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Effect of HBx on inflammation and mitochondrial oxidative stress in mouse hepatocytes

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Abstract. Hepatitis B virus x protein (HBx) serves an important role in the pathogenesis of the hepatitis B virus infection. Previous studies have reported that the interaction between HBx and hepatocyte mitochondria is an important factor leading to liver cell injury and apoptosis, ultimately inducing the formation of liver cancer. In the present study, a mouse model expressing HBx was constructed using hydrodynamic *in vivo* transfection based on the interaction between HBx and cytochrome *c* oxidase (COX) subunit III. The specific mechanism of HBx-induced oxidative stress in mouse hepatocytes and the subsequent effect on mitochondrial function and inflammatory injury was assessed. The results demonstrated that HBx reduced the activity of COX and the expression of superoxide dismutase and upregulated the expression of malondialdehyde, NF-κB and phospho-AKT, thus increasing oxidative stress. In addition, HBx induced an increase in interleukin (IL)-6, IL-1β and IL-18 expression levels, which created an inflammatory microenvironment in the liver, further promoting hepatocyte inflammatory injury. Therefore, it was proposed that HBx may affect hepatocyte mitochondrial respiration by reducing the activity of cytochrome *c* oxidase, leading to mitochondrial dysfunction and inducing hepatocyte inflammation and injury.

Introduction

Chronic hepatitis B virus (HBV) infection is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). At present, the mechanism by which HBV causes chronic hepatitis and liver cancer remains unclear; however, the hepatitis B virus x (HBx) protein may serve a role in this process (1). HBx is a multifunctional regulatory protein composed of 154 amino acids, with a molecular weight of 17 kDa (1). HBx is involved in a variety of signal transduction pathways affecting cell cycle progression, apoptosis and cancer progression (1). HBx can also affect mitochondria by altering the permeability of mitochondrial membranes, as HBx disrupts oxidative phosphorylation, interferes with the mitochondrial respiratory chain and inhibits ATP synthesis, leading to liver cell damage (2-6). Rahmani *et al* (2) have reported that HBx may be localized in the mitochondria, where it binds to the voltage-dependent anion channel 3 on the outer mitochondrial membrane, thus changing the mitochondrial membrane potential. Lee *et al* (3) have demonstrated that HepG2 cells that stably express HBx, causing the proton transfer to be blocked, induces the mitochondria of hepatocellular carcinoma cells in a sensitive state, thus leading to the formation of reactive oxygen species (ROS) and lipid peroxidation. Excessive ROS production then affects cell proliferation and differentiation, inducing apoptosis and gene mutations, thus promoting the occurrence of HCC. HBx can also induce the opening of the mitochondrial permeability transition pore and swelling of the mitochondrial matrix, mitochondrial membrane potential depolarization-induced release of cytochrome *c* apoptosis-inducing factor and calcium ions into the cytoplasm, and apoptosis by activation of the caspase signaling cascade (4-6). The mitochondrial permeability transition pore and swelling of the mitochondrial matrix induce apoptosis or necrosis, lead to cytoplasmic calcium overload, enhance HBV replication and contribute to liver inflammation (4-6). In addition, HBx can also induce liver disease by activating autophagy, mitochondria-dependent apoptosis pathways, mitochondrial division and fusion damage (7,8).

During the development of chronic hepatitis and cirrhosis, the mitochondrial respiratory chain is damaged and its function is significantly decreased (9). Cytochrome *c* oxidase (COX), composed of 13 subunits including COXI, COXII and COXIII, which are encoded by mitochondrial DNA, serves a key role in oxidative phosphorylation (10,11). The lack of functional order in COXIII hinders proton transfer and the accumulation of excess electrons and oxygen molecules leads to decreased ATP synthesis and mass ROS production (10,11) during chronic hepatitis and cirrhosis. It has been reported that intracellular HBx is primarily localized in mitochondria (12,13)

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and that HBx binds to COXIII, thereby upregulating ROS production (14-16).

Alterations in the levels of ROS in the mitochondria are common factors in the pathogenesis of inflammatory diseases and tumors (17). In acute liver inflammation, ROS primarily induces mitochondrial dysfunction through intracellular oxidative stress (17). Previous studies have demonstrated that the C-terminus of HBx causes mitochondrial DNA damage, resulting in the increase in ROS levels in liver cells. Accumulation of ROS can also upregulate HBx expression levels (13,18), indicating that mitochondria are the primary targets of ROS.

The NF-κB/AKT signaling pathway is a key inflammatory pathway involved in the development of cancer. Abnormal activation of NF-κB/AKT signaling in liver tissues can inhibit apoptosis and promote liver cell survival, which may lead to the development of liver cancer (19,20). It has been reported that the NF-kB signaling pathway serves a role in ROS-mediated liver injury (21). In addition, previous studies have demonstrated that the PI3K/AKT signaling pathway is involved in HBx-induced liver cancer formation and also serves a role in the regulation of antioxidant genes (22,23).

