Original Paper

Hepatitis B Virus X Protein Increases 8-Oxo-7,8-Dihydro-2′-Deoxyguanosine (8-Oxodg) Level via Repressing MTH1/ MTH2 Expression in Hepatocytes

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Key Words

Hbv X protein • 8-oxo-7 •8-dihydro-2′-deoxyguanosine • MTH1 (NUDT1) • MTH2 (NUDT15)

Abstract

Background/Aims: Chronic hepatitis B virus (HBV) infection markedly increases the risk of development of hepatocellular carcinoma (HCC). Among the seven viral proteins that HBV encodes, HBV X protein (HBx) appears to have the most oncogenic potential. The mitochondria-associated HBx can induce oxidative stress in hepatocytes, leading to the production of abundant reactive oxygen species (ROS). High levels of ROS usually induce oxidative DNA damage and 8-hydroxy-2′-deoxyguanosine (8-OHdG), also known as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is one of the major products of DNA oxidation and an important biomarker for oxidative stress and carcinogenesis. Cells have evolved a mechanism to prevent oxidized nucleotides from their incorporation into DNA through nucleotide pool sanitization enzymes of MTH1 (NUDT1), MTH2 (NUDT15), MTH3 (NUDT18) and NUDT5. However, little is known as to whether HBx can regulate the expression of those enzymes and modulate the formation and accumulation of 8-oxodG in hepatocytes. ***Methods:*** The level of 8-oxodG was assessed by ELISA in stable HBV-producing hepatoma cell lines, an HBV infectious mouse model, HBV and HBx transgenic mice and HBV-infected patients versus their respective controls. Expression of MTH1, MTH2, MTH3 and NUDT5 was determined by a real-time quantitative PCR and western blot analysis. Transcriptional regulation of MTH1 and MTH2 expression by HBx and the effect of HBx on MTH1 and MTH2 promoter hypermethylation were examined using a luciferase reporter assay and bisulfite sequencing analysis. ***Results:*** In comparison with controls, significantly higher levels of 8-oxodG were detected in the genome and culture supernatant of stable HBV-producing HepG2.2.15 cells, in the sera and liver tissues of HBV infectious mice and HBV or HBx transgenic mice, and in the sera of HBV-

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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infected patients. Expression of HBx in hepatocytes significantly increased 8-oxodG level and reduced the expression of MTH1 and MTH2 at both mRNA and protein levels. It was also demonstrated that HBx markedly attenuated the MTH1 or MTH2 promoter activities through hypermethylation. Furthermore, enhancement of 8-oxodG production by HBx was reversible by overexpression of MTH1 and MTH2. **Conclusion:** Our data show that HBx expression results in the accumulation of 8-oxodG in hepatocytes through inhibiting the expression of MTH1 and MTH2. This may implicate that HBx may act as a tumor promoter through facilitating the mutational potential of 8-oxodG thus connecting a possible link between HBV infection and liver carcinogenesis.

Introduction

Hepatitis B virus (HBV) infection causes various liver diseases including chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV encodes seven viral proteins, among which the HBV X protein (HBx) appears to have the most pathogenic potential as it can act on a wide variety of signaling pathways associated with apoptosis, cell proliferation and response to DNA damage [2-4]. As a multifunctional protein, HBx executes its actions by interaction with key transcriptional factors and/or epigenetic regulation of tumor suppressor genes critical for HBV-related hepatocarcinogenesis and metastases [5-8].

HBV infection can cause oxidative stress leading to sustained and elevated generation of reactive oxygen species (ROS) such as hydroxyl radical, superoxide radical anion and hydrogen peroxide (H_2O_2) [9, 10]. HBx has been shown to increase ROS level in human liver cell lines leading to oxidative liver injury [11, 12]. ROS can readily oxidize macromolecules in living cells, and thus genomic DNA and its precursor nucleotides are always in danger of oxidation by ROS. When DNA is attacked by oxidative stress such as ROS, the interaction of hydroxyl radical HO• with the nucleobases of the DNA strand such as guanine would result in the formation of C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2′-deoxyguanosine). Then by one electron abstraction, the 8-hydroxy-2′-deoxyguanosine (8-OH-dG) is formed and further undergoes keto-enol tautomerism leading to the formation of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) while 8-OHdG and 8-oxodG represent the same compound in the scientific literature [13, 14]. The existence of this oxidized guanine in genomic DNA can cause transversion mutation such as G-T or G-A binding, accumulation of which is closely related to the occurrence and development of cancers including HCC [15, 16]. In nuclear and mitochondrial DNA, 8-hydroxy-2-deoxyguanosine (8-OHdG), also known as 8-oxo-7, 8-dihydro-2-deoxyguanosine (8-oxodG) is one of the predominant forms of free radical-induced oxidative lesions, and has been widely used as a biomarker for oxidative stress and the risk of carcinogenesis [17].

Fortunately, mammalian cells have multiple repair systems such as base excision repair (BER) enzymes and nucleotide excision repair (NER) enzymes, which counteract DNA lesions deriving from endogenous sources such as ROS. In addition to directly attacking DNA bases, ROS can also cause oxidation of dNTPs, the precursors of DNA and the starting material to build DNA, which may pose a severe threat for genetic integrity when incorporated into nascent DNA. Removal of oxidized nucleotides from the ribonucleotide pool is offered by a series of "nucleotide pool sanitization enzymes" in mammalian cells including MTH1, MTH2, MTH3 and NUDT5 (homologs of *Escherichia coli* MutT proteins) that can hydrolyze nucleoside triphosphates such as 8-oxo-dGTP to their monophosphates thus avoiding their incorporation into DNA [18, 19]. Considering the kinetic parameters, the best known substrates for human MTH1 are 2-OH-dATP and 8-oxo-dGTP of which 2-OH-ATP is hydrolyzed more effectively [20, 21]. MTH2 displays a similar substrate specificity as MutT protein in *Escherichia coli* but might have a lower intrinsic activity [22]. MTH3 acts on 8-oxo-dGDP and 8-oxoGDP instead of 8-oxo-dGTP or 8-oxoGTP [23]. NUDT5 that possesses an intrinsic activity to cleave ADP sugars to AMP and sugar phosphate also has the ability to degrade 8-oxo-dGDP to the monophosphate [24]. Thus, it is reasonably assumed that

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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removal of 8-oxo-dGTP from the nucleotide pool by the sanitization enzymes is an important mechanism to diminish the mutational potential of oxidized nucleotides that can otherwise be erroneously incorporated into DNA [25].

However, no functional role has been ascribed to HBx in regulation of the expression of nucleotide pool sanitization enzymes nor are there studies addressing a causal association of HBx with the formation and accumulation of 8-oxodG in hepatocytes. In this study, we investigated whether HBx can regulate the expression of the MutT-related proteins and modulate the formation and accumulation of 8-oxodG in hepatocytes.

Materials and Methods

Cell culture and transfection

Human hepatoma cell line Huh7 (JCRB0403, Japan) and hepatoblastoma cell line HepG2 (HB-8065, American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen). HepG2.2.15 cells (CRL-11997, ATCC) harboring four copies of HBV DNA were cultured in DMEM (Invitrogen) with 380 µg/ml G418 (Geneticin, Invitrogen). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C. Transfection was carried out using Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 2.5 µg plasmid was mixed with 5 µl Lipofectamine 3000 and 5 µl P3000 enhancer reagent in 0.25 ml Opti-MEM reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA). After 15-min incubation at room temperature, the mixture was added to the cells growing in 6-well plates. 48 h after transfection, the transfected cells were collected for subsequent experiments.

