Gene 853 (2023) 147099

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Role of AHR, NF-kB and CYP1A1 crosstalk with the X protein of Hepatitis B virus in hepatocellular carcinoma cells

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ARTICLE INFO

Edited by: Ece Konac

Keywords:
Hepatocellular carcinoma
Hepatitis B virus X protein
Nuclear factor-kappa B
Aryl hydrocarbon receptor
Cytochrome P4501A1

ABSTRACT

In this study, it was aimed to elucidate the interaction between aryl hydrocarbon receptor (AHR), nuclear factor-kappa B (NF-kB), and cytochrome P4501A1 (CYP1A1) with hepatitis B virus X protein (HBX) in a human liver cancer cell line (HepG2) transfected with HBX. First, AHR, NF-kB, and CYP1A1 genes were cloned into the appropriate region of the CheckMate mammalian two-hybrid recipient plasmids using a flexi vector system. Renilla and firefly luciferases were quantified using the dual-luciferase reporter assay system to measure the interactions. Secondly, transient transfections of CYP1A1 and NF-kB (RelA) were performed into HBX–positive and HBX–negative HepG2 cells. The mRNA expression of CYP1A1 and NF-kB genes were confirmed with RT-PCR, and cell viability was measured by WST-1. Further verification was assessed by measuring the activity and protein level of CYP1A1. Additionally, CYP1A1/HBX protein–protein interactions were performed with co-immunoprecipitation, which demonstrated no interaction. These results have clearly shown that the NF-kB and AHR genes interact with HBX without involving CYP1A1 and HBX protein–protein interactions. The present study confirms that AHR and NF-kB interaction plays a role in the HBV mechanism mediated via HBX and coordinating the carcinogenic or inflammatory responses; still, the CYP1A1 gene has no effect on this interaction.

1. Introduction

Hepatitis B virus (HBV) infection is a significant health problem, as an acute and chronic infection is associated with hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Levrero and Zucman-Rossi, 2016). Chronic HBV infection is the leading risk factor for HCC, accounting for at least 50 % of total liver cancer cases, and is the second leading cause of cancer-associated death worldwide due to its high incidence and resistance to treatment (Kubo et al., 2016). Although HBV is considered an oncogenic virus, the detailed mechanism by which HBV causes HCC development has not been completely elucidated (Péneau et al., 2022). Evidence shows that the expression of the virus-encoded X

Abbreviations: 3MC, 3-Methylcholanthrene; AhR, Aryl Hydrocarbon Receptor; AP-1, Activator Protein-1; ATF, Activating Transcription Factor; B(a)P, Benzo(*a*)pyrene,; BCA, Bicinchoninic Acid; BSA, Bovine Serum Albumin; c-Myc, Myelocytomatosis; *Co-IP*, *Co-Immunoprecipitation*; CREB, cAMP Sensitive Element-Binding Transcription Factor; CYP1A1, Cytochrome P4501A1; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic Acid; EMEM, Eagle's Minimum Essential Medium; EROD, Ethoxyresorufin O-Deethylase; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; HBV, Hepatitis B Virus; HBX, Hepatitis B Virus X Protein; HCC, Hepatocellular Carcinoma; HepG2, Human Liver Cancer Cell Line; HLH, Helix-Loop-Helix; IFN-γ, Interferon-Gamma; IL-6, Interleukin-6; MEM, Minimum Essential Medium; NFAT, Nuclear Factor of Activated *T*-cells; NF-kB, Nuclear Factor-Kappa B; PAS, Per-Arnt-Sim; PMFS, phenylmethylsulfonyl fluoride; ROS, Reactive Oxygen Species; RT-PCR, Reverse Transcription Polymerase Chain Reaction; SD, Standard Deviation of the Mean; SDS-PAGE, Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis; STAT3, Signal Transducer and Activator of Transcription 3; TBK1, TANK-Binding Kinase-1; TCDD, Tetrachlorodibenzo-*p*-Dioxin; WST-1, Water-Soluble Tetrazolium 1; XREs, Xenobiotic Response Elements; ε-ACA, Epsilon-Aminocaproic Acid.

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https://doi.org/10.1016/j.gene.2022.147099
Received 29 July 2022; Received in revised form 22 November 2022; Accepted 29 November 2022
Available online 5 December 2022
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protein is consistently high in the livers of HCC patients chronically infected with HBV, and the HBV-X (HBX) protein plays a crucial role in developing HCC (Xu et al., 2019; Wei et al., 2019).

HBX contributes to hepatocarcinogenesis through various pathways, such as apoptosis, DNA repair, epigenetic changes, cell cycle, and transactivation (Tian et al., 2013; Ali et al., 2014; Liu et al., 2016). The HBX protein has been identified as a transactivator that activates a variety of viral and cellular promoters and enhancers (Kwon and Rho, 2003). It was shown that HBX has an increasing effect on HBV transcription and replication (Liu et al., 2016). Its transcriptional activity, which is required for viral replication, mediates a protein–protein interaction (Shen et al., 2020). It is known that transcriptional factors such as nuclear factor κB (NF-κB), activator protein-1 (AP-1), activating transcription factor (ATF)/cAMP-sensitive element-binding transcription factor (CREB), nuclear factor of activated *T*-cells (NFAT) and signal transducer and activator of transcription 3 (STAT3) interact with HBX. The interaction of NF-κB with HBX causes upregulation of the expression of some genes, such as interferon-gamma (IFN-γ), cellular myelocytomatosis (c-Myc), TANK-binding kinase-1 (TBK1), and interleukin-6 (IL-6) (Park et al., 2005; Su et al., 2001; Kim et al., 2010; Lee et al., 1998). As a result, HBX-induced NF-κB activation plays a role in HBV-associated liver diseases and the development of HCC.

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor belonging to the helix-loop-helix, Per-Arnt-Sim (PAS) protein family. It mediates biological responses to a variety of xenobiotics, such as benzo(a)pyrene, tetrachlorodibenzo-p-dioxin (TCDD), and 3-methylcholanthrene (3MC) (Nebert et al., 2004). AHR regulates the expression of many genes, such as cytochrome P4501A1, which is involved in the metabolism of xenobiotics, through binding consensus xenobiotic response elements (XREs) in the promoter regions of those genes (Ye et al., 2019). Although AHR is implicated primarily in xenobiotic homeostasis, recent studies have shown that it may interact with other signalling pathways involved in many other physiological processes, such as immune regulation and cell cycle regulation (Wright et al., 2017). NF-κB is a key component in regulating AHR expression and inducing AHR-dependent gene expression in immune cells, demonstrating the interaction of AHR and NF-κB signalling (Vogel et al., 2014). In addition, the AHR protein is part of a cytoplasmic complex that also includes two chaperone proteins, HSP90 and p23, as well as the XAP2 protein (HBV X-associated protein 2) or AIP (AHR Interacting Protein) (Flaveny et al., 2009). Thus, compelling evidence indicates the separate interaction between HBX- NF-κB, NF-κB -AHR, and AHR-HBX.

