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Serum withdrawal leads to reduced aryl hydrocarbon receptor expression and loss of cytochrome P4501A inducibility in PLHC-1 cells☆

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Abstract

Changes in the expression of the aryl hydrocarbon receptor (AHR) have been documented in several systems and in response to a variety of treatments. The significance of these findings is unclear, because the effects of such changes on subsequent responses to AHR ligands seldom have been measured. We tested the ability of changes in serum used in cell culture medium to alter expression of the AHR and induction of cytochrome P4501A (CYP1A) in PLHC-1 teleost hepatoma cells. Culture of early-passage cells in serum-free medium for 2 days led to a loss of CYP1A inducibility by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In contrast, culture in 10% delipidated calf serum increased the TCDD-induced levels of both CYP1A protein and enzymatic activity relative to levels in cells cultured in 10% complete calf serum. These effects were consistent between 8 and 24 hr post-treatment, indicating that the kinetics of induction were unaffected. In cells cultured in serum-free medium for 1 and 2 days there was a progressive loss of CYP1A inducibility. This loss of response paralleled a time-dependent decline in AHR protein, as measured by specific binding of [^{3}H]TCDD. Using an operational model for AHR action in PLHC-1 cells, the measured reduction in AHR could be shown to predict the loss of CYP1A induction. Expression of AHR protein was unaffected by culture in 10% delipidated serum. The effects of serum-free medium and delipidated serum were found only in early-passage cells; inducibility of CYP1A and expression of AHR protein in late-passage cells were unaffected by serum withdrawal. Comparison of early- and late-passage cells revealed a 2-fold greater rate of proliferation in the latter, suggesting that a growth advantage is coincident with loss of the serum-dependency of AHR expression. These results provide a quantitative link between changes in receptor expression and a downstream response, establishing a foundation for future studies of receptor expression and sensitivity to toxic responses *in vitro* and *in vivo*. © 2002 Published by Elsevier Science Inc.

Keywords: Aryl hydrocarbon receptor; Receptor expression; TCDD; Cytochrome P4501A; Receptor modeling; PLHC-1 cells

1. Introduction

Receptor theory predicts that the magnitude of response for receptor-mediated effects of toxicants depends, in part, on the concentration of receptors present in the cell. In some systems, maximal response occurs at concentrations of toxicant that produce sub-maximal receptor binding, and these systems are said to possess "spare receptors." This term applies only to the magnitude of the response, because any change in receptor content will affect the potency of the toxicant. Thus, a reduction in receptor expression initially will increase the concentration of toxicant necessary to elicit a response, while greater reductions should decrease potency further *and* diminish the maximum magnitude of response in the tissue [1] (and references therein).

TCDD and structurally related compounds cause altered gene expression and toxicity through activation of the AHR. Expression of the AHR can be altered under several conditions, including treatment with phenobarbital [2], *ortho*-substituted PCBs [3,4], TCDD [5], nocodazole [6], or transforming growth factor-β (TGF-β) [7], or upon serum withdrawal [8] or loss of a transcriptional regulator

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHR, aryl hydrocarbon receptor; CYP1A, cytochrome P4501A; PCB, polychlorinated biphenyl; TCDF, 2,3,7,8-tetrachlorodibenzofuran; LSC, liquid scintillation counting; MEM, minimum essential medium; EROD, ethoxyresorufin *O*-deethylase; S_0, serum-free medium; S_{10}, medium containing 10% calf serum; S_{DL}, medium containing 10% charcoal stripped, delipidated calf serum; GR, glucocorticoid receptor.

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[9]. The effect of changes in AHR expression upon response to AHR agonists has been determined in only a few of these cases [6,10]. The *in vivo* significance of altered AHR expression ultimately will depend upon how responses to AHR ligands are impacted, particularly the toxicity of AHR agonists. Thus, it is important to understand the effect of changes in AHR expression on downstream responses to ligand treatment.

In this study, the effect of serum on AHR expression and response to ligands was assessed. Serum withdrawal reduces AHR expression 4- to 10-fold in Swiss 3T3 murine fibroblasts [8], apparently by down-regulation of a tyrosine kinase. We wished to determine if serum-dependent regulation of AHR expression was present in other cell types and to measure the impact of a decline in the receptor on a downstream response. Induction of CYP1A was chosen as the response, because it is both well characterized and common throughout most of the vertebrate classes [11,12].

Previously, we reported that serum in cell culture medium alters the uptake and apparent potencies of AHR ligands [13]. To separate the effect of serum on ligand uptake from its effects on AHR expression, in the present study cells were cultured in medium containing different serum treatments *prior to* treatment with ligands in a common medium (Fig. 1).

Receptor expression and induction of CYP1A were measured in PLHC-1 cells, derived from a hepatocellular carcinoma of the teleost *Poeciliposis lucida* [14]. Binding of TCDD to the AHR and induction of CYP1A have been measured in this cell line, and a mathematical model of the stimulus–response relationship for this system has been developed [15]. The model predicts the presence of ''spare receptors'' for CYP1A induction by TCDD. It also allows effects of receptor expression on response to ligands to be modeled quantitatively, facilitating the generation of testable hypotheses regarding the relationship between receptor expression and response.

We report that culture of PLHC-1 cells in serum-free medium results in a progressive decline of CYP1A inducibility by TCDD (potency and magnitude), associated with decreased expression of the AHR. In contrast, culture in medium containing 10% delipidated serum increased maximal levels of induced CYP1A without changing the potency of induction by TCDD. This increase was not correlated with a change in AHR expression. Both of these phenotypes were dependent upon subclone and time in culture. The results are consistent with predictions from an operational model of AHR signaling.

