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Benzo[a]Pyrene, 3-Methylcholanthrene and β-Naphthoflavone Induce Oxidative Stress in Hepatoma Hepa 1c1c7 Cells by an AHR-dependent Pathway

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Polycyclic aromatic hydrocarbons have been shown to cause oxidative stress *in vitro* and *in vivo* in various animal models but the mechanisms by which these compounds produce oxidative stress are unknown. In the current study we have investigated the role of the aryl hydrocarbon receptor (AHR) in the production of reactive oxygen species (ROS) by its cognate ligands and the consequent effect on cyp1a1 activity, mRNA and protein expressions. For this purpose, Hepa 1c1c7 cells wild-type (WT) and C12 mutant cells, which are AHR-deficient, were incubated with increasing concentrations of the AHR-ligands, benzo[a]pyrene (B[a]P, 0.25–25 μM), 3-methylcholanthrene (3MC, 0.1–10 μM) and β-naphthoflavone (βNF, 1–50 μM). The studied AHR-ligands dose-dependently increased lipid peroxidation in WT but not in C12 cells. However, only B[a]P and βNF, at the highest concentrations tested, significantly increased H_2O_2 production in WT but not C12 cells. The increase in lipid peroxidation and H_2O_2 production by AHR-ligands were accompanied by a decrease in the cyp1a1 catalytic activity but not mRNA or protein expressions, which were significantly induced in a dose-dependent manner by all AHR-ligands, suggesting a post-translational mechanism is involved in the decrease of cyp1a1 activity. The AHR-ligand-mediated decrease in cyp1a1 activity was reversed by the antioxidant *N*-acetylcysteine. Our results show that the AHR-ligands induce oxidative stress by an AHR-dependent pathway.

Keywords: Aryl hydrocarbon receptor; Oxidative stress; Cytochrome P450; Drug metabolizing enzymes; Reactive oxygen species

Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; βNF, β-naphthoflavone; B[a]P, benzo[a]pyrene; C12, AHR-deficient Hepa 1c1c7 C12 cells; CYP450, cytochrome P450; CYP1A1 or cyp1a1, cytochrome P4501A1; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium base; DMSO, dimethyl sulfoxide; EROD, ethoxyresorufin O-deethylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; HSP90, 90 kDa heat-shock proteins; MDA, malondialdehyde; 3MC, 3-methylcholanthrene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-1, nuclear factor-1; NO, nitric oxide; 8-OH-dG, 8-hydroxydeoxyguanosine; PAH, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SSC, saline with sodium citrate; TCB, tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; WT, wild-type murine hepatoma Hepa 1c1c7 cells

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated basic helix-loop-helix transcription factor (bHLH) that controls the expression of a host of different genes whose functions are linked to the metabolism of dietary constituents, drugs and potentially hazardous agents, such as environmental contaminants.[1,2] The AHR exists as cytoplasmic aggregates bound to two 90 kDa heat-shock proteins (HSP90), the cochaperone p23 and the 43 kDa protein termed hepatitis B virus X-associated protein.[3–5] Upon ligand binding, the AHR dissociates from HSP90 and the ligand–receptor complex translocates to the nucleus. Then, the activated AHR dimerizes with the AHR nuclear translocator protein (ARNT), and binds to a class of promoter DNA sequences, called xenobiotic responsive elements, of target genes to activate their transcription.[6,7] In addition, ARNT forms a heterodimer with hypoxia inducible factor-1α to regulate hypoxia-inducible genes such as vascular

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endothelial growth factor, erythropoietin, and glycolytic enzymes and transporters.[8,9]

The AHR-regulated genes consist of three Phase I enzymes, cytochrome P4501A1 (CYP1A1), CYP1A2 and CYP1B1, and four Phase II xenobiotic metabolizing enzymes, including NAD(P)H:quinone oxidoreductase, glutathione S-transferase Ya subunit, cytosolic aldehyde dehydrogenase-3 and UDP-glucuronosyltransferase 1A6.[10] These enzymes are abundant in tissues that play an important role in first-pass metabolism, digestion, and drug metabolism, such as those of the gastrointestinal tract and the liver. For the most part, these enzymes could be considered to play an important role in drug metabolism. However, the induction of certain heme-thiolate proteins, such as CYP1A1, are considered to be potentially counterproductive to this process since CYP1A1 is capable of producing epoxides and dihydrodiol epoxides from aromatic and halogenated hydrocarbons.[11] The AHR has been shown to be involved in the mediation of a broad range of distinct toxic responses such as immune suppression, thymic involution, endocrine disruption, wasting syndrome, chloracne (keratinocyte proliferation), birth defects, and carcinogenesis.[12] The mechanism for these AHR-mediated pathophysiological conditions is not well understood.