Patients

A total of 100 patients with chronic hepatitis B enrolled in this study were outpatients of Mengchao Hepatobiliary Hospital of Fujian Medical University. These patients were positive for HBV DNA and had serum HBsAg and anti-HBc IgM positive results. The HBV viral load was quantified using Abbott real-time HBV PCR method (Abbott Laboratories, Chicago, IL). The plasma samples were processed by an automated sample preparation system, m2000sp, that uses a magnetic microparticle-based principle for purification of DNA. Amplification is carried out in the Abbott m2000rt system, and the HBV DNA concentration is calculated from the calibrators provided with the kit. The extraction system uses an initial sample input of 500 µl and final elution of 70 µl. This system targets the HBV surface gene for real-time detection with a PCR input volume of 50 µl, and the manufacturer's stated LLD for HBV DNA is 10 IU/ml (1 IU/ml ≈5.6 copy/ml) Samples that are detected with a viral load less than the LLD are showed by this system as <10 IU/ml and negative samples as not detected. No antiviral treatments were given previously to all patients included in the study. None of the patients consumed alcohol or were infected with HCV/HDV. The clinical part of this research was reviewed and ethically approved by the Institutional Ethics Committee of Fujian Medical University (Fuzhou, China). All patients had given informed and written consent.

Animal experiments

5 to 6 weeks old male C57BL/6J mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. HBV (HBV-Tg) or HBx$^{+/-}$ transgenic mice (HBx-Tg) and their wild-type (WT) littermates were used in this study [26]. Mice were housed in the Laboratory Animal Center, Fujian Medical University. They were kept with a 12 h fluorescent dark/light cycle at 24 ± 2°C temperature and 50 ± 5% relative humidity, given a normal laboratory diet and free access to tap water. The plasmid pAAV/HBV1.2 containing a 1.2-kb HBV genome [27] was dissolved in 2 ml physiological saline and injected into the tail veins of mice within 5 seconds following the hydrodynamics-based transfection protocol. Negative control mice were injected with physiological saline only. At the end of experiments, mice were sacrificed and their sera and livers were immediately collected and frozen in liquid nitrogen. The study was approved by the Laboratory Animal Welfare and Ethics Committee of Fujian Medical University.

Construction of plasmids

The HBx gene of HBV was cloned into pFLAG-CMV-2 (Sigma-Aldrich, St. Louis, MO, USA) to generate pFLAG-HBx (designated here as pHBx) as we previously described [28]. The plasmid pGL4.10-MTH1 with the MTH1 promoter driven firefly luciferase was constructed by ligation of the PCR-generated full length MTH1 promoter (nucleotides -2088 to +216, relative to the transcription start site) into the Kpn I and Nhe I (New England BioLabs, Beverly, MA) cleaved sites of the luciferase reporter plasmid pGL4.10 (Promega, Madison, WI). The plasmid pGL4.10-MTH2 with the MTH2 promoter driven firefly luciferase was constructed by ligation of the PCR-generated full length MTH2 promoter (nucleotides -1805 to +75, relative to the transcription start site) into the Sac I and Xho I (New England BioLabs, Beverly, MA) cleaved sites of the luciferase reporter plasmid pGL4.10 (Promega). All plasmids were prepared with Plasmid Maxi Purification Kit (Qiagen).

PCR-generated MTH1 or MTH2 cDNA was inserted into the EcoR I and Kpn I site of pcDNA3.1/myc-His(-) A (Invitrogen) to generate the resultant vectors pcDNA3.1/myc-His(-) A-MTH1 and pcDNA3.1/myc-His(-) A-MTH2, designated here as pcDNA3.1, pMTH1 and pMTH2, respectively. Paired primer sequences for PCR amplification of MTH1 (MTH1F and MTH1R) and MTH2 (MTH2F and MTH2R) are as follows: MTH1F: 5'-GGAATTCGGTCAGAGGCCACG CCC-3', MTH1R: 5'-GGGGTACCCACCGCTAGATAGCTT-3'; MTH2F: 5'-GGAATTCATTC CCCAACCTGATAG-3'; MTH2R:5'-GGGGTACCATGAGCAACAGAAATTTAT-3'.

Western blot analysis

Cell lysates were prepared using RIPA lysis buffer which contains a protease inhibitor cocktail (Roche). Total proteins were quantified by the Bradford method (Bio-Rad, Hercules, CA, USA). Protein from each sample was electrophoresed through 10–15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA) for 30 min at 150 V using a Transblot SD apparatus (Bio-Rad). Membranes were blocked in 5% milk in Tris-buffered saline for 1 h at room temperature. Blots were incubated at 4°C overnight with the specific antibodies. Membranes were then washed three times with the same buffer containing 0.05% Tween-20 and incubated with a horseradish peroxidase-coupled secondary antibody. Reactive proteins were visualized with the ECL Western blotting detection system (Amersham, Arlington Heights, IL, USA) and analyzed densitometrically by a ChemiImager (Alpha Innotech Corporation, San Leandro, CA, USA). The specific antibodies used included anti-MTH1 (PA5-52963, 1:1000 dilution; Pierce Company, Rockford, IL, USA), anti-MTH2 (SAB1102557, 1:1000 dilution; Sigma), anti-MTH3 (HPA028581, 1:200 dilution; Sigma), anti-NUDT5 (ab129172, 1:1000 dilution; Abcam), anti-β-tubulin (M20005L, 1:1000 dilution; Abmart, Shanghai, China). Proteins were detected by addition of alkaline phosphatase-conjugated secondary antibody. The bands were scanned using ImageQuant LAS 4000 mini (GE Healthcare, Waukesha, WI, USA). Intensities of band signals were quantified using the densitometric software Quantity One (Bio-Rad, Hercules, CA, USA), β-tubulin served as an internal control.

RNA extraction and real-time RT-PCR analysis

Total RNA was isolated from HepG2 or Huh7 cells using the TRIzol Reagent (Invitrogen). The integrity of extracted RNA was checked by agarose gel electrophoresis. A spectrophotometer (Thermo scientific, Waltham, MA, USA) was used to detect the RNA concentration and purity. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time RT-PCR was performed with the Mx3000P Real-Time PCR System (Agilent Technologies, PaloAlto, CA, USA) and the SYBR Premix Ex Taq Kit (TaKaRa). The assay was carried out in a final 20 μL reaction volume and performed using 2 × PCR Premix Ex Taq with 2 μL of sample cDNA. The forward and reverse primers were used for amplification with a PCR protocol consisting of a denaturation program (95°C for 3 min) and amplification and quantification program repeated 40 times (95°C for 10 s and 55°C for 45 s) and melting curve analysis. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The △Ct values from each cell line were obtained by subtracting the values for GAPDH Ct from the sample Ct. A 1-unit difference in Ct value represents a 2-fold difference in the level of mRNA. The paired forward and reverse primers were 5'-TGCACCACCAACTGCTTAGC-3' and 5'-AGCTCAGGGATGACC TTGCC-3' (for GAPDH), 5'-TTGAGTTCGTGGGCGAGC-3' and 5'-GCATGGGCGCATTT CG-3' (for MTH1), 5'-AGCATCCGCGTTGCGTCCT-3' and 5'-CGAGGTTTTAGGAGGTT-3' (for MTH2), 5'-GTGTGCTACGTGGTGCT-3' and 5'-AGGTCCGTGGGTACCAGG-3' (for MTH3), 5'-GTTCTCCAGCGGTCTGTATG-3' and 5'-CTTCGGCCTTGCGTTTTCG-3' (for NUDT5).

