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CHARACTERIZATION OF SERUM-INDUCED CYP1A1 EXPRESSION AND ACTIVITY IN MOUSE EMBRYO FIBROBLASTS

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CHARACTERIZATION OF SERUM-INDUCED CYP1A1 EXPRESSION AND ACTIVITY IN MOUSE EMBRYO FIBROBLASTS

By

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To My Family.

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The AhR is a ligand-activated transcription factor that mediates the toxic effects of environmental contaminants that include TCDD. Using a TCDD dose-response treatment in MEFs, we observed a super induction of CYP1A1 with newborn calf serum (NCS) in the presence (10nM/15nM) of TCDD. In addition to NCS, fetal bovine serum (FBS) also has the capability to yield a CYP1A1 super induction. These results suggest that components within the sera affect the activity of the AhR and consequent CYP1A1 expression. To pursue this idea, characterization of the serum factors were investigated. The findings indicated that serum factor(s) in both sera are heat sensitive at 50°C, withstand removal from charcoal stripping sera and are ≥ 10,000 kDa in size. Using RT-PCR, we found that NCS factors only, could super induce CYP1A1 at the gene level. Moreover, MEFs are the only cells observed in this study that are susceptible to CYP1A1 super induction.

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LIST OF ABBREVIATIONS

MEFs-Mouse embryo fibroblasts wild type
FBS-Fetal Bovine Serum
NCS- Newborn calf serum
CYP1A1 – Cytochrome P4501A1
ARNT- Aryl hydrocarbon receptor nuclear translocator
AhR- Aryl hydrocarbon receptor
pRb- Retinoblastoma protein
TCDD-2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin or dioxin
PAS -Per Arnt Sim
PCBs-polychlorinated biphenyls
PAHs-polyaromatic hydrocarbons

CHAPTER 1: INTRODUCTION

AhR acts as a ligand-activated transcription factor

The AhR is a member of the Per ARNT Sim (PAS) family of transcriptional regulators, which contain a basic helix loop-helix domain [1]. AhR is unique in that it is the only member conditionally activated by ligand's that include 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) [2]. In the absence of ligand, AhR is a cytosolic protein associated in a complex consisting of heat-shock protein 90 [3], the co-chaperone protein p23 [4] and the immunophilin-like AIP/ARA9/XAP2 protein [5-7] (Figure 1.1). As shown in Figure 1.1, upon ligand binding by TCDD, the AhR dissociates from the protein complex and translocates into the nucleus where it heterodimerizes with a second bHLH/PAS protein, known as the AhR nuclear translocator (ARNT) [1, 8, 9]. The dimer, then binds to DNA recognition sites, [10-11] referred to as xenobiotic response elements (XREs), located upstream of specific target genes such as CYP1A1 and alters its expression [2].

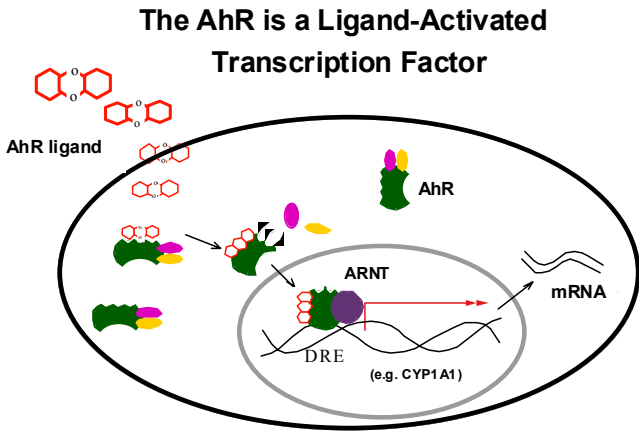


Figure 1.1 The AhR is a Ligand-Activated Transcription Factor. (Adapted from Whitlock et al. (1999)

TCDD is the most potent AhR ligand

Many coplanar aromatic compounds have been found to be AhR ligands. Some of these aromatic molecules include flavonoids, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and dioxins. TCDD (Figure 1.2) binds to the AhR with the highest affinity and is considered to be the prototypical ligand for the receptor. Moreover, TCDD is a persistent xenobiotic [12] and is produced as a byproduct of municipal waste incineration [13]. Unlike other AhR ligands, TCDD serves as a very poor substrate for cellular detoxification systems such as CYP1A1. TCDD acts by altering the transcription of selected XRE-containing genes [14] and almost all of its toxic effects are mediated through the AhR [15-17]. Studies have shown that TCDD is a chemical capable of inducing toxic effects, which lead to a variety of diseases.

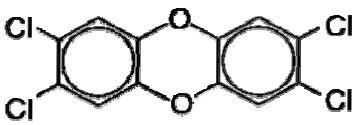


Figure 1.2 Chemical structure of TCDD (Adapted from www.stanford.edu/group/whitlock/dioxin.html (2005)

TCDD elicits toxic effects

TCDD persistence in cells allow it to be stored as fat and bioaccumulate up the food chain, potentially causing toxicity in both humans and animals [18]. Studies demonstrate that in the laboratory, TCDD elicits a variety of responses in experimental animals such as liver toxicity, immune suppression, hyperplasia, reproductive toxicity [13], in addition to changes in cell proliferation and differentiation [19]. With regard to the immune system, TCDD affects homeostasis of animals by inhibiting proliferation [20]. Teratogenetic effects elicited by TCDD are characterized by the reproduction demise due to the malformation of the genetalia [21]. TCDD exposure can also cause neurotoxicity preventing the brain infrastructure from to developing properly [21]. Tumor

promotion is linked to TCDD action as well [21]. The exact mechanism of carcinogenesis is not well understood, but researchers have noted that the presence of TCDD leads to increased cell proliferation. In humans, TCDD causes chloracne, a persistent cystic skin eruption[22]. Nonetheless, these observations suggest that TCDD has the capability to elicit a variety of toxic effects in both animals and humans.

Regulators of cell cycle progression (Checkpoint proteins and oncogenes)

G_1 to S phase of the cell cycle is regulated by checkpoint proteins that include tumor suppressors like pRb. pRb activity depends on its phosphorylated state. It is the main checkpoint protein from G_1 to S phase of the cell cycle. pRb is also apart of a family of pocket proteins that include p107 and p130, which all are regulators of the cell cycle [23]. As shown in figure 1.3, during G_1 phase of the cell cycle, pRb is in its hypophosphorylated (active) state [24]. In this state, pRb binds to E2F, which is a transcription factor involved in mediating transcription of S phase genes, then pRb represses it and inhibits cell cycle progression to S phase [24]. To allow cell cycle progression, pRb is phosphorylated to its hyperphosphorylated form (inactive) by cyclin dependent kinases (CDKs) and E2F is released to participate in transcription of S phase genes[24]. In this model, CDKs are positively regulated by cyclins and negatively regulated by CDK inhibitors such as p27kip1[24]. This paradigm is one of the biological basis of how G_1 to S phase of the cell cycle is regulated under normal conditions.

pRb is not only regulated by phosphorylation, but also through adenoviruses with oncogenic properties [25-26]. The human adenovirus early region 1A (E1A) protein binds to pRb resulting in the release of E2F[25-26]. It modulates transcription by interacting with cellular transcription factors such as E2F thereby facilitating unscheduled DNA synthesis [25,27-29]. Adenovirus E1A also interacts with p300, a protein that is structurally and functionally related to the cAMP binding protein (CBP) [30-31]. p300 is a non-DNA binding transcriptional coactivator that interacts with the adenovirus E1A to progress cells through G_1 phase to S phase of the cell cycle[32, 33]. When not interacting

with adenovirus E1A, p300 suppresses the cell cycle [34, 35]. Checkpoint proteins like pRb and transcriptional coactivators such as p300 are used to regulate the cell cycle during planned and unplanned DNA synthesis.

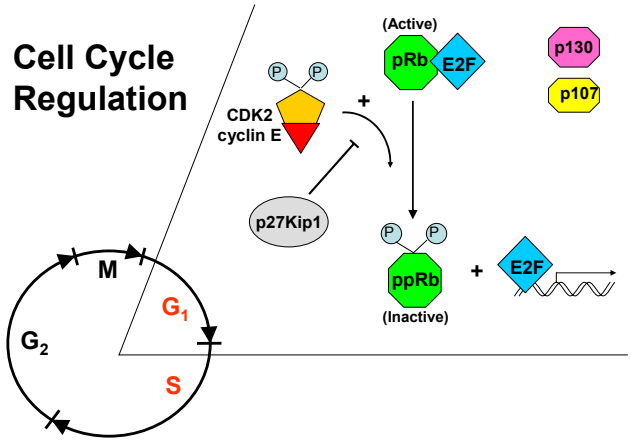


Figure 1.3 Model for pRb regulation of G_1 to S phase of the cell cycle.

AhR regulates cell cycle progression

Until recently, the AhR was known primarily for its role in mediating the toxic effects of dioxins and other related compounds, but recent observations have shown its involvement in cell cycle regulation. Several findings support the theory that the AhR is participates in cell cycle control. For example, AhR-defective mouse hepatoma cells exhibit prolonged doubling times compared to wild-type cells [36]. This prolonged response was attributed to delayed progression through G_1-phase [36]. Moreover, MEFs from AhR-null mice grow more slowly than MEFs from wild type mice [37]. To determine the mechanism by which AhR promotes cell cycle progression the association between p300 and the adenovirus E1A protein was examined. With regard to the AhR-null MEF cells, further investigations indicated that AhR contributes to p300-mediated induction of DNA synthesis by the adenovirus E1A protein [38]. Together, these

observations propose that, in the absence of an exogenous ligand such as TCDD, a functional AhR is critical for cell cycle progression.