Inflammatory mediators are the primary signal transducers in the tumor microenvironment and have been demonstrated to be involved in the development and progression of liver cancer. Numerous *in vivo* and *in vitro* studies have confirmed that the levels of inflammatory mediators, such as interleukin (IL)-6, IL-1β, IL-10, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) are often increased in the tissues and sera of patients with liver cancer (24-26). Pro-inflammatory IL-18 is involved in immune response regulation, and excessive IL-18 expression levels can lead to immune system deregulation, causing inflammatory damage to tissues and organs (27). The pro-inflammatory mediators (IL-6, IL-1β, IL-10, TNF-α, TGF-β and IL-18) can promote tumor growth, inhibit apoptosis, induce epithelial-to-mesenchymal transition and ultimately promote the invasion and metastasis of liver cancer.

Increasing attention has drawn to the relationship between malondialdehyde (MDA), superoxide dismutase (SOD) and mitochondria. Several studies on drugs (such as the impairment of mitochondrial function in mice with a low or excessive selenium diet) and the pathogenesis of diseases (for example, liver dysfunction) have been conducted by measuring the expression levels of MDA and SOD and observing the structural changes in mitochondria and demonstrating their association (28,29). Paradies *et al* (30) reported that decreased COX activity of myocardial mitochondria in rats was associated with increased lipid oxidation levels caused by increased MDA activity. Therefore, changes in MDA and SOD levels in the serum may reflect the state of mitochondria and indicate the degree of liver cell damage.

In the present study, a mouse model expressing HBx was established to observe acute hepatocyte injury in mice, and the levels of IL-6, IL-1β, IL-18, MDA and SOD and COX activity were determined. Changes in ROS levels, NF-κB, AKT and phosphorylated (p-) AKT were measured. The mechanism by which HBx affects mitochondrial function in hepatocytes, induces inflammation and damages hepatocytes was explored *in vivo*, providing new insight into the mechanism underlying HBV-related chronic hepatitis and hepatocellular carcinoma.

Materials and methods

Animals. The present study was approved by The Institutional Animal Care and Use Committee of Fujian Medical University (Fujian, China). Mice were euthanized using an intraperitoneal injection of 2% sodium pentobarbital at a dose of 100 mg/kg and death was confirmed by observing the ventilation, complete cardiac arrest and loss of reflexes. A total of 30 male ICR mice, aged 6-8 weeks and weighing 19-22 g, were purchased from Shanghai SLAC Laboratory Animal Technology Co. Ltd. The mice were maintained at 25˚C with a 12:12 h light/dark cycle and were provided with food and water, and access to food and water was arbitrary. The mice were randomly divided into 3 groups (n=10 per group) based on the administered treatment: i) Experimental; ii) null-plasmid control; and iii) blank control (plasmid solvent) groups. The solutions were injected into the caudal vein under high pressure.

Preparation of competent bacteria. *E. coli* DH5α cells from Tiangen Biotech Co., Ltd. were cultured on agar plates at 37˚C in an incubator overnight. A single colony was collected from the plate and the cells were cultured further with agitation at 37˚C and 250 rpm for 12 h. Subsequently, the bacteria were incubated on ice for 30 min followed by centrifugation at 4,000 x g and for 10 min at 4˚C. The supernatant was discarded, and 1 ml precooled 0.1 mol/l $CaCl_2$ was added to the obtained pellet. The mixture was mixed with a pipette.

Plasmid transformation and extraction. pcDNA3.1-HBx plasmid (stored in the laboratory) was added to a 200-μl suspension of susceptible *E. coli* DH5α cells, mixed by shaking gently, placed on ice for 30 min, subjected to heat shock at 42˚C for 2 min, and quickly moved to ice for 3-5 min. The cells were added to 800 μl LB medium and cultured with agitation at 37˚C and 250 rpm for 1 h. Bacterial cells were then streaked onto liquid culture medium using inoculation loops, followed by incubation of the plates at 37˚C for 18-24 h. The transformed bacteria were followed by plasmid extraction using an EndoFree Plasmid Maxi kit (Qiagen, Inc.).

