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The Activated Aryl Hydrocarbon Receptor Synergizes Mitogen-Induced Murine Liver Hyperplasia

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Abstract

Mechanisms of hepatocyte proliferation triggered by tissue loss are distinguishable from those that promote proliferation in the intact liver in response to mitogens. Previous studies demonstrate that exogenous activation of the aryl hydrocarbon receptor (AhR), a soluble ligand-activated transcription factor in the basic helix-loop-helix family of proteins, suppresses compensatory liver regeneration elicited by surgical partial hepatectomy. The goal of the present study was to determine how AhR activation modulates hepatocyte cell cycle progression in the intact liver following treatment with the hepatomitogen, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP). Mice were pretreated with the exogenous AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) 24 hours prior to treatment with TCPOBOP (3 mg/kg). In contrast to the suppressive effects of AhR activation observed during compensatory regeneration, TCDD pretreatment resulted in a 30–50% increase in hepatocyte proliferation in the intact liver of TCPOBOP-treated mice. Although pretreatment with TCDD suppressed CDK2 kinase activity and increased the association of CDK2 with negative regulatory proteins p21Cip1 and p27Kip1, a corresponding increase in CDK4/cyclin D1 association and CDK4 activity which culminated in enhanced phosphorylation of retinoblastoma protein, consistent with the increased proliferative response. These findings are in stark contrast to previous observations that the activated AhR can suppress hepatocyte proliferation *in vivo*, and reveals a new complexity to AhR-mediated cell cycle control.

Keywords

TCPOBOP; TCDD; AhR; liver; hyperplasia

1. Introduction

Liver cell proliferation is essential for restoring organ mass following trauma- or toxicant-induced tissue loss, and to maintain the metabolic capacity of the intact liver to meet physiological or pathological demands. During compensatory liver regeneration induced by

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surgical partial hepatectomy (PH), NF- κ B and Stat-3-mediated signaling cascades stimulate quiescent hepatocytes to enter the cell cycle, while c-met and EGF receptor signaling promote progress through the cell cycle until organ mass is restored (Fausto et al. 2006). In the absence of tissue injury or loss, hepatocyte proliferation in the intact liver can be stimulated through the mitogenic activation of nuclear receptors, such as the constitutive androstane receptor (CAR) (Columbano and Shinozuka 1996). Referred to as “direct hyperplasia,” such proliferation is Stat-3 independent and involves the transcriptional activation of numerous genes required for xenobiotic metabolism, down-regulation of apoptosis, and cell cycle progression (Columbano et al. 2005; Ledda-Columbano et al. 1998; Ledda-Columbano et al. 2000). Although the mechanisms that drive compensatory regeneration and direct hyperplasia are clearly distinct, less is known about the cellular and molecular processes that regulate hepatocyte proliferation during these responses.

Increasing evidence points to a role for the aryl hydrocarbon receptor (AhR) in regulating hepatocyte proliferation both in vitro and in vivo (Bauman et al. 1995a; Gottlicher and Wiebel 1991; Huang and Elferink 2005; Levine-Fridman et al. 2004; Mitchell et al. 2006; Weiss et al. 1996). The AhR is a soluble, ligand-activated transcription factor in the basic helix-loop-helix superfamily of proteins, typically associated with environmental stress responses. Upon binding exogenous or unidentified endogenous ligands, the AhR translocates to the nucleus, where it dimerizes with another bHLH protein, the Ah receptor nuclear translocator (ARNT), to form a transcriptionally active complex that modulates target gene expression (Rowlands et al. 1996). Treatment of hepatocyte-derived cell lines with the potent, exogenous AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits a G1 arrest that appears to be mediated through the induction of p27Kip1 (Kolluri et al. 1999), a negative regulator of S-phase progression. Exposure to TCDD initiates a similar G1 arrest in the mouse liver during compensatory liver regeneration stimulated by 70% PH (Bauman et al. 1995a; Mitchell et al. 2006). We have previously demonstrated that AhR-mediated suppression of liver regeneration coincides with decreased cyclin-dependent kinase (CDK)-2 activity (Mitchell et al. 2006), thus suppressing phosphorylation of the retinoblastoma protein (pRb) required for the transition into S phase. Moreover, reduced liver regeneration in TCDD-treated mice does not appear to be due to altered cytokine signaling during compensatory regeneration (Mitchell et al. 2006), suggesting that the consequences of AhR activation on hepatocyte proliferation may constitute a general response impacting the cell cycle check-point machinery.

