig. S1A). In addition, actinomycin D treatment showed no differences in turnover rates of Cyp1a1 mRNA between treatments (data not shown), demonstrating that the repressive effect of PMA is at the transcriptional level.

PMA and TCDD synergistically induce IL-6 expression, but not IL-8. In addition to regulating PKC signaling, PMA is known to increase the activity of the activator protein 1 (AP-1), NF-κB, and C/EBPβ transcription factors (30–33). To determine if one or more of these factors could be activated in MCF-7 cells during the aforementioned treatments, we measured IL-6 mRNA levels, the expression of which can be regulated by these transcription factors (34). Unexpectedly, IL-6 mRNA levels were greatly elevated when cells were cotreated with PMA and TCDD, compared with PMA alone (Fig. 1B). This synergistic induction of IL-6 expression occurred in a dose-dependent manner and showed a similar pharmacologic profile as seen for Cyp1a1 mRNA repression (Fig. 1C, a and b), with EC50 and IC50 values of ~1 nmol/L. Maximal regulation of Cyp1a1 and IL-6 mRNA expression was seen at ~10 nmol/L PMA. Interestingly, the synergistic induction of IL-6 is not directly applicable to all PMA-responsive genes. PMA treatment resulted in a pronounced induction of IL-8 (Fig. 1D) compared with IL-6, but no enhancement in expression was observed after TCDD cotreatment. The repression of Cyp1a1 mRNA induction, as well as the synergistic elevation of IL-6 occurs after exposure to nonsaturating concentrations of the AHR ligand TCDD (Fig. 2A, a and b), demonstrating that this transcriptional regulation is not merely a high-dose effect. Additionally, activation of the AHR by benzo(a)pyrene also results in PMA-mediated repression of Cyp1a1 and synergistic induction of IL-6 mRNA levels, showing that these effects are not unique to the metabolically resistant TCDD (Fig. 2B).

IL-1β and TCDD synergize to elevate IL-6 levels in an AHR-dependent manner. In many normal cell types, the physiologically relevant proinflammatory cytokine IL-1β is a potent inducer of IL-6 expression and synergistic induction of IL-6 mRNA is mediated by PMA treatment in combination with AHR activation. A and B, MCF-7 cells were plated in six-well dishes, serum-starved for 18 h, and treated with DMSO vehicle, 81 nmol/L PMA, 5 nmol/L TCDD, or TCDD and PMA. After 2 h, total RNA was isolated, cDNA was prepared, and relative Cyp1a1 and IL-6 mRNA levels were determined by quantitative real-time PCR. C (a and b) and D, Cyp1a1, IL-6, and IL-8 mRNA levels were determined after a 2-h treatment of increasing doses of PMA in combination with 1 nmol/L TCDD. Gene expression values are the average of triplicate samples normalized to cellular levels of gapdh mRNA. Error bars, SD between samples. Note that in C, Cyp1a1 and IL-6 values obtained in the absence of TCDD treatment were determined at 0 and 100 nmol/L PMA only.

Figure 1. The repression of Cyp1a1 expression and synergistic induction of IL-6 mRNA is mediated by PMA treatment in combination with AHR activation. A and B, MCF-7 cells were plated in six-well dishes, serum-starved for 18 h, and treated with DMSO vehicle, 81 nmol/L PMA, 5 nmol/L TCDD, or TCDD and PMA. After 2 h, total RNA was isolated, cDNA was prepared, and relative Cyp1a1 and IL-6 mRNA levels were determined by quantitative real-time PCR. C (a and b) and D, Cyp1a1, IL-6, and IL-8 mRNA levels were determined after a 2-h treatment of increasing doses of PMA in combination with 1 nmol/L TCDD. Gene expression values are the average of triplicate samples normalized to cellular levels of gapdh mRNA. Error bars, SD between samples. Note that in C, Cyp1a1 and IL-6 values obtained in the absence of TCDD treatment were determined at 0 and 100 nmol/L PMA only.
expression. Therefore, we wanted to determine whether activation of the AHR in combination with IL-1β treatment would result in synergistic induction of IL-6 expression. Indeed, cotreatment of cells with TCDD and IL-1β resulted in a substantial elevation of IL-6 mRNA expression over that observed with IL-1β alone (Fig. 2D, b). Similar to what is observed with PMA exposure, the cotreatment of MCF-7 cells with IL-1β and TCDD resulted in lower expression of Cyp1a1 mRNA than with TCDD alone (Fig. 2D, a). To prove that TCDD induced a synergistic increase in IL-6 mRNA in MCF-7 cells through AHR activation, cells were pretreated with the AHR partial antagonist α-naphthoflavone (α-NF; 0.5 μmol/L), followed by AHR activation with TCDD with or without PMA or IL-1β cotreatment. As seen in Fig. 2C, α-NF ablates the synergistic induction of IL-6 mRNA in the presence of TCDD, demonstrating the requirement for AHR activation.

