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Benz[a]anthracene and 3-Methylcholanthrene Induction of Cytochrome P-450 in C3H/10T1/2 Mouse Fibroblasts

Modulating Role of Cytotoxic 3-Methylcholanthrene Metabolites

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SUMMARY

The relative abilities of benz[a]anthracene, 3-methylcholanthrene (3MC), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to induce aryl hydrocarbon hydroxylase (AHH) in C3H/10T1/2 mouse fibroblasts were studied. Benz[a]anthracene (13 μM, 24-hr exposure) was found to be the most effective inducer of the three compounds; it elicited an 8- to 11-fold increase in AHH activity. No induction was seen after exposure of the cells to 13 μM 3MC, and exposure to 0.1 μM TCDD elicited only a 2-fold increase in AHH activity. The rank order of inducible AHH levels in C3H mouse liver microsomes was exactly reversed when these same three compounds were administered to intact C3H mice, the strain from which these C3H/10T1/2 cells were originally derived. In C3H/10T1/2 cell cultures, increasing concentrations of 3MC were able to suppress totally the induction seen with 13 μM benz[a]anthracene alone in a dose-related manner (3MC ID_{50} = 0.26 μM). A significantly higher concentration of 3MC was required to suppress induction when 26 μM benz[a]anthracene was added to the medium (3MC ID_{50} = 0.56 μM). The extent to which these ligands compete at the Ah receptor required for AHH induction was examined by sucrose density gradient analysis of a [^{3}H]TCDD receptor in C3H/10T1/2 cell and C3H liver cytosols. Stereospecific, saturable [^{3}H]TCDD receptors were found in both cytosol fractions, and in both cases a 100- to 130-fold excess of benz[a]anthracene, 3MC, or tetrachlorodibenzofuran totally abolished [^{3}H]TCDD binding. Cytotoxicity studies revealed more than 98% loss of viability in cells treated with 13 μM 3MC. Partial inhibition by carbon monoxide of the cytochrome P-450 oxidation of 3MC in the cells resulted in a 3-fold induction in AHH by 3MC. These data indicate that 3MC binds to the Ah receptor in C3H/10T1/2 cells and can act as an inducer of AHH activity. However, under the normal circumstances in which 3MC metabolism is not suppressed by carbon monoxide, 3MC is converted to a cytotoxic derivative(s) which kills the cells and thus prevents a normal induction response.

INTRODUCTION

Polycyclic aromatic hydrocarbons, such as 3MC[1] and BA, as well as halogenated aromatic hydrocarbons, such as TCDD and certain of its structural analogues, have been shown to induce microsomal cytochrome P-450 monooxygenation in essentially every rodent and human tissue which has been examined (1, 2). The ability of these compounds to induce AHH and other associated enzyme activities (3), as well as to induce two TCDD-associated toxic responses (4), is highly correlated with the presence of a cytosolic receptor which displays stereospecific, saturable binding of these compounds (5, 6). The Ah locus is presumed to be the structural gene for this receptor (5, 7). The cytosolic protein receptor, once occupied, shows time- and temperature-dependent translocation to the cell nucleus (7–9) and non-covalent association with DNA (10). The ability of a compound to initiate an inductive or toxic response at the Ah locus is closely correlated with the ability of the compound to occupy the cytosolic receptor (2, 4, 5). This ligand-receptor relationship has been demonstrated for several structurally dissimilar ligands which possess different affinities for the same cytosolic receptor (4, 5), as well as for

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[1] The abbreviations used are: 3MC, 3-methylcholanthrene; BA, benz[a]anthracene; AHH, aryl hydrocarbon hydroxylase; BP, benzo[a]pyrene: 3-OHBP, 3-hydroxybenzo[a]pyrene; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; ID_{50}, concentration which gives 50% inhibition of a response; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BME, Eagle's basal medium; FCS, fetal calf serum; BSA, bovine serum albumin.

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dissimilar cytosolic receptors in "Ah-responsive" and "Ah-nonresponsive" mice which have different affinities for the same ligand (4, 5).

Several reports have indicated cell culture systems in which increases in AHH and cytochrome P-450 are observed when an appropriate inducing agent is added to the culture medium. These culture systems (11) as well as the induction mechanism studies done with them (12) have been reviewed as indicated. The induction of cytochrome P-450 monooxygenase reactions has been described in primary fibroblast cultures derived from rodents or humans (13–16) as well as in stable embryonic mouse fibroblast cell lines (17–19). In each of the fibroblast cell cultures cited above, 3MC was reported to be either non-effective or minimally effective in inducing monooxygenase activity, whereas in the same cell populations, concentrations of BA variably optimized between 9 and 13 μM have proven highly effective in inducing 5- to 20-fold increases in AHH or cytochrome P-450 (13–20). A representative cell line exhibiting this phenomenon is the 10T1/2 fibroblast cell line, which was originally derived from disaggregated C3H mouse embryos (21) and which has since become a commonly used model for analysis of chemically induced *in vitro* neoplastic cell transformation (18, 22). As we have previously reported (19), 3MC is ineffective in inducing AHH in these 10T1/2 fibroblasts, and yet sizable increases (12- to 22-fold) in AHH activity and cytochrome P-450 content are observed after a 24 hr exposure to BA. Recent reports (18–22) also indicate that the frequency with which these cells are transformed in the presence of a standard substrate such as BP is highly dependent upon the prior induction achieved with BA treatment. As mentioned, these cells were derived from embryonic C3H mice (21); this strain of inbred mice has been previously shown (20) to be "responsive" to the inducing effects of both 3MC and TCDD in all of the C3H mouse tissues which were examined.