In the present study, we sought to determine how AhR activation affects hepatocyte proliferation during mitogen-induced hyperplasia in the intact liver. To this end, we tested the consequences of TCDD pretreatment in mice exposed to the non-genotoxic hepatomitogen 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP). Administration of TCPOBOP initiates a robust proliferative response in the intact liver, accompanied by changes in cell cycle regulatory protein expression and interactions that are distinct from those observed in the regenerating liver (Ledda-Columbano et al. 2000). In contrast to the suppressive effects of AhR activation observed during liver regeneration following PH, we demonstrate here that pretreatment with TCDD increases TCPOBOP-stimulated hepatocyte proliferation. The increased proliferative response following treatment with both TCDD and TCPOBOP is reflected in altered function of the G1 phase cell cycle regulatory protein complexes. These findings may shed light on mechanisms of AhR-mediated cell cycle control and provide insight into TCDD-induced carcinogenicity (Knerr and Schrenk 2006).

2. Materials and Methods

2.1. Chemicals

1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP; Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO and diluted in peanut oil to yield a 0.3 mg/ml working stock. TCDD (Cerilliant, Round Rock, TX) was dissolved in anisole and diluted in peanut oil at 2 mg/ml. Vehicle control consisted of peanut oil alone, spiked with a corresponding amount of DMSO or anisole.

2.2. Mice

All experiments were performed in compliance with the standards established for the care and use of laboratory animals at the University of Texas Medical Branch. Female C57Bl/6 mice, $Ahr^{fx/fx}$ mice (mice containing loxP sites that flank exon 2 of the AhR allele), and mice harboring an albumin promoter-driven *Cre* recombinase on a C57Bl/6 background (Cre^{Alb} mice) were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8–10 weeks of age. $Ahr^{fx/fx}$ mice were crossed with Cre^{Alb} mice to produce offspring in which the $Ahr^{fx/fx}$ allele was excised in parenchymal hepatocytes, effectively eliminating AhR protein expression in these cells after birth (Walisser et al. 2005). Excision of the AhR allele in the $Ahr^{fx/fx}/Cre^{Alb}$ mice was verified at the genomic, mRNA and protein levels (data not shown). Animals were housed in microisolator cages in a temperature- and light-controlled facility with free access to water and diet. Mice were gavaged with vehicle or TCDD (20 μ g/kg) 24 hr prior to treatment with TCPOBOP (3 mg/kg by gavage) and euthanized at the indicated time by isoflurane overdose followed by cervical dislocation (n=3). Livers were removed, weighed, and either fixed in 10% formalin for immunohistochemistry or immediately frozen in liquid nitrogen and stored at -80° C for later use.

2.3. BrdU Incorporation

To measure proliferation, mice were injected i.p. with 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) 2 hr before euthanasia. Preparation of liver tissue for immunohistochemical analysis was performed by the Research Histopathology Core Facility at UTMB. Assessment of BrdU incorporation in hepatocyte nuclei was performed as previously described (Mitchell et al. 2006).

2.4. Immunoprecipitation and Western Blotting

Frozen liver tissue was homogenized and prepared for immunoprecipitation and western blot as previously described (Mitchell et al. 2006). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): CDK2 (sc-163), CDK4 (sc-749), cyclin A (sc-751), cyclin E (sc-481), p21Cip1 (sc-397), p27Kip1 (sc-528), and P4501A1 (sc-9828). Cyclin D1 antibody (556470) was purchased from BD Biosciences (Franklin Lakes, NJ). Actin antibody was purchased from Millipore (Billerica, MA), and AhR antibody was purchased from BIOMOL International L.P. (Plymouth Meeting, PA).

2.5. Kinase assays

CDK2 kinase assays were performed as previously described (Mitchell et al. 2006). A similar procedure was used to measure CDK4 kinase activity using 20 μ g Rb (sc-4112; Santa Cruz Biotechnology) as a substrate.