KARGER

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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Luciferase reporter assay

Cells were transiently co-transfected with the MTH1/MTH2 promoter luciferase reporter constructs pGL4.10-MTH1 or pGL4.10-MTH2 and pHBx plasmids. The promoterless vector pGL4.10 and pcDNA3.1 served as the negative control. 48 h after transfection, cells were harvested and lysed in Glo Lysis Buffer (Promega), and 20 µl of cell lysate was used for the detection of intracellular luciferase activity with a luminometer (Orion II Microplate Luminometer; Berthold Detection Systems, Germany) using Bright-Glo Luciferase Assay System (Promega). Each transfection was performed in triplicate and the data were expressed as mean ± SD of three separate experiments.

Bisulfite DNA sequencing

DNA was extracted from Huh7 cells by DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Purified DNA quality and concentration were assessed using a Beckman Coulter DU-800 spectrophotometer and requiring a 260/280 ratio of greater than 1.7. Bisulfite conversion of the sample genomic DNA was done by using EpiTect Bisulfite kit (Qiagen). After thermal denaturation and sodium bisulfite DNA conversion, the DNA was applied to an EpiTect spin column where, using optimized buffers and a standard microcentrifuge or vacuum manifold, it was washed to remove all traces of sodium bisulfite and eluted. The eluted DNA (bisulfite-modified DNA) was amplified by PCR with Hotstart Taq (Qiagen) and MTH1/MTH2 promoter/exon1-specific primers. PCR was performed in the following thermal cycling program: 94°C 15 min, 42 cycles of 94°C 30 sec, 50°C 30 sec, 72°C 60 sec with additional 7 min at 72°C. The amplicons were visualized on 1.5% agarose gels under UV light, and then purified for subcloning into the pMD18-T vector (Takara, Japan). To determine the CpG methylation status of the 5-CpG island of the MTH1/MTH2 gene, 10 randomly selected clones from each cell line were sequenced.

Chemicals

5-Aza-2-deoxycytidine (DAC), Trichostatin A (TSA) and H_2O_2 were purchased from Sigma (Massachusetts, USA). Huh7 Cells were treated with 5 mM DAC for 72 h or 0.67 mM TSA for 24 h, or 0.1mM H_2O_2 for 48 h.

Quantification of 8-oxodG by ELISA

Genomic DNA was extracted from HepG2 or Huh7 cells using DNAzol Reagent (Invitrogen) according to the manufacturer's protocols. Briefly, cells grown in monolayer was lysed in DNAzol Reagent (0.75-1.0 ml of DNAzol Reagent/10 cm^2 culture plate area) by gently agitating and transferred into a 1.5 ml clear-colored tube. To precipitate DNA, 500 µl chilled absolute ethanol was added, and the tube was centrifuged at 12, 000 g for 15 min. The pellet was washed twice with 500 µl of 70% ethanol and centrifuged at the above condition for 3 min to remove residual salt. The pellet was air-dried for 5-15 seconds after removing the ethanol and dissolved in 8 mM NaOH. Extracted DNA was quantitated spectrophotometrically and adjusted to the final concentration at 200 µg/ml. 100× cations were added to DNA solution for final 1× concentration. The DNA in the mixture was digested by DNase I and alkaline phosphatase sequentially for 1 h at 37°C. For measurement of 8-oxodG in culture supernatant, the cell culturing medium was clarified for use after centrifugal removal of the cell debris. Tissue and serum samples were collected from mice or human as described above. After samples clarification, 8-oxodG detection was performed by an HT 8-oxodG ELISA Kit II (Trevigen, Gaithersburg, MD, USA). Briefly, the samples and the different amounts of a standard 8-oxodG solution were added in triplicate to 96 pre-coated wells of the plate. Then, anti-8-oxodG monoclonal solution that binds competitively to 8-oxodG was added to all wells; PBST (1× PBS containing 0.1% Tween 20) was used to wash the excess anti-8-oxodG antibody. The diluted goat anti-mouse IgG-HRP conjugate was added to all wells. PBST was used to wash the excess antibody. The pre-warmed TACS-Sapphire colorimetric substrate was added to all wells. 0.2 M HCl was used to stop the reactions. Absorbance was measured in a microplate reader (BioTek; Midland, ON, Canada) at 450 nm, and quantitation of 8-oxodG was calculated from the standard curve.

Statistical analysis

Data were presented in form of mean ± SD. Statistical analyses were performed using two-tailed Student's *t*-test. p value < 0.05 was considered as statistical significance.

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Cellular Physiology and Biochemistry

