Molecular Pharmacology Fast Forward. Published on August 4, 2006 as doi:10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #27474

Calpain mediates the dioxin-induced activation and down-regulation of the aryl hydrocarbon receptor

Yolanda R. Dale and Sakina E. Eltom

Department of Biomedical Sciences Division of Cancer Biology (S.E.E), Graduate Program in Pharmacology (Y.R.D.), Meharry Medical College, 1005 Dr. D.B. Todd Blvd, Nashville, TN 37208

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title:

The role of calpain in the regulation of the AhR

Corresponding Author:

Sakina Eltom, D.V.M., Ph.D.

1005 Dr. D.B. Todd Blvd

Nashville, TN 37208

Tel/Fax: (615) 327-5713

seltom@mmc.edu

Text pages: 22

Tables: 0

Figures: 5

References: 41

Abstract: 212 words

Introduction: 417 words

Discussion: 892 words

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, Ah receptor nuclear translocator; TCDD/dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive elements; CYP1A1, cytochrome 450 1A1; PAH, polyaromatic hydrocarbons

ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic-helix-loop-helix (bHLH) transcription factor that binds polyaromatic hydrocarbons (PAH), such as dioxin, and mediates their toxicity. Binding of PAH to AhR in the cytoplasm triggers a poorly defined transformation step of the receptor into a nuclear transcription factor. In this study, we show that the calcium-dependent cysteine protease, calpain, plays a major role in the ligand-induced transformation and signaling of AhR. Fluorescence imaging measurements showed that TCDD treatment elevates intracellular calcium, providing the trigger for calpain activation, as measured towards BOC-LM-CMAC, a calpain-specific substrate. Inhibition of calpain activity by the MDL28170 blocked the TCDD-induced nuclear translocation of AhR in Hepa1c1c7 mouse hepatoma cell line. Treatment of the human metastatic breast carcinoma cell line MT-2 with MDL28170 and PD 150606, two calpain-selective inhibitors, completely abolished the TCDD-induced transactivation of AhR as assessed by transcription of *CYP1A1* gene. Previous studies have established that following TCDD-induced transactivation, the AhR undergoes a massive depletion; we show here that selective calpain inhibitors can block this step, suggesting that the ligand-induced down-regulation of the AhR is calpain-dependent. The data presented support a major role for calpain in the AhR transformation, transactivation and subsequent down-regulation, and provide a possible explanation for many of the reported phenomena of ligand-independent activation of AhR.

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix (bHLH) transcription factor that regulates the adaptive and toxic responses to a variety of environmental carcinogens, including polyaromatic hydrocarbons (PAH), such as dioxin (Poland and Knutson, 1982). In the absence of ligand, the AhR resides predominantly in the cytoplasm in a conformation stabilized by chaperone proteins hsp90, XAP2, and p23 (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer and Perdew, 1999). The current working model for the AhR signaling proposes that ligand binding to AhR facilitates the dissociation of the chaperone proteins and AhR transformation into a form that readily translocates to the nucleus. In the nucleus, AhR dimerizes with the related bHLH Ah receptor nuclear translocator (ARNT) protein (Kazlauskas et al., 2001), and binding of this heterodimer to DNA recognition motifs designated as xenobiotic-responsive elements (XREs) results in enhanced transcription of the Ah-responsive genes (Jones et al., 1985), typified by *CYP1A1* and *CYP1A2* (Gonzalez et al., 1984). The protein products of these *CYPs* are catalytically active in metabolizing, not only many endogenous compounds such as β-estradiol, but also many drugs, dietary components, mutagens, carcinogens and environmental pollutants (Conney, 1982). Following transcriptional activation, the liganded-AhR undergoes a rapid degradation (Pollenz, 1996; Prokipcak and Okey, 1991; Reick et al., 1994). Studies have suggested that this ligand-induced down regulation of AhR is proteasome-dependent (Davarinos and Pollenz, 1999), and that the nuclear export is required for this process (Song and Pollenz, 2002).
Although forced dissociation of the chaperone proteins, especially hsp90 by geldanamycin, allows for the nuclear translocation of the receptor it is not sufficient for its transactivation (Song and Pollenz, 2002).

In vitro studies have shown the AhR to be a substrate for the calcium-dependent protease calpain (Poland and Glover, 1988), which is a family of cytosolic calcium-dependent cysteine proteases. Calpain is involved in regulating many cellular processes including proliferation, differentiation, cell motility and metastasis through regulation of signal transduction and cleavage of many target cellular regulatory proteins (Potter et al., 1998; Wang, 1990). Of the several calpain isoforms, calpain I (μ̃-calpain), and calpain II (m-calpain) are ubiquitous enzymes, activated with low and high calcium concentrations,

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

respectively. Since the treatment of cells with PAH can elicit a rapid increase in intracellular calcium (Hanneman et al., 1996; Tannheimer et al., 1997), we hypothesize that this increase in intracellular calcium could provide the trigger to activate calpain. Here we have studied the involvement of calpain in the transformation process required for the nuclear translocation, transactivation, and subsequent degradation of AhR.

MATERIALS AND METHODS

Materials:

Calpain inhibitor III (MDL 28170), PD 150606, carbobenzoxyl-L-leucyl-L-leucyl-leucinal (MG-132), epoxomicin, and Ionomycin were purchased from Calbiochem (San Diego, CA). Dioxin (TCDD) was purchased through NCI Chemical Carcinogen Repository- Midwest Research Institute (Kansas City, MO). The calpain substrate BOC-LM-CMAC and Fluo-4-AM were purchased from Molecular Probes (Eugene, OR). Real time PCR kit was purchased from Bio-Rad (Hercules, CA). The rabbit polyclonal anti-AhR antibodies (Poland and Glover, 1990; Pollenz et al., 1994) were a kind gift from Dr. Christopher Bradfield, University of Wisconsin (Madison, WI). The Sager MT-2 metastatic cell line was derived from a patient with an infiltrating intraductal carcinoma, and was kindly provided by Dr. Vilma Band from Northwestern University (Chicago, IL).

Cell Culture and stimulation of MT-2 cells

The metastatic 21MT-2 cell line were grown in DFCI-1 medium as described previously (Band et al., 1990). For experiments, MT-2 cells were seeded in six-well plates at a density of 5×10^5 and grown for 24 hrs. Cells were pre-incubated with inhibitors MG-132, epoxomicin or MDL 28170 for 2 hr and maintained during 3 hr TCDD treatment. TCDD, MG-132, MDL 28170, and epoxomicin were solublized in DMSO, with an equivalent volume added to control cells (maximum of 0.1% v/v).