2.6. Quantitative Real-Time PCR

Total RNA was isolated from frozen mouse liver tissue using an RNAqueous Kit (Ambion, Foster City, CA) according to the manufacturer's protocol. cDNA was synthesized using

SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Transcript levels were measured as previously described (Mitchell et al. 2006) by quantitative real-time PCR (qRT-PCR) at the real-time PCR core facility at the UTMB Sealy Center for Cancer Biology or at the UTMB Molecular Genomics Core.

2.7. Statistical Analysis

Where appropriate, data depict mean values $\pm$ SEM. Statistical significance was determined using Prism 4 software (GraphPad Software, San Diego, CA). For comparison between vehicle- and TCDD-pretreated mice, data were evaluated by two-way analysis of variance (ANOVA) followed by a Bonferroni's post-hoc test with $p \leq 0.05$. In some instances, data within a single treatment group were analyzed using a one-way ANOVA followed by a Dunnett's posthoc test with $p \leq 0.05$.

3. Results

3.1. TCDD treatment increases TCPOBOP-induced hepatocyte proliferation

To determine how AhR activation influences mitogen-induced hyperplasia in the intact liver, mice were pretreated with vehicle or TCDD 24 hr prior to TCPOBOP administration and euthanized 12–72 hours later. As shown in Figure 1A, TCPOBOP treatment elicited an increase in liver mass that was first evident at 24 hr and well established by 36 hr in vehicle-pretreated mice. Pretreatment with TCDD potentiated this effect of TCPOBOP at every time point tested. Increased liver-body weight ratios in TCDD-pretreated mice were not due to a direct induction of hepatomegaly by TCDD, as TCDD treatment alone had no effect on liver weight (Fig 1A, 0 hr; supplemental data).

Using incorporation of the thymidine analogue BrdU to measure DNA replication, the increase in liver weight correlated with hepatocyte proliferation, which was detected as early as 18 hr after TCPOBOP treatment and peaked at 36 hr (Fig. 1B and 1C). TCDD-pretreated mice displayed levels of proliferation that were comparable to vehicle controls at 18 hr, but were almost two-fold higher at 36 and 48 hr. Hepatocyte proliferation was independently assessed based on expression of cyclin A, an S-phase-specific cell cycle regulatory protein (Fig. 1D). In vehicle-pretreated mice, increased expression of cyclin A was detected 36 hr after TCPOBOP. Levels of cyclin A were markedly increased in TCDD-pretreated mice and remained elevated 36 to 72 hr after TCPOBOP administration. TCDD treatment alone increased neither BrdU incorporation nor cyclin A expression (Mitchell et al., 2006, and data not shown), indicating that the observed increase in S-phase progression is not due to an additive effect of TCDD- and TCPOBOP-induced cell proliferation.

3.2. TCPOBOP administration increases AhR-mediated activity

Given the prior findings that TCDD-induced AhR activity could promote a cell cycle arrest in both hepatoma cells and in the regenerating liver (Bauman et al. 1995; Kolluri et al. 1999; Mitchell et al. 2006; Weiss et al. 1996), we sought to characterize AhR activity in the presence of TCPOBOP. Although the level of AhR protein initially decreased in TCDD pretreated mouse liver in keeping with previously reported proteolytic degradation (Ma and Baldwin 2000; Pollenz 2002), it was restored to a level comparable among vehicle- and TCDD-treated mice within 12–24 hr after treatment with TCPOBOP (Fig. 2A). AhR level appears to transiently increase beyond the steady state level detected in untreated animals consistent with previous agonist binding studies (Benedetti et al., 1994). With regard to AhR activity, there was no overt effect of TCPOBOP treatment on the TCDD-induced expression of the AhR-regulated gene *Cyp1a1* (Fig. 2B). However, TCPOBOP administration in the absence of TCDD transiently increased *Cyp1a1* transcript levels with an approximate 70-fold increase observed at 12 hr. This transient increase in *Cyp1a1* expression was also

