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ACCELERATED COMMUNICATION

Leukocyte Activation Induces Aryl Hydrocarbon Receptor Up-Regulation, DNA Binding, and Increased *Cyp1a1* Expression in the Absence of Exogenous Ligand

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SUMMARY

The aryl hydrocarbon receptor (AhR) functions as a transcription factor after ligand binding by halogenated aromatic hydrocarbons. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic halogenated aromatic hydrocarbon, is dependent on binding to the AhR to mediate a broad range of toxic effects. Immune suppression is one of the most sensitive sequela associated with TCDD exposure, yet, paradoxically, resting leukocytes express a relatively low amount of AhR. Here we report that activation of leukocytes produced a 6-fold increase in AhR steady state mRNA levels and a concordant increase in AhR protein expression. Furthermore, leukocyte activation induced AhR translocation, DNA binding to a dioxin response element, and CYP1A1 transcription in the absence of TCDD. Activated leukocytes exhibited an even greater enhancement of dioxin response element binding by the AhR in the presence of TCDD than in the absence of TCDD. These studies suggest that the mechanism responsible for the sensitivity of immunocompetent cells to TCDD may be directly associated with a marked increase in AhR expression, which accompanies leukocyte activation.

The AhR is a cytosolic protein that has no known endogenous ligands; however, it is bound by a large family of chemicals known as HAHs (1). The most toxic member of this family of compounds is TCDD, which is, in certain rodent species, carcinogenic (2), teratogenic (3), and immunosuppressive (4). Less is known about the adverse effects produced by TCDD in man, although the most extensively characterized lesion is chloracne (5). The putative mechanism by which TCDD and other HAHs mediate their broad range of toxicity is believed to be through the translocation of the TCDD-AhR complex to the nucleus, where it forms a dimer with a second protein, ARNT, to regulate gene expression by acting as a transcription factor. The first gene identified whose expression was found to be regulated directly by the TCDD-AhR complex, and thus far the most extensively characterized, is cytochrome P450 1A1. Based on this well established mechanism of gene regulation by TCDD, it has been widely presumed that the mechanism for immune suppression by TCDD is likewise mediated through the AhR. However, even though the immune system is one of the most sensitive target organs to TCDD-mediated toxicity, leukocytes possess relatively low amounts of AhR compared with other TCDD-sensitive tissues, such as the liver (6). One notable difference between leukocytes and other previously examined TCDD-sensitive tissues is that leukocytes are quiescent cells that are critically dependent on activation, proliferation, and differentiation before mediating their respective immune effector functions. In light of this unique feature, the objective of the present studies was to determine if leukocyte activation results in a change in the regulation of *Ahr* expression and, if so, what effect this change has on the ability of the AhR to function as a DNA binding protein in the presence and absence of TCDD.

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DMSO, dimethyl sulfoxide; DRE, dioxin-responsive enhancer; EMSA, electrophoretic mobility shift assay; HAH, halogenated aromatic hydrocarbon; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hsp90, 90-kDa heat shock protein; Io, ionomycin; PMA; phorbol-12-myristate-13-acetate; RT-PCR, reverse transcriptase-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Materials and Methods

Chemicals and media. Chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO). All enzymes used in the quantitative RT-PCR were purchased from Promega (Madison, WI), including the *Taq* DNA polymerase. TCDD was purchased from AccuStandard (New Haven, CT). The purity of TCDD was >99% as determined by gas chromatography/mass spectrometry. All media and their components were purchased from Gibco BRL (Grand Island, NY). Complete RPMI 1640 is RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 5% bovine calf serum (Hyclone, Logan, UT).

Animals. Virus-free female B6C3F1 mice, 5–6 weeks of age, were purchased from the Charles River Laboratories (Boston, MA). On arrival, mice were randomized, transferred to plastic cages containing a sawdust bedding (5 mice per cage) and quarantined for 1 week. Mice were provided with food (Purina Certified Laboratory Chow; Ralston Purina, St. Louis, MO) and water *ad libitum*. Animal holding rooms were kept at 21–24° and 40–60% relative humidity with a 12-hr light/dark cycle.

Splenocyte isolation and treatment. Mice were euthanized, spleens were removed aseptically, placed in complete RPMI 1640, and made into a single cell suspension by removing the connective tissue and capsule. Cells were washed once in RPMI 1640, adjusted to 5×10^6 cells/ml in complete RPMI 1640 and aliquoted into 60 × 15 mm petri dishes. Cells were treated with either vehicle (0.1% DMSO), PMA (80 nM), and/or ionomycin (1 μM) and cultured at 37° and 5% $C0_2$. At the designated times, splenocytes were harvested and total RNA or cell lysates for protein were prepared.