Intracellular Calcium Measurements

MT-2 cells cultured in 24-well plates (2×10^4) in DFCI medium were loaded with 5 μM Ca^{2+}-sensitive dye Fluo-4-AM (Molecular Probes, Eugene, Oregon) for 45 min at 37 °C. Following pre-incubation, cells were rinsed three times with DFCI medium to remove free dye and continued to incubate for 30 min in medium alone to allow complete de-esterification of AM esters. Fluo-4-loaded cells were then stimulated with 1 nM or 10 nM TCDD, 10 μM ionomycin, or vehicle only for 25 min. Changes in intracellular calcium were measured as captured fluorescence images of cells using the IX50 Olympus Fluorescence microscope (excitation at 385 nm, emission at 512 nm).

Calpain Activity Assay

Calpain activity in MT-2 cells was assessed by fluorescence microscopy using the calpain substrate BOC-LM-CMAC (Molecular Probes, Eugene, OR). The non-fluorescent cell-permeable substrate is conjugated by intracellular thiols into a membrane impermeable form allowing substrate accumulation within the cell (Carragher et al., 2004). Proteolytic cleavage of BOC-LM-CMAC by calpain results in blue fluorescence. Cells in 24-well plates were pretreated with 15 μM calpain inhibitor MDL28170, MG-132 or epoxomicin followed by TCDD or ionomycin treatment. Briefly, cells were incubated with 50 μM BOC-LM-CMAC for 20 min at 37 ºC. Fluorescence intensity corresponding to calpain activity was visualized and images were capture with an Olympus IX50 fluorescence microscope using a digital camera with MagnaFire software, and quantified by the NIH ImageJ software. The image exposure settings were identical within each experiment. Data for each experiment were normalized to Ionomycin values (set as 100%)

Immunocytochemical Staining and Fluorescence Microscopy

Cells growing on cover slips in 6-well plates were washed in PBS and then fixed by incubation in a (1:1) methanol: acetone solution at 4ºC for 30 min and subsequently air-dried. For staining, cells were rinsed and hydrated with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) and transferred to clean 6 well plates. The cover slips were incubated at room temperature for 1 hr in 4% milk solution in TBST to block nonspecific binding. The cover slips were then incubated at room temperature for 1 hr in 1 μg/ml anti-AhR polyclonal antibody (BEAR-4) in 2% milk solution in TBST while rocking. Cover slips were then washed three times (15 min each) with TBST. A 1:200 dilution of fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit antibodies in 2% milk with TBST was added to the cover slips in reduced light, and incubated at room temperature for 1 hr. The cover slips were then washed extensively and mounted onto glass slides using mounting solution containing DABCO as an anti-fading agent.

Preparation of Total Cell Lysates and Immunoblotting

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

Following treatments, cell monolayers were lyzed in 1 ml Trizol, which allowed for simultaneous isolation of RNA and protein. Following lysis, both RNA and protein were isolated according to the vendor's instruction. The protein pellets were resuspended in 2% SDS and sonicated briefly to dissolve. The protein concentration in cell extracts was determined using the BCA kit, per the manufacturer's instructions. Equivalent amounts of protein (10 μg) were separated by Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred for 2 hr at 175V to Polyvinylidene fluoride (PVDF) membranes. The membranes were blocked at room temperature for 1 hr in 4% nonfat milk in TBST with subsequent incubation with BEAR-3 anti-AhR (1 μg/ml). After brief washing, blots were incubated with the corresponding HRP-coupled anti-rabbit or anti-mouse secondary antibody (1:20,000) for 1 hr followed by additional washing in TBST and TBS. Reactive protein bands were visualized using enhanced chemiluminescence reagents. Band density was quantified by UVP Bio-Imaging System using LabWorks™ Image Acquisition Analysis Software (UVP Laboratory products, Upland, CA). Subsequently, blots were probed with actin monoclonal antibodies (1:4000) for normalization of protein loading. The relative levels of AhR protein were then normalized to the level of β-actin to generate normalized values for the relative concentration of AhR in each sample.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Following lysis with Trizol, total RNA was isolated according to manufacturer's protocol. cDNA was prepared from 2 μg of mRNA using random hexamer primers and Moloney murine leukemia virus reverse transcriptase. CYP1A1 PCR amplification was done using forward primer sequence 5'TAG ACA CTG ATC TGG CTG CAG3' and the reverse primer sequence 5'GGG AAG GCT CCA TCA GCA TC3'. House keeping gene glyceraldehyde 3-phosphate dehydrogenase was amplified as an internal control using forward primer 5'ACC ACA GTC CAT GCC ATC AC3' and reverse primer 5'TCC ACC ACC CTG TTG CTG TA3'. PCR products were visualized in agarose gels stained with ethidium bromide, and bands were quantified by densitometric scanning, as described previously (Eltom et al., 1999).

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

Real Time RT-PCR

Real time quantitative PCR was performed using an iCycler iQ Real time PCR detection system (Bio-Rad, Hercules, CA). Complementary DNA (cDNA) generated from TCDD-treated MT-2 cells was serially diluted to establish a standard curve (20,000 pg to 0.2 pg). Reactions were performed in triplicates using 2.5 μl of cDNA per 25 μl reaction containing iQ SYBR green super mix and CYP1A1 primers (5'-CTA TGA CCA CAA CCA CCA AGA ACT G-3' forward primer and 5'-AGG TAG CGA AGA ATA GGG ATG AAC TC-3' reverse primer) with the following PCR parameters: 95ºC for 5 min followed by 45 cycles at 95ºC for 15 s, 60ºC for 30 s and 72º C for 15 s. Reactions for detection of the endogenous control gene, ribosomal 18s rRNA, were run in parallel for each cDNA template as a reference for normalization using the following primers: 5'-CGG ACA GGA TTG ACA GAT TGA TAG C-3' 18S forward primer and 5'-TGC CAG AGT CTC GTT CGT TAT CG-3' 18S reverse primer. A melting curve analysis was performed for each amplification run to ensure the specificity of product amplification.

Statistical Analysis

The data from different experiments were analyzed using GraphPad Prism Software (San Diego, CA) by one-way analysis of variance ANOVA and Tukey-Kramer multiple comparison tests of values from control versus treated samples

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

TCDD Mobilizes Calcium in MT-2 cells

Exposure of human T cells and primary human epithelial cells to PAH leads to the mobilization of intracellular calcium. Because this phenomenon was not observed in the MCF-10A mammary epithelial cell line, which have low concentrations of AhR (Tannheimer et al., 1997), it was important to determine if TCDD is capable of evoking an increase in intracellular calcium in the MT-2 human metastatic breast cancer cell line, which have high concentrations of AhR. We measured $[Ca^{2+}]_i$ in MT-2 cells in the presence of TCDD, using Fluo-4 AM as a probe. In MT-2 cells, TCDD resulted in an increase in $[Ca^{2+}]_i$ that was comparable to values of the calcium ionophore, Ionomycin, which results in a profound increase in $[Ca^{2+}]_i$, when compared to the solvent control (DMSO) (Fig 1).