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DOI: 10.1159/000495166

Published online: 15 November 2018

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Results

HBV infection increases 8-oxodG level

The effect of HBV on regulating the 8-oxodG level was first evaluated in the stable HBV-producing hepatoma cell line HepG2.2.15, which had been proven to have stable HBV expression and replication in the culture system [29]. An 8-oxodG standard curve was generated to calculate the 8-oxodG sample concentrations using the polynomial equation
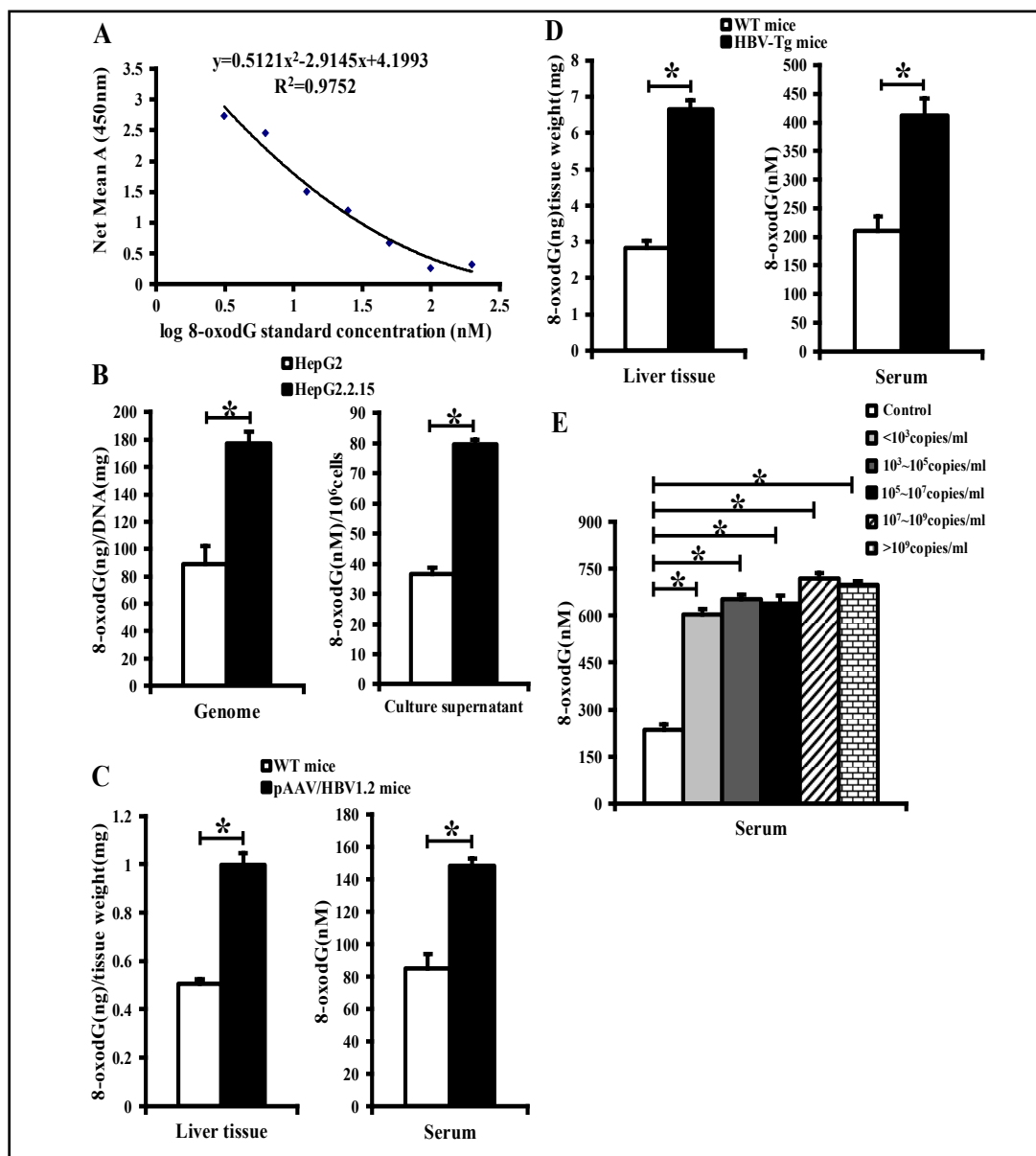
Fig. 1. Effect of HBV infection on regulation of 8-oxodG level. (A) 8-oxodG standard curve where the relative absorbance was plotted versus the log of standard 8-oxodG concentrations via 2-fold serial dilutions starting from 200 nM. (B) The level of 8-oxodG in the genome and culture supernatant of HBV-transfected HepG2.2.15 and the parental HepG2 cells. (C) The level of 8-oxodG in the liver tissue and serum of the HBV infectious mice with hydrodynamic injection of pAAV/HBV1.2. (D) The level of 8-oxodG in the liver tissue and serum of HBV transgenic (HBV-Tg) and wild-type (WT) mice (n=10). (E) The serum level of 8-oxodG in the HBV-infected patients with various HBV DNA copy number (n=30/group). *P<0.05.

with R^2 = 0.98 (Fig. 1A). The result showed that 8-oxodG level was significantly elevated in the genome or culture supernatant of HepG2.2.15 cells compared to HepG2 cells (Fig. 1B). To extrapolate the *in vitro* result, an HBV infectious mouse model was generated by hydrodynamic injection of a liver-targeted pAAV/HBV1.2 containing a 1.2-kb HBV genome into the tail veins of male C57BL/6 mice [27]. Three days after injection, the amount of 8-oxodG in the liver tissues and serum were measured that demonstrated a higher level in the HBV infectious mice than in the control mice (Fig. 1C).

We also examined the level of 8-oxodG in the in the liver tissues and the serum of HBV-Tg mice and in the serum of HBV-infected patients. As expected, the 8-oxodG level was significantly higher in the serum and liver tissues of mice as compared to that in the wild-type mice (Fig. 1D). Likewise, the level of 8-oxodG was markedly elevated in the serum of HBV-positive patients relative to non-HBV infected subjects although no significant difference in the 8-oxodG level was observed among the HBV-positive patients of different HBV copy numbers (Fig. 1E). These data suggest that an increase in 8-oxodG level may result from HBV infection and might play an important role in HBV infection-induced hepatic diseases.

HBx enhances the level of 8-oxodG and potentiates oxidative radical-induced DNA damage

Since HBx is the most pathogenic HBV viral protein, and has been shown to increase ROS level leading to oxidative liver injury [11, 12]. We went further to check if HBx is actually responsible for the 8-oxodG increase upon HBV infection. Huh7 and HepG2 cells were transfected with empty vector of pcDNA3.1 or pHBx and the levels of 8-oxodG were measured. As shown in Fig. 2A and B, ectopic expression of HBx in Huh7 and HepG2 cells significantly increased 8-oxodG level in the genome and culture supernatant of the cells as compared to the empty vector-transfected controls. To address directly whether HBx impairs cellular defenses against ROS-mediated injury, the cells were exposed to H_2O_2 and the levels of 8-oxodG measured. Although H_2O_2 increased the production of 8-oxodG in both genome and culture supernatant of the control cells, HBx-expressing cells produced even higher 8-oxodG level (Fig. 2A and B). This result indicates that HBx can further enhance the free radical-induced oxidative DNA damage.

We also analyzed the level of 8-oxodG in HBx-Tg mice, showing that 8-oxodG was significantly increased in the liver tissues and the serum of HBx-Tg mice relative to the wild-type mice at the same age (Fig. 2C).

HBx represses MTH1 and MTH2 expression through downregulation of their transcription

To examine the effect of HBx on the expression of MutT-related genes including MTH1, MTH2, MTH3 and NUDT5, Huh7 and HepG2 cells were transfected with HBx-expressing plasmid pHBx or empty vector pcDNA3.1 and checked for both mRNA and protein levels. As shown in Fig. 3A, B, HBx expression significantly reduced MTH1 and MTH2 mRNA levels in Huh7 and HepG2 cells. However, there was no significant change in the expression of hMTH3 or NUDT5 between HBx-expressing and empty vector-transfected Huh7 and HepG2 cells. Similar results were obtained when the protein levels were measured (Fig. 3C, D). To determine whether HBx inhibited the MTH1 or MTH2 promoter activities, a luciferase reporter plasmid pGL4.10-MTH1 or pGL4.10-MTH2 containing the human MTH1 or MTH2 promoter sequence was co-transfected with pHBx or pcDNA3.1 into Huh7 or HepG2 cells. The results revealed that the MTH1 or MTH2 promoter activities were markedly diminished in the HBx-expressing cells relative to the respective controls (Fig. 3E, F).

