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Cadmium interference with ERK1/2 and AhR signaling without evidence for cross-talk

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Abstract

Cadmium (Cd) is a toxic metal that enters the food chain. Following oral ingestion, the intestinal epithelium may in part protect against Cd toxicity but is also a target tissue. Using human enterocytic-like Caco-2 cells, we have previously shown that Cd may induce a concentration and time-dependent increase in MTT (3-[4,5-dimethyl-2-thiazol-2-yl]-2,5-diphenyltetrazolium bromide assay) activity in differentiated cultures exclusively. This effect was insensitive to estrogen antagonist, and it was not related to cell proliferation but to enhanced protein synthesis that involves ERK1/2 activation. Because some studies have suggested cross-talk between AhR and ERK signaling pathways we have hypothesized that Cd may indirectly lead to AhR activation. Western blot analysis and immunofluorescence data show that: i) contrary to Cd and insulin, dioxin (TCDD) or benzo[a]pyrene did not increase ERK phosphorylation; ii) TCDD and Cd both increased the nuclear translocation of AhR; iii) TCDD but not Cd or insulin increased CYP450 1A1/2 expression; iv) there was no correlation between phospho-ERK and AhR activation. In conclusion, the Cd-induced hormesis-like effect on MTT activity in the Caco-2 cells is a differentiation stage-specific phenomenon that is not observed with xenobiotics acting as AhR ligands. ERK phosphorylation is not a prerequisite to AhR activation and it does not necessarily lead to AhR activation. Cd does not stimulate the transcriptional activity of AhR but it favors its nuclear translocation which may have an impact on cell's sensitivity to AhR activators.

Introduction

Cadmium (Cd) is a highly toxic metal that enters the food chain. Chronic oral absorption of contaminated food products and water may represent an important exposure source in addition to contaminated air and cigarette smoking[1]. Cadmium is classified as a type I carcinogen by the International Agency for Research on Cancer, but depending on the concentration and time of exposure, it may also induce apoptosis (for review see Thévenod and Lee[2]). Immunosuppression, anemia, osteoporosis and renal dysfunction are well-known long-term toxic effects of Cd[3–5]. Cadmium also affects cell cycle progression by inhibiting or stimulating cell proliferation[6–9], and may also have some estrogenic effects[10,11]. In addition to promoting the production of reactive oxygen species[12], Cd affects numerous signaling cascades, including the serine/threonine extracellular signal-regulated kinases (ERKs) ERK1/2 (p44/p42MAPK), c-JUN N-terminal kinase (JNK), or p38 kinase in various cell types[2]. In previous studies we have shown that a 24-h exposure to 10 μM Cd that did not affect cell viability activated ERK and p38 kinase signaling cascades in the enterocytic-like cells Caco-2[13]. This effect, which was insensitive to estrogen antagonist, was correlated with an unexpected hormesis-like increase in MTT activity. However, cell proliferation was not affected, and increase in MTT activity was rather correlated with stimulated protein synthesis[13]. In this previous study we have investigated steps of activation upstream ERK. Now, because it is possible that ERK may in turn modify AhR activity (see below), the present study focuses on the consequences that the Cd-induced ERKs activation may have on AhR signaling.

The aryl hydrocarbon receptor (AhR) is a ligand-activated helix–loop–helix protein that exists as cytoplasmic aggregates bound to two 90-kDa heat shock proteins (HSP90), the cochaperone p23 and the 43-kDa hepatitis B virus X-associated protein. Upon interaction with xenobiotics such as 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene (BaP), the

agonist-receptor complex dissociates from HSP90 and translocates to the nucleus where it associates with the aryl hydrocarbon receptor nuclear translocator (Arnt). The whole complex then acts as a transcription factor that binds to DNA promoter sequences termed xenobiotic responsive element (XRE), or dioxin responsive element (DRE), thereby enhancing the transcription of the target genes. Among these genes are those encoding a number of drug metabolizing enzymes, including the phase I enzyme cytochrome P4501A1 (CYP1A1), which oxygenates aryl hydrocarbons during the detoxification process[14]. The AhR also plays an important role in cell and tissue homeostasis by participating in other signaling mechanisms controlling cell proliferation and differentiation, as well as organ development without requiring exogenous ligand[15–17]. Phosphorylation sites have been identified in the C-terminal region of AhR[18]; some phosphorylation events (involving PKC or other phosphoproteins) would be critical either for the DNA binding step[18,19] or for subsequent transcriptional AhR activity[20,21]. However it is not clear whether TCDD modify AhR phosphorylation[18]. Some studies have provided evidence that ERK might phosphorylate AhR. ERK activation has been shown to be critical for the TCDD-induced activation of AhR as well as increased CYP1A1 expression[19,22,23]. Exogenous AhR ligands, i.e. TCDD or BaP, apparently activate ERK in an AhR-independent manner, which in turn would activate the AhR. These conclusions mainly came from the observation that PD98059 or U0126, two inhibitors of MEK upstream ERK, abolished AhR activation or CYP1A1 expression. Given that some data also suggests that PD98059 may directly inhibit TCDD binding to AhR[24], whereas U0126 could bind to and activate AhR[25], the absolute requirement of ERK in AhR activation can be criticized. Whether ERK may participate in AhR activation through phosphorylation remains unclear. If that were the case, one would expect increased AhR activity as a consequence of Cd-induced activation of ERK. Some studies have shown that Cd may influence the expression or activity of one of the most extensively studied

AhR-target gene, namely CYP1A1. However data vary considerably. In rats treated with Cd by gavage or in drinking water, Cd decreased intestinal Cyp1a1 mRNA[11]. In vitro, mouse hepatoma cells exposure to 25 µM Cd for 6 h increased cyp1a1 mRNA without modifying the protein level or CYP1A1 activity[26]. However, a 48-h exposure to a micromolar level of Cd did not modify Cyp1a1 gene expression in the liver cell line HepG2[27]. Similar exposure conditions failed to modify CYP1A1 protein level in primary culture of goldfish hepatocytes[28], and exposure to 1 µM Cd for 5 days led to 20% lower Cyp1a1 mRNA levels in the Caco-2 cells[11].

We thus pursue our study on the effects of Cd on the intestinal cells by investigating the possibility of cross-talk between ERK and AhR signaling in the Caco-2 cells and by testing the hypothesis that Cd may activate AhR indirectly via ERK1/2 phosphorylation. The Caco-2 cells represent a widely used in vitro model to study intestinal function because they undergo spontaneous enterocytic differentiation. The growth-related differentiation of these cells has been well characterized[29,30]. An exponential growth phase is observed until dish culture confluence is reached. Then a stationary growth phase is observed: cells form a polarized monolayers with tight junctions between adjacent cells and the onset of differentiation. The present data show that basal level of nuclear AhR varies with the differentiation status. Contrary to Cd, TCDD does not increase ERK1/2 phosphorylation in the differentiated Caco-2 cells. However TCDD increases AhR location in the nucleus and CYP1A1 overexpression. In contrast, Cd stimulates AhR nuclear translocation but does not induce CYP1A1 expression. Cadmium effect on AhR location was not inhibited by the AhR antagonist resveratrol.