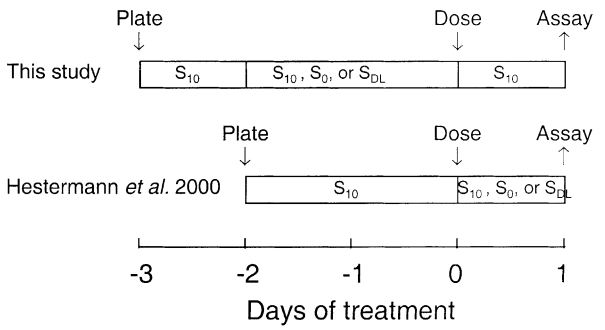

Fig. 1. Cell treatment protocol in the current study as compared with that used in a previous study [13].

2. Materials and methods

2.1. Chemicals and solutions

2,3,7,8-Tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin ([^{3}H]TCDD, purity ≥97%, specific activity 27 Ci/mmol) was obtained from Chemsyn Science Laboratories. Unlabeled TCDD and TCDF were obtained from Ultra Scientific. Peroxidase-conjugated goat anti-mouse IgG was from Schleicher & Schuell. Luminescent peroxidase substrate was from Tropix. Resorufin and ethoxyresorufin were from Molecular Probes. All other reagents were obtained from the Sigma Chemical Co.

PBS consisted of 0.8% NaCl, 0.115% Na_2HPO_4, 0.02% KCl, 0.02% KH_2PO_4, pH 7.4. Phosphate buffer was 50 mM Na_2HPO_4 with pH adjusted to 8.0 with 50 mM NaH_2PO_4. Sample treatment buffer was 0.25 M Tris–HCl, pH 6.8, 40% (v/v) glycerol, 4% (w/v) sodium lauryl sulfate, 0.008% (w/v) bromphenol blue, and 5% (v/v) 2-mercaptoethanol. TCDD, TCDF, and PCB solutions were prepared in DMSO as described previously [16]. Concentrations of [^{3}H]TCDD solutions were verified by LSC on a Beckman LS5000TD.

2.2. Culture and treatment of cells

PLHC-1 cells [14] were cultured at 30° in MEM containing Earle's salts, nonessential amino acids, L-glutamine, and 10% calf serum (Sigma C6278, lot 106H4628), as described previously [17]. For CYP1A induction experiments, cells were suspended to 0.5 to 1×10^6/mL and seeded into 48- or 96-well plates (Costar) at 0.5 or 0.2 mL/well, respectively. One day later the complete medium (MEM with 10% serum) was removed and replaced with fresh experimental medium, as illustrated in Fig. 1. Media used in the experiments included MEM without serum (S_0), MEM with 10% calf serum (S_{10}), and MEM with 10% delipidated, charcoal stripped calf serum (Sigma C1696) (S_{DL}). After 1 or 2 days, the medium was changed to MEM + 10% calf serum (S_{10}), and the cells were treated with TCDD (1 pM to 100 nM) dissolved in DMSO or a solvent blank (2.5 or 1.0 μL/well). DMSO concentrations were ≤0.5% (v/v) in all treatments. Following treatment, plates were incubated at 30° for 24 hr unless otherwise indicated. With the exception of the delipidated serum, all serum used was from a single lot. None of the treatments reduced cell viability, as assessed by Trypan Blue exclusion.

For [^{3}H]TCDD specific binding experiments, cells were seeded into 24-well plates (Corning) at 2×10^6 cells in 1 mL culture medium per well. One day later the complete medium (MEM with 10% serum) was removed and replaced with fresh experimental medium. After 2 days, the specific binding of [^{3}H]TCDD was determined as described below.

2.3. EROD and protein assays

CYP1A-catalyzed EROD activity was measured using a multiwell fluorescence plate reader by a modification of the method of Kennedy *et al*. [18]. Increases in this activity accurately reflect induction of CYP1A by TCDD in PLHC-1 cells [16]. Volumes given are for 48-well plates; halve for assays in 96-well plates. Cells were rinsed once with room temperature PBS, and the EROD reaction was then initiated with the addition of 2 μM 7-ethoxyresorufin in phosphate buffer (200 μL/well). The reaction was stopped after 8 min (resorufin production is linear with respect to time over this period [16]) with the addition of 150 μL of ice-cold fluorescamine solution (0.15 mg/mL in acetonitrile). After a 15-min incubation, resorufin and fluorescamine fluorescence were measured. Resorufin and protein concentrations were determined from standard curves prepared in the same plate. BSA was used for the protein standard curve. In some experiments, the EROD reaction was followed kinetically over 8 min, as described previously [16]. EROD data were fit to a modified Gaussian function for determination of dose–response relationships, as described previously [16,18].

2.4. Measurement of CYP1A protein

CYP1A protein was measured by immunblotting of whole cell lysates, essentially as described earlier [16]. Cells cultured and treated in 48-well plates were solubilized at 4° for 15 min in 100 μL of sample treatment buffer, with gentle agitation. Lysates were transferred to Eppendorf tubes and boiled for 5 min to complete solubilization and inactivate proteases.