It is known that the AHR/P450 pathway plays a crucial role in maintaining physiological homeostasis. It has been shown that inflammatory stimuli such as LPS induce the expression of AHR in human dendritic cells associated with an AHR-dependent increase of CYP1A1 (cytochrome P4501A1). Further analyses confirmed that LPS-mediated induction of AHR is NF-κB dependent (Vogel et al., 2020). CYP1A1 metabolizes xenobiotics, including procarcinogens, clinical drugs, environmental pollutants, and essential endogenous substances. Thus, alterations of CYP1A1 activity affect the metabolic effect of both endogenous and exogenous substances, leading to some physiological or pathological changes in humans, such as carcinogenesis.

HBX has been shown to interact with various transcription factors at many host target genes and activate transcription (Feng et al., 2016; Zhang et al., 2012). Thus, identifying the natural effectors of HBX and the pathways that contribute to developing HCC can help advance effective treatments for patients with HCC. Based on this information, we hypothesize that the HBX protein increases the risk of cancer formation by interacting with other proteins involved in cancer and inflammation, such as NF-κB, AHR, and CYP1A1. In this study, we investigated direct and indirect interactions of HBX with NF-κB, AHR, and CYP1A1 proteins to test this hypothesis.

2. Materials and Methods

2.1. Cell culture

HBX-positive HepG2 cells were transfected with the eukaryotic expression vector pCDNA3-HBX (Satiroglu-Tufan et al., 2010). Both HBX-positive and HBX-negative HepG2 cells were cultured in type-1 rat tail collagen (SERVA Electrophoresis GmbH, Germany)-coated tissue culture dishes or plates in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 0.1 mM minimum essential medium (MEM) non-essential amino acids, 1 mM sodium pyruvate and 100 U/0.1 mg penicillin/streptomycin. The cells were incubated in a humidified atmosphere with 5 % CO_2 at 37 °C and sub-cultured twice a week.

2.2. Plasmid preparation

pCMV6-AC-GFP-CYP1A1 (RG205760) and pCMV6-AC-GFP-RelA (RG226840) plasmids were purchased from OriGene (Rockville, MD, USA). Transformation of plasmids into DH5α competent cells was carried out, and glycerol stocks were prepared. These plasmids were purified using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. pGEM-T easy vector system

Total RNA was extracted from HBX-positive HepG2 cells using the Qiagen RNeasy Plus Universal Kit. cDNA synthesis was performed using the Easy Script Plus cDNA Synthesis kit according to the manufacturer's instructions. The pGEM-T and pGEM-T Easy vectors are linearized vectors. Semi-quantitative two-step RT-PCR was performed by using gene-specific primers. The sequences of the primers for NF-κB (RelA) were 5′-AAA CGC GAT CGC GAC ATG GTG GTC GGC TTC GC-3 (forward primer) and 5′-AAA CGT TTA AAC CTG ACT GTA CCC CCA GAG ACC TCA T-3 (reverse primer), and those of the primers for AHR were 5′-AAA CGC GAT CGC ATG AAC AGC AGC AGC GCC AAC ATC ACC-3′ (forward primer) and 5′- AAAC GTTTAAAC GAT ACA TCG ACA CGG CCC CAG CAT AAC AT −3′ (reverse primer), and those of the primers for CYP1A1 were 5′- AAA CGC GAT CGC ATG CTT TTC CCA ATC TCC ATG TCG GCC-3′ (forward primer) and 5′- AAA CGC GAT CGC CTA AGA GCG CAG CTG CAT TTG GAA GTG CTC-3′ (reverse primer). Following purification of the PCR amplicons using the Wizard S.V. Gel and PCR Clean-Up System (Promega, USA), we cloned the amplicons into the pGEM-T Easy vector (Promega, USA) following the manufacturer's instructions. Then, ligation was performed using the One-Shot TOP10 Competent Cells Kit; plasmid isolation was performed as described above using the PureLink Quick Plasmid Miniprep Kit.

2.4. Mammalian two-hybrid assays

A Checkmate Mammalian Two-Hybrid System (Promega) was used to detect protein–protein interactions between AHR, NF-κB, and CYP1A1. Three genes encoding the AHR, NF-κB and CYP1A1 proteins, which we thought may interact, were cloned into pBIND and pACT vectors containing the DNA binding domain of GAL4 and the activation domain of VP16, respectively, to form fusion proteins. pBIND-Id and pACT-MyoD vectors encoding GAL4:Id and VP16:MyoD fusion proteins were used as control vectors. In addition, the pGL4.71 plasmid, which lacks the binding site, was used as a negative expression control.

The AHR, NF-κB, and CYP1A1 DNA products were generated by inserting them into pBIND and pACT vectors digested with *Sgf*I and *Pme*I. Then, the transformation was performed using the One-Shot TOP10 Competent Cells Kit; plasmid isolation was performed according to the manufacturer's instructions using the PureLink Quick Plasmid Miniprep Kit.

2.5. Luciferase assay

HepG2-HBX cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and allowed to adhere overnight. Then, they were transfected with Fugene HD reagent (Promega) using the pGL4.31(luc2P/Gal4UAS/Hygro) plasmid containing the Renilla gene for luciferase normalization. After 24 h, the cellular lysate was assayed for luciferase activity using the Dual-Glo Luciferase assay system (Promega). Luminescence was measured using a Synergy HTX luminometer (BioTek). Luciferase activity was normalized to Renilla activity.

2.6. Generation of CYP1A1- and NF-κB-overexpressing HepG2 and HepG2-HBX cells

HBX-positive and HBX-negative HepG2 cells were transiently transfected with pCMV6-AC-GFP-CYP1A1 and pCMV6-AC-GFP-RelA plasmids using Lipofectamine LTX & Plus reagent (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was determined by fluorescence microscopy (Olympus BX53). HepG2-CYP1A1, HepG2-RelA, HepG2-HBX-CYP1A1, and HepG2-HBX-RelA mRNA expression was confirmed by semi-quantitative RT-PCR.

2.7. RNAi-mediated silencing of CYP1A1 in CYP1A1-transfected HepG2 and HepG2-HBX cells

Transfection of cells with siRNA was performed using Turbofectin 8.0 solution (OriGene) following the supplier's instructions using CYP1A1 Human siRNA Oligo Duplex from OriGene (I.D.s: CYP1A1: 1543, CAT: SR301093). After siRNA transfection, RNA was isolated, and the expression level of CYP1A1 mRNA was determined by semi-quantitative RT-PCR.

2.8. RNA isolation and RT-PCR

Total RNA was extracted from cells using a Qiagen RNeasy Plus Universal Kit. cDNA synthesis was performed using the Easy Script Plus cDNA Synthesis Kit (ABM, Canada) according to the manufacturer's instructions. Semi-quantitative two-step RT-PCR was performed by using gene-specific primers. Specific primers for CYP1A1 (forward primer, 5′- AAG TCA GCT GGG TTT CCA GA −3′; reverse primer, 5′- TCT GGA AAC CCA GCT GAC TT −3′) and NF-κB (forward primer, 5′- AGC AGC GTG GGG ACT ACG AC −3′; reverse primer, 5′- AGG CTG GGG TCT GCG TAG GG −3′) were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The PCR products were analysed by electrophoresis on 1.5 % agarose gels containing ethidium bromide. mRNA levels were determined by measuring the RT-PCR product's band intensity on each agarose gel and are reported relative to GAPDH expression.

