NF-κB IS INVOLVED IN CROSS-TALK BETWEEN AH-RECEPTOR AND SIGNAL TRANSDUCTION PATHWAYS.

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Introduction

Persistent environmental pollutants such as halogenated aromatic hydrocarbons (HAHs) and non-halogenated polycyclic aromatic hydrocarbons (PAHs) are known to cause a diverse range of toxic and biological effects in a variety of species and tissues. Exposure to various HAHs, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent HAH, produces such effects as tumor promotion, teratogenicity, lethality, wasting, immuno-, hepato- and dermal toxicity as well as alterations in the expression of a battery of genes[1]. These effects are mediated by the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor which binds TCDD and other agonists in the cytosol. Following ligand binding, the receptor complex translocates into the nucleus where it dissociates from its associated subunits and dimerizes with the Ah receptor nuclear translocator (ARNT) protein, a resident nuclear protein factor. Formation of the AhR:ARNT heterodimer converts the AhR complex into its high affinity DNA binding form, which binds to its specific DNA recognition sequence, the dioxin responsive element (DRE), stimulating the expression of assorted downstream genes. Several recent studies have shown that there is cross-talk between the AhR pathway and other signal transduction pathways, most notably that of protein kinase C (PKC). The PKC activator, phorbol-12-myristate-13-acetate (PMA) has been shown to synergistically increase the ability of AhR ligands (TCDD and b-naphthoflavone) to induce AhR-dependent gene expression[2]. The focus of studies on the PMA enhancement effect to date has been upon characterization of the effects of PKC on the AhR, with little emphasis on the role that other PKC- or PMA-dependent signal transduction pathways play in these effects. Because PMA is also a potent activator of nuclear factor-κB (NF-kB), a pleiotropic transcription factor that regulates over 60 proinflammatory genes involved in immune response[3], this study examines the involvement of NF-kB in AhR signal transduction.

Materials and Methods

Cell Culture and Luciferase Assays

The recombinant mouse hepatoma cell line, H1L1.1c2, was derived from the Hepa1c1c7 line and grown and maintained as described in detail[4]. These cells contain a stably integrated DRE-driven firefly luciferase reporter gene plasmid, pGudLuc1.1, whose transcriptional activation occurs in a time-, dose-, ligand-, and AhR-dependent manner[4]. For chemical treatment, H1L1.1c2 cells were grown to 80 % confluency in 6-well tissue culture plates and incubated with the indicated chemical(s) in triplicate for 4 hours at 37 °C. When the NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC) was used, it was added 90 minutes prior to addition of the other chemical(s) indicated and when the PKC inhibitor chelerythrine chloride (CHEL) was used, this chemical was added 15 minutes prior to the addition of the other chemical(s) indicated. After incubation the cells were washed twice with 1X PBS and lysed. Luciferase activity was determined using Dynatech ML3000 Microplate Luminometer or an Anthos Lucy 2 Microplate Luminometer with automated injection of Promega stabilized luciferase substrate as previously described[4]. Luciferase activity was normalized to sample protein concentration,

determined using the fluorescamine protein assay[5] and bovine serum albumin as the standard and was calculated as relative light units (RLU) per mg of protein.

Results and Discussion

The PKC activator PMA synergistically enhanced TCDD-induced reporter activity in H1L1.1c2 cells two- to three-fold greater than TCDD treatment alone, as previously observed[2]. In addition, pre-treatment of the same cell line with the PKC inhibitor, chelerythrine chloride, inhibited both TCDD-induced reporter activity as well as the enhancement of this activity with PMA treatment, indicating the specific involvement of the PKC pathway in AhR-dependent gene expression (Fig. 1, top). In a parallel experiment, pre-treatment of these cells with the NF-κB inhibitor PDTC resulted in inhibition of both TCDD-induced reporter activity and enhancement by PMA, supporting a role for NF-κB in normal AhR-dependent gene expression and the PMA enhancement effect (Fig. 1, bottom). Two other NF-κB inhibitors, caffeic acid phenethyl ester and capsaicin had very similar effects on TCDD induction of AhR-dependent gene expression (data not shown).