Samples and CYP1A standards (purified CYP1A from scup, *Stenotomus chrysops*) were analyzed by denaturing gel electrophoresis on 8–12% acrylamide gradient minigels (Novex). Twenty five microliters of each sample (equivalent to 15 μg protein) was used. Proteins were transferred electrophoretically onto 0.2 μm nitrocellulose and incubated sequentially with blocking solution (Schleicher & Schuell), monoclonal antibody 1-12-3 (anti-scup CYP1A [19]) at 1 μg/mL, and alkaline phosphatase conjugated goat anti-mouse IgG at 1:5000 dilution. Following each antibody incubation, membranes were washed three times each with Tris-buffered saline and water. Color was developed by enhanced chemiluminescence as directed by the supplier (Tropix) using Kodak X-AR film. Images were acquired with a Kodak DCS200 digital camera and Adobe Photoshop, and band intensities were quantified by densitometry using NIH Image software. Values for CYP1A equivalents were determined from a standard curve using the scup CYP1A.

2.5. [^{3}H]TCDD binding

Specific binding of [^{3}H]TCDD in PLHC-1 cells was measured by a whole-cell filtration assay [20]. One day after seeding in 24-well plates, medium was changed as appropriate for the experiment. One or two days later the medium was changed to 0.5 mL of MEM + 10% calf serum, and cells were treated with 1 nM [^{3}H]TCDD in the presence or absence of 200 nM TCDF and incubated for 2 hr at 30°. This time was determined to be sufficient to achieve a steady state of bound radioligand (unpublished data). Following the incubation, the medium was removed, and cells were rinsed with 0.5 mL of ice-cold PBS and then detached with 0.5 mL of trypsin. The trypsin was inactivated by the addition of 0.5 mL of ice-cold culture medium (with 10% serum), and cells from each well were collected under vacuum on a prewetted, 25 mM Whatman GF/F filter. Filters were then washed four times with 2.5 mL of acetone that had been precooled to −80°. Replicates were processed in batches of 12 on a Millipore 1225 filter manifold. Radioactivity remaining on the filter was quantified by LSC. Specific binding was measured in triplicate as the difference of each of three total binding (without TCDF) replicates and the average of three non-specific binding (with TCDF) replicates in each medium. Protein concentrations were determined by fluorescamine assay (as above) in duplicate wells fed each medium and treated with DMSO alone.

3. Results

3.1. Effect of culture medium on CYP1A inducibility

PLHC-1 cells were cultured for 2 days in one of three media: serum-free MEM (S_0), MEM with 10% calf serum (S_{10}), or MEM with 10% delipidated calf serum (S_{DL}). The medium then was changed to S_{10} (to ensure equal kinetics of TCDD uptake [13]), and the cells were treated with TCDD. CYP1A-catalyzed EROD activity was assayed 24 hr later (Fig. 2). Prior culture in S_0 abolished induction of EROD by TCDD, while prior culture in S_{DL} led to a significant ($P < 0.05$) increase in the magnitude of induction relative to S_{10}. Similar results were obtained when the medium was changed to S_0 for TCDD treatment (not shown), although EC_{50} values were reduced, consistent with our previous results [13].

The effects of culture medium on EROD induction were confirmed at the level of CYP1A protein expression by immunoblot (Fig. 3). At concentrations of TCDD below 1 nM, the levels of CYP1A were undetectable by this

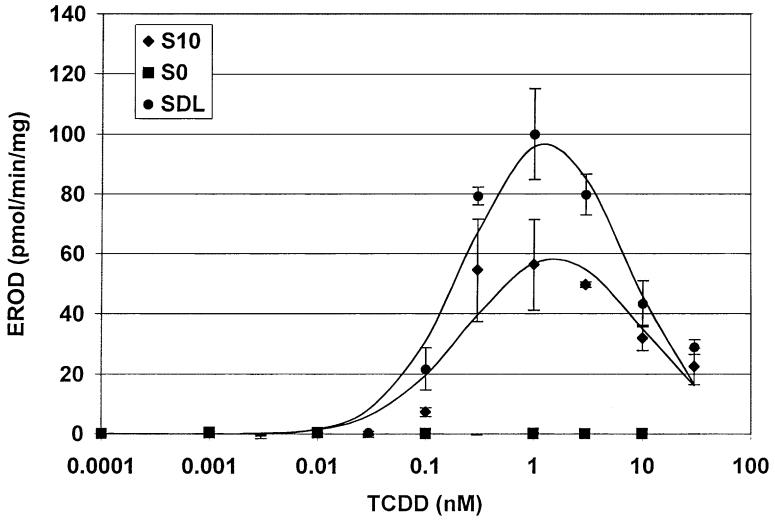

Fig. 2. Effect of culture medium on EROD inducibility by TCDD. PLHC-1 cells were seeded in 48-well plates and cultured for 2 days in the medium indicated. The medium was then changed to S_{10}, and cells were treated with TCDD. EROD activity was assayed 24 hr later. Values are means ± SEM of triplicate wells. The modified Gaussian fits to these data are plotted. Results are representative of 6 independent experiments. Key: S10: S_{10}; S0: S_0; and SDL: S_{DL}.

method. At concentrations of TCDD where CYP1A was detectable, there was greater induction in cells cultured in S_{DL} than in S_{10}, and little or no induction in cells cultured in S_0. A small amount of CYP1A protein expression was seen in cells cultured in S_0 and treated with large concentrations of TCDD (≥10 nM; Fig. 3). At those high