Exposure of cells to TCDD Activates Calpain

Calpain is activated by elevated intracellular calcium, resulting in auto-proteolytic cleavage to further enhance its activity (Mathiasen et al., 2002). To explore whether TCDD-induced changes in intracellular calcium could lead to calpain activation, whole-cell calpain activity assay was preformed in MT-2 cells using the cell-permeable substrate BOC-LM-CMAC. TCDD exposure resulted in a robust increase in calpain activity, comparable to the levels induced by ionomycin, the ionophore that was used as a positive control (Fig. 2). These data indicate that TCDD could activate calpain. The activation of calpain by TCDD was strongly inhibited by MDL 28170, a potent calpain inhibitor. Fig 2 also shows that MG-132, which has previously been reported to inhibit both proteasomes and calpain (Mathiasen et al., 2002), mimicked the effect of MDL 28170 on TCDD-induced calpain activity**.** Epoxomicin, which inhibits proteasomes only (Meng et al., 1999), had no effect on calpain activity. These results support the conclusion that TCDD is involved in the activation of calpain in MT-2 cells. The data further establish the selectivity of both MG-132 and MDL 28170 in inhibiting calpain, in agreement with published reports (Mathiasen et al., 2002; Potter et al., 1998).

TCDD-induced nuclear accumulation of AhR is calpain-dependent

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

To explore whether calpain might be involved in the transformation of AhR leading to its translocation from the cytoplasm into the nucleus, subcellular localization of AhR was analyzed in Hepa-1, the murine hepatoma cell line following treatment with TCDD. Hepa-1 cells were used here because they have no detectable nuclear AhR levels under basal conditions, unlike the human metastatic MT-2 cells, which have substantial nuclear AhR levels in the absence of ligand treatment. TCDD treatment results in enhanced fluorescence nuclear accumulation accompanied by reduction in the cytoplasmic staining, indicative of the receptor nuclear translocation. As shown in Fig 3, AhR is predominantly localized within the cytoplasm in DMSO-treated control cells, as shown by fluorescence immuno-staining (Fig. 3A). Treatment with MDL 28170 prior to TCDD exposure resulted in predominant cytoplasmic staining, indicating that calpain influences the localization of the AhR following treatment with TCDD.

Inhibition of Calpain blocks transcriptional activity of AhR

The expression of *CYP1A1*, a gene that is transcriptionally regulated by AhR, is induced by increases in intracellular calcium, but the mechanism of this induction is poorly defined (Le Ferrec et al., 2002). Interestingly, the disruption of hsp90-AhR complex facilitates nuclear localization of the AhR yet fails to induce gene expression. Based on the data presented in Fig. 3, we presume that calpain-mediated events may be important for AhR-induced transactivation. To this end, we measured CYP1A1 gene transcription in MT-2 cells treated with MDL 28170 to specifically block the actions of calpain. As shown in Fig. 4A, MT-2 cells have low constitutive levels of CYP1A1 mRNA, and TCDD treatment resulted in a significant increase in CYP1A1 mRNA, that was suppressed to basal levels by the calpain inhibitors MDL 28170 and PD 150606.

Real time RT-PCR was additionally used to provide more quantitative analysis of CYP1A1 mRNA expression. The results in Fig. 4B, showed that treatment with TCDD for 3h resulted in approximately 40-fold increase in CYP1A1 expression over DMSO-treated control. MDL 28170 completely blocked the TCDD ligand-induced *CYP1A1* gene transcription. Similarly, MG-132, which also inhibits calpain, inhibited TCDD-induced CYP1A1 gene expression to the same degree, as MDL28170, while epoxomicin, a proteasome inhibitor, had no effect on the TCDD-induced CYP1A1

expression. Therefore, we conclude that inhibition of calpain preferentially reduces the ability of TCDD to induce the expression of CYP1A1.

AhR degradation following TCDD treatment

The data presented thus far demonstrated that TCDD mobilizes calcium, triggering calpain activation, which could transform the AhR into a transcription factor, possibly by limited cleavage of the receptor. Subsequent to enhancing CYP1A1 transcription, AhR is shuttled from the nucleus to the cytoplasm where it is degraded. To assess whether calpain contributes to this degradation, TCDD-induced AhR degradation was analyzed in the presence and absence of calpain inhibitor MDL 28170. Treatment of MT-2 cells with TCDD resulted in greater than 75% reduction in AhR protein levels (Fig 5A), and pretreatment with MDL 28170 completely blocked this degradation. However, these treatments with ligand and protease inhibitors, although had an effect on the AhR levels, did not have any effect on the levels of ARNT, the AhR partner for transcriptional activation (Fig 5A).

Recent reports have implicated proteasomes in the degradation of the AhR therefore the proteasome inhibitors MG-132 and epoxomicin were tested. Epoxomicin, a potent and selective proteasome inhibitor, has no cross-activity against non-proteasomal proteases such as calpain (Meng et al., 1999), while MG-132 interferes equally with both proteasomes and calpain (Davarinos and Pollenz, 1999; Mailhes et al., 2002). As shown in Fig. 5B, epoxomicin treatment did not protect the receptor from this TCDD-induced down-regulation whereas MDL 28170 effectively protected the AhR from TCDD-induced down-regulation (Fig. 5B). Unlike epoxomicin, MG-132 inhibited the TCDD-induced degradation of the AhR, to a comparable level of the MDL 28170 (Fig. 5C). These results demonstrate that the degradation of the AhR is a calpain-dependent process.

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

PAHs, such as dioxin, elicit a wide range of toxic effects including carcinogenesis. One way that AhR mediates the toxic responses of these chemicals is through its ability to enhance the transcription of *CYP1A1* gene in many tissues. The protein product of CYP1A1 catalyzes the bioactivation of these chemicals as well as some endogenous hormones producing reactive metabolites that cause DNA damage and initiate neoplasia. Data presented in this report provide compelling evidence that the Ca^{2+}- dependent protease, calpain is a critical player in driving this AhR-mediated process.

For AhR to direct the ligand-induced CYP1A1 transcription it has been established that ligand binding to AhR results in a sequence of events starting with the receptor dissociation from chaperone proteins, such as hsp90 and adoption of conformational changes that allow the AhR to translocate into the nucleus to bind DNA and activate the transcription of CYP1A1 (Whitlock, 1999). However, the mere dissociation of the hsp90 from the receptor complex, although allowing for AhR nuclear translocation, is not sufficient to induce the receptor transactivation (Song and Pollenz, 2002). This observation suggests that additional processing of AhR is required following its dissociation from chaperone proteins. Our data clearly implicate calpain in the transformation required for the AhR nuclear translocation and subsequent transactivation. Inhibition of calpain completely blocked the transcription of CYP1A1, indicating that calpain is required for the transformation of AhR into a transcriptional factor. We demonstrate that the trigger for activation of calpain is provided by elevation in intracellular calcium by AhR ligands such as TCDD, which was previously reported in other cell systems.