observed during liver regeneration following PH (Mitchell et al. 2006), and is thought to be due to transient endogenous activation of the AhR associated with the proliferative response. Although TCPOBOP is not an AhR agonist, we verified that this TCPOBOP-mediated induction of *Cyp1a1* in vehicle-pretreated mice occurred through an AhR-dependent mechanism. AhR conditional knockout $Ahr^{fx/fx}/Cre^{Alb}$ mice (CKO)—lacking AhR expression in the hepatic parenchyma specifically—were pretreated with vehicle or TCDD prior to TCPOBOP administration. In the absence of TCDD, TCPOBOP treatment elicited a modest 3-fold increase in *Cyp1a1* transcript at 12 hr in the CKO mice (Fig. 2C), considerably less than the 70-fold increase observed in wild type mice at this same time point (Fig. 2B). As expected, AhR CKO mice pretreated with TCDD exhibited a markedly attenuated increase in *Cyp1a1* transcript (Fig. 2C), four orders of magnitude lower than that observed in wild type mice in the absence of TCPOBOP (Fig. 2B, 0 hr). Accordingly, induction of the *Cyp1a1* gene product cytochrome P4501A1 was markedly diminished in TCDD-treated AhR CKO mice compared to wild type mice (Fig. 2D, compare lanes 2 and 4 with 6 and 8). The residual *Cyp1a1* mRNA induction detected in the CKO mouse liver is attributed to AhR activity in the hepatic non-parenchymal cell population represented by the stellate, endothelial and Kupffer cells.

3.3. AhR activation alters TCPOBOP-stimulated CDK2 protein interactions and suppresses CDK2 kinase activity

We previously showed that exposure to TCDD reduces CDK2/cyclin E complex formation in the regenerating liver following 70% PH, accompanied by reduced CDK2 kinase activity and suppression of S-phase progression (Mitchell et al. 2006). Hence, we initially examined whether CDK2 was also a sensitive target of AhR-mediated TCDD action during TCPOBOP-stimulated liver hyperplasia. Whereas levels of CDK2 remained relatively unchanged between treatment groups, the formation of CDK2/cyclin E complexes was increased in TCDD-pretreated mice 36–72 hr after TCPOBOP treatment (Fig. 3A). TCDD also increased CDK2/cyclin A complex formation, consistent with increased S-phase progression in TCDD-pretreated mice. The data also revealed that CDK2-cyclin complexes became increasingly associated with p21Cip1 and p27Kip1 in TCDD-pretreated mice 36–72 hr after TCPOBOP. These are canonical members of the Cip/Kip family of cell cycle inhibitory proteins that typically suppress S-phase entry by inhibiting G1 kinase activity (Sherr and Roberts 1995). Their recruitment to the CDK2-cyclin E/A complexes at times beyond 36 hr post-TCPOBOP treatment correlates with a loss of CDK2 kinase activity (Fig. 3B). The evidence reveals that the hyperplastic effect of TCPOBOP treatment coincides with a modest increase in CDK2 activity at 24 and 36 hr in vehicle-pretreated mice. In mice pretreated with TCDD, maximal CDK2 activity occurred at 24 hr, but was completely abolished thereafter coincident with pronounced p21Cip1 and p27Kip1 binding to the CDK2 complex (Fig. 3A). Thus, despite increased CDK2-cyclin E/A complex formation, CDK2 kinase activity fails to explain the enhanced S-phase progression in TCDD-pretreated mice subsequently administered TCPOBOP to induce liver proliferation.

3.4. The TCDD-enhanced hyperplastic response is associated with altered CDK4 complex formation

Since analysis of the CDK2 complex did not reveal a basis for the TCDD-enhanced hyperplasia induced by TCPOBOP, we next examined CDK4 and its recruitment of cyclin D1 (Fig. 4). Previous reports demonstrate that cyclin D1 is rapidly upregulated following TCPOBOP treatment (Columbano and Shinozuka 1996). As a member of the G1 cyclin family, cyclin D1 partners with CDK4 and CDK6 to phospho-inactivate the retinoblastoma protein (pRb), thereby facilitating entry into S phase. While cyclin D1 protein levels increased slightly 12 hr after TCPOBOP administration, no TCDD effects were readily evident (Fig. 4A). We also measured p21Cip1 and p27Kip1 protein expression normalized

against actin expression, and observed an approximate 2-fold increase in expression of these proteins by 24–36 hr. However, marked TCDD-dependent changes in expression were not evident, and thus did not offer an explanation for the increased liver hyperplasia.