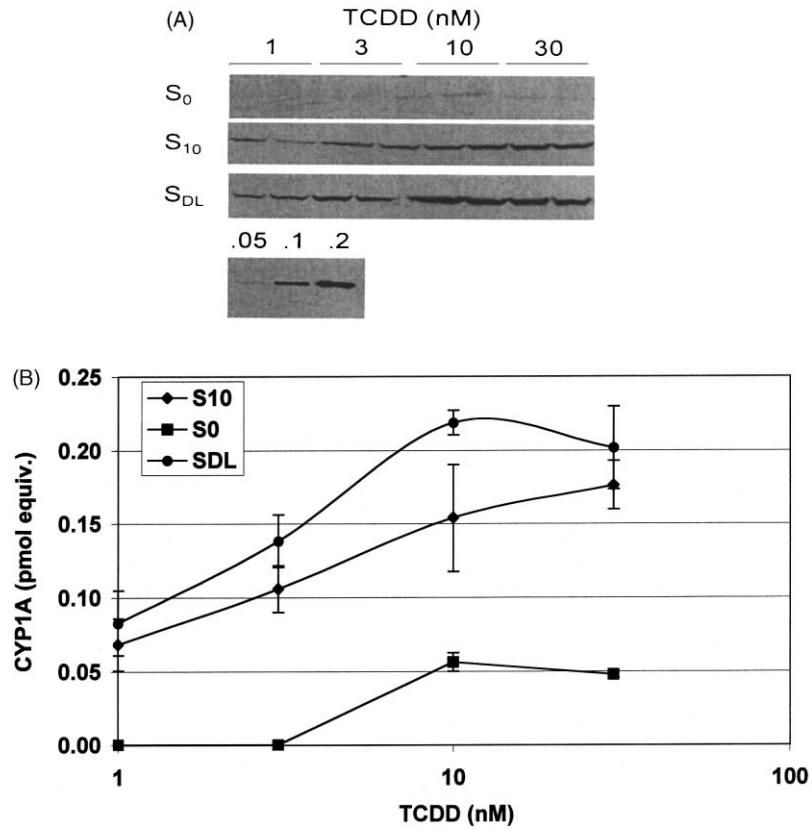

Fig. 3. Effect of culture medium on CYP1A protein induction. Cells were cultured and treated as in Fig. 2. Cell lysates were harvested 24 hr after treatment with TCDD and analyzed by SDS–PAGE and immunoblotting with mAb 1-12-3. (A) Digital image of blot with medium and TCDD treatments indicated. The bottom row shows scup CYP1A standards with pmol amounts indicated. (B) Integrated densities of individual bands were converted to CYP1A equivalents. Values are means of duplicate samples; error bars indicate the range of values. Results are from a single experiment. Symbol key is defined in the legend of Fig. 2.

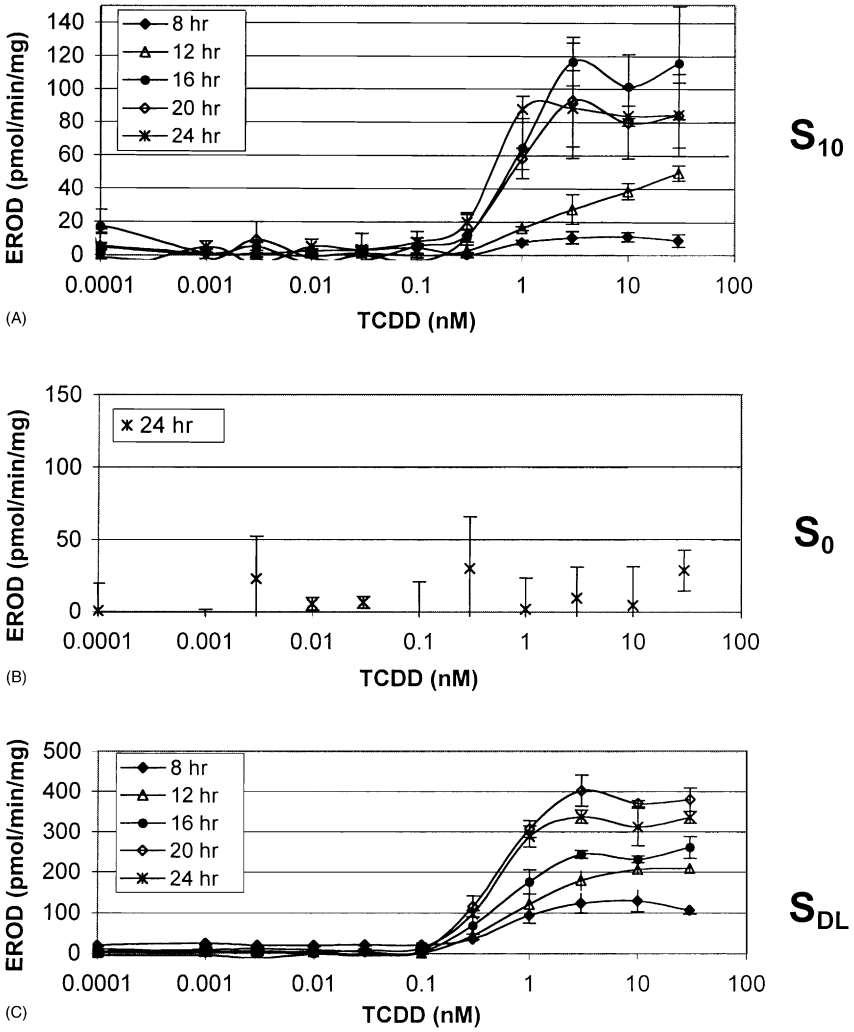

Fig. 4. Time course of induction of EROD activity. PLHC-1 cells were cultured and treated as in Fig. 2, and EROD activity was assayed at the indicated times after TCDD treatment. Values are means ± SEM of triplicate wells. The modified Gaussian fits to these data are plotted. Cells were cultured in (A) S_{10}, (B) S_0, or (C) S_{DL}. There was no induction in cells cultured in S_0 for 8–20 hr (not shown). Note the difference in scales on the *y*-axis. Results are representative of 2 independent experiments.

concentrations of halogenated aromatic hydrocarbon, the inducing compound also can act as an inhibitor of EROD [17,21,22], so the enzymatic activity of the small amount of CYP1A present would not be evident (Fig. 2).

