All-trans retinoic acid modulates AHR signaling and its downstream target gene, CYP1A in human hepatoma cells

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Abstract

The aryl hydrocarbon receptor (AHR) was identified for its mediating toxicological role in response to variety of the polycyclic aromatic hydrocarbon family of environmental contaminants however, recent data indicate that the AHR can be activated with different types of endogenous and exogenous chemicals. The aim of this study was to gain more information about the mechanisms that regulate expression of the AHR target gene, CYP1A1 by All-trans retinoic acid (ATRA) in human hepatoma cells (HepG2 and Huh7). The human hepatoma cell line (HepG2-XRE-Luc) carrying cytochrome P4501A1 (CYP1A1) response elements, HepG2 and Huh7 cells were exposed to different doses of ATRA (1-50 µM) and CYP1A1 transcription and enzymatic activities, as well as gene expression were measured. Our results showed that ATRA is able to induce CYP1A1 in an AHR-dependent manner using CH223191 as an AHR antagonist. The result showed that different doses of ATRA have no significant effects on cell viability. CYP1A1 enzyme and transcription activities as well as *CYP1A1* mRNA for all treated group showed a significant elevation by ATRA. To better understand the mechanism underlying AHR activation by ATRA more molecular studies are needed.

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1. Introduction

Aryl hydrocarbon receptor (AHR) a members of the Per ARNT-Sim (HLH-PAS) transcription factor family that mediates expression of cytochrome P450 (CYP1) isoforms participates in the detoxification or bio-activation of xenobiotics such as aromatic halogenated hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) as well as natural compounds such as phy-tochemical and estradiol (1). One example of natural compound with highest affinity with AHR is the 6-formylindolo[3,2-b]carbazole (FICZ) which is initially characterized as a photooxidation product of tryptophan amino acid, and introduced as an endogenous ligand of AHR. Combined with CYP1A1 inhibitors, FICZ at high concentrations can cause sustained CYP1A1 induction, which leads to oxidative stress, apoptosis, and toxicity (2-11). Also another high affinity AHR ligand, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) binds and activates AHR most potently among xe-

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nobiotics (1, 12). In addition to its high toxicity and bioaccumulation, TCDD also has a long half-life of about 7.5 years in humans. Due to its long half-life, CYP1A1 induction by TCDD is very long-lasting in the body(13).

A retinoid, vitamin A (VA) belongs to a group of lipid-soluble retinoids, which also includes retinol and retinal. Retinoic acid (RA), the bioactive form of retinol, is available in three forms: all trans retinoic acid (ATRA), 9-cis retinoic acid (9cis-RA), and 13-cis retinoic acid (13cis-RA)(14). During the digestion of vitamin A-rich foods, retinoic acid is produced endogenously (15). There is also evidence that retinoids play a role in the pathophysiology of several liver diseases that are compatible with them, including fatty liver, portal fibrosis, cirrhosis, and liver cancer. Moreover, dioxin-induced hepatotoxicity has been reported to cause bone lesions and developmental abnormalities, including cleft palate formation which is similar to the symptoms of vitamin A overdose.(15). The immune system and vision normal function depend on vitamin A (16). As a growth stimulant, VA maintains bones and epithelial tissue, and promotes mucosal epithelial secretion. With a VA deficiency, the epithelial cells of the respiratory tract become keratinized, resulting in reduced immunity and an elevated risk of infection. Through nuclear receptors, VA regulates a variety of gene targets, improving immune function and inducing cytokine production in immune cells. (16). In vitro studies shown that a high dose of VA may have anti-tumor effects on cancer cell lines, due to its ability to maintain a sufficient level of natural killer cells in the circulation ,In clinical trials, VA consumption at high doses has been demonstrated to reduce the risk of liver cancer in Chinese subjects (16).

The aim of this study was to gain more information about the mechanisms that regulate expression of the AHR target gene, CYP1A1 by ATRA in human hepatoma cells.

2. Materials and Methods
2.1. Chemicals
Chemicals were obtained from the following suppliers: FICZ (6-formylindolo(3,2b)carbazole), 2,5-diphenyltetrazolium bromide (MTT) and CH223191 were purchased from Sigma-Aldrich Germany.