Interestingly, previous reports have shown that deletion of the P/S/T domain in the carboxyl terminal of AhR leads to an increase in its transcriptional activity (Kumar et al., 2001). Although calpain prefers Leu or Val as a second residue on the N-terminal side of cleavage site (Wang, 1990), it also recognizes hydrophilic sequences enriched in Pro, Glu, Asp, Ser, and Thr (or PEST sequences) near cleavage sites (Wang et al., 1989). Therefore, it is conceivable that activated calpain could cleave the carboxy-terminal P/S/T domain of AhR resulting in its transcription activation. This truncated form of AhR may be recognized by the importin receptors of the nuclear membrane in a ligand-dependent or

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

ligand-independent manner. Intriguingly enough, such a truncated form of AhR (~90 kDa) was recovered from nuclei of TCDD-treated Hepa-1 cells, which was slightly smaller than the cytosolic AhR in these cells (~95 kDa) (Eltom, unpublished data).

Following transcriptional activation, the liganded-AhR undergoes a rapid degradation leading to a massive depletion (Prokipcak and Okey, 1991). Some studies have suggested that this process is proteasome-dependent (Davarinos and Pollenz, 1999). Conversely, studies by Poland and Glover (Poland and Glover, 1988) reported fragments of AhR following increases in calcium concentration, which they attributed to calpain activation. To distinguish the role of the two protease-systems the current study utilized highly selective inhibitors of both calpain and proteasomes, with no overlapping reactivity. Although the inhibitor MG-132 was used as a proteasome inhibitor to implicate proteasomes in the degradation of the AhR, this report in agreement with others have identified MG-132 as a strong inhibitor of calpain (Mailhes et al., 2002). Moreover, classical calpain inhibitors, which were used to discredit calpain in the AhR degradation process, have been shown to have less affinity for calpain I and II (Bang et al., 2004). In our study, MDL 28170, the most potent inhibitor of calpain, demonstrated that calpain is responsible for the transcriptional activation of AhR and subsequent degradation. In addition, MG-132, which exhibits a cross-specificity for calpain and proteasomes, has blocked the TCDD-induced degradation to the same extent as MDL 28170. On the other hand, the proteasome selective inhibitor, epoxomicin failed to prevent TCDD-induced degradation of the receptor. These lines of evidence suggest that calpain is also responsible for the agonist-dependent degradation of AhR.

Thus, the activation of calpain by TCDD contributes to each of the sequential steps of the AhR-mediated transcriptional activation of CYP1A1 and the subsequent AhR degradation. We propose a model where TCDD elevates intracellular calcium, triggering calpain activation to both initiate and terminate he AhR signaling. In such model, calcium is the second messenger subsequent to TCDD exposure and calpain is the downstream effector molecule. Transcriptional activation of CYP1A1 by AhR has been reported in response to agents that do not bind AhR such as caffeine (Goasduff et al., 1996), oltipraz (Le Ferrec et al., 2002) and omeprazole (Quattrochi and Tukey, 1993). AhR is also

activated by disturbing cellular adhesion to extra-cellular matrix (Sadek and Allen-Hoffmann, 1994), increasing intracellular Ca^{2+} (Reiners et al., 1990), or disturbing cytoskeleton (Scholler et al., 1994). The common factor between all these phenomena of ligand-independent activation of AhR is the increase in the intracellular calcium, providing the second messenger to activate AhR through the effector enzyme, calpain. Further work is required to delineate the exact mechanisms of how calpain activates AhR.

Recent studies have established that AhR, independent of PAH ligands, is directly responsible for inducing cancers of the stomach (Andersson et al., 2002) and pancreas (Koliopanos et al., 2002) and the progression of breast carcinoma (Eltom, unpublished data). The involvement of calpain in the activation of AhR identifies calpain as a potential therapeutic target for AhR associated cancers. The advantage of this strategy is that calpain exists in a latent form requiring sustained elevation in intracellular calcium.

Acknowledgements The authors acknowledge Professors Ifeanyi Arinze, Fred Guengerich and Lee Limbird for their invaluable critical reviewing of this manuscript.

References

Andersson P, McGuire J, Rubio C, Gradin K, Whitelaw ML, Pettersson S, Hanberg A and Poellinger L (2002) A constitutively active dioxin/aryl hydrocarbon receptor induces stomach tumors. *Proc Natl Acad Sci U S A* **99**:9990-5.

Band V, Zajchowski D, Swisshelm K, Trask D, Kulesa V, Cohen C, Connolly J and Sager R (1990) Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res* **50**:7351-7357.

Bang B, Baadsgaard O, Skov L and Jaattela M (2004) Inhibitors of cysteine cathepsin and calpain do not prevent ultraviolet-B-induced apoptosis in human keratinocytes and HeLa cells. *Arch Dermatol Res* **296**:67-73.

Carragher NO, Fonseca BD and Frame MC (2004) Calpain activity is generally elevated during transformation but has oncogene-specific biological functions. *Neoplasia* **6**:53-73.

Carver LA and Bradfield CA (1997) Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. *J Biol Chem* **272**:11452-11456.

Conney AH (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res* **42**:4875-917.

Davarinos NA and Pollenz RS (1999) Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. *J Biol Chem* **274**:28708-28715.

Eltom SE, Zhang L and Jefcoate CR (1999) Regulation of cytochrome P-450 (CYP) 1B1 in mouse Hepa-1 variant cell lines: A possible role for aryl hydrocarbon receptor nuclear translocator (ARNT) as a suppressor of CYP1B1 gene expression. *Mol Pharmacol* **55**:594-604.

Goasduff T, Dreano Y, Guillois B, Menez JF and Berthou F (1996) Induction of liver and kidney CYP1A1/1A2 by caffeine in rat. *Biochem Pharmacol* **52**:1915-1919.

Gonzalez FJ, Mackenzie PI, Kimura S and Nebert DW (1984) Isolation and characterization of full-length mouse cDNA and genomic clones of 3-methylcholanthrene-inducible cytochrome P1-450 and P3-450. *Gene* **29**:281-292.

Hanneman WH, Legare ME, Barhoumi R, Burghardt RC, Safe S and Tiffany-Castiglioni E (1996) Stimulation of calcium uptake in cultured rat hippocampal neurons by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology* **112**:19-28.

Jones PB, Galeazzi DR, Fisher JM and Whitlock JP, Jr. (1985) Control of cytochrome P1-450 gene expression by dioxin. *Science* **227**:1499-1502.

Kazlauskas A, Sundstrom S, Poellinger L and Pongratz I (2001) The hsp90 chaperone complex regulates intracellular localization of the dioxin receptor. *Mol Cell Biol* **21**:2594-2607.