Quantitative RT-PCR. Quantitative RT-PCR was performed as described previously (6) with several modifications. Briefly, total RNA from each sample was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH). Total RNA (100 ng) and internal standard (recombinant RNA) were reverse-transcribed simultaneously in the same reaction tube. The AhR PCR reaction consisted of PCR buffer, 4 mM $MgCl_2$ and 2.5 units of *Taq* DNA polymerase (Promega). Samples were cycled 35 times; each cycle consisted of 94° for 15 sec, 59° for 30 sec, and 72° for 45 sec. PCR products were visualized by ethidium bromide staining and quantified by assessing the absorbance for both of the DNA bands using a Gel Doc 1000 video imaging system (Bio Rad, Hercules, CA). The number of transcripts were calculated from a standard curve generated by using the density ratio between the gene of interest and the different internal standard concentrations used. Primers for the *Cyp1a1* gene were a generous gift from Dr. D. L. Morris (J.D. Searle). The CYP1A1 PCR was conducted under the same conditions as described above for the AhR, except 400 ng of total RNA was utilized per reaction and the annealing temperature was 56°.

Western blot analysis. Western blot analysis was performed on whole cell lysates from splenocytes. Cell lysates were prepared in buffer (25 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 20 mM sodium molybdate) and subsequently resolved by a denaturing SDS-PAGE with 7.5% polyacrylamide (National Diagnostics, Manville, NJ). The proteins were resolved by electrophoresis and transferred to nitrocellulose (Amersham, Arlington Heights, IL). Protein blots were blocked for 2 hr at 22° in Blotto buffer (Tris-buffered saline with 0.1% Tween 20 and 5% low-fat skim milk). Immunoblot analysis was perform using primary antibodies to the AhR, ARNT as perviously characterized by Pollenz *et al.* (7), and cytochrome P450IA1 (Oxford Biomedical Research, Oxford, MI). Immunochemical staining was performed as described previously (6), except that the anti-AhR antibody and anti-ARNT were diluted to 1 μg/ml in antibody dilution buffer (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.05% gelatin, 0.1% Nonidet P-40, and 0.5% bovine serum albumin in borate buffer saline). The CYP1A1 monoclonal antibody was diluted 1:100 in antibody dilution buffer. Detection was performed using the enhanced chemiluminescence method (Amersham). Absorbance for the protein of interest was measured by densitometry using a model 700 imaging system (Bio Rad).

EMSA and EMSA/Western blot analysis. Splenocyte nuclear proteins were isolated and analyzed by EMSA as described previously (6) with several modifications. The nuclear proteins (6–8 μg) were incubated with 200 ng of poly(dI-dC)(BMB) at 22° for 15 min. Radiolabeled DRE oligomer (8) was added (80,000 dpm) and incubated at 22° for another 35 min. The binding of protein to the DNA oligomer was resolved by a 4% nondenaturing PAGE gel, dried on 3-mm filter paper (Whatman, Clifton, NJ), and autoradiographed. Final reaction concentrations were as follows: 25 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 110 mM KCl. To show specific DRE binding, 2 pmol of cold DRE oligomer was added to the reaction mixture. EMSA-Western analysis was conducted as described previously (9) with one modification. EMSA conditions for DRE binding were used as described, except that 10 pmol of cold DRE oligomer was added to the reaction mixture in place of ^{32}P-labeled DRE oligomer before resolution by electrophoresis and nitrocellulose blotting. Immunochemical staining for AhR and detection were performed as described.

Results and Discussion

Recent evidence has implicated a role for the AhR in cell cycle progression and cellular differentiation (10–12). Because leukocytes are quiescent cells that are critically dependent on activation, proliferation, and differentiation before mediating their respective immune effector functions and because they are markedly sensitive to inhibition by TCDD, they represent a unique model for studying the AhR. In the present studies, we examined whether leukocyte activation results in a change in the regulation of *Ahr* expression and, if so, what effect this change has on the ability of the AhR to function as a DNA binding protein in the presence and absence of TCDD. Initially, splenocytes were activated with PMA/Io treatment, and the amount of AhR mRNA expressed was measured by quantitative RT-PCR. Time-course studies showed that activated splenocytes exhibited a rapid and robust increase in steady state *Ahr* expression beginning at 4 hr after PMA/Io treatment that peaked at approximately 8 hr and then gradually returned to a basal level of expression by 48 hr (Fig. 1). *Ahr* expression was increased by approximately 6-fold in PMA/Io-activated splenocytes at 8 hr (peak time) compared with freshly isolated, resting splenocytes (i.e., time 0). Isolated splenocytes not activated with PMA/Io exhibited a relatively constant basal level of AhR mRNA during the first 2 hr of culture that then modestly decreased during the next 22 hr. The decrease in AhR mRNA in unstimulated splenocytes correlated closely with a gradual loss in cell viability (data not shown). Follow-up experiments showed that PMA or Io treatment alone induced a modest increase in AhR mRNA in splenocytes; the increase was of a lesser magnitude but had kinetics similar to those induced when the stimuli were combined (Fig. 1).

