**HEP-21-2028.R2**

**SUPPLEMENTARY MATERIAL**

**MITOCHONDRIAL STRESS IN ADVANCED FIBROSIS AND CIRRHOSIS ASSOCIATED WITH CHRONIC HEPATITIS B, CHRONIC HEPATITIS C OR NONALCOHOLIC STEATOHEPATITIS**

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**Footnotes**

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Concept, set-up and design (AM, TA); acquisition and analysis of data (DL, AM, IT, CMB, MR, SN, AS, TA); writing original and final drafts (AM, TA, DL); all authors were involved in critical review of the manuscript and approved the final version of the manuscript.

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**SUPPLEMENTAL METHODS**

**Analyses flow chart**

Different amounts of DNA, RNAs and/or proteins were obtained from distinct groups of patients and controls depending on the size of liver fragments that remained available after histological studies (Suppl. Fig. 1).

In patients with CHB, RNA, DNA and proteins were simultaneously obtained in 42 cases (31 with F0-F2 and 11 with F3-F4), and both DNA and proteins obtained in 13 patients. The remaining 91 patients have only mRNA (64 cases), DNA (25 cases) or proteins (4 cases). mtDNA damage search, mRNAs levels, immunoblotting, and liver ATP content were then assessed in distinct groups of 65, 104, 44, and 30 patients with CHB, respectively (Suppl. Fig. 1).

The 33 patients with CHC had only total RNA, and all the 12 patients with NASH had both RNA, DNA and only 7 among 12 had RNA, DNA and proteins. mtDNA damage has been reported in CHC and therefore was not searched in the present study (Suppl. Ref1, 2, 34).

Seven (7) of the 24 controls subjects (non-CHB, non-CHC, non-NASH) had RNA, DNA and proteins simultaneously, and 8 had both RNA and DNA while 16 had both DNA and proteins.

mRNAs and/or proteins expression were analyzed for the main genes involved in mitochondrial function (MT-CO1, MT-CO2 and COX4I1 subunits for complex IV; MT-ATP8 subunit for complex V), mitochondrial biogenesis (PPARGC1α, TFAM), mitochondrial fusion/fission (MFN1/2, OPA-1, UTRN), mtUPR (HSPD1, HSPA9, LONP1 protease), mitophagy (PINK1, PRKN), oxidative stress (iNOS, MnSOD), and genes for the inflammatory cytokines (TNFα, IL6, IL1β) (Suppl. Fig. 1) using specific primers (Suppl. Table 2) and/or antibodies (Suppl. Table 3). All these parameters were analyzed in normally distributed groups of patients and controls for age, ALT, steatosis, or HBV DNA.

**Real-time RT-qPCR assessment for liver mRNA levels**

Frozen liver fragments from controls and patients with CHB, CHC or NASH were dissolved in 1mL of iced-Trizol and crushed during 60s at 30Hz using Eppendorf-TissusLyser II. Chloroform was added, and the aqueous phase was collected after centrifugation. Total RNAs was precipitated at -20°C overnight using 2-propanol and 1 µg of glycogen RNA grade. RNA was washed in 75% ethanol and the pellet obtained was resuspended in DEPC RNAse Free water. Total RNA (0.25-1µg) treated with DNase I was retro-transcribed using random primers and Superscript II reverse transcriptase kit. mRNA levels were estimated by real-time RT-qPCR using specific primers (Suppl. Table 2) and SYBR Green I Master Mix. GAPDH was used as a reference gene, and the 2−ΔΔCq method was used to estimate the relative mRNA expression following MIQE guidelines (Suppl. Ref 3).

**Liver ATP determination and Western blotting analysis**

Frozen liver biopsies from patients with CHB were homogenized in a lysis buffer (either 50 mmol/L Tris-HCl, pH 7.2, 250 mmol/L NaCl, 0.1% NP-40, 2 mmol/L EDTA, 10% glycerol; or 70 mmol/L sucrose, 220 mmol/L Mannitol 2 mmol/L, HEPES-KOH 0,1 mmol/L EDTA II, pH 7.4) containing protease and phosphatase inhibitors cocktail.

To measure hepatic ATP levels, 10 protein pools each from 3 patients with CHB (30 µg of liver proteins from each patient) and 4 pools of 3 controls each were constituted and permitted 10 determinations of liver ATP levels in patients with CHB and 4 determinations in the non-CHB controls. Thus, equal amounts of liver proteins from 30 patients were mixed to constitute 5 pools of patients with CHB and F0-F2 and 5 pools with F3-F4. Similarly, 12 controls were pooled into 4 mixes. These 14 liver protein pools were then ground in liquid nitrogen and transferred into 100 µl of ice-cold 1 M perchloric acid. After centrifugation at 4 °C, supernatants were neutralized with 5 M K2CO3 and centrifuged again at 4 °C. The pellets were resuspended in phosphate-buffered saline (PBS) containing 0.2N NaOH and used to determine protein content. The supernatant was used to measure ATP with Luminescent ATP Detection Assay Kit on a luminescence plate reader following the manufacturer’s recommendations.

For Western blotting, homogenates were centrifuged, and the supernatant was collected. Total protein concentrations were determined by the Bradford reagents. Proteins (25-75 µg) were mixed with Laemmli buffer and subjected to SDS–PAGE. The proteins were blotted on nitrocellulose membranes using a semi-dry transfer system and the transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 20% ethanol). The membranes were blocked overnight in Tris-buffered saline (TBS) containing 10% BSA 0.05 % tween20 and incubated with specific antibodies (Suppl. Table 3). After 3 washes in TBS-Tween 0.05%, blots were incubated with horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated secondary antibody.

**Cell culture and transfection**

The human hepatoma HepG2 cell line was purchased from ATCC (Rockville, MD). Cells were cultured at 37°C in a 5% CO2/95% air atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovin serum, streptomycin (0.1 mg/ml) and penicillin (100 U/ml). HepG2 cells were transfected using JetOptimus DNAtransfection reagent (Polyplus-transfection) following the manufacturers' protocol.

The plasmid Payw1.2-HBV (gift from Christine Neuveut, Institut Pasteur, Paris) containing more than one full-length HBV genomes of the ayw subtype cloned into pGEM7 Zf vector (Promega Corporation, Madison, WI) and expressing HBV pregenomic RNA (Suppl. Ref. 4) was transiently transfected into HepG2 cells. The expression of HBV pregenomic RNA was confirmed by PCR using specific primers (Suppl. Table 1).

The plasmids pcDNA3.1Flag-HBx (Addgene, #42596) and pcDNA3.1Flag-HBx(G124L, I127A) (Addgene, #42598) were respectively used to express wild type and mutated HBx protein under Cytomegalovirus promotor (41).