Lack of pronounced TCDD-dependent alterations in cell cycle protein levels did not preclude the possibility that assembly or activity of cyclin-CDK4 complexes required for S-phase progression differed in response to TCDD exposure. Indeed, it is the composition and activity of such complexes, rather than the quantity of the individual components that dictate kinase activity to promote cell cycle progression. The interaction with D-type cyclins promotes CDK4 activity, which is suppressed when p27Kip1 is bound to the complex (Sherr and Roberts 1995). However, the role of p21Cip1 in regulating CDK4 is intricate, as p21Cip1 facilitates the assembly of D-type cyclins with CDK4 and CDK6 (Cheng et al. 1999), yet also functions to inhibit CDK4 activity at higher stoichiometric ratios (Sherr and Roberts 1995). Immunoprecipitation studies revealed increased levels of cyclin D1 bound to CDK4 in TCDD-pretreated mice (Fig. 4B). This effect persisted over the first 48 hr after TCPOBOP administration, during which time p21Cip1 binding to CDK4 diminished in mice pretreated with TCDD. The interaction of p27Kip1 with CDK4 did not reveal a discernable pattern among treatment groups until 72 h post-TCPOBOP treatment, when TCDD appeared to increase binding. These data are consistent with enhanced CDK4 activity in TCDD-pretreated mice exposed to TCPOBOP.

3.5. TCDD augments TCPOBOP-stimulated CDK4 kinase activity

We next directly measured CDK4 activity in order to ascertain whether TCDD could modulate the kinase activity. As shown in Figure 5A, using a recombinant pRb fragment as a CDK4 substrate, kinase activity in vehicle-pretreated mice was clearly detectable by 48 hr after TCPOBOP. In contrast, robust kinase activity was detected much earlier in TCDD-pretreated mice, first evident 24 hr after TCPOBOP administration. The functional significance of elevated CDK4 kinase activity was further addressed by measuring hepatic levels of endogenous phosphorylated pRb using an antibody specific for phosphorylation at the serine 780 residue, which is mediated exclusively by CDK4 (Kitagawa et al. 1996). As shown in Figure 5B, phospho-Ser 780 is first detectable at 24 hr in TCDD-pretreated mice, reaching maximal levels by 48 hr after TCPOBOP administration. Collectively, these findings demonstrate elevated CDK4 activity in mice pretreated with TCDD consistent with the enhanced liver hyperplasia seen in these animals. In light of a recent report that the AhR forms a complex with CDK4 to promote cell cycle progression in human breast cancer cell lines (Barhoover et al. 2010), we also investigated the possibility that enhanced CDK4 activity correlated with increased association between the AhR and CDK4. While results demonstrate that an interaction exists between the AhR and CDK4 in the liver, especially at 12 and 36 hr after TCPOBOP treatment, TCDD had no overt effect on this interaction (Fig. 5C) reminiscent of those reported by Barhoover and colleagues (2010). Moreover, fluctuations in AhR/CDK4 interactions fail to explain the marked increase in CDK4 activity detected at 24 and 36 hr in mice pretreated with TCDD (Fig. 5A).

4. Discussion

The present study demonstrates that AhR-mediated modulation of hepatocyte proliferation is not limited to the regenerative response induced by PH, but also occurs in the intact liver following exposure to the potent hepatomitogen, TCPOBOP. In contrast to the suppressive effects of AhR activation in the regenerating liver, however, AhR activity promoted hepatocyte proliferation in mice treated with TCPOBOP, as evidenced by an increased liver to body weight ratio, increased BrdU labeling of hepatocytes, elevated levels of the S-phase protein, cyclin A, and greater CDK4 activity. Although hepatomegaly has been associated with TCDD exposure previously (Morris et al., 1992), our results illustrate that TCDD alone

neither increased the liver-to-body weight ratio nor induced hepatocyte proliferation (Fig. 1). Therefore, the TCDD-induced growth effects observed are contingent upon the presence of a distinct proliferative signal, such as following PH (Mitchell et al., 2006) or the action of the hepatomitogen TCPOBOP. It is noteworthy that, as a phenobarbital (PB)-like compound, TCPOBOP is an agonist for the constitutive androstane receptor (CAR), and the TCPOBOP-mediated mitogenic effects are absolutely dependent on CAR (Wei et al., 2000).