An abundance of evidence in the literature suggests that oxidative stress represents an important underlying cause of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced toxicity,[13] a response mediated primarily by the AHR.[14] Several studies have reported that TCDD increases reactive oxygen species (ROS) formation, lipid peroxidation, and DNA damage in various tissues of rodents.[15–17]

It is well established that acute high dose exposure to TCDD results in an increase in oxidative stress in various tissues and species.[18] Recent data suggest that TCDD causes sustained oxidative stress in the liver of C57BL/6 mice, characterized by increases in the ratio of oxidized to reduced glutathione (GSSG/GSH) and in urinary 8-hydroxydeoxyguanosine (8-OHdG), a by-product of oxidative DNA damage.[19] Similarly, a TCDD-induced AHR-dependent increase in 8-OHdG has been demonstrated in cultured Hepa 1c1c7 cells.[20] One possible mechanism of TCDD-mediated ROS production may involve cytochrome P450s (CYP450),[20] specifically those under AHR control, such as CYP1A1 and CYP1A2.[6] However, there is recent data showing that CYP1A2 may not be involved.[21]

The objectives of this study are to: (1) investigate the role of the AHR in the production of ROS by its cognate ligands, and (2) determine whether or not there is a correlation between AHR-ligand-induced oxidative stress and cyp1a1 activity, mRNA and protein expressions.

MATERIALS AND METHODS

Materials

Benzo[a]pyrene (B[a]P), 3-methylcholanthrene (3MC), Dulbecco's modified Eagle's medium base (DMEM), 7-ethoxyresorufin, fluorescamine, glucose, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, and anti-goat IgG peroxidase secondary antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Tris hydrochloride and agarose were purchased from EM Science (Gibbstown, NJ). Amphotericin B, resorufin, β-naphthoflavone (βNF), and 100X vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, QC). Gentamicin sulfate, penicillin-strepromycin, L-glutamine, MEM non-essential amino acids solution, Fetal Bovine Serum, TRIzol reagent, and the random primers DNA Labeling system were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N-nylon membranes were from Amersham Canada (Oakville, ON). [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by PerkinElmer (Boston, MA). Bromophenol blue, β-mercaptoethanol, glycine, acrylamide, N′N′-bis-methylene-acrylamide, ammonium persulphate, nitrocellulose membrane (0.45 μm), and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A1 goat anti-mouse polyclonal primary antibody (G-18) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Skim milk was obtained from DIFCO Laboratories (Detroit, MI). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Cell Culture

Hepa 1c1c7 cell lines (wild-type (WT) and C12, generously provided by Dr O. Hankinson, University of California, Los Angeles, CA) were maintained in DMEM medium supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin and 25 ng/ml amphotericin B. Cells were grown in 75 cm^2 tissue culture flasks at 37°C in a 4% CO_2 humidified environment.

Chemical Treatments

Cells were treated in serum free medium with AHR-ligands dissolved in DMSO. In all treatments, the DMSO concentration did not exceed 0.05% (v/v). *N*-acetylcysteine (NAC) was dissolved in PBS and adjusted to pH 7.4 with NaOH. In some experiments, cells were first pretreated with 10 mM NAC for 6 h before the addition of AHR-ligands, where appropriate. For enzyme activity and protein assays, the duration of chemical exposure was 24 h.

However, RNA was extracted only 6 h after chemical treatment.

Cytotoxicity Assay

Cell viability, as an indicator of cytotoxicity, was determined by measuring the capacity of Hepa cells to reduce MTT to formazan, as previously described.[23] MTT is reduced to the blue-colored formazan by the mitochondrial enzyme succinate dehydrogenase, which is considered a reliable and sensitive measure of mitochondrial function. Briefly, WT and C12 cells were seeded into 96-well microtiter tissue culture plates and incubated for 24 h at 37°C in a 4% CO_2 humidified incubator. The medium was replaced with serum free medium, and subsequently treated with increasing concentrations of AHR-ligands. The cells were further incubated for 24 h. The medium was again removed and replaced with serum free medium containing 1.2 mM of MTT dissolved in PBS (pH 7.2). After 2 h of incubation, the formed crystals were dissolved with isopropanol. The intensity of the colour in each well was measured at a wavelength of 550 nm using the BIO-TEK Instruments EL 312e microplate reader.

EROD Assays

The cyp1a1-dependent 7-ethoxyreorufin *O*-deethylase (EROD) activity was performed on intact, living cells as described previously.[24] Enzymatic activity was normalized for cellular protein content which was determined using a modified fluorescent assay.[25]

RNA Extraction and Analysis

After incubation with the test compounds for the specified time periods, total cell RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. Northern blot analysis of total RNA was performed as described elsewhere.[22] Briefly, aliquots of RNA were separated in a denaturing (2.2 M formaldehyde) agarose (1.1%) gel and transferred to Hybond-N nylon membranes. The RNA was fixed to the membranes by baking at 80°C for 2 h. Prehybridization of the membranes was carried out in a solution containing 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate), 50% deionized formamide, 5 × Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS) and 100 μg/ml sheared salmon sperm DNA for 4 h at 42°C. Hybridization with the [^{32}P]-labeled cDNA probes was carried out in the same solution, minus Denhardt's reagent, for 16–24 h at 42°C. The membranes were then washed twice at room temperature in 2 × SSC, 0.5% SDS for 15 min. This was followed by a 30 min wash in 0.1 × SSC, 0.5% SDS at 42°C and a final 30 min wash in 0.1 × SSC, 0.5% SDS at 65°C for 30 min. The washed membranes were sealed in plastic wrap and exposed to Kodak X-OMAT MS film in the presence of an intensifier screen at −80°C. Hybridization signals were compared to those obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

The cDNA probes for mouse cyp1a1 and GAPDH mRNAs were generously provided by Dr John R. Bend (University of Western Ontario, London, ON). All probes were [^{32}P]-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

Western Immunoblotting Analysis

Cells treated for 24 h with AHR ligands were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail. The cytosolic fractions were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 *g* for 10 min at 4°C. Proteins (25 μg) were resolved by denaturing electrophoresis, as described previously.[22] Briefly, the cytosol supernatants were dissolved in 1X sample buffer, boiled for 5 min, separated by 7.5% SDS-PAGE bis-acrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4°C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base). After blocking, the blots were incubated with a primary polyclonal goat anti-mouse cyp1a1 antibody for 2 h at room temperature in TBS containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody was carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (Amersham, Arlington Heights, IL).