Similar to AhR defective cells, TCDD can cause a G_1 cell cycle arrest in variety of cells types [39-41]. These data, suggest that AhR normal function is altered or masked in the presence of TCDD. Therefore, activating the AhR with a persistent agonist (TCDD) prevents the AhR from participating in its normal function of cell cycle regulation. These findings prompted several investigations that emphasized on determining the mechanism by which the AhR mediates a TCDD-induced G_1 arrest. With our understanding the importance of pRb , a tumor suppressor that serves as the main checkpoint from G_1 to S phase [23] and given that the AhR mediates a TCDD- facilitated G_1 arrest, research has focused on the interaction between pRb and AhR.

Recent efforts determined that a direct interaction exist between the AhR and pRb [13]. To explore the functional significance of the AhR-pRb interaction, experiments were facilitated and found that maximal TCDD-induced G_1 arrest (Figure 1.4) and CYP1A1 induction in rat 5L hepatoma cells were dependent on pRb binding through the LXCXE motif in AhR [42]. It is this sequence found in the AhR that confers pRb binding [42, 43]. Accordingly, the results concluded that pRb acts as a coactivator for AhR function [44]. Moreover, the model for TCDD-induced G_1 arrest in 5L cells also proposes that the AhR–pRb interaction likely facilitates expression of p27Kip1, a negative regulator of G_1 to S phase [42]. To further investigate the mechanism by which AhR mediates G_1 arrest, Huang and Elferink (2005) used small interfering RNA to downregulate ARNT to show that TCDD-induced G_1 arrest is dependent on the ARNT protein [44]. This study also found that coactivation only accounts for partial contribution to AhR mediated G_1 arrest suggesting that corepression exists as well (Figure 1.5).

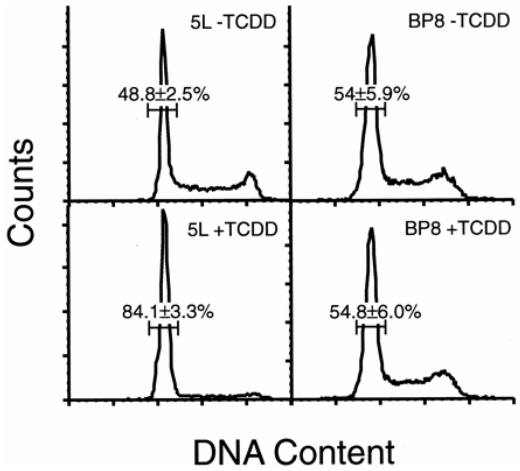


Figure 1.4 TCDD induced G1 arrest in rat 5L hepatoma cells. Asynchronous 5L (+AhR) and BP8 (-AhR) cell cultures were grown in the presence or absence of 10 nM TCDD for 24 h, fixed in ethanol and stained with propidium iodide. DNA content was determined using a FACSCalibur cytometer equipped with CellQuest and ModFit software. The steep slope represents G_1 phase of the cell cycle. (Adapted from Elferink et al. (2001)

Other studies have proposed different mechanisms by which pRb and AhR interact in order to cause cell cycle arrest at G_1 phase. With the understanding that pRb regulates E2F, Puga et al. (2000) has shown that AhR acts as a corepressor with pRb to inhibit E2F-dependent transcription and cell cycle arrest (Figure 1.5). However, there is an even more detailed role for AhR functioning as a corepressor with pRb and thus regulating cell cycle control through p300. Work by Marlowe et al. (2004) demonstrated that the AhR displaces p300 from E2F-dependent promoters and represses S phase specific gene expression. These results along with the above discussion indicate that there is either corepression and/ or coactivation of both pRb and AhR as a result of their association between each other [42-45] (Figure 1.5).

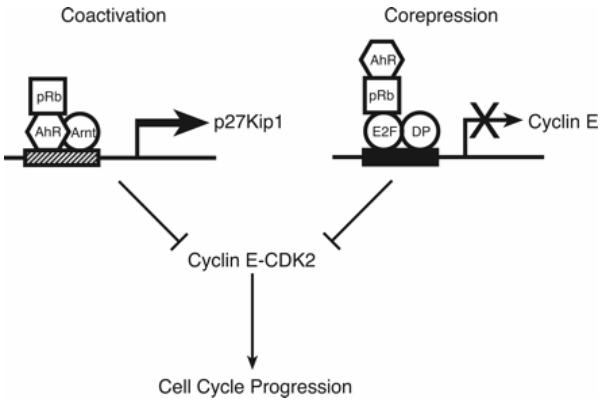


Figure 1.5 Two models for AhR-mediated G_1 cell cycle arrest. **(Adapted from Huang et al. (2005)**

AhR is involved in apoptosis

Cells undergo many processes that consist of proliferation, differentiation, and apoptosis. AhR has been shown to regulate some of these cell functions, specifically, cell cycle control and the regulation of genes that promote or prevent apoptosis.

AhR regulates many factors, particularly certain cytokines, which include transforming growth factor beta (TGFβ). TGFβ is associated with the induction large amounts of fibrosis [46-48], inhibition of cell proliferation in the liver of rodents [49] and accelerated amounts of apoptosis [46]. Previous studies, shown that the AhR-/- mice exhibit an increased amount of fibrosis in the liver as well as upregulation of TGFβ and found that the AhR +/+ mice are able to suppress TGFβ and prevent apoptosis [50]. This data suggests that in the absence of TCDD, AhR is involved in cell cycle control of signal transduction pathways which include TGFβ [50].

More recent studies propose that normal, disease free hepatocytes do not undergo apoptosis as readily as abnormal and diseased hepatocytes [51]. In fact, apoptosis in normal, disease free hepatocytes is very uncommon [51]. In addition, diseased hepatocytes are susceptible to Fas-mediated apoptosis [52]. These observations lead to studies that showed a different role of the AhR involvement in apoptosis. The findings indicated that in the absence of an exogenous ligand, AhR promotes apoptosis in

hepatocytes through Fas-stimulation [52]. Furthermore, this study supports the role for AhR involvement in regulating expression of genes engaged in apoptotic signaling.

AhR gene expression is controlled by signal transduction protein pathways that include Mybbp1 and p21

Signal transduction protein pathways have been found to control AhR gene expression. Transduction pathways that contain Myb-binding protein 1a (Mybbp1), a nuclear protein that associates with transcription factors, has been found to increase AhR-dependent gene expression by associating with the acidic activation domain (AAD) of the AhR[53]. In contrast, cell check point proteins like p21 (Ha-ras oncogene) is capable of downregulating both AhR function as well as CYP1A1 induction [54]. These two findings reveal new aspects of AhR gene regulatory control.

AhR has a number of potential physiological and biological roles

Speculations contend that AhR could serve as a tumor suppressor or oncoprotein [55]. Various studies have documented that AhR controls cell cycle regulation by mediating the effects of TCDD. Conversely, in the absence of this ligand, AhR has contrasting roles in the cell. For instance, AhR promotes and inhibits cell proliferation in various cell lines, and in vivo animal models. Some of AhR roles are parallel to those of tumor suppressor proteins like pRb. More importantly, pRb is not only coactivator but also a corepressor partner of AhR in a cell cycle arrest paradigm [42, 43]. Therefore, pRb has been one research focus for identifying the physiological role of the receptor. However, there other experimental investigations that can contribute to finding AhR functions in the cell.

AhR has been found to promote apoptosis upon Fas-stimulation and prevent apoptosis by downregulating TGFβ. This association between the AhR and genes involved in cell death suggests a physiological role for the receptor in regulating both pro-apoptotic and anti-apoptotic signals. Ultimately, identifying the physiological role of

the AhR will help broaden the understanding of AhR biology.

CYP1A1 is a drug metabolizing enzyme

The CYP1A1 gene encodes for cytochrome P4501A1, a phase I microsomal enzyme whose functions include the detoxification and oxygenation of certain lipophilic aromatic hydrocarbons present in combustion products [56] during their metabolic processing to water-soluble derivatives. Furthermore, under some conditions, CYP1A1 can produce chemically reactive, toxic and mutagenic metabolites resulting in xenobiotic-induced cancers [12]. This gene is found in most tissues of the body including endothelial cells of blood vessels, epithelial cells of the skin and gastrointestinal tract, fetus and embryo [56], but is highly expressed in the liver [57]. However, the expression of CYP1A1 is normally low or absent in the quiescent (G_0) cells, but is mostly induced following treatment with TCDD.

TCDD is a poor substrate for detoxification systems like CYP1A1

TCDD is a xenobiotic with the greatest ability to induce transcription of CYP1A1 in cells. However, it happens to be a poor substrate for cellular detoxification systems such as those found in CYP1A1 [18]. This could be due to the fact that TCDD has a persistent characteristic which does not allow to be metabolically broken down but able to accumulate in cells [18]. Moreover, its half-life has been found to be about 14 years in humans [58] and in water or soil, it has been found to be 1 to 4years [59-60], which could account these observations. In any case, all of TCDD toxic effects are mediated through the AhR.