To ascertain whether the increase in the steady state AhR mRNA observed in activated leukocytes led to an increase in AhR protein, Western blot analysis was performed. Whole cell-lysates prepared from PMA/Io-activated or resting splenocytes showed a concordant increase in cellular AhR protein (Fig. 2). A similar rate of increase was observed in both of the allelic forms of the AhR, approximately 104 kDa and 98 kDa, which are codominantly expressed in the B6C3F1 mouse (F1: C57/Bl6 × C3H) (13). The results clearly indicated an increase in AhR that exhibited peak protein

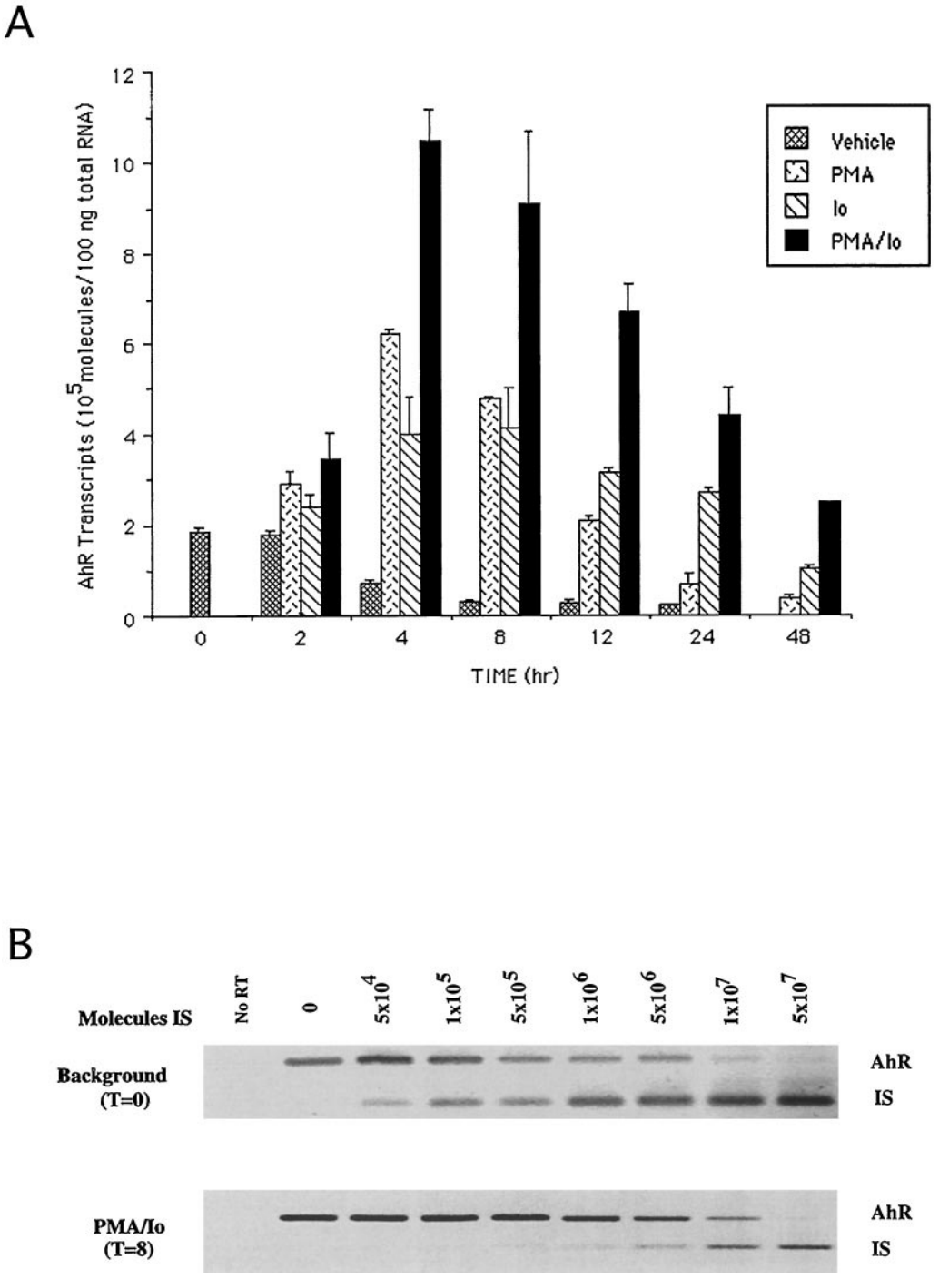

Fig. 1. Kinetics for up-regulation of AhR mRNA expression in PMA/Io-activated splenocytes. Splenocytes (5×10^6 cells/ml) were incubated either with vehicle (0.1% DMSO), PMA (80 nM), Io (1 μM), or PMA plus Io for 0–48 hr, at the end of which time the cells were harvested and total RNA was isolated. A, *Bars*, magnitude of AhR mRNA expressed in splenocytes after the specified treatment as determined by quantitative RT-PCR. Results are averaged from two independent experiments. B, Representative agarose gels showing quantitative RT-PCR products for *ahr* expression in splenocytes using a recombinant RNA internal standard (*IS*) at time 0 and at 8 hr after PMA/Io-activation.

expression between 12 and 24 hr and then gradually decreased to basal levels by 72 hr. A minor band of lower molecular mass that cross-reacted with anti-AhR antibody was routinely observed in our preparations and increased simultaneously with the AhR after PMA/Io activation of splenocytes. The identity of this minor band that cross-reacted with anti-AhR antibody, although