HBx expression does not affect DNA methylation of MTH1 or MTH2 promoter
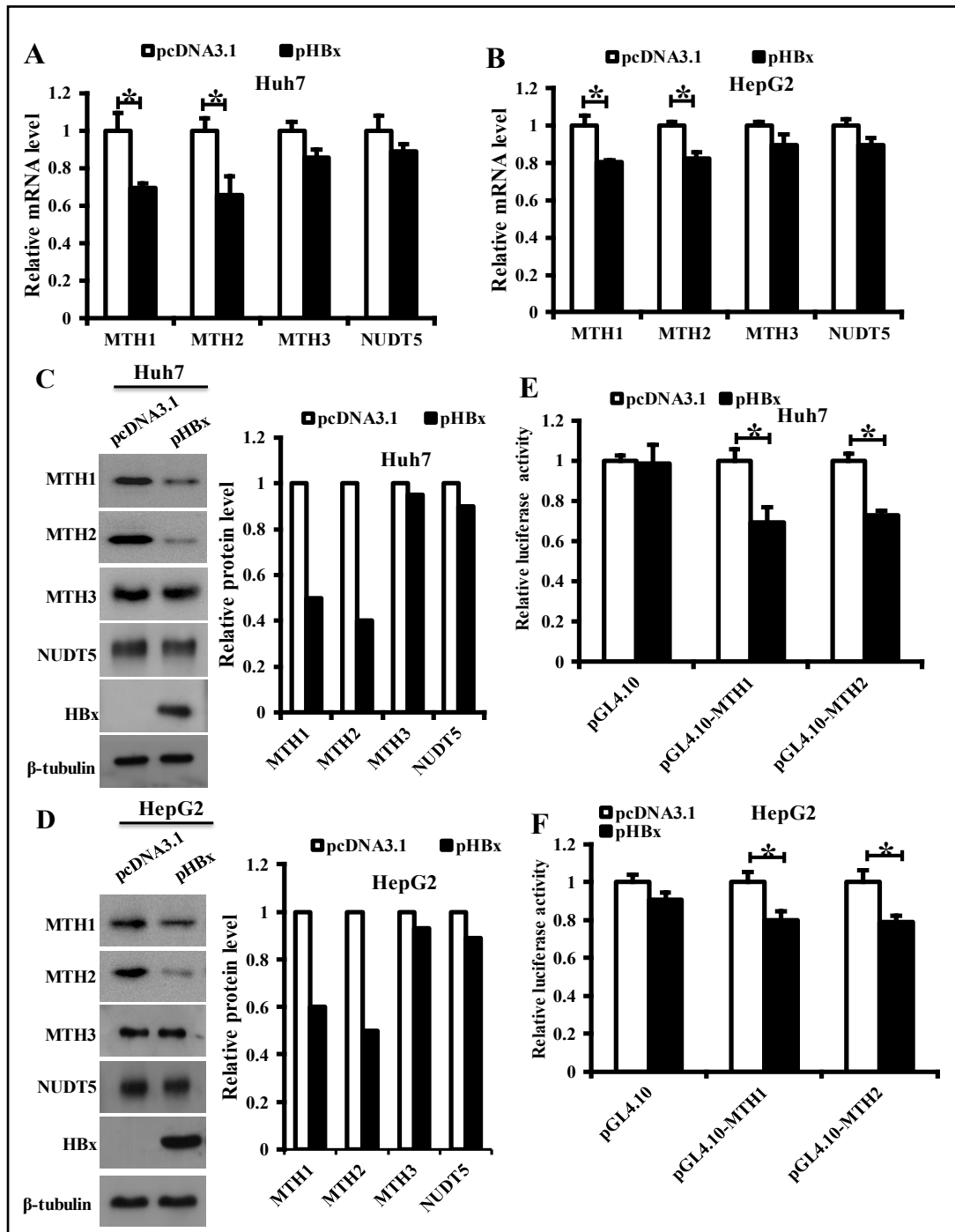
Epigenetic changes that involve DNA methylation and alterations of chromatin structure can transcriptionally silence many genes. Owing to the transcriptional suppression of MTH1 or MTH2 in HBx-expressing cells, we speculated that HBx may induce DNA methylation that silenced MTH1 or MTH2 expression. Indeed, treatment of HBx-expressing Huh7 cells with a demethylating agent DAC but not histone deacetylase inhibitor TSA effectively restored the transcripts of both MTH1 (Fig. 4A) and MTH2 (Fig. 4B).

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Fig. 2. HBx expression increased 8-oxodG level and sensitized hepatoma cells to H_2O_2-induced DNA damage. (A-B) The level of 8-oxodG in the genome and culture supernatant of HBx-expressing Huh7 and HepG2 cells with or without H_2O_2 treatment. Huh7 and HepG2 cells were transfected with pcDNA3.1 or pHBx followed by exposure to H_2O_2 for 24 h. (C) The level of 8-oxodG in the liver tissue and serum of HBx-Tg and WT mice (n=10). *P<0.05.
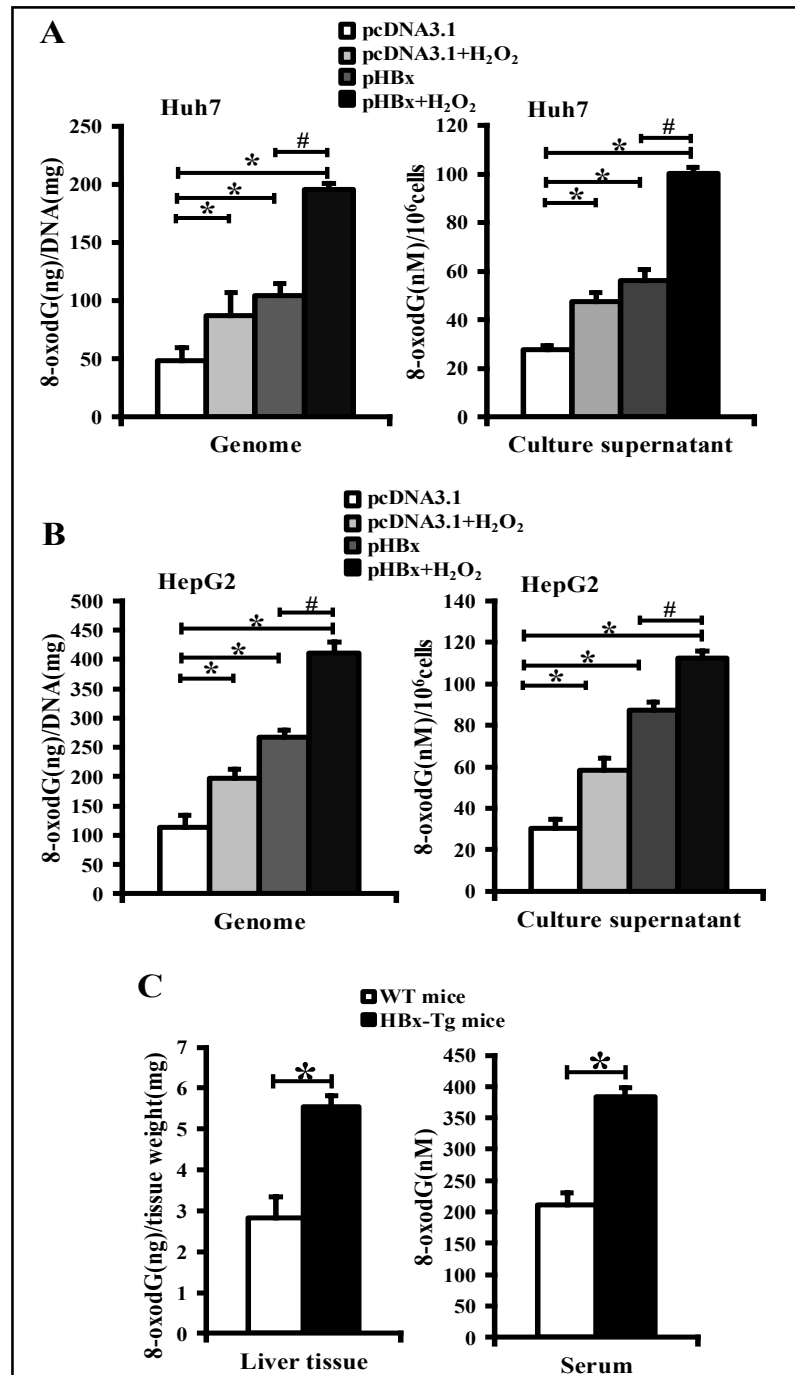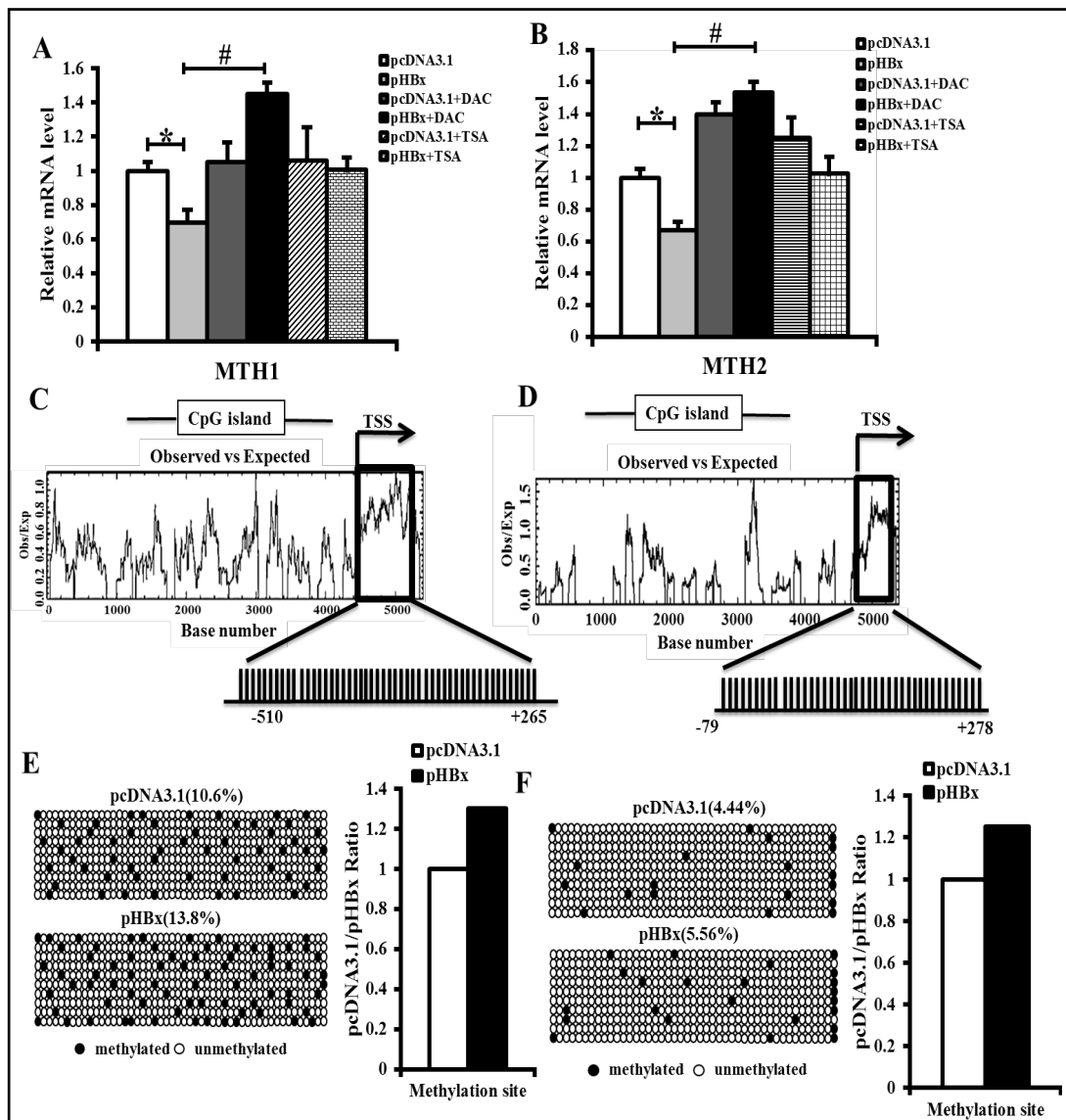

