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Protocatechuic acid inhibits hepatitis B virus replication by activating ERK1/2 pathway and down-regulating HNF4α and HNF1α *in vitro*

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ABSTRACT

Aims: Protocatechuic acid (PCA) is a phenolic compound found in many antiviral Chinese herbal medicines. HNF4α and HNF1α, the members of hepatocyte nuclear factor (HNF) family, play an important regulatory role in the gene transcription of hepatitis B virus (HBV). Previous studies found that PCA inhibited HBV antigen secretion and HBV DNA replication in HepG2.2.15 cells, but its anti-HBV mechanism has not been fully understood. We aim to illustrate the anti-HBV mechanism of PCA.

Materials and methods: MTT was used to estimate cytotoxicity. The content of HBsAg or HBeAg was detected using an enzyme-linked immunosorbent assay kit. HBV DNA in cell-free culture media was detected by PCR kit. HNF1α and HNF4α mRNA expression was detected by real-time PCR. HNF1α, HNF4α and ERK1/2 protein expression was detected by western blotting and HBV promoter activity was tested by luciferase reporter assay.

Key findings: Our results demonstrated that PCA inhibited the gene transcription and protein translation of HNF1α and HNF4α in Huh7 and HepG2.2.15 cells, as well as the promoter activities of HBV X and preS1 in Huh7 cells transfected with the luciferase reporter plasmid of HBV promoter. Further study suggested that PCA induced the phosphorylation of extracellular-signal-related kinase (ERK) 1/2, and thereby inhibited HNF4α and HNF1α expression in HepG2.2.15 cells to exert its antiviral activity.

Significance: To our knowledge, this study is the first to reveal the anti-HBV mechanism of PCA. Our results demonstrate that PCA inhibits HBV replication by activating ERK1/2 pathway and subsequently down-regulating HNF4α and HNF1α in HepG2.2.15 cells.

1. Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus and causes acute and chronic hepatitis in humans. HBV replication and its gene expression is not only regulated by the virus itself but also governed by a variety of host factors [1]. It has been reported that hepatocyte nuclear factors (HNFs) are able to bind to HBV promoters including *pre*S1 promoter (*pre*S1P), *pre*S2 promoter (*pre*S2P), X promoter (XP) and core promoter (CP), and therefore play an important regulatory role in HBV gene transcription [2–4]. Among HNFs, HNF1 has two isoforms named as HNF1α and HNF1β [2], and HNF1α is an important regulatory factor controlling hepatic glucose and amino acid balance [5], liver development, differentiation and normal structure maintenance [6]. HNF4 has three isoforms named as HNF4α, HNF4β and HNF4γ [7], and HNF4α is considered to be a central regulator of HBV gene replication and expression by binding to and trans-activating the three distinct nuclear receptor response elements located in the enhancer I (Enh I), the enhancer II (Enh II) and the pre-core regions of HBV genome [8–11].

Protocatechuic acid (PCA) is a phenolic compound (Fig. 1) found in many antiviral Chinese herbal medicines, such as *Phyllanthus urinaria*, *Salvia mitiorrhiza* and *Schisandra chinensis*. Our previous studies found that PCA inhibited HBV antigen secretion and HBV DNA replication dose- and time-dependently [12]. To elucidate the underlying anti-HBV mechanism of PCA, we further inspected the effects of PCA on the expression of HNF4α and HNF1α, the activities of HBV promoters and the extracellular-signal-related kinase (ERK) 1/2 pathway in Huh7 and HepG2.2.15 cells in this study.

2. Materials and methods

2.1. Chemicals and reagents

PCA with the purity of 98.5% was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Lamivudine was purchased from Ruode Hengxing Technologies

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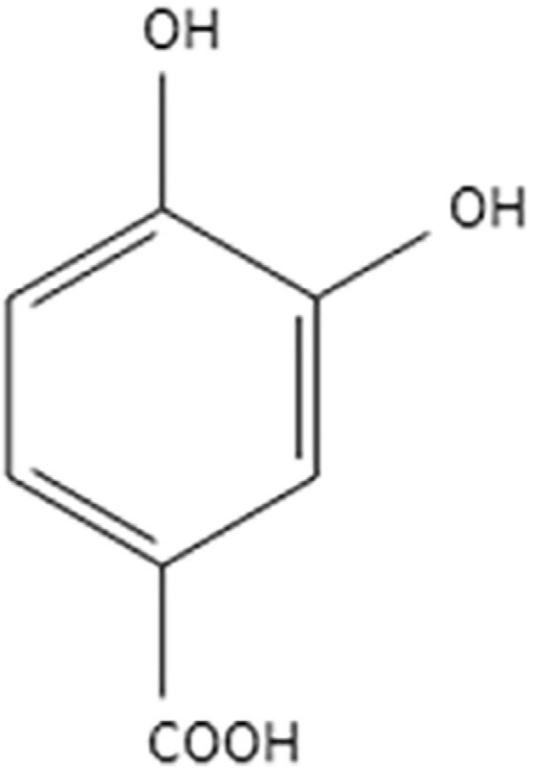

Fig. 1. The chemical structure of PCA.

Co., Ltd. (Beijing, China). U0126 was purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Cell culture

Huh7 cells (obtained from the cell preservation center of Wuhan University, China) were cultured at 37 °C in a humidified 5% CO_2/air atmosphere in Dulbecco's modified Eagle's culture medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 10 units/ml penicillin and 10 μg/ml streptomycin (Jinuo biomedical technology Co., China). HepG2.2.15 cells (obtained from the cell preservation center of Wuhan University, China) were cultured under the same condition as described above, with the addition of 380 μg/ml geneticin G418 (Genview, USA) in the medium to select HepG2.2.15 cell line which can stably express HBV DNA [13].

