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POSSIBLE INVOLVEMENT OF THE AH RECEPTOR IN THE INDUCTION OF CYTOCHROME P-450IA1 UNDER CONDITIONS OF HYDRODYNAMIC SHEAR IN MICROCARRIER-ATTACHED HEPATOMA CELL LINES

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SUMMARY: The exposure of two hepatoma cell lines, Hep G2 and Hepa-1, to moderate hydrodynamic shear, in microcarrier-attached suspension cultures, resulted in the transient induction of cytochrome P-450IA1 (CYP1A1). Both cell lines have been characterized with respect to their Ah receptor (AhR) concentrations and induce CYP1A1 in response to exposure to xenobiotics such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Using an AhR antagonist, α-naphthoflavone (α-NF) and a protein kinase C (PKC) inhibitor, staurosporine (ST), in the Hep G2 cell line, the induced CYP1A1 activity was modulated in the same manner as when the cells were coexposed to TCDD and either α-NF or ST. Exposure of the Hep G2 cell line to TCDD and shear resulted in both enhancement of the induced CYP1A1 activity in addition to a competitive response. Finally, using the wild type and AhR defective Hepa-1 cell lines, it was demonstrated that a functional AhR was required for shear-induced CYP1A1 expression. The data obtained in the three cell lines indicate a role for the AhR in the induction of CYP1A1 by shear in agitated microcarrier cultures.

The induction of CYP1A1 is a model response for exposure to the environmental contaminant TCDD both *in vivo* and *in vitro* (1-3). The mechanism of CYP1A1 induction has been studied extensively and is known to require the binding of TCDD to the cytosolic AhR-hsp 90 complex followed by binding to the Ah receptor nuclear translocating protein (ARNT) and subsequent release of the hsp 90 dimer (4-8). Translocation of the ARNT-AhR-TCDD complex into the nucleus and its binding to

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ABBREVIATIONS: α-NF, α-naphthoflavone; AhR, Ah receptor; ARNT, Ah receptor nuclear translocating protein; CYP1A1, cytochrome P-450IA1; DRE, dioxin-responsive element; EOR, ethoxyresorufin; EROD, ethoxyresorufin-O-dealkylase; K_d, equilibrium dissociation constant; PKC, protein kinase C; ST, staurosporine; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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dioxin-responsive elements (DREs) upstream of the CYP1A1 gene results in transcription of CYP1A1 mRNA.

In the absence of TCDD, we observed the induction of CYP1A1 in agitated microcarrier cultures of Hep G2 cells (manuscript in preparation). Microcarrier cultures allow the cultivation of anchorage-dependent cell lines in suspension. One of the advantages of microcarrier suspension cultures is the ease of observing the kinetics of cellular responses in a homogeneous culture. However, one of the major disadvantages of microcarrier cultures is the increase in cell damage due to hydrodynamic shear stress (9-13).

The shear-induced CYP1A1 expression has been shown to involve the arachidonate cascade in two human hepatoma cell lines, Hep G2 and Mz-Hep-1 (manuscript in preparation). However, the exact mechanism by which arachidonic acid or one of its metabolites is involved in the induction of CYP1A1 is not known. The cell lines that were used in previous studies and in the present study have been characterized with respect to AhR concentrations and all three cell lines have inducible CYP1A1 activity. The Hepa-1 cells have approximately 35,000 AhR sites per cell and the equilibrium dissociation constant (K_d) for binding of ^{3}H-TCDD to the AhR has been reported to be approximately 1.2 nM (14, 15). The Mz-Hep-1 cell line has approximately 30,000 AhR sites per cell with a K_d of 5.4 nM (15). The Hep G2 cells line has fewer AhR sites per cell compared to both the Hepa-1 and Mz-Hep-1 cell lines, 14,000 sites per cell, and the K_d is also higher than the two other cell lines, approximately 9 nM (14).

In order to examine the possible role of the AhR in the mechanism of shear-induced CYP1A1 induction three strategies were used. First, two compounds that have been shown to decrease TCDD-induced CYP1A1 expression were used. The first compound is α-NF, an AhR antagonist (16-19). The second compound is the PKC inhibitor ST (20-23), which has been shown to decrease cytosolic AhR concentrations after prolonged exposures to ST (24). Second, the effect of coexposure of the Hep G2 cell line to TCDD and two forms of increased shear was examined. Third, two clones of the mouse hepatoma cell line Hepa-1, the wild-type and AhR defective mutant, were used to further investigate the possible role of the AhR in shear-induced CYP1A1 expression (25-27).