The fact that MTH1 or MTH2 mRNA expression can be restored upon DAC treatment prompted us to investigate whether MTH1 or MTH2 promoter was hypermethylated in HBx-expressing cells. Using EMBOSS CpG plot software, after depositing MTH1 and MTH2 gene promoter sequence we identified 50 and 45 CpG dinucleotides present in the MTH1 (Fig. 4C) and MTH2 (Fig. 4D) promoter region, respectively. Through extensive bisulfite sequencing analysis, we found that the extent of MTH1 or MTH2 CpG island methylation did not differ significantly in the HBx-expressing Huh7 cells as compared to the control cells (Fig. 4E and F). This result suggests that HBx-induced suppression of MTH1 or MTH2 is not attributable to a transcriptional block caused by the methylation of their promoters.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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Fig. 3. Effect of HBx expression on the transcripts of MTH1, MTH2, MTH3 and NUDT5. (A-B) mRNA levels of the four nucleotide pool sanitization enzymes in Huh7 and HepG2 cells transfected with pcDNA3.1 or pHBx. GAPDH served as an internal control. (C-D) Protein levels of the four nucleotide pool sanitization enzymes in Huh7 and HepG2 cells transfected with pcDNA3.1 or pHBx. β-tubulin served as a loading control. (E-F) hMTH1 and hMTH2 promoter activities in the HBx-expressing and control cells. Huh7 and HepG2 cells were co-transfected with 0.2 μg of the MTH1 or MTH2 promoter luciferase reporter construct, 0.5 μg pHBx and 10 ng of pRL-SV40. pcDNA3.1 and pGL4.10 served as the negative control. Luciferase activities were measured 48 h after transfection. The relative luciferase units (RLU) were obtained by comparison with the pGL4.10 plasmid, which was set to 1. Each transfection was performed in duplicate and the data were expressed as the mean ± SD of three separate experiments. *$p<0.05$.

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Fig. 4. Effect of HBx expression on hypermethylation of MTH1 or MTH2 promoter. (A-B) The relative MTH1 or MTH2 mRNA level in Huh7 cells after treatment with DAC or TSA. Cells were treated with 5 mM DAC for 72 h or 0.67 mM TSA for 24 h. MTH1 or MTH2 mRNA expression was measured by qPCR. Results were shown as mean ± SD of three independent experiments performed in duplicate. *P<0.05 versus pcDNA3.1. #p<0.05 versus pHBx. (C-D) The putative CpG islands containing 50 CpG dinucleotides for hMTH1 and 45 CpG dinucleotides for hMTH2 before the transcription start site (TSS). (E-F) Methylation status of the MTH1 and MTH2 CpG islands in Huh7 cells. Bisulfite DNA sequencing was performed to determine the methylation status of the CpG islands of the hMTH1 or hMTH2 promoter in cells transfected with pHBx or pcDNA3.1 control.