In some cases, 5 µM of the mitochondrial-targeted superoxide scavenger Mito-Tempo[(2-(2,2,6,6-Tetramethylpiperidin-1-Oxyl-4-Ylamino)-2-Oxoethyl) Triphenylphosphonium Chloride, Monohydrate] (Life Sciences, Villeurbanne, France) or 5 µM of the specific iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamidine (1400W) (30) were added to the cultured HepG2 cells 30 minutes before transfection. A second dose of the treatment was added 16 h after transfection.

Cells were collected at 24, 48, 72 or 96 h after transfection. Anti-HBx antibody (ab39716; Suppl. Table 1) was used to confirm HBx expression.

The empty plasmids pGEM7 Zf and pcDNA3.1 were used as transfection controls.

**Cytotoxicity detection of lactate dehydrogenase activity (LDH) in the cell culture media**

Damaged cells release the LDH enzyme into the extracellular medium. LDH activity was determined in cell culture media using the colorimetric (450 nm) Cytotoxicity Detection Kit (LDH) (MAK066-1KT, Sigma-Aldrich) following the manufacturer’s recommendations. At the indicated time-points the medium was changed and  
LDH released into the medium per 24 h was calculated and expressed as LDH activity (U/mL).

Fresh medium was added every 24 h post-transfection. At 24, 48, 72 and 96 h post-transfection, medium was taken for lactate dehydrogenase (LDH) activity determination and cells were recovered for total DNA and proteins extraction. mtDNA damage, iNOS, MnSOD and mitochondrial oxidative stress were analyzed.

**Mitochondrial formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in transfected HepG2 cells**

To measure the nitration of the mitochondrial respiratory complexes, mitochondria were isolated by differential centrifugation using the Kit MITOISO2-1KT (Sigma, Aldrich).

To assess mitochondrial ROS and RNS formation, HepG2 homogenates or purified mitochondria were prepared 24 h after Payw1.2-HBV or pcDNA3.1Flag-HBx or pcDNA3.1Flag-HBx (G124L, I127A) transfection, and incubated with 10 mM succinate or 5 mM each of malate and glutamate in the presence of either the MitoSOX Red reagent (Invitrogen), a mitochondria-targeted superoxide indicator, or H2DCF-DA, which forms the green fluorescent 2’,7’-dichlorofluorescein in the presence of hydrogen peroxide and other peroxides, or DHR 123, a mitochondrial avid cationic dye oxidized by peroxynititrite and other ROS to rhodamine 123.

Each well of a 96-well plate was filled with respiration buffer (130 mM sucrose, 50 mM KCl, 5 mM MgCl2, 5 mM KH2PO4, 0.05 mM EDTA, 5 mM HEPES, pH 7.4) containing 2 µM H2DCF-DA, and mitochondria (5-20 mg protein/ml). The reaction was started by the addition of 10 mM succinate or 5 mM each of glutamate and malate. Fluorescence was recorded at 480/520 nm with a FLUOstar fluorimeter for 60 min at 30 °C.

Mitochondrial superoxide anion was determined by incubating mitochondria with either malate:glutamate or succinate in the presence of MitoSOX Red reagent (Invitrogen), a mitochondria superoxide indicator which exhibited red fluorescence in the presence of superoxide but not other ROS or RNS. The MitoSOX red fluorescence was recorded at 510/580 nm.

The formation of peroxynitrite was determined in total HepG2 lysates by measuring the ONOO− dependent oxidation of dihydrorhodamine (DHR) 123 to rhodamine 123. DHR is a cell-permeant mitochondrial-avid analog of H2DCF-DA. Following malate/glutamate or succinate addition, homogenates were incubated with 5 μM DHR 123 for 1 h at 37 °C. The fluorescence of rhodamine 123 was measured at excitation 485 nm, emission 530 nm.

**Nitrite/Nitrate levels in cell culture medium**

Medium nitrite/nitrate concentrations were determined using the Griess reaction, as previously described (17-19). The produced NO was measured in culture medium of the transfected cells using a colorimetric Nitrite/Nitrate Assay Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Absorbance at 540 nm was recorded with microplate reader (Tecan Infinite M200 Pro, Tecan Group Ltd., Switzerland).

**MnSOD and Cu,ZnSOD activities, total and mitochondrial Glutathione (GSH)  levels in HepG2 cells.**

Native gel assays were used to assess MnSOD and Cu,ZnSOD activities. HepG2 proteins (50 µg) were resuspended in 40% glycerol and 0.025% bromophenol blue and loaded on a non-denaturing 15% polyacrylamide gel. Migration was performed at 4°C for 12 h at 120 V, and superoxide dismutase activity was assessed within the gel, as described (18). The total and the mitochondrial contents of reduced glutathione were determined as has been described (17,18).

**Complex I activity and protein levels and detection of 3-Nitrotyrosine residues in proteins of respiratory chain complexes I, III and V.**

Hepatic mitochondria were isolated and submitochondrial particles were prepared by two cycles of freezing/thawing. Complex I was assayed by following the oxidation of NADH in the presence of decylubiquinone (17,18).

Alterations in the levels of mitochondrial complexes I, III and V were examined using blue native polyacrylamide gel electrophoresis (BN-PAGE) (17,18). Mitochondrial extracts were treated with n-dodecyl-β-D-maltoside, a mild detergent that allows the separation of respiratory chain complexes, whereas their subunit assembly and activity remain intact. BN-PAGE was performed using 5 to 13% polyacrylamide gel gradient with a 4% stacking gel. The anode buffer contained 50 mM Bis-Tris and the cathode buffer contained 50 mM Tricine, 15 mM Bis-Tris, and 0.02% of the Serva Blue G dye. Protein samples (25 µg) in 2 µl of 5% Serva Blue G, 750 mM aminocaproic acid, and 50 mM Bis-Tris/HCl, pH 7.0, were applied to a 10-well gel. Electrophoresis was conducted first at 100 V at 4°C until the samples entered the stacking gel, then the voltage was increased to 200 V. When the dye front reached the middle of the gel, the initial cathode buffer containing 0.02% Serva Blue G was replaced by a cathode buffer containing 0.002% Serva Blue G. The banding pattern was scanned and quantified by densitometry to measure the level of protein loaded on the gel for each complex. Immediately after electrophoresis, the separated complexes on BN-PAGE were transferred electrophoretically to nitrocellulose membranes for Western blot detection of 3-nitrotyrosine using anti-3-nitrotyrosine antibody (Suppl. Table 2). Membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated overnight at 4°C with a monoclonal antibody against 3-nitrotyrosine. Membranes were washed several times and incubated with HRP-conjugated secondary antibody for 1 h. Protein bands were visualized by enhanced chemiluminescence.