Koliopanos A, Kleeff J, Xiao Y, Safe S, Zimmermann A, Buchler MW and Friess H (2002) Increased arylhydrocarbon receptor expression offers a potential therapeutic target for pancreatic cancer. *Oncogene* **21**:6059-6070.

Kumar MB, Ramadoss P, Reen RK, Vanden Heuvel JP and Perdew GH (2001) The Q-rich subdomain of the human Ah receptor transactivation domain is required for dioxin-mediated transcriptional activity. *J Biol Chem* **276**:42302-42310.

Le Ferrec E, Lagadic-Gossmann D, Rauch C, Bardiau C, Maheo K, Massiere F, Le Vee M, Guillouzo A and Morel F (2002) Transcriptional induction of CYP1A1 by oltipraz in human Caco-2 cells is aryl hydrocarbon receptor- and calcium-dependent. *J Biol Chem* **277**:24780-24787.

Ma Q and Whitlock JP, Jr. (1997) A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Biol Chem* **272**:8878-8884.

Mailhes JB, Hilliard C, Lowery M and London SN (2002) MG-132, an inhibitor of proteasomes and calpains, induced inhibition of oocyte maturation and aneuploidy in mouse oocytes. *Cell Chromosome* **1**:2.

Mathiasen IS, Sergeev IN, Bastholm L, Elling F, Norman AW and Jaattela M (2002) Calcium and calpain as key mediators of apoptosis-like death induced by vitamin D compounds in breast cancer cells. *J Biol Chem* **277**:30738-30745.

Meng L, Mohan R, Kwok BH, Elofsson M, Sin N and Crews CM (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc Natl Acad Sci U S A* **96**:10403-10408.

Meyer BK and Perdew GH (1999) Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization. *Biochemistry* **38**:8907-17.

Poland A and Glover E (1988) Ca2+-dependent proteolysis of the Ah receptor. *Arch Biochem Biophys* **261**:103-111.

Poland A and Glover E (1990) Characterization and strain distribution pattern of the murine Ah receptor specified by the Ahd and Ahb-3 alleles. *Mol Pharmacol* **38**:306-312.

Poland A and Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**:517-554.

Pollenz RS (1996) The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Mol Pharmacol* **49**:391-398.

Pollenz RS, Sattler CA and Poland A (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. *Mol Pharmacol* **45**:428-438.

Potter DA, Tirnauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, Mier JW, Maki M and Herman IM (1998) Calpain regulates actin remodeling during cell spreading. *J Cell Biol* **141**:647-662.

Prokipcak RD and Okey AB (1991) Downregulation of the Ah receptor in mouse hepatoma cells treated in culture with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Can J Physiol Pharmacol* **69**:1204-1210.

Quattrochi LC and Tukey RH (1993) Nuclear uptake of the Ah (dioxin) receptor in response to omeprazole: transcriptional activation of the human CYP1A1 gene. *Mol Pharmacol* **43**:504-508.

Reick M, Robertson RW, Pasco DS and Fagan JB (1994) Down-regulation of nuclear aryl hydrocarbon receptor DNA-binding and transactivation functions: requirement for a labile or inducible factor. *Mol Cell Biol* **14**:5653-5660.

Reiners JJ, Jr., Cantu AR and Pavone A (1990) Modulation of constitutive cytochrome P-450 expression in vivo and in vitro in murine keratinocytes as a function of differentiation and extracellular Ca2+ concentration. *Proc Natl Acad Sci U S A* **87**:1825-1829.

Sadek CM and Allen-Hoffmann BL (1994) Cytochrome P450IA1 is rapidly induced in normal human keratinocytes in the absence of xenobiotics. *J Biol Chem* **269**:16067-16074.

Scholler A, Hong NJ, Bischer P and Reiners JJ, Jr. (1994) Short and long term effects of cytoskeleton-disrupting drugs on cytochrome P450 Cyp1a-1 induction in murine hepatoma 1c1c7 cells: suppression by the microtubule inhibitor nocodazole. *Mol Pharmacol* **45**:944-954.

Song Z and Pollenz RS (2002) Ligand-dependent and independent modulation of aryl hydrocarbon receptor localization, degradation, and gene regulation. *Mol Pharmacol* **62**:806-816.

Tannheimer SL, Barton SL, Ethier SP and Burchiel SW (1997) Carcinogenic polycyclic aromatic hydrocarbons increase intracellular Ca2+ and cell proliferation in primary human mammary epithelial cells. *Carcinogenesis* **18**:1177-1182.

Wang KK (1990) Developing selective inhibitors of calpain. *Trends Pharmacol Sci* **11**:139-42.

Wang KK, Villalobo A and Roufogalis BD (1989) Calmodulin-binding proteins as calpain substrates. *Biochem J* **262**:693-706.

Whitlock JP, Jr. (1999) Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* **39**:103-125.

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes:

This research was funded in part by NIH grants RR03032 15, CA91408, and Department of Defense grant DAMD17-02-01-0483 to SEE. Yolanda Dale was supported through R25 GM59994 and T32 CA09592.

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1. The effect of TCDD on intracellular free calcium concentration and calpain activation in MT-2 cells. MT-2 cells were pre-loaded with 5 μM Fluo-4,AM for 45 min at 37°C. Fluo-4 loaded cells were then stimulated with varying concentrations of TCDD, or 10 μM Ionomycin, and control cells received DMSO for 15 min. Fluorescence images of cells viewed by the IX50 Olympus Fluorescence microscope (Excitation at 385 nm, emission at 512 nm) were captured by MagnaFire digital camera. Relative fluorescence values for the 20 min time point was quantified using the NIH ImageJ analysis software and values for each experiment were normalized to Ionomycin values; plotted values are average of three independent experiments.

Figure 2. Activation of calpain in MT-2 cells following exposure to TCDD and ionomycin in the absence and presence of calpain and proteasome inhibitors. Fluorescence intensity corresponding to calpain activity was captured at 20 min by fluorescence microscopy (magnification X 200). Blue fluorescence is indicative of calpain activity. The average fluorescence units of the images were quantified using the ImageJ software from several fields, and plotted values are the average of n =3 independent experiments. *, $p < 0.05$; comparing drug treatments to vehicle control.

Figure 3. The effect of calpain on the subcellular localization of AhR in Hepa-1 cells. Murine Hepa cells grown on cover slips were pre-treated with calpain inhibitor, MDL 28170 (15 μM) or its vehicle DMSO for 2 hr, then exposed for 3 hr to 1 nM TCDD or its vehicle DMSO. Cells were fixed and immuno-stained for AhR followed by FITC-conjugated secondary antibodies as described in the methods. Cells were viewed using the IX50 Olympus Fluorescence microscope and scored for cytoplasmic and nuclear staining form multiple fields. A representative of the cellular staining for AhR is presented in 3A. Cells were scored for cytoplasmic and nuclear staining from multiple fields from the two experiments. Average numbers from two different experiments on the two different cell types (Hepa-1 and MT-2) were normalized to a percentage within each experiment and the average values were plotted in 3B.