CYP1A1 controls AhR Activity

CYP1A1 function extends beyond its role as a drug metabolizing enzyme, it has also been found to regulate AhR activity in hepatoma cell lines. It is proposed that a

critical CYP1A1 function is the modulation of AhR activity by regulating the level(s) of physiological receptor agonists involved in cell cycle regulation (Figure 1.6) [61].Within cell culture; AhR can promote cell cycle progression and mediate the effects of TCDD-induced G_1 arrest through CYP1A1 negative regulation [61]. CYP1A1 negatively regulates the AhR by metabolically removing endogenous ligands, thereby allowing AhR to facilitate cell cycle progression [61]. This hypothesis is supported by evidence that showed AhR activation was sustained by a potent CYP1A1 antagonist 1-(1-propynyl) pyrene (1-PP) [61]. Moreover, this study showed that in the absence of dioxin, the serum factor(s) in FBS can stimulate CYP1A1 in hepatoma cell lines particularly, 5L cells (Figure 1.7a) [63]. Although the contributing factor is yet to be identified, it appears have several characteristics; it is heat labile (Figure 1.7c), 10,000 kilo daltons in size and able to persist even when serum was charcoal stripped and dialyzed (Figure 1.7b) [61].

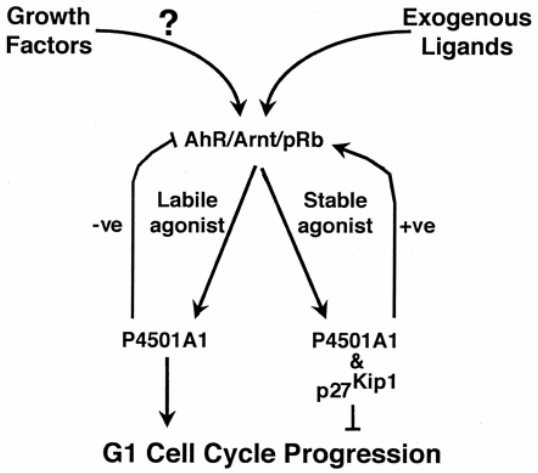


Figure 1.6 Model depicting the proposed mechanism whereby the AhR regulates transition through G_1 phase of the cell cycle. **(Adapted from Levine-Fridman et al (2004)**

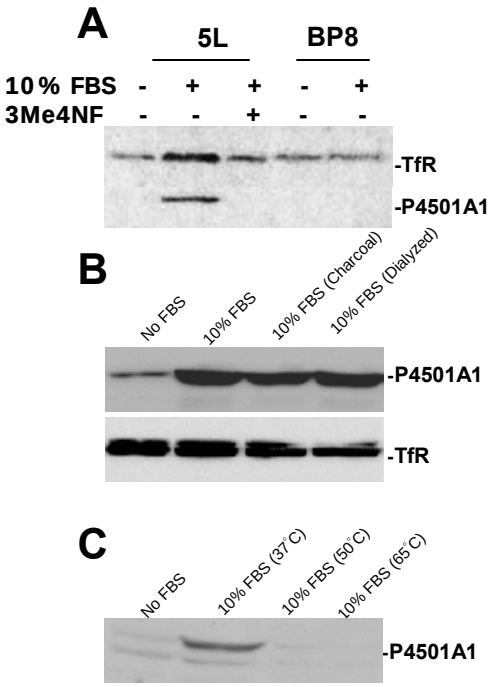


Figure 1.7 AhR-mediated expression of P4501A1 is induced in 5L hepatoma cells. **A.** Subconfluent asynchronous 5L (+AhR) and BP8 (-AhR) cell cultures were serum-arrested with 0.1% FBS/24 h. Fresh media containing 0.1% FBS (–FBS) or 10% FBS (+FBS) was added for 4 h in the absence of the AhR antagonist 3Me4NF (–) or presence of 1 μM 3Me4NF (+). Total cell lysates were prepared and analyzed by Western blotting for P4501A1 and TfR (loading control). **B/C.** Serum factors induce CYP1A1 protein expression in 5L cells. **B.** Subconfluent asynchronous 5L cell cultures were serum-arrested in DMEM without FBS for 24 h. Fresh media containing 10% normal FBS, 10% charcoal-stripped FBS, or 10% dialyzed FBS was added for 8 h. Total cell lysates were prepared and analyzed by Western blotting for P4501A1 and TfR. **C.** Serum-arrested 5L cell cultures were released for 8 h with media containing 10% normal FBS treated at the indicated temperature for 1 h, and total lysates analyzed by Western blotting for CYP1A1 protein. (Adapted from Levine-Fridman et al 2004)

Conclusions

AhR has numerous roles in cell cycle regulation, apoptosis, and mediating toxic effect of environmental contaminant. However, the physiological AhR ligand has not been identified even though naturally occurring AhR ligands are known. Some of these naturally occurring agonists that activate the AhR, include lipoxin A4 [62], bilirubin [63], indole and tryptophan metabolites such as indole-3-carbinol found in vegetables such as broccoli and Brussels sprouts [64]. Despite being able to activate the AhR, these agonists have fairly small potency, restricted distribution and are expressed in low levels of cells; which prohibits them as likely physiological AhR ligands [2]. Thus far, investigations of possible physiological AhR ligands have not been found but previous studies shown that serum can induce expression of CYP1A1. These studies suggest a serum factor(s) in sera elicit the production of an endogenous AhR ligand. In view of these findings, our goal in this study was to characterize the serum factors in both the NCS and FBS that elicit CYP1A1 super induction in MEFs, which could facilitate the identification of an endogenous AhR ligand.

CHAPTER 2: METHODS

Materials:

Mouse embryo fibroblasts (MEFs) on a C57BL/6 background were given to us as a gift from Dr. Nicolas Dyson, Harvard Medical School (Charlestown, MA.). New born calf serum (NCS) and Super Script Reverse Transcriptase (SSIIRT) were obtained from Invitrogen (Carlsbad, CA.). Fetal bovine serum (FBS) was acquired from Hyclone (Logan, UT). TCDD was purchased from AccuStandard Incorporated (Newhaven, CT.). Taq Polymerase was obtained from Fisher Scientific (Pittsburgh, PA). The RNAqueous Midi Kit was obtained from Ambion (Austin, TX). The forward and reverse primers for mouse CYP1A1 **(Forward/5` CCACATCCGGGACATCACAGACAG 3`/ Reverse/ 5`GCAGCAAGATGGCCAGGAAGAGAA 3`)** and mouse GAPDH **(Forward/5`GACTGTGGATGGCCCCTCTGG 3`/Reverse/ 5` CACCCTGTTGCTGTAGCCGTATTC 3`)** were purchased from Sigma-Genosys (Woodlands, TX). The CYP1A1 antibodies were obtained from DAIICHI Pure Chemical Company (Tokyo, Japan). Lastly, the dextran-coated activated-charcoal was obtained by Sigma-Aldrich (Milwaukee, WI.).

Cell culture:

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% NCS or FBS, 100 U/ml penicillin and 100µg/ml streptomycin in 5% CO_2 atmosphere at 37°C.

The experimental paradigm for studying G_1 arrest: Asynchronous cells in culture were washed with phosphate buffer saline (PBS) twice before being serum-starved with DMEM + 0% serum for 72 hours to synchronize all cells in G_0 phase [61]. Fresh media containing DMEM + 0% serum (baseline control) or DMEM + 10% serum (NCS or FBS) in the presence of TCDD diluted in dimethyl sulfoxide (DMSO) or DMSO alone (vehicle control) is added to cells in culture for 24 hours. The DMEM + 10% serum triggers a synchronized reentry into the G_1 phase of the cell cycle. This design is used in all experimental approaches in this study.

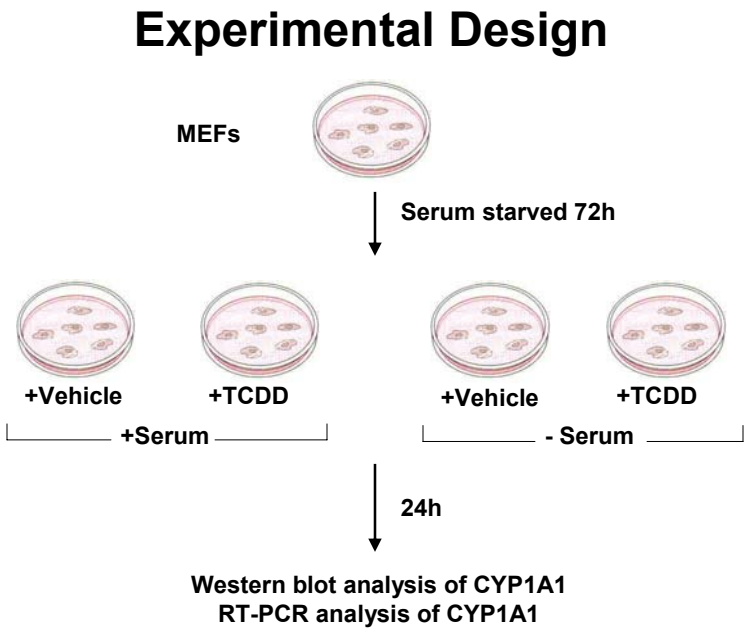


Figure 2.1 Experimental Design.

Confluency of Cells:

Subconfluent cell culture levels were used in all experiments except in the heat denaturing experiment with NCS treated samples. This experiment had confluent cell culture levels (Figure 2.2).

Sub-Confluent **Confluent**

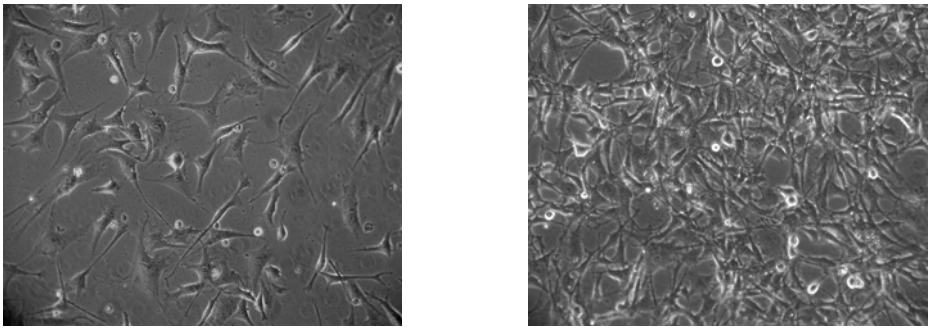


Figure 2.2 Pictures of Asynchronous Sub-Confluent and Confluent MEFs.