**SUPPLEMENTAL RESULTS**

**mtDNA toplogy changes in patients with CHB**

The distribution of the main mtDNA forms was analyzed by Southern Blot (Suppl.Fig.3B). Southern blots were hybridized with the radio-labeled 6.080-kb mtDNA probe, and the percentages of supercoiled, circular, and linear (full-length) mtDNA were determined in 5 control subjects and 10 patients with CHB (5 with F3-F4 fibrosis and 5 with F0-F2) (Suppl. Fig. 3C). Supercoiled mtDNA corresponds to unnicked mtDNA; circular mtDNA may correspond either to un-nicked DNA or to mtDNA molecules with single strand break(s), whereas the linear 16.5-kb mtDNA is a form that has been cut on both strands (Suppl. Fig. 3B).

In non-CHB controls, the percentages of supercoiled, circular, and linear 16.5-kb mtDNA were 51% ± 9%, 44% ± 14%, and 4% ± 2 %, respectively (mean ± SEM, n=5) (Fig. 3C). In patients with CHB, the proportion of supercoiled mtDNA (10% ± 5%) was only 20% of that in control subjects (*p<0.05*) (Suppl. Fig. 3C). The percentage of relaxed-circular form was 64% ± 14%, whereas the proportion of full-length linear mtDNA was 8.5-fold that of controls (Suppl. Fig. 3C). Similarly, while the uncut mtDNA almost disappeared (less than 2%) in patients with F3-F4, it was 12% in patients with F0-F2. Whereas the proportion of full length linear mtDNA was 48% in F3-F4, it was only 14% in F0-F2 fibrosis (*p<0.05*) (Suppl. Fig. 3C). Combined with the 66% decrease in mtDNA levels in F3-F4 fibrosis (Suppl. Fig. 3C), these percentages indicated that the supercoiled form had almost disappeared, and the circular form had been halved (in absolute amounts), whereas most mtDNA had been converted to linear forms (partly to the full-length linear form) and mostly to lower-sized mtDNA fragments. mtDNA smears were observed below the 16.5-kb linear mtDNA in two patients with CHB and advanced fibrosis, confirming degradation into lower-sized linear fragments.

**mRNA and/or protein levels for the mitochondrial biogenesis markers PPARGC1α and TFAM and for the mitochondrial import marker TOMM20 in patients with CHB, CHC or NASH**

The mitochondrial transcription factor A (TFAM) is involved in both mtDNA transcription and replication, and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1α) is involved in mtDNA replication (7).

Compared to control subjects, both TFAM and PPARGC1α mRNAs levels significantly dropped in patients with CHB whatever the degree of fibrosis (Fig. 5A, C). TFAM mRNA significantly decreased also in patients with CHC as compared to controls, while PPARGC1α mRNA levels were unchanged (Fig. 5A, C). Compared to controls, patients with NASH and cirrhosis had significantly higher levels of PPARGC1α mRNA but lower levels of TFAM mRNA (Fig. 5A, C).

While TFAM and PPARGC1α mRNAs levels were similar in patients with CHB and F3-F4 (n=20; 0.23±0.13 and n=20; 0.43±0.25 respectively) as compared to those F0-F2 (n=60; 0.29±0.15, and n=60; 0,58±0.45, respectively), these levels significantly decreased in patients with CHC and F3-F4 (n=20; 0,44±0.19 and n=20; 0,63±0.31 respectively) compared to patients with CHC and F0-F2 (n=60; 0.68±0.29, *p<0.05* and n=60; 1.71±1.19, *p<0.01* respectively) (Fig. 5A, C).

Patients with NASH and cirrhosis had significantly higher TFAM (n=12; 0.39±0.19, *p<0.05*) and PPARGC1α (n=12; 2.82±1.21, *p<0.01*) mRNAs levels than patients with CHB and advanced fibrosis-cirrhosis (Fig. 5A, C). Patients with NASH had also significant increases in PPARGC1α (*p<0.*001) mRNA levels but similar TFAM mRNA levels when compared to patients with CHC and advanced fibrosis-cirrhosis (Fig. 5A, C).

Compared to controls subjects (n=7, 1.59±0.75), TFAM/β-actin ratios significantly decreased in patients with CHB and F3-F4 (n=7; 0.74±0.25, *p<0.*05) but unchanged in patients with CHB and F0-F2 (n=10; 1.11±0.37, *p=0.26*) or in patients with NASH and cirrhosis (n=12; 1.26± 0.39, *p=0.62*) (Fig. 5B).

TOMM20 is involved in the mitochondrial import machinery (7). TOMM20 protein/β-actin ratios significantly decreased in patients with CHB and F0-F2 fibrosis but were not altered in patients with CHB and F3-F4 or in patients with NASH and cirrhosis as compared to controls subjects (Suppl. Fig. 4B).

**Altered expression of the mitochondrial fusion marker mitofusin1 and the fission marker OPA1 in patients with CHB and F3-F4**

Mitofusin1/β-Actin and OPA1/β-Actin ratios were significantly decreased by 1.8 and 1.5-fold in patients with CHB and F3-F4 (n=5; 0.70±0.37 and n=5, 0.71±0.36, respectively) compared to those with F0-F2 (n=6; 1.31±0.49, *p<0.05* and n=7; 1.09±0.32, *p<0.05* respectively) (Suppl. Fig 4C, D).

**HBV replication and wild-type HBx or mutated HBx(G124L, I127A) expression** **in human HepG2 cells *in vitro***

Transfection with pcDNA3.1Flag-HBx expressing wild-type HBx protein, pcDNA3.1Flag-HBx (G124L, I127A) expressing mutated HBx or Payw1.2 expressing whole HBV genome confirmed the expression of HBx protein (Suppl. Fig. 6A) or HBV pregenomic RNA (HBV pgRNA) (Suppl. Fig. 6B).

**Time course of LDH leakage in HepG2 cells replicating HBV or expressing wild-type HBx protein *in vitro***

LDH activity was assessed in the extracellular medium as cell survival marker at 24, 48, 72 and 96 h after transfection (Suppl. Fig. 6C). Medium LDH activity was minimal in non-transfected cells, in cells transfected with empty vectors, or in cells transfected for 24 h with pcDNA3.1Flag-HBx or with Payw1.2 vectors (Suppl. Fig. 6C). LDH activity dramatically increased at later time-points indicating that necrotic damage and cell death occurred after longest times of HBx expression or HBV replication. The % of attached cell (survivals) significantly decreased starting at 48 h post-transfection with vectors expressing HBx or HBV. We therefore kept 24 h time-point to further investigate the effects of HBx and HBV on mtDNA damage, mitochondrial oxidative stress, mitochondrial antioxidant system and mitochondrial function.