2.9. Cell survival assay

The effect of CYP1A1 and RelA transfection on cell viability was determined using the WST-1 (Boster Biological Technology, USA) assay according to the manufacturer's instructions.

2.10. Enzyme assay

HBX-positive and HBX-negative HepG2 cells transiently transfected with CYP1A1 were seeded at a density of 2×10^5 cells/well in a 6-well plate and allowed to adhere overnight. The cells were then scraped from the culture dishes in lysis buffer (0.1 M phosphate buffer Tris-HCl (pH 7.8), 0.2 % Triton X 100, 2 mM EDTA, 0.5 mM PMFS, 0.3 mM ε-ACA, and 1 mM DTT) and homogenized mechanically by sonication. The total cellular protein concentration was determined by the BCA assay using BSA as the standard. CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) activity was assayed as described by Sen and Arinc (Sen and Arinç, 1998).

2.11. Gel electrophoresis and western blotting

SDS-PAGE and Western blotting were performed as described previously (Sen and Arinç, 1998). The proteins were detected using Pierce ECL Western Blotting Substrate (Pierce, Rockford, IL, USA). The protein bands were visualized and imaged using Gel Quant Image Analysis Software on a DNR Light BIS Pro Image Analysis System (DNR Bio-Imaging Systems ltd., Jerusalem, Israel). The protein bands were quantified using Image Studio Lite Ver 5.2 software.

2.12. Co-immunoprecipitation (Co-IP) with CYP1A1

Co-IP of CYP1A1 in HBX-positive HepG2 cells was performed using a Pierce Co-Immunoprecipitation Kit following the manufacturer's instructions. Briefly, the CYP1A1 antibody was cleared of impurities using a Pierce Antibody Clean-Up Kit, and purified CYP1A1 was coupled to AminoLink Plus coupling resin. Lysates of HepG2-HBX cells transiently transfected with CYP1A1 were cleared using Control Agarose Resin. Purified HepG2-HBX-CYP1A1 cell lysate was incubated with CYP1A1-conjugated agarose resin overnight at 4 °C. Upon elution, the proteins were separated on SDS-PAGE gels as described above.

2.13. Statistical analysis

Statistical analyses were carried out using the Minitab 13 statistical software package (Minitab, Inc., State College, PA, USA). All outcomes are expressed as the means and standard deviation of the mean (SD). Comparisons between groups were carried out using Student's *t*-test, and $p < 0.05$ was selected as the level required for statistical significance.

3. Results

3.1. Interaction of AHR/NF-κB/CYP1A1 with HBX in HepG2 cells

This study aimed to elucidate the interaction of AHR, NF-κB, and CYP1A1 with HBX, which may underlie the carcinogenic and inflammatory occurrences of HBV. For this purpose, the AHR, NF-κB, and CYP1A1 genes were first cloned into the pGEM-T Easy vector. Then, the resulting plasmid and PCR products of the NF-κB, AHR, and CYP1A1 genes were cloned into acceptor plasmids of the mammalian two-hybrid system, which is a powerful system used to detect interactions *in vivo*. In the mammalian two-hybrid system, a pBIND vector containing a GAL4 DNA binding domain and a pACT vector, which activates HSV VP16 transcription and contains an SV40 promoter domain expressing luciferase, are applied. AHR, NF-κB, and CYP1A1 were cloned into either the pACT or pBIND acceptor vector, and then luciferase activity was measured using a dual-luciferase reporter assay system. The pGL4.31 (luc2P/Gal4UAS/Hygro) vector was designed as a reporter of transcriptional activation of the firefly luciferase reporter gene and was used to assess the interaction between proteins luciferase activity. A pGL4.73 (hRluc/SV40) vector containing the hRluc reporter gene and an SV40 promoter lacking the Gal4 DNA binding site was used as a negative control vector. In addition, pBIND-Id and pACT-MyoD control vectors encoding GAL4:Id and VP16:MyoD fusion proteins, respectively, were used as positive control vectors.

The positive control reaction resulted in firefly luciferase activity levels well above the background levels, indicating successful transfection. Transfection of pACT-HBX and pBIND-AHR along with the reporter pGL4.31 yielded 7.34-fold higher luciferase activity than co-transfection of pACT-HBX and pBIND-AHR along with reporter pGL4.71, confirming successful transfections and expression. Co-transfection of pACT-AHR with pBIND-HBX along with the reporter pGL4.31 into HepG2 cells resulted in a 3.23-fold increase in luciferase

activity compared to that observed upon transfection with pACT-HBX and pBIND-AHR along with the reporter pGL4.31. Additionally, co-transfection of pACT-HBX + pBIND-NF-κB + pGL4.31 resulted in a 5.62-fold increase in luciferase activity compared to that observed following co-transfection of pACT-HBX + pBIND- NF-κB + pGL4.31. This result showed that the interactions yielded higher luciferase activity if HBX was cloned into the pBIND acceptor plasmid. Comparatively, low luciferase activity was observed when AHR-BIND + NF-κB-ACT + pGL4.31 or AHR-ACT + NF-κB-BIND + pGL4.31 were transiently transfected into HepG2 cells. In addition, the interaction between AHR and NF-κB was not affected by any acceptor plasmid loaded with AHR or NF-κB (Fig. 1). Neither pACT-HBX and pBIND-CYP1A1 nor pACT-CYP1A1 and pBIND-HBX, as well as pACT- NF-κB and pBIND-CYP1A1 nor pACT-CYP1A1 and pBIND- NF-κB along with reporter pGL4.31, yielded no luciferase activity. It clearly states that neither HBX nor NF-κB does not interact with the CYP1A1 directly. The results clearly demonstrated a direct interaction between AHR or NF-κB with HBX and between AHR and NF-κB in hepatocellular carcinoma cells.

3.2. HBX induced NF-κB signalling in HepG2 cells

To determine whether HBX can affect the expression of NF-κB, we further studied the expression of the NF-κB gene in HBX-negative and HBX-positive HepG2 cells. As shown in Fig. 2, the NF-κB mRNA expression of HepG2 and HepG2-HBX cells was determined by RT-PCR. The results indicated that the expression of NF-κB was up-regulated in HepG2-HBX cells compared to HepG2 cells at the transcriptional level. In addition, the significant increase in NF-κB expression in the HepG2-NF-κB and HepG2-HBX-NF-κB cell lines after transfection indicated successful transfection.