Measurement of H_2O_2 Production

H_2O_2 production was examined by measuring the conversion of the cell permeant probe, 2′,7′-dichlorofluorescein diacetate (DCF-DA) to its fluorescent product, 2′,7′-dichlorofluorescein.[26] WT or C12 cells plated onto 96-well plates were grown for 24 h to 90% confluence. Thereafter, the cells were incubated with AHR-ligands in the presence of DCF-DA

(5 μM). The fluorescence readings were taken after 1 h incubation at 37°C using the Baxter 96-well plate reader (excitation and emission wavelengths of 485 and 535 nm, respectively).

Measurement of Lipid Peroxidation

WT or C12 cells, plated at 1×10^6 cells/10 cm dish, were grown for 72 h to 90% confluence and harvested after treatment with AHR-ligands for 3 h. Cells were centrifuged for 2 min at 500 *g*, the supernatant was removed, and the residual pellet was washed with PBS. Malondialdehyde (MDA) formation, an indicator of the degree of lipid peroxidation, was determined in cells using the thiobarbituric acid assay as previously described.[27,28]

Statistical Analysis

All results are presented as mean ± SEM. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* tests. EROD activity from cells pretreated with NAC was compared to the respective EROD activities from cells treated only with AHR-ligands using a *t*-test. The differences were considered significant when $p < 0.05$.

RESULTS

Effect of AHR-ligands on Cell Viability

AHR-ligands were tested for potential cytotoxicity in WT and C12 cells, after 24 h exposure. In general, WT cells were more sensitive to the toxic effects of AHR-ligands although the AHR-ligands differentially affected cell viability in both cell types. B[a]P was the most toxic, while βNF was the least toxic, to both cell types. The cytotoxicity of B[a]P was apparent even at the lowest concentration tested (0.25 μM), as demonstrated by the degree of inhibition of MTT reduction, indicating loss of mitochondrial function. Concentrations of 3MC greater than 1 μM were cytotoxic to WT, but not C12 cells. With respect to βNF, both cell lines exhibited the same degree of cell viability, which was significantly reduced only at the highest concentration tested (50 μM) (Fig. 1).

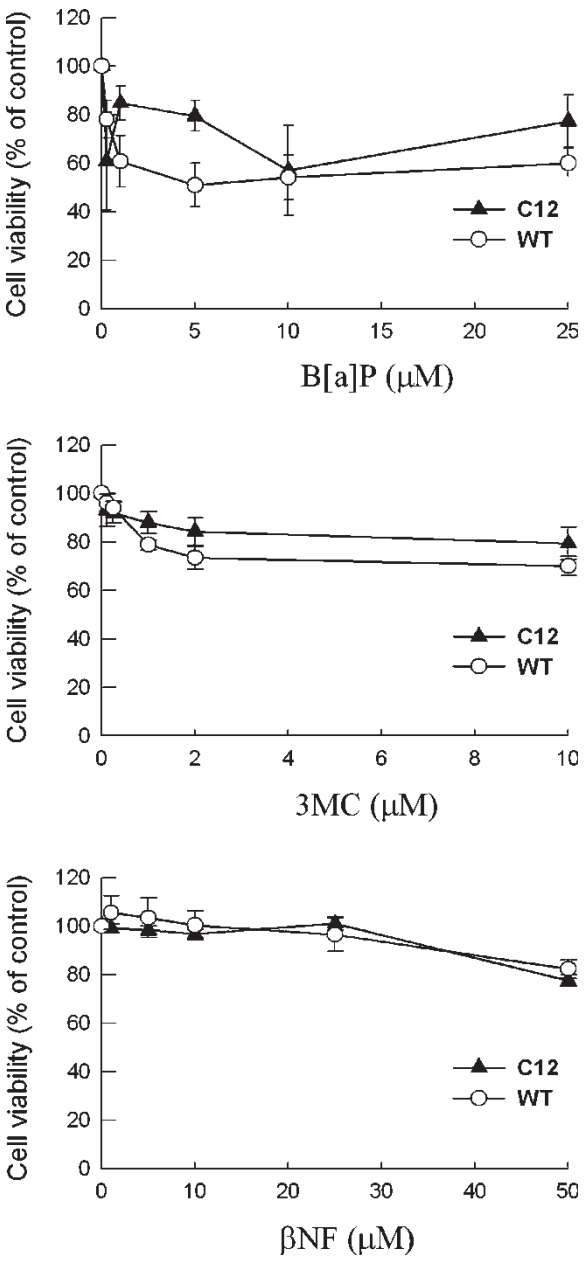

FIGURE 1 Effect of AHR-ligands on Hepa 1c1c7 WT and C12 cell viability as a function of AHR-ligand concentration. The cells were treated with various concentrations of B[a]P (0.25–25 μM), 3MC (0.1–10 μM) or βNF (1–50 μM) for 24 h. Cell viability was assessed using the MTT assay, as described in materials and methods. Data are expressed as percent of untreated control, which is set at 100%, ± SEM ($n = 8$).

