Original Article

P2Y\textsubscript{11}R regulates cytotoxicity of HBV X protein (HBx) in human normal hepatocytes

Changjiang Lei\textsuperscript{1}\textsuperscript{*}, Ying Fan\textsuperscript{2}\textsuperscript{*}, Xiulan Peng\textsuperscript{3}\textsuperscript{*}, Xiaojun Gong\textsuperscript{1}, Liwei Shao\textsuperscript{1}

Departments of 1General Surgery, 2Cardiology, 3Oncology, The Fifth Hospital of Wuhan, Wuhan 430000, Hubei, China. *Equal contributors.

Received January 25, 2019; Accepted March 19, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Hepatitis B infection is a major global health problem and a primary cause of hepatocellular carcinoma (HCC). While various antiviral treatments have been explored, there is not yet a reliable method for preventing the progression of chronic hepatitis B infection into HCC. Hepatitis B virus X protein (HBx) plays a major role in viral replication, chronic inflammation and the pathogenicity of chronic liver disease. Modulation of purinergic receptors using their specific agonists has become a popular new strategy for modifying disease processes. In the present study, we investigated the involvement of the P2Y\textsubscript{11} receptor using its specific antagonist NF157 in some key aspects of HBx-induced liver disease in human MIHA hepatocytes, including mitochondrial dysfunction due to compromised mitochondrial membrane potential (MMP), oxidative stress resulting from overproduction of reactive oxygen species (ROS) and decreased antioxidant glutathione (GSH), production of proinflammatory cytokines and chemokines such as interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, and chemokine (C-X-C motif) ligand 2 (CXCL2), as well as activation of cellular signaling pathways including the p38/mitogen-activated protein kinase (p38/MAPK) and nuclear factor-κB (NF-κB) pathways. Our findings present a novel new strategy for the treatment and prevention of chronic liver infection and subsequent morbidities induced by HBx via specific antagonism of the P2Y\textsubscript{11} purinergic receptor.

Keywords: Hepatitis B virus (HBV), HBx, hepatocellular carcinoma, purinergic receptors, P2Y\textsubscript{11}, liver disease, NF157

Introduction

The human hepatitis B virus (HBV) is a member of the Orthohepadnavirus genus of the Hepadnaviridae hepatotropic double-stranded DNA virus family [1]. Currently, the estimated global population of hepatitis B patients with chronic liver infection (CHB) is around 400 million. HBV infection is also responsible for acute hepatitis, cirrhosis of the liver, and can lead to more severe diseases such as hepatocellular carcinoma (HCC) [2]. HBx plays a key role in viral replication and pathogenesis and acts as a major regulator of apoptosis in hepatocytes by modulating protein interactions and the cell cycle. Furthermore, HBx has been shown to induce expression of oncogenes and proinflammatory cytokines, as well as cause mitochondrial dysfunction which induces and oxidative stress environment [3, 4]. In the pathogenesis of hepatitis B, HBx has been shown to cause mitochondrial dysfunction by translocating to mitochondria and disrupting mitochondrial membrane potential (MMP) by modulating the mitochondrial permeability transition pore. Additionally, activation of nuclear factor-κB (NF-κB) plays a role in the ability of HBx to alter MMP, wherein HBx-induced activation of NF-κB prevents depolarization of the mitochondrial membrane while inactivation of NF-κB induces depolarization [4]. In terms of oxidative stress, an imbalance in oxidants vs. antioxidants, excess generation of reactive oxygen species (ROS) has been shown to contribute to the development of HCC [5, 6].

Considerable research has been conducted around the involvement of HBx-induced expression of interleukin (IL)-6 in hepatitis B. IL-6 is mainly produced by activated monocytes in response to viral infection and regulates differentiation of pro- and anti-inflammatory cells.
P2Y\textsubscript{11}R regulates cytotoxicity of HBx

Additionally, as the major clearance mechanism of IL-6, impaired liver function results in elevated levels of IL-6 which in turn causes liver inflammation and destruction of immune cells through IL-6-induced proliferation and differentiation of cytotoxic T-cells [7-9]. Additionally, elevated serum levels of IL-6 is considered to be a predictor for future development of HCC [10]. Meanwhile, chemokines such as monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-X-C motif) ligand 2 (CXCL2) have been shown to modulate liver inflammation in patients with CHB by regulating immune cell recruitment [11, 12]. In the context of CHB, HMGB-1 has been shown to shift the balance between regulatory T (Treg) and T helper 17 (Th17) cells to a Th17-dominant state through activation of the toll-like receptor 4 (TLR-4)/IL-6 pathway, thereby further promoting liver damage and inflammation [13]. Under normal conditions, NF-κB is sequestered in the cytoplasm by its inhibitor IκBα and is activated upon degradation of IκBα and phosphorylation of p38 mitogen-activated protein kinase, which is essential for NF-κB-dependent gene expression [14, 15]. Activation of NF-κB not only induces expression of IL-6 but is also a key regulator of inflammation that leads to the development HCC and other cancers [16].