Figure 4. Inhibition of TCDD-induced CYP1A1 transcription by treatments that suppress calpain activity. *(A)* RT-PCR analysis of the effect of calpain inhibitors on the TCDD- induced CYP1A1

expression in MT-2 cells. Cells were pre-exposed to 15 μM PD150606 or 15 μM MDL 28170, or their vehicle (DMSO) for 2 hr and then treated with TCDD for additional 3 hr. The relative expression of CYP1A1 was quantified as described in the material and methods and normalized to the level of GAPDH or 18S. A representative gel image of one PCR analysis is presented for illustration. The bars represent the average ± S.D. for three independent experiments and duplicate cultures, which were PCR-assayed in duplicates. * denotes a $p < 0.05$, comparing DMSO and TCDD treated cells. † denotes a $p < 0.05$, comparing treatments with TCDD alone to MDL28170/ PD150606-TCDD treated cells. *(B)* Differential effect of inhibitors of calpain and proteasome on TCDD-induced CYP1A1 expression. Triplicate cultures of MT-2 cells were pre-treated with the different inhibitors or the vehicle for 2 hr followed by exposure to TCDD for an additional 3 hr. The relative expression of CYP1A1 mRNA was normalized to the levels of 18s mRNA, which was run simultaneously with CYP1A1 as described in materials and methods. The bars represent the average ± S.D. of two independent experiments. * denotes $p < 0.05$ comparing TCDD to basal levels. † denotes $p < 0.05$ comparing TCDD to the MDL28170/ MG-132- TCDD.

Figure 5. Inhibition of calpain suppresses the TCDD-induced degradation of the AhR. **(*A*)** Triplicate culture plates of MT-2 cells were pre- exposed to 15 μM MDL28170 or the vehicle (DMSO) for 2 hr at 37ºC followed by 1 nM TCDD or DMSO for additional 3 hr. A representative Western blot image of one experiment is presented in the upper panel for illustration. Bars represent the average ± S.D. values from three independent experiments. * denote $p < 0.05$, comparing TCDD treated cells to DMSO controls. †, $p < 0.05$, comparing MDL 28170-TCDD treated cells to TCDD-treated. ***(B)*** MT-2 cell cultures were pretreated with 15 μM MDL28170, 25 μM MG-132 or vehicle (DMSO) for 2hr followed by a 3 hr TCDD treatment. *, $p < 0.05$, comparing TCDD to DMSO. †, $p < 0.05$, comparing TCDD to MDL28170-TCDD and MG-132-TCDD. ***(C)*** Western blot analysis of AhR following calpain and proteasome inhibition. Duplicate cultures of MT-2 cells were pretreated with proteasome inhibitor epoxomicin (25 μM) or calpain inhibitor MDL28170 (15μM) followed by 3 hr TCDD (1 nM) or DMSO. *, $p < 0.05$, compared to DMSO control. †, $p < 0.05$, comparing TCDD to MDL28170/epoxomicin-TCDD. The bars represent the average ± S.D. for three independent experiments.

Figure 1

Molecular Pharmacology Fast Forward. Published on August 4, 2006 as DOI: 10.1124/mol.106.027474
This article has not been copyedited and formatted. The final version may differ from this version.

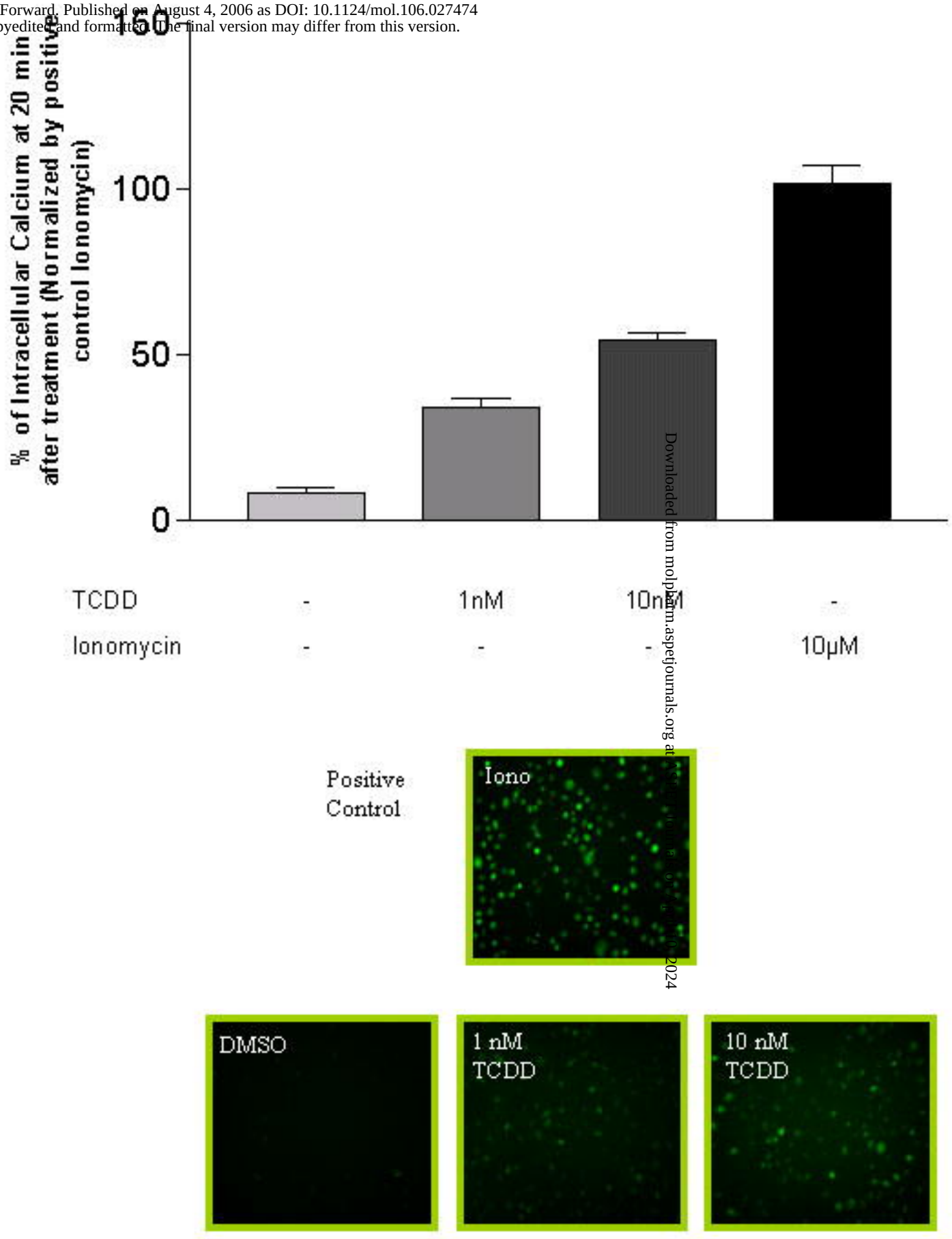
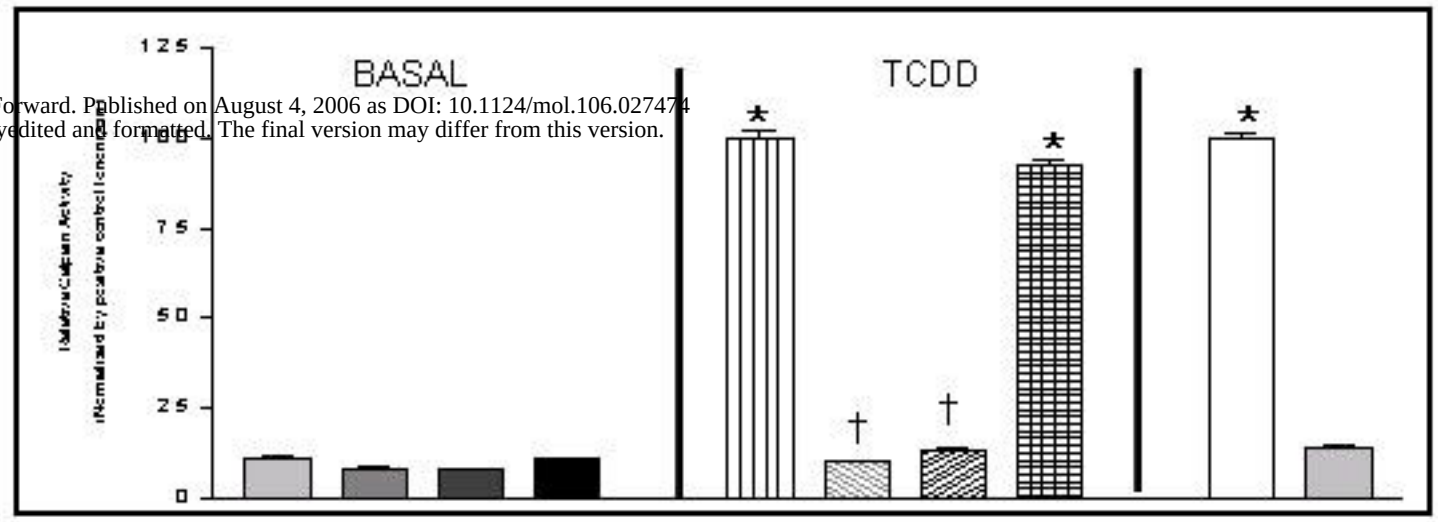


