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Polynuclear aromatic hydrocarbon carcinogens as antiestrogens in MCF-7 human breast cancer cells: role of the Ah receptor

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Treatment of MCF-7 cells with 1.0 μM 3-methylcholanthrene (MC) caused a decrease in cell proliferation and [^{3}H]thymidine uptake whereas no effects were observed at a lower (0.1 μM) concentration. Co-treatment of the cells with 1 nM 17β-estradiol plus 0.1 or 1.0 μ MC resulted in a significant inhibition of 17β-estradiol-induced growth and [^{3}H]thymidine uptake. MC also inhibited the 17β-estradiol-induced secretion of the 52 kDa protein (procathepsin D) in MCF-7 cells and caused a concentration-dependent decrease in the nuclear estrogen receptor (ER) as determined by either velocity sedimentation analysis or immunoquantitation with human ER antibodies. The effects of several different polynuclear aromatic hydrocarbon (PAH) congeners on the nuclear ER in MCF-7 cells were also determined. Only those congeners which bound to the aryl hydrocarbon (Ah) receptor, namely benzo[*a*]pyrene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene and MC, caused a decrease in nuclear ER levels. In contrast, benzo[*ghi*]perylene, a congener which did not bind to the Ah receptor did not affect nuclear ER levels in MCF-7 cells. Moreover, with some congeners the decrease in nuclear ER levels could be observed without any significant induction of ethoxyresorufin *O*-deethylase activity, a P4501A1-dependent monooxygenase. These data suggest that the Ah receptor liganded with MC and related PAHs induced a broad spectrum of antiestrogenic responses in MCF-7 cells and complements the results of previous studies which report the antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and other halogenated aromatics which are also Ah receptor agonists.

Introduction

3-Methylcholanthrene (MC*) is a member of the polynuclear aromatic hydrocarbon (PAH) family of compounds which exhibit a broad spectrum of biochemical, toxic and genotoxic responses in laboratory animals and mammalian cells in culture (1–5). For example, MC, benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene and other PAHs have been characterized as carcinogens in laboratory animals and may be involved in the etiology of human cancers associated with exposure to PAH mixtures in coal tar and smoke (6–8). The carcinogenic activity of these compounds is dependent on their metabolic activation to reactive intermediates which alkylate critical target genomic sites (1–3,9). In addition, many of the carcinogenic PAHs such as MC, benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene exhibit high binding affinity for the aryl hydrocarbon (Ah) receptor (10). The Ah receptor is an intracellular protein which has been widely identified in mammalian cells and tissues (11). It binds with high affinity not only to PAHs, but also to other structural classes of aromatic compounds, including the toxic halogenated aromatic hydrocarbons, typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (12–15). TCDD, MC and related isostereomers induce *CYP1A-1* gene expression and the molecular mechanism of this response has been extensively investigated (16–20). The results of these studies suggest that TCDD or MC reversibly binds to the Ah receptor; the liganded Ah receptor complex is transformed to a heterodimer which undergoes nuclear translocation and interacts with specific binding genomic sites in the 5′-flanking region of the *CYP1A-1* gene. These sites have been designated as xenobiotic or dioxin responsive elements and the interaction of the nuclear Ah receptor complexes with these sites is necessary for induced gene transcription. The nuclear Ah receptor complex liganded with TCDD, MC and related compounds acts as a ligand-responsive transcription factor (21) which is involved in the induction of Ah-responsive genes.

TCDD elicits a broad spectrum of age, species-, sex- and organ-/cell-specific responses which are also believed to be mediated through the Ah receptor (13,15,17). For example TCDD inhibits several 17β-estradiol-induced responses in the female rodent and in human breast cancer cell lines (22,23). The Ah receptor has been identified in the target issues (e.g. rat uterus) and cells (24–27) and, based on structure–activity studies with related congeners, the results support a direct or indirect role for the Ah receptor in this process. The effects of other Ah receptor agonists as antiestrogens have not been determined. Therefore, this study is focused on the activity of MC and related PAHs on several estrogen-induced responses in MCF-7 human breast cancer cells.

Materials and methods

Chemicals and biochemicals

MC, 1,2-benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, benzo[*ghi*]perylene, NADP, NADPH, rhodamine B, bovine serum albumin (BSA) and transferrin were purchased from Sigma Chemical Co. (St. Louis, MO). α-Naphthoflavone (αNF) was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Radiolabeled [^{3}H]17β-estradiol (143 Ci/mmol) and [^{3}H]thymidine (73.5 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). Hydroxylapatite was purchased from Bio-Rad Laboratories (La Jolla, CA) and all other chemicals were of the highest quality available from commercial sources.