Materials and Methods

Cell culture

The enterocytic-like Caco-2 cell line (obtained from Dr. A. Zweibaum[31]), was used between passages 201 and 249 as in our previous studies showing an hormetic effect of Cd on MTT activity[13]. Stock cultures were grown in 75-cm^2 flasks (Corning, Inc., Corning, NY, USA) at 13 x 10^3 cells/cm^2, and were kept at 37ºC in a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle minimum essential medium (DMEM) containing 25 mM glucose (Gibco Life Technologies, Co, Grand Island, NY, USA), supplemented with 15% inactivated (56ºC for 30 min) fetal bovine serum (FBS) (Wisent Inc., St-Bruno, QC, Canada), 0.1 mM non-essential amino acids, penicillin–streptomycin (50 U/ml to 50 mg/ml) (Gibco Life Technologies) and 19 mM $NaHCO_3$. Non-essential amino acids L-aspartic acid and glycine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), whereas L-alanine, L-asparagine, L-glutamic acid, L-proline, and L-serine were obtained from GibcoBRL. For subcultures for the experiments, the cells were passaged by trypsinization (0.05% trypsin–0.53 mM EDTA) (Gibco Life Technologies) for 15 min and seeded in 100 x 20 mm diameter culture dishes (Sarstedt, Nümbrecht, Germany), 96-well plates (Sarstedt), 0.15 mm Delta T dishes (Bioptechs, Inc., Butler, PA, USA) or 0.15 mm Nunc Lab-tek II chambered coverglass (ThermoFisher Scientific, Waltham, MA, USA). The medium was changed every 2 days, and the cell cultures were maintained for 20 days to allow functional differentiation[29]. Indeed, the Caco-2 cells undergo an exponential growth phase until the culture dish confluence is reached, which is around day 6-7 of culture at the above mentioned cell seeding density. Then proliferation stops and the cell monolayer begins to differentiate[29,30]. In the present study, 6-day-old cells refer to confluent but undifferentiated cell monolayers, whereas 20-day-old cells are differentiated cell monolayers.

MTT activity measurements

MTT (3-[4,5-dimethyl-2-thiazol-2-yl]-2,5- diphenyltetrazolium bromide) (Sigma Aldrich) assay estimates MTT (yellowish solution) reduction to blue formazan crystals. In this study, MTT activity was not used to evaluate cell viability but as an indicator of Cd-like hormesis effect (related to ERK1/2 activation and increased protein synthesis) revealed in our previous study[13]. Cells grown for 6 and 20 days on 96-well plates were rinsed twice with FBS-free DMEM prior to incubation with various concentrations of Cd (added as $CdCl_2$, Sigma Aldrich, 99.99% purity) ranging from 0 to 30 μM (prepared from 1 and 10 mM sterile stock solutions in ultra pure water) for 24 h. In parallel experiments, cells were incubated with 1 or 2 nM TCDD (Sigma Aldrich) or with 2 or 4 μM BaP (Sigma Aldrich) for 24 h, well-known xenobiotic AhR agonists. At the end of the incubation period, 10 mL MTT (0.5 mg/mL final concentration) were added to each well, and cells were incubated for 1.5 h at 37ºC. The medium was discarded and changed to 200 μL DMSO to dissolve the formazan crystals before measuring absorbance at 575 nm with a Tecan SpectraFluor Plus microplate spectrophotometer (Esbe Scientific Industries, Inc., Markham, Canada). MTT data are expressed relative to their respective controls measured following a 24-h incubation in FBS-free DMEM.

Immunoblotting

Six and 20-day-old cell cultures were rinsed twice with FBS-free DMEM before incubation in the presence of 1 or 2 nM TCDD, 0.2 or 0.4 μM BaP, 10 μM Cd, 100 nM bovine insulin (Sigma Aldrich) used as a well-known activator for ERK1/2, with or without 20 μM of the ERK1/2 inhibitor U0126 (Sigma Aldrich) and 100 μM resveratrol (Sigma Aldrich) used as an AhR antagonist, in FBS-free medium for 24 h at 37ºC. Cells were then harvested in an ice-cold hypotonic lysis buffer, passed through a 26-G needle and centrifuged at 10 000 g for 15 min at 4ºC. The pellet was discarded and 20 μg protein of the supernatant was mixed with 62.5 mM

Tris–HCl buffer (2% SDS, 10% glycerol, 5% â-mercaptoethanol, pH 6.8), heated to 95ºC for 5 min, and separated by electrophoresis in 10% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE). Proteins were detected immunologically following electrotransfer to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked via a 1-h incubation with Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% non-fat milk at room temperature. The membranes were incubated overnight at 4ºC with the following primary antibodies (Cell Signaling Technology, Inc. Danvers, MA): p44/42MAPkinase (9102), phospho-p44/42MAPkinase (Thr202/Tyr204) (9101), α-actin (4967) and CYP1A1/2 (3112249, Assay BioTech, USA). The membranes were then incubated with a mixture of secondary antibodies (Cell Signaling Technology, Inc. Danvers, MA, USA): horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000) (7074) and HRP-conjugated anti-biotin (1:1000) (7075) for 1 h. Biotinylated molecular weight markers (7727, Cell Signaling) were used and membranes were developed by adding the substrate HyGLO (Denville Scientific, Metuchen, NJ, USA) and visualized using a Chemiluminescence-Fluorescence and Advanced Fluorescence Fusion FX7 instrument (Montreal Biotech Inc. Laboratory Equipment). Densitometry values for the treated cell samples were normalized relative to that of â-actin and compared to control cells using ImageJ 1.48p software (Wayne Rasband National Institutes of Health, USA).

Immunofluorescence

Six and 20-day-old cells, maintained in 0.15-mm Nunc Lab-tek II chambered coverglass or 0.15-mm Delta T Dish were rinsed twice with FBS-free DMEM before incubation in the presence of 10 µM Cd, 1 nM TCDD, alone or in combination, 100 nM insulin, with or without 20 µM U0126 or 100 µM resveratrol in FBS-free medium for 24 h at 37ºC. Cells were then rinsed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, exposed to 0.2%

Triton X-100 for 5 min at 4ºC (for intracellular detection), blocked with 5% BSA solution for 1 h at room temperature, and incubated overnight at 4ºC with the primary antibody AhR (1:200) (H-211, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). The next day, cells were incubated for 1 h at room temperature with Alexa Fluor® 568 conjugated goat anti-rabbit IgG (H+L, A-11011, Life technologies), then exposed for 5 min also at room temperature, to 4',6-diamidino-2-phenylindole (DAPI, blue) (P36935, Life technologies), and finally visualized with a Nikon A1R confocal laser microscope system (Japan, 2011). As a control, fluorescence have been verified in cells stained with Alexa Fluor® 568 conjugated goat anti-rabbit IgG alone. In this case, the baseline fluorescence was almost undectable. The intensity of fluorescence of the regions of interest (nuclei) was evaluated using ImageJ 1.48p software and then normalized to that measured in control cells maintained for 24 h in the absence of FBS.

Statistical analysis

Data are expressed as mean ± SD estimated on 3 to 5 independent cell cultures. Normality was visually assessed. Statistical analyses between two cell treatments were performed with the two-tailed Student's *t*-test for unpaired data on small samples with Welch's correction using Instat 4 software (GraphPad Software, San Diego, CA, USA). Statistical significance was assessed at $p \leq 0.05$.