unknown, has been previously reported by other laboratories and is believed to be an AhR degradation product (13). Collectively, our results indicate that activation of splenocytes with PMA/Io leads to an increase in steady state AhR mRNA that could be mediated either through an increase in *Ahr* gene expression or mRNA stabilization. However, irrespective of the actual mechanism, the increase in AhR mRNA resulted in a concordant increase in AhR protein. Interestingly, it has been reported previously that the immunoinhibitory effects exerted by TCDD are of greatest magnitude when TCDD is present within the first 24 hr after antigenic stimulation, that period of time corresponding to when *Ahr* expression is greatest as suggested by our present results (14).

Because the ligand bound AhR must first dimerize with ARNT before binding DREs, *Arnt* expression was evaluated in resting and PMA/Io-activated splenocytes. Quantitative RT-PCR showed no significant change in the amount of

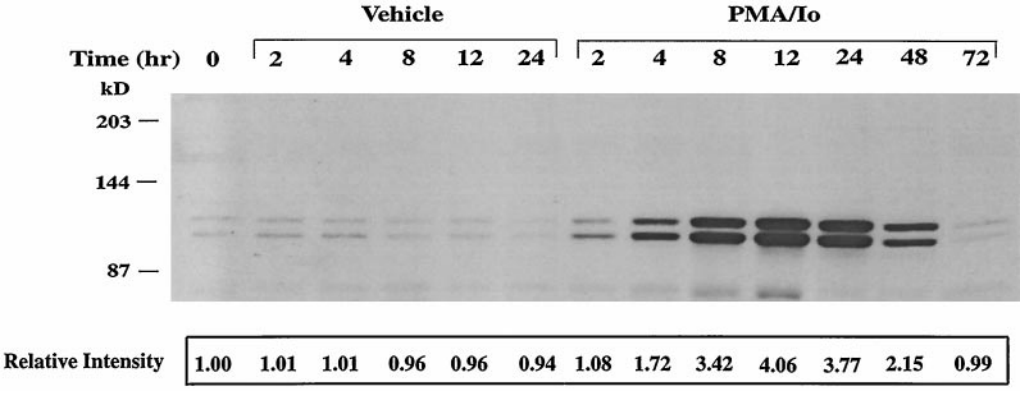

Fig. 2. Kinetics of AhR protein up-regulation in PMA/Io-activated splenocytes. Splenocytes (5×10^6 cells/ml) were incubated either with vehicle (0.1% DMSO) or PMA (80 nM) plus Io (1 μM) and harvested at 0–72 hr. Western blot analysis for AhR was performed on whole cell lysates (100 μg) that were resolved on 7.5% SDS-PAGE gels. The magnitude of AhR expression was assessed by combining the absorbance for both the 104-kDa and 98-kDa forms of the receptor as measured by densitometry. The relative intensity for the 0 time treatment group was arbitrarily assigned a value of 1.00 to which all other treatment groups are compared.