3.3. HBX regulates the expression of CYP1A1 but does not interact with it

After demonstrating that AHR and NF-κB interact with HBX in HCC cells, the pCMV6-AC-GFP-CYP1A1 vector was transiently transfected into HepG2-pcDNA3 and HepG2-pcDNA3-HBX cells to elucidate the underlying mechanism of the carcinogenic response (Fig. 3A). Fig. 3B depicts the levels of CYP1A1 mRNA in each cell line, as determined by semi-quantitative RT-PCR. The expression of CYP1A1 was significantly higher in HepG2-HBX cells than in HepG2 cells. Moreover, transfection of CYP1A1 resulted in further increases in CYP1A1 expression at the mRNA level.

To determine whether the inhibition of CYP1A1 expression could suppress the growth of HepG2-HBX cells, the effects of RNAi-mediated CYP1A1 gene silencing were analysed in HepG2-HBX cells. First, RNAi-mediated CYP1A1 silencing was confirmed in HepG2 cells by measuring CYP1A1 mRNA expression in HepG2-HBX, HepG2-HBX-CYP1A1, and HepG2-HBX-CYP1A1-siRNA cells. After CYP1A1 siRNA transfection, CYP1A1 mRNA expression was significantly decreased in HepG2-HBX-CYP1A1-siRNA cells compared to HepG2-HBX-CYP1A1 cells (Fig. 4A).

The effect of either overexpression or silencing of CYP1A1 on HepG2-HBX cell viability was determined by the WST-1 assay. No significant effect was observed on the proliferation of HepG2-HBX-CYP1A1 and

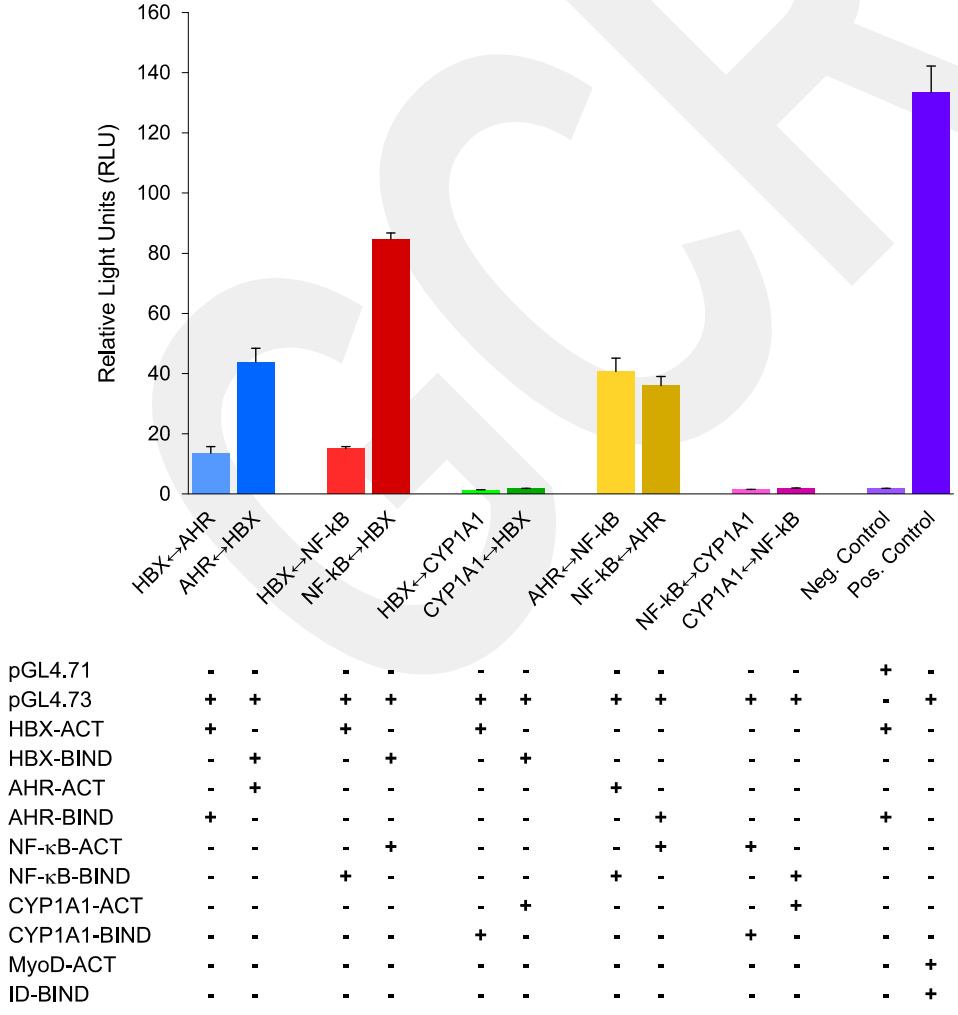

Fig. 1. Expression of luciferase by AHR-HBX, NF-κB-HBX, and AHR-NF-κB in HepG2 cells. Transient transfection of different combinations of AHR, NF-κB, and HBX using acceptor plasmids and pGL4.31 or pGL4.71 luciferase reporter plasmids. pACT-MyoD + pBIND-Id + pGL4.31 was used as a positive control. A pGL4.71 luciferase reporter plasmid lacking a binding region was used as a negative expression control. The results are the mean of two independent experiments with duplicates ± standard deviation.