To determine if the different media affected the timing of CYP1A induction, EROD was measured from 8 to 24 hr after the addition of TCDD (Fig. 4). Induction of EROD was measurable by 8 hr in cells previously cultured in S_{10} or S_{DL}, and rates continued to increase until 16–20 hr after treatment (Fig. 4, A and C). Rates decreased slightly from this maximum at 24 hr. In contrast, no induction was measured in cells cultured in S_0 at any point up to 24 hr post-treatment (Fig. 4B).

3.2. Timing and mechanism of loss of inducibility

Cells were cultured in S_{10}, S_0, or S_{DL} for 1 or 2 days, after which the medium was changed to S_{10}, and the cells were treated with TCDD. EROD activity was measured 24 hr after TCDD treatment (Fig. 5). In cells cultured for 1 or 2 days in S_{DL}, TCDD-induced EROD activities were greater than those in cells cultured in S_{10}, with no apparent change in EC_{50} values. After a single day in S_0, the EC_{50} for induction of EROD had increased about 5-fold compared to cells in S_{10}, but the maximum level of induction was unchanged (Fig. 5A; Table 1). After 2 days, the EC_{50} increased further, and the maximal level of induction was lower than that observed after 1 day in S_0. In contrast to previous results (Fig. 2), TCDD caused a slight induction of EROD activity after 2 days in S_0 (Fig. 5B). Subsequent experiments also showed subtle variability in the kinetics of loss of inducibility.

One possible mechanism for the progressive decline in inducibility in cells cultured in S_0 could be a time-dependent loss of AHR protein or function. Levels of AHR protein were measured by specific binding of [^{3}H]TCDD in PLHC-1 cells cultured in the different media for 1 or 2 days (Fig. 6). The amount of AHR showed a significant decrease

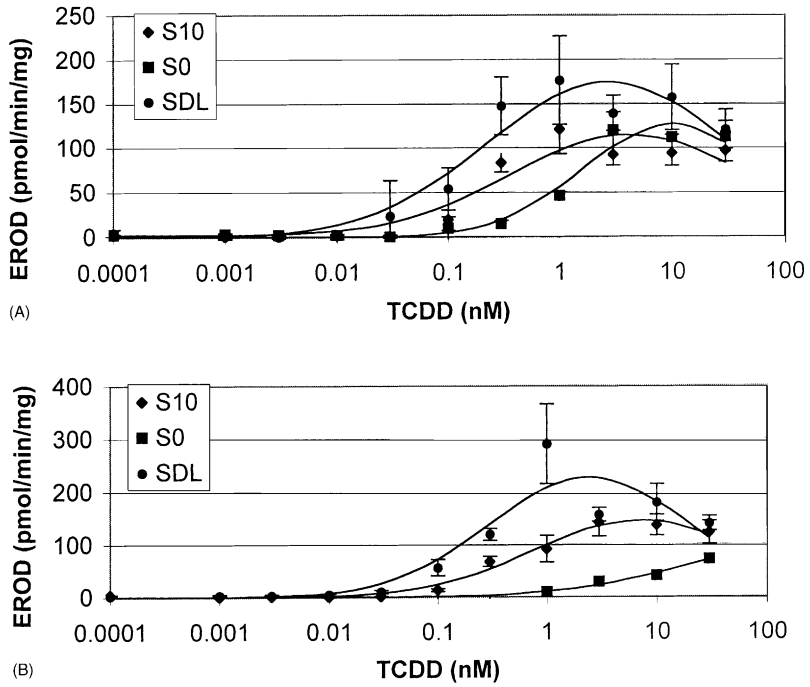

Fig. 5. Effect of 1 or 2 days of culture in different media on EROD induction. Cells were cultured, treated, and assayed as in Fig. 2, except that medium change and treatment took place after (A) 1 or (B) 2 days of culture in the indicated media. Values are means ± SEM of triplicate wells. The modified Gaussian fits to these data are plotted. Results are representative of 2 independent experiments. Symbol key is defined in the legend of Fig. 2.

in cells cultured in S_0 as compared with cells cultured in S_{10} or S_{DL} (Fig. 6A). As with the induction response, the decrease in AHR content was time-dependent. A significant reduction was already present after 1 day in S_0, and after 2 days, specific binding was barely detectable (Fig. 6B). Interestingly, there was no difference in AHR content between cells cultured in S_{10} and S_{DL} (Fig. 6A).

3.3. Heterogeneity of serum-dependent CYP1A inducibility

The down-regulation of AHR and the loss of CYP1A inducibility following serum withdrawal were detected only in a subset (4 of 8) of PLHC-1 subclones tested. In subclones in which it occurred, the AHR down-regulation and loss of inducibility did not persist beyond 35 passages.