As a member of the P2Y family of G protein-coupled receptors, P2Y\textsubscript{11}R is specifically activated by adenine nucleotides, including ATP and UTP [17]. The role of purinergic receptors as potential targets for regulating inflammation and immune response is only recently beginning to receive major attention, and the possibility of purinergic receptor modulation as an anti-viral therapy has only been suggested within the past year [17, 18]. However, there is still no sufficient treatment for chronic HBV infection, and thus, it is imperative that new therapeutic strategies be sought. In the present study, we explored the involvement of the P2Y\textsubscript{11} purinergic receptor (P2Y\textsubscript{11}R) using its specific antagonist NF157 by transfecting HBx protein (HBx) into human MIHA hepatocytes. To our knowledge, this study is the first to test the specific involvement of P2Y\textsubscript{11}R in the effects of HBx in human hepatocytes.

Materials and methods

Cell culture, transfection, and luciferase assay

Human MIHA hepatocytes were purchased from ATCC, USA. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) at 37°C in a 5% CO\textsubscript{2} incubator. The medium was supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin. HBx gene sequence from a previously reported plasmid was subcloned into the pcDNA3.1 vector. The plasmid was transfected into MIHA hepatocytes using the EndoFree Plasmid Mega Kit (Qiagen, USA). After 24 h, cells were treated with NF157 (Tocris Bioscience, UK) at the concentrations of 25 and 50 μM [19] for 24 h.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated from human MIHA hepatocytes using Qiazol reagent (Qiagen, USA) in accordance with the manufacturer's instructions. cDNA was produced using RT-PCR analysis with an Advantage RT-for-PCR Kit (Takara). Expression of target genes at the mRNA level was determined by real time PCR analysis with 2 μL cDNA aliquots using a SYBR Green Master Mix (Applied Biosystems, USA) on a 7500 Real-time PCR system. The fold change of the target gene was calculated by normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the 2\textsuperscript{ΔΔCt} method. The following primers were used in this study: human P2Y\textsubscript{11}R: 5’-CGT GAG CTG AGC CAA TGTG-3' (sense) and 5’-GGG TGG GAA AGG CGA CTGC-3' (antisense); human IL-6: 5’-AGGGCTCTTCGGGAAATGTA-3' (sense) and 5’-TGCCCAGTGGACAGGTTTC-3' (antisense); human MCP-1: 5’-CATCCACGTGTTGGCTCA-3' (sense) and 5’-GATCATTTGCTGGTGAGT-3' (antisense); human CXCL2: 5’-GACAGAGTCTGCGCTC-3' (sense); 5’-GCCTTGCTTGTTCCAT-3' (antisense); human GAPDH: 5’-ACTGGCGTCTTCACCACCAT-3' (sense); 5’-AAGGCC ATGCCA GTGAGCTT-3' (antisense).

Western blot analysis

After the necessary treatment, proteins were extracted from MIHA hepatocytes using cell lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Protein concentrations were determined using a commercial BCA kit (Thermo Fisher Scientific, USA). Equal aliquots of protein (20 μg/lane) were loaded and separated on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). After blocking with 5% non-fat milk, membranes were sequentially incubated with primary antibod-


Enzyme linked immunosorbent assay (ELISA)

MIHA hepatocytes were transfected with HBx plasmid. At 24 h post-transfection, cells were treated with NF157 at the concentrations of 25 and 50 μM for 24 h. The culture supernatants of MIHA hepatocytes were collected and assayed for IL-6, MCP-1, CXCL2, and HMGB1 at the protein level using commercial ELISA kits in accordance with the manufacturer’s instructions.

Determination of mitochondrial membrane potential (MMP)

After the necessary treatment, MMP in MIHA hepatocytes was assessed using tetramethylrhodamine methyl ester (TMRM) staining (Invitrogen, USA). Briefly, cells were washed with PBS 3 times and probed with 20 nmol/L TMRM for 30 min at 37°C. Cells were then washed 3 times with PBS and fluorescent signals were visualized using a fluorescence microscope (Zeiss, Germany). Quantification of MMP was performed with the Image J software. Firstly, we defined the regions of interest (ROI) in the fluorescent images and counted the average number of cells in the ROI. Then we calculated the integrated density value (IDV) of green fluorescence in ROI. Average MMP=IDV/Cell numbers.