Cell culture growth

MCF-7 human breast cancer cells were grown in DME/F12 media (Gibco, Gaithersburg, MD) supplemented with 2.2 g/l tissue grade sodium bicarbonate, 0.2 g/l bovine serum albumin, 10 mg/l transferrin, 5% fetal calf serum and a 1% antibiotic–antimycotic solution. Stock cultures were grown in 150 cm^2 culture flasks and incubated at 37°C in 95% air and 5% CO_2. For enzyme assays, 5 × 10^6 cells in 5 ml of media were passaged to 25 cm^2 culture flasks. For nuclear experiments, 5 × 10^7 cells in 50 ml of media were passaged to 150 cm^2 culture flasks. Solutions of the treatment chemicals dissolved in dimethylsulfoxide (DMSO) were added to the tissue culture flasks so that the

*Abbreviations: MC, 3-methylcholanthrene; PAH, polynuclear aromatic hydrocarbon; Ah, aryl hydrocarbon; TCDD, 2,3,7,8-tetradecanoyl-phorbol-13-acetate; XRE, xenobiotic responsive element; DRE, dioxin responsive element; BSA, bovine serum albumin; αNF, α-naphthoflavone; DMSO, dimethylsulfoxide; EROD, ethoxyresorufin *O*-deethylase; ER, estrogen receptor; DCC, dextran-coated charcoal; TCA, trichloroacetic acid.

final concentration of DMSO did not exceed 0.1%. Cultured MCF-7 cells were harvested and assayed for enzyme induction 24 h after treatment unless otherwise specified and 12 h after treatment for the nuclear extraction studies.

Induction of ethoxyresorufin O-deethylase (EROD) activity

The cells were harvested and the EROD activity was determined by the method of Pohl and Fouts as previously described (28). Protein concentrations were determined by the method of Lowry *et al.* (29).

Preparation of nuclear extracts

MCF-7 cells were rinsed with 20 ml of phosphate-buffered saline (PBS) to remove any traces of the chemical treatments and harvested by trypsinization. Six 150 cm^2 culture flasks were used per treatment group. Cells from each treatment group were collected in warm PBS, pooled into 50 ml polypropylene conical centrifuge tubes and centrifuged at 800 *g* (2°C) for 10 min. The resulting pellet was resuspended in 20 ml of DME/F12 media, syringed five times to ensure a single-cell suspension and aliquoted equally into two 25 cm^2 culture flasks. The cellular suspension was treated with either 1 nM [^{3}H]17β-estradiol or 1 nM [^{3}H]17β-estradiol plus a 200-fold excess of diethylstilbesterol (for determination of nonspecific binding) for 60 min at 37°C in a shaking water bath. Following the 60 min radioligand treatment, the cell suspension was transferred to 50 ml polypropylene conical centrifuge tubes and centrifuged at 800 *g* (62°C) for 10 min. The resulting pellet was then rinsed twice in TEGD buffer (10 mM Tris–HCl, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol pH 7.6) and pelleted by centrifugation at 800 *g* (2°C) for 10 min. The washed cell pellet was resuspended in TED buffer (10 mM Tris–HCl, 1.5 mM EDTA, 1 mM dithiothreitol pH 7.6) and transferred to a 2 ml Wheaton homogenizing tube. The samples were homogenized with 15 passes of a Teflon pestle/drill apparatus. The homogenate was transferred to a 15 ml polypropylene conical centrifuge tube to which 2 ml of TEGDM buffer (10 mM Tris–HCl, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 20 mM sodium molybdate pH 7.6) was added. The sample was centrifuged at 100 *g* (2°C) for 10 min and the resulting pellet rinsed twice with 5 ml of TEGDM. After the final wash, the pellet was resuspended in 2 ml of TEGDM containing 0.4 M KCl, to extract the receptor–ligand complex, in a 5 ml polycarbonate Beckman centrifuge tube. The suspension was incubated at 2°C for 1 h before centrifugation at 105 000 *g* (2°C) for 1 h. The resulting supernatant was used for sucrose density gradient analysis or direct liquid scintillation counting.