Table 1
Effect of culture medium and time on TCDD EC_{50} and maximal EROD induction

Day	Medium	TCDD EC_{50} (nM)	Maximum activity (pmol/min/mg)
1	S_{10}	0.24 ± 0.094	114 ± 11
	S_0	1.20 ± 0.230	126 ± 7.8
	S_{DL}	0.15 ± 0.049	174 ± 13
2	S_{10}	0.15 ± 0.080	145 ± 5.7
	S_0	ND[a]	ND[a]
	S_{DL}	0.20 ± 0.100	226 ± 29

These data are derived from the curves shown in Fig. 5, which were obtained by modified Gaussian fits [16,18] to data from triplicate wells at each TCDD concentration, in one experiment. Values are shown with standard error of regression determined from the curve fits. Results are representative of 2 independent experiments that gave similar results.

[a] Neither EC_{50} nor maximum activity could be determined due to insufficient data for a curve fit.

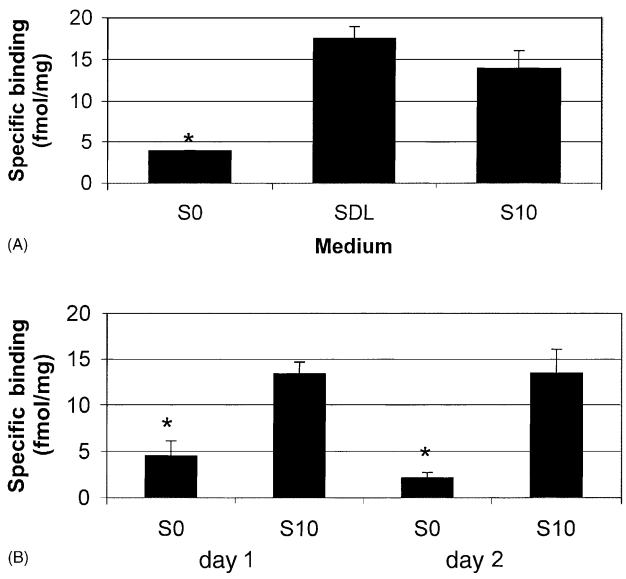

Fig. 6. Effect of time in culture medium on AHR expression. PLHC-1 cells were cultured in the indicated media, changed to S_{10}, and incubated with 1 nM [^{3}H]TCDD in the presence or absence of 200 nM TCDF for 2 hr. [^{3}H]TCDD binding was measured by a whole-cell filtration assay [20]. Specific binding is shown as the average ± standard error of three replicates. Key: (∗) indicates significantly less specific binding than cells in S_{10} ($P < 0.01$, t-test, df = 4). (A) Cells were cultured in the indicated media 2 days prior to assay. (B) Cells were cultured for 1 or 2 days. AHR expression in cells cultured in S_0 was not significantly different between 1 and 2 days ($P = 0.12$, df = 4). Results are representative of 2 independent experiments. Key: S10: S_{10}; S0: S_0; and SDL: S_{DL}.

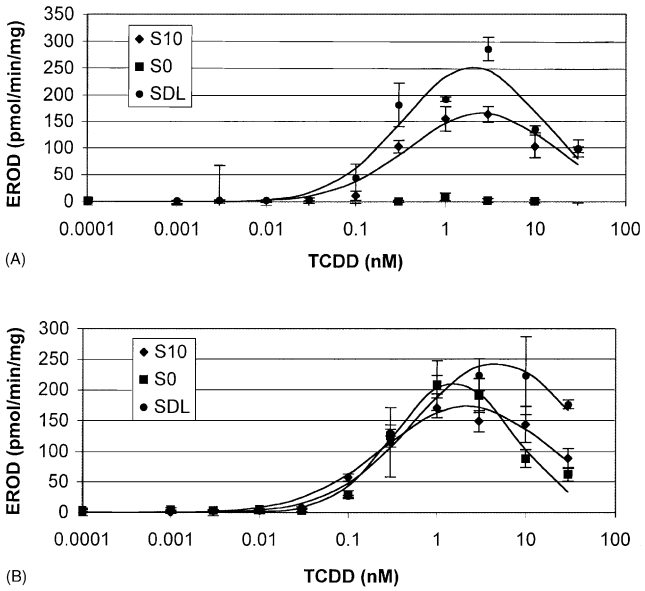

Fig. 7. Response of early- and late-passage cells to culture medium. Cells were cultured, treated, and assayed as in Fig. 2. (A) Early-passage cells (passage number: 10). (B) Late-passage cells (passage number: 42). Points are means ± SEM of triplicate wells. The modified Gaussian fits to these data are plotted. Results are representative of 4 independent experiments. Symbol key is defined in the legend of Fig. 2.

Later-passage cells revealed no significant differences in induction of EROD, regardless of culture medium (Fig. 7). The transition between the phenotypes depicted in Fig. 7 (termed "early" and "late" passage) was abrupt; typically, only three passages separated serum-dependent and serum-independent phenotypes in the same subclone of cells. This abrupt transition suggested that the serum-independent phenotype (i.e. late-passage cells) might have a growth advantage as compared with the serum-dependent phenotype. Accordingly, rates of proliferation for early- and late-passage cells were determined (Fig. 8). Late-passage cells (doubling time of 28 hr) proliferated at twice the rate of early-passage cells (doubling time of 56 hr).

3.4. Modeling receptor expression and inducibility

In PLHC-1 cells cultured in S_0, the decline in inducibility of CYP1A protein and activity paralleled a decline in specific binding of [^{3}H]TCDD, presumably reflecting a decline in expression of AHR protein. With the operational model of AHR function developed previously [15], it is possible to predict the effects of changes in receptor content on measured responses. EROD responses predicted for different levels of AHR expression are shown in Fig. 9A. The model predicts that a reduction in receptor expression as great as 85% would still allow near-maximal induction of CYP1A by TCDD, albeit with a 6-fold increase in the EC_{50} (Fig. 9; Table 2). Greater reductions in receptor content led to steady declines in the maximal level of induction.