In this work, we examine the reasons for this disparity between the inducing effects of 3MC and BA in 10T1/2 cells and C3H mice, using BA-3MC competitive induction experiments, cytosol receptor analysis, and cytotoxicity determinations.

MATERIALS AND METHODS

Chemicals. Chemicals and materials were obtained from the following sources: BA, dextran, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, Hepes, EDTA, and dithiothreitol from Sigma Chemical Company (St. Louis, Mo.); 3MC from Tridom Chemicals, Inc. (Hauppauge, N. Y.); BP from Aldrich Chemical Company (Milwaukee, Wisc.), recrystallized from ethanol before use; [^{3}H]TCDD (39 Ci/mmole), TCDBF, and TCDD were the generous gift of Dr. Alan Poland, McArdle Laboratory for Cancer Research, University of Wisconsin; activated charcoal (PX21) was a generous gift of Amoco Research (Chicago, Ill.); sucrose from Schwarz/Mann (Orangeburg, N. Y.); glycerol from Fisher Scientific Company (Fairlawn, N. J.); trypsin, Fungizone, penicillin-streptomycin, BME, and fetal bovine serum were obtained from Grand Island Biological Company (Grand Island, N. Y.).

Cells and cell culture. The establishment and characterization of the C3H/10T1/2CL8 cell line (referred to here as 10T1/2 cells) has previously been described (21); the cells for these experiments were kindly supplied by Dr. Charles Heidelberger, University of Southern California. Cells to be assayed for AHH activity were grown in 5% CO_2 at 37° in 100-mm dishes (Falcon) containing 20 ml of BME supplemented with 10% fetal calf serum, penicillin-streptomycin (80 units, 80 μg/ml), and Fungizone (2 μg/ml). Cultures were induced with hydrocarbons for 24 hr and then harvested in the following manner: the medium was removed and the cells were rinsed with phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.2); the cells were then scraped in the absence of trypsin and centrifuged at 100 × *g* for 10 min. The cell pellet was resuspended in phosphate-buffered saline (4°) to a final volume of 0.5 ml. The cells were then sonicated on ice (microtip, 2 setting, Branson sonifier, 10 sec). AHH activity was determined by measuring fluorescence at 396-nm excitation and 522-nm emission by the method of Nebert and Gelboin (23), except that 2 ml of the hexane phase were extracted with 2 ml of 1 N NaOH.

Mouse liver microsomes and cytosol. C3H mice received i.p. injections of BA or 3MC (80 mg/kg) in corn oil or TCDD (10 μg/kg) in *p*-dioxane, or were untreated (control), and then were killed after 40 hr for the preparation of liver microsomes by our previously described procedure (24); aliquots of the microsomal suspension were frozen in liquid nitrogen and subsequently assayed for AHH activity.

For preparation of mouse liver cytosol, livers from untreated C3H mice were perfused *in situ* with 4° HEDG/KCl buffer (25 mM Hepes/1.5 mM EDTA/1 mM dithiothreitol/10% glycerol/100 mM KCl, pH 7.2) and then minced and homogenized (Teflon-glass) in 5 volumes of the same buffer. The supernatant of a 10,000 × *g* (15 min) centrifugation was centrifuged at 105,000 × *g* for 60 min. The protein concentration of the supernatant cytosol was determined (25) with bovine serum albumin as the standard. The cytosol was used immediately to measure [^{3}H]TCDD binding, because freezing prior to the binding assay promoted receptor aggregation.

Cell cytosol. Roller bottles (850 cm^2, Corning) containing approximately 25 × 10^6 10T1/2 fibroblasts were rinsed and scraped in the presence of 4° phosphate-buffered saline; cells were pelleted at 1,000 × *g* for 10 min. The washed cell pellet was resuspended in 4° HEDG/KCl buffer at approximately 5 × 10^7 cells/ml. The cells were then sonicated (microtip, 2 setting, Branson sonifier, four 15-sec bursts, 30 sec between bursts) until all cells were lysed as evidenced by microscopic examination. The 10,000 × *g* (15 min) supernatant was further centrifuged at 105,000 × *g* for 60 min. The resulting supernatant (cytosol) was used for protein determination and immediate [^{3}H]TCDD receptor binding analysis.

Sucrose density gradient analysis. Cytosol (1 ml) (10 mg of protein) was treated *in vitro* with 1 nM [^{3}H]TCDD (added in 10 μl of *p*-dioxane) either alone or in the presence of 100 nM TCDBF, 130 nM BA, or 130 nM 3MC for 1 hr at 4°. Unbound and loosely bound [^{3}H]TCDD

was removed by adding the 1-ml samples to dextran-charcoal pellets (10 mg of charcoal, 1 mg of dextran pelleted from HEDG/KCl buffer). The dextran-charcoal pellets were resuspended on a Vortex mixer and the samples were incubated for 15 min at 4° before the charcoal-dextran was removed by centrifugation at 2,000 × *g* for 20 min. Aliquots (0.6 ml) of supernatant were layered onto linear (5%–20%) sucrose density gradients prepared in HEDG/KCl buffer. The gradients were centrifuged at 4° for 16 hr at 48,000 rpm in a Beckman SW 50.1 rotor. After centrifugation, 40 fractions from each gradient were collected into mini-scintillation vials. Radioactivity in each fraction was determined by liquid scintillation counting and corrected for counting efficiency. In experiments to determine an approximate sedimentation coefficient for the radioactive peak, BSA (4.6 S) and catalase (11.3 S) were used as internal sedimentation markers.