TCDD Dose Response Experiment:

Using the model previously described, a TCDD-dose response experiment was performed to test if the AhR is functional in MEFs, 5L hepatoma cells, FL183 B hepatoma cells, and RAT 2 hepatoma cells. These subconfluent asynchronous cells in culture were serum-starved with DMEM + 0% serum for 72 hours. Then, fresh media was added for 24 hours containing DMEM + 0% serum or DMEM + 10% serum (NCS or FBS) in the presence of 6nM, 10nM or 15nM of TCDD or DMSO. Cell lysates were prepared and analyzed by western blotting for CYP1A1 and loading control, actin.

Heat Denaturing Experiment:

The heat labile experiment was done to determine if the serum factor contributing to the super induction of CYP1A1 was heat sensitive. This experimental design requires serum (NCS or FBS) to be preheated at temperatures of 37°C, 50°C and 65°C for 1 hour prior to treatment with cells. The heated serum is diluted in DMEM + 100 U/ml penicillin and 100µg/ml streptomycin to a 10% solution. Confluent (NCS) or subconfluent (FBS) (Figure 2.2) asynchronous cell cultures were serum-arrested in DMEM without NCS or FBS for 72 hours. Serum-arrested cell cultures were starved again or released for 24 hours with media containing, 0% serum (NCS or FBS), 10% serum (NCS or FBS) or normal 10% serum (NCS or FBS) treated at 37°C, 50°C and 65°C for 1 h in the

presence or absence of 10nM TCDD. Total lysates were prepared and analyzed by Western blotting for CYP1A1 and loading control, actin.

Charcoal and Dialyzed Serum Experiment:

In this model CYP1A1 super inducement in charcoal-stripped serum and dialyzed serum will be assayed to eliminate the possibility that the serum factor is an AhR agonist contaminant and to determine the approximate size of the serum factor. Flow diagrams (Figures 2.3, 2.4) are depicted below describing the charcoal stripping and dialyzing serum methods.

Subconfluent asynchronous cell cultures were serum-arrested in DMEM without NCS or FBS for 72 hours. Fresh media containing 0% serum (NCS or FBS), 10% normal serum (NCS or FBS), 10% charcoal-stripped serum (NCS or FBS), or 10% dialyzed serum(NCS or FBS) in the presence (+) or absence (-) of 10nM TCDD was added for 24 hours. Total cell lysates were prepared and analyzed by Western blotting for CYP1A1 and actin or TfR.

Western Blot Analysis:

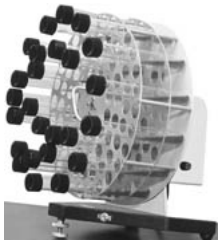
All experiments were analyzed by western blot using antibodies specific for CYP1A1, Actin or TfR (loading controls). For total cell lysates, subconfluent or confluent cultures (60-mm plates) were washed once in PBS, and the cells were harvested by scraping in 150-300 µl SDS-polyacrylamide gel electrophoresis loading buffer and boiled for 10 minutes. The nuclear extracts were arranged according to the method illustrated by Denison et al. (1988). Protein was fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane that was blocked for 1 h at RT in 4% (w/v) dry milk in Tris-buffered saline, pH 7.5, and 0.1% (v/v) Tween 20 (2). Membranes were incubated with primary

Flow Diagram on How to Charcoal Strip Serum

1. Add 1 gram of dextran- coated activated charcoal to 50 ml of serum in a tube.

Charcoal **Serum** **Tubes**

2. Put tube on a tube on a roller drum overnight at 4°C and let the mixture rotate gently.



Roller drum

3. Then, put tube in centrifuge for 15 minutes at 2000 rpm at 4°C.

4. Sterilize serum by filtering it through a .22µm membrane filter in a vacuum filter apparatus.

Membrane paper **Vacuum filter**

5. Then, the charcoal stripped serum is diluted in DMEM + 100 U/ml penicillin and 100 µg/ml streptomycin to a 10% solution.

Figure 2.3 Flow diagram of the protocol on how to charcoal strip serum. (Pictures provided from Image search from www.google.com (2005)

Flow Diagram on How to Dialyze Serum

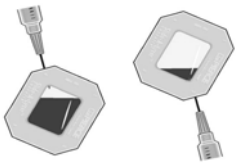
1. To add serum (NCS or FBS)
A. Fill the syringe with the serum.
B. Insert the tip of the needle through one of the syringe ports as shown in picture below.
C. Inject serum slowly.

Picture of correct technique to add serum.

2. To Dialyze Membrane
A. Remove Slide-A-Lyzer® Cassette from its pouch and attach into the groove of a buoy.
B. Immerse cassette in PBS buffer and let dialyze overnight at 4°C on a stirrer.

Picture of dialyzed membrane.

3. Removing Serum (FBS or NCS)
A. Turn the unit so that needle is on the bottom and allow the NCS or FBS to collect near the port.
D. Withdraw the serum into the syringe.
E. Then, the dialyzed serum is diluted in DMEM + 100 U/ml penicillin and 100µg/ml streptomycin to a 10% solution.



Picture of correct technique to remove serum.

Figure 2.4 Flow diagram of the protocol on how to dialyze serum. (Pictures adapted from Pierce (2004)

antibodies for 4 h at RT or overnight at 4°C and with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT, and visualized using an enhanced chemiluminescence detection method according to the manufacturer's protocol.

RNA Isolation:

To isolate RNA from MEFs required various steps that first included adding 1ml of lysis/binding solution to plated MEFs, then scrapping and collecting them in a tube. Then, vortex thoroughly too completely disrupt the pelleted MEFs. Once cells are disrupted, homogenize MEFs to reduce lysate viscosity and to shear DNA. Afterward, perform a clarifying centrifugation (15,000 rpm for 5 minutes at 4°C), to remove particulate debris from lysate samples. Transfer the clarified lysate to a 10 ml tube and add an equal volume of 64% ethanol and mix by gentle inversion three times. Next, pass lysate mixture through a glass fiber filter. After that, attach an 18 gauge needle to a large disposable syringe (5 ml) and aspirate the lysate up into the syringe through the needle. Then, remove the needle from the syringe and attach a glass fiber filter unit and slowly pass the lysate through the filter unit into a waste tube. Subsequently, unclog filter by attaching it to a large empty syringe, then force air through the filter once or twice to expel the white foam. Next, remove the filter, retract the plunger of the syringe, then reattach the filter and force air through the filter unit once or twice until all white foam is gone. After that, remove the filter unit, then aspirate 2ml of wash solution 1 containing guanidinium thiocyanate, up into the syringe and slowly pass the wash solution 1 through the filter into a waste tube. Afterwards, remove the filter and retract the plunger of the syringe and reattach the filter to force air through the filter. Then, remove the filter unit and aspirate 1.4ml of wash solution 2/3 containing 64% ethanol, up into the syringe to pass the wash solution 2/3 through the filter into a waste tube. Remove the filter unit, retract the plunger of the syringe, reattach the filter, and actively force air through the filter to remove residual wash solution 10 times until no drops of wash solution are seen. Next, add a 30ml aliquot of elution solution in a tube and place the tube into a glass

beaker with water and heat on a hot plate until temperature reaches 100°C. Then, remove the plunger from a 5 ml disposable syringe and attach the filter and place the assembly on top of an open collection tube and add 0.5 ml of hot elution solution to the syringe. Lastly, insert the plunger and force the elution solution through the filter into the elution tube and repeat this step 2 more times.

RT- PCR:

RT-PCR was performed to determine if the CYP1A1 super induction is seen at the transcriptional level. The starting procedure in RT-PCR is transforming the total RNA into cDNA libraries. The first step in RT is to add 1 µl Oligo(dT), 1 µg total RNA *(*5µl), 1 µl dNTP Mix (10 mM each), and 6 µl Sterile of distilled water to a nuclease-free microcentrifuge 1.5ml tube. Next, heat mixture to 65°C in water bath for 5 min and chill on ice for 30 seconds. Then, collect the contents of the tube by a 1 minute centrifugation. Afterwards, add 4 µl 5X First-Strand Buffer and 2 µl 0.1 M DTT to the tube, then mix contents of the tube gently by inversion. Subsuequently, incubate mixture at 42°C for 2 minutes. At this point, add 1 µl (200 units) of SuperScript™ II RT and mix by pipetting gently up and down. Then, inactivate the reaction by heating it to 70°C for 15 minutes. Lastly, add 230 µl of sterile of distilled water.

At this point in time, the PCR procedures are performed. The first step in PCR is to make a 50 µl solution mix by adding; 31 µl of sterile of distilled water; 5 µl of cDNA; 5 µl of 10X Buffer; 3 µl of 25mM $MgCl_2$; 1 µl of 10nM DNTP; 1 µl of mouse CYP1A1 forward primer (5` CCACATCCGGGACATCACAGACAG 3`); 1 µl of mouse CYP1A1 reverse primer (5`GCAGCAAGATGGCCAGGAAGAGAA 3`);1 µl of mouse GAPDH forward primer (5`GACTGTGGATGGCCCCTCTGG 3`);1 µl of mouse GAPDH reverse primer (5` CACCCTGTTGCTGTAGCCGTATTC 3`); and lastly adding 1 µl of Taq Polymerase.
Once PCR mixture is made, perform a 1 minute centrifugation to mix and allow solution to settle at bottom of tube. Then, set the experiment at 35 cycles (94°C for 15 seconds to denature, 65°C for 30 seconds for primer annealing, and 72°C at 2 minutes for extension).