2.2. Cell culture and chemical treatments
Three human hepatoma cell lines (HepG2, HepG2-XRE-Luc and Huh7) were used in the experiments. The HepG2-XRE-Luc cell line containing a pTX.DIR-luciferase reporter under the control of a model CYP1A1 promoter, consisting of two XRE sequences of the rat CYP1A1 gene (17) was kindly provided by K. Gradin, Karolinska Institutet. All cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/ mL streptomycin, and 100 IU/mL penicillin under an atmosphere containing 5% CO_2 at 37 °C. The XRE-HepG2 medium was additionally supplemented with 800 μg/mL geneticin. Treatments were started by replacing the growth medium with fresh medium without FBS containing different concentrations of the test compounds (1 to 50μM of ATRA and/or 10 nM CH223191). The lengths of treatments varied and are indicated in the results section.

2.3. MTT analyses of cell viability
To determine the effects of ATRA on cell viability, the cells were plated into 96-well plates and grown for 24 h and were treated with different concentrations of ATRA for another 24 h. After the incubation period, the treatments were terminated by removing the medium and rinsing the cells with PBS. The cells were exposed to the MTT dye for 4 h and the blue purple formazan crystal products were dissolved in DMSO for quantification by measuring the absorption at 570 nm.

2.4. Analyses of CYP1A1 enzyme activity
Whole cell EROD activities were measured to estimate CYP1A1 activity. Cells were seeded at high density in 96-well plates and grown to over-confluence. At the indicated time points, the medium was removed and the cells were rinsed with PBS. The EROD activity was determined as previously described (5). Data were normalized to cellular protein content determined with the DC protein assay kit.

2.5. Analyses of CYP1A1 reporter activity

Table 1. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequences
CYP1A1 F	-CATCCCTATTCTTCGCTACCTACCC-
CYP1A1 R	GTGCTCCTTGACCATCTTCTGC--
GAPDH F	-GAAGGTGAAGGTCGGAGTC-
GAPDH R	-GAAGATGGTGATGGGATTTC-

Transcription of the CYP1A1 gene was estimated using a luciferase report assay HepG2-XRE-Luc cells as previously described (5). Luciferase activity was normalized to cellular protein content determined with the DC protein assay kit.

2.6. Analyses of CYP1A1 gene expression

Total RNA was extracted from using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, after the addition of 1 ml Trizol to 2×10^6 cells, 300 µl chloroform was added and cells were homogenized by pipetting. Then the mixture was centrifuged at 10,700 g for 15 min, the supernatant was collected, mixed with 500 µl of ice-cold isopropanol, and was centrifuged at 10,700 g for 10 min. The resulting precipitated RNA pellet was washed once with 75% ice-cold ethanol and re-suspended in 40 µl of DEPC-treated water. The RNA concentration and purity were estimated using Nanodrop (ACT gene, USA). Then, 1 µg of RNA was used for the synthesis of the first-strand cDNA using the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). For real-time RT-PCR, samples were assayed in triplicate by adding 1 µl of cDNA in a reaction mixture of 25 µl containing 12 µl SYBR Green master mix, 10 µl nuclease-free water, 1 µl of forward, and 1 µl of reverse primers. Primers were designed using AlleleID® version 7.84 and their characteristics are shown in Table 1. All assays were carried out under the following condition: 1 cycle of pre-denaturation (15 min at 95˚C) followed by 40 cycles of denaturation (15 s at 95 ˚C), annealing (30 s at 60˚C), and extension (30 s at 72 ˚C), respectively. Melt curve analysis was performed to confirm the specificity of the amplified products. Gene expression levels were calculated via the 2-ΔΔCT method and normalization was done against β-actin threshold cycle (Ct) values. The data were presented as the fold change of the target gene expression normalized and relative to the reference sample.

2.7. Statistics

All studies have been performed in at least in two independent experiments. All measurements were performed with 2-4 replicates and data are expressed as the mean±SD, and show results from one representative experiment. For comparison between more than two experimental groups one-way ANOVA with Tukey post hoc test was used. For comparison between two experimental groups, two-tailed t-tests were used. Probability p values <0.05 were considered statistically significant.