Effect of AHR-ligands on cyp1a1 Activity, MRNA and Protein Levels in Hepa 1c1c7 Cells

To assess the functional implications of exposure to increasing concentrations of the AHR-ligands: B[a]P, 3MC, and βNF, cyp1a1-dependent EROD activity was measured in Hepa 1c1c7 cells (Fig. 2). In WT cells, the three AHR-ligands caused a significant induction in EROD activity at all concentrations tested, compared with vehicle-treated control cells. However, the induction was not dose dependent and differed with the AHR-ligand treatment. With B[a]P treatment, the greatest induction in EROD activity occurred with the 0.25 and 1 μM concentrations. However, with concentrations greater than 1 μM, there was a decrease in the EROD activity inductive capacity with increasing concentrations (Fig. 2). The greatest induction in EROD activity with 3MC

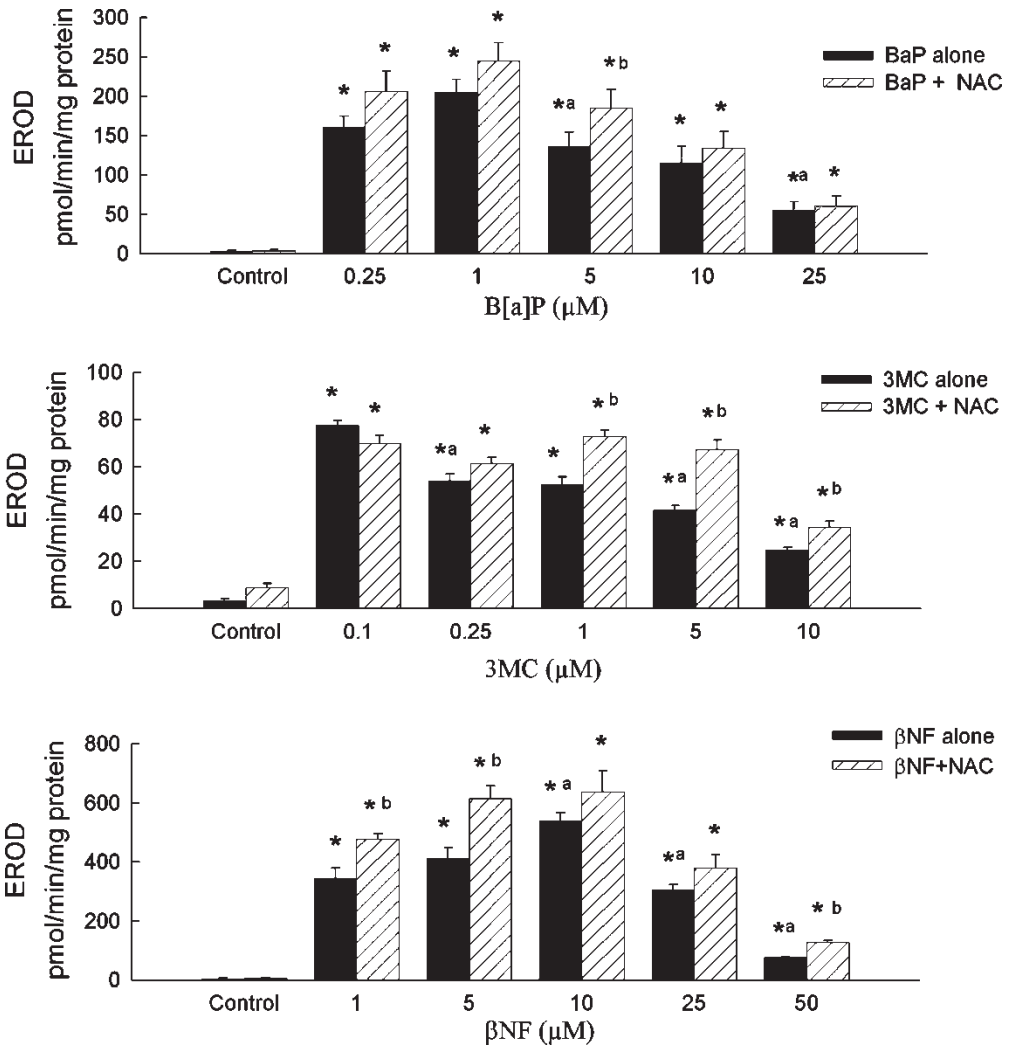

FIGURE 2 Effect of AHR-ligands in the absence or presence of *N*-acetylcysteine on EROD activity in Hepa 1c1c7 cells. Cells were treated with B[a]P (0.25–25 μM), 3MC (0.1–10 μM) or βNF (1–50 μM), with and without pretreatment with *N*-acetylcysteine (10 mM), for 24 h prior to EROD assay. EROD activity was measured in intact living cells using a 96-well plate fluorescent assay as described in materials and methods. Values are presented as mean ± SEM ($n = 8$). $*p < 0.05$ compared to control, $^{a}p < 0.05$ compared to the next lower concentration, $^{b}p < 0.05$ pretreatment with NAC compared to treatment with AHR-ligand alone.

treatment occurred at the lowest concentration tested (0.1 μM). Yet at higher concentrations, the increase in EROD activity was inversely correlated with the concentration of 3MC. On the other hand, βNF (1–10 μM) caused dose-dependent increase in EROD activity. However, at the highest concentrations tested (25 and 50 μM) there was also a decrease in the level of EROD induction, compared with the induction achieved at the lower concentrations of βNF. Not surprisingly, none of the AHR-ligands caused a significant induction in EROD activity in C12 cells.