Activity of cytochrome C oxidase

Cytochrome C oxidase activity of human MIHA hepatocytes was assayed to index mitochondrial function. Cell lysates were prepared and sonicated for 10 s on ice. Cell lysates were then reacted with 1% reduced cytochrome C and the OD value at 550 nm was measured at 30°C to index cytochrome C oxidase activity.

Measurement of reactive oxygen species (ROS)

ROS levels in MIHA hepatocytes were examined using the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, cells were washed with PBS 3 times and probed with 5 μM DCFH-DA for 30 min at 37°C. Cells were then washed 3 times with PBS and fluorescent signals were visualized using a fluorescence microscope (Zeiss, Germany). Quantification of ROS was performed with the Image J software. Firstly, we defined the regions of interest (ROI) in the fluorescent images and counted the average number of cells in the ROI. Then we calculated the integrated density value (IDV) of green fluorescence in ROI. Average ROS=IDV/Cell numbers.

Promoter luciferase activity assay for NF-κB

Transcriptional activity of NF-κB was determined by measuring promoter luciferase activity. Briefly, cells were co-transfected with NF-κB promoter and a firefly promoter pRL-TK using Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA). After the indicated treatment, cell lysates were prepared and the luciferase activities of NF-κB and renilla promoters were measured using a dual luciferase reporter assay system (Promega, USA).

Statistical analysis

All the experiments were repeated at least for 3 times. Experimental data are expressed as means ± standard error of measurement (S.E.M). Statistical analysis for comparisons among different group was performed using the analysis of variance (ANOVA) test with the software SPSS (version 17), followed by the Tukey post-hoc test. The criterion for statistical significance was P < 0.05.
Results

HBx increases expression of P2Y_{11}R in hepatocytes

First, we set out to confirm the ability to overexpress HBx in human MIHA hepatocytes. As shown in Figure 1A, transfection of MIHA hepatocytes with HBx-encoding plasmid resulted in successful overexpression of HBx. Next, we assessed whether HBx overexpression leads to increased expression of P2Y_{11}R. As demonstrated by the results of real-time PCR and western blot analyses, transfection with HBx led to significant elevation of P2Y_{11}R expression by approximately 3-fold at the mRNA level and 2.5-fold at the protein level, respectively (P < 0.01) (Figure 1B and 1C). These findings imply that P2Y_{11}R may indeed be involved in HBx-mediated pathologies in chondrocytes.

Inhibition of P2Y_{11}R ameliorates HBx-induced mitochondrial dysfunction and oxidative stress

Next, we investigated the effects of P2Y_{11}R blockade on HBx-induced oxidative stress in MIHA hepatocytes. Here, we first measured generation of ROS induced by transfection with HBx in the presence or absence of 25 and 50 µM P2Y_{11}R antagonist NF157. As shown in Figure 3A, HBx induced roughly 3.33-fold higher generation of ROS, which was rescued by treatment with 25 and 50 µM NF157 to only 2.3- and 1.75-fold basal levels, respectively. Additionally, we investigated the antioxidant properties of NF157 by measuring the effect of HBx transfection of the level of GSH. As shown in Figure 3B, HBx transfection reduced the level of GSH by roughly half, which was restored to approximately 70% and 90% of the basal level, thus indicating a strong antioxidant effect of P2Y_{11}R antagonism by NF157.

Inhibition of P2Y_{11}R reduced HBx-induced expression of proinflammatory cytokines and chemokines

Proinflammatory cytokines and chemokines play major roles in a myriad of diseases, including HBV. To determine the effects of P2Y_{11}R blockade on the expression of some key proinflammatory mediators involved in HBV, we transfected normal human MIHA hepatocytes with HBx-encoding plasmid. At 24 h post-transfection, cells were treated with 25 and 50 µM
P2Y\textsubscript{11}R regulates cytotoxicity of HBx

Figure 2. Inhibition of P2Y\textsubscript{11}R with its specific antagonist NF157 ameliorated HBx-induced mitochondrial dysfunction in normal human MIHA hepatocytes. Normal human MIHA hepatocytes were transfected with HBx-encoding plasmid. At 24 h post-transfection, cells were treated with NF157 at the concentrations of 25 and 50 μM for 24 h. A. Intracellular levels of MMP were determined by TMRM; Scale bar, 50 μm; B. Cytochrome C oxidase activity (*, #, $, P < 0.01 vs. previous column group, n=5-6).