MATERIALS AND METHODS

Materials: Crystal violet, citric acid, α-NF, ST, and Cytodex 3 microcarriers were purchased from Sigma Chemical Co. (St. Louis, MO). MEM, S-MEM, α-MEM, RPMI 1640, trypsin, and FBS were purchased from Gibco BRL Life Technologies (Grand Island, NY). Ethoxyresorufin (EOR) and resorufin were purchased from Molecular Probes (Eugene, OR). TCDD was received as a gift from Paracelsian, Inc. (Ithaca, NY). Spinner flasks (50 ml) and spinner plates were purchased from Bellco (Vineland, NJ).

Cell Culture: Hep G2 cells, obtained from ATCC (Rockville, MD), is a continuous human hepatoma cell line. The Hep G2 cell line was derived from a tumor biopsy (28). The Hep G2 cells were grown in MEM supplemented with 10% FBS at 37 ^oC in 95% air and 5% CO_2 in T-flask cultures. Cells were passaged using split ratios of

1:4 at 6-day intervals. Cells were used for experiments within 20 passages. One T-75 flask was used to inoculate one 50-ml spinner flask with a working volume of 40 ml S-MEM and a microcarrier density of 3 g/l using the procedure of Forestell *et al.* (29). The spinner flasks were equipped with a round-tipped impeller with 0.6 cm diameter and 4 cm length. Porous foam closures were used to ensure adequate oxygen supply. Medium was exchanged in the spinner flasks after 2 days. Cells were grown in spinner flask at 70 rpm for 3 days before being used in an experiment at which point the cells on the microcarriers were approximately 80% confluent.

Wild-type and AhR defective Hepa-1 cell lines, obtained from Paracelsian Inc. (Ithaca, NY), are continuous mouse hepatoma cell lines. The Hepa-1 cells were grown in RPMI 1640 supplemented with 5% FBS at 37 ^{o}C in 95% air and 5% CO_2 in T-flask cultures. Cells were passaged using split ratios of 1:10 at 3-day intervals. Cells were used for experiments within 20 passages. One T-75 was used to inoculate one 50-ml spinner flask with a working volume of 40 ml. The microcarrier inoculation procedure followed was the same as for the Hep G2 cells as was the spinner flask configuration. Medium was exchanged on day 2 and cells were used on day 3 for experiments and were approximately 90% confluent.

Spinner Flask Experiments: Cells, together with the medium, were transferred from the growth spinner flask to a freshly washed and autoclaved flask and placed in the same incubator and on the same spinner plate at 70 rpm for the standard control experiments. The cells were transferred slowly down the arm of the spinner flask with a standard 10-mL pipet. The effects of increasing shear on the response were studied by increasing the agitation rate and using a modified spinner flask equipped with an additional paddle-like impeller, of length 4.5 cm and width 1.5 cm, mounted on the impeller shaft.

Determination of Cell Number: The method of van Wezel was used to determine cell number (30). 0.5-ml aliquots of immobilized cells were washed twice with D-PBS. The D-PBS was then removed and the cells were resuspended in 0.1 M citric acid. After 10 minutes, cells were stained with a 1:1 ratio of cell suspension to 0.15% crystal violet in 0.1 M citric acid. After staining for 1 hour, cells were counted using a hemacytometer.

Measurement of CYP1A1 Activity: CYP1A1 activity was measured as ethoxyresorufin-O-dealkylase (EROD) activity by a modification of the method of Burke and Mayer for suspended cells (31, 32). 0.5-ml aliquots of cells were washed twice with D-PBS. The D-PBS was removed and the cells resuspended in 2-ml of reaction buffer (0.05 M Trizma base, 0.01 M $MgCl_2$, 0.2 M sucrose). EROD activity was measured after addition of 2 µl of 0.5 mM EOR. The reaction was performed in a SLM-Aminco AB2 Spectrofluorometer (SLM-Aminco, Urbana, IL) at 37 ^{o}C in a stirred cuvette holder. The change in fluorescence intensity with respect to time was converted to pmol resorufin formed per minute using a standard curve for resorufin fluorescence intensity.