To test the hypothesis that oxidative stress induced by AHR-ligands plays a role in decreasing the cyp1a1 inductive capacity at high concentrations of AHR-ligands, the cells were treated with the antioxidant NAC before treatment with the AHR-ligands. As can be seen in Fig. 2, the induction of EROD activity was greater in cells pretreated with NAC, compared to cells treated with AHR-ligands alone. This suggests that ROS may play a role in limiting the induction of EROD activity by the AHR-ligands at high doses.

To investigate other mechanisms that may be responsible for the decrease in the inductive capacity of the AHR-ligands at high doses, the effect of AHR-ligands on cyp1a1 mRNA levels in WT cells was assessed by Northern blot analysis, as shown in Fig. 3. In the concentration ranges tested, all of the AHR-ligands caused a dose-dependent increase in cyp1a1 mRNA levels 6 h after treatment, compared with vehicle-treated control cells. In contrast to the cyp1a1 activity results, the greatest induction in cyp1a1 mRNA levels was achieved with the highest concentration, for all AHR-ligands tested. Hence, transcriptional mechanisms do not seem to be involved in mediating the decrease in the inductive capacity of AHR-ligands at high doses. It is more likely that post-transcriptional mechanism(s) are

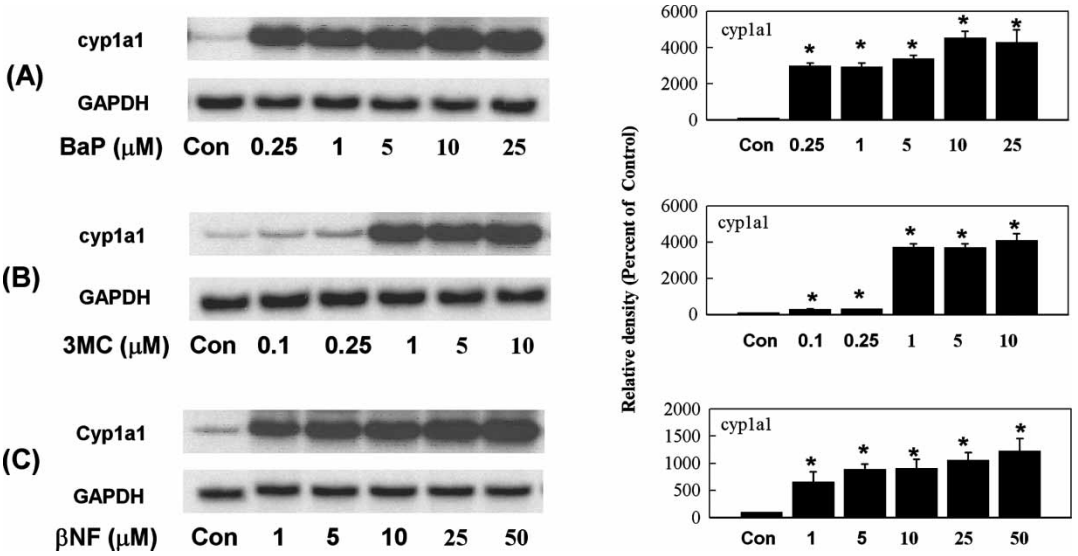

FIGURE 3 Effect of AHR-ligands on cyp1a1 mRNA levels in Hepa 1c1c7 WT cells. Cells were treated with B[a]P (0.25–25 μM), 3MC (0.1–10 μM) or βNF (1–50 μM) for 6 h prior to assay. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [^{32}P]-labeled cDNA probe specific for mouse cyp1a1. The blots were subsequently stripped and re-hybridized, sequentially with a cDNA probe specific for GAPDH, which was used as a loading control. Graphs represent the average optical density (±SEM) of cyp1a1 mRNA bands from three different experiments, as a percent of vehicle-treated control. Only one representative blot is shown. $*p < 0.05$ compared to control.

involved. For reference purposes, the blots were stripped and rehybridized with a cDNA probe for GAPDH mRNA as a loading control for all of the Northern blots presented in this study.

To assess whether the inhibitory effects of AHR-ligands on the cyp1a1 activity was attributed to pre- or post-translational mechanisms, quantitative measurement of cyp1a1 protein using Western blot analysis was carried out. Our results clearly show that all three AHR-ligands caused a dose-dependent induction in cyp1a1 protein expression, which correlates well with the mRNA levels (Fig. 4).