IL-1β represses TCDD-mediated induction of CYP1A1 protein levels. It was imperative to determine if the changes observed in Cyp1a1 and IL-6 mRNA expression were also manifested at the protein level. As expected, TCDD-induced CYP1A1 expression in MCF-7 cells was lower in cells cotreated with PMA (10 and 81 nmol/L) or IL-1β compared with TCDD alone. This decrease in CYP1A1 levels was observed after short-term treatment (5.5 hours) and was maintained for at least 20 hours, with ~50% reduction in the presence of IL-1β (Fig. 3A and B). Note that the nonspecific bands in Fig. 3A are due to the overall low levels of CYP1A1 expression compared with what is seen after 20 hours (Fig. 3B). Additionally, PMA treatment resulted in a rapid and sustained elevation in AHR protein levels. Whether this is due to increased AHR synthesis or stabilization is not clear but, despite the higher receptor levels present, CYP1A1 expression in the presence of TCDD remains lower than without PMA exposure. Interestingly, despite the PMA-mediated elevation of AHR levels even after 20 hours of treatment, the combination of TCDD and PMA mediates a marked reduction in AHR levels.

IL-1β and TCDD synergize to mediate a sustained induction of IL-6 mRNA and protein. Remarkably, the synergistic induction and accumulation of IL-6 mRNA was sustained after a single treatment with TCDD and IL-1β for at least 72 hours (Fig. 3C). Whereas IL-1β treatment alone resulted in a modest induction of IL-6 mRNA levels (10.6-fold, 10.7-fold, and 24.3-fold at 24, 48, and 72 hours, respectively), the cotreatment of serum-starved MCF-7 cells with TCDD and IL-1β caused a much greater level of expression (50-fold, 95-fold, and 154-fold at 24, 48, and 72 hours, respectively). The accumulation of IL-6 protein in the media correlated well with mRNA expression, and importantly the concentrations of IL-6 in TCDD/IL-1β cotreated cell media were found at physiologically significant levels (Fig. 3D).

RELA expression is required for IL-1β–induced repression of TCDD-induced Cyp1a1 expression. As previously mentioned, PMA and IL-1β signal transduction cascades are able to alter the
activity of the AP-1, NF-κB, and C/EBPβ transcription factors. After a 5.5-hour treatment with PMA (10 and 81 nmol/L) or IL-1β, enhanced expression of C/EBPβ and c-JUN protein levels was observed. Cotreatment with TCDD did not result in any additional changes in the induction of C/EBPβ and c-JUN protein expression. RELA levels were also unaffected by any of the treatment conditions (Fig. 4A). Individual reduction of c-JUN and C/EBPβ expression by siRNA had no effect on the ability of PMA or IL-1β to inhibit the induction of Cyp1a1 mRNA (Supplementary Fig. S1B). However, the repression of Cyp1a1 mRNA expression by IL-1β, but not PMA, was alleviated by two different siRNA oligonucleotides directed against RELA (Fig. 4B). This result is not surprising, given that previous reports have described RELA/NF-κB repression of Cyp1a1 induction through tumor necrosis factor-α (TNF-α) and lipopolysaccharide signaling (11). A representative image of siRNA silencing of constitutive and PMA-induced (12 hours) c-JUN and C/EBPβ or constitutive RELA in MCF-7 cells is shown to confirm that siRNA oligonucleotides were efficient at reducing expression of target proteins (Supplementary Fig. S1C). Additionally, ChIP assay shows that the decrease in Cyp1a1 mRNA expression is mirrored by an overall decrease in AHR occupancy at the Cyp1a1 enhancer element (Fig. 4C).