Hepa1c1c7 cells have been reported to contain relatively high constitutive levels of NF-κB activity. However, using an NF-κB-responsive reporter, we were able to demonstrate that PMA treatment still results in a significant and rapid increase in of NF-κB-dependent gene expression in these cells. Reduction in both normal and PMA-enhanced AhR-dependent gene expression following NF-κB inhibition strongly supports a role for NF-κB in AhR signal transduction. To confirm the DRE-dependence of this effect we examined the ability of PDTC to eliminate the PMA enhancement effect on TCDD induction in Hepa1c1c7 cells transfected with pGudLuc2DRE3, a luciferase reporter gene under AhR-dependent control of a single DRE. Similar to the results obtained in H1L1.1c2 cells, NF-κB inhibition with PDTC eliminated the PMA-dependent enhancement of TCDD-induced luciferase activity from the single-DRE luciferase reporter plasmid. These results confirm that the repressive effect of NF-κB inhibitors on AhR-dependent gene expression is DRE-dependent. The ability of NF-κB inhibitors to block the PMA enhancement effect raises the possibility that NF-κB may actually be the key factor responsible for the enhancement in TCDD induction, however, the contribution of PKC can not be eliminated. To determine whether increased NF-κB activity is solely responsible for the enhancement effect, we examined the ability of tumor necrosis factor-α (TNF-α), a potent NF-κB activator, to enhance the TCDD induction response. The inability of TNF-α to enhance TCDD-induced gene expression suggests that the PMA-dependent enhancement of AhR signaling occurs via a mechanism other than simply activation of NF-κB. There may be a sufficient pool of constitutively activated NF-κB in Hepa1c1c7 cells to allow AhR signaling to proceed and activation of additional NF-κB has no additional effect. This is further supported by the inability of the super-repressor IκBαM to inhibit AhR-dependent gene expression. Since overexpression of the super-repressor did not completely eradicate all NF-κB activity, the remaining active NF-κB appears to have been sufficient to allow AhR-dependent gene expression to proceed. This may have been made possible by the existence of comparable levels of IκB-α and IκB-ß in Hepa1c1c7 cells, both of which preferentially interact with dimers that contain c-Rel or RelA (p65). It has been proposed that one mechanism that would prevent IκB-a from inhibiting IκB-ß-released NF-κB is that IκB-ß-associated NF-κB would be modified, possibly by phosphorylation, so that it could not be targeted for inhibition by IκB-α[3].

Differential alterations in the level of nuclear accumulation and DNA binding could account, at least in part, for changes in AhR- and NF-κB-dependent gene expression in the presence of PMA, PDTC, and TCDD. To examine the effect on AhR, nuclear extracts were prepared from H1L1.1c2 cells pre-incubated in the absence or presence of 200 μM PDTC for 90 minutes or CHEL for 15 minutes followed by the addition of DMSO or 1 nM TCDD with and without 81 nM PMA for an additional 1

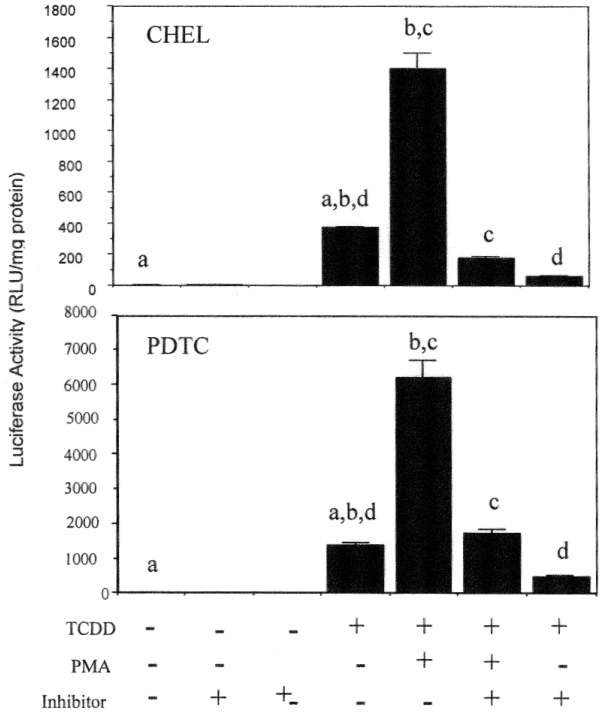

Figure 1. Effects of 81 nM phorbol-12-myristate-13-acetate (PMA), 4 mM chelerythrine chloride (CHEL), and 200 mM pyrrolidinedithiocarbamate (PDTC) on AhR-dependent gene expression.

hour incubation. Gel retardation analysis of these nuclear extracts revealed that neither PMA nor PDTC treatment affected the amount of nuclear AhR-DRE binding whereas there was a decrease in nuclear AhR-DRE binding with CHEL treatment. These results indicate that the PMA enhancement effect is not due to an increase in nuclear AhR complexes and the repression by PDTC and CHEL results from distinctly different mechanisms.

Normal and PMA-enhanced AhR-dependent gene expression are both dependent upon and modulated by PKC and NF-κB, however, the mechanism involved in the cross-talk between these pathways remains to be elucidated. The vast array of cellular effects resulting from PMA treatment or PKC activation provides many avenues by which AhR signaling may be affected or regulated. For instance, in cells containing very low basal NF-κB activity, one can easily envision that AhR functionality and TCDD responsiveness would be reduced. Thus, it would be expected that PMA treatment of cells containing low basal NF-κB activity would not only stimulate AhR functionality (via increased NF-κB activity), but it would also enhance AhR signaling via the PMA effect. Because PKC modulates the action of several co-activators common to both NF-κB and AhR complexes, it is also tempting to speculate that the activation of both NF-κB and the co-activators results in some form of cooperative interaction between these complexes and the AhR, resulting in increased transcriptional activation. We expect that cross-talk between second messenger and AhR signaling pathways will dramatically impact the biochemical and toxic potency of TCDD and HAHs and that further

characterization of these interactions may provide insights in the critical events responsible for the differential responsiveness of species and tissues to these chemicals.

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