Carbon monoxide experiments. Separate gas-flow meters, attached to either CO or O_2 cylinders, were connected to a common manifold, and the CO/O_2 gas mixtures in the combined effluent were determined from the readings of the individually calibrated flow rates. Inducing agents were added to pre-confluent 10T1/2 cultures in T75 flasks (Falcon), containing 20 ml BME and 10% FCS, and the flasks were immediately gassed with 4 flask volumes of the appropriate gas mixture; the gas stream was filtered through a 0.2-μM Millipore filter. Immediately after gassing, the flask caps were tightly sealed, and the flasks were incubated in the dark for 24 hr at 37° prior to cell harvest for AHH and cytotoxicity determinations. For determination of CO inhibition of AHH activity in liver microsomal preparations, incubations without microsomes or substrate (BP) were bubbled with the appropriate CO/O_2 mixture for 2 min; microsomes and BP were then added and the sealed tubes were incubated in the dark for 3 min to determine AHH activity.

Cytotoxicity determination. Monolayers of 10T1/2 cells, after a 24-hr exposure to the indicated inducing agent, were trypsinized and the cell number was determined using a Coulter counter (Model ZB1). Fresh BME/10% FCS (2 ml) (without inducers) containing either 2000 or 4000 control or inducer-treated cells was added to replicate wells of a 24-well cell culture plate (Linbro). The plates were incubated at 37° for 4–5 days in a humidified 5% CO_2 atmosphere. The subconfluent cultures in each well were then trypsinized and the cell suspension was counted. The number of cells in the wells containing control cultures (i.e., 10T1/2 cells untreated prior to seeding into wells) typically increased 10- to 15-fold over the 5-day growth period. Cell numbers less than the controls were indicative of the degree of hydrocarbon toxicity induced in the cells prior to seeding.

RESULTS

Characterization of AHH induction in 10T1/2 cultures. The level of induction which is achieved by BA treatment during any single 24-hr period of 10T1/2 cell growth was found to vary with the age of the cell culture (Fig. 1). Each day after seeding, 13 μM BA was added to specific dishes, and 24 hr later the treated and control

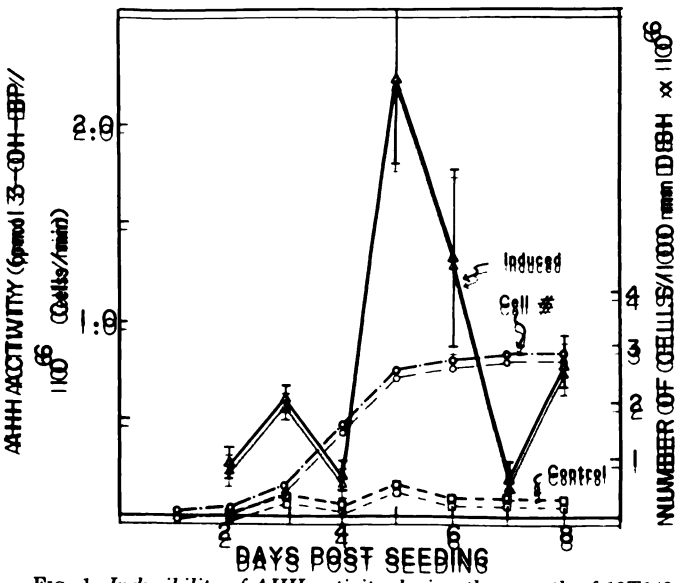

FIG. 1. *Inducibility of AHH activity during the growth of 10T1/2 cells*

Dishes (100 mm) were seeded with 0.1 × 10^6 cells. Each day, three dishes were treated with 13 μM BA dissolved in acetone, and two dishes were treated with acetone alone at a final concentration of 0.5%. After 24 hr, cells were harvested, counted, and assayed for AHH activity as described under Materials and Methods. □–□, Control AHH activity; △—△, BA-induced AHH activity; ○–○, number of cells per 100-mm dish. Error bars indicate the mean and standard deviation of three analyses from three dishes.

dishes were analyzed for cell number and AHH activity. The greatest induction (12- to 15-fold) was always seen in cultures which entered confluence during the 24-hr BA treatment period. Typically, cells reached confluence on day 4–5 (Fig. 1), but occasionally on day 5–6, hence the somewhat greater variation in induced AHH levels during these two time periods. After confluence, induced AHH activity was greatly reduced. This cell density-dependent induction phenomenon has been previously observed by us and others in 10T1/2 (18, 26), 3T3 (17), and human fibroblast[2] cultures. To maximize our sensitivity to induction, all subsequent studies were done with the inducer added on day 4 and the cells harvested on day 5.

Comparison of AHH induction in 10T1/2 cells and C3H mouse livers. The 10T1/2 cells used represent a cloned cell line originally derived from a C3H mouse embryo (21). Because of past observations showing that 3MC was ineffective as an inducer in 10T1/2 (18, 19) and 3T3 (17) cells, in comparison to the pronounced inducing effect of BA, we wanted to compare the relative inducing abilities of these two agents as well as TCDD in both the 10T1/2 cells and the parent C3H mice. The results in Fig. 2 clearly indicate that, although BA treatment yielded a 9.1-fold AHH increase in the 10T1/2 cells, 3MC was ineffective and TCDD gave only a 2.0-fold increase. In the C3H mice, the rank-ordered levels of induced liver AHH were very different, with 3MC now clearly (5.6-fold) more effective than BA (1.9-fold) in inducing AHH, and TCDD (7.8-fold) being the most effective (Fig. 2).

[2] D. Ho and W. Fahl, unpublished observation.