Both RELA and AHR expression are required for TCDD-mediated and IL-1β-mediated synergistic induction of IL-6. Whether RELA or AHR expression is required for high level of induction of IL-6 after treatment with IL-1β + TCDD was tested through the use of siRNA-mediated gene silencing in MCF-7 cells. Targeted knockdown of the AHR mRNA revealed that AHR protein level was almost totally abated (Supplementary Fig. S1D). Under these conditions, TCDD-induced Cyp1a1 mRNA expression is lost (Supplementary Fig. S1E). Silencing of RELA expression eliminates TCDD-mediated synergistic induction of IL-6 (Fig. 4D, a). Interestingly, AHR silencing also led to a dramatic repression of constitutive and IL-1β-mediated expression of IL-6. This observation suggests that the AHR may play a role in regulating the relatively low amount of constitutive IL-6 expression in MCF-7 cells. The role of RELA in mediating IL-6 production was assessed using siRNA-mediated targeted knockdown. Silencing of RELA expression in MCF-7 cells leads to a greatly reduced level of IL-1β–mediated, TCDD-mediated, or TCDD + IL-1β–mediated induction of IL-6 (Fig. 4D, b). In addition, IL-8 expression was examined and its expression was also greatly diminished after RELA knockdown (Supplementary Fig. S1F). These results support a critical role for RELA in regulating IL-6 expression in MCF-7 cells, but does not resolve the question as to whether the role of the AHR in the synergistic response is mediated through the presence of NF-κB at the IL-6 promoter.

Similar modes of Cyp1a1 and IL-6 gene regulation are observed in ECC-1 cells as are seen in MCF-7 cells. To show that the previously described effects on CYP1A1 and IL-6 expression are not restricted to MCF-7 cells, we repeated these experiments in the endocervical cancer cell line ECC-1. As seen in Fig. 5, cotreatment of ECC-1 cells with TCDD and IL-1β resulted in repression of Cyp1a1 (Fig. 5A) mRNA levels and synergistic induction of IL-6 (Fig. 5B) compared with TCDD alone. Levels of CYP1A1 (Fig. 5C) and IL-6 (Fig. 5D, b) protein expression correlate well with mRNA expression (Fig. 5A and D, a) and are very similar to what is observed in MCF-7 cells. The level of synergistic induction of secreted IL-6 in ECC-1 cells is particularly dramatic compared with TCDD or IL-1β alone. It should be noted, however, that not all tumor cell lines tested were responsive. In T-47D and HepG2 cells, Cyp1a1 mRNA was highly inducible by TCDD, but no repression after cotreatment with PMA or IL-1β was observed, and IL-6 mRNA expression in both lines was largely undetectable (data not shown).

Increased chromatin remodeling mirrors IL-6 expression changes, and the AHR is recruited to the IL-6 promoter. To assess if the synergistic induction of IL-6 is reflected through chromatin remodeling, ChIP assays were performed using acetylated histone-4 as a marker. Expectedly, levels of acetylated histone-4 are highest when cells are cotreated with TCDD and either PMA or IL-1β (Fig. 6A). Additionally, ChIP assay also shows that the AHR itself is recruited to the IL-6 promoter in a ligand-dependent manner (Fig. 6B). These results strongly suggest that AHR regulates IL-6 transcription at the chromatin level.

Figure 3. Repression of CYP1A1 and IL-6 synergistic induction by combinatorial TCDD and PMA/IL-1β treatment was assessed at the protein level. A and B, MCF-7 cells were plated in 10 cm² dishes, serum-starved for 18 h, and treated with DMSO vehicle, 10 nmol/L PMA, 81 nmol/L PMA, and 10 ng/mL IL-1β alone, or in combination with 1 nmol/L TCDD for 5.5 or 20 h. Whole-cell extracts were prepared, and exposed levels of AHR and CYP1A1 were assessed by immunoblot. Cellular levels of p23 were used as a loading control. C and D, MCF-7 cells were plated in 12-well dishes, serum-starved for 22 h, and treated with DMSO vehicle, 10 ng/mL IL-1β, 1 nmol/L TCDD, or TCDD and IL-1β for 24, 48, and 72 h, respectively. G, IL-6 mRNA levels were determined by quantitative real-time PCR. D, at each time point media was collected and used to quantify IL-6 protein levels by ELISA. Gene expression and protein values are the average of quadruplicate samples. IL-6 mRNA values are normalized to cellular levels of gapdh mRNA. Error bars, SD between samples. In A, the arrow is CYP1A1 and the * is a nonspecific band.
THP-1 conditioned media in combination with AHR activation causes enhanced expression of IL-6 and repression of Cyp1a1. It is important to show that AHR activation can synergize with a mixture of inflammatory signals that may be released into the microenvironment of a tumor. Therefore, the human THP-1 monocytic cell line was differentiated into cytokine-secreting macrophage-like cells by sequential treatment with PMA and LPS. Media collected from the differentiated THP-1 cells were added to serum-starved MCF-7 cells in combination with DMSO vehicle or 1 nmol/L TCDD. As seen in Fig. 6C, AHR activation, in combination with the conditioned media, results in a 3.5-fold enhancement of IL-6 mRNA expression over the induction observed with unconditioned media in the presence of TCDD or conditioned media alone. Additionally, signaling through conditioned media exposure resulted in the attenuated ability of TCDD to induce Cyp1a1 expression (Fig. 6D). This experiment shows that the AHR-dependent synergistic induction of IL-6 could occur in a cellular microenvironment where inflammatory signaling is prevalent, as observed in many cancers.