RESULTS AND DISCUSSION

The first strategy employed to study the role of the AhR in shear-induced CYP1A1 expression examined the effect of two drugs α-NF and ST that have been shown to reduce the response of TCDD-induced CYP1A1 expression in several hepatoma cell lines (16, 18, 19, 22-24). Figure 1A demonstrates that 1 µM α-NF effectively blocks the induction of CYP1A1 by 1 nM TCDD up to 15 hours in the Hep G2 cell line. Merchant et al. found similar results using the MCF-7 cell line (19). Coexposure of MCF-7 cells to 1 nM TCDD and α-NF at concentrations ranging from

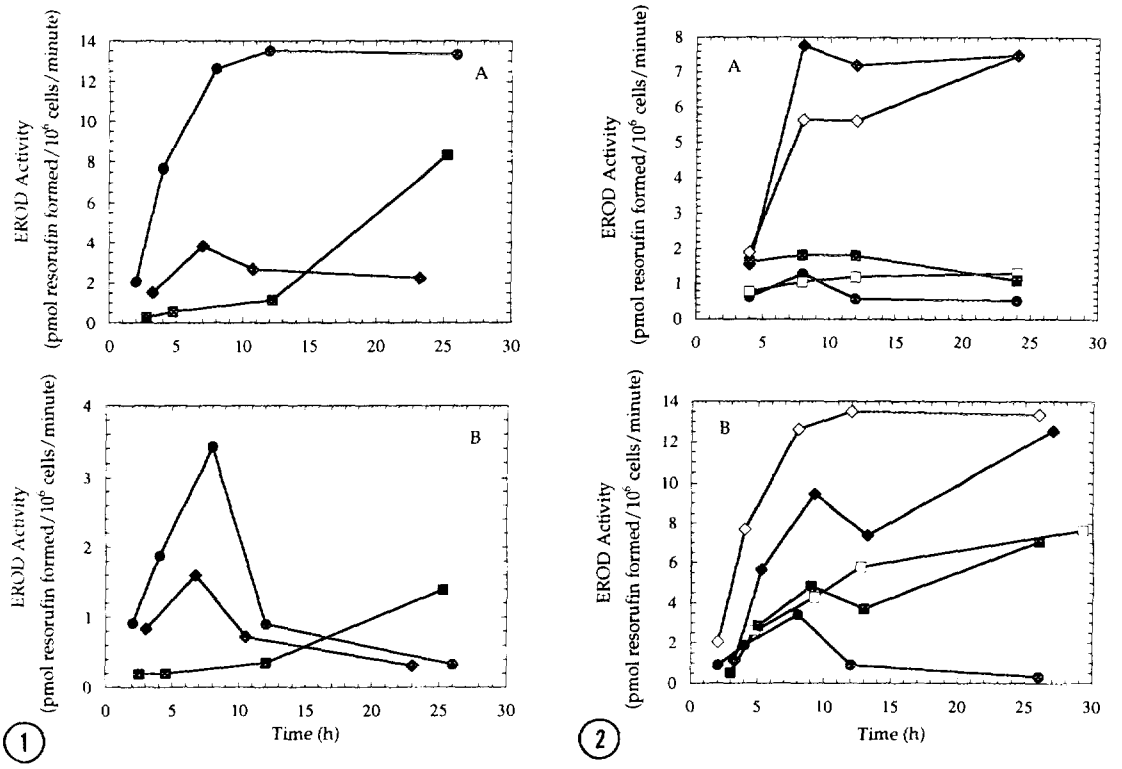

Figure 1. The effect of 1 μM α-NF (■) and 100 nM ST (◆) on the kinetics of CYP1A1 induction for microcarrier-attached Hep G2 cells exposed to (A) 1 nM TCDD (●) and (B)hydrodynamic shear (●). Cells were either treated with either α-NF directly or ST for 4 hours before either TCDD exposure or initiating the shear stress. The shear stress was initiated by transferring the cells from the growth spinner flask to a fresh spinner flask equipped with an additional paddle-like impeller and then the flasks were agitated at 100 rpm. The volume of α-NF and ST added did not exceed 0.05% (v/v). Each point represents the average of duplicate samples for a typical experiment. Each experiment was repeated at least twice.