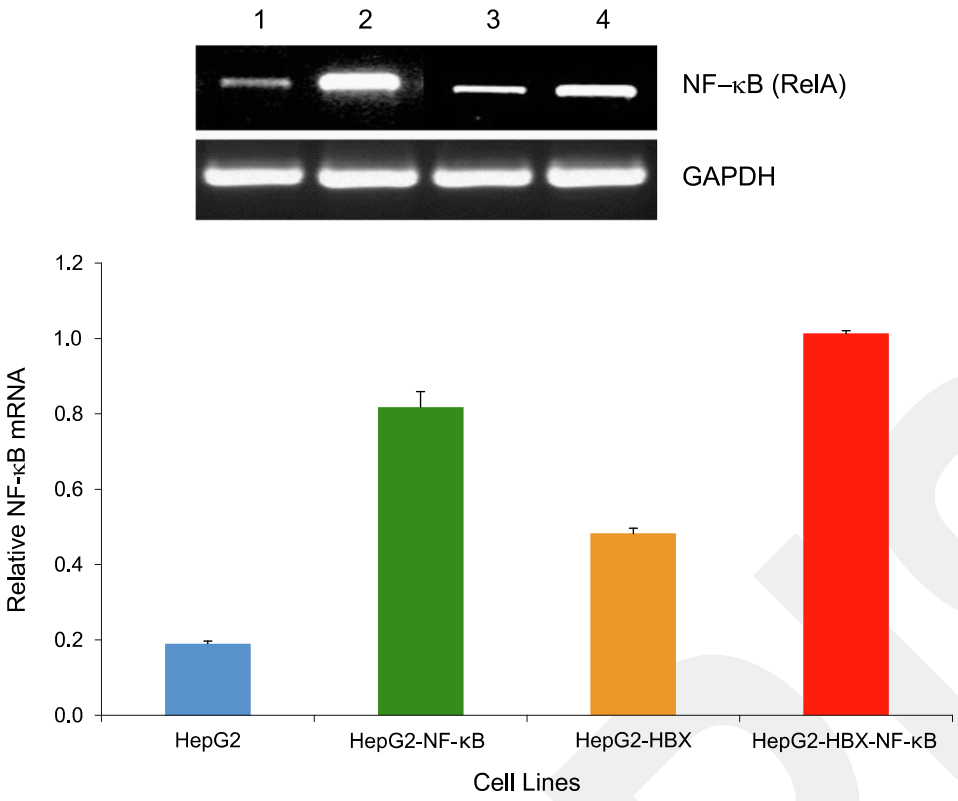

Fig. 2. The mRNA expression levels of NF-κB (RelA) in HepG2 and HepG2-HBX cells. Assays were carried out as described in the Materials and Methods section. (A) Representative agarose gel showing NF-κB (RelA) mRNA expression, as analysed by semi-quantitative RT-PCR. Lane 1, HepG2 cells; lane 2, NF-κB-transfected HepG2 cells; lane 3, HepG2-HBX cells; lane 4, NF-κB-transfected HepG2-HBX cells. The wells contained an equal amount of mRNA. The bar graphs show the fold change in the average intensity of the bands obtained by RT-PCR. The results are the mean of three independent experiments with triplicates ± standard deviation.

HepG2-HBX-CYP1A1-siRNA cells compared to control cells. This indicates that neither CYP1A1 overexpression nor silencing exerted a toxic effect on cells and had no effect on the cell proliferation or inhibition of HBX-mediated HepG2 cells (Fig. 4B).

3.4. CYP1A1 activity (Ethoxyresorufin O-deethylase (EROD)) measurement

To further confirm the expression of CYP1A1 in CYP1A1-transfected or CYP1A1-silenced cells, 7-ethoxy-resorufin-O-deethylase (EROD) activity was measured in HepG2, HepG2-CYP1A1, HepG2-HBX, HepG2-HBX-CYP1A1, and HepG2-HBX-CYP1A1-siRNA cell homogenates. Increased EROD activity was detected in HepG2 and HepG2-HBX cells transiently transfected with CYP1A1, demonstrating successful CYP1A1 transfection. Relatively high EROD activity was observed in HepG2-HBX-CYP1A1 cells compared to HepG2-CYP1A1 cells. HepG2-HBX-CYP1A1 siRNA cells exhibited a decrease in EROD activity compared to that of HepG2-HBX-CYP1A1 cells. This result indicates that CYP1A1 gene expression was suppressed by siRNA (Fig. 5).

3.5. Determination of CYP1A1 protein expression

The protein expression of CYP1A1 in HepG2, HepG2-CYP1A1, HepG2-HBX, and HepG2-HBX-CYP1A1 cells was determined by Western blotting. The results showed no CYP1A1 protein expression in HepG2 cells but an exceptionally higher level of CYP1A1 protein in HepG2-HBX cells. Furthermore, there was a significant increase in CYP1A1 protein expression in both HepG2-CYP1A1 and HepG2-HBX-CYP1A1 cells compared to individual non-transfected cells (Fig. 6).

3.6. Co-immunoprecipitation/SDS-PAGE analysis

Increases in CYP1A1 mRNA expression, activity, and protein expression were observed in HepG2-HBX cells compared to HepG2 cells. For this reason, we carried out a co-immunoprecipitation/SDS-PAGE analysis to further evaluate the interaction between HBX and CYP1A1. HepG2-HBX-CYP1A1 cell lysates were incubated with an anti-CYP1A1 antibody and analysed by SDS-PAGE. The co-immunoprecipitation study showed no clear protein band, indicating no significant association between HBX and CYP1A1 or that this interaction was not detectable by co-immunoprecipitation (Fig. 7).

4. Discussion

Chronic HBV infection is a global public health problem closely associated with the occurrence and development of HCC, which is associated with significant morbidity and mortality (Ringehan et al., 2017). The most critical proposed mechanism for HBV-mediated hepatocarcinogenesis is the expression of a multifunctional viral protein called HBX (Vogel et al., 2020). This protein has drawn significant attention due to its role as a transacting factor in regulating the host cell machinery. Accumulating vital evidence has shown that HBX contributes to developing HCC through HBX-host interactions. HBX interferes with numerous cellular activities by transactivating many genes involved in cell cycle control, induction of proto-oncogenes, cell growth, DNA repair, and apoptosis (Ali et al., 2014; Liu et al., 2016). Despite controversial reports, the activation of NF-κB signalling by HBX has been shown to play an essential role in developing HCC. It is one of the most critical factors for HCC development (Sun and Karin, 2008; Ayub et al., 2013). However, although there have been many studies on the HBX-NF-κB interaction, the mechanism by which HBX induces HCC has not been fully elucidated. Given the role of HBX in HCC, the present research was conducted to identify natural mediators of HBX by investigating the coordination of crosstalk of NF-κB, AHR, and CYP1A1 with HBX in HBV-infected cells.

HBX has previously been shown to interact with various proteins and transcription factors, resulting in the modulation of various proteins (Slagle and Bouchard, 2016). To elucidate the mechanism underlying the development of carcinogenesis following HBV infection, a mammalian two-hybrid system was applied. AHR, NF-κB, CYP1A, and