Characterization of AhR expression and activity revealed that the AhR retains normal responsiveness to TCDD in the presence of TCPOBOP. This assertion is based on the TCDD-induced expression of *Cyp1a1* as well as the TCDD-induced turnover of AhR protein, which has been previously attributed to proteasome-mediated degradation (Ma and Baldwin 2000; Pollenz 2002). While it has been shown that PB treatment increases AhR expression in rat liver cytosol (Okey and Vella 1984), TCPOBOP treatment failed to elicit a similar increase in AhR levels in the mouse liver. However, the combination of TCDD and TCPOBOP did increase *Cyp1a1* mRNA levels above those found in TCDD-treated mice that were not given TCPOBOP, albeit inconsistently (Fig. 2B), consistent with studies reporting an additive effect between CAR and AhR ligands on cytochrome P450 activity (Gielen and Nebert 1971; Parkinson et al. 1980). However, what was particularly intriguing was the transient 70-fold induction of *Cyp1a1* gene expression following TCPOBOP administration in the absence of TCDD. This robust *Cyp1a1* induction by TCPOBOP is AhR dependent, since expression is markedly attenuated in the AhR CKO mouse liver (Fig. 2C). The residual mRNA expression in response to TCPOBOP or TCDD is attributed to AhR activity in the non-parenchymal cells that eluded excision of the region encoding exon 2 in the AhR gene flanked by the loxP elements, as has been previously demonstrated (Walisser et al., 2005). While we cannot formally exclude the possibility that TCPOBOP is acting as an AhR agonist to induce *Cyp1a1* expression, we attribute the transient increase in *Cyp1a1* expression to AhR activity in response to endogenous cues during hepatocyte proliferation (Mitchell and Elferink 2009). In support of this, we previously showed that both *Cyp1a1* mRNA and protein expression increase during liver regeneration following PH in the absence of exogenous agonists (Mitchell et al. 2006). Accordingly, the lack of *Cyp1a1* induction follow 4 days of TCPOBOP treatment (Pacyniak et al, 2007; Petrick and Klaasen, 2007), when the proliferative response is over, supports the contention that increased *Cyp1a1* expression is not a direct effect of TCPOBOP on AhR function. The link between AhR function and orderly cell cycle progression is supported by several studies demonstrating that a functional AhR is essential for optimal proliferation in hepatocytes (Elizondo et al. 2000; Ma and Whitlock 1996). Hence, it is conceivable that the observed transient increase in AhR activity during hepatocyte proliferation, stimulated either by PH or TCPOBOP administration, exposes important endogenous signaling events occurring during hepatocyte proliferation. However, the mechanistic basis for such endogenous AhR activation in hepatocytes remains poorly characterized.

Our analysis of the G1 phase CDKs served to identify functional differences in key molecular targets that conform to the altered proliferative response. The initial focus on CDK2 activity is borne from our previous finding that TCDD inhibits this kinase during liver regeneration (Mitchell et al. 2006). The observation that pretreatment with TCDD suppresses CDK2 activity during peak TCPOBOP-stimulated liver hyperplasia by 36 hr is consistent with our previous finding that TCDD disrupts CDK2 activity. However, the protein-protein interactions that confer TCDD-mediated suppression appear to differ between PH-induced liver regeneration and TCPOBOP-stimulated hyperplasia. Specifically, we previously demonstrated that, following PH, TCDD-treated mice display decreased formation of active CDK2/cyclin E and CDK2/cyclin A complexes rather than the more conventional sequestration of the inhibitors p21Cip1 and p27Kip1 into the CDK/cyclin complex observed in this study (Fig. 3). These latter observations are consistent with the

reported role of p27Kip1 in driving AhR-mediated cell cycle arrest in hepatocyte cell lines (Kolluri et al. 1999). Significantly, the TCDD mediated inhibition of CDK2 activity is at odds with the TCDD enhanced growth response induced by TCPOBOP (Fig. 1). By contrast, assessment of the CDK4/cyclin D1 complex and measurement of its kinase activity are entirely congruent with the proliferative response, and suggest that CDK4 compensated for diminished CDK2 activity to inactivate pRb and promote cell cycle progression (Fig. 5). The importance of cyclin D1 in hepatocyte proliferation is further illustrated by Columbano and coworkers (Ledda-Columbano et al. 2002) who demonstrated that lack of cyclin D1 delays hepatocyte entry into S-phase. Barhoover et al. (2010) recently demonstrated that the AhR interacts with CDK4 in a TCDD-dependent manner in the MDA MB-231 and MCF-7 breast cancer cell lines. Specifically, these researchers showed that growth arrest coincided with a TCDD-induced dissociation of AhR/CDK4 complex and decreased kinase activity. At this time it is unclear how these findings reconcile with those reported here, given that TCDD promotes CDK4 activity in the whole liver following mitogen stimulation. Moreover, the significance of the AhR/CDK4 interaction is uncertain: whilst we could also demonstrate an interaction, it was neither TCDD responsive, nor uniform during the proliferative process (Fig. 5C), thus casting some doubt on the functional universality of this interaction.