RNA extraction and reverse transcription PCR analysis. Total RNA from mouse liver tissue was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (2 μg) was reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The conditions for the RT of RNA into DNA were as follows: 60 min at 42˚C, 5 min at 70˚C, and storage at 4˚C. The primer sequences of each gene are listed in Table I. The thermo cycling conditions of PCR were as follows: Pre-denaturation at 95˚C for 5 min; followed by 30 cycles of denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec and 72˚C for 1 min; and a final extension step at 72˚ for 7 min. PCR was performed using a Taq PCR Master mix (Thermo Fisher Scientific, Inc.). A total of 10 μl PCR product with 5 μl marker (cat. no. MD110; Tiangen Biotech Co., Ltd.) was loaded onto a 2% agarose gel containing 1.5 μl gold view (Beijing Solarbio Science & Technology Co., Ltd.). Gel electrophoresis was performed at 100 V for 15-30 min. The results of the electrophoresis were scanned using a UVP scanner with Grab-IT

Table I. Primer sequences.

Gene	Sequences (5'→3')
HBx-flag	
Forward	ATGCAAGCTTATGGCTGCTAGGCTGTACTG
Reverse	TGCGAATTCTTAGGCAGAGGTGAAAAAGTT
β-actin	
Forward	GGCATCGTGATGGACTCCG
Reverse	GCTGGAAGGTGGACAGCGA

HBx, hepatitis B x protein.

(Gel Doc 100; Bio-Rad Laboratories, Inc.) and analyzed by Gelpro32 (Media Cybernetics, Inc.).

Western blot analysis. Liver tissues were lysed in RIPA buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (Beyotime Institute of Biotechnology) and kept on ice for 30 min, followed by centrifugation at 12,000 x g for 15 min at 4˚C. Protein concentration was measured using a Bicinchoninic Acid (BCA) Protein Assay kit (Beyotime Institute of Biotechnology). Total protein samples (60 μg) were loaded onto a 12% gel, resolved using SDS-PAGE, transferred to a nitrocellulose filter membrane and blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min at room temperature. The membranes were incubated overnight at 4˚C with the following primary antibodies: Rabbit anti-human HBx (1:1,000; cat. no. ab39716; Abcam), rabbit anti-mouse NF-κB (1:1,000; cat. no. 8242; Cell Signaling Technology, Inc.), rabbit anti-mouse AKT (1:1,000; cat. no. 4685; Cell Signaling Technology, Inc.), rabbit anti-mouse p-AKT (1:500; cat. no. 4060; Cell Signaling Technology, Inc.) and rabbit anti-mouse GAPDH (1:1,000; cat. no. 2118; Cell Signaling Technology, Inc.). The membrane was then washed three times (10 min each) with TBST and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. ZB-2301; OriGene Technologies, Inc.) for 60 min at room temperature. After washing with TBST, the membrane was incubated with an ECL chemiluminescence kit (1:1; OriGene Technologies, Inc.) for 1 min. A auto-exposure (ChemiDoc MP System; Bio-Rad Laboratories, Inc.) was used to expose the target strip and analyzed by Gelpro32.

Construction and identification of a HBx expression mouse model. Hydrodynamics-based transfection is an *in vivo* gene transfection method based on the principle of making the venous system and hepatic sinus of mice hyperemic and generating high venous pressure, which permits the transfer of plasmids into liver cells by rapidly injecting a large volume of plasmid solution into the tail vein (31,32). The present study used this method to transfect mice with pcDNA3.1-HBx, empty pcDNA3.1 plasmids and plasmid solvent, according to the manufacturer's protocol using a TransIT *In Vivo* Gene Delivery system (Mirus Bio). A total of ~250 μg of plasmid was added to the *in vivo* polymer solution in a 15-ml centrifuge tube that does not contain nucleotide enzymes, and the tube was allowed to stand at room temperature for 5 min. The solution was then injected into the mice via the caudal vein at a constant speed within 4-8 sec. After 24 h, the mice were euthanized and eyeball blood and liver tissues were collected; eyeball blood was stored at -20˚C, and liver tissue was cryopreserved at -70˚C.

Isolation of mitochondria and measurement of COX activity. Mitochondria were isolated using the Mitochondrial Isolation kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Following lysis of ~100 mg of liver tissue, cell debris and nuclei were removed by centrifugation at 700 x g for 10 min at 4˚C, followed by centrifugation at 3,000 x g for 15 min at 4˚C to pellet the mitochondria-enriched fraction and then at 12,000 x g for 5 min at 4˚C to pellet the isolated mitochondria. Protein concentrations were measured using the BCA Assay kit (Beyotime Institute of Biotechnology) and adjusted to 1 μg/μl. COX activity was determined using the Cytochrome *c* Oxidase Assay kit (Genmed Scientifics Inc,) according to the manufacturer's instructions and measured by an ELx800 microplate reader (BioTek Instruments Inc.) at 550 nm.