2.3. Cytotoxicity

The cytotoxicity of PCA was measured by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Briefly, Huh7 or HepG2.2.15 cells were seeded in 96-well culture plates (5×10^3 cells/well) in 100 μl of culture medium and grown for 24 h. After incubation with different concentrations of PCA for 24 h or 48 h, 20 μl of MTT solution (5 mg/ml) was added to each well and continuously incubated in the dark at 37 °C for 4 h. After the supernatant was removed, 200 μl of DMSO was added to each well to dissolve the precipitated formazan. The absorbance was measured with a microplate reader (BIO-RAD, USA) at 570 nm.

2.4. Determination of HBsAg, HBeAg and HBV DNA

After HepG2.2.15 cells were incubated respectively with PCA (0, 0.1, 1 and 10 μg/ml) and lamivudine (2.5 μg/ml, used as the positive control) for 48 h, the cell-free culture medium was collected. Additionally, HepG2.2.15 cells were pre-treated by U0126 (10 μM) for 4 h and therewith treated by PCA (10 μg/ml) for 48 h was used as the negative control. The content of HBsAg or HBeAg was detected using an enzyme-linked immunosorbent assay (ELISA) kit (KHB Shanghai Kehua Bio-Engineering Co., Ltd., China). HBV DNA in the cell-free culture media was detected by real-time PCR kit (DA AN GENE CO., Guangzhou, China) according to the manufacturer's instructions. PCR amplification was executed at 94 °C for 2 min, followed by 40 cycles of denaturing at 94 °C for 20 s, annealing at 55 °C for 20 s, and extending at 72 °C for 20 s.

2.5. Real-time PCR for HNF1α and HNF4α

Huh7 or HepG2.2.15 cells were seeded in 6-well plates (5×10^5 cells/well) and grown for 24 h. After incubation with PCA (0.1, 1 and 10 μg/ml) for 24 h or 48 h, the total RNA was extracted from the cells using TRIzol reagent (Ambion, USA) as per the manufacturer's instructions. Two micrograms of total RNA were reversely transcribed to cDNA using Rever Tra Ace qPCR RT Kit (TOYOBO, JPN). The cDNA was further amplified using SYBR Green PCR Master Mix (Applied Biosystems, USA), and the amplified DNA was quantified using a CFX Connect™ Real-Time System (BIO-RAD, USA). 5′-TCACCCACACTGTG CCCATCTACGA-3′ (forward) and 5′-CAGC GGAACCGCTCATTGCCAA TGG-3′ (reverse) for *β-actin* (used as the control), 5′-GCAGGCTCAAG AAATGCTTC-3′ (forward) and 5′-AGTGCCGAGGGACAATGTAG-3′ (reverse) for *HNF4α*, 5′-TCTACAACTGGTTTGCCAACC-3′ (forward) and 5′-GGCTTCTGTACTCAGCAGGC-3′ (reverse) for *HNF1α* were used as primers in the real-time PCR. Fold increase of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ relative expression method [14].

2.6. Western blotting for HNF1α, HNF4α and ERK1/2

In order to explore the effect of PCA on the protein expression of HNF4α and HNF1α in Huh7 and HepG2.2.15 cells, the cells seeded in 6-well plates (5×10^5 cells/well) were grown for 24 h and then incubated with PCA (0.1, 1 and 10 μg/ml) for 48 h. Afterward, cells were collected and lysed using RIPA (Beyotime, China). Protein (50 μg) was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were respectively incubated with the primary antibody of mouse monoclonal anti-β-actin (Santa Cruz Biotechnology USA), rabbit polyclonal anti-HNF4α and anti-HNF1α (Boster, China) over night.

To study the effect of PCA on ERK1/2 pathway in HepG2.2.15 cells, the cells seeded in 6-well plates (5×10^5 cells/well) were grown for 24 h and then incubated with PCA (10 μg/ml) for 48 h, or pre-incubated with U0126 (specific inhibitor for ERK1/2 phosphorylation) for 4 h [15, 16] and subsequently incubated with PCA (10 μg/ml) for 48 h. Cells were collected and lysed using RIPA. Protein (50 μg) was separated by 10% SDS-PAGE and the expression of total ERK1/2, phosphorylated ERK1/2, HNF4α and HNF1α were analyzed by western blotting. Rabbit monoclonal antiphospho-ERK1/2 (Cell Signal, USA), rabbit polyclonal anti-HNF4α and anti-HNF1α (Boster, China), anti-ERK1/2 (Wanleibio, China) and mouse monoclonal anti-β-actin (Santa Cruz Biotechnology Inc., USA) were used as the primary antibodies.

The immunoblot signals were examined using goat anti-rabbit IgG conjugated with horseradish peroxidase (KPL, USA) and visualized using an enhanced chemiluminescence detection kit (Beyotime, China).

2.7. Luciferase reporter assay for the HBV promoter activity

To study the effect of PCA on HBV promoter activity, the ectogenic plasmids of pGL3-HBV-*preS1*P, pGL3-HBV-*preS2*P, pGL3-HBV-XP, pGL3-HBV-CP and pRL-TK (kindly provided by State Key Laboratory of Virology, Wuhan University, China) were transiently transfected into Huh7 cells respectively by using Sofast™ Transfection Reagent according to the manufacturer's protocol (Sunma, China). pRL-TK reporter plasmid (20 ng) was added to normalize the transfection efficiency.