Once PCR is completed, the PCR products were fractionated on a 1.2% agarose gel and visualized by ethidium bromide staining. Images were captured digitally using a gel documentation system (Alpha Innotech, San Leandro, CA) and the bands quantified using ChemiImager 5500 analysis tools.

Quantitative Analysis for Western Blot and RT-PCR:

The CYP1A1 densities were quantified by using ChemiImager 5500 (Alpha Innotech Corporation) analysis tools- spot density feature. Quantification of CYP1A1 products were statistically analyzed and graphed by Graph Pad Prism 4. A one way-ANOVA using Tukey or Bonferroni multiple comparison tests was used. $*p < 0.05$ is considered statically significant. n=3 in all experiments.

CHAPTER 3: THE CYP1A1 SUPER INDUCTION IN MEFS

INTRODUCTION:

Increasing evidence indicates that the AhR is involved in cell cycle regulation. Previous studies have shown that the TCDD-induced G_1 arrest in hepatoma cell lines requires the interaction between AhR and the cell cycle regulator pRb [42]. pRb is a member of the family of pocket proteins that has been shown to be involved in cell cycle control[23] . Moreover, pRb is able to bind to LXCXE motifs similarly to those found in the AhR [42]. Work by, Classon et al (2000) demonstrated that the pocket proteins which include pRb, p107 and p130 all have combinatorial roles in E2F-mediated cell cycle control[65]. Accordingly, we tested the hypothesis that in the absence of pRb, other pocket proteins (namely p107 and p130) mediate a TCDD-induced G_1 arrest. To this end, we obtained mouse embryo fibroblasts (MEFs) derived from pRb knock out mice (MEF RB-/-) and treated these cells with serum in the presence of TCDD.

Interestingly, exposure to TCDD failed to elicit a G_1 arrest in these cells (Figure 3.1 A), despite our findings that p107, p130 (Figure 3.2), AhR and ARNT all appear to be expressed in these cells. To address why p107 and p130 failed to mediate a G_1 arrest in MEF RB-/-, we compared these cells to the wildtype MEFs that contained the pRb gene. MEFs were treated with TCDD and 10% NCS and cell cycle analysis was performed by flow cytometry. Results indicated that MEF RB-/- as well as MEFs do not G_1 arrest following TCDD treatment (Figure 3.1 A,B). Moreover, when MEF RB-/- were transfected with a pRb construct, treatment with TCDD still failed to elicit a G_1 arrest.

This finding that MEF RB-/- and MEFs do not arrest in the presence of TCDD directly contradicts observations made in previous studies with hepatocyte cell lines exposed to TCDD. Furthermore, the lack of G_1 arrest is not due to impaired AhR because CYP1A1 is expressed in MEF RB-/- and MEFs, which indicates that the AhR is functionally active in these cells. For instance, one possible reason we did not see a

TCDD-mediated G_1 arrest in these cells is that concentration of TCDD is not high enough. Consequently, further investigation was conducted to determine, if indeed, the concentration of TCDD had an effect on these cells. Hence, a dose- response experiment was performed on MEF RB-/- and MEFs using doses of TCDD ranging from 6nM-15nM. We found that increasing the amount of TCDD was still not sufficient enough to induce a G_1 arrest in these cells. However, western blot analysis did reveal a super induction of CYP1A1 in MEFs treated with higher concentration of TCDD (10nM, 15nM). Based on previous studies that showed a serum-induced expression of CYP1A1, this phenomenon prompted us to explore the serum factors in both NCS and FBS that contribute to the CYP1A1 super induction in MEFs, which may possibly identify a physiological ligand for the AhR.

RESULTS:

To study the mechanism by which AhR mediates G_1 arrest in MEFs, we used an experimental paradigm implemented by Levine-Fridman et al (2004) in which cells are serum-starved (DMEM + 0% Serum) for three days in order to synchronize the cell in the G_0 (quiescent) phase of the cell cycle. Upon release with serum, the cells are allowed to participate in the cell cycle.

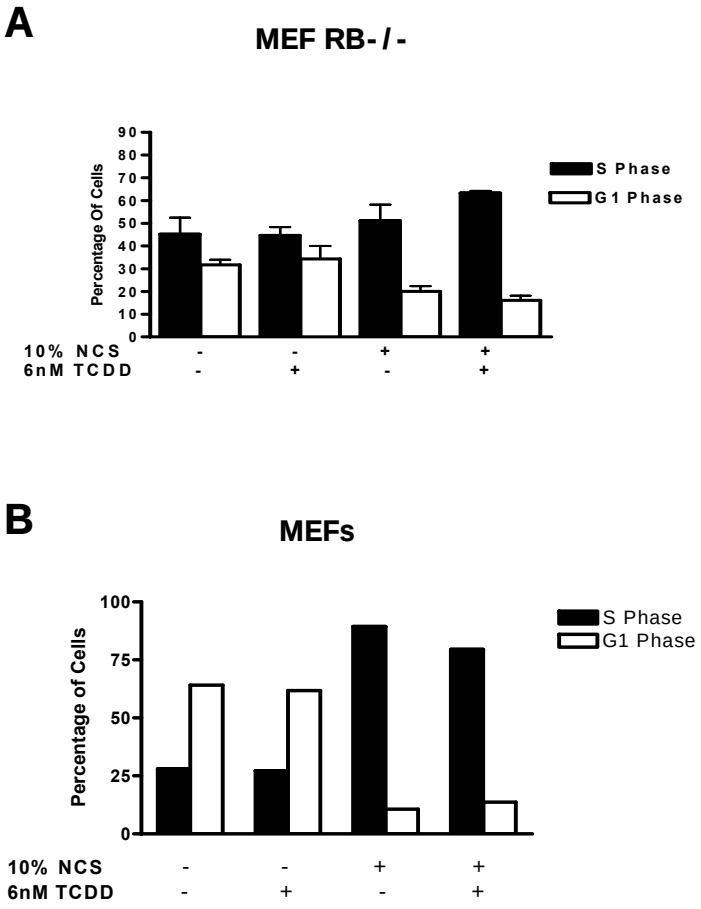


Figure 3.1 MEF RB-/- and MEFs do not G1 arrest in the presence of TCDD. A. Flow analysis of MEF RB-/- are representative of three independent experiments .
B. Flow analysis of MEFs represents one experiment. Subconfluent asynchronous MEF and MEF RB-/- cell cultures were serum-starved (DMEM + 0% NCS) for 72 hours. Fresh media containing DMEM + 0% NCS (-NCS) or DMEM + 10% NCS (+ NCS) in the presence 6nM of TCDD or DMSO (control) was added to MEFs for 24hours. Nuclei were stained with low and high salt buffer containing propidium iodide. The DNA content was determined using a FACSCalibur cytometer equipped with CellQuest and ModFit software. Quantification of DNA content were statistically analyzed and graphed by Graph Pad Prism 4. Two way-ANOVA using a Bonferroni multiple comparison test. *$p<0.05$ is significant.

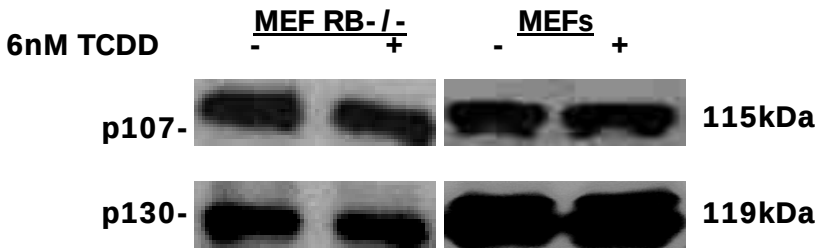


Figure 3.2. p107 and p130 are expressed in MEF RB-/- and MEFs. Subconfluent asynchronous MEF and MEF RB-/- cell cultures were serum-starved (DMEM + 0% NCS) for 72 hours. Fresh media containing DMEM + 0% NCS (-NCS) or DMEM + 10% NCS (+ NCS) in the presence 6nM of TCDD or DMSO (control) was added to MEFs for 24hours. Total cell lysates were prepared and analyzed by western blotting for p107 and p130.

A serum factor in NCS elicits a CYP1A1 super induction in MEFs

Recently, studies were performed to determine if the pocket proteins pl07 and p130 are acting in the absence of pRb by coactivating the AhR and serving as cell cycle regulators in MEF RB-/- cells. However, MEF RB-/- cells did not G_1 arrest in the presence of TCDD (Figure 3.1 A). To further examine the reasoning for this finding, we decided to compare the MEF RB-/- to the MEF wild types (MEFs). Surprisingly, the MEFs that possess pRb, did not G_1 arrest in the presence of TCDD and 10% NCS(Figure 3.1 B). To additionally examine the basis for not seeing a G_1 arrest, a TCDD dose-response experiment was completed on both the MEF RB-/- and MEFs to not only determine if saturating amounts of TCDD were needed to arrest these two cell lines, but also to determine if the AhR was fully functional. Both MEF cell lines were NCS released and NCS-starved (control) in the presence of 6nM-15nM concentrations of TCDD or DMSO (control) for 24 hours. Yet, the MEFs and MEF RB-/- still did not G_1 arrest with increasing amounts of TCDD (Data not shown), even though AhR was found to be functional in these cells. Moreover, our results indicated that these cell lines were

obviously not suitable for studying AhR-mediated G_1 arrest. Nevertheless, MEFs treated with NCS and 10nM or 15nM concentrations of TCDD could elicit a super induction of CYP1A1 compared to MEFs and TCDD alone (Figure 3.3). In addition, previous studies showed that serum alone could elicit CYP1A1 in 5L rat hepatoma cells. Despite, the finding that MEFs did not G_1 arrest, these cells were interesting enough to study the characterization of the serum factor that causes a CYP1A1 super induction.