In situ fluorescence assay for reactive oxygen species measurement. *In situ* fluorescence staining was used to detect the ROS levels in the frozen sections of mouse liver tissue. Frozen sections (10 mm) were stained using a ROS *in situ* fluorescence staining kit (GenMed) and placed at room temperature. Briefly, the section surface was covered with 500 μl pre-cooled cleaning solution, which was then carefully removed. Subsequently, 200 μl staining and diluent dye solutions were carefully added to cover the section surface, and the sections were incubated at 37˚C for 30 min. The sections were treated again with 500 μl of pre-cooled cleaning solution after removal of the other reagents, followed by removal of the pre-cooled cleaning solution. The sections were then transferred to glass slides, covered and observed under a Nikon Eclipse TE 2000-U inverted fluorescence microscope at x100 magnification (Nikon Corporation); enhanced green fluorescence was observed at 499 nm excitation and 515 nm emission wavelengths.

H&E staining. The control group and the experimental group model mice were injected with pcDNA3.1-HBx, empty pcDNA3.1 plasmids and plasmid solvent solution into the tail vein for 24 h, and liver tissues sized 1.0x1.0x0.3 cm were obtained, fixed with 4% paraformaldehyde for 24 h at room temperature. Subsequently, the tissue sections were dehydrated in 80, 90, 95 and 100% gradient ethanol for 2-4 h. The embedded liver tissue was cut into 3-μm sections, using the microtome (Thermo Fisher Scientific, Inc.), and dried for H&E staining. Following 20 min of baking at 60˚C, xylene I and II were used to dewax the tissues for 10 min each. Subsequently, tissues were incubated in 100, 95, 85, 75% gradient ethanol for 5 min each, and in distilled water for 5 min to complete the dewaxing process. Hematoxylin (cat. no. C0390; Beijing Noblelight Technology Co., Ltd.) staining was performed for 5 min at room temperature, after washing with water. Eosin staining (cat. no. C0390, NobleRyder) was conducted for 5 sec, and the sections were subsequently fully washed (33).

Sections were then dehydrated using 75, 85, 95 and 100% ethanol (2 min each). Xylene I, II transparent for 5 min neutral gum seal is intended for fixation. Sections were observed and images were captured using a Leica DM2000 light microscope (Leica Microsystems, Inc.; magnification, x20).

Detection of MDA, SOD, IL-6, IL-1β and IL-18 in serum. Inflammatory cytokine expression in mouse serum was detected using mouse ELISA kits (Mouse MDA/SOD/IL-6/IL-1β/IL-18 ELISA kits; cat. nos. M6000B, DYC3419-2, 7625, MLB00C; R&D Systems, Inc.). The serum was thawed at room temperature and thoroughly mixed. A wash buffer diluted 1:20 with distilled water was used. In the ELISA plate, the wells were divided into standard, sample and blank wells. A total of 50 μl of the different concentrations of the standard (10 μl of the test sample and 40 μl of the diluent) were added to the standard and sample wells, respectively. Nothing was added to the blank well. Subsequently, 100 μl of the horseradish peroxidase-labeled detection antibody was added to the standard and sample wells. The reaction plate membrane aperture was sealed, and the plate was incubated at 37˚C for 60 min. After the incubation period, unbound components were discarded, and the plate was patted dry with absorbent paper. The wells were filled with washing liquid, and the plate was allowed to rest for 1 min. The washing liquid was then discarded, and the plate was patted dry with absorbent paper. The substrates (50 μl each) were added, and the plate was incubated at 37˚C in the dark for 15 min, followed by the addition of 50 μl termination solution to each well for 15 min. The optical density was measured at 450 nm using an ELx800 microplate reader (BioTek Instruments, Inc.).

Statistical analysis. Data were analyzed using GraphPad Prism statistical software version 5.0 (GraphPad Software, Inc.) and ImageJ 2x (National Institutes of Health). Data are expressed as the mean ± standard deviation (unless otherwise stated) from at least three independent experiments. One-way ANOVA was used for statistical analysis, followed by Tukey's post hoc test to assess statistical differences among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection efficiency. Transfection of pCDNA3.1-HBx notably increased the mRNA (Fig. 1A) and protein (Fig. 1B) expression levels in mouse liver tissues. HBx expression was not observed in the control or empty vector-transfected liver tissues. These results demonstrated that the HBx gene was successfully transfected into the livers of experimental mice.

Effect of HBx on the mitochondrial function in mouse hepatocytes. MDA levels in serum of mice in the experimental group transfected with pcDNA3.1-HBx were significantly higher compared with the control group and mock group, whereas SOD levels were significantly lower compared with the control group and mock group (P<0.01; Fig. 2A).