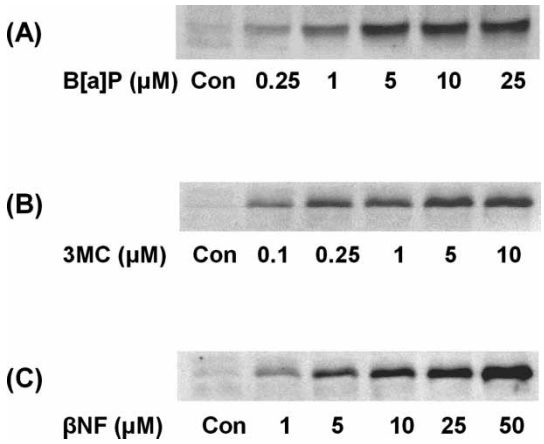

FIGURE 4 Effect of AHR-ligands on cyp1a1 protein level in Hepa 1c1c7 WT cells. Cells were treated with B[a]P (0.25–25 μM), 3MC (0.1–10 μM) or βNF (1–50 μM). Cells were then harvested 24 h after treatment and the cytosolic fractions were extracted. Proteins were resolved by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and sequentially incubated with a primary anti-mouse cyp1a1 antibody and a peroxidase-conjugated IgG secondary antibody. Western blots were performed at least three times but only one representative result is shown.

Effect of AHR-ligands on H_2O_2 Production

To determine whether or not the production of ROS by AHR-ligands is mediated by an AHR-dependent pathway, ROS production was assayed in WT and C12 cells using DCF-DA as a probe. When oxidized within the cell by ROS, especially H_2O_2, DCF-DA yields the fluorescent compound dichlorofluorescein (DCF). Figure 5 shows that only B[a]P and βNF at the highest concentration tested, 25 and 50 μM, respectively, significantly increased H_2O_2 production in WT but not in C12 cells (Fig. 5). In C12 cells, all AHR-ligands caused a dose-dependent inhibition of H_2O_2 production (Fig. 5).

Effect of AHR-ligands on Lipid Peroxidation

Consequently, we investigated whether exposure to AHR-ligands would trigger an increase in lipid peroxidation in these hepatoma cell lines. Dose-dependent increases in MDA, an indication of elevated lipid peroxidation, occurred in WT but not in C12 cells after B[a]P, 3MC or βNF treatment for 3 h (Fig. 6). Of interest, 3MC caused a dose-dependent inhibition of lipid peroxidation in C12 cells.

DISCUSSION

Cellular response to oxidative stress involves changes in the expression of many genes, including

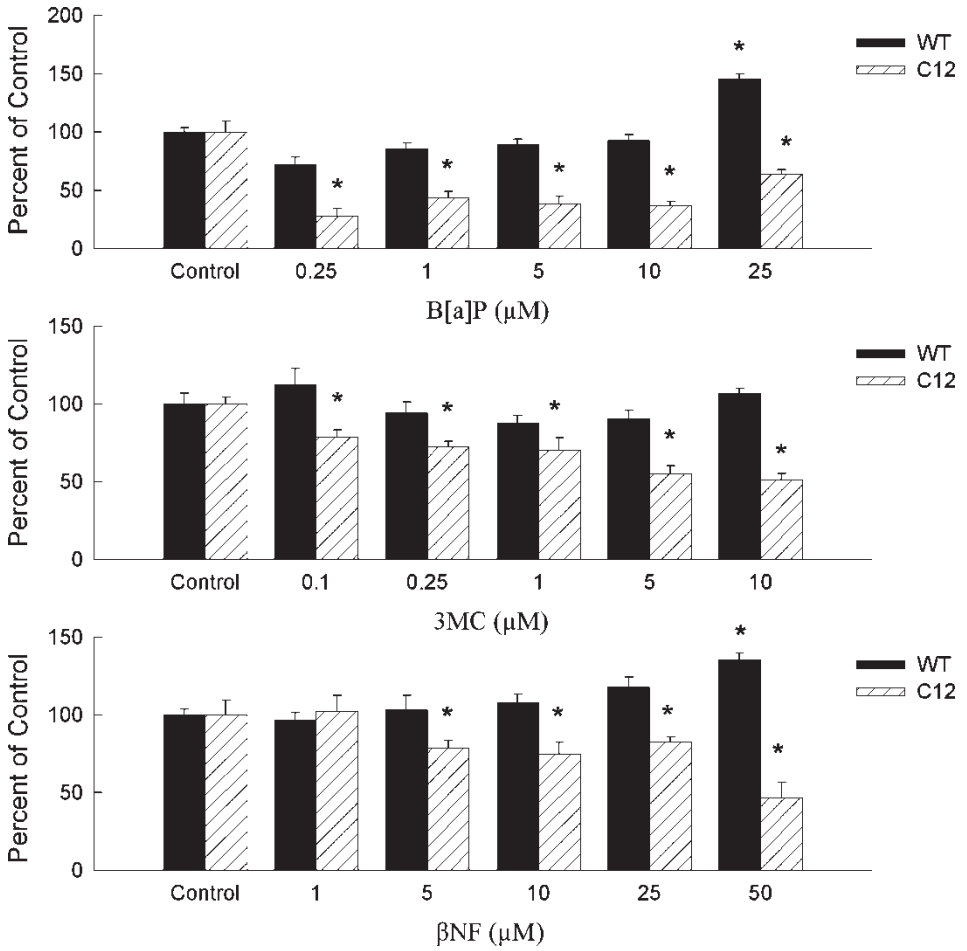

FIGURE 5 Effect of AHR-ligands on intracellular H_2O_2 production in Hepa 1c1c7 WT and C12 cells. Cells were plated onto 96-well plates for 24 h. Thereafter, the cells were incubated with B[a]P (0.25–25 μM), 3MC (0.1–10 μM) or βNF (1–50 μM) in the presence of 2′,7′-dichlorofluorescin diacetate (5 mM). The fluorescence readings were taken 1 h after incubation at 37°C. Data are expressed as mean ± SEM ($n = 12$), $*p < 0.05$ compared to control.