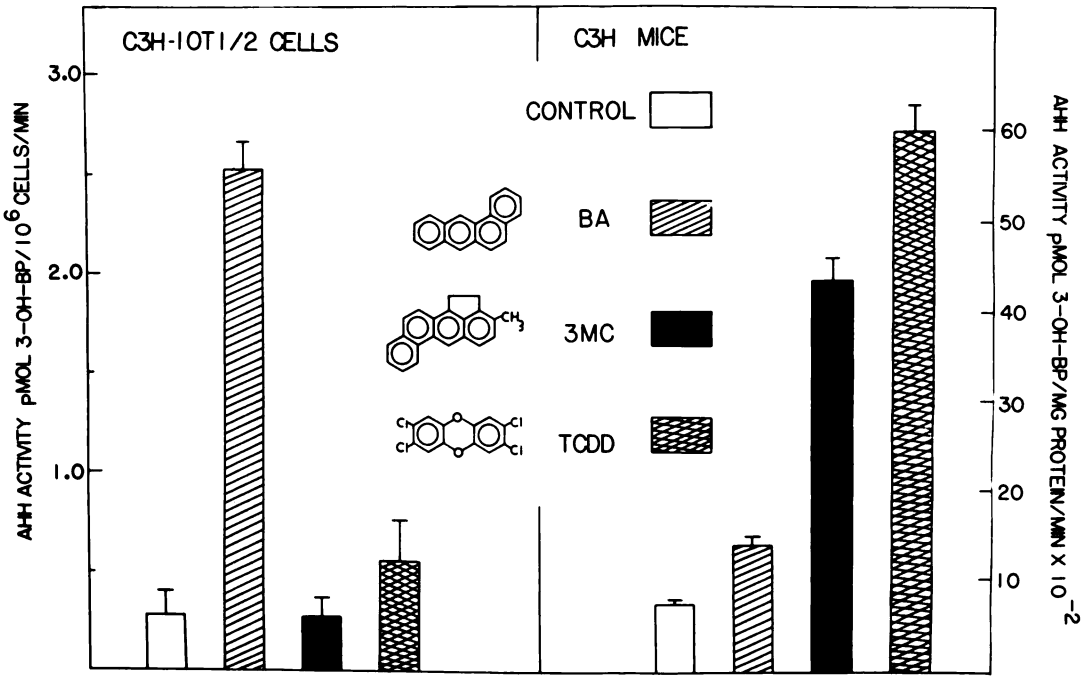

FIG. 2. *Comparison of the levels of AHH activity induced in C3H/10T1/2 cells and C3H mouse liver by aromatic hydrocarbons*

C3H/10T1/2 cells: Dishes (100 mm) were seeded with 0.1×10^6 cells. On day 4 postseeding, three dishes were treated with 13 μM BA, 13 μM 3MC, 0.1 μM TCDD, acetone, or *p*-dioxane. After 24 hr, the cells were harvested, counted, lysed, and assayed for AHH activity as described under Materials and Methods. C3H mouse liver: C3H mice received i.p. injections of BA or 3MC (80 mg/kg) in corn oil or TCDD (10 μg/kg) in *p*-dioxane and were killed 40 hr later for preparation of liver microsomes. Microsomal incubations (0.1 mg/ml) were carried out for 3 min as described under Materials and Methods.

Simultaneous addition of BA and 3MC to 10T1/2 cultures. In order to determine whether the ineffective inducer, 3MC, was capable of competitively interacting with BA in the 10T1/2 cells, increasing amounts of 3MC were added to dishes along with the standard amount of BA (13 μM). The results in Fig. 3 indicate that 3MC was capable of totally blocking the normal BA-induced increase in 10T1/2 AHH activity (3MC ID_{50} = 0.26 μM). By doubling the concentration of BA initially added to the cells (26 μM), the 3MC ID_{50} was then also doubled to 0.56 μM (Fig. 3).

Ah receptor ligand-binding characteristics in 10T1/2 cell and C3H mouse liver cytosols. The above results suggested a competitive inhibition of BA induction by 3MC in the 10T1/2 cells, whereas a sustained maximal induction in liver AHH was seen when BA and 3MC were administered together to C3H mice (data not shown). In order to clarify the seemingly contradictory antagonist-agonist roles of 3MC in the cell culture and mouse liver systems, we examined the relative abilities of 3MC and BA to occupy the Ah receptor in cytosols prepared from both 10T1/2 cells and C3H mouse livers. In the absence of available [^{3}H]BA for ligand binding studies, [^{3}H]TCDD was used, and we then determined whether each of the ligands of interest (i.e., 3MC, BA, or TCDBF) was capable of displacing the radiolabeled ligand, and thus by inference of occupying the receptor in the cell culture or mouse liver cytosols.

Incubation of isolated cytosol from 10T1/2 cells with 1 nM [^{3}H]TCDD for 1 hr at 4° produced a ligand-receptor complex which, after charcoal-dextran treatment, was identifiable by sucrose density gradient analysis (Fig. 4).

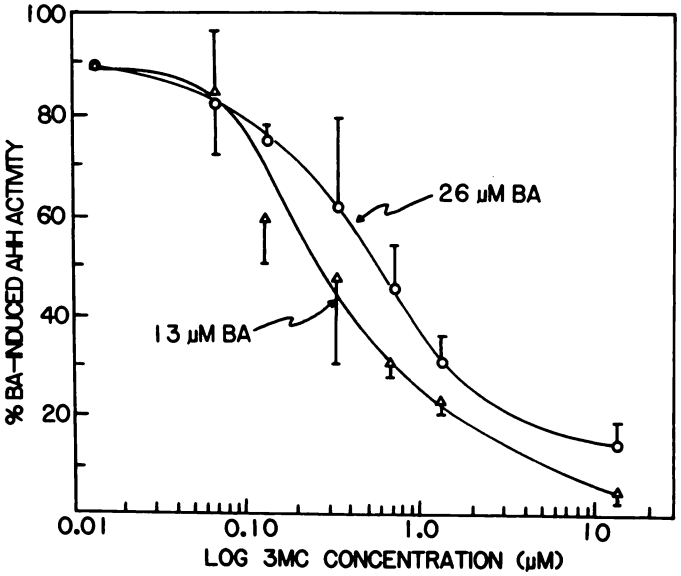

FIG. 3. *Inhibition of AHH induction by BA in 10T1/2 cells as a result of simultaneous 3MC addition*