Figure 3. Inhibition of P2Y\textsubscript{11}R with its specific antagonist NF157 ameliorated HBx-induced oxidative stress in normal human MIHA hepatocytes. Normal human MIHA hepatocytes were transfected with HBx-encoding plasmid. At 24 h post-transfection, cells were treated with NF157 at the concentrations of 25 and 50 μM for 24 h. A. Intracellular ROS was determined by the DCFH-DA assay; B. Reduced GSH (*, #, $, P < 0.01 vs. previous column group, n=5-6).

NF157 and analyzed via real-time PCR and ELISA to determine the mRNA and protein expression levels, respectively, of IL-6, MCP-1 and CXCL2. As shown in Figure 4A, transfection with HBx significantly increased expression of IL-6, MCP-1, and CXCL2 by approximately 4.1-, 3.8- and 5.6-fold, respectively, all of which were reduced by NF157 treatment in a dose-dependent manner. Notably, the higher dose of NF157 reduced expression of all three of these inflammatory mediators to less than 2-fold basal levels.

The chemokine HMGB-1 is involved in regulating inflammation and liver injury in chronic hepatitis B. To investigate the involvement of P2Y\textsubscript{11}R in modulating the expression of HMGB-1, we transfected normal human MIHA hepatocytes with HBx-encoding plasmid. At 24 h post-transfection, cells were incubated with 25 or 50 μM NF157 for another 24 h. The results of ELISA analysis in Figure 5 show that HBx increased expression of HMGB-1 by more than 5-fold basal levels, which was reduced by NF157 in a dose-dependent manner. Here, the higher dose of NF157 reduced HMGB-1 expression to less than 2-fold basal levels. These findings demonstrate the potential of P2Y\textsubscript{11}R antagonism by NF157 to rescue the imbalance between Treg and Th17 cells in chronic hepatitis B induced by HBx.

Inhibition of P2Y\textsubscript{11}R reduced HBx-induced activation of NF-κB

Activation of the NF-κB pathway resulting from phosphorylation of p38 protein and IκBα is an attractive target for regulating inflammation in various diseases including hepatitis B. Controlled regulation of NF-κB activation has been
P2Y<sub>11</sub>R regulates cytotoxicity of HBx

mentioned as a potential treatment target for comorbidities of HBV infection, such as hepatocellular injury, liver fibrosis and HCC [19]. To determine the potential of P2Y<sub>11</sub>R blockade to modulate NF-κB activity induced by HBx, normal human MIHA hepatocytes were transfected with HBx-encoding plasmid. At 24 h post-transfection, cells were treated with NF157 at the concentrations of 25 and 50 μM for 24 h. Secretion of HMGB1 was determined by ELISA assay (*, #, $, P < 0.01 vs. previous column group, n=6).

Finally, we explored the effects of NF157 on HBx-induced activation of NF-κB by assessing nuclear translocation of p65 protein and luciferase activity of NF-κB. Normal human MIHA cells were transfected with HBx-encoding plasmid. At 24 h post-transfection, cells were treated with 25 or 50 μM NF157 for 6 h. As demonstrated by the results in Figure 8, HBx increased the level of nuclear p65 to roughly 3.3-fold basal levels, which was reduced to approximately 1.8- and 1.5-fold basal levels by 25 and 50 μM NF157, respectively. Additionally, HBx increased NF-κB luciferase activity to a remarkable approximate 120-fold basal levels. This
P2Y₁₁R regulates cytotoxicity of HBx

HBx-induced increase in NF-κB luciferase activity was reduced by roughly 50% and 75% by 25 and 50 μM NF157, respectively. Thus, blockade of P2Y₁₁R by its specific antagonist NF157 may prevent excessive activation of NF-κB by down-regulating phosphorylation of p38 protein and IκBα.

Discussion

HBx is a multifunctional HBV sequence which is highly conserved in all HBV transcripts which is essential for HBV replication [20]. HBV mainly comprises HBx protein, a 154 amino acid-containing protein with a molecular weight of 16.5-kDa. HBx has been shown to enhance HBV replication and encode the HBx protein to mediate carcinogenesis of HCC [21]. Notably, HBx-knockdown HBV mutant exhibits impaired replication, decreased p300 recruitment, and hypo-acetylation of cccDNA-bound histones [22]. While there has been considerable research on the biological function of HBx, little is known regarding the role of HBx in immune-mediated liver damage associated with HBV infection.