Results and discussion

Contrary to Cd, AhR activators do not induce a hormesis effect on MTT activity

In a previous study with have shown that a 24-h exposure to Cd level that do not affect cell viability increases MTT activity in differentiated Caco-2 cells[13]. This effect was shown to be the result of Cd-induced activation of signaling cascade involving ERK. To test whether AhR

ligands may also elicit similar response, the effects of TCDD and BaP were tested and compared to that of Cd.

MTT activity was measured following a 24-h exposure to increasing concentrations of Cd in confluent but undifferentiated 6-day-old cells, and in 2-week post-confluent differentiated cell cultures. A 150% increase in MTT activity was observed at 10 – 15 µM Cd in 20-day-old cells, whereas levels similar to control were observed at higher concentrations (Fig. 1A). This effect was not observed in undifferentiated cultures. Contrary to Cd, TCDD or BaP did not stimulate MTT activity independently of days in culture (Fig. 1B, C).

MTT activity measurement was originally used as an indicator of cell survival and the chemosensitivity of drug-resistant tumor cells[32]. It has been widely used in cytotoxicity studies and to measure cell proliferation. However, we have previously shown that this Cd-induced hormesis effect on MTT activity was not related to stimulated cell proliferation: [^{3}H]-thymidine incorporation was no increased, Cd did not modify the proportion of the cell population in the G_1, S or G_2-M phases, aphidicolin (which inhibits DNA polymerase) did not affect the Cd-induced increase in MTT activity whereas cyclohemixide (a protein synthesis inhibitor) completely abolished it[13]. Moreover, tamoxifen, used as an estrogen antagonist, did not modify the increase in MTT activity. Thus, the estrogenic activity of Cd leading to increased cell proliferation that has been reported by others in human breast cancers cell lines is potentially unlikely to be responsible for the observed increased MTT activity in the Caco-2 cells[10,33]. Cadmium effect on MTT activity in Caco-2 cells would rather be the result of increased protein synthesis. Such transient increase in MTT activity without correlation with cell proliferation as also been reported by others[34,35]. The present study shows that the Cd-induced hormesis-like effect on MTT activity in Caco-2 cells is a stage-specific differentiation phenomenon that is not observed with xenobiotics known as AhR ligands.

AhR activators do not increase ERK1/2 phosphorylation

Cadmium-induced MTT activity in Caco-2 cells has been related to ERK activation[13]. Indeed, 4.5-fold increases in phospho-ERK1/2 levels were observed in 20-day-old differentiated cells following a 24-h exposure to 10 μM Cd (Fig. 2B, D), whereas Cd failed to activate ERK1/2 in undifferentiated cultures (Fig. 2A, C). As expected, ERK1/2 inhibitor U0126 completely eliminated Cd effects and lowered the phosphorylated protein levels below to those estimated in unexposed control cells in both differentiated and undifferentiated cells. No increase in phospho-ERK1/2 was observed in differentiated cells exposed to either 1 and 2 nM TCDD or 0.2 and 0.4 μM BaP. Of note, control level of phospho-ERK (normalized to that of β-actin) was 6 times higher in undifferentiated 6-day-old cells compared to differentiated 20-days-old cultures (data not shown) which is accordance with the growth-related differentiation process of the Caco-2 cells. Indeed, as it is observed along the crypt-villus axis in the intestine, phospho-ERK1/2 are mainly present in undifferentiated cells and a dramatic decrease in active ERK1/2 is observed as soon as the Caco-2 cells begin to differentiate[36,37].

We have previously shown that the Cd-induced MTT activity in differentiated Caco-2 cells was abolished by the presence of U0126, which reveals correlation between Cd-induced MTT stimulation and ERK1/2 activation[13]. Such correlation has also been observed in embryo lung fibroblasts and HEK293 cells following a 24-h exposure to only 0.05 – 1 μM Cd[38,39]. A number of studies reported that 1 – 10 μM Cd may rapidly increase ERK phosphorylation within a few hours or even minutes[40–44]. Our previous study showed that 10 μM Cd increases MTT activity after 12 h with optimal induction at 24 h and levels back down to the control values at 48 h[13]. The kinetic of Cd-induced phosphorylation of ERK1/2 was not studied, but the observed correlation between the variation in MTT activity and the level of phospho-ERK1/2 suggests that

ERK1/2 activation by Cd in differenciated Caco-2 cells would be a transient phenomenon with optimal induction for 10 – 15 μM Cd at around 24 h under our experimental conditions.

Contrary to Cd, TCDD did not stimulate ERK1/2 phosphorylation in Caco-2 cells under our experimental conditions. This result is in accordance with the lack of MTT induction by TCDD (Fig. 1). However, some studies have shown ERK activation in mouse hepatoma cells RAW264.7 following a 10-min or 2-h exposure to 10 nM and 1 nM TCDD, respectively[19,22]. Increased levels of phospho-ERK were also observed in the human ovarian granulosa cells HO23 exposed to 10 nM TCDD for 15 min[45]. Moreover, some studies have shown that ERK activation by TCDD is not sustained. In murine leukemia cells exposed to 10 nM TCDD, significant increases in ERK (mostly ERK1) phosphorylation were observed at 4 h with levels decreasing to control values at 12 h[46]. In the human T-cell line Jurkat T, the transient activation of ERK was most effective for ERK2 and began 30 min after exposure to 10 nM TCDD with levels of phosphorylation no longer higher than control values after 12 h[47]. Thus the possibility that TCDD could rapidly activate ERK1/2 transiently with different kinetics of induction compared to Cd cannot be excluded.

ERK1/2 activation is not a prerequisite to AhR activation

To evaluate whether ERK activation is a prerequisite to or favors AhR activation, we studied the receptor's nuclear translocation via confocal microscopy. Cell nuclei were stained with blue DAPI, and AhR was conjugated to the red fluorochrome Alexa Fluor® 568. As shown in Figure 3A, Alexa staining in undifferentiated cells was rather diffused throughout the cell monolayers. A weak nuclear localization of AhR was observed in unexposed undifferentiated control cells, and cell treatment with Cd, TCDD, BaP or insulin did not increase AhR location in the nuclei. As shown in Figure 3B, the staining pattern obtained in differentiated cell cultures was

quite different: AhR location to the nuclei was more pronounced, even in control cells. Also, in differentiated cells, exposure to Cd and TCDD led to 2- and 1.5-fold increases in the nuclear localization of AhR, respectively, without any additive effect (Fig. 3C). Since TCDD did not increased phospho-ERK in the Caco-2 cells (Fig. 2), these data suggest that ERK activation would not be a prerequisite to AhR activation. Moreover, cell treatment with 100 nM insulin, which led to a 15-fold increase in phospho-ERK1/2 (data not shown), did not modify AhR nuclear location. Thus conversely, ERK1/2 phosphorylation does not necessarily lead to AhR activation.

The present study also reveals differences in cells response as a function of the days in culture. It is thus tempting to conclude that the lack of effect of TCDD in 6-day-old cultures is related to the cell developmental stage. However, although most studies on TCDD, BaP and CYP1A1 in Caco-2 cells have been conducted in differentiated cultures, a few studies have shown that TCDD may increase EROD activity or CYP1A1 expression in 1 to 2-day-old (undifferentiated) cells[48,49]. Based on our knowledge, there is no evidence showing an AhR-independent mechanism for the TCDD-induced expression of CYP1A1. Thus the results reported by de Waard et al.[48] and Perdrew et al.[49] suggest that TCDD may activate AhR in undifferentiated Caco-2 cells as well. We may hypothesize that the cells' responsiveness to AhR activating ligands varies with culture conditions. In most studies, cells were treated using standard medium containing serum. Cadmium binds to bovine serum albumin, and we have previously shown that Cd complexation by BSA considerably lowers the total cellular accumulation of metal[50]. For this reason, serum-free exposure conditions have been used for cell treatment with Cd as well as with TCDD and BaP. In the absence of serum, Caco-2 cell sensitivity to TCDD-induced AhR activation seems to vary with the differentiation status. As for

the Cd-induced increases in MTT and ERK activities, stimulation of the nuclear translocation of AhR would be a stage-specific differentiation phenomenon.