MTH1 or MTH2 overexpression reverses HBx-mediated increase of 8-oxodG

To determine whether the effect of HBx expression on the level of 8-oxodG was primarily due to its suppression on MTH1 and/or MTH2, HBx-expressing Huh7 cells were forcibly overexpressed with pMTH1 or pMTH2 or both, and then tested for changes in the 8-oxodG level. The successful expression of HBx, MTH1 and MTH2 in the respective molecularly engineered cells was confirmed by Western blot analysis using the specific antibodies (Fig. 5A). Fig. 5B and C documented that overexpression of either MTH1 or MTH2 counteracted HBx-induced accumulation of 8-oxodG in the genome and culture supernatant of HBx-

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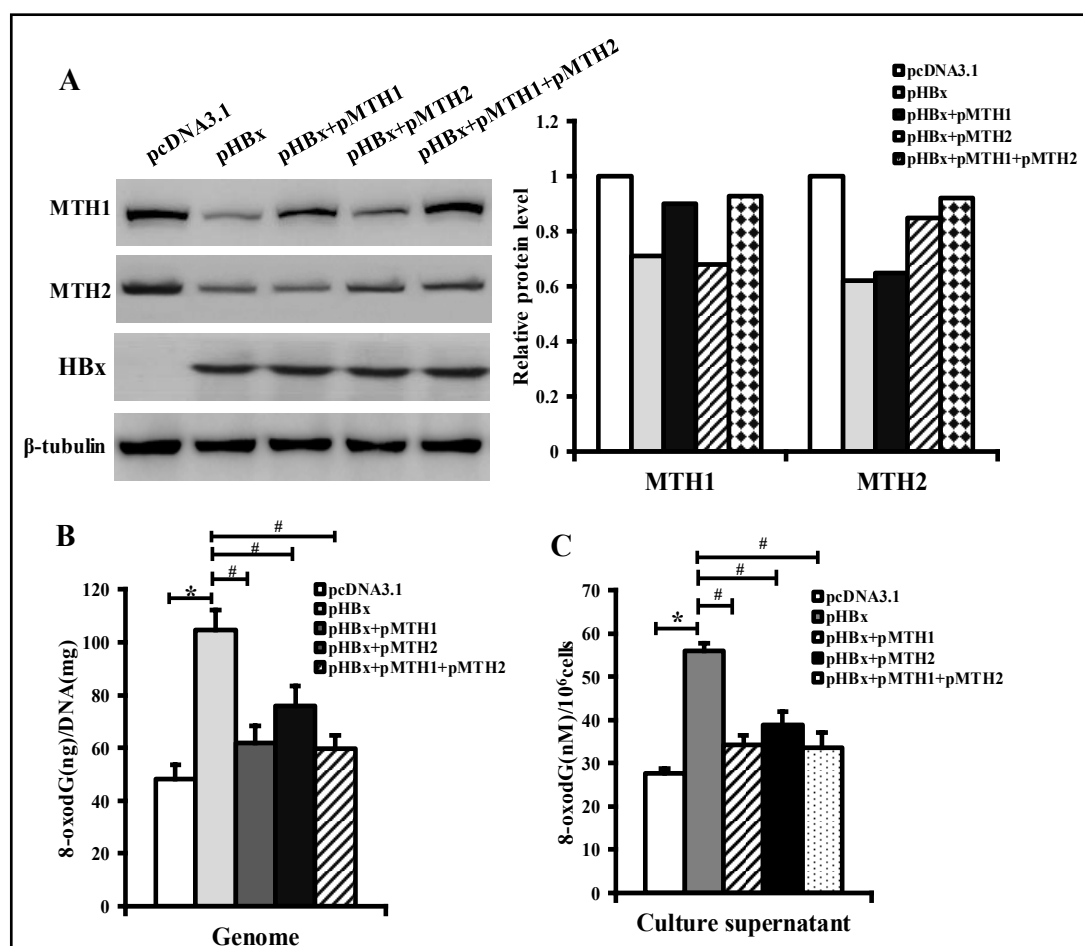
Fig. 5. HBx-induced increase of 8-oxodG was reversible by overexpression of MTH1 or MTH2. (A) Expression of HBx, MTH1 and MTH2 in the differently transfected Huh7 cells was confirmed by Western blot analysis. (B-C) The level of 8-oxodG in the genome and culture supernatant of the indicated cells. *P<0.05 versus pcDNA3.1. #p<0.05 versus pHBx.

expressing Huh7 cells while expression of both MTH1 and MTH2 did not produce a greater effect. These results indicate that the effect of HBx on 8-oxodG level is mediated, at least in part, through regulation of MTH1 and MTH2 expression.

Discussion

Oxidative stress and excessive generation of ROS can cause damage to proteins, lipids and DNA thus contributing to dysfunction, mutation, and development of a wide variety of diseases including cancer and neurodegeneration [30, 31]. Among four kinds of DNA bases, guanine (G) was the most vulnerable to ROS attacking because of its lowest redox potential. 8-oxodG is a frequently used biomarker of oxidative DNA damage and oxidative stress. If left uncorrected, it can be incorporated into DNA and cause mispair with adenine during DNA replication, leading to the induction of A:T to C:G transversion mutations [18].

Accumulation of 8-oxodG has been found in the liver of patients with chronic hepatitis [32, 33], cirrhosis [34], and HCC [35]. An immunochemical study has revealed that 8-hydroxy-2'-deoxyguanosine expression level in the noncancerous region was associated with postoperative recurrence of HCC in the remnant liver [36]. Furthermore, the number of 8-oxodG positive hepatocytes in HCC was greater than that in chronic hepatitis [36],

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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implicating that 8-oxodG-positive hepatocytes may have a high-grade malignancy potential in HCC and that 8-oxodG could thus be a useful marker of HCC development. Although 8-oxodG has been considered a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection [37], the importance of 8-oxodG in the pathogenesis of chronic liver diseases due to HBV infection remains to be clarified. In the present study, we found a significantly higher level of 8-oxodG present in the stable HBV-producing HepG2.2.15 cells, in the liver tissues and serum of HBV infectious mice and HBV or HBx transgenic mice, as well as in the serum of HBV-infected patients. These results might suggest a pivotal role of 8-oxodG in HBV-mediated pathogenesis of chronic liver diseases including HCC.