Dishes (100 mm) were seeded with 0.1×10^6 cells. On day 4 postseeding, the dishes received either BA alone or BA with increasing concentrations of 3MC, 13 μM BA (Δ——Δ), or 26 μM BA (○——○). After 24 hr, the cells were harvested, counted, lysed, and assayed for AHH activity as described under Materials and Methods. Error bars indicate the mean and standard deviation of three assays on three dishes. BA-induced (100%) AHH activity equals 2.6 pmoles of 3-OHBP/10^6 cells/min; control (0%) AHH activity equals 0.3 pmole of 3-OHBP/10^6 cells/min.

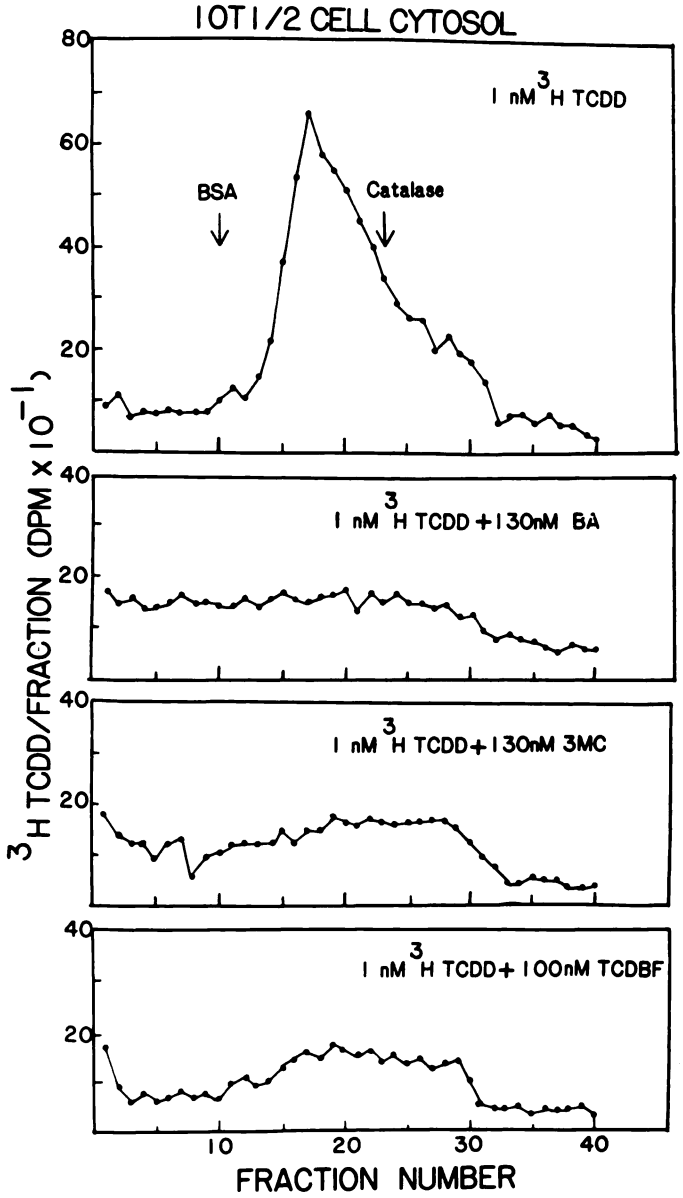

FIG. 4. *Sucrose density gradient detection of a specific, high-affinity TCDD receptor in cytosols from C3H/10T1/2 cells and evidence of ligand competition*

Ten milligrams of 10T1/2 cell cytosol protein were incubated with 1 nM [^{3}H]TCDD in a 1-ml incubation in the absence or presence of additional ligands at the specified concentrations. Incubations were carried out for 1 hr at 4°. Unbound [^{3}H]TCDD was removed by charcoal-dextran incubation and centrifugation; the supernatant was applied to linear 5%–20% sucrose gradients prepared with HEDG buffer containing 100 mM KCl. The positions of the internal sedimentation markers, BSA (4.6 S), and catalase (11.3 S) are indicated in the *top* gradient.

Parallel sucrose density gradients containing BSA and catalase or hemoglobin and catalase indicated a sedimentation coefficient of 7–8 S for the 10T1/2 receptor, similar to that previously reported for mouse liver and cells (9, 27) and observed by us with C3H mouse liver cytosol (data not shown). If incubations containing 10T1/2 cytosol and 1 nM [^{3}H]TCDD also received BA (130 nM), 3MC (130 nM), or TCDBF (100 nM), then each of the three unlabeled compounds was able to displace the stereospecifically receptor-bound [^{3}H]TCDD in both the 10T1/2 cell cytosol (Fig. 4) as well as in the C3H mouse liver cytosol (Table 1). This would indicate that all four of these compounds are ligands for the Ah receptor in both the 10T1/2 cells and C3H mouse livers, and as such might be expected to initiate the Ah receptor-mediated induction response in both systems (5, 28).