The detection of COX using enzymatic kinetics demonstrated that COX activity in the experimental group transfected with pcDNA3.1-HBx was significantly lower compared with

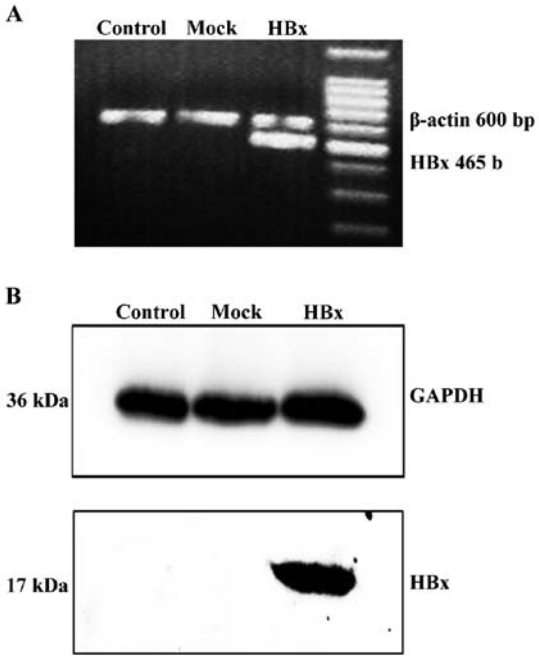

Figure 1. Identification of HBx expression in the mouse model. (A) mRNA and (B) protein expression levels of HBx. Control, blank control group treated with plasmid solvent; Mock, null plasmid control group; HBx, experimental group; M, marker; HBx, hepatitis B virus x protein.

in the control group and mock group (P<0.001; Fig. 2B), indicating that COX activity was reduced by HBx, which was in agreement with our previous experimental results in HL-7702 cells stably expressing HBx (15).

Oxidative stress was determined by measuring the ROS levels in frozen sections using *in situ* fluorescence staining. In the experimental HBx group, compared with the pcDNA3.1 plasmid-transfected group and the control group, the ROS levels in the liver tissues were significantly increased (P<0.05; Fig. 2C and D).

ROS-mediated effects of HBx on NF-κB and p-AKT expression in mouse hepatocytes. The results of western blot analysis demonstrated that the expression levels of NF-κB and p-AKT were significantly increased in the experimental HBx group, which was significant compared with the empty plasmid group and the blank group (P<0.01; Fig. 3).

Detection of HBx-induced inflammatory damage in hepatocytes. Edema and inflammatory cell infiltration were observed in the liver tissue of the HBx group by HE staining (Fig. 4). The serum levels of the inflammatory cytokines IL-6, IL-1β and IL-18 in the experimental group were significantly higher compared with the control group and mock group (P<0.001; Fig. 5A-C). This indicated that HBx may induce the synthesis and secretion of IL-6, IL-1β and L-18, thus promoting inflammatory damage in liver cells.

Discussion

Under normal physiological conditions, ROS levels are in a stable state, and their production and clearance maintain a certain dynamic balance. When the body is invaded by bacteria