**HBV and HBx protein induced iNOS, decreased MnSOD activity and increased the nitration of tyrosine residues in mitochondrial complexes I, III and V in cultured HepG2 cells *in vitro***

Twenty-four hours post-transfection, both HBV and HBx significantly induced iNOS protein (iNOS/β-actin ratios were 2.59 ± 0.30 and 4.33 ± 0.26, respectively) compared to corresponding empty vectors (0.52 ± 0.14 and 1.25 ± 0.35, respectively, *p<0.05*) (Suppl. Fig. 7A). Although HBV significantly induced MnSOD protein (Suppl. Fig. 7B), it significantly inactivates MnSOD activity (*p<0.05*) (Suppl. Fig. 7C). However, HBx did not induce MnSOD protein, rather it decreased residual MnSOD activity (Suppl. Fig. 7C). Cu,ZnSOD activity remained unchanged.

There were no changes in the protein levels of mitochondrial respiratory chain complexes as separated by blue native polyacrylamide gel electrophoresis 24 h after HBV or HBx transfection (Suppl. Fig. 7D). The separated respiratory chain complexes were transferred onto nitrocellulose membranes and revealed with an antibody against 3-nitrotyrosine (Suppl. Fig. 7D). In pGEM or pcDNA3.1 transfected HepG2 cells, 3-nitrotyrosine residues were not detected or less presented in the proteins of complexes I, III and V, but significantly increased after the transfection with Payw1.2 expressing whole HBV genome or with pcDNA3.1FlagHBx (Suppl. Fig. 7D).

**HBV and wild-type HBx but not mutated HBx (G124L, I127A) increased mitochondrial ROS and RNS formation, depleted mtDNA and mitochondrial GSH and decreased complex I activity *in vitro***

HBx has been shown interact with the mitochondrial voltage-dependent anion channel (54), and to bind to the mitochondrial Bcl-2 family members (Bcl-2 and Bcl-xL) to cause the mitochondrial transition pore (MTP) opening and the fall of the mitochondrial membrane potential Δpsi (48). HBx containing G124L and I127A substitutions prevented HBx binding to mitochondrial Bcl-2 and Bcl-xL and thus prevented HBx-induced MTP opening and Δpsi collaps (48). pcDNA3.1Flag-HBx(G124L, I127A) was therefore used to further explore the possibility that Hbx-induced mitochondrial stress might involve its binding to Bcl-2 and Bcl-xL.

Twenty-four hours after transfection, both HBV and wild-type HBx significantly increased the formation of superoxide anion and hydrogen peroxide by purified mitochondria isolated from transfected HepG2 cells (Suppl. Fig 8A, Suppl. Table 4). HBV and wild type HBx also significantly increased the peroxynitrite formation by whole HepG2 cells homogenates (Suppl. Table 4).

Consequently, HBV and wild type HBx significantly depleted mtDNA at 24 h post-transfection. mtDNA/nDNA ratios were 0.78 ± 0.17 in pGEM-transfected HepG2 cells *and* 0.29 ± 0.12 in HepG2 replicating HBV, and were 1.17 ± 0.19 in HepG2 transfected with pcDNA3.1 and 0.49 ± 0.09 in HepG2 cells expressing wild type HBx protein (Suppl. Fig. 8B,C). However, mtDNA deletions were not detected in cells replicating HBV or expressing HBx protein up to 72 h post-transfection (Suppl. Fig 8D).

Culture medium nitrite/nitrate concentrations were 4.4 ± 0.6 µM and 3.8 ± 1.2 µM after transfection with the corresponding control plasmids and increased significantly to 8.85 ± 1.21 µM and 7.34 ± 0.89 µM in culture supernatants from HepG2 replicating HBV or expressing wild type HBx, respectively (*p<0.05*) (Suppl. Table 4).

Both mitochondrial GSH and total GSH were significantly depleted in HepG2 cells replicating HBV or expressing wild type HBx. Mitochondrial GSH was 8.84 ± 1.12 and 3.63 ± 0.58 nmol/mg protein in pGEM and payw1.2-HBV transfected HepG2, respectively (p<0.05) and was 9.24 ± 1.12 and 4.21 ± 1.10 nmol/mg protein in pcDNA3.1 and pcDNA-FlagHBx transfected HepG2, respectively *(p<0.05*) (Suppl. Table 4).

Finally, plasmids expressing HBV or wild type HBx significantly decreased the complex I activity by 46% and 37%, respectively, as compared to the corresponding empty vectors (Suppl. Table 5).

Importantly, HBx(G124L, I127A) failed to increase mitochondrial ROS formation, failed to deplete mtDNA or mitochondrial GSH and preserved complex I activity compared to wild type HBx (Suppl. Fig. 7A,C; Suppl. Tables 4,5).

**Mito-Tempo or 1400W protected against HBx- and HBV-induced mtDNA depletion and against respiratory complex I impairment**

To gain further insight into the type and origin of the reactive species involved in mtDNA depletion, we treated transfected cells with Mito-TEMPO (superoxide scavenger) or with 1400W (selective iNOS inhibitor).

Twenty-four hours after transfection, Mito-Tempo fully prevented HBx- and HBV-mediated mtDNA depletion, while 1400W partially protected against HBx- and HBV-mediated complex I inactivation in HepG2 cells (Suppl. Table 5).

**SUPPLEMENTAL DISCUSSION**

Impaired mitochondrial function leads to liver steatosis, a lesion ascribed as consequence of altered lipids metabolism and mitochondrial β-oxidation of fatty acids (7). NASH is a condition characterized by the presence of fat droplets associated with liver fibrosis, necroinflammation, lipid peroxidation and underlying lipotoxicity (Suppl. Ref 5-7). Lipid metabolic changes play a critical role in NASH development (7). The common 4977 bp mtDNA deletion appeared progressively with normal age and has been reported in patients with hepatic steatosis (7, 11, Suppl Ref 8-10). Despite their old age, patients with NASH and cirrhosis had only one mtDNA deletion as compared to patients with CHB and F3-F4 (Table 1, Suppl. Table 4). This age-related mtDNA deletion may favor steatosis in patients with NASH. Conversely, lipid hydroperoxides may cause mtDNA damage in patients with NASH which in turn might aggravate hepatic steatosis and the development and progression of fibrosis (7,50). All the 7 patients with NASH and mtDNA deletion developed significant hepatic steatosis (≥66% of fat containing hepatocytes in 5 and >33%-<66% in 6). Conceivably, subjects with mtDNA deletions (and possibly many other mtDNA mutations) may poorly reoxidize NADH (reduced nicotinamide adenine dinucleotide) in the mitochondria leading to hepatic steatosis as a consequence of impaired oxidation of fatty acids (7). In the present study, mtDNA deletions were associated with decreases in the expression of mtDNA-encoded genes which may block electron flow within respiratory chain complexes, thus causing an excess of reducing equivalents NADH in the liver. Such alterations in the mitochondrial redox state inhibit the oxidized nicotinamide adenine dinucleotide (NAD+)-dependent fatty acids dehydrogenases involved in the β-oxidation of fatty acids (7). In NASH, the increased hepatic free fatty acids then uncouple respiration from ATP formation (7). Mitochondria-derived ROS oxidize the unsaturated lipids to cause lipid peroxidation. Extensive lipid peroxidation was observed in animal models of steatohepatitis and lipid peroxidation products alter mtDNA and react with mitochondrial proteins, including respiratory chain complexes (7).These effects tend to partially block the transfer of electrons in the respiratory chain. Even when adaptive changes such as mitochondrial biogenesis occur, any block in the flow of electrons along the respiratory chain may cause the formation of high amounts of ROS (7). Moreover lipid peroxidation and ROS consume antioxidant enzymes and vitamins thus compromising ROS scavenging, causing further lipid peroxidation, increasing mitochondrial damage, and higher ROS formation in NASH (7). Reactive lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) increase hepatic fibrogenesis by two mechanisms. MDA and 4-HNE enhance the hepatic production of transforming growth factor β1 (TGF-β1), which activates hepatic stellate cells to evolve into collagen secreting myofibroblasts. MDA and 4-HNE also directly enhance collagen production by hepatic stellate cells (7).