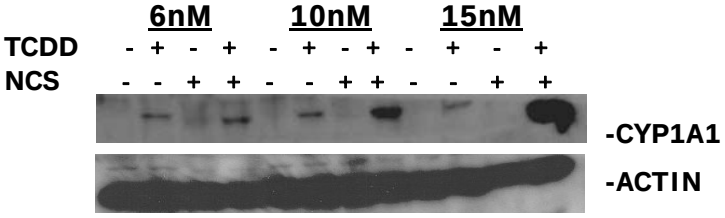


Figure 3.3 A serum factor in NCS elicits a CYP1A1 super induction in MEFs. Subconfluent asynchronous MEF cell cultures were serum-starved (DMEM + 0% NCS) for 72 hours. Fresh media containing DMEM + 0% NCS (-NCS) or DMEM + 10% NCS (+ NCS) in the presence 6nM, 10nM, and 15nM of TCDD or DMSO (control) was added to MEFs for 24hours. Total cell lysates were prepared and analyzed by western blotting for CYP1A1 and Actin (loading control).

FBS can cause a CYP1A1 super induction in MEFs

Since, FBS was used in previous studies that described serum-induced CYP1A1 expression, FBS was used in this study to determine whether NCS was the only conventionally used serum that could elicit a CYP1A1 super induction in the presence of saturating amounts of TCDD.

Using the same experimental model as described above, MEFs were treated with 6nM-15nM of TCDD in the presence FBS and in the absence of FBS. We found that FBS could indeed cause a CYP1A1 super induction in MEFs (Figure 3.4). Our findings

indicate that both FBS and NCS are suitable for characterization of the serum factors that cause CYP1A1 super induction in MEFs. To follow the pursuit further, we next set out to determine if MEFs were the only cells that CYP1A1 super induction could occur in.

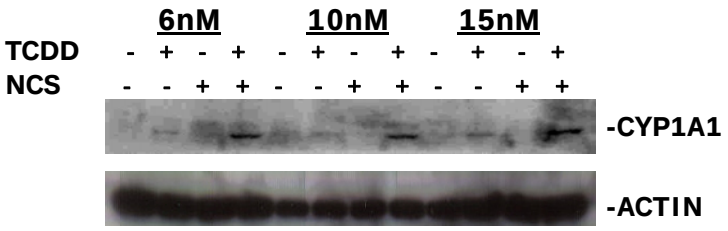


Figure 3.4 A serum factor in FBS elicits a CYP1A1 super induction in MEFs. Subconfluent asynchronous MEF cell cultures were serum-starved (DMEM + 0% FBS) for 72 hours. Fresh media containing DMEM + 0% FBS (-FBS) or DMEM + 10% FBS (+ FBS) in the presence 6nM, 10nM, and 15nM of TCDD or DMSO (control) was added to MEFs for 24hours. Total cell lysates were prepared and analyzed by western blotting for CYP1A1 and Actin (loading control).

MEFs are the only cells in this study by which a CYP1A1 induction can occur

Investigations were carried out to determine if other cell lines besides MEFs were suitable for studying characterization of CYP1A1 super induction. Looking at, RAT 2 hepatoma cells, mouse FL183 B hepatoma cells, and rat 5L hepatoma cells, we found that 10% FBS and increasing concentrations of TCDD failed to elicit a CYP1A1 super induction in these cell lines (Figure 3.5). The RAT 2 hepatoma cells did not give any signal for CYP1A1, in contrast to the 5L and FL183 B hepatoma cells which gave a robust signal for CYP1A1 but not a super induction. However, the FL183 B hepatoma cells did not have a serum-induced signal. Conjectures as to the fact that some of these cell lines did not have serum-induced expression of CYP1A1 and the finding that all

these cell lines do not super induce CYP1A1, will need to be further investigated. Despite the finding that other cell lines are not able to study characterization of the serum factors in NCS and FBS, the MEFs appear to be fitting for the task.

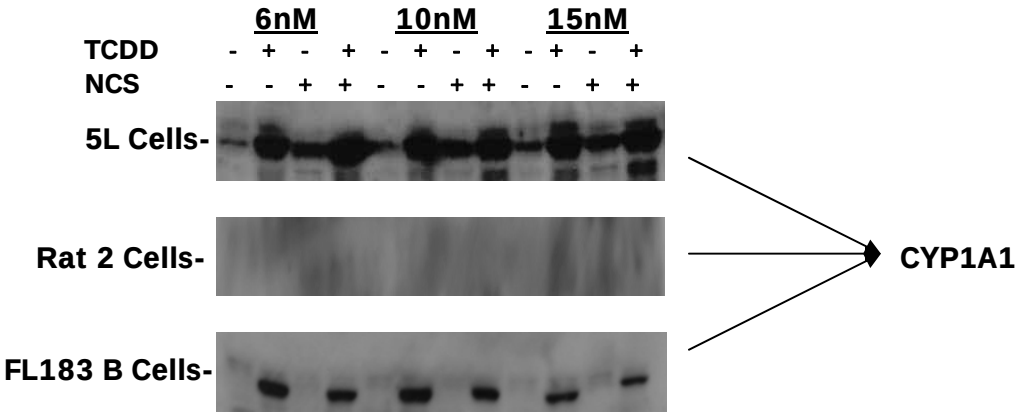


Figure 3.5 The serum factor in FBS does not elicit a CYP1A1 super induction in 5L, RAT 2, or FL183 B hepatoma cells. Subconfluent asynchronous 5L, Rat 2, or FL183 B cell cultures were serum-starved (DMEM + 0% FBS) for 72 hours. Fresh media containing DMEM + 0% FBS (-FBS) or DMEM + 10% FBS (+ FBS) in the presence 6nM, 10nM, and 15nM of TCDD or DMSO (control) was added to MEFs for 24hours. Total cell lysates were prepared and analyzed by western blotting for CYP1A1 and Actin (loading control).

Characterization of the Serum Factors that cause a CYP1A1 super induction in MEFs

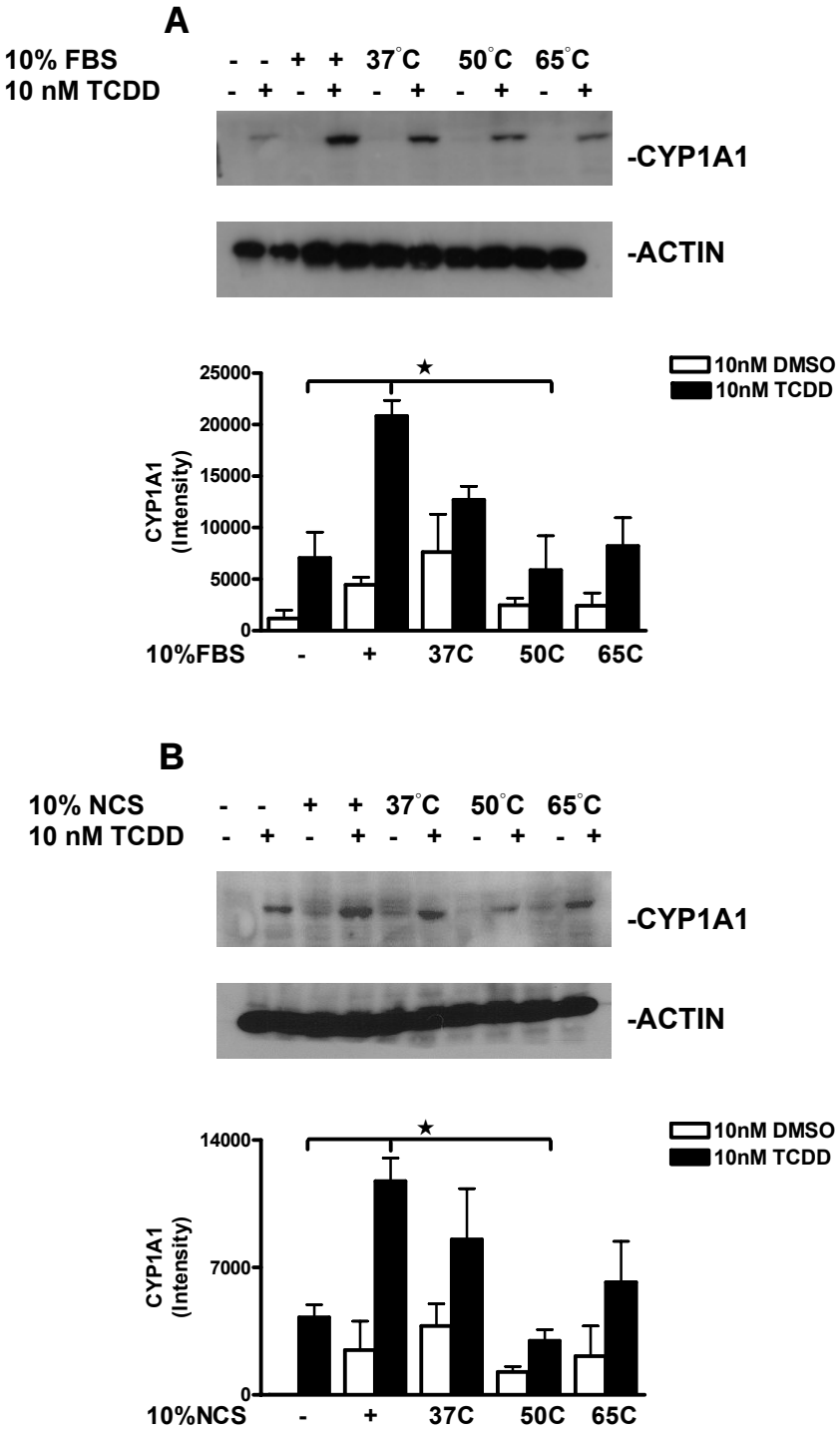
To further characterize the serum factors in NCS and FBS, we determined several key characteristics; their approximate sizes, if they are heat sensitive, if they are AhR agonist contaminants in serum and lastly if the CYP1A1 super induction is seen at the transcriptional level resembling the translational level of induction.