3MC-induced loss of cell viability. 3-MC, which has been used to initiate the transformation of 10T1/2 cells in culture, has also been shown to be cytotoxic in these cultures (18, 21). This might be particularly true within the 24-hr period which was selected here (Fig. 1) for maximal induction of a polycyclic hydrocarbon-metabolizing cytochrome P-450 species (i.e., catalyzing 3-OHBP formation) which probably also mediates the oxidation of 3MC to potentially toxic hydroxyl, epoxide, or dihydrodiol-epoxide metabolites (29). The results of Fig. 5 indicate that 13 μM BA alone is not toxic to these cells; however, when 3MC is also added to these dishes, a dose-dependent loss in cell viability is seen which reflects the dose-dependent loss in the ability of BA to induce AHH in these cells. The 3MC-induced loss in the ability of these cells to grow occurs at a slightly lower concentration (ID_{50} = 0.20 μM) than the 3MC-induced loss in ability to induce AHH maximally (ID_{50} = 0.32 μM) (Fig. 5).

Inhibition of 3MC cytotoxicity. If cytotoxic 3MC metabolites were rendering the cells incapable of responding to the 3MC or BA-occupied Ah receptor in 10T1/2 cells, then inhibition of the cytochrome P-450-mediated oxidation of 3MC should result in AHH induction in the presence of 3MC. Experiments in which 7,8-benzoflavone or metyrapone was added with 3MC to the cell culture medium, at concentrations shown to give greater than 90% inhibition of liver microsomal AHH, were unsuccessful inasmuch as these compounds were either cytotoxic or inducers themselves, thus preventing any conclusions about associated 3MC effects. Poland and Kappas (30) previously showed that a CO content of 83% in the atmosphere above monolayer cell cultures was capable of producing a significant inhibition of aminopyrine me-

TABLE 1

Polycyclic hydrocarbon competition with [^{3}H]TCDD for binding to the Ah receptor in C3H mouse liver cytosol

C3H mouse liver cytosol (2 mg) was incubated with 1 nM [^{3}H]TCDD in a 1-ml incubation (HEDG/KCl buffer) in the absence or presence of additional ligands at the specified concentrations. Incubations were carried out for 1 hr at 4°. Unbound [^{3}H]TCDD was removed by charcoal-dextran incubation and centrifugation. Aliquots (0.6 ml) of the supernatant were removed and the radioactivity was measured. Values shown represent the means of duplicate determinations.

Incubation	Total [^{3}H] TCDD bound/ 2 mg of cytosol	Stereospecific [^{3}H]TCDD bound[a]/2 mg of cytosol
	dpm	*dpm*
1 nM [^{3}H]TCDD	6766	3281
1 nM [^{3}H]TCDD + 100 nM BA	4067	582
1 nM [^{3}H]TCDD + 100 nM 3MC	3666	181
1 nM [^{3}H]TCDD + 100 nM TCDBF	3485	0

[a] Stereospecific binding (i.e., minus nonspecific binding) is defined as those counts remaining in the presence of a 100-fold molar excess of TCDBF.

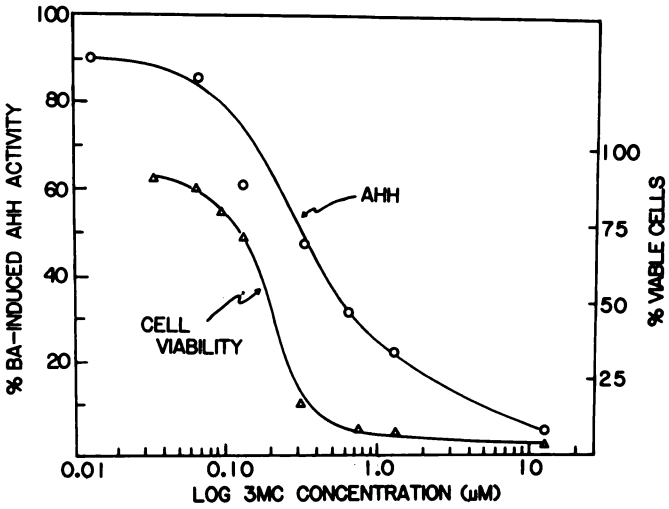

FIG. 5. *Inhibitory effect of 3MC on AHH induction by BA in 10T1/2 cells: associated loss in cell viability*

Dishes (100 mm) were seeded with 0.1×10^6 cells. On day 4 postseeding, the dishes received either 13 μM BA alone or 13 μM BA with increasing concentrations of 3MC. After 24 hr, the cells were trypsinized and counted. Either 2000 or 4000 cells were seeded into replicate wells of 24-well plates for cytotoxicity determination (see Materials and Methods); the remaining cells from each dish were lysed and assayed for AHH activity. Treatment with 13 μM BA alone (100% AHH activity) gave an AHH activity of 2.4 pmoles of 3-OHBP/10^6 cells/min; the viability of these cells was equivalent to acetone controls (i.e., 100% value).

tabolism by cytochrome P-450. The results in Fig. 6 indicate that CO/O_2 gas mixtures of 6:1 (i.e., 86% CO) were capable of giving a 50–70% reduction in AHH activity catalyzed by the induced C3H mouse liver microsomes. A further increase in CO content to 95% of the gaseous phase gave only marginally better inhibition of the cytochrome P-450 oxidation of BP (60–75%), a value which should be representative of the inhibition one would expect for 3MC oxidation.