However, this might not be the case in HBV-infected patients because hepatic steatosis is a relatively uncommon in CHB patients unlike patients with NASH or CHC (35, Suppl. Ref 5, 11 and the present study). Only 12 (8%) over the 146 patients with CHB had significant steatosis (>33% of fat-engorged hepatocytes) compared to 24% in CHC and 92% in NASH in the present study (Table 1). mtDNA depletion, strand breaks, and the high prevalence of mtDNA deletions and their diversity in patients with CHB as compared to controls or to patients with NASH all suggest that HBV infection may instead cause massive oxidative damage to liver mtDNA which progressively accumulates in patients with advanced fibrosis. These observations also suggest that mitochondrial dysfunction and altered mitochondrial dynamics would speed-up the progression of CHB-related fibrosis. Indeed, mtDNA damage is a hallmark of oxidative stress (7). Although ROS were not measured in our patients, the mtDNA damage detected in livers from patients with CHB or with NASH and advanced fibrosis suggested that significant oxidative stress rises in these livers. In line with this hypothesis, we demonstrated that transient replication of HBV or expression of HBx protein increased the mitochondrial formation of superoxide anion and peroxynitrite radicals (Suppl. Table 4).

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**SUPPLEMENTAL TABLES**

**Supplemental Table 1.** Oligonucleotide Primers.

|  |  |  |  |
| --- | --- | --- | --- |
| **Symbol** | **Name** | **Primer** | **Sequence** |
| **Primers used for qPCR** | | | |
| GADPH | D-glyceraldehyde-3-phosphate dehydrogenase | Forward | 5'-GAA GGT GAA GGT CGG AGT CA-3' |
| Reverse | 5'-ATT GAA GGG GTC ATT GAT GG-3' |
| MT-CO1 | Mitochondrial Cytochrome c oxidase 1 | Forward | 5'-CCT GAC TGG CAT TGT ATT AGC-3' |
| Reverse | 5'-TTT TGG CGT AGG TTT GGT CT-3' |
| MT-CO2 | Mitochondrial Cytochrome c oxidase 2 | Forward | 5'-AGA TGC AAT TCC CGG ACG T-3' |
| Reverse | 5'-CAT GTC CAG CAT CCT CTT GGT-3' |
| COX4i1 | Mitochondrial Cytochrome c oxidase 4 | Forward | 5'-ACC GCG CTC GTT ATC ATG TG-3' |
| Reverse | 5'CAT GTC CAG CAT CCT CTT GGT-3' |
| LONP1 | Lon Peptidase 1 Caseinolytic | Forward | 5'-GAA GTT GGT TGA GCT GCT GAG-3' |
| Reverse | 5'-ATC TCA TGG ATC TGG GCA AA-3' |
| HSPD1 | Mitochondrial heat shock protein 60 | Forward | 5'-CCC ACA GTC TTT CGC CAG AT-3' |
| Reverse | 5'-GGA CTT CCC CAA CTC TGC TC-3' |
| HSPA9 | Mitochondrial heat shock protein 70 | Forward | 5'-CTG AAG AAG ACC GGC GAA AGA-3' |
| Reverse | 5'-AGC TTG TTG CAC TCA TCA GCA-3' |
| TFAM | Mitochondrial transcription factor A | Forward | 5'-TTT GCA TCT GGG TTC TGA GCT TTA A-3' |
| Reverse | 5'-CGA GGT GGT TTT CAT CTG TCT TG-3' |
| PPARGC1α | Peroxisome proliferator-activated receptor gamma coactivator-1α | Forward | 5'-TCC TTT CTC TCG CCC AAC ACG ATC T-3' |
| Reverse | 5'-GCA TCC GAC AGG ACA AAC AGT GGA-3' |
| PINK1 | Serine/threonine-protein kinase PINK1 | Forward | 5'-GGG GAG TAT GGA GCA GTC AC-3' |
| Reverse | 5'-CAT CAG GGT AGT CGA CCA GG-3' |
| PRKN | E3 ubiquitin-protein ligase parkin | Forward | 5'-TAC GTG CAC AGA CGT CAG GAG-3' |
| Reverse | 5'-GAC AGC CAG CCA CAC AAG GC-3' |
| TNFα | Tumor necrosis factor alpha | Forward | 5'-CTCTTCTGCCTGCTGCACTTTG-3' |
| Reverse | 5-ATGGGCTACAGGCTTGTCACTC-3' |
| IL1β | Interleukin-1 beta | Forward | 5’-CCG GGG GTA TAC TAC GG-3’ |
| Reverse | 5’-GGT TCA TTC GGG AGG ATC CTA TTG GTG CGG-3’ |
| IL6 | Interleukin-6 | Forward | 5'-AGA CAGCCACTCACCTCTTCAG-3' |
| Reverse | 5’-TTC TGC CAG TGC CTC TTT GCT G-3’ |
|  | | | |
| **Primers used for the detection of mtDNA deletions** | | | |
| Primers A | (nt 8150 - nt 8166) | 5’-CCG GGG GTA TAC TAC GG-3’ | |
| Primers B | (nt 14276 nt 14247) | 5’-GGT TCA TTC GGG AGG ATC CTA TTG GTG CGG-3’ | |
| **Primers used for the sequencing of mtDNA deletions** | | | |
| Primers C | (nt 13330-nt 13350) | 5’-CAC GCC TTC TTC AAA GCC ATA-3’ | |
| Primers D | (nt 8335-nt 8355) | 5’-ATT AAG AGA ACC AAC ACC TCT-3’ | |
| **Primers used for the detection of HBV pregenomic RNA after Payw1.2 transfection** | | | |
| HBV pgRNA | Forward | 5’-TGT CAA CAC TAA TAT GGG CCT AA-3’ | |
| Reverse | 5’-AGG GGC ATT TGG TGG TCT AT-3’ | |