Modulation of purinergic receptors has been explored in various diseases outside of diabetes, including Adenosine 5′-triphosphate (ATP) is a damage-associated molecular pattern molecule (DAMP) and plays an important role in intracellular communication and apoptosis. Release of ATP into the extracellular space induces a signaling cascade by binding to the ubiquitously expressed P2Y receptors including the Gq-11/G protein coupled P2Y₁₁ receptor [23-26]. Notably, purinergic signaling has recently been suggested as a treatment target for HCC [27], however research is still emerging on the roles of the specific members of the P2Y receptor family in CHB infection. In the present study, we explored the potential role of P2Y₁₁R in HBx-mediated HBV infection by transfecting normal human MIHA hepatocytes with HBx-encoding plasmid and then exposing infected cells to treatment with the specific P2Y₁₁R antagonist NF157. Our findings show that P2Y₁₁R is indeed overexpressed in hepatocytes following HBx transfection (Figure 1). This led us to further investigate the effects of P2Y₁₁R blockade...
P2Y_{11}R regulates cytotoxicity of HBx

using NF157 on various characteristics of HBV infection, including mitochondrial dysfunction, oxidative stress, production of cytokines and chemokines, and activation of the NF-κB pathway. Importantly, we found that antagonism of P2Y_{11}R significantly reduced these markers of HBV infection.

Mitochondrial dysfunction and oxidative stress are well-recognized as playing key roles in the inflammatory and immune responses responsible for liver damage [28, 29]. Here, we found that antagonism of P2Y_{11}R significantly ameliorated reduced MMP and oxidant-antioxidant imbalance induced by HBx transfection (Figures 2 and 3). This suggests a potential role of NF157 in preventing liver damage induced by HBx, which is a major risk factor for HCC. Another important factor in the pathogenesis of hepatitis B infection and subsequent liver damage is expression of proinflammatory cytokines and chemokines. Of these, IL-6 overexpression induced by HBx has been shown to play a major role in HBV-induced liver damage by inhibiting liver regeneration through disrupting the cell cycle [30]. Additionally, recruitment of immune cells to the liver exacerbates the disease state by sustaining inflammation and inflicting further liver damage. Our findings indicate that antagonism of P2Y_{11}R by its specific inhibitor NF157 significantly downregulated expression of IL-6, as well as two major chemokines, MCP-1 and CXCL2 (Figure 4). We also explored the effects of P2Y_{11}R antagonism on expression of HMGB1, which has been shown to drive HCC metastasis by promoting cell migration and invasion. Furthermore, an inverse relationship was demonstrated between expression of HMGB1 in tumor cytoplasm and general prognosis in patients with HCC [30]. Our findings show that antagonism of P2Y_{11}R caused a remarkable decrease in HBx-induced expression of HMGB1, thus implicating this strategy as a potential preventative treatment against HCC tumor metastasis (Figure 5). Activation of the p38 mitogen kinase pathway has been shown to be involved in numerous aspects of HBx-driven disease, including HCC metastasis and invasion, virus replication, and enhanced cell proliferation and survival [31-33]. Here, we found that blockade of P2Y_{11}R significantly rescued HBx-induced phosphorylation of p38, thereby suggesting a potential important role of P2Y_{11}R in these p38-driven processes (Figure 8).
Finally, we investigate the effects of P2Y$_{11}$R antagonism on activation of NF-κB via phosphorylation of IkBα, its inhibitor. NF-κB signaling is one of the most widely studied targets for modulating the inflammatory process in a myriad of diseases. However, it has been shown that NF-κB also plays a vital role in suppressing apoptosis, and thus, complete blockade of NF-κB is not considered as a viable target in HCC [34]. Here, we show that P2Y$_{11}$R antagonism by NF157 rescues phosphorylation of IkBα to near basal levels, and downregulates HBx-induced activation of NF-κB without completely inactivating NF-κB (Figures 7 and 8).

Taken together, our findings indicate the potential of P2Y$_{11}$R blockade using its specific antagonist NF157 to modulate the pro-inflammation, pro-liver injury and pro-HCC carcinogenic processes induced by HBx in human hepatocytes. Our findings potentiate a novel new treatment target in viral infections including CHB. Further study is required to better understand the importance of P2Y$_{11}$R and other purinergic receptors in the processes of viral replication and invasion.

Acknowledgements

This study was supported by the Wuhan City Health Bureau of Medical Research project (No. WX18C02), Hubei Province health and family planning scientific research project (No. WJ20-19M008).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaojun Gong and Liwei Shao, Department of General Surgery, The Fifth Hospital of Wuhan, NO.122, Xian Zheng Street, Hanyang District, Wuhan 430000, Hubei, China. Tel: +86-027-84812419; Fax: +86-027-84812419; E-mail: lztzn3@sina.com (XJG); caill9306@163.com (LWS)

References


P2Y11R regulates cytotoxicity of HBx