3. Results
3.1. Effect of ATRA on cell viability

We evaluated the effect of different doses of ATRA on cell viability using the MTT assay. HepG2 and Huh7 cells were treated with different concentration of ATRA (1-50µM) for 24 h. after incubation period, we measured cell viability according to the materials and methods. The result showed that different dose of ATRA have no significant effects on cell viability (Figure 1).
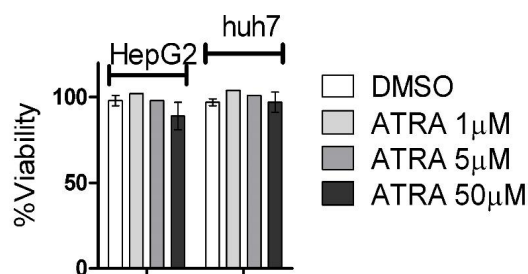
3.2. Effect of ATRA on CYP1A1 enzyme activity

Figure 1. Effect of ATRA on cell viability. HepG2 and Huh7 cells were treated with DMSO or 1, 5, or 50 µM concentrations of ATRA for 24 h. Cell viability was measured with the MTT assay. Different doses of ATRA have no significant effect on cell viability. Data are expressed as mean±SD.
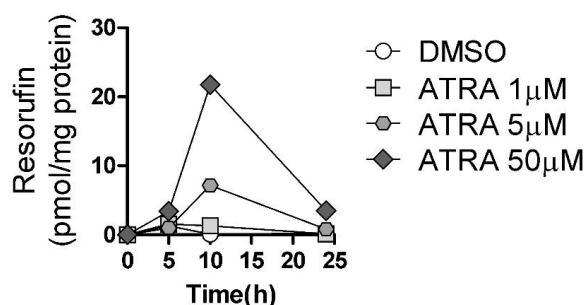
Figure 2. Time-course and dose-response effect of ATRA on EROD activity. CYP1A1 enzyme activity was measured in Huh7 cells treated for 5, 10, or 24 hr with ATRA (1 to 50 µM). EROD activity (pmol resorufin/ mg protein) was determined according to the materials and methods. *CYP1A1* enzyme activity was reached to a maximal levels after 10 hr. However, the amount of enzyme declined after 24 hr. Data are expressed as mean±SD.

We measured CYP1A1 enzyme activity in Huh7 cells treated for 5, 10, or 24 h with ATRA (1-50µM). Then we determined the activity of EROD (pmol resorufin/mg protein). CYP1A1 enzyme activity was reached to a maximal levels after 10 hr. However, the amount of enzyme decreased after 24 hr (Figure 2).

3.3. Effect of ATRA and FICZ on CYP1A1 reporter activity

We measured AHR activation in HepG2-XRE-Luc treated for 5, 7.5, 24 and 48 hr with increasing concentrations of ATRA (1-50µM) that led to a maximum increased in CYP1A1 transcription activity after a transient period of inhibition (7.5 hr). However, after 24 hr, CYP1A1 transcription activity also declined (Figure 3).

3.4. Effect of ATRA and FICZ on CYP1A1 gene expression

We detected relative expression levels of *CYP1A1* mRNA by reverse transcription-quantitate polymerase chain reaction in Huh7 cells. The result showed a significant increase in the mRNA levels of *CYP1A1* treated with 5µM of ATRA which was reduced by CH223191 (10 nM) (Figure 4).

4. Discussion

Aryl hydrocarbon receptor (AHR) and its nuclear translocator (ARNT) are well-known transcription factors between species. However, there are very limited studies on the physiological function of AHR, especially on the regulation of AHR
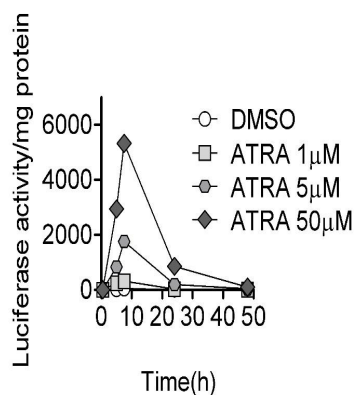