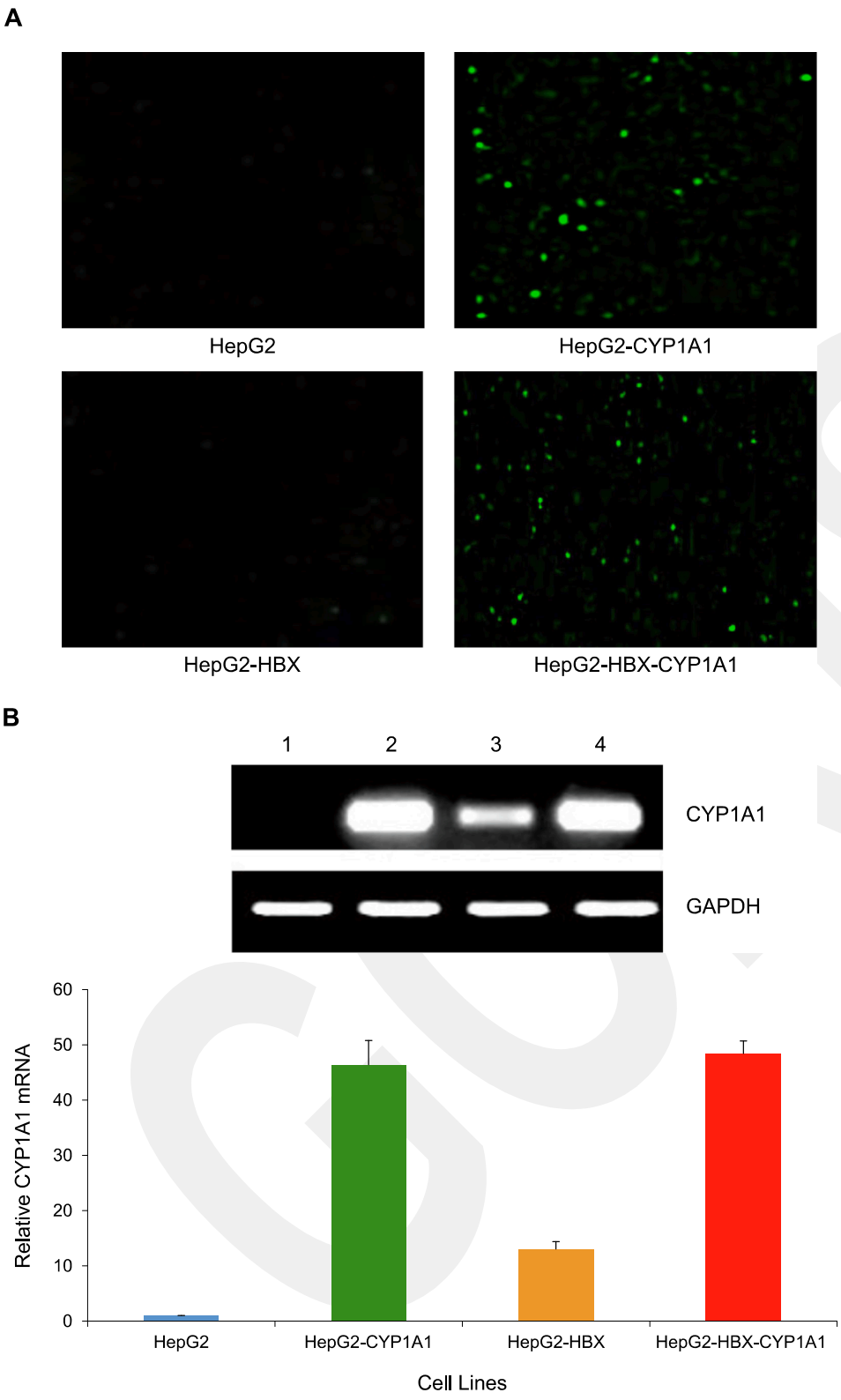

Fig. 3. A. Fluorescence microscopy images using a 10X objective after transient transfection of the pCMV6-AC-GFP-CYP1A1 plasmid into HepG2 and HepG2-HBX cells. **B.** The expression level of CYP1A1 mRNA in HepG2 and HepG2-HBX cells. Assays were carried out as described in the Materials and Methods section. Representative agarose gel showing CYP1A1 mRNA expression, as analysed by semi-quantitative RT-PCR. Lane 1, HepG2 cells; lane 2, CYP1A1-transfected HepG2 cells; lane 3, HepG2-HBX cells; lane 4, CYP1A1-transfected HepG2-HBX cells. The wells contained an equal amount of mRNA. The bar graphs show the fold change in the average intensity of the bands obtained by RT-PCR. The results are the mean of three independent experiments with duplicates ± standard deviation.

HBX genes were cloned into appropriate regions of the recipient plasmids, their interactions in HepG2 cells were detected, and luciferase activity was measured. The results showed that HBX interacted with both NF-κB and AHR and activated downstream signalling in HepG2 cells. It has been reported that NF-κB is expressed in HCC-9204 cells and that its localization in cells is related to the expression of HBX. Additionally, HBX induces the translocation of NF-κB into the nucleus in HCC-9204 cells (Guo et al., 2001). Thus, our results and the available current literature suggest that HBX may dimerize with NF-κB in the cytosol and translocate into the nucleus, inducing the expression of inflammatory genes. It has been shown that HBX induces NF-κB by activating apoptosis, either through forming reactive oxygen species (ROS) or overexpression of c-myc, by an unknown mechanism (Su et al., 2001). The present study further revealed that this activation involves a direct