ARNT mRNA in activated versus resting splenocytes over a 24 hr time period (data not shown). However, as shown previously, resting splenocytes exhibited a significantly greater amount of ARNT transcripts compared with AhR (6, 15). The marked difference in the magnitude of expression between these two genes in a variety of tissues (15) has led to speculation that ARNT may be a multifunctional protein that acts as a partner for a number of different transcriptional regulators. This premise is supported by the recent finding that ARNT, under *in vitro* conditions, forms DNA binding complexes with several helix-loop-helix Per-Arnt-AhR-Sim family members including AhR, SIM, and ARNT (16). Interestingly, in the present studies, the difference in the magnitude of mRNA expression between AhR and ARNT was minimal at the peak time of AhR expression (i.e., 8 hr) in PMA/Io-activated splenocytes (Fig. 3). Consistent with the relatively constant expression of ARNT mRNA, Western blot analysis also showed a fairly constant level of ARNT protein in splenocytes during the first 24 hr of culture, irrespective of PMA/Io activation. A modest increase in ARNT protein was observed in PMA/Io activated splenocytes beginning at 24 hr and peaking at approximately 48 hr (Fig. 4).

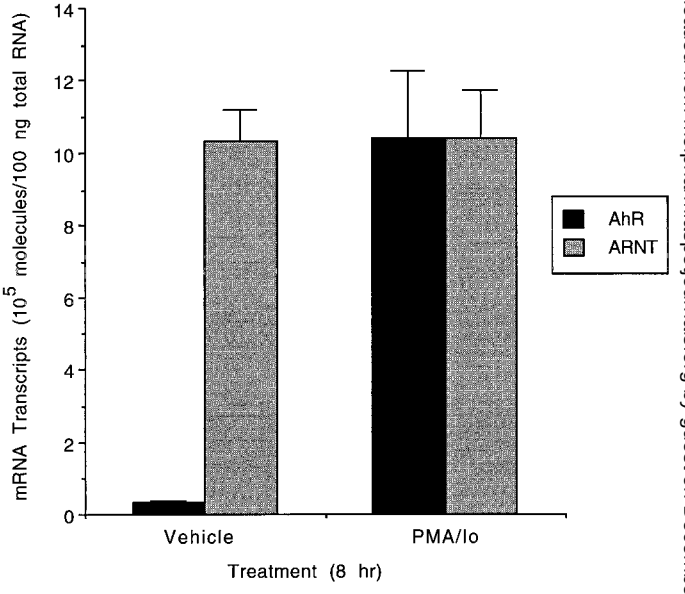

Fig. 3. A comparison of the magnitude of ARNT and AhR mRNA expression in PMA/Io-activated splenocytes. Splenocytes (5×10^6 cells/ml) were incubated either with vehicle (0.1% DMSO) or PMA (80 nM) plus Io (1 μM) and harvested at 8 hr, at the end of which time the cells were harvested and total RNA was isolated. *Bars,* magnitude of ARNT and AhR mRNA expressed in splenocytes as determined by quantitative RT-PCR. Results are averaged from two independent experiments.

Because of the large increase in AhR protein in PMA/Io-activated splenocytes, the magnitude of DRE binding was compared by EMSA in activated versus resting splenocytes in both the presence and the absence of TCDD (Fig. 5A). As expected, TCDD induced DRE binding in resting splenocytes; little or no DRE binding was observed in the absence of TCDD. Conversely, in PMA/Io-activated splenocytes, DRE binding was readily induced in the absence of TCDD and was further increased in the presence of TCDD. AhR binding to the DRE in both the presence and the absence of TCDD was confirmed by EMSA/Western analysis (Fig. 5B). In light of the marked increase in DRE binding observed in activated leukocytes in the absence of exogenous ligand, studies were initiated to determine whether evidence for changes in AhR-mediated transcriptional regulation could be obtained. As shown in Fig. 6, *Cyp1a1* is tightly regulated in leukocytes and its basal expression was below the level of detection by RT-PCR. After PMA/Io activation, leukocytes exhibited significant expression of CYP1A1 mRNA, a gene that is transcriptionally regulated through AhR binding to multiple DRE consensus recognition motifs present in its promoter/enhancer region. Western blotting revealed measurable CYP1A1 protein at approximately 24 hr that continued to increase during the next 24 hr. The increase in CYP1A1 protein confirms that the DRE binding of the AhR was sufficient to up-regulate the expression of a gene under DRE control in the absence of an exogenous ligand. It is notable that an increase in CYP1A1 expression in the absence of exogenous ligands has been demonstrated previously (17, 18); however, because different cell-types were used under different experimental conditions (compared with the present studies), it is unclear whether a similar mechanism is involved.