When 10T1/2 cell monolayers were exposed to 95% CO/5% O_2 alone for 24 hr, the atmosphere was shown to be non-cytotoxic, and the AHH value obtained from the single acetone/CO-treated flask was not significantly different from the acetone/air-treated flask (Fig. 7). In addition, the data shown in Fig. 7 now allowed us to draw certain conclusions regarding inducer effects: (a) BA was effective in inducing AHH to similar levels in both of the CO atmospheres and in the normal 95% air/5% CO_2 atmosphere; (b) the viability of cells exposed to 3MC for 24 hr in air/CO_2 was extremely low (2%), and the viability rose with increasing CO content in the atmosphere over the cell monolayers; and (c) as cells exposed to 3MC retained viability, a concomitant induction of AHH was now observable. In the 3MC-treated cells, the appearance of the 3MC-induction response (in the 86% and 95% CO flasks) occurred more rapidly than the appearance of viability; this reinforces the results of Fig. 5, in which the cells lost viability faster than AHH inducibility. Likewise, under conditions (i.e., 3MC-95% CO/5% O_2 flask) in which we would anticipate a 50–70% inhibition of 3MC oxidation in the 10T1/2 cells (assuming similar CO inhibition of a 3MC-oxidizing cytochrome P-450 isozyme; Fig. 6) we *do* regain 51% of the induced AHH activity which is normally seen in the BA-air/CO_2 flask. This suggests that 100% inhibition of the 3MC-metabolizing cytochrome P-450 in these 10T1/2 cells should then yield a normal 3MC induction response equal in magnitude to that seen with BA.

DISCUSSION

Several reports have shown that 3MC, while being an effective inducer of cytochrome P-450-dependent AHH in intact rodents, is incapable of eliciting AHH induction in 10T1/2 and other commonly used rodent or human cell cultures. In contrast, BA has been persistently observed to elicit multifold induction of AHH in the same cell culture models. Our interest in this work was to probe this seeming disparity in agonist function for two compounds which have previously been shown stereospecifically to bind to the mouse liver Ah receptor (5), a receptor which has now been demonstrated to mediate the induced expression of those genes regulated by the Ah locus. Ligand binding studies using cytosol from both 10T1/2 cell cultures and C3H mouse livers indicate that 3MC, while inducing no increase in 10T1/2 AHH activity (Fig. 2), was fully capable of occupying the 10T1/2 cytosol Ah receptor. However, cytotoxicity analysis of 3MC-treated 10T1/2 cell cultures indicated a striking correlation between a 3MC-induced loss of cell viability and a concomitant inability of the cells to mount an induction response after exposure to 3MC (Fig. 5). Finally, after partially blocking 3MC-induced cytotoxicity through cytochrome P-450 inhibition, 3MC then induced AHH in the 10T1/2 cells to a level which represented

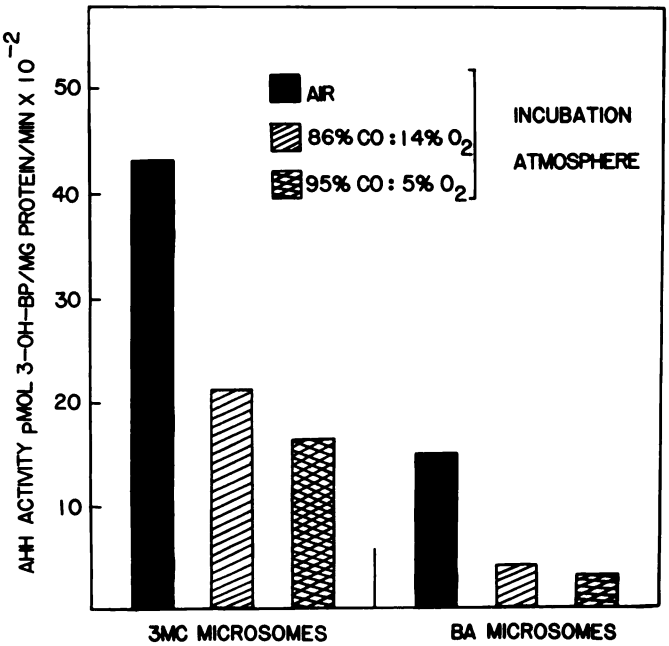

FIG. 6. *Inhibition of mouse liver microsomal AHH activity by carbon monoxide*

Liver microsomes were prepared from C3H mice that had previously received injections of BA or 3MC (see Materials and Methods). Incubations (1 ml) without microsomes or BP were gently bubbled with the specified CO/O_2 mixtures for 2 min. Microsomes (0.1 mg) and substrate (15 nmoles) were then added in small volumes, and the tubes were sealed and incubated in the dark for 3 min. Control tubes were bubbled with air prior to assay.

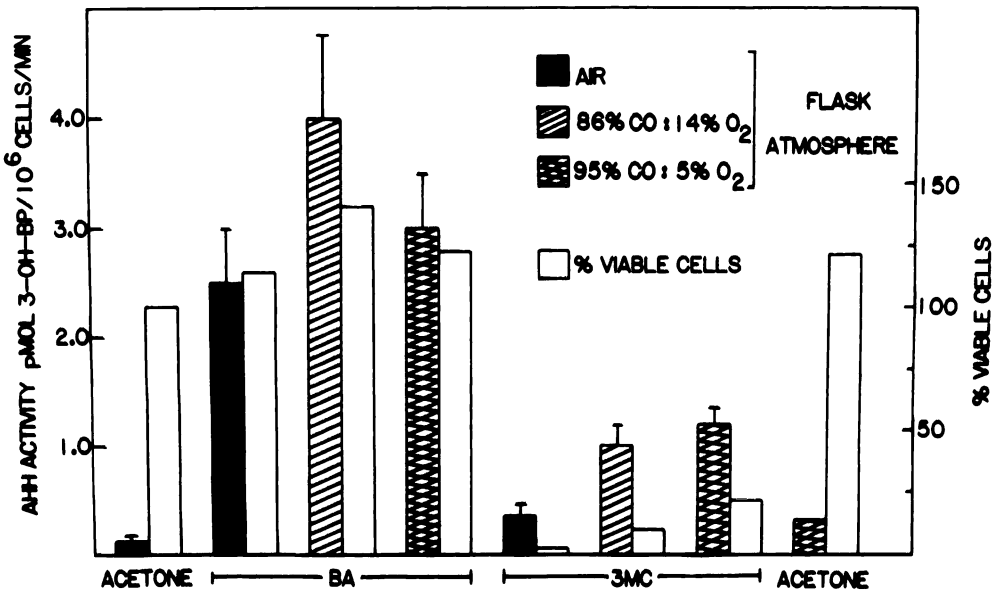

FIG. 7. *Effect of carbon monoxide on the inducing ability and cytotoxic effect of 3MC or BA in 10T1/2 cells*