those involved in the repair and the protection of cells against additional oxidative damage.[29] Among these genes sensitive to oxidative stress are those of the phase I xenobiotic metabolizing enzymes, such as CYP450.[30,31]

In the present study we have demonstrated that the AHR-ligands, B[a]P, 3MC and βNF dose-dependently increased lipid peroxidation in WT and not in C12 mutant cells, which are AHR-deficient. However, only B[a]P and βNF at the highest concentration tested significantly increased the H_2O_2 production in WT but not in C12 cells. Hence, the present findings demonstrate the role of the AHR in the production of ROS by its cognate ligands. It has been previously shown that nitric oxide (NO) and several ROS such as H_2O_2, hydroxyl and superoxide radicals induce lipid peroxidation.[32] The present results suggest that several ROS are implicated in the increase in lipid peroxidation and the decrease in cyp1a1 activity-mediated by the AHR-ligands. This multiplicity of ROS may explain why, only the highest doses of B[a]P and βNF increased H_2O_2 production although B[a]P, 3MC and βNF significantly increased lipid peroxidation in a dose-dependent manner.

Several studies have implicated the AHR in controlling the TCDD-induced oxidative stress response. This is supported by the observation that disruption of the *AHR* gene protects mice from the acute toxicity of TCDD.[33,34] Furthermore, C57BL/6 mice, which possess a high-affinity AHR, produce more ROS than DBA/2 mice, which have a low-affinity AHR.[35,36] In Hepa 1c1c7 cells, an increase in 8-OHdG was seen following TCDD treatment of WT but not of the mutant Hepa 1c1c7 C4, which lack the ARNT protein, the dimeric partner of the AHR that is necessary for transcription.[20] Recently it has been reported that *AHR*(−/−), but not *cyp1a1*(−/−) or *cyp1a2*(−/−) knockout mice were protected from TCDD-induced production of mitochondrial ROS and a resultant oxidative stress response, suggesting that ROS production is dependent on the AHR but not CYP1A1 or CYP1A2.[14]

The mechanism by which AHR-ligands increase cellular oxidative stress is unknown. The CYP450s