Discussion

PMA is a potent activator of PKC that eventually leads to a loss of PKC protein levels. Studies using a dioxin responsive element (DRE)-driven luciferase reporter vector have indicated that activation of PKC enhances AHR-mediated transcriptional activity (28). Interestingly, inhibition of PKC activity through the use of inhibitors represses AHR activity. In contrast, studies examining the influence of PMA treatment on AHR ligand–mediated induction of CYP1A1 have revealed that PMA leads to a decrease in transcriptional activity (29, 35). At the time, this was attributed to the ability of PMA to lead to PKC down-regulation. However, there are other possible explanations, such as PMA exposure leads to activation of AP-1 or NF-kB. This concept is supported by the fact that activation of inflammatory signaling pathways by either IL-1β, TNF-α, or LPS can suppress AHR ligand inducible CYP1A1 activity in Hepa 1 cells (36, 37). The results in Figs. 1 and 2 confirm that both PMA and IL-1β treatments lead to repression of TCDD-induced Cyp1a1 mRNA levels in MCF-7 cells. Using siRNA gene expression knockdown, the possible role of several key transcriptional mediators of inflammatory signaling in Cyp1a1 transcriptional repression was tested. Reduced expression of only RELA expression resulted in recovery of TCDD-mediated Cyp1a1 mRNA levels after IL-1β treatment (Fig. 4). However, knockdown of RELA expression had no effect on Cyp1a1 transcription after cotreatment with TCDD and PMA. This suggests that PMA and IL-1β use distinct mechanisms to repress Cyp1a1 transcription. ChIP analysis revealed that IL-1β cotreatment with TCDD reduced the amount of AHR present at the Cyp1a1 promoter. This result, coupled with the RELA siRNA experiment would suggest that RELA interferes with the recruitment of the AHR/ARNT complex to the Cyp1a1 promoter. This observation is consistent with earlier studies demonstrating that the AHR and RELA can suppress each other’s transcriptional activity (11, 38). Reduced levels of histone acetylation and AHR occupancy on the Cyp1a1 promoter were also observed with PMA treatment; however, the actual mechanism that mediates this effect remains to be determined.

TCDD and many polycyclic aromatic hydrocarbons (PAH) are potent AHR ligands that can mediate tumor initiation and
promotion. TCDD exhibits its ability to promote almost solely through continual activation of the AHR. In contrast, benzo[a]pyrene and a number of other PAH are complete carcinogens that can behave both as initiators of DNA damage and as tumor promoters. It is generally accepted that this promotional phase involves the continued accumulation of DNA damage upon repeated exposures to PAH, although the AHR is also known to regulate the expression of genes involved in cell cycle progression (as reviewed in ref. 39). Whether prolonged activation of the AHR plays a significant role beyond the induction of cytochrome P-450s that metabolize PAH to reactive intermediates is poorly understood. Data presented here clearly suggest that chronic activation of the AHR in some tumors would lead to elevated IL-6 levels that can act in a paracrine or autocrine manner to enhance tumor cell outgrowth, survival, or metastasis.

IL-6 has been shown to have tumor promotional effects in numerous tumor cell types, including breast, prostate, ovarian, and malignant cholangiocytes (40). In these cells, IL-6 promotes tumor cell progression through the enhancement of proliferation, cell migration and angiogenesis, decreased apoptosis, or increased resistance to chemotherapeutic agents. An increased rate of proliferation of MCF-7 cells has been observed in three-dimensional cell culture using Matrigel in the presence of bone marrow stromal cell conditioned media (41). This observation was later shown to be due to the presence of IL-6 in the media, and this effect was specific for ERα-positive breast tumor cell lines (24). These studies, coupled with the data presented here, would suggest that the combination of inflammatory cytokines and AHR activation might also enhance MCF-7 cell outgrowth, although this hypothesis remains to be tested. It should be noted, however, that some studies have correlated IL-6 expression in cancer cells with antitumor properties (42, 43). In fact, Lee et al. (16) suggest that, in the LNCaP prostate cancer line, protumorigenic IL-6 signaling properties are only acquired after the acquisition of constitutive IL-6 expression, whereas early paracrine signaling induces growth arrest and differentiation.