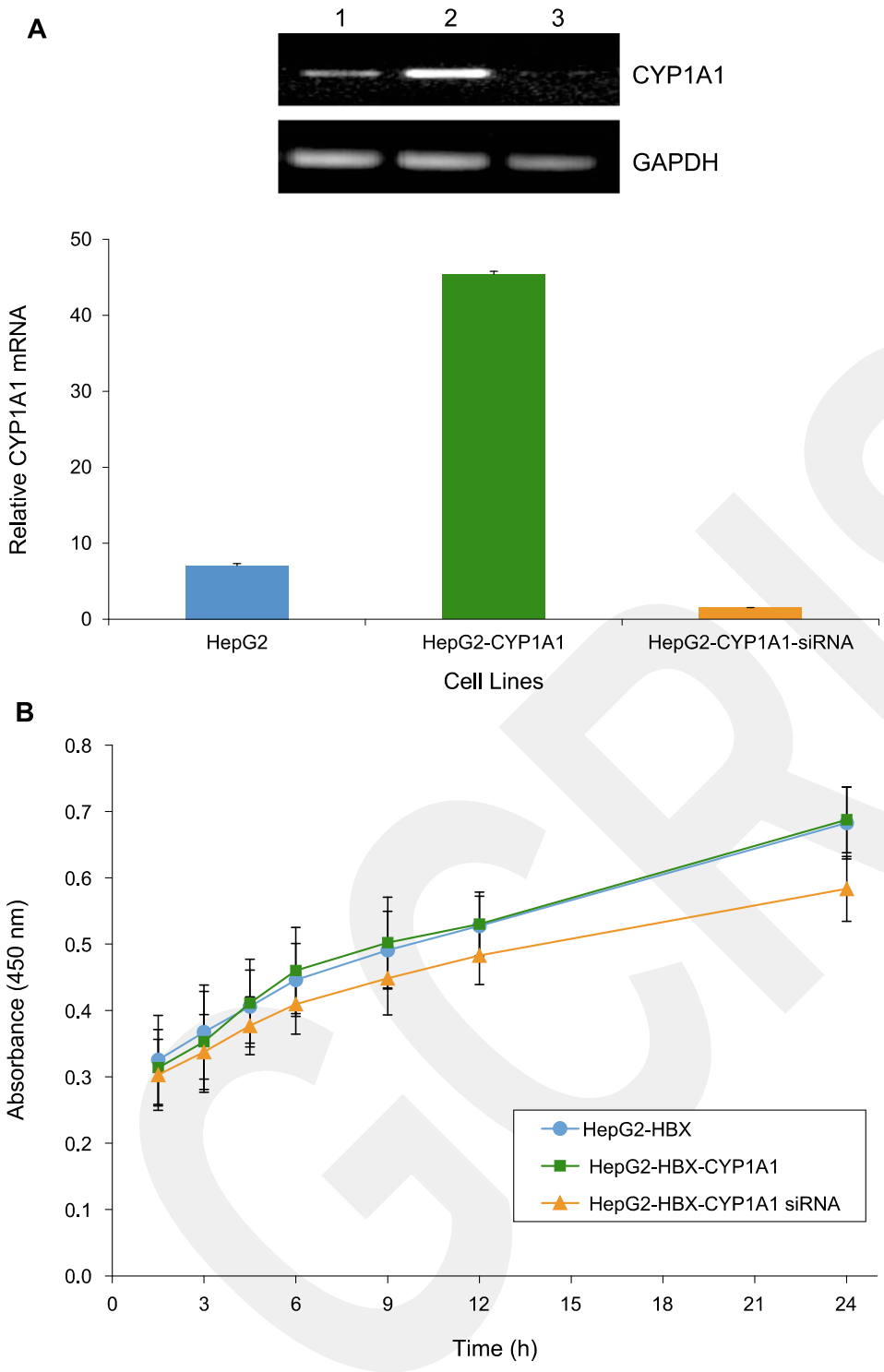

Fig. 4. A. The mRNA expression levels of CYP1A1 after CYP1A1 overexpression or siRNA-mediated CYP1A1 silencing in HepG2-HBX cells. Assays were carried out as described in the Materials and Methods section. Representative agarose gel showing CYP1A1 mRNA expression, as analysed by semi-quantitative RT-PCR. Lane 1, HepG2-HBX; lane 2, HepG2-HBX + CYP1A1; lane 3, HepG2-HBX + CYP1A1 siRNA. The bar graphs show the fold change in the average intensity of the bands obtained by RT-PCR. The results are presented as the mean of three independent experiments ± standard deviation. **B.** The effect of CYP1A1 overexpression or siRNA-mediated CYP1A1 silencing in HepG2-HBX cells on cell growth. Growth curves for HepG2-HBX, HepG2-HBX-CYP1A1, and HepG2-HBX-CYP1A1-siRNA cells cultured in the complete medium comprising 10 % FBS. The curves were generated from average data from two independent experiments, each of which was performed in triplicate.

protein–protein interaction between HBX and NF-κB. Therefore, activation of NF-κB signalling by HBX via protein–protein interactions plays a vital role in inflammation and the development of HCC, as shown in other studies (Sun and Karin, 2008; Ai et al., 2019; Liang et al., 2019; Chen and Yang, 2008; Liu et al., 2010; Liu et al., 2014).

AHR is a ligand-activated transcription factor that can be switched on by a broad spectrum of compounds, including exogenous molecules such as xenobiotics and natural compounds found in foods, and molecules formed by endogenous metabolisms such as arachidonic acid metabolites and kynurenine pathway metabolites (Larigot et al., 2018). After ligand binding, AHR migrates to the nucleus, forms a heterodimer complex with ARNT, and regulates a family of detoxification enzymes, especially CYP1A1 and a number of target genes (Denison and Nagy, 2003; Zhao et al., 2019). Since its discovery in the 1980s, AHR has received significant attention in the field of toxicology because it mediates the impacts of many carcinogens (Esser et al., 2015). AHR is a crucial partner in many regulatory processes affecting cell proliferation and apoptosis, the immune system, chemical carcinogenesis, tumour promotion, and metabolic diseases (Murray et al., 2014; Ma et al., 2009). Thus, for the first time, we studied the interactions between AHR and HBX to investigate the possible role of AHR signalling in HBV-induced hepatocellular carcinogenesis. A clear physical interaction

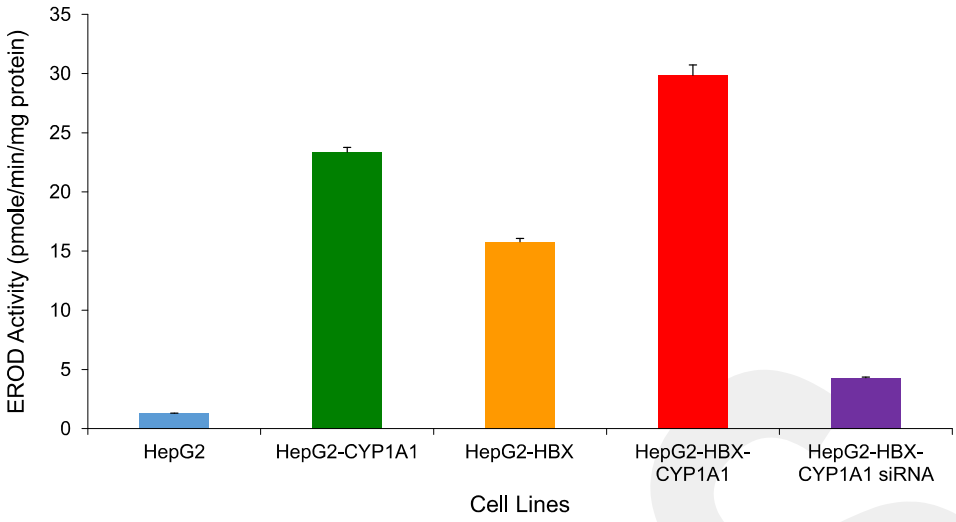

Fig. 5. The activity of CYP1A1 overexpression or siRNA-mediated CYP1A1 silencing in HepG2 and HepG2-HBX cells. The assay was carried out as described in the Materials and Methods section. The bar graphs show activity as determined by the EROD assay. The results demonstrate the average of two independent experiments performed in triplicate.

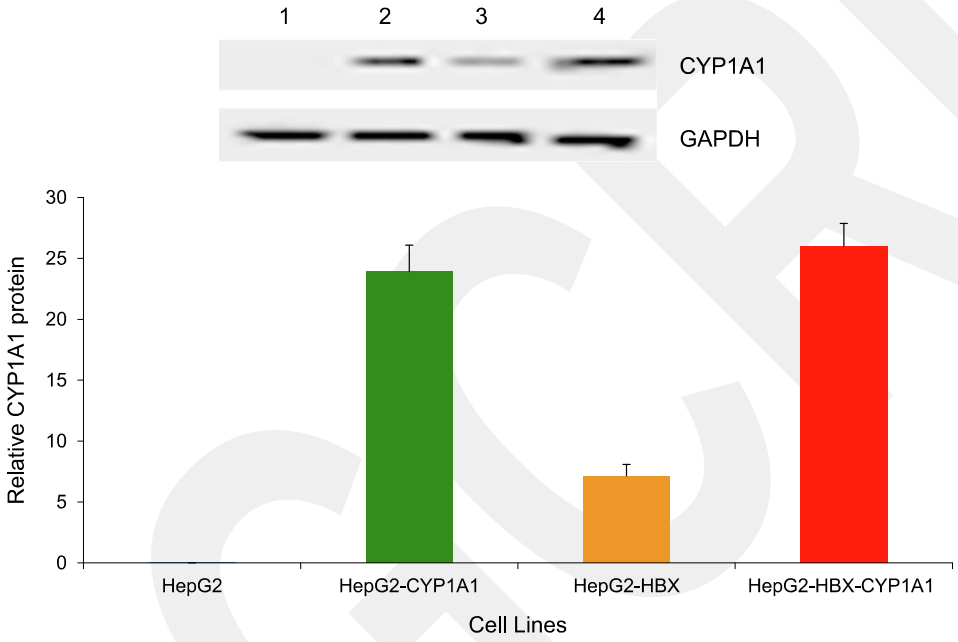

Fig. 6. The protein expression levels of CYP1A1 in HepG2 and HepG2-HBX cells. Lane 1, HepG2 cells; lane 2, CYP1A1-transfected HepG2 cells; lane 3, HepG2-HBX cells; lane 4, CYP1A1-transfected HepG2-HBX cells. The wells contained an equal amount of protein, i.e., 100 μg. Transfection and Western blot analysis were carried out as described in the Materials and Methods section. Proteins were detected using a chemiluminescent substrate for 3 min, and bands were visualized and recorded using a DNR Light BIS Pro Image Analysis System. The bar graphs show the mean intensity of the bands obtained by Western blot analysis. The results are the mean of three independent experiments with duplicates ± standard deviation.