Sucrose density gradient analysis

Aliquots (1 ml) of the supernatant were briefly treated with dextran/charcoal solution (0.2 mg charcoal and 0.02 mg dextran) in order to remove any unbound or loosely bound [^{3}H]17β-estradiol. The dextran/charcoal was removed after a 15 min incubation by centrifugation at 1000 *g* (2°C) for 10 min. Aliquots (300 μl) of the supernatant were layered onto a linear 5–25% sucrose density gradient prepared in TEGDM buffer containing 0.4 M KCl. Gradients were then sealed and centrifuged at 404 000 *g* (2°C) for 2.5 h in a Beckman VTi-65 vertical rotor. The gradient was fractionated into 30 fractions with 4 drops/fraction. Radioactivity of each fraction was determined by liquid scintillation counting.

Measurement of nuclear estrogen receptor (ER) by monoclonal antibody kit from Abbott Laboratories (83-5167/R5)

Nuclear extracts were prepared as described above. Lyophilized standards were prepared as described in the instructions enclosed in the kit. Each sample was assayed in triplicate, and 100 μl of nuclear extract were used per well. Each sample was diluted with the specimen diluent provided in the kit. One estrogen receptor bead was placed into each well containing either the sample extract, the standard or the control. The trays were then incubated for 16 ± 1 h at 2°C. Following the incubation period, each bead was washed twice with 6–8 ml of deionized water. ER conjugate was put into each well and incubated in water bath at 37°C for 1 h. After the incubation with the ER conjugate, the beads were again rinsed twice with 6–8 ml of deionized water. The beads were transferred to test tubes, and 300 μl of *O*-phenylenediamine–2 HCl substrate solution was added. The absorbance was read at 492 nm using a Beckman DU-70 spectrophotometer with deionized water as a blank. Concentrations of the nuclear estrogen receptor were calculated from a standard curve, with the results being reported as fmol of nuclear estrogen receptor/mg nuclear protein.

Cell proliferation assays

A cellular suspension containing 25 000 cells/ml was made with DME/F12 supplemented with 3% 2 × dextran-coated charcoal (DCC) CPSR-2 and 5% 2 × DCC FCS (both sera had previously been stripped twice with an 0.05% dextran, 0.5% charcoal solution for 30 min at 56°C); 50 000 cells (2 ml of the suspension) were then seeded into a six-well tissue culture plate and incubated at 37°C with a 5% CO_2 and 95% air atmosphere. After 24 h, the medium was replaced with DME/F12 plus 3% 2 × DCC CPSR-2 and 1% 2 × DCC FCS medium containing the appropriate treatment chemicals. The media and chemicals were changed every second day. At the end of seven days, the cells were harvested and counted using a hemocytometer.

[^{3}H]Thymidine incorporation assay

The cells for the [^{3}H]thymidine incorporation assay were grown and treated in the same conditions as outlined in the cell proliferation assay section with the following exceptions: (1) 100 000 cells were seeded per well, and (2) the dosing period consisted of only 4 days. After dosing for 4 days, the cells were pulsed for 1 h with 1 μCi [^{3}H]thymidine per well at 37°C prior to harvesting. The medium was subsequently removed and the wells were rinsed twice with cold PBS. Two milliliters of cold 10% trichloroacetic acid (TCA) was added to each well, and the plates were placed on ice for 4 h to precipitate the protein. The cells were then scraped from the wells, rinsed twice with 2 ml of 10% TCA and transferred to 15 ml polypropylene conical centrifuge tubes. The 15 ml tubes were centrifuged at 2500 *g* (2°C) for 10 min. The supernatant was decanted and the pellets rinsed twice with 10% TCA, followed by a final rinse with 1 ml of cold absolute ethanol (2500 *g* at 2°C for 10 min). After the final rinse, the pellets were resuspended in 0.3 M sodium hydroxide solution to solubilize the protein. The protein was allowed to solubilize for 18 h at 20°C. The samples were vortexed and three 50 μl aliquots transferred to scintillation vials; 2.5 μl of 2 N hydrochloric acid was added to neutralize the base; 5 ml of Ecoscint A scintillation cocktail was also added to the scintillation vial. Radioactivity was determined by liquid scintillation counting.

Detection of the 52 kDa protein (cathepsin D)

MCF-7 cells were grown and maintained in RPMI 1640 medium supplemented with 2 × DCC-FCS. On day 1 cells were passaged into 150 cm^2 culture flasks containing DME/F12 medium supplemented with 5% 2 × DCC-FCS and 3% 2 × DCC CPSR-2. On day 3 cells were seeded into six-well tissue culture plates at a density of 50 000 cells/ml of the DME/F12 medium. On day 4 the medium was changed to the above medium containing 1% 2 × DCC-FCS. On day 5 cells were washed with PBS and treated with fresh medium which contained the appropriate chemicals. After 42 h the cells were washed with PBS and 750 μl of serum-free DME/F12 medium plus the appropriate chemicals was added. After 6 h the medium was removed for further analysis. The treatment chemicals were dissolved in absolute ethanol (1% v/v). The protein concentration in each sample was determined by using the method employed by Lowry *et al.* (29).