Figure 3. Time-course and dose-response effects of ATRA on CYP1A1 transcription activity. HepG2-XRE-Luc cells were treated with vehicle (DMSO), 1, 5 or 50 µM of ATRA for 5, 7.5, 10, 24 and 48 hr. Luciferase activity (unit luciferin/mg protein) was measured in HepG2- XRE-Luc cells. ATRA treatment led to a maximum increased in the CYP1A1 transcription activity after a transient period of inhibition (7.5 hr). However, after 24 hr, CYP1A1 transcription activity declined. Data are expressed as mean±SD.
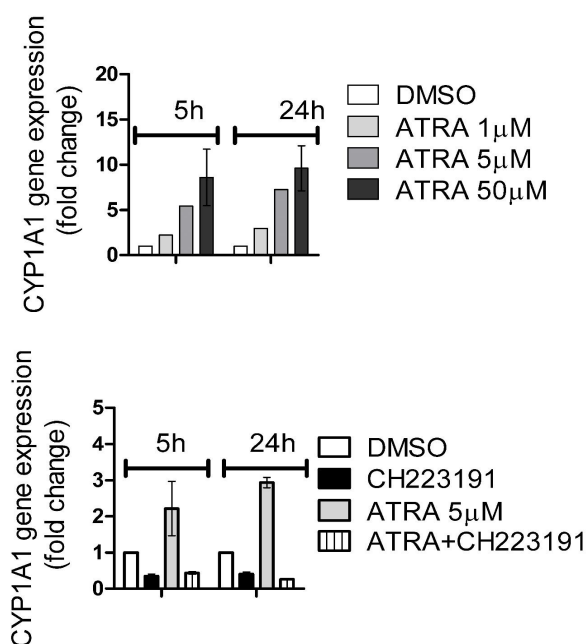
Figure 4. Effect of ATRA on *CYP1A1* gene expression. Relative expression levels of *CYP1A1* m RNA were detected by reverse transcription-quantitate polymerase chain reaction in Huh7 cells. The result of *CYP1A1* treated with 5μM of ATRA showed a significant increase in the m RNA levels. However, ATRA+CH223191 (10nM) treatment led to inhibition of CYP1A1 gene expression.

by endogenous compounds. Hence, we examined ATRA effects on AHR-induced cytochrome P450 (CYP) 1A1 gene transcription as a model of AHR-regulated transcriptional mechanisms in human hepatoma cells.

Here, we incubated the human hepatoma cell line (HepG2-XRE-Luc) carrying the CYP1A1 response elements, HepG2 and Huh7 cells with different doses of ATRA. Activation of AHR in HepG2-XRE-Luc cells by increasing ATRA caused a further increase in the CYP1A1 transcriptional activity measured after a transient period and decreased CYP1A1 transcriptional activity after 24 hr (Figure 3).

Data on the consequences of retinoid receptor activation on CYP1A1 expression are inconsistent; Fallone et al. showed that ATRA at physiological concentrations could reduce AHR-mediated gene transcription by inhibiting ARNT uptake into related regions(18). Ohno et al. also showed that ATRA is able to induce weakly *CYP1A1* gene expression in keratinocytes and in hepatocytes (19) While other studies showed an increase(20, 21).

In this study, we investigated the effects

of ATRA on CYP1A1 gene expression in human hepatocytes (HepG2 and Huh7). In our study, the relative expression levels of *CYP1A1* mRNA expression levels were detected by reverse transcriptase-polymerase chain reaction in Huh7 cells. *CYP1A1* results showed a significant increase in mRNA levels for all treated groups (Figure 4).

Downstream signaling of the CYP1A1 promoter can be mediated by different synergistic activation processes, either by binding AHR proteins to independent binding sites, or by sharing sites in the gene promoter. Activation of each path by upstream signaling allows for synergistic effects. Participation and collaboration at several AHR junctions as well as the synergistic behavior of AHR-dependent signaling have been interpreted in detail at the gene promoter level in recent years(22) .The available data now suggest the existence of cross-mechanisms between AHR and retinoid receptors. Indeed, cooperation between the AHR and RAR / RXR signaling pathways has been reported by Gambone et al. (23). It has also the retinoid response elements (RARE) has been recognized in the CYP1A1 promoter gene(20, 21).

5. Conclusion

From these and other studies, it appears that ATRA can induce AHR activation, and increases *CYP1A1* gene expression, but the full mechanism is not yet fully understood and more molecular studies including ligand binding affinity, AHR translocation as well as AHR-ARNT dimer binding to the xenobiotic response elements (XRE) studies are needed.

Conflict of Interest

None declared

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