Figure 2. The kinetics of CYP1A1 induction in microcarrier-attached Hep G2 cells coexposed to TCDD and hydrodynamic shear. Cells were transferred to new spinner flask either identical to the growth flasks (A) or to flasks equipped with a flat-edged baffle and agitated at 100 rpm (B). The control shear response (●) is compared to TCDD treatment alone or TCDD and shear coexposure. (A) Two concentrations of TCDD were used:0.05 nM (□) and 0.5 nM (◇). (B) The two concentrations of TCDD used were 0.1 nM (□) and 1.0 nM (◇). For both figures, the coexposure of shear and the lower concentration of TCDD (■) is compared to coexposure to shear and the higher concentration of TCDD (◆). Each point represents the average of duplicate samples for a typical experiment.

0.01 to 1 μM, resulted in decreased EROD activity from 80% down to 10% of control values, respectively. The decrease in EROD activity was shown to correlate directly with decreased P-450IA1 mRNA and ^{3}H-TCDD-AhR nuclear complex. Similar experiments were also performed in the Hep G2, H-4-II-E, and Hepa-1 cell lines by Merchant and coworkers (18). In the case of the Hep G2 cells, coexposure with 1 nM

TCDD and 1 μM α-NF resulted in 4% of the control EROD activity, 19% of the control nuclear ^{3}H-TCDD-AhR levels and 37% of control DRE binding to nuclear extracts (18).

Pretreatment of the Hep G2 cells with 100 nM ST also decreased the induction of CYP1A1 by TCDD to approximately 25% of the control reponse (see Figure 1A). Singh and Perdew (1993) observed that exposure of Hepa-1 cells to 75 nM ST for eight hours resulted in a 50% decrease in the amount of cytosolic AhR. In addition, no morphological or cytotoxic effects were observed at 75 nM ST whereas 250 nM ST did elicit cytotoxic effects. Furthermore, the physicochemical properties of the remaining AhR were unchanged after ST treatment and the total protein concentration in the cell was not altered. Similar findings were observed by Carrier et al. (1992) in the Hepa-1 cell line. The concentration of ST required to inhibit 50% of CYP1A1 mRNA induction by TCDD was 60 nM. Unlike α-NF where it is known that it forms an inactive complex with the AhR, it is unclear as to how ST decreases AhR levels. Singh and Perdew (1993) hypothesized that ST may alter AhR gene regulation, or it may inhibit post-translational modifications of the receptor required for stabilization, or that phosphorylation may be involved with the receptor's ability to complex with other proteins required for its stability. There have also been studies that have implied a role for PKC in the modulation of the DNA binding activity of the AhR but not in the transactivating activity (33). However, futher investigations support the hypothesis of Singh and Perdew that PKC may play a role in the phosphorylation of the AhR and/or its associated subunits prior to ligand binding and not in the AHR transformation and DNA binding (23).

In order to determine if the stress-induced CYP1A1 shared a similar mechanism to the TCDD-induced CYP1A1, the effects of α-NF and ST were examined (see Figure 1B). The shear response was initiated by transferring Hep G2 cell to a modified spinner flask and agitating at a higher rate. As seen in Figure 1B, the shear response is transient with a maximum induction 10 to 20 times higher than the basal enzyme activity. Similar to the effect of α-NF and ST on TCDD-indcued CYP1A1, the shear-induced enzyme induction was blocked by α-NF for approximately 12 hours and ST attenuated the response to approximately 40% of the control response but retained the same kinetics of induction.

The next set of experiments evaluated the effects of coexposing microcarrier-attached Hep G2 cells to TCDD and hydrodyanmic shear (see Figure 2). Two methods were used to induce the shear response. The first method involved transferring the cells from their growth flask to a fresh spinner flask (Figure 2A). For the second case, the fresh flask was equipped with a paddle-like impeller and a higher agitation rate was used (Figure 2B). In the case of transferring the cells to a fresh flask and not changing the agitation rate from the growth conditions, the maximum EROD activity was approximately 1.3 pmol resorufin formed/10^{6} cells/minute. Coexposure of the cells to 0.05 nM TCDD and shear resulted in an additive effect followed by a convergence to the same value of EROD activity as observed for TCDD exposure alone after 24 hours when the shear response subsides. Coexposure using a higher TCDD concentration also

resulted in an additive response followed by a convergence to the same level of CYP1A1 induction as when the cells are exposed to TCDD alone.