U0126 but not resveratrol inhibits the Cd-induced nuclear translocation of AhR

In order to discriminate between direct (as ligand) and indirect (via ERK) effect on AhR, the receptor nuclear translocation induced by either Cd or TCDD was studied in the presence of resveratrol, used as antagonist of AhR, and U0126. Resveratrol inhibited TCDD-induced but not Cd-induced nuclear translocation of AhR, which confirm that contrary to TCDD, Cd does act as a ligand for AhR (Fig. 4). Although only Cd activated ERK (Fig. 2), nuclear translocation of AhR induced by Cd and TCDD were both inhibited by U0126 (Fig. 4). This, in addition with the lower AhR nuclear location measured in cells exposed to U0126 alone, suggests that: i) ERK1/2 activation would not be a prerequisite to AhR activation but basal ERK1/2 activity would be necessary or ii) a rapid transient (not observed) activation of ERK1/2 precedes AhR activation. Interestingly, higher basal levels of nuclear AhR (almost 2-fold) were observed in differentiated cells compared to undifferentiated cultures, whereas the opposite was measured for the basal levels of phosphorylated ERK1/2 (data not shown). No correlation could be observed between phospho-ERK and AhR activity. The possibility that the Cd-induced AhR activation involves elements (other than ERK1/2) that need to be identified deserved to be investigated.

Cd-induced AhR nuclear location does not necessarily stimulate CYP1A1/2 expression

To get further insights on Cd effect on AhR signaling, we tested whether AhR target genes could be induced by estimating the level of CYP1A1/2 in differentiated cells. Western blot analysis showed a 5-fold induction of CYP1A1/2 expression in differentiated cells treated with 1 nM TCDD (Fig. 5). ERK1/2 activation would not be required since U0126 did not lower the

stimulatory effect of TCDD (Fig. 5), and TCDD itself did not increase ERK1/2 phosphorylation (Fig. 2). Conversely, Cd that increased the level of phospho-ERK1/2 and the nuclear translocation of AhR failed to induce CYP1A1 (Fig. 5). Lack of CYP1A1 induction by Cd has also been reported by others. In mouse hepatoma cells that respond to TCDD stimulation in minutes, Cd (20 μM) failed to induce CYP1A1 expression and AhR activity as estimated using a luciferase reported gene[52]. In contrast, some authors did observe increases in CYP1A1 mRNA in mouse hepatoma cells exposed to 5 – 25 μM Cd, but without modification in the CYP1A1 protein level[26,53]. In Caco-2 cells, a 5-day exposure to 1 μM Cd, which succeeded in inducing metallothionein mRNA (a well-known metal biomarker), reduced CYP1A1 mRNA by 20%[11]. This study was probably conducted on undifferentiated cells because exposure began 24 h post seeding. One may thus wonder how differentiated cultures would have responded. However, similar results were obtained in vivo, in the intestinal tissues of rats receiving Cd orally[11].

Given the lack of CYP1A1 induction by Cd, we must specify that an increased translocation of AhR to the nucleus does not guarantee its transcriptional activity. Indeed, in an attempt to better understand the role protein kinase plays in AhR activation, numerous studies dissected the steps of AhR signaling, from ligand binding to gene expression. These studies showed that AhR may indeed translocate to the nucleus without being an active transcription factor. Phosphorylation steps, with the possible involvement of PKC, would be required either for AhR-ARNT binding to XRE[54,55] or for the subsequent transcriptional activity of the dimer[18,20,21]. Although our previous data suggested the involvement of PKC in Cd-induced ERK activation in Caco-2 cells[13], it would not be sufficient to induce the transcriptional activity of AhR. Furthermore, some studies have shown that stress-induced signaling may also modulate CYP1A1 expression (for review see Delescu et al.[56]), and it has been suggested that oxidative stress may be involved in Cd-induced CYP1A1 expression[57]. One may therefore hypothesize that, whatever

the AhR stimulation, the Cd-induced CYP1A1 expression observed by some investogators involves AhR-independent mechanisms triggered by oxidative stress signaling. However, our previous data suggest that a 24-h exposure to 10-μM Cd is unlikely to induce oxidative stress in differentiated Caco-2 cells[58]. Assuming an oxidative stress-dependent mechanism, this could explain the absence of CYP1A1 induction despite AhR stimulation observed in the present study.

Conclusion

The present study was motivated by some of our previous results showing that Cd may induce a concentration and time-dependent increase in MTT activity in differentiated Caco-2 cell cultures exclusively. This effect was insensitive to estrogen receptor antagonist, and it was not related to cell proliferation but to enhanced protein synthesis that involves ERK1/2 activation. Because some studies have suggested that phospho-ERK could activate AhR we have hypothesized that Cd-induced increase in phospho-ERK may result in AhR activation. The present study demonstrates that the Cd-induced hormesis-like effect on MTT activity in Caco-2 cells is a stage-specific differentiation phenomenon that is not observed with xenobiotics acting as AhR ligands. The nuclear location of AhR is more pronounced in differentiated cells than undifferentiated cultures, whereas the opposite was measured for phospho-ERK1/2. TCDD promotes the nuclear translocation of AhR and stimulates CYP1A1/2 expression in differentiated cells. Contrary to Cd, TCDD does not stimulate ERK phosphorylation. Conversely, Cd that increases the phospho-ERK level and AhR nuclear location does not enhance CYP1A1 expression. Insulin, which considerably increases ERK phosphorylation, has no effect on AhR nuclear location. Thus, ERK activation is not a prerequisite to AhR activation and does not necessarily lead to AhR activation. Cadmium does not stimulate the transcriptional activity of

AhR but increases its nuclear location that could result in increased cell's sensitivity to AhR activators.

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Legend to figures

Figure 1. MTT activity concentration-response curve as a function of increasing concentrations of (A) Cd, (B) TCDD or (C) BaP in 6- and 20-day-old cells. Cells were grown in the presence of FBS for 6 or 20 days, and were then exposed to increasing concentrations of Cd, TCDD or BaP in the absence of FBS for 24 h. Data shown are means ± SD estimated on 5 to 7 independent cell cultures. * Significantly different ($p \leq 0.05$) compared to the respective control values measured in unexposed cells of the same culture day maintained for 24 h in FBS-free medium.

Figure 2. Effect of Cd, TCDD and BaP on ERK activation as investigated by Western blot analysis as described in the Material and Methods Section. Cells were cultured for 6 (A, C) or 20 days (B, D) in the presence of FBS and were then incubated for 24 h in FBS-free medium in the presence or absence of 1 or 2 nM TCDD, 0.2 or 0.4 nM BaP, 10 µM Cd alone or in combination with 20 µM U0126. A, B: representative blot of ERK1/2 phosphorylation. C, D: Levels of ERK and phospho-ERK (optical density, O.D. normalized to β-actin) expressed relative to that of unexposed cells maintained for 24 h in the absence of FBS. Data shown are means ± SD estimated on 3 independent cell cultures.