Serum factor in both the NCS and FBS are heat sensitive

Our experimental model of heat sensitivity is consistent with previous studies [63] that heated serum at 37°C, 50°C, and 65°C to determine if the serum factor was heat sensitive. As shown in Figure 3.6 A-B, serum factors in both the FBS and NCS that was heated at temperatures higher than 37°C are heat labile in MEFs. This data is represented by a statistical significant decline (*p<0.01) of CYP1A1 super induction in MEFS treated with heated serum at 50°C (Figure 3.6 A, B). More importantly, there seems to be a regain of basal level CYP1A1 when MEFs are treated with serum heated at 65°C. These observations propose that there might be several components in both the FBS and NCS that contribute to the CYP1A1 super induction.

Figure 3.6 The serum factor(s) in both the NCS and FBS is heat labile at 50°C. A. FBS treated **B**. NCS treated. Asynchronous MEF cell cultures were serum-arrested in DMEM without NCS or FBS for 72 hours. Serum-arrested MEF cell cultures were released for 24 hours with media containing, 0% serum (NCS or FBS), 10% serum (NCS or FBS) or normal 10% serum (NCS or FBS) treated at the indicated temperature for 1h in the presence or absence of 10nM TCDD. Total lysates analyzed by Western blotting for CYP1A1 and Actin. Data was quantified by ChemiImager 5500 (Alpha Innotech Corporation) using analysis tools (spot density). Quantification of CYP1A1 products (normalized against Actin) were statistically analyzed and graphed by Graph Pad Prism 4. One way- ANOVA using Tukey (FBS) or Bonferroni (NCS) multiple comparison tests. *p <0.05 is significant. This data is represented by 3 independent experiments.

Figure 3.6

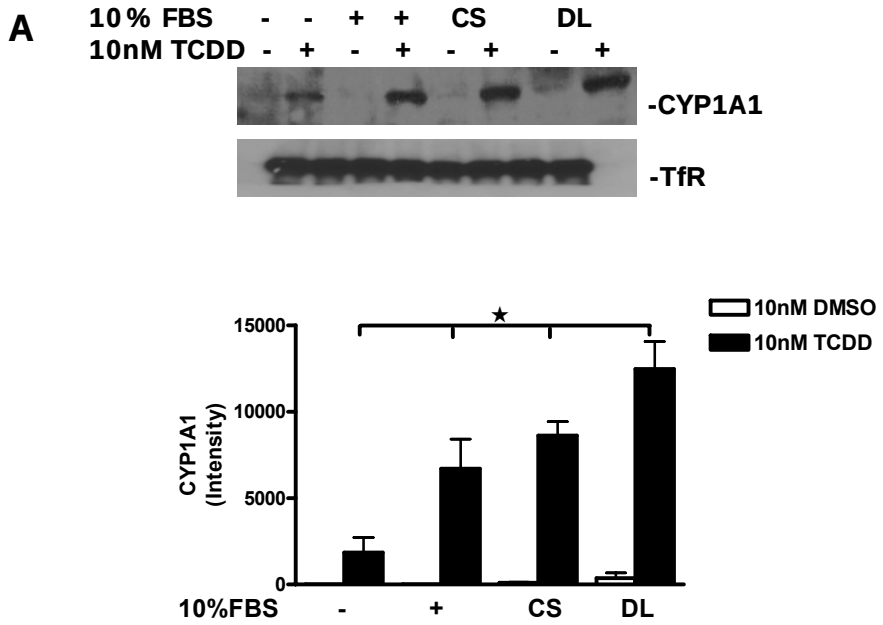

A
10% FBS - - + + 37˚C 50˚C 65˚C
10 nM TCDD - + - + - + - + - +
-CYP1A1
-ACTIN
10nM DMSO
10nM TCDD
CYP1A1 (Intensity)
25000
20000
15000
10000
5000
0
10%FBS - + 37C 50C 65C
B
10% NCS - - + + 37˚C 50˚C 65˚C
10 nM TCDD - + - + - + - + - +
-CYP1A1
-ACTIN
10nM DMSO
10nM TCDD
CYP1A1 (Intensity)
14000
7000
0
10%NCS - + 37C 50C 65C

The serum factors in both the NCS and FBS persist even when serum is charcoal stripped and dialyzed

Experiments were performed to determine the possibility that there could be an AhR agonist contaminant in both the NCS and FBS that elicits a CYP1A1 super induction by charcoal stripping both serums [63]. To find the approximate size of the serum factors in both the NCS and FBS, the serums were dialyzed with a 10,000 MW cut off. As shown in Figure 3.7A, B, CYP1A1 super induction persists even when NCS or FBS is charcoal stripped or dialyzed. This data indicates that the serum factors are approximately ≥ 10,000 kilo daltons in size and that the serum response persisted when MEFs were treated charcoal stripped and dialyzed serums in presence of TCDD. The findings also point out that the charcoal stripped, dialyzed, and the 10% serum samples in the presence of TCDD is indistinguishable from each other and they were statistically significant (*p<0.01) from MEFs treated with 0% serum and TCDD (Figure 3.7 A, B).

Figure 3.7 The serum factor in both the NCS and FBS persists when serum is charcoal-stripped or dialyzed. A. FBS treated **B**. NCS treated. Subconfluent asynchronous MEF cell cultures were serum-arrested in DMEM without NCS or FBS for 72 hours. Fresh media containing 0% serum (NCS or FBS), 10% normal serum (NCS or FBS), 10% charcoal-stripped serum (NCS or FBS), or 10% dialyzed serum (NCS or FBS) in the presence (+) or absence (-) of 10nM TCDD was added for 24 hours. Total cell lysates were prepared and analyzed by Western blotting for CYP1A1 and TfR. Data was quantified by ChemiImager 5500 (Alpha Innotech Corporation) using analysis tools (spot density). Quantification of CYP1A1 products (normalized against TfR) were statistically analyzed and graphed by Graph Pad Prism 4. One way- ANOVA using Tukey (NCS) or Bonferroni (FBS) multiple comparison tests. *p <0.05 is significant. This data is represented by 3 independent experiments.

Figure 3.7

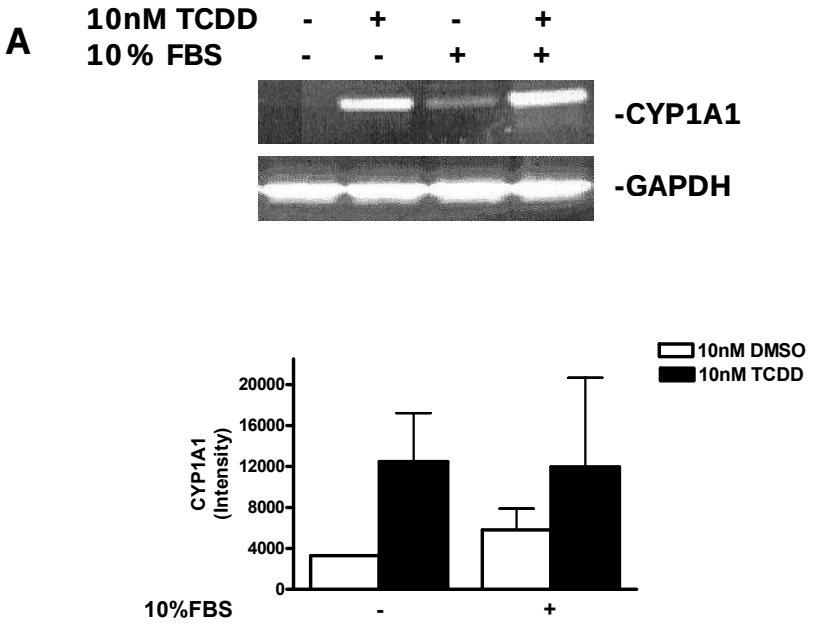

A
10% FBS - - + + CS DL
10nM TCDD - + - + - + - +
-CYP1A1
-TfR
10nM DMSO
10nM TCDD
CYP1A1 (Intensity)
15000
10000
5000
0
10%FBS - + CS DL
B
10% NCS - - + + CS DL
10nM TCDD - + - + - + - +
-CYP1A1
-TfR
10nM DMSO
10nM TCDD
CYP1A1 (Intensity)
14000
7000
0
10%NCS - + CS DL

CYP1A1 super induction at the transcriptional level

Thus far, we have looked at CYP1A1 super induction on the translational level, but CYP1A1 is regulated at the transcriptional level, so it would be ideal to look for this super induction at the gene level as well. RT-PCR experiments were performed on MEFs that revealed that NCS, not FBS, could statistical significantly (*p<0.05) super induce CYP1A1 at the transcriptional level.(Figure 3.8 A,B). However, further investigations will have to be completed to determine the reason that FBS did not cause a super induction at the transcriptional level. In any case, these results clearly indicate that serum factors in NCS are able to super induce CYP1A1 on both the translational level and transcriptional level.