The above findings have at least three significant implications. First, if the mechanism responsible for immune suppression by HAHs, such as TCDD, is mediated through the

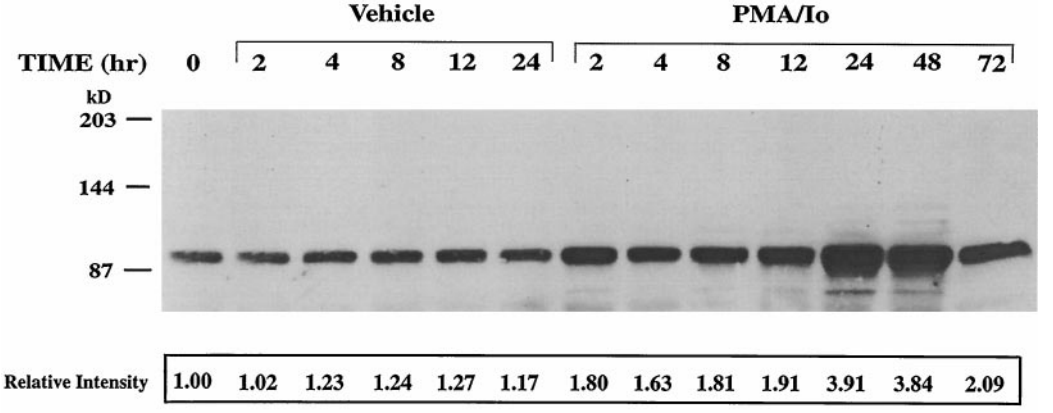

Fig. 4. Western blot analysis for ARNT in PMA/Io-activated splenocytes. Splenocytes (5×10^6 cells/ml) were incubated either with vehicle (0.1% DMSO) or PMA (80 nM) plus Io (1 μM) and harvested at 0–72 hr. Western blot analysis for ARNT was performed on whole cell lysates (100 μg) that were resolved on 7.5% SDS-PAGE gels. The magnitude of ARNT expression was assessed by densitometry. The relative intensity for the 0 time treatment group was arbitrarily assigned a value of 1.00 to which all other treatment groups are compared.