**Supplemental Table 2.** Reagents and Antibodies.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibodies** |  | **Supplier** | **Catalog no.** | **Clone no.** |
| Anti-MTCO1 antibody |  | Abcam | ab14705 | 1D6E1A8 |
| Anti-MTCO2 antibody |  | Abcam | ab110258 | `12C4F12 |
| Anti-COX4, mitochondrial antibody |  | Abcam | ab1102272 | 1A12A12 |
| PGC-1α |  | Santa Cruz Biotechnology | sc-517380 | 4A8 |
| Anti-TFAM rabbit pAb |  | Calbiochem® | DR1071 |  |
| Parkin Antibody |  | Cell Signalling | 2132S |  |
| PINK1 |  | Cell Signaling | 6946 | D8G3 |
| LonP1 |  | Cell Signaling | 56266 | D7L8M |
| OPA1 (D6U6N) Rabbit mAb 80471 |  |  |  |  |
|  | Cell Signaling | 80471 | D6U6N |
| Tom20 (D8T4N) Rabbit mAb 42406 |  |  |  |  |
|  | Cell Signaling | 42406S | D8T4N |
| HSP60 (D6F1) XP® Rabbit mAb 12165 |  |  |  |  |
|  | Cell Signaling | 12165 | D6F1 |
| mtHSP70 monoclonal antobody (JG1) |  | Enzo Life Sciences | ALX-804-077-R100 | JG1 |
| MT-ATP8 Polyclonal Antibody |  |  |  |  |
|  | Invitrogen | PA5-68103 |
| iNOS monoclonal antibody |  | BD Transduction Laboratories/BD Bioscience | 610329 |  |
| Rabbit Anti-Human MnSOD / SOD2  Anti-HBx antibody |  | Tebu-Bio  Abcam | 119-16935  ab39716 |  |
| Monoclonal Anti-β-Actin antibody produced in mouse |  | Sigma-Aldrich | A5441 | AC-15 |
| Anti-mouse IgG, HRP-linked Antibody 7076 |  | Cell Signaling | 7076S |  |
| Anti-rabbit IgG, HRP-linked Antibody 7074 |  | Cell Signaling | 7074 |  |
| Anti-mouse IgG, AP-linked Antibody 7074 |  | Cell Signaling | 7056 |  |
|  |  |  |  |  |
| **Reagents** | | | | |
| **Name** | | **Catalog no.** | **Supplier** | |
| pcDNA3.1Flag-HBx  pcDNA3.1Flag-HBx(G124L, I127A)  SYBR™ Safe DNA Gel Stain  Expand Long Template PCR system | | 42596  42598  S33102  116818 | Addgene  Addgene  Thermoscientific  Roche | |
| UltraPure™ Agarose | | 16500500 | Thermoscientific | |
| Hybond-N1 nylon membrane | | 11209299001 | Roche | |
| TRIzol™ Reagent | | 15596018 | Invitrogen | |
| 2-Propanol | | I9516 | Sigma Aldrich | |
| Chloroform | | C2432 | Sigma Aldrich | |
| Ethanol | | 1.08543 | Sigma Aldrich | |
| DEPC-Treated Water | | AM9906 | Invitrogen | |
| Ambion™ DNase I (RNase-free) | | AM2222 | Invitrogen | |
| Random Hexamers (50 µM) | | N8080127 | Invitrogen | |
| Transcriptase inverse SuperScript™ II | | 18064071 | Invitrogen | |
| LightCycler® 480 SYBR Green I Master | | 4887352001 | Roche | |
| Corning® Costar® Spin-X® centrifuge tube filters | | CLS8162-96EA | Costar | |
| BigDye™ Terminator v3.1 Cycle Sequencing Kit | | 4337458 | Applied Biosystems | |
| QIAamp DNA Blood Mini Kit Print | | 51104 | Qiagen | |
| PureLink™ RNase A (20 mg/mL) | | 12091021 | Invitrogen | |
| Luminescent ATP Detection Assay Kit | | ab113849 | Abcam | |
| Quick Start™ Bradford Protein Assay | | 5000201 | Biorad | |
| Novex™ Tris-Glycine SDS Sample Buffer (2X) | | LC2676 | Invitrogen | |

**Supplemental Table 3.** Prevalence of diverse mtDNA deletions in patients with CHB, NASH and controls.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Patients and controls** | **Number** | **No. of patients with each mtDNA deletion** | | | | **No. of Patients**  **with at least one mtDNA deletion, (%)** |
| **4977-bp** | **5385-bp** | **2451-bp** | **4881-bp** |
| Controls  CHB F0-F4  CHB F0-F2  CHB F3-F4  NASH (F4) | 24  65  46  19  12 | 5  46  30  16  7 | 0  2  0  2  0 | 0  21  12  9  0 | 0  5  0  5  0 | 5 (21%)  56 (86%)\*  30 (65%)  18 (95%)#  7 (58%)†‡ |

\*, CHB *vs* Controls*, қ2=34.68; p<0.005*; #, CHB and F3-F4 fibrosis *vs* CHB and F0-F2 fibrosis*, қ2=20.04; p<0.005);* †, NASH *vs* Controls, *kc2 = 5.06, p<0.025*; ‡, CHB and F3-F4 fibrosis *vs* NASH and cirrhosis (F4), *k2 =6.93, p<0.005.* Chi-square (*Ƙ2*) test; Chi-square corrected for continuity (*kc2*) test.

**Supplemental Table 4.** ROS and RNS Formation, Mitochondrial and Total GSH and Culture Medium Nitrite/Nitrate Levels in HepG2 cells Replicating HBV or Expressing Wild-type or Mutated HBx and Corresponding Control Vectors at 24 h post-transfection.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **ROS/RNS formation** (% of corresponding vector) | **pGEM** | **Payw1.2 (HBV)** | **pcDNA3.1** | **pcDNA3.1Flag-HBx** | **pcDNA3.1Flag-HBx(G124L,I127A)** |
| Mitochondria + MitoSox + Mal/Glu | 99 ± 9 | 172 ± 13\* | 98 ± 10 | 146 ± 12\* | 112.81 ± 14† |
| Mitochondria + MitoSox + succinate | 100 ± 12 | 190 ± 18\* | 100 ± 12 | 161 ± 12\* | 119 ± 21† |
| Mitochondria + H2DCF + Mal/Glu | 100 ± 14 | 147 ± 10\* | 102 ± 11 | 138 ± 8\* | 104 ± 18† |
| Mitochondria + H2DCF + succinate | 100 ± 10 | 223 ± 29\* | 101 ± 7 | 166 ± 14\*# | 121 ± 12† |
| Homogenate + DHR123 + Mal/Glu | 98 ± 19 | 155 ± 11\* | 98 ± 18 | 159 ± 19\* | 105± 8† |
| Homogenate + DHR123 + succinate | 102 ± 15 | 188 ± 23\* | 100 ± 15 | 184 ± 22\* | 114 ± 15† |
| **Mitochondrial GSH** (nmol/mg protein) | 8.84 ± 1.12 | 3.63 ± 0.58\* | 9.24 ± 1.12 | 4.21 ± 1.10\* | 8.88 ± 1.33† |
| **Total GSH** (nmol/mg protein) | 25.40 ± 10 | 19.01 ± 4.48 | 20.25 ± 2.8 | 18 ± 4.74 | ND |
| **Medium nitrite/nitrate** (µM) | 4.42 ± 0.56 | 9.76 ± 1.22\* | 3.63 ± 1.05 | 7.28 ± 0.85 \* | ND |