In the case of shear induced by using a baffled spinner flask and increased agitation, the maximum EROD activity was approximately 3.5 pmol resorufin formed/10^6 cells/minute, as illustrated in Figure 2B. Unlike the case of the lower shear response, the shear-induced EROD appears to be controlling the overall response. At 0.1 nM TCDD, there is not an additive effect but rather the stress-induced CYP1A1 appears to control the response up to 8 hours followed by a switch to a TCDD controlled response after 12 hours. Increasing the TCDD concentration to 1 nM resulted in a competitive effect. The coexposure case showed a lower response than observed for TCDD alone. However, when the shear response subsides after 12 hours, the coexposure case rebounds to the same response as observed for TCDD expoure alone.

The results from the coexposure experiments indicate that shear induced the formation of a product that can either compete with or contribute to the TCDD-induced CYP1A1 expression. When the shear response is low, there is an additive effect when cells are treated with TCDD and exposed to shear. However, the kinetics of induction in the coexposure cases parallel those of CYP1A1 induction due to shear alone. Increasing the shear results in increased product formation responsible for the shear-induced CYP1A1 and thus we observe different patterns of CYP1A1 induction in the presence of both TCDD and shear. The competitive and additive effects could be occurring at the level of AhR binding. The product formed by hydrodynamic shear may have a higher affinity for the receptor and since the shear response is transient, the TCDD response can dominated once the shear response subsides.

Finally the wild-type and AhR mutant of the Hepa-1 cell line were used to futher examine the possibility of the involvement of the AhR in the shear response. Figure 3 illustrates the induction of CYP1A1 in the wild-type Hepa-1 cells. Unlike the Hep G2 cells, there is siginificant variation in the basal activity in the microcarrier-attached

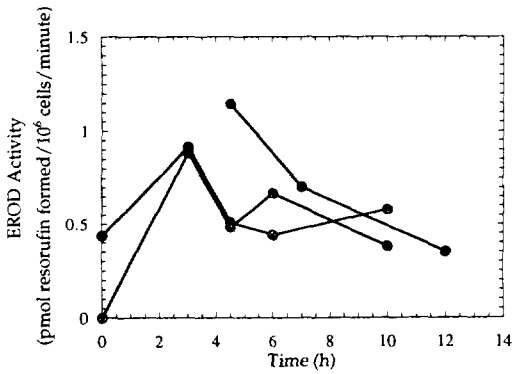

Figure 3. The kinetics of shear-induced CYP1A1 expression in microcarrier-attached wild-type Hepa-1 cells. The data represent the average of duplicate samples.

cultures (data not shown). The high EROD activities observed before the cells are transferred may indicate that the cells are more shear sensitive. The increased shear sensitivity may be related to the higher number of AhR per cell and its increased affinity for TCDD compared to the Hep G2 cell line. Finally there was no detectable EROD activity in the mutant cells either before or after the cells were transferred to fresh spinner flasks. The nonresponsiveness of the LA1 cell line to shear was paralleled by the inability of 1 nM TCDD to induce a response compared to the wild-type cells (data not shown).

Currently, no endogenous ligand has been identified for the AhR. Possible candidates include indoles and photooxidized derivatives of tryptophan (34-37). However, the data obtained from the three strategies employed to elucidate a role for the AhR in shear-induced CYP1A1 expression are consistent with the hypothesis that shear results in the formation of an endogenous ligand for the AhR and subsequently results in the induction of CYP1A1. The AhR antagonist α-NF and the PKC inhibitor ST both decreased the shear-induced CYP1A1 level, similar to the experiments involving TCDD exposure. Coexposure to TCDD and shear resulted in a modulation of TCDD-induced CYP1A1 levels, either augmented or suppressed, depending on the relative strength of the shear-induced CYP1A1 activity, indicating either an additive or competitive effect at the level of AhR binding. Finally, the AhR mutant Hepa-1 cell line, which has a defective AhR, was resistant to the effects of hydrodynamic shear whereas the wild-type Hepa-1 cells were shear sensitive with respect to CYP1A1 induction suggesting that a functional AhR is required to observed shear-induced CYP1A1 expression.

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