Figure 2



TCDD		-	-	-	-	+	+	+	+	-	-
Ionomycin		-	-	-	-	-	-	-	-	+	+
Inhibitors											
Calpain	MDL28170	-	+	-	-	-	+	-	-	-	+
Calp /Pro	MG-132	-	-	+	-	-	-	+	-	-	-
Proteasome	Epoxomicin	-	-	-	+	-	-	-	+	-	-

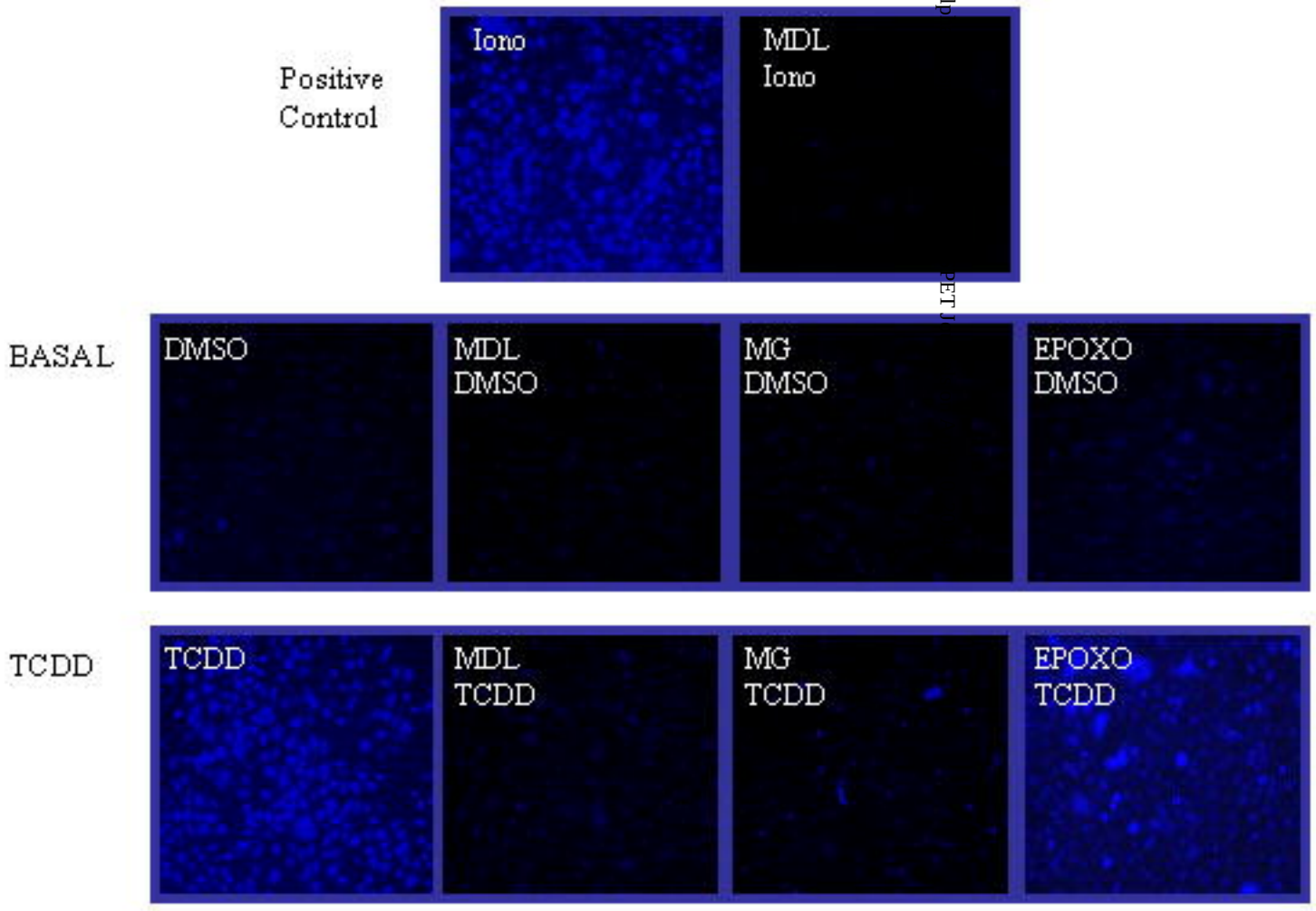
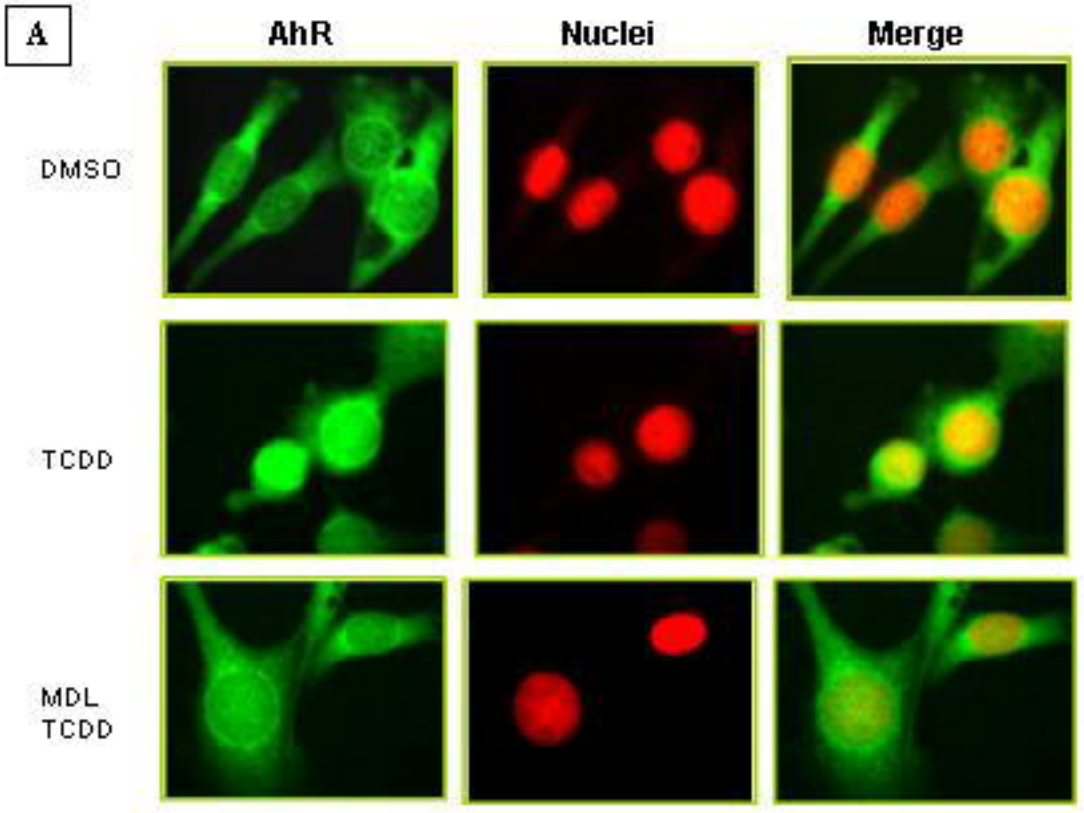