Double staining procedure

The secreted proteins were analyzed by SDS–PAGE. Five micrograms of each sample were loaded onto a 12% acrylamide gel slab with a 3% stacking gel. Gels were washed in 12% TCA overnight and incubated in the pretreatment solution for 60 min followed by 30–60 min of staining in the ISS ProBlue™ staining solution (30). The time for staining varied with the amount of protein on the gel. The gels were then washed five times for 2 min with deionized water followed by fixing the gels in a 50% methanol, 10% acetic acid aqueous solution for 30 min. The fixation was further continued overnight in a 5% methanol, 7% acetic acid aqueous solution. The gel was treated with 10% glutaraldehyde solution for 30 min followed by four 30 min washes with deionized water. The gels were then stained with ammoniacal silver stain solution for 15 min with vigorous shaking. The excess solution was washed off the gel using five 1 min washes with an excess of deionized water. The bands were developed using the developing solution (0.5 g sodium citrate, 0.5 ml of 37% formaldehyde solution) until the desired intensity was reached. The stain was fixed onto the gel using the Kodak Rapid Fix™ solution with mild agitation for 5 min. The excess rapid fix was washed off using deionized water. The gel was then dried and subjected to further analysis using a scanning laser densitometer.

Statistics

All the treatment groups were performed in triplicate and the results are presented as means ± SD. The significant differences between different treatment groups were determined by ANOVA or Student's *t*-test.

Results

The results in Table I summarize the effects of 1 nM 17β-estradiol, 0.1 and 1 μM MC and their combinations on the proliferation and [^{3}H]thymidine uptake in MCF-7 human breast cancer cells. 17β-Estradiol significantly increased cell proliferation and [^{3}H]thymidine uptake; 0.1 μM MC alone did not increase cell growth of [^{3}H]thymidine uptake whereas 1.0 μM MC inhibited both growth and incorporation of [^{3}H]thymidine into cellular DNA. In the co-treatment studies, MC (0.1 and 1.0 μM) caused a concentration-dependent decrease in 17β-estradiol-induced cell proliferation and [^{3}H]thymidine uptake in MCF-7 cells.

The effects of MC on the 17β-estradiol-induced secretion of the 52 kDa protein (procathepsin) in MCF-7 cells was also

Table I. Effects of MC on the 17β-estradiol-stimulated cell proliferation and [^{3}H]thymidine uptake in MCF-7 cells[a]

Treatment	Concentration (μM)	[^{3}H]Thymidine (d.p.m./μg protein)	Cell no ($\times 10^{-4}$)
Control (DMSO)	0	2260 ± 586	22 ± 4.0
17β-estradiol	0.001	4000 ± 416[b]	55 ± 3.0[b]
MC	0.1	1730 ± 351	29 ± 4.0
MC	1.0	1060 ± 88.0[c]	13 ± 0.3[c]
17β-estradiol + MC	0.001 + 0.1	2324 ± 647[d]	34 ± 6.0[d]
17β-estradiol + MC	0.001 + 1.0	917 ± 340[d]	11 ± 1.0[d]

[a]The MCF-7 cells (50 000) were treated with the solvent (DMSO) vehicle control, 17β-estradiol, MC and MC plus 17β-estradiol and the media and treatments were changed very 48 h. The cells were counted after 7 days using a hemocytometer. The [^{3}H]thymidine assays were determined after growth of MCF-7 cells (100 000 seeded) for 4 days using a 1 h pulse of [^{3}H]thymidine; the DNA was isolated from the cells as described in the Materials and methods and quantitated by liquid scintillation counting.
[b]Significantly higher ($P < 0.01$) than values for control cells.
[c]Significantly lower ($P < 0.01$) than values for control cells.
[d]Significantly lower ($P < 0.01$) than values for cells treated with 17β-estradiol alone.

