ACCELERATED COMMUNICATION

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) and Graduate Program in Pharmacology (Y.R.D.), Meharry Medical College, Nashville, Tennessee
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ABSTRACT
The aryl hydrocarbon receptor (AhR) is a ligand-activated basic-helix-loop-helix transcription factor that binds polyaromatic hydrocarbons (PAH), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 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 toward \( t \)-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin, a calpain-specific substrate. Inhibition of calpain activity by the \( N \)-benzyl-oxycarbonyl-Val-Phe-aldehyde (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 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (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 after TCDD-induced transactivation, the AhR undergoes a massive depletion; we show here that selective calpain inhibitors can block this step, which suggests 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.

The AhR is a ligand-activated basic helix-loop-helix transcription factor that regulates the adaptive and toxic responses to a variety of environmental carcinogens, including polyaromatic hydrocarbons (PAH), such as TCDD (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 aryl hydrocarbon receptor nuclear translocator (ARNT) protein (Kazlauskas et al., 2001), and binding of this heterodimer to DNA recognition motifs designated as xenobiotic-responsive elements 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 cytochrome P450 genes are catalytically active in metabolizing not only many endogenous compounds such as \( \beta \)-estradiol, but also many drugs, dietary components, mutagens, carcinogens,
and environmental pollutants (Conney, 1982). After transcriptional activation, the liganded-AhR undergoes a rapid degradation (Prokipcak and Okey, 1991; Reick et al., 1994; Pollenz, 1996). Studies have suggested that this ligand-induced down-regulation of AhR is proteasome-dependent (Davarnos 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 calpain (Poland and Glover, 1988), a member of 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 (Wang, 1990; Potter et al., 1998). Of the several calpain isoforms, calpain I (μ-calpain), and calpain II (m-calpain) are ubiquitous enzymes, activated with low and high calcium concentrations, respectively. Because 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. In this study, we have examined the involvement of calpain in the transformation process required for the nuclear translocation, transactivation, and subsequent degradation of AhR.

Calpain Activity Assay. Calpain activity in MT-2 cells was assessed by fluorescence microscopy using the calpain substrate BOC-LM-CMAC (Invitrogen). The nonfluorescent cell-permeable substrate is conjugated by intracellular thiolos 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. In brief, cells were incubated with 50 μM BOC-LM-CMAC for 20 min at 37°C. Fluorescence intensity corresponding to calpain activity was visualized, and images were captured with an Olympus IX50 fluorescence microscope using a digital camera with MagnaFire software, and quantified by ImageJ software (http://rsb.info.nih.gov/ij/). 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 six-well plates were washed in phosphate-buffered saline and then fixed by incubation in a (1:1) methanol/aceton solution at 4°C for 30 min and subsequently air-dried. For staining, cells were rinsed and hydrated with TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20) and transferred to clean six-well plates. The cover slips were incubated at room temperature for 1 h in 4% milk solution in TBST to block non-specific binding. The cover slips were then incubated at room temperature for 1 h 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-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 h. The cover slips were then washed extensively and mounted on glass slides using mounting solution containing 1,4-diazabicyclo[2.2.2]octane as an anti-fading agent.

Preparation of Total Cell Lysates and Immunoblotting. After treatments, cell monolayers were lysed in 1 ml of TRizol, which allowed for simultaneous isolation of RNA and protein. After lysis, both RNA and protein were isolated according to the vendor’s instructions. The protein pellets were resuspended in 2% SDS and sonicated briefly to dissolve. The protein concentration in cell extracts was determined using a BCA assay kit, per the manufacturer’s instructions. Equivalent amounts of protein (10 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred for 2 h at 175 V to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 1 h 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 horseradish peroxidase-coupled anti-rabbit or anti-mouse secondary antibody (1: 20,000) for 1 h 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 Inc., Upland, CA). Thereafter, 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. After 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 CAG-3’ and the reverse primer sequence 5’-GGG AAC CTA GCC TCA ACA TC-3’. Housekeeping gene glyceraldehyde 5-phosphate dehydrogenase was used as an internal control. Forward primer was 5’-TCC ACC CTG TTT CTG TA-3’. 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).

Real Time RT-PCR. Real-time quantitative PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). cDNA generated from TCDD-treated MT-2 cells was serially diluted to establish a standard curve (20,000–0.2 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’/H11032 forward primer and 5’-AGG TAG CGA AGA ATA GGG ATG AAC TC-3’ reverse primer) with the following PCR parameters: 95°C for 5 min followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s. Reactions for the 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 AGG TAG CGA AGA ATA GGG ATG AAC TC-3’/H11032 forward primer and 5’-TGC CAG AGT CTC GTT CGT TAT CG-3’ 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 Prism software (GraphPad Software, San Diego, CA) by one-way analysis of variance and Tukey-Kramer multiple comparison tests of values from control versus treated samples.