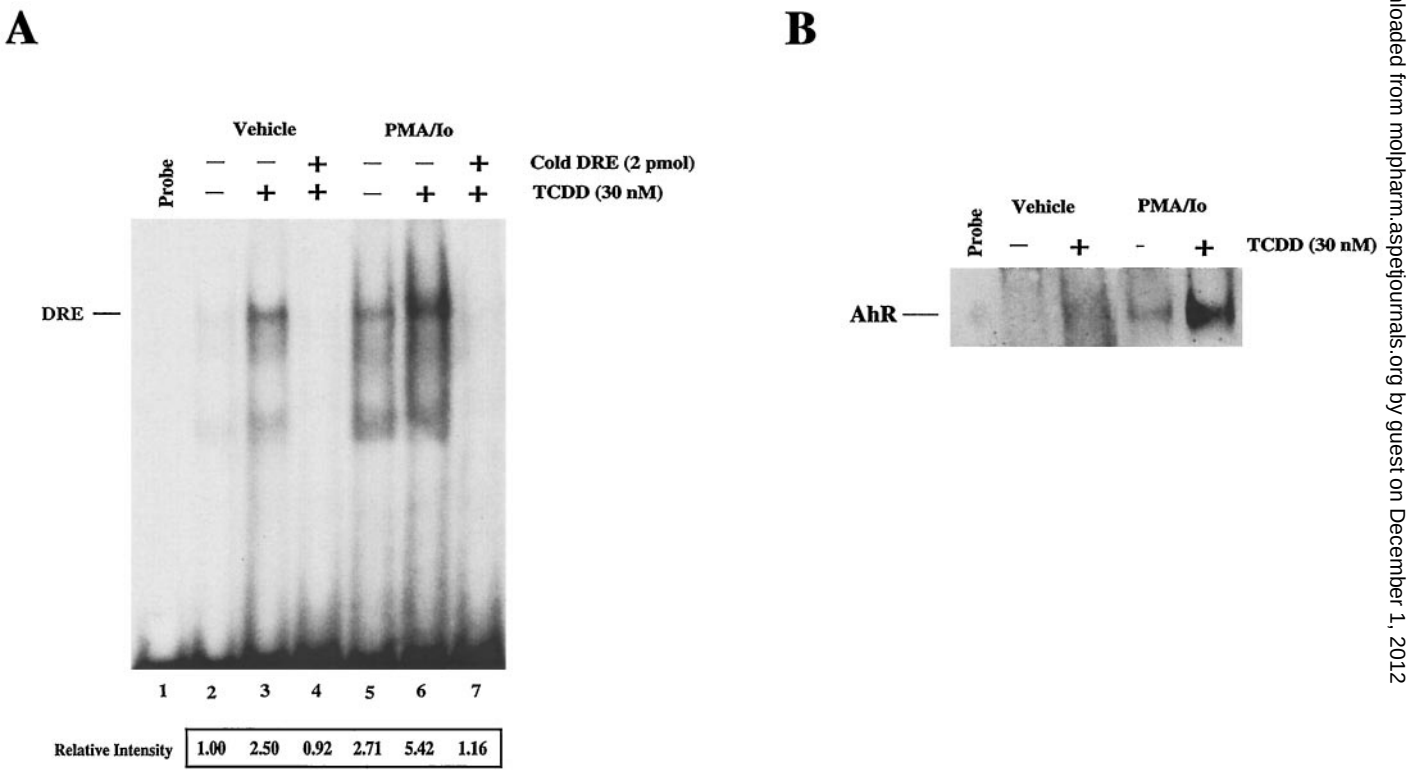

Fig. 5. PMA/Io activation of splenocytes increased AhR binding to a DRE. Splenocytes were incubated for 12 hr in the presence of vehicle (0.1% DMSO) or PMA (80 nM) plus Io (1 μM). Vehicle and PMA/Io-treated cells were then aliquoted into two groups and incubated for an additional 3 hr in the presence or absence of TCDD (30 μM). A, Representative EMSA analysis of splenocyte isolated nuclear proteins (8 μg) for binding to a ^{32}P-labeled DRE oligonucleotide derived from the mouse *Cyp1a1* promoter/enhancer region. *Lane 1,* probe alone; *lane 2*, resting splenocytes in the absence of TCDD; *lane 3*, resting splenocytes in the presence of 30 nM TCDD for 3 hr; *lane 4*, same as lane 3 but with the addition of 2 pmol of cold DRE oligonucleotide; *lanes 5–7,* same as 2–4 except the mouse splenocytes were stimulated with PMA/Io for 12 hr. B, DRE-EMSA Western analysis was used to confirm that the AhR is part of the protein complex bound to the DRE. DRE-EMSA Western analysis was performed (9) on the same nuclear preparation used in A transferred onto nitrocellulose and probed with anti-AhR antibody (1.6 μg/ml).

up-regulation of the AhR and increased AhR/DRE interactions, activated leukocytes may be significantly more sensitive to immune modulation by HAHs than are resting leukocytes, as suggested previously (14, 19). Second, the increase in AhR expression and DNA binding after cell activation suggests that the AhR may play a role in cell cycle and/or some aspect of cell differentiation. These results are consistent with a previous study by Hayashi *et al.* (10) that showed an induction of AhR mRNA in a number of monocytic cell lines during differentiation. Moreover, it recently has been demonstrated that AhR defective Hepa 1c1c7 cell line exhibited a much slower doubling time compared with the wild-type cells (11). Interestingly, introduction of antisense AhR cDNA into the wild-type cells increased their doubling time to a rate comparable with that of the AhR defective cells. Likewise, Weiss and coworkers have implied that there is a