Table II. Effects of MC, 17β-estradiol and MC plus 17β-estradiol on the secretion of the 52 kDa protein in MCF-7 cells[a]

Treatment (concentration, μM)	Relative levels of secreted 52 kDa protein (% of control)
Control (DMSO)	100 ± 11
17β-estradiol (0.001)	278 ± 24[b]
MC (0.1)	97 ± 8
MC (1.0)	88 ± 7
17β-estradiol (0.001) + MC (0.1)	165 ± 2[c]
17β-estradiol (0.001) + MC (1.0)	105 ± 9[c]

[a]The MCF-7 cells (50 000) were seeded in multiwell culture plates and after attachment were treated with the solvent (DMSO) vehicle control and the various chemicals for 42 h; the cells were then washed and treated with the same chemical in serum-free media for 6 h. The secreted proteins were separated by SDS–PAGE, double-stained with ISS ProBlue™ and a silver stain and finally quantitated by densitometric analysis as described in the Materials and methods section.
[b]Significantly higher ($P < 0.01$) than values for control cells.
[c]Significantly lower ($P < 0.01$) than values for cells treated with 17β-estradiol alone.

determined (Table II) by densitometric analysis of the double-stained gel used to separate the proteins secreted into the media from the cells treated with different chemicals (Figure 1). The levels of the secreted 52 kDa protein in the cells treated with 1 nM 17β-estradiol were 278% of the control cell values wheres in cells treated with 0.1 or 1.0 μM 3-MC there were no significant changes in the 52 kDa protein levels compared with the solvent-treated control cells. In contrast, in the cells co-treated with MC plus 17β-estradiol, MC caused a significant concentration-dependent decrease in the levels of the 17β-estradiol-induced protein.

The concentration-dependent effects of MC on nuclear ER levels were determined using both radioligand binding assays (velocity sedimentation analysis) (Figure 2) and immuno-quantitation with a commercially available monoclonal antibody kit. The absolute values for the nuclear ER for the control cells differed according to the method of analysis and some of these differences may be due in part to the cell passage number since nuclear ER levels tend to decrease with increasing passage number. The results in Table III show that the percentage

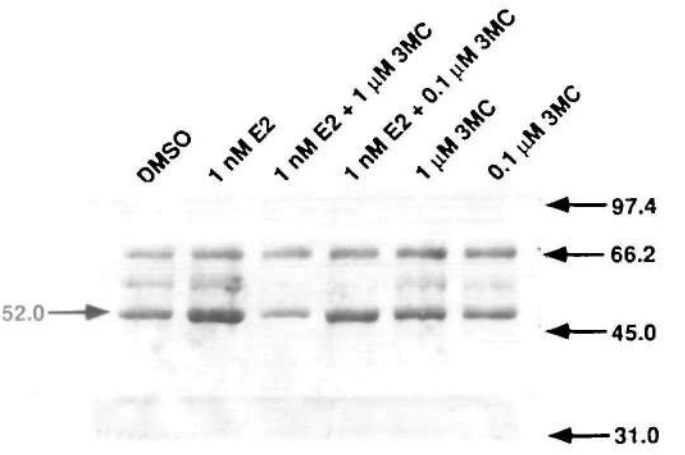

Fig. 1. Double staining of the SDS–PAGE separation of media proteins from MCF-7 cells treated with 17β-estradiol, MC and their combinations. The separation and staining procedures are given in the Materials and methods section. In this chromatogram, aliquots of the media from cells treated with (left to right) solvent (DMSO), 1 nM 17β-estradiol (E2), 1 nM 17β-estradiol plus 1 μm MC, 1 nM 17β-estradiol plus 0.1 μM MC, 1.0 μM MC and 0.1 μM MC were applied to the gel and separated by SDS–PAGE. The position of the molecular weight markers are also noted.

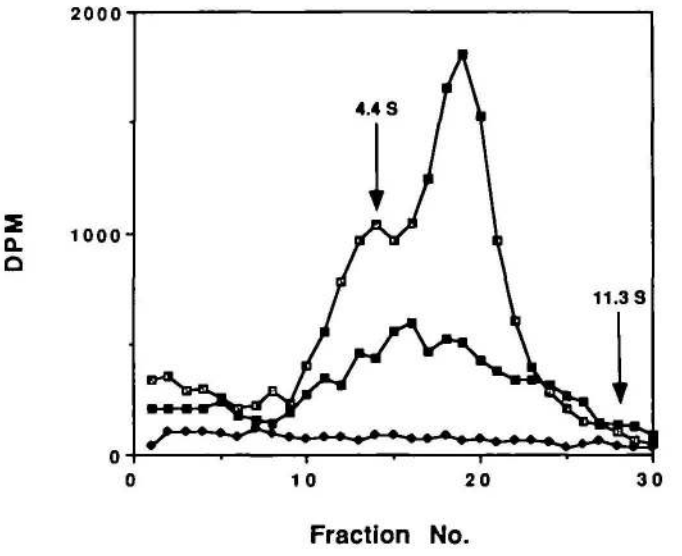

Fig. 2. Velocity sedimentation analysis of nuclear extract from MCF-7 cells treated with 1 nM [^{3}H]17β-estradiol (□), 1 nM [^{3}H]17β-estradiol plus 0.1 μM MC (■), and 1 nM [^{3}H]17β-estradiol plus a 200-fold molar excess of unlabeled diethylstilbestrol (◆). The sucrose-density gradient centrifugation procedure is noted in the Materials and methods section; [^{14}C]catalase and bovine serum albumin were used as molecular weight markers as described (34).