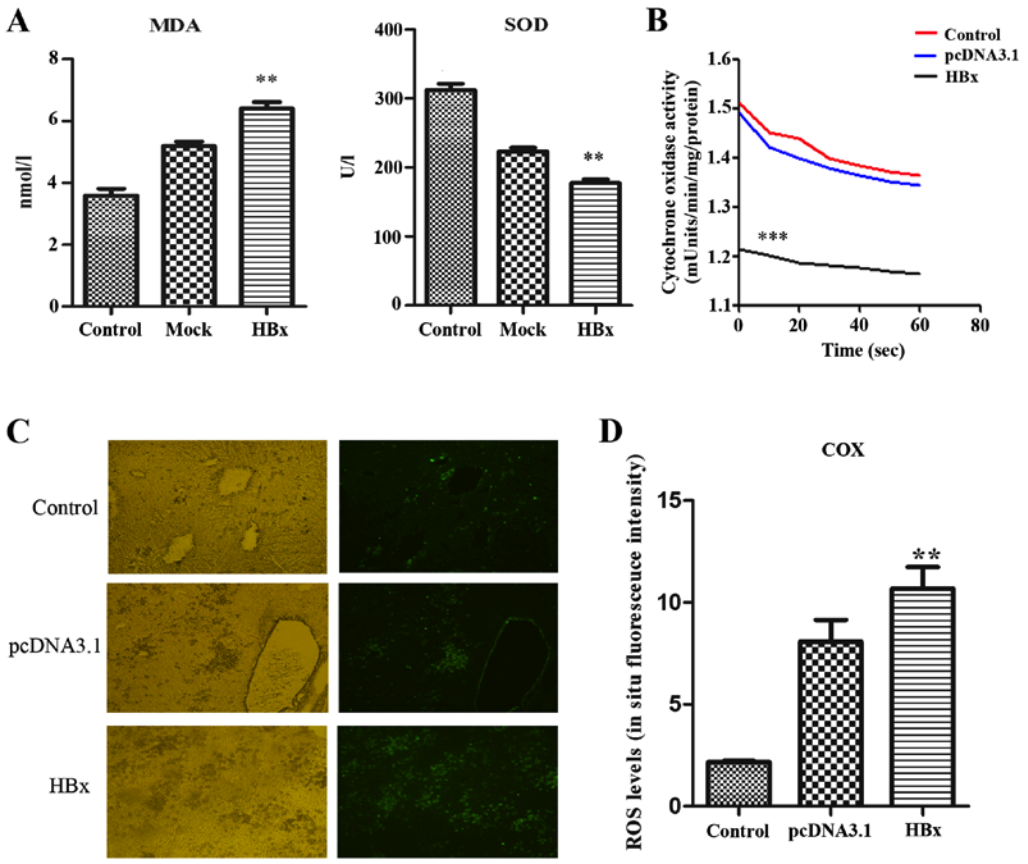

Figure 2. Effect of HBx on mitochondrial function in mouse hepatocytes. (A) Changes in oxidative stress and COX in the sera of mice demonstrated that HBx significantly increased MDA and decreased SOD levels in the sera of mice. n=10. **P<0.01 vs. Control; (B) HBx reduced COX levels. n=10. ***P<0.001 vs. Mock. (C) and (D) HBx increased ROS levels in liver tissues observed by fluorescence microscopy. Magnification, x100. **P<0.01 vs. Control and Mock. Control, blank control group treated with plasmid solvent; Mock, null plasmid control group; HBx, experimental group; HBx, hepatitis B virus x protein; MDA, malondialdehyde; SOD, superoxide dismutase; COX; Cytochrome c oxidase.

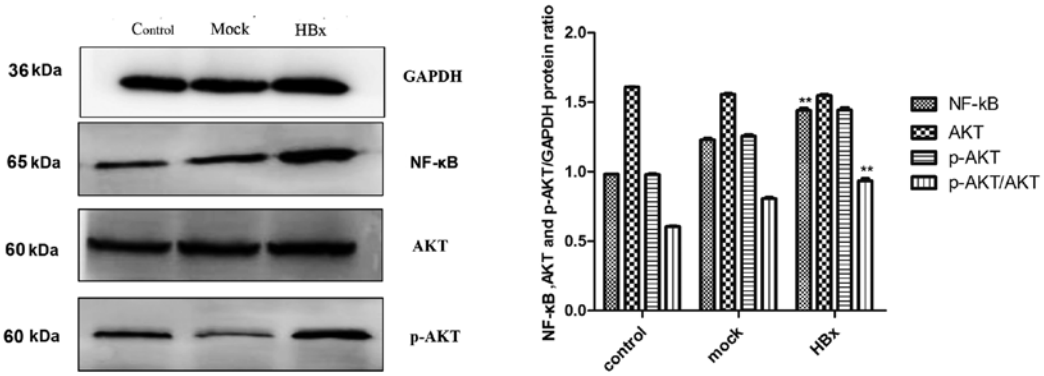

Figure 3. ROS-mediated effects of HBx on NF-κB and p-AKT expression in mouse hepatocytes. HBx upregulated the expression of NF-κB and p-AKT. **P<0.01 vs. Control and Mock. Control, blank control group treated with plasmid solvent; Mock, null plasmid control group; HBx, experimental group; HBx, hepatitis B virus x protein; kDa, kilodaltons; p-AKT, phosphor-AKT.

or viruses, ROS are actively produced as part of the immune response. Another major source of ROS production is the oxidative metabolism of mitochondria. The increase in ROS levels can affect cell signaling pathways and cell growth (34). In particular, ROS influences the NF-κB signaling pathway and activates the MAPK and STAT3 signaling pathways via the release of IL-1β, IL-6 and TNF-α (35-37). However, excessive production of inflammatory cytokines induced by HBx can stimulate cells to produce a large amount of ROS, further stimulating NF-κB to produce inflammatory mediators (for example: IL-1β, IL-6 and TNF-α), forming a cycle and thus accelerating the inflammatory injury of liver cells and HBV replication (37).