The AHR and the ER have been shown to interact functionally, as well as physically, and reciprocally modulate each other's transcriptional activities (44, 45). It is well understood that the ER acts as a repressor of IL-6 transcription (46, 47). Therefore, it is possible that the AHR is decreasing the ability of the ER to repress IL-6, thereby resulting in more responsive cytokine-induced expression. However, it should be noted that the ER-positive T-47D cell line did not display synergistic induction of IL-6, implying that ER alone may not be the sole factor required for the observed synergy (data not shown). Clearly, additional studies are warranted to determine whether the ER plays a role in the synergistic induction of IL-6 mediated by the AHR.

Figure 5. Cyp1a1 and IL-6 regulation by combinatorial TCDD and IL-1β treatment is also evident in the ECC-1 endocervical cancer cell line. A and B, ECC-1 cells were plated in six-well dishes, incubated with 1% dextran/charcoal-stripped serum for 24 h, and treated for 2 h with DMSO vehicle, 10 ng/mL IL-1β, 1 nmol/L TCDD, or TCDD and IL-1β. Cyp1a1 and IL-6 mRNA levels were determined by quantitative real-time PCR. C, ECC-1 cells were plated in 10 cm² dishes, serum starved for 24 h, then treated with DMSO vehicle, 10 nmol/L PMA, 81 nmol/L PMA, and 10 ng/mL IL-1β alone or in combination with 1 nmol/L TCDD for 5 h. Whole-cell extracts were prepared, and AHR, CYP1A1, and p23 protein levels were assessed by immunoblot. D (a and b), ECC-1 cells were plated in six-well dishes, serum starved for 24 h, and treated with DMSO vehicle, 10 ng/mL IL-1β, 1 nmol/L TCDD, or TCDD and IL-1β for 24, 48, and 72 h. At each time point, media was collected and then total RNA was isolated. D (a), IL-6 mRNA levels were determined by quantitative real-time PCR. D (b), IL-6 protein levels in media were determined by ELISA. Gene expression and protein values are the average of triplicate samples. Cyp1a1 and IL-6 mRNA values are normalized to cellular levels of gapdh mRNA. Error bars, SD between samples.
Regulation of IL-6 transcription is complex and can be directly modulated by the presence of NF-κB, C/EBPβ, C/EBPδ, and AP-1 at the promoter. Studies have indicated that these transcription factors, especially NF-κB and C/EBPβ, can synergistically induce IL-6 transcription (34, 48). Interestingly, the mode of IL-8 transcriptional regulation is similar, but no synergistic activation of IL-8 was observed after AHR activation. This suggests that the regulation of IL-6 is unique within the context of the cell lines used here and not applicable to all genes regulated by these common transcription factors. Studies have indicated that these transcription factors, especially NF-κB, C/EBPβ, and AP-1 at the promoter, are critical for AHR and RELA in synergistic induction of IL-6. Synergistic induction can be obtained via exposure to differentiated monocyte-conditioned media. A and B, serum-starved 15 cm² dishes of MCF-7 cells were treated for 60 min with DMSO vehicle, 10 nmol/L PMA, and 10 ng/mL IL-1β alone, or in combination with 1 nmol/L TCDD. ChIP assay was performed using an anti–acetylated histone-4 or anti-AHR rabbit polyclonal antibodies. The IL-6 promoter was amplified by PCR. A cathepsin D rabbit polyclonal antibody was used as a negative control. ChIP assays were repeated several times with essentially the same results obtained each time. C and D, MCF-7 cells were plated in six-well dishes and serum-starved for 24 h, after which half of the media was replaced with unconditioned or conditioned THP-1 media. Cells were immediately treated with solvent control (DMSO) or 1 nmol/L TCDD for 2 h. Levels of Cyp1a1 and IL-6 mRNA were determined by quantitative real-time PCR. Gene expression data are normalized to cellular levels of gapdh mRNA. Error bars, SD between samples.

Figure 6. Increased chromatin remodeling and AHR presence on the promoter mirror the AHR-dependent synergistic induction of IL-6. Synergistic induction can be obtained via exposure to differentiated monocyte-conditioned media.
No potential conflicts of interest were disclosed.

Acknowledgments

References


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