Cells have evolved various defensive mechanisms to mitigate the mutational potential of 8-oxo-G that include nucleotide pool sanitization enzymes (MTH1, MTH2, MTH3 and NUDT5) to avoid incorporation of oxidized nucleotides into DNA, base excision repair (BER) of 8-oxo-G in DNA (involving MUTYH, OGG1, Pol λ, and other components of the BER machinery), and faithful bypass of 8-oxo-G lesions during replication (using a switch between replicative Pols and Pol λ) [25]. Among these, removal of 8-oxo-dGTP from the nucleotide pools to prevent its incorporation into DNA is an important pathway for safekeeping the genome from 8-oxo-G induced mutagenicity. To date there are 4 nucleotide pool sanitization enzymes, i.e. MTH1, MTH2, MTH3 and NUDT5, that can catalyze the hydrolysis of 8-oxo-dGTP or 8-oxo-dGDP to 8-oxo-dGMP. The degradation product 8-oxo-dGMP is unusable for DNA synthesis since it cannot be reutilized to produce either 8-oxodGDP or 8-oxodGTP via phosphorylation by guanylate kinase as 8-oxo-dGMP is not the substrate of this enzyme [38]. Therefore, 8-oxo-dGTPase (MTH1) is considered as an antimutagenic enzyme that sanitizes the cellular nucleotide pool and whose inhibition is expected to increase incorporational mutagenicity of 8-oxo-dGTP. A mechanism of carcinogenesis induced by 8-oxo-dGTPase inhibition and concomitant enrichment of 8-oxodG in DNA was actually proposed and experimentally supported two decades ago [39, 40]. Several carcinogenic metals such as cadmium(II) were shown to inhibit 8-oxo-dGTPase activity and increase nuclear 8-oxo-dG level in both cell free systems and cultured cells as well as in a target organ for cadmium(II)-induced mutagenesis [40-42]. Current evidence has suggested that MTH1 is the most important determinant to nucleotide pool sanitization of 8-oxo-dGTP in mammalian cells while the other three enzymes might play a backup role for this nucleotide [25]. MTH1 hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate, thus preventing their incorporation into DNA. In the present study, we found that levels of 8-oxodG were concomitantly increased both in culture medium and in the "genome" of the HBx-harboring cells. Unlike any 8-oxoG containing nucleotides, 8-oxodG can readily cross the cell membrane making it possible to be detected in the urine or serum of patients who have diseases associated with oxidative stress [43, 44]. Therefore, it is not beyond expectation that impaired MTH1 with decreased 8-oxo-dGTPase activity due to HBx expression should redirect 8-oxoguanine of free nucleotide pool from degradation to DNA incorporation. Except for MTH1, there is no data on regulation of the levels of the other nucleotide pool sanitization enzymes MTH2, MTH3 or NUTD5 [25]. It has been reported that a single-nucleotide polymorphism (SNP) in the human MTH1 gene alters splicing patterns of hMTH1 transcripts, and that a novel hMTH1 polypeptide with an additional mitochondrial targeting signal is produced from the altered hMTH1 mRNAs; thus, intracellular location of hMTH1 is likely to be affected by a SNP [45]. Upregulation of 8-oxo-dGTPase activity of MTH1 protein was seen in the brain, testes and kidneys of mice exposed to (137) Cs gamma radiation [46]. A higher level of MTH1 has generally been observed in cancerous cells compared to normal cells [47]. For instances, the expression of MTH1 mRNA in tumors including renal-cell carcinomas [48], brain tumors [49], lung cancers [50, 51], gastric cancer [52] and esophageal squamous cell carcinomas [53] is considerably higher than that in adjacent normal tissues. These observations suggest that tumor cells rely strongly on sanitization of their nucleotide pools for proper growth. Therefore, MTH1 has been explored as a potential cancer therapeutic target, and indeed inhibition of MTH1 by small molecules TH588 and TH287, the first-in-class nudix hydrolase family inhibitors,

causes incorporation of oxidized dNTPs in cancer cells leading to DNA damage, cytotoxicity and therapeutic responses in patient-derived mouse on xenografts [54, 55]. Unfortunately, this finding has been seriously challenged by recent studies demonstrating that MTH1 is dispensable for cancer cell survival [56, 57]. Thus, it remains to be established how much cancer cells really rely on MTH1 activity. In this study, we found that ectopic expression of HBx in HepG2 and Huh7 cells markedly suppressed the transcripts of MTH1 and MTH2 but not MTH3 or NUDT5 through inactivation of the promoter activity. While the upregulation of MTH1 in certain types of cancer cells is presumed to be a result of increased oxidative stress [48-50, 52], it should also be recognized that regulation of the different key proteins participating in repair or removal of 8-oxo-G is highly diversified and complex [25]. MTH1/2 can be regulated on many different levels and the differences in findings regarding inducibility of MTH1/2 transcripts or protein levels might well be due to differences between cell and tissue types as well as the respective stresses. More detailed understanding of the exact mechanisms in the context of cell-type and stress-type specific differences is warranted in future investigations.

Cytoprotective mechanisms against excessive endogenous ROS are also provided by a variety of antioxidant and detoxifying enzymes [58]. Our prior work has demonstrated that HBx can induce epigenetic silencing of NAD(P)H:quinone oxidoreductase 1 (NQO1) in hepatoma cells by the promoter hypermethylation via recruitment of DNA methyltransferase DNMT3A to the promoter region of NQO1 gene, thus impairing mitochondrial function and increasing ROS generation with consequent increases in oxidative stress-induced hepatocytic cell death [59]. In the present study, we showed that HBx was able to transcriptionally downregulate MTH1 and MTH2 expression, and the demethylating agent DAC but not histone deacetylase inhibitor TSA could restore their transcription. This result seems to point toward the conception that HBx-induced suppression of MTH1 or MTH2 is ascribed to a transcriptional block via the methylation of their promoters. However, EMBOSS analysis and DNA bisulfite sequencing revealed that both the number of CpG islands and the extent of their methylation in the MTH1 or MTH2 promoter region were not significantly increased in the HBx-expressing cells as compared to their respective control cells. We speculated that HBx might induce DNA methylation on the MTH1 or MTH2 related transcription factors rather than on the MTH1 or MTH2 promoter itself. Another intriguing observation from this study is that overexpression of either MTH1 or MTH2 appeared to decrease the elevated 8-oxodG level in the genome and culture supernatant of the HBx-expressing cells to a similar magnitude. In mammalian cells, MTH1 protein is the best characterized of the suggested MutT homologues and is the most prominent sanitizer of the cellular oxidized dNTP pool known to date with 8-oxo-dGTPase activity. However, a previous study has also shown that MTH2 was able to convert 8-oxo-dGTP and 8-oxo-dGDP to 8-oxo-dGMP [22]. The activity of MTH2 towards 8-oxo-dGTP has led to the assumption that it may contribute to sanitizing the oxidized dNTP pool despite much lower activity than MTH1 towards 8-oxo-dGTP or 2-OH-dATP [23, 60]. More recently, Carter *et al.* reported that MTH2 was structurally distinct from MTH1 and had considerably lower activity to oxidized guanine species than to the undamaged nucleotides [61]. Moreover, knockdown of MTH2 did not alter the level of 8-oxo-dG incorporation into DNA or cancer cell survival, suggesting that MTH2 might not sanitize 8-oxo-dGTP in the cells [61]. Regardless, a most recent study using CRISPR/Cas9 approach to knock out MTH1, MTH2 or both in HeLa S3 cells demonstrated an increased sensitivity to hydrogen peroxide whereas overproduction of MTH1 and MTH2 almost completely suppressed the mutator phenotype of *mutT*-deficient cells, suggesting that both MTH1 and MTH2 are involved in the maintaining genome stability in human cells against oxidative stress [62]. While their respective contribution to sanitize the nucleotide pool is yet to be confirmed and the molecular basis of how HBx regulates MTH1 or MTH2 gene expression remains to be determined in the future studies, the observation that overexpression of MTH1 or MTH2 reversed HBx-induced accumulation of 8-oxodG implicates that the effect of HBx increasing 8-oxodG level is mediated at least partially through regulation of MTH1 and MTH2 expression.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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Conclusion

HBx expression enhances the cellular accumulation of 8-oxodG in hepatocytes via inhibition of the expression of nucleotide pool sanitization enzymes MTH1 and MTH2, which may facilitate 8-oxodG mediated mutagenesis for hepatocarcinogenesis and progression owing to HBV infection.

Abbreviations

HBx (HBV X protein); HBV (chronic hepatitis B virus); HCC (hepatocellular carcinoma); ROS (reactive oxygen species); 8-oxodG (hydroxy-2-deoxyguanosine); 8-oxodG (8-oxo-7, 8-dihydro-2-deoxyguanosine); NER (nucleotide excision repair); DAC (5-Aza-2-deoxycytidine); TSA (Trichostatin A).

Disclosure Statement

The authors have no competing interests.

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and Biochemistry

Cell Physiol Biochem 2018;51:80-96

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:80-96

DOI: 10.1159/000495166
Published online: 15 November 2018

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