In conclusion, the findings provide evidence that AhR activity potentiates mitogen-induced hepatocyte proliferation through a mechanism that involves deregulation of G1 kinase activity. However, the notion that the AhR, in response to TCDD, can suppress hepatocyte proliferation after PH, yet augment the proliferative response following exposure to a potent mitogen such as TCPOBOP, serves to illustrate the complex and thus far poorly understood role of the AhR in cell proliferation. Clearly, future studies that dissect these molecular events will enlighten us to the physiological role of the AhR in tissue homeostasis and simultaneously help to uncover the basis for TCDD-induced tumor promotion and toxicity in general (Mandal 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AhR	Aryl hydrocarbon receptor
BrdU	bromodeoxyuridine
CKO	conditional knockout
CDK	cyclin-dependent kinase
PH	partial hepatectomy
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)] benzene

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Figure 1. TCDD treatment increases TCPOBOP-induced hepatocyte proliferation

C57Bl/6 mice were pretreated with 20 μ g/kg TCDD or vehicle 24 hr prior to administration of 3 mg/kg TCPOBOP. Following a 2-hr pulse with BrdU, mice were euthanized at the indicated times relative to TCPOBOP administration (n=3). (A) Data represent mean ($\pm$ SEM) liver-to-body weight ratios. * $p \leq 0.05$ when compared to vehicle-pretreated mice at same time point. ^ $p \leq 0.05$ when compared to vehicle-pretreated mice at 0 hr. (B) Representative photomicrographs (200 $\times$) depicting non-proliferative nuclei (dark blue) and BrdU positive nuclei (dark brown) from vehicle- and TCDD-pretreated mice prior to TCPOBOP administration. (C) Quantitation of BrdU-positive hepatocyte nuclei presented as a mean ($\pm$ SEM) percentage from 4 separate fields per sample (at least 2000 nuclei per

mouse; n=3 mice in each treatment group at each time tested). * $p \leq 0.05$ when compared to vehicle-pretreated mice at same time point. This experiment was repeated twice (n=3–5 mice), and similar BrdU staining and liver-body weight ratios were obtained each time. (D) Representative western blot analysis of cyclin A protein levels in unpooled liver homogenates (50 µg protein/lane) from vehicle- or TCDD-pretreated mice euthanized at the indicated times following TCPOBOP administration.

Figure 3. AhR activation alters TCPOBOP-stimulated CDK2 protein interactions and suppresses CDK2 kinase activity

Mice were treated as described in Figure 1. CDK2 was immunoprecipitated from liver homogenates as described in the Materials and Methods. (A) Representative western blot analysis of immunoprecipitated CDK2, as well as CDK2-bound proteins cyclin E, cyclin A, p21Cip1 and p27Kip1. (B) Kinase activity of immunoprecipitated CDK2 was tested in vitro using histone H1 as a substrate, as described in the Materials and Methods. No activity was detected in liver homogenates that were immunoprecipitated with IgG control (data not shown). Data are representative of three experiments.

Figure 5. TCDD augments TCPOBOP-stimulated CDK4 kinase activity

(A) Kinase activity of immunoprecipitated CDK4 was tested in vitro using an pRb fragment (amino acids 769–921) as a substrate, as described in the Materials and Methods. No activity was detected in liver homogenates that were immunoprecipitated with IgG control (data not shown). (B) Representative western blot analysis of phospho-Ser780 pRb in unpooled liver homogenates (100 µg protein/lane). Equivalent results were obtained when this assay was repeated with liver tissue from additional mice within each treatment group (n=3). NS, represents a non-specific immunoreactive protein. (C) Liver homogenates were immunoprecipitated with anti-CDK4 as described in the Materials and Methods. Data depict western blot analysis of immunoprecipitated CDK4 protein and coimmunoprecipitated AhR protein.