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 has low concentrations of AhR (Tannheimer et al., 1997), it was important to determine whether TCDD is capable of evoking an increase in intracellular calcium in the MT-2 human metastatic breast cancer cell line, which has high concentrations of AhR. We measured [Ca2+]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 [Ca2+]i that was comparable with values of the calcium ionophore ionomycin, which results in a profound increase in [Ca2+]i, compared with 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 with 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. Figure 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 (Potter et al., 1998; Mathiasen et al., 2002).

TCDD-Induced Nuclear Accumulation of AhR Is Calpain-Dependent. 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 after 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 before TCDD exposure resulted in predominant cytoplasmic staining, indicating that calpain influences the localization of the AhR after 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). It is noteworthy that 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 also used to provide more quantitative analysis of CYP1A1 mRNA expression. The results in Fig. 4B show that treatment with TCDD for 3 h resulted in an increase in CYP1A1 expression of approximately 40-fold over DMSO-treated control. MDL 28170 completely blocked the TCDD ligand-induced CYP1A1 gene transcription. Likewise, MG-132, which also inhibits calpain, inhibited TCDD-induced CYP1A1 gene expression to the same degree as MDL28170, whereas 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 after TCDD Treatment.** The data presented thus far demonstrate that TCDD mobilizes calcium, triggering calpain activation, which could transform the AhR into a transcription factor, possibly by limited cleavage of the receptor. After 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 the 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, although these treatments with ligand and protease inhibitors had an effect on the AhR levels, they had no 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 nonproteasomal proteases such as calpain (Meng et al., 1999), whereas 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

![Fig. 2. Activation of calpain in MT-2 cells after 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, 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.](image-url)
results demonstrate that the degradation of the AhR is a calpain-dependent process.

Discussion

PAHs, such as TCDD, 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 Ca2+-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 insufficient to induce the receptor transactivation (Song and Pollen, 2002). This observation suggests that additional processing of AhR is required after 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 com-

Fig. 3. The effect of calpain on the subcellular localization of AhR in Hepa-1 cells. Murine Hepa cells grown on cover slips were pretreated with calpain inhibitor, MDL 28170 (15 μM), or its vehicle DMSO for 2 h, then exposed for 3 h to 1 nM TCDD or its vehicle DMSO. Cells were fixed and immunostained for AhR followed by fluorescein isothiocyanate-conjugated secondary antibodies as described in the methods. Cells were viewed using the IX50 Olympus Fluorescence microscope and scored for cytoplasmic and nuclear staining from 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.

Fig. 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, 15 μM MDL 28170, or their vehicle (DMSO) for 2 h and then treated with TCDD for additional 3 h. The relative expression of CYP1A1 was quantified as described under Materials and Methods and normalized to the level of glyceraldehyde 3-phosphate dehydrogenase 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. *, p < 0.05, comparing DMSO- and TCDD-treated cells. †, 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 pretreated with the different inhibitors or the vehicle for 2 h followed by exposure to TCDD for an additional 3 h. The relative expression of CYP1A1 mRNA was measured by real-time reverse transcription-PCR and normalized to the levels of 18S mRNA, which was run simultaneously with CYP1A1 as described under Materials and Methods. The bars represent the average ± S.D. of two independent experiments. *, p < 0.05 comparing TCDD to basal levels. †, p < 0.05 comparing TCDD to the MDL28170/MG-132-TCDD.
pletely 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.

Previous reports have shown that deletion of the P/S/T domain in the carboxyl-terminal domain 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 carboxyl-terminal P/S/T domain of AhR resulting in its transcriptional activation. This truncated form of AhR may be recognized by the importin receptors of the nuclear membrane in a ligand-dependent or -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) (S. E. Eltom, unpublished data).

After transcriptional activation, the liganded-AhR undergoes a rapid degradation leading to a massive depletion (Prokipcak and Ökey, 1991). Some studies have suggested that this process is proteasome-dependent (Davarinos and Pollenz, 1999). On the other hand, Poland and Glover (1988) reported fragments of AhR after increases in calcium concentration, which they attributed to calpain activation. To distinguish the role of the two protease systems, the current study used 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, has identified MG-132 as a strong inhibitor of calpain (Mailhes et al., 2002). Moreover, classic 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 in which TCDD elevates intracellular calcium, triggering calpain activation to both initiate and terminate the AhR signaling. In such a 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 extracellular matrix (Sadek and Allen-Hoffmann, 1994), increasing intracellular Ca\(^{2+}\) (Reiners et al., 1990), or disturbing cytoskeleton (Scholler et al., 1994). The common factor among 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 by which 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 (S.E. 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.

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References


Address correspondence to: Dr. Sakina Eltom, 1005 Dr. D.B. Todd Blvd, Nashville, TN 37208, Tel/Fax: (615) 327-5713, seltom@mmc.edu