Figure 3.8 The serum factor in NCS, not FBS, elicits a CYP1A1 Super induction at the transcriptional level. A. FBS treated **B**. NCS treated. Subconfluent asynchronous MEF cell cultures were serum-arrested in DMEM without NCS or FBS for 72 hours. Fresh media containing 0% serum (NCS or FBS) or 10% normal serum (NCS or FBS) in the presence (+) or absence (-) of 10nM TCDD was added for 24 hours. Total RNA was extracted. RT-PCR was performed on dT-primed, reverse-transcribed RNA (1 μg) followed by PCR using primers specific for mouse CYP1A1 and mouse GAPDH. PCR products were fractionated on a 1.2% agarose gel and visualized by ethidium bromide staining. Gel images were captured digitally using a CCD camera and the band signals were quantified by ChemiImager 5500 (Alpha Innotech Corporation) using analysis tools (spot density). Quantification of CYP1A1 products (normalized against GAPDH and baseline control) were statistically analyzed and graphed by Graph Pad Prism 4. One way-ANOVA using Bonferroni multiple comparison tests. *p <0.05 is significant. This data is represented by 3 independent experiments.

Figure 3.8


A
10nM TCDD - + - +
10% FBS - - + +
-CYP1A1
-GAPDH
10nM DMSO
10nM TCDD
CYP1A1 (Intensity)
20000
16000
12000
8000
4000
0
10%FBS
-
+
B
10nM TCDD - + - +
10% NCS - - + +
-CYP1A1
-GAPDH
★
10nM DMSO
10nM TCDD
CYP1A1 (Intensity)
16000
8000
0
10%NCS
-
+

DISCUSSION AND CONCLUSION:

Both serums (NCS or FBS) provide evidence for CYP1A1 super induction

The serum factor(s) in both sera (NCS or FBS) are heat sensitive at 50°C (Figure 3.6 A, B), withstand removal from charcoal stripping and are ≥ 10,000 kilo-daltons in molecular weight (Figure 3.7 A, B). Hence, the serum factor is not a small lipophilic organic AhR contaminant agonist in sera. Furthermore, both the NCS and the FBS can super induce CYP1A1 on the protein level in MEFs. These results indicate that in the presence of TCDD, sera can mediate a CYP1A1 super induction in MEFs, but the exact mechanism is still not known.

Super induction of CYP1A1 could be occurring differently than hypothesized

Initially, we hypothesized that serum factor(s) can function by binding to a cell surface receptor(s) to trigger activation of the AhR, possibly by promoting the release or synthesis of an endogenous AhR agonist: The data obtained in the studies using MEFs is incompatible with the original hypothesis. The original hypothesis has its basis in recent finding using hepatoma cells (Levine-Fridman et al. (2004) where TCDD and FBS alone each induced CYP1A1 equally and in combination displayed no further induction. The findings in this study show that MEFs treated with TCDD and sera (NCS or FBS) elicit a super induction of CYP1A1. However, TCDD alone appears to induce CYP1A1 to a far greater extent than seen with either NCS or FBS. Hence, the premise that the serum response reflects endogenous agonist activity is irreconcilable with the data. There are other possible explanations of how this super induction of CYP1A1 might be occurring in MEFs. One reason could be due to a serum-mediated post-translational modification such as phosphorylation of the AhR protein that strengthens AhR transcriptional activity. Conversely, previous studies have found that super induction of CYP1A1 can occur by mechanisms that imply inactivation of an AhR repressor, which could help explain how this phenomenon occurs in MEFs. These studies found that Cyclohexamide, a protein

translational inhibitor can produce CYP1A1 super induction at the transcriptional level by squelching inhibitory effects of a labile putative AhR repressor [66-68]. Conceivably, the activity of NCS or FBS may similarly trigger super induction by suppressing the activity of a repressor protein in MEFs. Distinguishing between these two scenarios will require further studies examining whether the AhR is post-translationally modified by serum, or is the consequence of protein turn-over.

Super induction of CYP1A1 is seen at the transcriptional level in MEFs

Both NCS and FBS serum factors induce CYP1A1 super induction on the protein level in MEFs (Figure 3.3, 3.4). However, at the transcriptional level, only NCS serum factor(s) appears to induce CYP1A1 super induction (Figure 3.8). This finding can be attributed to several possible explanations. The failure to detect super induction at the RNA level in FBS-treated MEFs may be real, or may reflect limitations of the semi-quantitative nature of the RT-PCR method. The marked expression variation between replicate experiments of FBS-treated MEFs further cloud meaningful data interpretation. Therefore, these studies would need to be repeated, preferably using quantitative real-time PCR. Another likely reason that FBS did not super induce CYP1A1 at the gene level could be the fact that translation is regulated by protein synthesis and degradation, independent of gene transcription regulation. Furthermore, there may possibly be increased protein synthesis and decreased degradation in FBS treated samples, which facilitates a CYP1A1 super induction only on the protein level. Other possible explanations might be there was too much variability between experiments that caused a lost in super induction of CYP1A1. Whatever the case may be for the lost of CYP1A1 super induction in FBS-treated MEFs, further investigation will have to be done by subsequent experiments. In the case of the NCS-treated MEFs, the serum factors can super induce CYP1A1 on both the gene and protein levels, we can attribute this observation to a few possible explanations.

NCS is different from FBS in that it is serum taken from new born calves, ten days or less in age. This NCS serum factors might be different from FBS due to further development of the calf, which could be the reason that NCS has the capability to super induce on both the transcriptional and translational levels. Others reasons that NCS elicits this CYP1A1 super induction on both the gene and protein levels could be that at both levels, CYP1A1 is regulated at the same rates, which could account for the parallel levels of induction. In conclusion, these observations whereby NCS serum factors are able to super induce CYP1A1 on both the gene and protein level provides additional support to the theory that the AhR is post-translationally modified by serum, or is the consequence of protein turn-over.

Serum-induced CYP1A1 expression

Experiment differences in serum- (NCS or FBS) treated MEFs, in the absence of TCDD, can be accredited to several likely explanations. Experiments that were facilitated to determine whether the CYP1A1 super induction persisted even when charcoal stripped and dialyzed yielded result analysis differences in serum-treated MEFs compared to the same treatment type in other experiments (Heat denaturing and RT-PCR). Result findings indicated that there was low or not any induction of serum (NCS or FBS) -induced CYP1A1 in the charcoal stripped and dialyzed experiment (Figure 3.7 A, B). However, in the RT-PCR experiment (Figure 3.8 A, B) and the heat denaturing experiment (Figure 3.6 A, B) there was a serum-induced expression of CYP1A1. One possible reason for this account might be the variability between experiments. Another reason could be confluency levels of MEFs between experiments. In the heat denaturing experiment, MEFs were confluent (Figure 2.2) that were treated with NCS alone, which could attribute to the increased protein in those samples, but the FBS-treated MEFs in this experiment were subconfluent (Figure 2.2), which can not explain the increase in protein. More importantly, there was a serum (NCS or FBS)-induced CYP1A1 expression in the RT-PCR experiment with subconfluent cultures, which may be due to the fact that the

gene transcription regulation is independent of protein regulation. Despite these small discrepancies between experiments, the data still indicates that serum factors in both the FBS and NCS super induce CYP1A1 expression.

Cell lines whereby CYP1A1 super induction does not occur

CYP1A1 super induction does not occur in RAT 2, mouse FL183 B, and rat 5L hepatoma cell lines (Figure 3.5). More surprisingly, FBS, in the absence of TCDD, does not induce CYP1A1 expression in FL183 B and RAT 2 cells. In fact, not even TCDD and FBS could elicit a signal for CYP1A1 in RAT 2 cells. Based on previous experiments done in the lab, it was proposed that a great amount of RAT 2 cells are needed to visualize CYP1A1 induction and CYP1A1 is expressed in very low levels in these cells. For the reason that the FL183 B cells did not express CYP1A1 in the presence of FBS (alone) could be that in these cells the serum factors could not bind to the cell surface protein since there was not a receptor; or may be the serum factors are binding to cell surface receptors, but the receptors do not signal or neutralize the signal that activates the endogenous AhR ligand. Further investigations will have to be completed to determine the reason for these observations in the RAT 2 and FL183 B hepatoma cells.

However, the serum factor in FBS is not specie specific nor is it tissue specific. These observations are linked to the fact that serum can induce CYP1A1 expression in rat 5L hepatoma cells, in mouse embryonic fibroblasts (MEFs), human colon CaCo2 carcinoma cells [69] and human HepG2 hepatoma cells [70]. These findings suggest that certain cell lines are suitable for serum-induced CYP1A1 expression whereas other cell lines are not and more studies will have to be conducted to determine the reason for this result.

FUTURE DIRECTIONS:

We hope by characterizing the serum factor(s) responsible for CYP1A1 super induction, it could facilitate the identification of the physiological ligand for AhR which is not known as of yet. Furthermore, by determining the mechanism underlying the super induction of CYP1A1 could provide a better understanding of how AhR regulates cell cycle progression. In the future, further investigations will be done to characterize the serum factor(s) by using surface-enhanced laser desorption/ionization- time of flight (SELDI-TOF) mass spectrometry. The SELDI-TOF technology will help in the identification of multiple unknown proteins in serum [71] by comparing them with known proteins in gene bank databases. Finding these unknown proteins can lead to the discovery of a new AhR endogenous ligand and further build the AhR biology field.

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