Figure 3. AhR nuclear translocation in (A) undifferentiated and (B) differentiated cells as investigated by immunofluorescence as described in the Material and Methods Section. Cells were cultured for 6 and 20 days in the presence of FBS and were then incubated for 24 h in FBS-free medium in the absence or presence of 10 µM Cd or 1 nM TCDD, alone or in combination, or 100 nM insulin. Cells were then incubated with AhR antiboy and Alexa Fluor® 568 conjugated goat anti-rabbit IgG. The nuclei were stained with DAPI and fluorescence was detected by

confocal microscopy. (C) Quantitation of AhR nuclear translocation in undifferentiated (black columns) and differentiated cells (dashed columns) as investigated by immunofluorescence. The intensity of fluorescence (arbitrary fluorescence units, F.U.) of the regions of interest (nuclei) was evaluated using ImageJ 1.48p software and then normalized to that measured in unexposed cells maintained for 24 h in the absence of FBS. Data shown are means ± SD estimated on 3 to 5 independent cell cultures. * Significantly different ($p \leq 0.05$) compared to 6-day-old undifferentiated cells.

Figure 4. AhR nuclear translocation in differentiated cells as investigated by immunofluorescence as described in the Material and Methods Section. (A) Cells were cultured for 20 days in the presence of FBS and were then incubated for 24 h in FBS-free medium in the absence or presence of 10 µM Cd and 1 nM TCDD with or without 20 µM U0126 and 100 µM resveratrol. Cells were then incubated with AhR antibody and Alexa Fluor® 568 conjugated goat anti-rabbit IgG. The nuclei were stained with DAPI and fluorescence was detected by confocal microscopy. (B) Quantitation of AhR nuclear translocation in differentiated cells as investigated by immunofluorescence. The intensity of fluorescence (arbitrary fluorescence units, F.U.) of the regions of interest (nuclei) was evaluated using ImageJ 1.48p software and then normalized to that measured in unexposed cells maintained for 24 h in the absence of FBS. Data shown are means ± SD estimated on 3 to 4 independent cell cultures. * Significantly different ($p \leq 0.05$) compared to the respective cell treatment in the absence of U0126 and resveratrol.

Figure 5. Effect of Cd, TCDD and insulin on CYP1A1/2 expression and ERK activation as investigated by Western blot analysis as described in the Material and Methods Section. Cells were culture for 20 days in the presence of FBS and were then incubated for 24 h in FBS-free

medium in the presence or absence of 1 nM TCDD, 10 μM Cd, 100 nM insulin alone or in combination with 20 μM U0126. A: representative blot of CYP1A1/2 and ERK1/2 phosphorylation. Levels of (B) CYP1A1/2 and (C) phospho-ERK1/2 (optical density, O.D., normalized to β-actin) expressed relative to that of unexposed cells maintained for 24 h in the absence of FBS. Data shown are means ± SD estimated on 3 independent cell cultures. * Significantly different ($p \leq 0.05$) compared to the respective cell treatment in the absence of U0126.