Fig. 9B shows the EROD induction data from Fig. 5 superimposed on the curves predicted from the model. The

Table 2
Effect of receptor number on EC_{50} and maximum response

AHR content (% of max)	EC_{50} (nM)	Response (% of max)
100	0.27	98
33	0.78	95
15	1.60	89
3	5.70	62
1	9.70	35
0.1	14.20	5

Values were calculated as follows: Maximal response $= (R_T/K_E)/1+(R_T/K_E)$ $EC_{50} = K_d/1 + (R_T/K_E)$. See the legend of Fig. 9 for definitions and values.

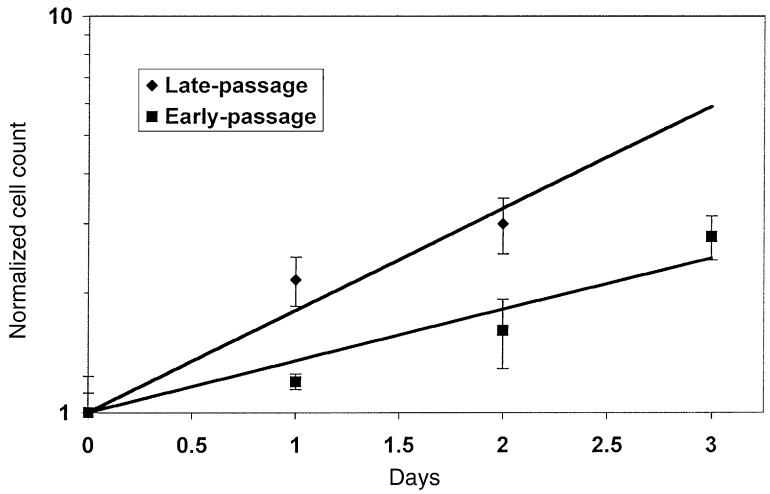

Fig. 8. Proliferation in early- and late-passage cells. PLHC-1 cells were seeded in 25 cm^2 flasks at 2.4×10^5/cm^2 and cultured in S_{10}. Cells were counted at daily intervals with a hemacytometer. Counts were normalized to the value on day 0, and are means ± SEM for triplicate flasks. Exponential fits are shown (proliferation had stopped in late-passage cells by day 3). The doubling times are 28 and 56 hr for late- and early-passage cells, respectively. Passage numbers are as in Fig. 7. Results are representative of 2 independent experiments.

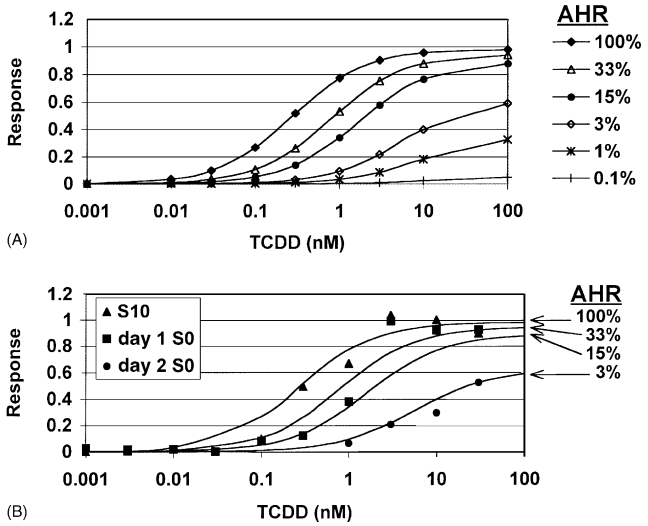

Fig. 9. Influence of receptor number on induction of CYP1A. (A) Predicted EROD response to TCDD treatment for various levels of AHR expression. (B) EROD data from Fig. 5 (points) are shown with selected induction curves (lines) from panel A. Predicted responses were calculated from the following equation, with $R_T = 110$ fmol/mg at 100% AHR, $K_d = 15$ nM, and $K_E = 2$ fmol/mg: $E = [R_T] \times [A]/K_d \times K_E + ([R_T] + K_E)[A]$, where E is the fractional response, R_T is cellular AHR content, $[A]$ is TCDD concentration, K_d is the equilibrium dissociation constant, and K_E is an efficacy constant [15]. These predicted curves do not account for inhibition of EROD activity. Symbol key is defined in the legend of Fig. 2.

EROD activities have been normalized to the maximal values in cells cultured in S_{10} for each day. The AHR content in cells cultured in S_0 declined 67 and 85% compared to cells cultured in S_{10} after 1 and 2 days, respectively (Fig. 6). Induction curves predicted by the model for full AHR expression and for a 67% reduction in AHR fit the induction data from S_{10} and day 1 in S_0 very well. However, a ~97% decline in AHR is necessary to bring the predicted curve in alignment with the induction data from day 2. Because the receptor content measured after 2 days in S_0 is near the limit of detection, a 97% reduction is not significantly different from the measured value.

4. Discussion

This report explores the connection between AHR expression and cellular response to AHR agonists. We have demonstrated a loss of inducibility of CYP1A in early-passage PLHC-1 cells cultured in serum-free medium for 2 days, while cells cultured in medium with 10% delipidated serum showed an increase in the maximal level of induction over cells cultured in the typical 10% calf serum. Neither the loss of inducibility in cells cultured in S_0 nor the increase in induced CYP1A in cells cultured in S_{DL} was due to a change in the kinetics of induction. The loss of inducibility paralleled a loss of AHR over the same period. These effects were evident in early- but not late-passage PLHC-1 cells.