between AHR and HBX was demonstrated in HepG2 cells, indicating the induction of AHR battery n HBV-infected host machinery. However, though highly significant, this interaction is not as strong as the HBX-NF-κB interaction. It is reasonable to suggest that HBX has a higher affinity for NF-κB than AHR and would interact first with NF-κB in HepG2 cells. Therefore, the HBX-AHR interaction may regulate the expression of a range of genes mediated by canonical AHR signalling, leading to increased toxicity of exogenous chemical carcinogens and, in turn, may have a role in the development of hepatocellular cancer following HBV infection.

Additionally, the mammalian two-hybrid analysis showed that AHR and NF-κB cooperatively interacted in hepatocellular carcinoma cells. It is known that the NF-κB and AHR interaction leads to co-transactivation of the c-myc promoter in breast cancer cells and thus may contribute to the malignant proliferation of breast epithelial cells (Kim et al., 2000). It has been shown that the AHR-NF-κB dimer can bind to DNA response elements, including the XRE and NF-κB binding sites, suggesting that target genes of both the AHR and NF-κB pathways are activated. The interaction characteristics appear to depend on the nature of NF-κB subunits and other physiological partners in the cells (Vogel et al., 2005; Vogel et al., 2011; Øvrevik et al., 2014). Thus, co-transactivation of AHR/NFκB crosstalk with HBX might be an essential factor for developing HBV-induced HCC.

It is accepted that AHR has crucial roles in the development, progression, and metastasis of cancer, particularly in the placenta, liver, and lung (Moretti et al., 2020; Natividad et al., 2018). However, the molecular mechanisms regulating AHR expression in most diseases are largely unknown. Therefore, after demonstrating that AHR and HBX interact in HepG2 cells, we investigated the interaction between HBX and CYP1A1 to elucidate the mechanism of AHR-regulated gene expression and the underlying mechanism of the carcinogenic response in HCC. It is well known that CYP1A1 plays a crucial role in cancer metabolism. Significant increases in CYP1A1 activity and protein and mRNA expression were observed in HepG2-HBX cells compared to

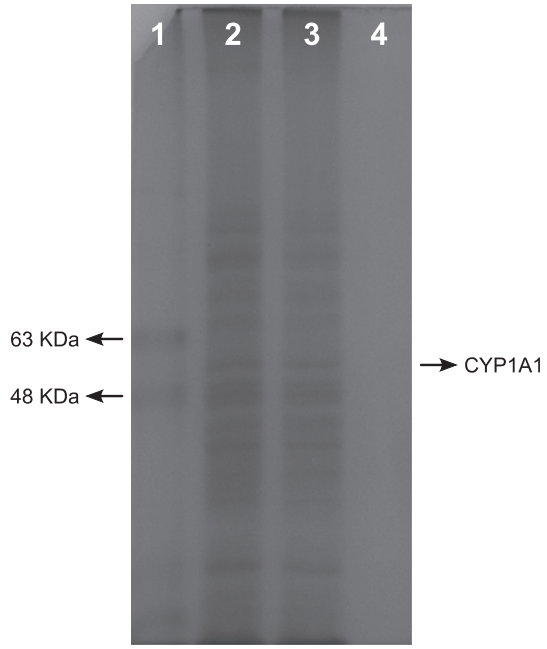

Fig. 7. Co-immunoprecipitation of HBX and CYP1A1. Co-immunoprecipitation/SDS-PAGE analysis was performed to assess the specific association between HBX and CYP1A1. The assay was carried out as described in the Materials and Methods section. Lane 1, marker; lane 2, HepG2-HBX-CYP1A1 cell lysate; lane 3, CYP1A1 immunoprecipitated with an antibody against HBX; lane 4, proteins eluted in the immunoprecipitate.

HepG2 cells. However, these increases did not confirm a physical interaction between CYP1A1 and HBX. Therefore, we performed co-immunoprecipitation to assess the interaction between HBX and CYP1A1 and visualized this interaction by SDS-PAGE analysis, but no physical relationship was detected. Moreover, we investigated whether overexpression or suppression of CYP1A1 influences cell proliferation or cell death. Although it was not significant, a slight increase in cell proliferation was observed after CYP1A1 overexpression, which was suppressed by CYP1A1-siRNA in HepG2-HBx cells. The lack of a significant effect of CYP1A1 on cell proliferation or cell death might be explained by the absence of procarcinogens to be converted into toxic metabolites that affect cell growth in the culture medium. These results suggest that HBX up-regulates CYP1A1 expression through AHR-dependent signalling cascades in HepG2 cells in the absence of direct physical interaction between HBX and CYP1A1 and may implicate a role in HBX-mediated liver cancer development.

In conclusion, in this study, we showed that AHR/NF-κB and HBX physically interact in HepG2 cells, leading to the activation of canonical or non-canonical NF-κB and AHR signalling pathways. While no interaction was observed between CYP1A1 and HBX, which is downstream of the canonical AHR pathway, it is thought that HBX-AHR interaction-dependent induction of CYP1A1 expression might participate in HBX-mediated development of hepatocarcinoma in liver cells. To our knowledge, this is the first report presenting compelling evidence that HBX coordinates either direct protein–protein interactions or indirect interactions between AHR, NF-κB, and CYP1A1 in HBV-infected liver cells during the development of hepatocellular carcinoma and inflammation. As a result, these interactions may be a therapeutic target for developing novel drugs for HBV-driven hepatocellular carcinogenesis and hepatocellular inflammation.

5. Ethical disclosure

This work does not contain any studies with human participants or animals.

6. Fund***ing

This work was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) [Grant No 111T612]; and Pamukkale University Department of Scientific Research Projects (PAU-BAP) [grant number 2011FBE053 and 2014FBE028].

7. Data statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Gurbet Celik-Turgut: Visualization. **Nazmiye Olmez:** . **Tugba Koc:** Investigation. **Ozden Ozgun-Acar:** Investigation. **Asli Semiz:** Investigation. **Yavuz Dodurga:** . **Naciye Lale Satiroglu-Tufan:** Funding acquisition. **Alaattin Sen:** Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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