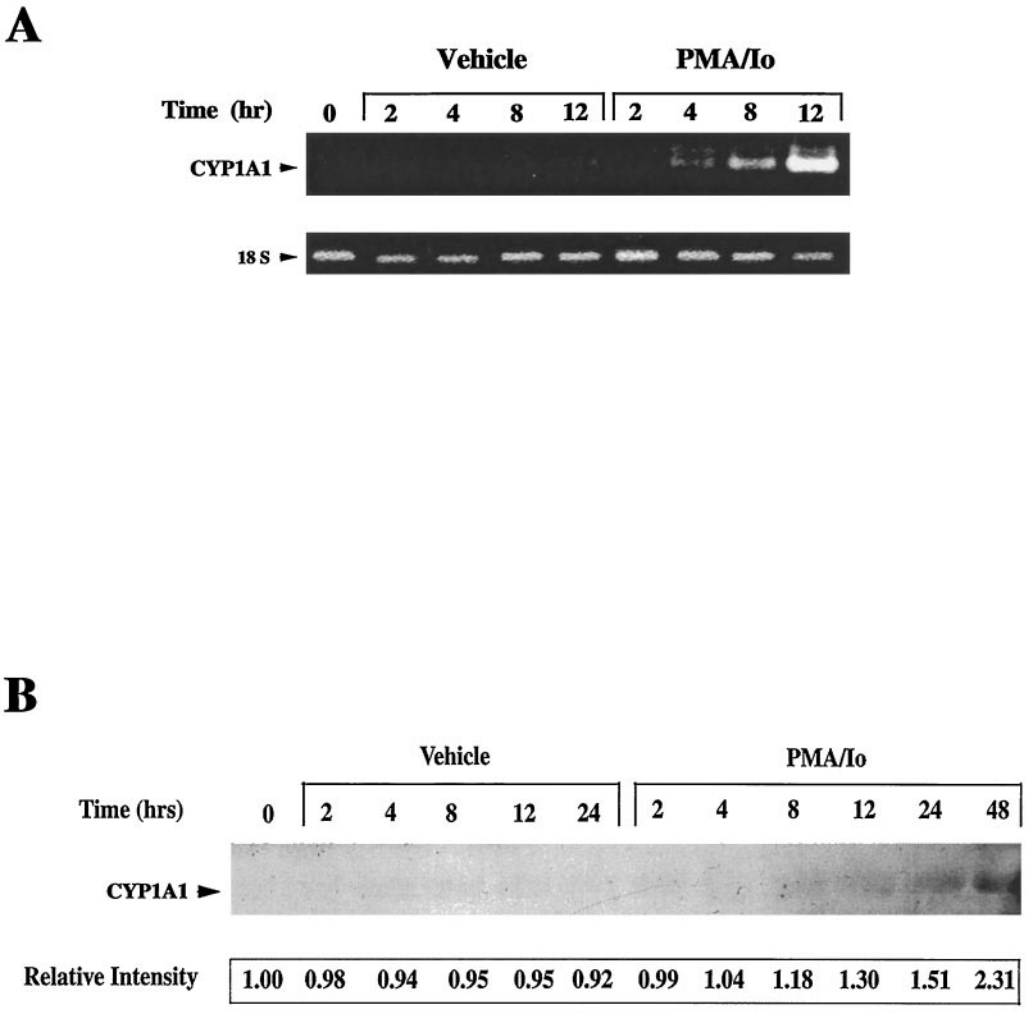

Fig. 6. PMA/Io activation of splenocytes increased CYP1A1 steady state mRNA expression and protein expression. Splenocytes were incubated in the presence of vehicle (0.1% DMSO) or PMA (80 nM) plus Io (1 μM) over the indicated time period. A, RT-PCR was performed to detect a 228-base pair CYP1A1 product. B, Western blotting for CYP1A1 was performed on whole cell lysates (100 μg) that were resolved on 7.5% SDS-PAGE gels. The magnitude of Cyp1A1 expression was assessed by densitometry. The relative intensity for the 0 time treatment group was arbitrarily assigned a value of 1.00 to which all other treatment groups are compared. Results are representative from one of two independent experiments.

role for the AhR in cell cycle control (12). In the present studies, the mechanism responsible for the increase in AhR after leukocyte activation by PMA/Io is also unclear but seems to be associated with signaling events initiated by an increase in protein kinase C activity. Moreover, because at least one activator protein-1 site has been identified in the promoter enhancer region for the murine *Ahr* gene (20), one possibility is that PMA/Io treatment can increase AhR transcription through enhanced activator protein-1 DNA binding. Also consistent with a protein kinase C-mediated increase in AhR, it has been recently reported that PMA treatment of the human hepatoma cell line 101L in combination with TCDD significantly enhanced *Cyp1a1* promoter activity compared with TCDD treatment alone (21). Third, and perhaps most significantly, in the absence of TCDD, leukocyte activation by PMA/Io treatment induced nuclear translocation of the AhR, binding to DRE consensus recognition motifs, and induction of CYP1A1 mRNA and protein expression, all of which are also readily induced by exogenous ligands. Similarly, Vanden Heuvel *et al.* (22) demonstrated that mitogenic activation of human peripheral blood lymphocytes, in the absence of TCDD, induced CYP1A1 expression, which is consistent with AhR DNA binding independent of an exogenous ligand. It is tempting to speculate that the increase in CYP1A1 by activators of leukocytes such as PMA/Io or mitogens, in the absence of TCDD, is caused by the binding of an endogenous ligand to the AhR. However, at least one other explanation exists for the induction of CYP1A1 in activated leukocytes, which also takes into account the increase in AhR that was observed with PMA/Io treatment. Pongratz *et al.* (23) showed that AhR DNA binding readily occurred in the absence of exogenous ligand after *in vitro* disruption of the hsp90-AhR complex. In our studies, AhR DNA binding in the absence of exogenous ligand was most pronounced at 12 hr after PMA/Io treatment, the peak time of AhR protein expression. If the rate of AhR protein synthesis were to exceed the rate at which the newly synthesized receptor associates with the hsp90, the result would be similar to that observed after disruption of the hsp90-AhR complex in the studies by Pongratz *et al.* Under these conditions, it may be possible for AhR to undergo nuclear translocation and DNA binding in the absence of ligand in the intact cell. Collectively, these findings suggest that an increase in AhR may be a general consequence of leukocyte activation and may also account for the temporal aspects associated with the sensitivity of the immune system to TCDD.

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