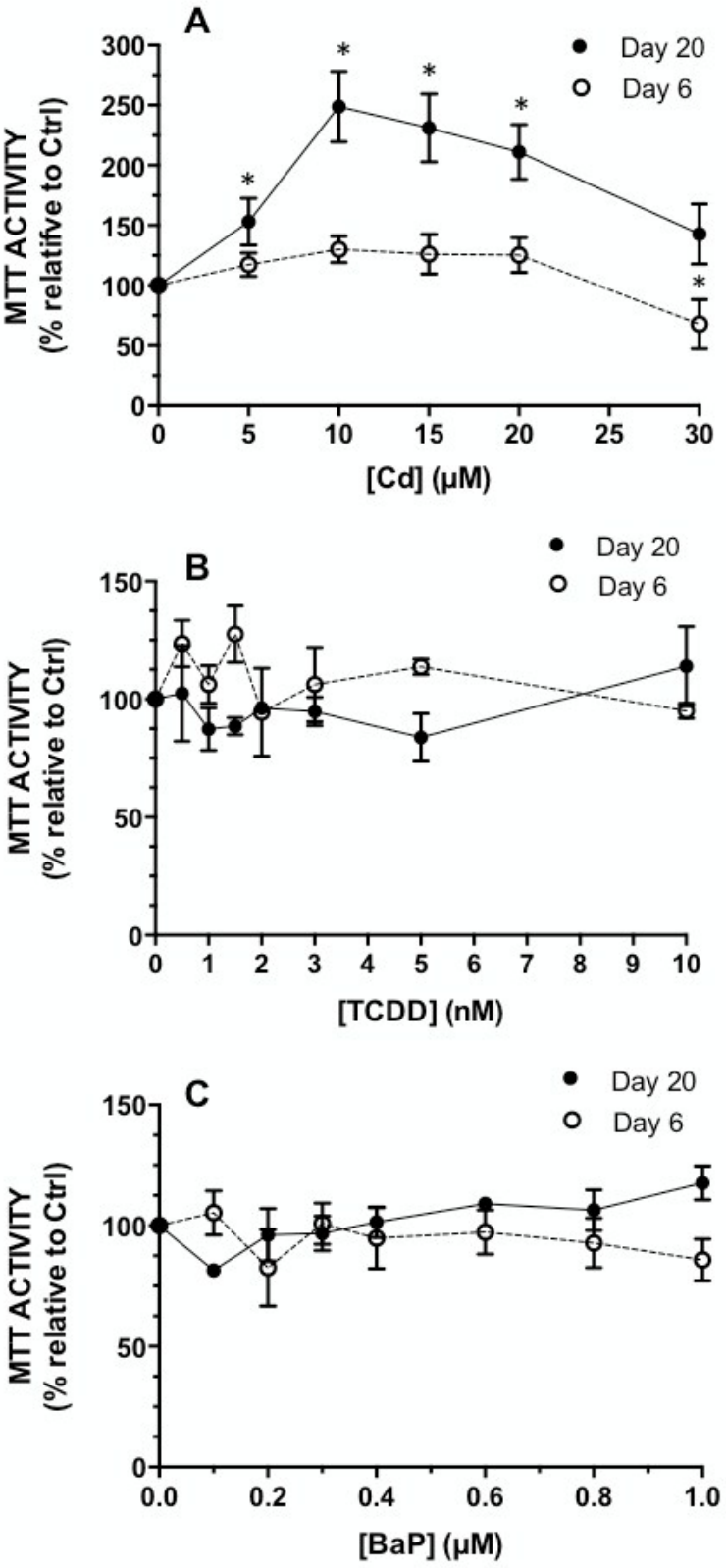


Figure 1

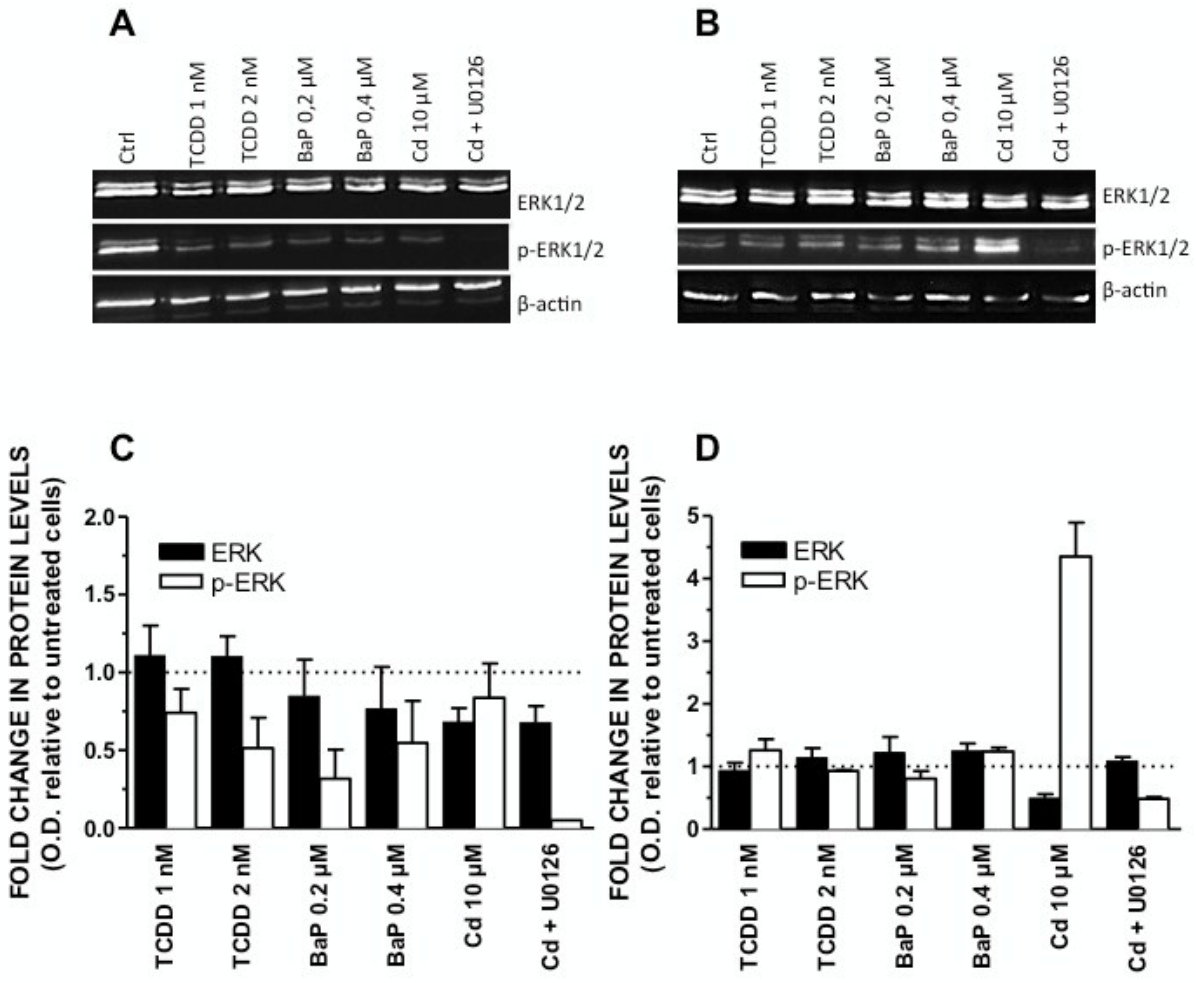


Figure 2

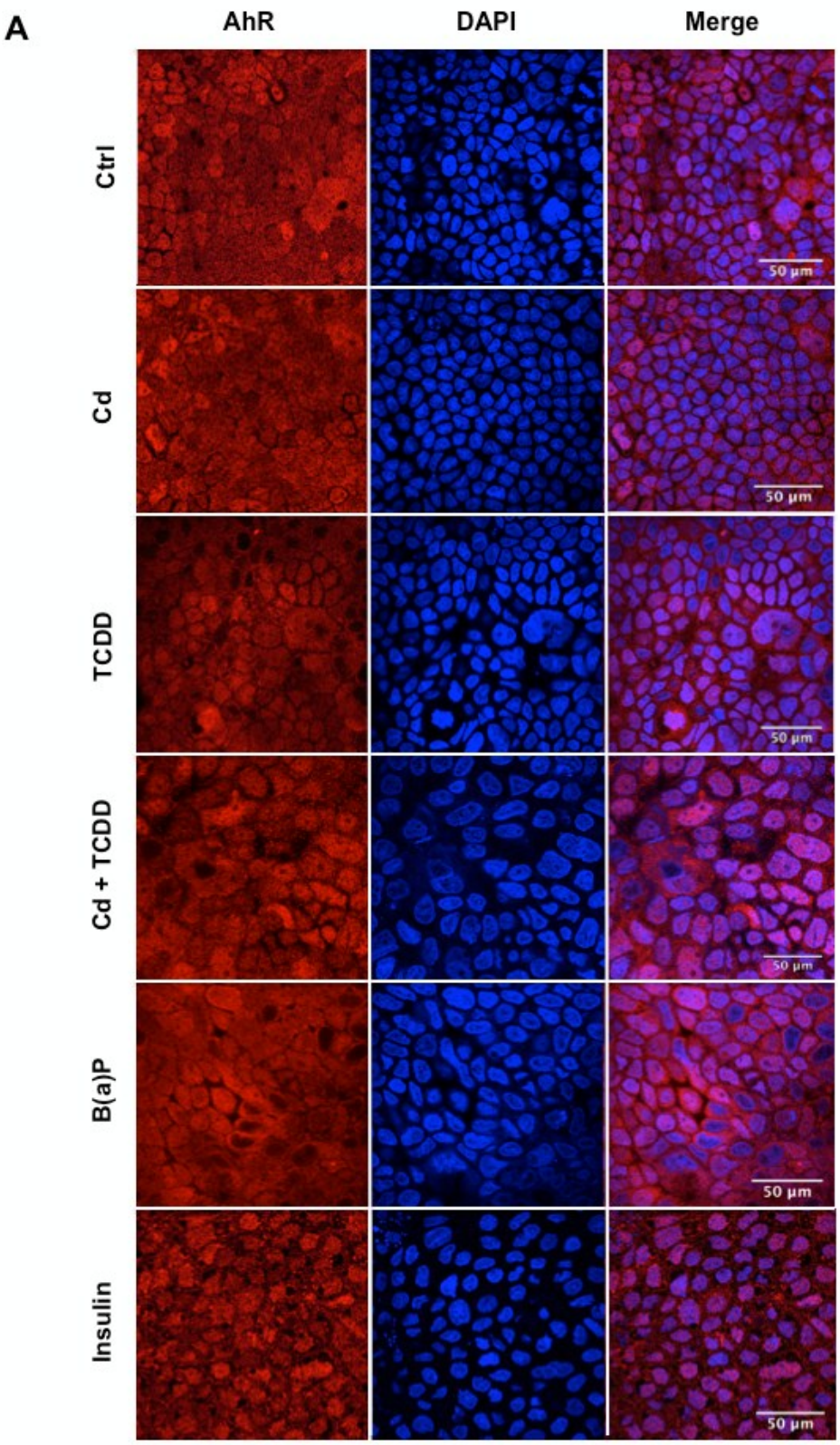


Figure 3

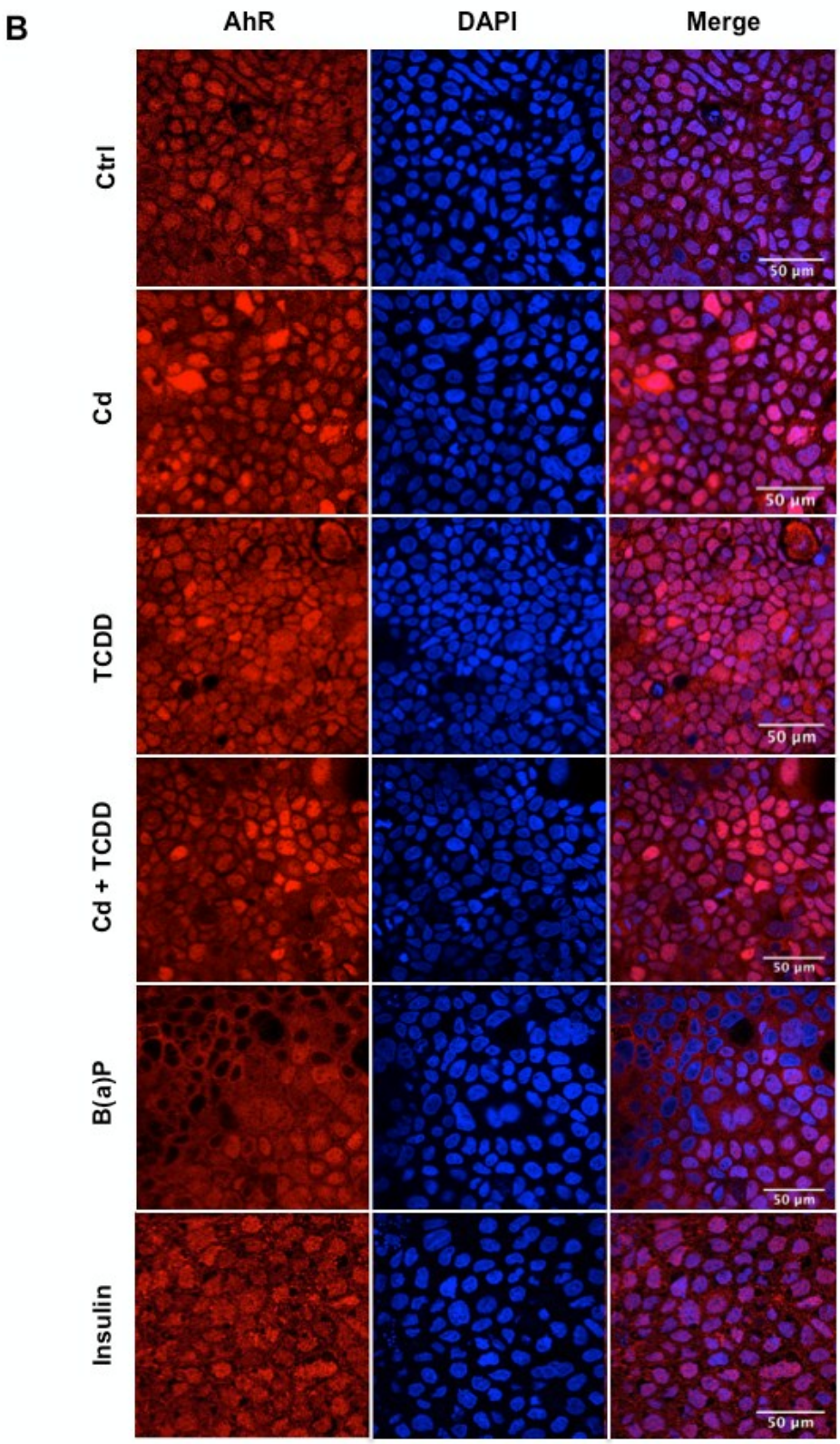


Figure 3