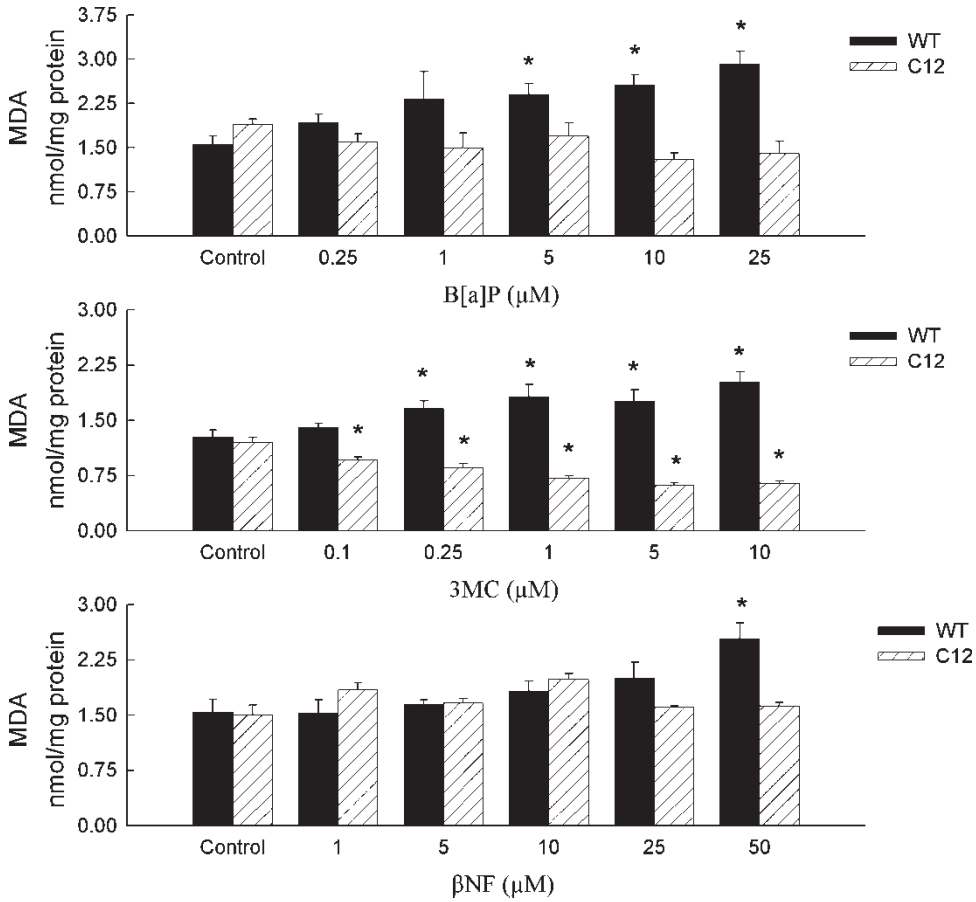

FIGURE 6 Effect of AHR-ligands on lipid peroxidation in Hepa 1c1c7 WT and C12 cells. Cells plated at 1×10^6 cells/10 cm dish, were grown for 24 h to 90% confluence and harvested after treatment with B[a]P (0.25–25 μM), 3MC (0.1–10 μM) or βNF (1–50 μM) for 3 h. MDA formation, an indicator of the degree of lipid peroxidation, was determined as described in Materials and Methods. Data are expressed as mean ± SEM ($n = 12$), $*p < 0.05$ compared to control.

sequentially transfer two electrons to oxygen from microsomal NADPH-P450 oxidoreductase, or from mitochondrial adrenodoxin, with subsequent formation of an oxygenated substrate and water.[37,38] Although electron transfer is normally a well-coupled process, superoxide and H_2O_2 may be released, particularly in the presence of CYP1A1 ligands that are poorly metabolized. For example, Schlezinger *et al.*[39] showed that the TCDD-like planar 3,3′,4,4′-tetrachlorobiphenyl (TCB) increases CYP1A1-dependent microsomal ROS production in both fish and rodents. The mechanism of ROS production was speculated to be due to blockage of the normal catalytic cycle of CYP1A1 by the non-metabolizable ligand TCB, resulting in release of ROS.

Interestingly, our results not only prove that AHR-ligands-stimulated production of ROS is dependent on the presence of a functional AHR, we have also demonstrated a decrease in H_2O_2 production by B[a]P, 3MC, and βNF and a decrease in MDA production by 3MC in C12 cells. Similar observations have been previously seen when AHR knockout mice were treated with dioxin.[14] It has been presumed that dioxin decreases the rate constant of cytochrome *c* oxidase by an AHR-dependent mechanism and the absence of the AHR causes an increase in cytochrome *c* oxidase activity.[14] The increase in cytochrome *c* oxidase activity may be further potentiatd in C12 cells in the presence of AHR-ligands, leading to an increase in oxygen consumption and a decrease in ROS.

Also in the current study, increases in lipid peroxidation and H_2O_2 production by AHR-ligands were accompanied by decreases in the AHR-ligands' inductive capacity of cyp1a1 catalytic activity. In agreement with our results, it has been reported that AHR-ligands such as 3,3′,4,4′-TCB and other planar polychlorinated biphenyls at high doses suppress CYP1A activity and content.[40,41] In the fish model, scup (*Stenotomus chrysops*), high doses of TCB cause a sharp and selective decline in hepatic CYP1A content. This effect was found to occur at a post-transcriptional level.[42]

Contrary to the activity results, cyp1a1 mRNA and protein expression were significantly induced by all AHR-ligands in a dose-dependent manner. Thus, the results provided here present us, with the first

evidence that the limited induction of cyp1a1 activity at high concentrations of AHR-ligands is caused by post-translational mechanism(s). Since pre-treatment with NAC provided partial protection for the decrease in cyp1a1 activity, it is likely that ROS induced by high doses of AHR-ligands play a role in modifying cyp1a1 enzyme function.

Several studies have reported the ability of ROS to inhibit CYP1A1. It has recently been shown that ROS such as H_2O_2 suppress CYP1A1 and CYP1A2 expression.[43,44] Reduction of CYP1A1-dependent activity during oxidative stress may be part of an adaptive response by the cells to minimize their damage. The suppression of CYP1A1 activity presumably both lowers the activation of carcinogens and reduces the production of more ROS.[20] The exact mechanism by which H_2O_2 down-regulates the CYP450 remains unclear, but it has been suggested that H_2O_2 formed in the hemoprotein active centre can interact with the enzyme-associated Fe^{2+} leading to heme destruction and enzyme inactivation.[45,46] Morel and Barouki[44] have reported that the *CYP1A1* gene is repressed by oxidative stimuli via modulation of the binding of nuclear factor-1 (NF-1) to the *CYP1A1* promoter. In addition it has been suggested that, lipid peroxidation, probably through its aldehydic by-products, decreases CYP1A1 activity.[31]

Another potential mediator that may be involved in the suppression of cyp1a1 activity is NO. NO is capable of binding to the heme protein of CYP450 enzymes, blocking the binding of O_2 and thus inhibiting the activity of enzyme.[47] Also, in the presence of ROS, NO can form highly reactive species, including peroxynitrite, capable of oxidizing amino acids critical to the functioning of the enzyme with resultant irreversible loss of catalytic activity.[48]

In Summary, the present study reports three significant observations. First, the AHR-ligands induce oxidative stress by an AHR-dependent pathway. Second, the increase in ROS production is closely associated with the decrease in cyp1a1 activity. Finally, the decrease in the level of cyp1a1 activity induction by the AHR-ligands at high concentrations is caused by post-translational mechanism(s).

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