Flasks (T75) were seeded with 0.1×10^6 cells. On day 4 postseeding, cells were treated with 13 μM BA, 13 μM 3MC, or acetone. Immediately after adding the hydrocarbons, the flasks were flushed with 4 volumes of the specified gas mixtures and tightly sealed. The flasks were incubated in the dark at 37° for 24 hr; the cells were then trypsinized and counted. Cells were seeded into 24-well plates for cytotoxicity determination, and the remaining cells from each flask were lysed and assayed for AHH activity. The inducing agent which was added to specific flasks for 24 hr is indicated *below* the bar graphs. The *patterned bars* indicate the type of gas mixture which was present in the flasks during the 24-hr induction period. The *open bars* indicate the percentage of cells in the flasks which were viable after the 24-hr induction period.

about 50% of the maximal achievable response normally seen after BA treatment (Fig. 7).

TCDD, which was the most effective inducer of AHH in the C3H mice, gave only a 2-fold increase in enzyme activity in the cells over a range (10^{-11} to 10^{-7} M) of TCDD concentrations in the medium (Fig. 2). The reason for its marginal effect within the cell culture is unknown. Knutson and Poland (31) reported a similar 2-fold AHH induction under culture conditions (10^{-7} M TCDD) in which there was no evidence of any TCDD-associated toxicity to the 10T1/2 cell cultures. However, Okey *et al.* (32) found 5- to 7-fold increases in 10T1/2 AHH activity after TCDD exposure, suggesting that the low and variable TCDD induction response may relate simply to cell culture conditions or perhaps the relative affinity of TCDD for cell membranes or the plastic tissue culture vessel.

Experiments in which 3MC was added to dishes containing 13 μM BA showed that 3MC could block the BA-mediated induction response in a dose-dependent manner (Fig. 3). Doubling the starting BA concentration resulted in an approximate doubling of the 3MC ID_{50} (Fig. 3). Both the shape of the curves and the concentration-dependent displacement at first suggested to us that 3MC may be competitively displacing BA from the Ah receptor in these cells and as such acting as a nonfunctional ligand or as a BA antagonist. Figure 4 and Table 1 clearly show that both 3MC and BA are capable of binding to the [^{3}H]TCDD receptor molecule in both 10T1/2 and C3H mouse liver cytosol. However, the generality of this BA-3MC induction phenomenon (e.g. refs. 13–19), as well as the subsequent striking viability results, has led us to favor rather an explanation involving 3MC metabolite cytotoxicity as compared with our original working hypothesis involving an aberrant receptor molecule in these cells, as well as all other cells (principally mammalian fibroblasts) in which this BA-3MC phenomenon had been observed. Our results, however, do not disprove the aberrant receptor hypothesis. We do not know the relative binding constants for 3MC and BA at the Ah receptor in the 10T1/2 cells. Okey *et al.* (32) have recently reported concentrations of 3MC and BA which were capable of displacing 50% of the stereospecifically bound [^{3}H]TCDD in 10T1/2 cytosol fractions. Their data indicated roughly a 10-fold higher receptor affinity for 3MC than for BA, relative affinities which are similar to the values previously reported for C57BL/6J mouse liver cytosol (5).

Results of the experiment shown in Fig. 5 strongly implicated the formation of toxic 3MC derivatives as a cause for the lack of AHH inducibility, and suggested that in the absence of these metabolites 3MC may well act as an inducer of AHH activity. By using CO, a well-characterized inhibitor of cytochrome P-450-mediated oxidations, we were able to maintain reasonable cell viability in the presence of 13 μM 3MC and to identify a 3- to 5-fold increase in 10T1/2 AHH activity. The high viability values and the full BA induction of AHH which was achievable in the presence of 95% CO indicated that CO itself had no effect on these cell processes and thus allowed observation of the 3MC induction response.

The ability of BA but not 3MC to act as an inducer of AHH activity, which appears to be generally observable in most rodent and human fibroblast cultures, clearly is not seen among essentially all tested primary hepatocyte and hepatoma cultures. In these cultures, we (33) and others (34–36) have routinely observed the ability of 3MC to induce cytochrome P-450 and the associated

monooxygenase activities. Laine *et al.* (37) have also recently shown that 3MC can induce AHH in primary fetal liver cells derived from C3H mouse fetuses. Given the above observations, one could ask why in the hepatocyte populations the Ah receptor-mediated induction response predominates over any 3MC cytotoxic response, whereas in fibroblast cultures (e.g., 10T1/2) the cytotoxic response is predominant. These findings are in agreement with earlier tumorigenicity experiments in which 3MC induced 99% fibrosarcomas with no hepatomas when injected into C3H mice (38). Comparative studies of cellular nucleophile concentrations and conjugation enzyme levels in fibroblast and hepatocyte populations may provide evidence that explains the reason for the organ specificity of 3MC toxicity.

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