Note: Mitochondrial ROS and RNS formation was evaluated by recording the relative fluorescence of specific fluorogenic dyes. Mitochondrial superoxide formation was detected with MitoSOX Red, and mitochondrial peroxide formation with H2DCF after incubation of mitochondria with either malate and glutamate (mal/glu) or succinate. The formation of peroxynitrite and other ROS in a whole homogenate was evaluated with the mitochondrial-avid dye, DHR123. Values are expressed as mean ± SEM for 3-8 determinations in each condition. \*Different from corresponding empty vector*, p<0.05*. #Different from HBV*, p<0.05. Mann-Whitney U-test.* †,different from wild type HBx, *p<0.05*. DHR123, dihydrorhodamine 123; GSH, reduced glutathione; H2DCF-DA, 2’,7’-dichlorodihydrofluorescein diacetate; mal/glu, malate/glutamate; RNS, reactive nitrogen species; ROS, reactive oxygen species; ND, not determined.

**Supplemental Table 5.** Effects of HBV Replication, Wild-type HBx or Mutated HBx(G124L,I127A) Expression on mtDNA Levels and Complex I Activity and Effects of the Mitochondrial-Targeted Superoxide Scavenger Mito-Tempo [(2-(2,2,6,6-Tetramethylpiperidin-1-Oxyl-4-Ylamino)-2-Oxoethyl)-Triphenylphosphonium Chloride, Monohydrate] and Specific Inducible Nitric Oxide Synthase Inhibitor (1400W) on HBx- and HBV-Induced Mitochondrial DNA Depletion and Complex I Impairment in HepG2 cells.

|  |  |  |
| --- | --- | --- |
|  | **mtDNA/nDNA hybridization ratio**  **(% corresponding vector)** | **Complex I activity**  **(% corresponding vector)** |
| pcDNA3.1 | 100 ± 08 | 99 ± 12 |
| pcDNA3.1Flag-HBx | 48 ± 12\* | 62 ± 9\* |
| pcDNA3.1Flag-HBx(G124L,I127A) | 88 ± 10# | 92 ± 14# |
| pcDNA3.1FlagHBx (HBx) + Mito-Tempo | 86 ± 05# | 91 ± 10# |
| pcDNA3.1FlagHBx (HBx) + 1400W | 79 ± 12# | 80 ± 11 |
| pGEM | 101 ± 09 | 100 ± 11 |
| Payw1.2 (HBV) | 46 ± 05\* | 54 ± 08\* |
| Payw1.2 (HBV) + Mito-Tempo | 81.84 ± 06§ | 89.03 ± 12§ |
| Payw1.2 (HBV) + 1400W | 69.84 ± 12\*§ | 62.03 ± 06\* |

Mito-TEMPO (5 µM) or LNAME (5 µM) were added to the cultured HepG2 cells 30 minutes before transfection. A second dose of the treatment was added 16 h after transfection. Values represent means ± SEM for 3-8 determinations. \*Different from corresponding empty vector*, p<0.05*. #Different from wild type HBx, *p<0.05.**§*Different from HBV*, p<0.05. Mann-Whitney U-test.*

**SUPPLEMENTARY FIGURE LEGENDS**

**Supplemental Figure 1.** **Analyses flow chart.** Different amounts of DNA, RNAs and/or proteins were obtained from distinct groups of patients and controls depending on the size of liver fragments that remained available after histological studies. In patients with CHB, RNA, DNA and proteins were simultaneously obtained in 40 cases (29 with F0-F2 fibrosis and 11 with F3-F4 fibrosis), and both DNA and proteins obtained in 13 patients. The remaining 93 patients have either DNA, mRNA or proteins. The remaining 93 patients have only mRNA (64 cases), DNA (25 cases) or proteins (4 cases). mtDNA damage search, mRNAs levels, immunoblotting, and liver ATP content were then assessed in distinct groups of 65, 104, 44, and 30 patients with CHB, respectively. The 33 patients with CHC had only total RNA, and all the 12 patients with NASH had simultaneously RNA, DNA and proteins. Eight (8) of the 24 controls subjects (non-CHB, non-CHC, non-NASH) had both RNA, DNA and proteins, while 16 had simultaneously DNA and proteins. mRNAs and/or proteins expression were analyzed for the main genes involved in mitochondrial function (MT-CO1, MT-CO2 and COX4I1 subunits for complex IV; MT-ATP8 subunit for complex V), mitochondrial biogenesis (PPARGC1α, TFAM), mitochondrial fusion/fission (MFN1/2, OPA-1, UTRN), mtUPR (HSPD1, HSPA9, LONP1 protease), mitophagy (PINK1, PRKN), oxidative stress (iNOS, MnSOD), and genes for the inflammatory cytokines (TNFα, IL6, IL1β).

**Supplemental Figure 2. Characterization and map of mtDNA deletions.** (A) DNA sequences flanking the four mtDNA deletions characterized in the present study. For some DNA samples that gave an unexpected mtDNA PCR product, the mtDNA band was excised, recovered, and sequenced by single-primer PCR amplification in the presence of dye terminators. Sequences are those of the L-strand, reading 5’ to 3’. Brackets and numbers indicate the deletion boundaries. The deletions start within the genes for subunit 8 of MT-ATP8 (A8), subunit 3 of cytochrome *c* oxidase (MT-CO3) or subunit 3 of reduced nicotinamide adenine dinucleotide dehydrogenase (ND3). The four deletions end up within the gene coding for subunit 5 of reduced nicotinamide adenine dinucleotide dehydrogenase (ND5). All deletions are flanked by long or short direct repeats (underlined). All remove the sequence between the direct repeats plus one of these repeats. (B) mtDNA map showing the 37 mtDNA-encoded genes. The position of the four mtDNA deletions and the corresponding affected genes were shown. D-LOOP: the regulatory region Displacement loop; OH: origine of replication for the heavy strand; Ol: Origine of replication for the light strand; RRN12S, RRN16S: Ribosomal RNA 12S and 16S; CO1, 2, 3 : Subunits 1, 2 and 3 of cytochrome c oxidase (complex IV); ATP6, 8: subunits 6 and 8 of ATP synthase (complex V); ND1, 2, 3, 4L, 4, 5 and 6, subunits 1, 2, 3, 4L, 4, 5 and 6 of reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (complex I); CYTB: cytochrome b (complex III). The 22 transfer RNA (TRN) genes for the following amino acids: F, V, L1 (codon UUA/G), I, Q, M, W, A, N, C, Y, S1 (UCN), D, K, G, R, H, S2 (AGC/U), L2 (CUN), E, T and P are indicated.