decreases in nuclear ER levels in MCF-7 cells treated with different concentrations of MC were similar using both assay systems. The results in Table IV summarize the effects of αNF and several different PAH congeners on nuclear ER levels in MCF-7 cells. Benzo[*a*]pyrene, benz[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene cause a concentration-dependent decrease in nuclear ER levels whereas benzo[*ghi*]perylene was inactive at the same concentrations (0.1 and 1.0 μM). The results also showed that 1 μM αNF, an Ah receptor antagonist, did not decrease nuclear ER levels but significantly protected against the MC-induced decrease of the nuclear ER.

The recovery of nuclear ER levels after treatment with MC was also determined in MCF-7 cells. The cells were initially

Table III. Concentration-dependent decrease of the nuclear ER in MCF-7 cells treated with MC[a]

Treatment (dose, μM)	Nuclear ER levels (fmol/mg protein) (% control)	Immunoreactive nuclear ER levels (fmol/mg protein) (% control)
DMSO (control)	160 ± 9.0 (100)	312 ± 12 (100)
MC (0.001)	167 ± 8.0 (104)	316 ± 12 (101)
MC (0.01)	146 ± 5.0 (91)	317 ± 3.7 (102)
MC (0.1)	99 ± 4.0 (62)[b]	221 ± 28 (71)[b]
MC (1.0)	51 ± 1.0 (32)[b]	189 ± 16 (61)[b]
MC (10.0)	48 ± 1.0 (30)[b]	94 ± 5.0 (30)[b]

[a]The MCF-7 cells were grown in 150 cm^2 flasks (6 per treatment group) and treated with the various chemicals. After isolation of the cells, they were suspended in growth media for 2 h at 37°C in the presence of 1 nM [^{3}H]17β-estradiol in the absence or presence of 200 nM diethylstilbesterol. Nuclear extracts were obtained from the suspended cells and the specifically-bound nuclear ER levels were determined by velocity sedimentation analysis or by immunoassay procedures as described in the Materials and methods section.
[b]Significantly lower ($P < 0.01$) than values for control cells.

Table IV. Effects of MC, αNF, MC plus αNF, Benz[*a*]anthracene Benzo[*a*]pyrene, 7,12-Dimethylbenz[*a*]anthracene and Benzo[*ghi*]perylene on nuclear ER levels in MCF-7 cells[a]

Treatments (concentration, μM)	Nuclear ER levels (% control)
DMSO	100 ± 12
Benzo[*a*]pyrene (0.1)	75 ± 10[b]
Benzo[*a*]pyrene (1.0)	30 ± 9[b]
Benz[*a*]anthracene (0.1)	51 ± 8[b]
Benz[*a*]anthracene (1.0)	24 ± 6[b]
7,12-Dimethylbenz[*a*]anthracene (0.1)	44 ± 9[b]
7,12-Dimethylbenz[*s*]anthracene (1.0)	30 ± 2[b]
Benzo[*ghi*]perylene (0.1)	113 ± 3
Benzo[*ghi*]perylene (1.0)	99 ± 3
DMSO	100 ± 8
αNF (1)	100 ± 3
MC (0.1)	38 ± 2[b]
αNF (1) + MC (0.1)	70 ± 6[c]

[a]The cell growth, treatment protocols and nuclear ER levels (velocity sedimentation analysis) were determined as described in Table III and the Materials and methods section.
[b]Significantly lower ($P < 0.01$) than values for control cells.
[c]Significantly higher ($P < 0.01$) than values for cells treated with 0.1 μM MC alone.

treated with 0.1 μM MC 12 h before the addition of 1 nM [^{3}H]17β-estradiol and nuclear ER levels were determined 1 h after addition of the radiolabeled hormone. The recovery experiment were determined by increasing the MC pretreatment times from 12 to 18, 24, 30, 36 and 48 h. The results show that there was a time-dependent recovery of the cells from MC and, after 36 or 48 h exposure to MC, no significant decreases in ER levels were observed (Table V).