Figure 3


A
AhR
Nuclei
Merge
DMSO
TCDD
MDL
TCDD

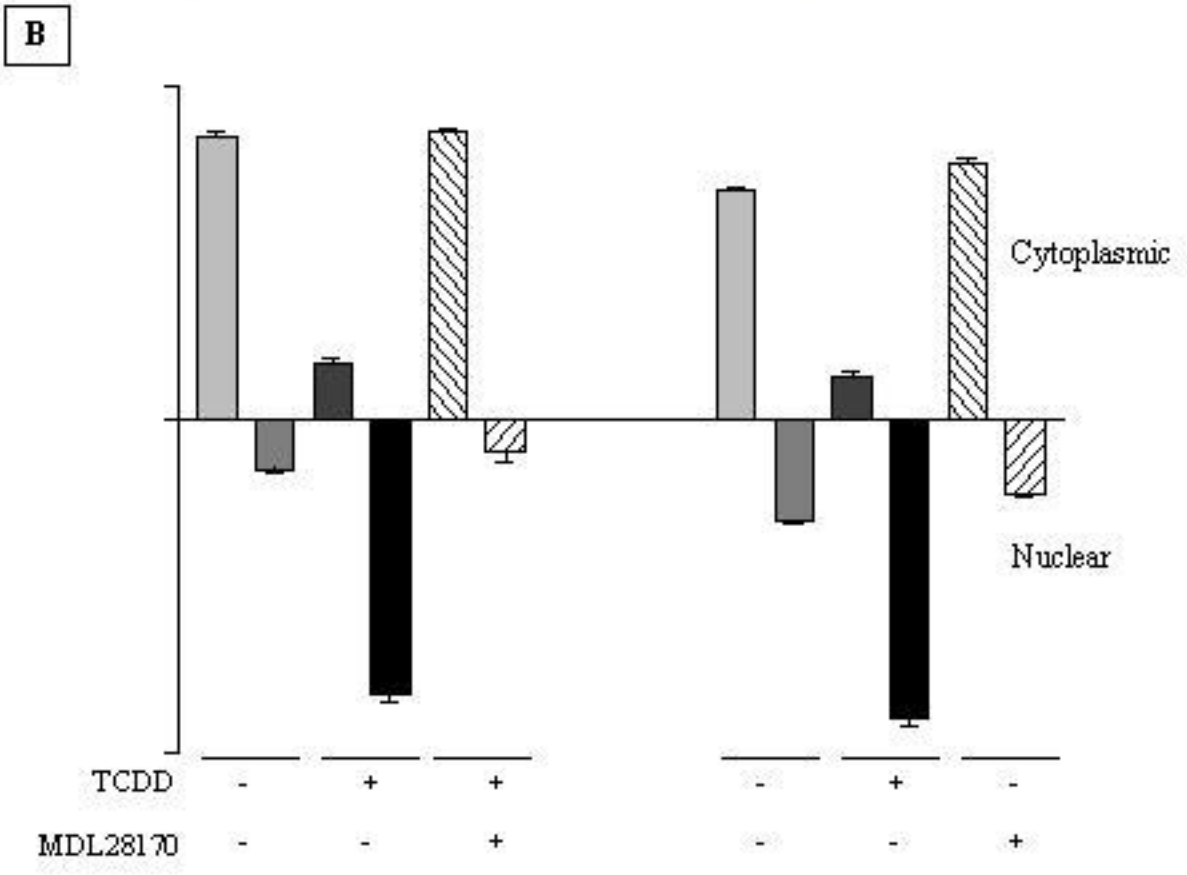

B
Cytoplasmic
Nuclear
TCDD - + + - + -
MDL28170 - - + - - +

Figure 4