Previous studies have demonstrated that the HBx protein interacts with the mitochondrial COXIII subunit, causing mitochondrial damage and affecting the biological activity of liver cells (14-16). In the present study, HBx was demonstrated to alter the mitochondrial oxidative respiratory chain

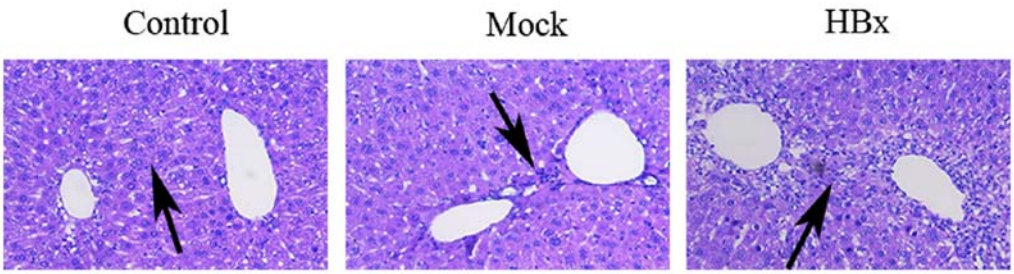

Figure 4. Hepatocyte inflammatory injury mediated by HBx. HBx promoted inflammatory damage in liver cells, and edema and inflammatory cell infiltration were observed in the liver tissue of the HBx experimental group. Shown by black arrow. Magnification, x20. Control, blank control group treated with plasmid solvent; Mock, null plasmid control group; HBx, experimental group; HBx, hepatitis B virus x protein.

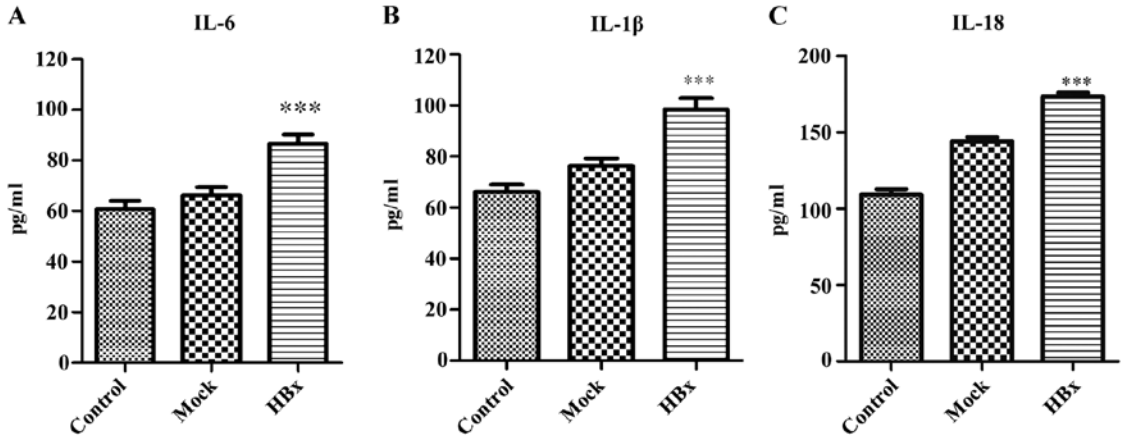

Figure 5. HBx induces the expression of pro-inflammatory cytokines IL-6, IL-1β and IL-18. (A) HBx promoted a significant increase in IL-6 levels in the sera of mice (n=10). ***P<0.001 vs. Control and Mock. (B) HBx increased the levels of IL-1β in the sera of mice. n=10. ***P<0.001 vs. Control and Mock. (C) HBx increased IL-18 levels in the sera of mice. n=10. ***P<0.001 vs. Control and Mock. Control, blank control group treated with plasmid solvent; Mock, null plasmid control group; HBx, experimental group; IL, interleukin.

activity by reducing COX activity, that was in agreement with our previous experimental results in HL-7702 cells stably expressing HBx (15). This resulted in altered intracellular ROS levels and increased expression levels of NF-κB and p-AKT. Therefore, HBx may have increased NF-κB protein expression levels, which subsequently promoted an increase in hepatocyte ROS levels, thus damaging hepatocytes (37). HBx may also upregulate p-AKT protein expression levels as a mechanism to minimize oxidative damage in hepatocytes, although it was observed in this study that the antioxidant effect of p-AKT was insufficient to reverse the liver damage caused by HBx. However, HBx-induced upregulation of MAPK, NF-κB and PI3K is considered an important factor in the development of HCC (34,38,39), and abnormal activation of NF-κB in liver cancer tissue has been reported to inhibit apoptosis and promote liver cell proliferation, contributing to cancer development (38).

Previous studies have demonstrated that the PI3K/AKT pathway serves a role in HBx-induced HCC formation and the activation of nuclear factor erythroid 2-related factor 2 (NRF2), a key transcription factor regulating antioxidant genes during oxidative stress and maintaining intracellular redox homeostasis (40,41). In addition, SOD is a protease that scavenges for excess oxygen free radicals, serving a role in maintaining the generation and scavenging balance of oxygen free radicals. Therefore, the decreased serum levels of SOD observed in experimental mice of the present study supported the notion that the antioxidant capacity in the HBx group was enhanced.