The effects of several PAH congeners on the induction of EROD activity in MCF-7 cells was also determined (Table VI). These data show that after treatment of the cells for 12 h, <10% maximal induction was observed for 0.1 and 1.0 μM MC and 1.0 nM TCDD whereas higher levels of induced EROD activity were observed for 1 μM benz[*a*]anthracene and 1 nM TCDD 12 and 24 h after treatment of the cells.

Table V. Time-Dependent effects of 0.1 μM MC on nuclear ER levels in MCF-7 human breast cancer cells[a]

Treatment	Pretreatment time (h)	Nuclear ER levels	
		fmol/mg protein	% control
Control (DMSO)	–	221 ± 3.1	100 ± 1.4
MC	12	134 ± 4.0[b]	61 ± 2.3[b]
MC	18	136 ± 17[b]	61 ± 13[b]
MC	24	174 ± 5.8[b]	79 ± 3.3[b]
MC	30	206 ± 4.4[b]	93 ± 2.1[b]
MC	36	223 ± 12	101 ± 5.4
MC	48	216 ± 4.5	98 ± 2.1

[a]The cell growth, treatment protocols and nuclear ER levels (velocity sedimentation analysis) were determined as described in Table III and the Materials and methods section. The standard pretreatment time for MC was 12 h; however, in this experiment cells were also pretreated for 18, 24, 30, 36 or 48 h with MC prior to addition of the radiolabeled hormone.
[b]Significantly lower ($P < 0.01$) than values for control cells.

Table VI. Effects of selected PAHs and 2,3,7,8-TCDD on the induction of EROD activity in MCF-7 human breast cancer cells[a]

Treatment (concentration, μM)	Duration (h)	EROD activity (pmol/min/mg)
MC (0.1)	12	5 ± 1[b]
MC (1.0)	12	4 ± 1[b]
Benz[*a*]anthracene (0.1)	12	ND
Benz[*a*]anthracene (1.0)	12	36 ± 2[b]
Benzo[*a*]pyrene (0.1)	12	ND
Benzo[*a*]pyrene (1.0)	12	ND
7,12-Dimethylbenz[*a*]anthracene (0.1)	12	ND
7,12-Dimethylbenz[*a*]anthracene (1.0)	12	ND
Benzo[*ghi*]perylene (0.1)	12	ND
Benzo[*ghi*]perylene (1.0)	12	ND
2,3,7,8-TCDD (0.001)	12	8 ± 0.1
2,3,7,8-TCDD (0.001)	24	85 ± 18
DMSO (control)		ND

[a]The MCF-7 cells were treated with different concentrations of the individual PAH or 2,3,7,8-TCDD for 12 h or with 2,3,7,8-TCDD for 24 h and the EROD activity was determined as described (28). ND, not detectable.
[b]Significantly higher ($P < 0.01$) than values for control cells.

Discussion

Human breast cancer cell lines have been widely used as models for investigating the effects of various mitogenic and antimitogenic agents on cell proliferation and gene transcription (31–33). MCF-7 cells were selected for this study since these cells express both the estrogen and the Ah receptor and are responsive to agonists which bind to these receptor proteins (27). Like TCDD and several structurally related halogenated aryl hydrocarbons (34–36), MC elicits a similar spectrum of antiestrogenic responses. For example, 0.1 μM MC inhibited 17β-estradiol-induced cell proliferation and [^{3}H]thymidine uptake and the secretion of the 52 kDa protein (Tables I and II). In addition, 1.0 μM MC also inhibited constitutive MCF-7 cell growth and [^{3}H]thymidine uptake. The results in Table III also demonstrate that MC decreased nuclear ER levels as determined by velocity sedimentation analysis and immunoreactive protein which was measured using a human ER antibody kit.

The antiproliferative effects caused by MC are not unique since many different structural classes of compounds elicit some of the same responses. For example, recent studies have reported

that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), protein kinase C activators and several other differentiation-inducing agents including retinoic acid, sodium butyrate, dimethylformamide, hexamethylene bisacetamide, forskolin, the calcium ionophore A23187, TCDD and related isostereomers inhibit MCF-7 cell growth and decrease nuclear ER levels in MCF-7 cells (35–42). TPA also inhibited 17β-estradiol-induced growth and decreased 17β-estradiol-induced immunoreactive progesterone receptor levels (39–42), and similar results have been observed for both TCDD and MC (33,43). In contrast, TPA did not inhibit estrogen-induced pS2 or procathepsin D gene expressions (39), and it was concluded that the growth inhibitory properties of TPA were not due to inhibition of estrogen-induced gene transcription. In contrast, the results shown in Table II indicate that MC, like TCDD, inhibited the estrogen-induced secretion of procathepsin D in MCF-7 cells. Moreover, *in vivo* studies in this laboratory (R.Dickerson, unpublished results) have shown that MC, like TCDD, inhibited the 17β-estradiol-induced uterine wet weight increase and uterine progesterone receptor levels in female Sprague–Dawley rats. Thus, both MC and TCDD exhibited a broad spectrum of antiestrogenic activities which more closely resembled the ER antagonists ICI 164,384 (44,45) than TPA and the differentiation-inducing agents.