**Supplemental Figure 3.** (A) mtDNA/nDNA ratio as assessed by qPCR amplification of mtDNA-encoded MT-ND1 gene and nDNA-encoded β-globin gene in 6 control subjects and 12 patients with CHB (6 F0-F2 and 6 F3-F4). (B and C) Proportions of the three main mtDNA topology forms. Liver DNA (1.5-3 μg) was separated on agarose gels without intercalating dyes. Separated DNA was transferred on H+-Hybond nylon membranes and southern blots were hybridized with α-dCTP32-radiolabeled 6.08-kb mtDNA probe. mtDNA Southern blots from one control and two patients with CHB were shown (B). The control subjects mainly contain supercoiled and circular mtDNA molecules. DNA from patients with CHB shows disappearance of supercoiled mtDNA and increased linear mtDNA. (C) Relative proportions of the three main mtDNA forms. The percentages of supercoiled, full-length linear and circularforms of mtDNA were determined by densitometry analysis of Southern blot autoradiographs. Results are means ± SEM for 5 control subjects, 5 patients with F0-F2 and 5 patients with F3-F4 fibrosis. \*Different from control subjects, *p<0.05;* Mann-Whitney U-test. Bp, base pairs; CHB, Chronic Hepatitis B; mtDNA, mitochondrial DNA; MT-ND1, NADH dehydrogenase 1; nDNA, nuclear DNA.

**Supplemental Figure 4.** **Liver mRNA levels for nDNA-encoded cytochrome *c* oxidase 4 (COX4i1) and protein levels for TOMM20, MFN1 and OPA1.** (A) Cox4i1 mRNA levels were measured in controls (n=8), patients with CHB and F3-F4 (n=16) or F0-F2 (n=51), patients with CHC and F3-F4 (n=12) or F3-F4 (n=19) and patients with NASH and cirrhosis (n=12). (B) TOM20/β-actin ratios in control subjects (n=7), patients with CHB and F3-F4 fibrosis (n=7) or with F0-F2 fibrosis (n=13) and patients with NASH and cirrhosis (n=8). (C) MFN1/β-actin ratios in patients with CHB and F3-F4 (n=5) or F0-F2 (n=6). (D) OPA1/β-actin ratios in patients with CHB and F3-F4 (n=5) or F0-F2 (n=7). #Different from control subjects, *#p<0.05, ##p<0.01.* \*Different from F0-F2 fibrosis or F3-F4 of different etiology, *\*p<0.05, \*\*\*p<0.001.* Mann-Whitney U-test. *Mann-Whitney U-test.*

**Supplemental Figure 5. Protein and/or mRNA levels for the mitochondrial biogenesis markers TFAM and PPARGC1α.** (A) TFAM mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 (n=60), patients with CHC and F3-F4 (n=22) or F0-F2 (n=60), patients with NASH and F4 (n=12) and controls (n=8). (B) TFAM protein levels were compared in patients with CHB and F3-F4 (n=7) or F0-F2 (n=10), patients with NASH and F4 (n=7) and controls (n=7). (C) PPARGC1α mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 (n=60), patients with CHC and F3-F4 (n=12) or F0-F2 (n=21), patients with NASH and F4 fibrosis (n=12) and controls (n=8). #, ##, ###Different from controls *(p<0.05, 0.01, 0.001); \*,\*\*,\*\*\**F3-F4 compared to F0-F2 fibrosis within the same etiology or comparison of F3-F4 between etiologies, *\*p<0.05, \*\*p<0.01, \*\*\*p<0.001,* Mann-Whitney U-test. U-test. Ctrl, Controls.

**Supplemental Figure 6.** (A) Wild type or mutated HBx protein expression in HepG2 cells after 24h transfection with pcDNA3.1Flag-HBx or pcDNA3.1Flag-HBx(G124L,I127A). (B) Agarose gel showing pgRNA expression 24 h after transfection with payw1.2 vector. (C) Time course of lactate dehydrogenase activity (LDH) in the cell culture medium of HepG2 cells replicating HBV or expressing wild type HBx protein.

**Supplemental Figure 7. Inducible nitric oxide synthase (iNOS) protein and manganese superoxide dismutase (MnSOD) protein and activity, and nitration of the respiratory chain complexes in HepG2 cells overexpressing HBV or HBx protein.** (A) Representative immunoblot of the iNOS and iNOS/β-actin ratios quantification. (B) Representative immunoblot of the MnSOD protein and quantification of MnSOD/β-actin ratios. (C) Representative in-gel activity and quantification of the MnSOD and Cu,ZnSOD activities. (D) Equal amounts of HepG2 mitochondrial proteins (30 µg) underwent blue native-gel (BNG) electrophoresis to separate intact oxidative phosphorylation complexes. Separated complexes were transferred to nitrocellulose membranes for Western blot detection of 3-nitrotyrosine residues in the proteins of complex I, III, and V with an anti-3-nitrotyrosine antibody. Shown are representative gels of five independent experiments in each condition. Results are mean ± SEM from 5 independent determinations. \*Different from the corresponding empty vector, *p< 0.05*. \*\*Different from the corresponding empty vector, *p< 0.01*. *Mann-Whitney U-test.*

**Supplemental Figure 8.** **Effects of HBV or HBx on superoxide production and on mtDNA levels and integrity.** (A) Real time mitochondrial superoxide production in isolated mitochondria incubated with 5 mM of each glutamate and malate. (B) and (C) Representative slot blots and mtDNA/nDNA ratios in HepG2 cells expressing HBV (B), wild type HBx or mutated HBx (C). (D) Agarose gel for mtDNA deletions search. HepG2 cells DNA was amplified with primers A and B (Suppl. Table 1) and PCR products were run on agarose gels (1%). Data are mean±SEM from 3-8 replicates. \**p<0.05;* \*\**p<0.01, \**\*\**p<0.001*. *Mann-Whitney U-test.* NT, Non-transfected; mtDNA, Mitochondrial DNA; nDNA, Nuclear DNA; RFU, Relative fluorescence unit.