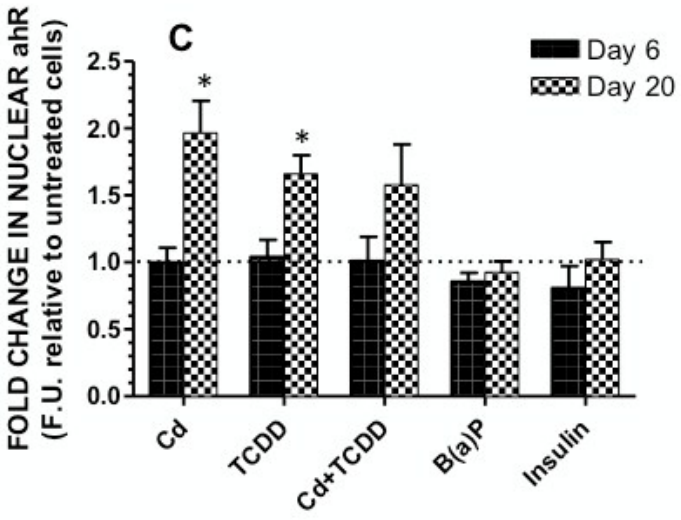


Figure 3

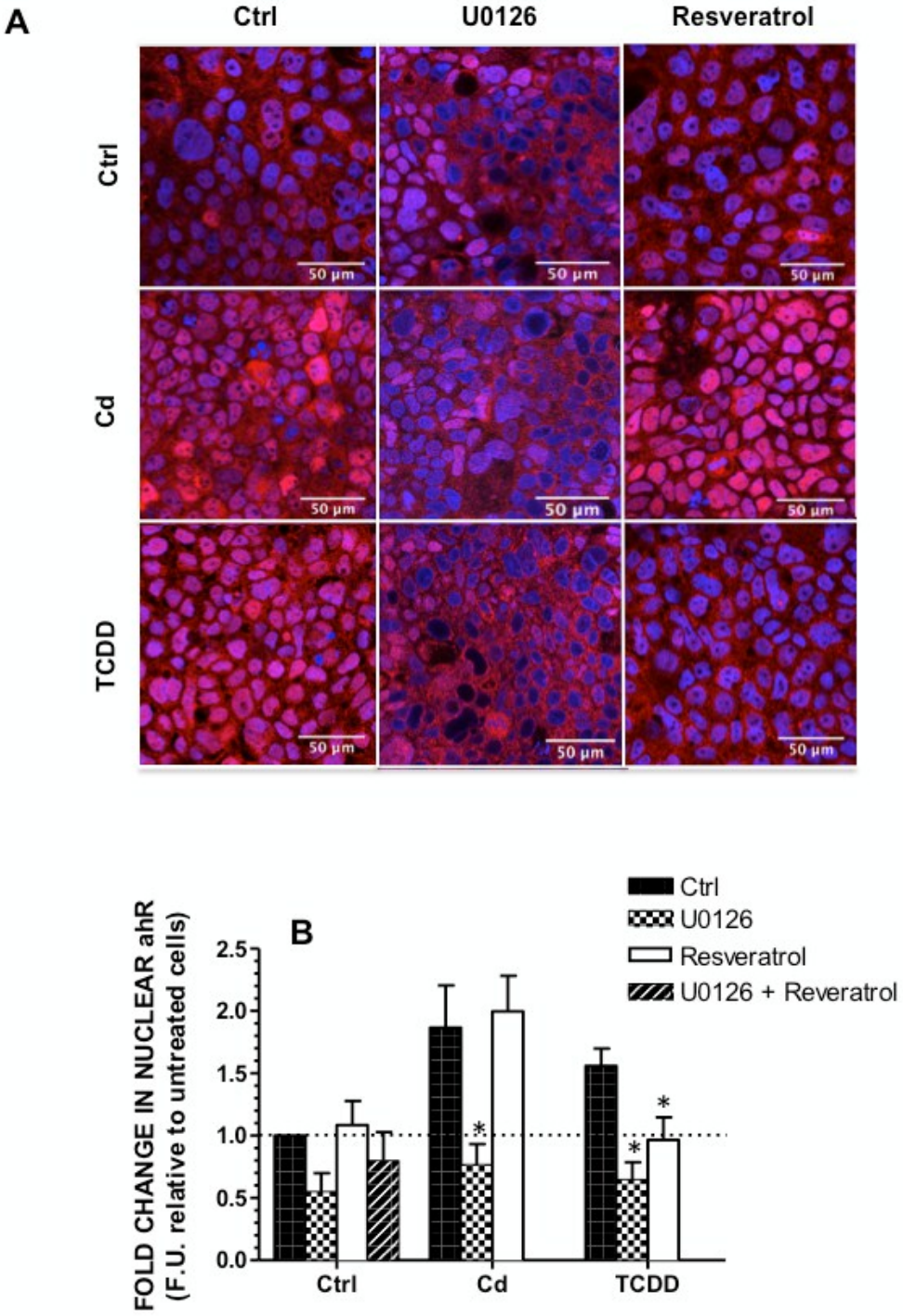


Figure 4

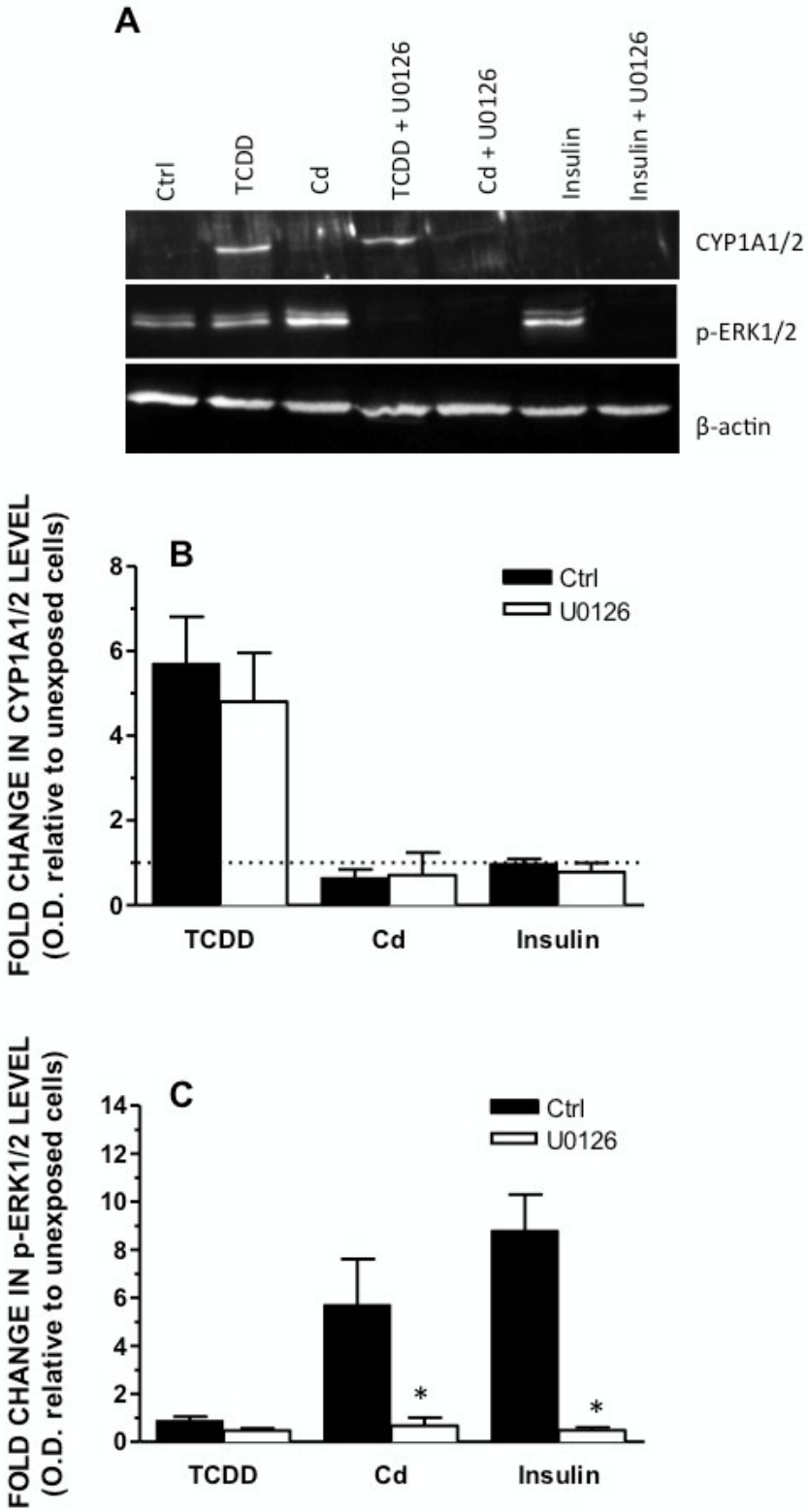
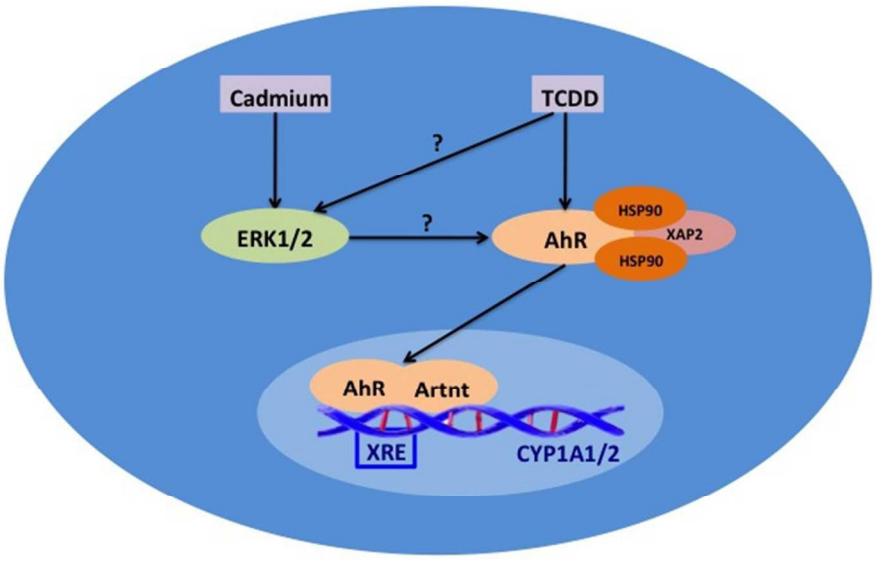


Figure 5

The possibility that Cd may activate AhR indirectly via ERK1/2 phosphorylation was tested as a function of enterocytic differentiation status in the human Caco-2 cells.



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