MC and TCDD have been extensively used as prototypes for investigating the molecular biology of *CYP1A1* gene expression (16–20). Structure–activity relationships among the polynuclear and halogenated aromatic hydrocarbons have shown a correlation between the structure Ah receptor binding versus structure-induction (P4501A1) activities for both sets of compounds (10,15). MC, benzo[*a*]pyrene, benz[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene all bind to the Ah receptor and induce aryl hydrocarbon hydroxylase and EROD activity (10). The results summarized in Table IV also show that these Ah receptor agonists cause a decrease in nuclear ER levels. In contrast, benzo[*ghi*]perylene, a ligand which does not exhibit significant binding to the Ah receptor (10), does not cause a decrease in nuclear ER levels in MCF-7 cells. The effects of αNF on the antiestrogenic activity of MC also supports the role of the Ah receptor in the cross-talk between the two endocrine systems. αNF, an Ah receptor antagonist (46,47), significantly inhibited the MC-induced decrease in nuclear ER levels (Table IV) and these data coupled with similar correlations between the structure–binding and structure–antiestrogenicity relationships for TCDD and related halogenated aryl hydrocarbons support a role for the Ah receptor in this process.

The recovery of nuclear ER levels in MCF-7 cells treated with MC was also investigated. Twelve hours after treatment with 0.1 μM MC, a 39% decrease in nuclear ER levels was observed (Table V) and significant reduction of the nuclear ER was maintained in cells treated with MC for up to 24 h. However, in cells exposed to MC for 30 h, the nuclear ER levels were decreased by $<10\%$ and returned to control levels 36 h after initial exposure to MC. These results suggest that the effects of MC on nuclear ER levels are reversible and this may be due to the metabolism of MC in MCF-7 cells.

At least two different mechanisms have been proposed for the Ah receptor-mediated antiestrogenic responses in human breast cancer cell lines. Safe *et al.* have proposed that the antiestrogenic responses require the induction of unknown genes whose products inhibit estrogen-induced gene transcription (22,23). In contrast, Gierthy *et al.* have demonstrated that TCDD-induced *CYP1A1* gene expression results in increased 17β-estradiol metabolism and they propose that the induced metabolism of the hormone is responsible for decreased hormone responsiveness (48,49). The results in Table VI summarize the induction of EROD activity by MC, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, benzo[*ghi*]perylene and TCDD 12 h after addition of the inducer and also 24 h after the addition of TCDD. At this time point, benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene did not induce EROD activity and $<10\%$ of the maximum induced activity was observed for MC. These data are consistent with the hypothesis that induced *CYP1A1* gene expression is not required for the Ah receptor-mediated antiestrogenic responses. It should be noted that 1 μM benz[*a*]anthracene induced exceptionally high levels of EROD activity within 12 h (Table VI) and this was accompanied by the largest decrease (76%) in nuclear ER levels (Table IV). These data confirm that, at least for the higher concentration of benz[*a*]anthracene, the induction of P4501A1 and the subsequent increased metabolism of 17β-estradiol may also significantly contribute to the observed antiestrogenic responses.

Epidemiological studies have reported that cigarette smoking is associated with diverse antiestrogenic responses in women and these include a reduction in the risk from endometrial cancer, earlier onset of menopause and increased osteoporosis (50–55). The effects of cigarette smoking on breast cancer in women is unclear; however, the results suggest that any protective effects are minimal (56–58). Michnovicz *et al.* (59) have suggested that increased estradiol-2-hydroxylase activity in women who smoke may be responsible for the antiestrogen activity of cigarette smoking; this result is consistent with the induction of both *CYP1A1* and *CYP1A2* gene expression by PAHs in cigarette smoke since P4501A1 and P4501A2 enhance 17β-estradiol metabolism (48,49,60). However, based on the results of the present study, antiestrogenic activity of the PAHs may also be due to Ah receptor-mediated responses which are independent of induced *CYP1A1* and *CYP1A2* gene expression.

Current studies in this laboratory are focused on delineating the mechanism of Ah receptor-mediated antiestrogenicity and identifying the induced gene products associated with this response.

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