Previously, we reported that serum in the culture medium reduces the uptake of TCDD and other halogenated AHR ligands by PLHC-1 cells [13]. The present results demonstrate that serum also has effects on CYP1A induction, distinct from its effect on ligand uptake. Cells cultured in S_0 or S_{DL} and returned to S_{10} for short-term ($\leq$24 hr) ligand exposure exhibited induction properties different from those of cells cultured in S_{10}. This suggests that the mechanisms of the observed effects involve changes in protein expression or another relatively slow (i.e. several hours to days) response, rather than changes in ion flux, enzyme activity, or similarly quick (i.e. minutes to hours) response.

4.1. Loss of CYP1A inducibility and receptor expression

We have demonstrated previously the existence of "spare" aryl hydrocarbon receptors for induction of CYP1A by TCDD in PLHC-1 cells [15]. Theoretically, the presence of spare receptors implies that a small reduction in receptor content will increase the EC_{50} for the response, but not decrease significantly the maximal level of induction. A larger decrease in receptor content would further increase the EC_{50} and should also reduce the maximal response (Fig. 9A). The results presented here (Figs. 5 and 9) provide an experimental demonstration of the progressive loss of AHR function predicted from receptor theory.

A relationship between AHR expression and function has also been suggested by previous studies. A comparison of wild-type and AHR-deficient Hepa-1 cells [23] showed that a 3-fold reduction in AHR content was correlated with a 16-fold increase in EC_{50} and a 5-fold decline in the maximal induction of CYP1A1. Another study in Hepa cells showed that treatment with the microtubule inhibitor nocodazole reduced AHR ligand-binding capacity and CYP1A1 inducibility, although this result might have been secondary to alterations in cell cycle progression [6]. Finally, increases in the expression of the AHR by treatment with 2,2′,4,4′,5,5′-hexachlorobiphenyl increased the potency, but not the efficacy, of TCDD as an inducer of EROD activity in mice [10]. Our results support and extend this previous work by showing the progressive nature of the effect of altering AHR expression on CYP1A inducibility, and by comparing experimental results to those predicted from an operational model of AHR signaling.

4.2. Increased CYP1A induction in PLHC-1 cells cultured in delipidated serum

Receptor theory predicts that, in a system with spare receptors, an increase in the maximal CYP1A induction response such as we observed in cells cultured in S_{DL} could not be caused by changes (increases) in receptor

expression. Consistent with this, no change in receptor expression was seen after culture in S_{DL} as compared with S_{10} (Fig. 6A). Thus, the increased induction of CYP1A protein and activity after culture in S_{DL} must be the result of another mechanism. Since the EC_{50} of induction was not changed by culture in S_{DL}, any proposed mechanism must affect signaling following binding of ligand to the AHR.

The increased induction of CYP1A observed here in cells cultured in S_{DL} is reminiscent of the potentiation of induction previously observed upon co-treatment of PLHC-1 cells with AHR agonists and GR agonists [24,25]. The former study found increased expression of CYP1A when cells were treated with dexamethasone and TCDD versus TCDD alone. Potentiation also was observed with the hormonal GR agonists cortisol and prednisone, and it was inhibited by GR antagonists. The potentiation of induction ranged from 2- to 20-fold, depending on the concentrations of each compound used, and showed variability between experiments, as was seen here (compare maximal EROD rates for cells cultured in S_{DL} in Figs. 2 and 4). Such potentiation has been shown to involve an increased rate of CYP1A mRNA transcription, and has been hypothesized to involve binding of the GR to response elements in the CYP1A gene [26].

Another possible mechanism for the enhanced CYP1A induction in cells cultured in S_{DL} is a loss of transcriptional ''squelching.'' Squelching is the process in which activated receptors compete for a limited supply of co-activators [27,28], and is well established for steroid hormone receptors. The supplier's process for removal of lipids from serum also removes steroid hormones. This would reduce activation of hormone receptors, leading to an increase in co-activators available for the AHR [29]. The switch from S_{DL} to S_{10} for AHR agonist exposure complicates this hypothesis, since steroid hormones would be reintroduced at the time of TCDD treatment.

4.3. Effects of passage number and subclone

The reduction in AHR expression and CYP1A inducibility reported here occurred in only 50% of the subclones of PLHC-1 we studied, and did not persist beyond 35 passages in the subclones in which they occurred. Fig. 8 shows that the late-passage cells have a growth advantage over early-passage cells. Whether the entire population of cells makes this transition or the relative numbers of a subpopulation increase is unclear. The fact that half of the subclones were responsive to TCDD after culture in S_0 even at very early passages (e.g. passage 10) suggests that heterogeneity may have existed in the parent line. This apparent heterogeneity is consistent with earlier results showing differences among subclones in the effects of TCDD on cell proliferation [30]. Taken together, these observations suggest a link between cell proliferation and AHR function, because a subpopulation of cells with faster proliferation also shows the loss of a control on AHR expression.

In this report, a lack of inducibility of CYP1A in cells grown in serum-free medium has been connected to a concurrent loss of AHR. The operational model of AHR signaling developed previously has been used to show that responses measured at intermediate points of AHR expression are consistent with our understanding of the relationship between receptor levels and downstream responses. Future work should focus on comparing CYP1A induction and additional responses to determine how changes in receptor expression may influence the sensitivity of various toxic endpoints to AHR ligands, as well as determining the mechanism(s) responsible for differences between early- and late-passage cells and among subclones.

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