Previous studies have demonstrated that, under oxidative stress, NRF2/ARE can induce the expression of cell protective genes in HBV-positive cells (40,42). Papaiahgari *et al* (40) have confirmed that ROS activates NRF2 via the PI3K/AKT signaling pathway. Increased ROS activates mitotic pathways through oxidative inactivation of PTEN, a tumor suppressor that serves a role in AKT dephosphorylation, therefore permitting AKT phosphorylation and activation and accelerating HepG2 cell growth, which is closely associated with the development of liver cancer in HBx transgenic mice (43). The increased expression of NF-κB and p-AKT in the experimental group of the present study indicated that the NF-κB and AKT signaling pathways may serve a role in ROS-mediated HBx-induced inflammatory hepatocyte injury. The present study demonstrated that the expression levels of NF-κB and p-AKT were increased in the experimental group; in addition, the expression levels of NF-κB and p-AKT in liver cells were significantly decreased following treatment with ROS inhibitors (data not shown). These results suggested that HBx may activate NF-κB and AKT signaling through multiple pathways mediated by ROS. Therefore, ROS may serve an important role in HBx-induced hepatocyte inflammatory injury.

During HBV infection, the immune response may lead to liver cell injury as, HBx increases the expression of MHC-I and MHC-II by activating MHC promoters and forming of HBx-MHC antigen-peptide complexes, which ultimately activate cellular and humoral immune responses, respectively (44). Lara-Pezzi *et al* (45,46) have demonstrated that HBx upregulates the expression levels of TNF-α in hepatocytes by activating the nuclear factor of activated T cells in the cellular immune response. TNF-α mediates the activation of $CD8^+$ cytotoxic T cells, which eliminates infected cells by releasing toxic particles and activating death receptor pathways. In addition, $CD8^+$ cytotoxic T lymphocytes may damage the membranes of liver cells, which results in liver cell injury and initiates apoptosis.

A previous study has demonstrated that HBx stimulates the synthesis and secretion of IL-6, a major pro-inflammatory cytokine, in a toll-like receptor adaptor protein myeloid differentiation factor 88 (MYD88)-dependent manner (47). Quétier *et al* (48) reported that HBx transgenic mice overexpressed IL-6, increased hepatic proliferation and delaying hepatocyte regeneration. A possible explanation for this may be that HBx also activates signaling proteins downstream of MYD88, including NF-κB, which was upregulated in the experimental group of the present study and may have contributed to hepatocyte injury (47).

HBx selectively regulates other pro-inflammatory cytokines, such as IL-1β, IL-18 and IL-23, and participates in the regulation of immune cell interactions (49,50). These cytokines serve a role in the development and progression of liver cancer. IL-1β promotes neutrophil migration to the liver, phagocytosis and pathogen elimination, regulates tumor growth and is associated with the invasion and metastasis of liver cancer (51,52). In addition, Chen *et al* (38) have demonstrated that HBx increases IL-1 secretion and induces NF-kB activation by interacting with an evolutionarily-conserved signaling intermediate in the Toll pathway. IL-18 is a pro-inflammatory cytokine that mediates the inflammatory cascade reaction and is a factor in acute liver injury (53). IL-18 levels in the sera of patients with hepatocellular carcinoma are significantly increased, which suggests that HBx may promote the occurrence and development of hepatocellular carcinoma by regulating IL-18 (53,54). Overall, these studies suggest that pro-inflammatory cytokines serve a role in HBx-induced hepatic inflammatory injury.

In the present study, H&E staining of liver tissue and the levels of IL-6, IL-1β and IL-18 in the sera of the experimental group demonstrated that edema and inflammatory cell infiltration had occurred in the central part of the portal tissue. The levels of IL-6, IL-1β and IL-18 were also significantly higher compared with the control group. In addition, HBx increased the expression levels of TNF-α, receptor-interacting protein kinase (RIP)3 mRNA and RIP3 protein (date not shown). This was consistent with the results from a previous study, which demonstrated that TNF-α activates RIP3, inducing programmed necrosis of cells and aggravating cellular inflammatory responses (data not shown).

In summary, the present study demonstrated that HBx upregulated the expression levels of IL-6, IL-1β IL-18, NF-kB and p-AKT, increased the level of oxidative stress and ultimately contributed to an inflammatory microenvironment in the liver. HBx reduced the activity of COX and may affect mitochondrial respiration in liver cells, resulting in mitochondrial dysfunction and subsequent inflammatory damage of liver cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study will be provided by the corresponding author on reasonable request.

Authors' contributions

DL designed the experiments. LL and DZ performed the experiments and wrote the manuscript. ZZ, WX, ZC, YH and XZ analyzed the experimental results.

Ethics approval and consent to participate

The present study was approved by The Institutional Animal Ethics Committee of Fujian University of Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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