

Basic Study

Activation of AMPK/MnSOD signaling mediates anti-apoptotic effect of hepatitis B virus in hepatoma cells

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Abstract

AIM: To investigate the anti-apoptotic capability of the hepatitis B virus (HBV) in the HepG2 hepatoma cell line and the underlying mechanisms.

METHODS: Cell viability and apoptosis were measured by MTT assay and flow cytometry, respectively. Targeted knockdown of manganese superoxide dismutase (MnSOD), AMP-activated protein kinase (AMPK) and hepatitis B virus X protein (HBx) genes as well as AMPK agonist AICAR and antagonist compound C were employed to determine the correlations of expression of these genes.

RESULTS: HBV markedly protected the hepatoma cells from growth suppression and cell death in the condition of serum deprivation. A decrease of superoxide anion production accompanied with an increase of MnSOD expression and activity was found in HepG2.215 cells. Moreover, AMPK activation

contributed to the up-regulation of MnSOD. HBx protein was identified to induce the expression of AMPK and MnSOD.

CONCLUSION: Our results suggest that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway, which may provide a novel pharmacological strategy to prevent HCC.

Key words: Hepatitis B virus; Reactive oxygen species; Apoptosis; Manganese superoxide dismutase; AMP-activated protein kinase

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Core tip: Hepatitis B virus markedly protected the cells from growth suppression and cell death in the condition of serum deprivation. A decrease of superoxide anion production accompanied with an increase of manganese superoxide dismutase (MnSOD) expression and activity was found in HepG2.215 cells. Moreover, AMP-activated protein kinase activation contributed to the up-regulation of MnSOD. Hepatitis B virus X protein was identified to promote the expression of AMPK and MnSOD.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed malignant cancers worldwide, while 50% of cases and deaths occurred in China^[1]. Chronic hepatitis B virus (HBV) infection has been internationally recognized as one of the major risk factors for the development of HCC^[2]. An estimated 350 million people were chronically infected and 600000 hepatitis B-related deaths occurred every year all over the world^[3]. Accumulated evidence has shown that HBV proteins, particularly hepatitis B virus X protein (HBx) and surface protein (HBs), are implicated in hepatocyte carcinogenesis^[4]. However, the mechanisms underlying HBV-induced malignant transformation remain ambiguous.

Apoptosis, also named programmed cell death, plays a crucial role in the development and homeostasis in normal tissue^[5]. Recently, studies have indicated that defect or insufficient apoptosis may contribute to carcinogenesis, tumor progression and resistance of tumor cells to chemo-radiotherapy^[6-8]. For that reason, escape of apoptosis has been iden-

tified as one of prominent hallmarks of cancer^[9]. Reactive oxygen species (ROS), as toxic products of cell metabolism, can cause cell apoptosis by leading to cellular DNA damage and subsequently activating apoptotic signaling pathways^[10]. In cancer, tumor niches characterized with poor nutrient and oxygen usually possess oxidative stress with excessive ROS formation^[11,12]. Mitochondrial ROS (mtROS) especially superoxide anion, a natural by-product of electron transport chain activity, is the main source of cellular ROS^[13]. Thus, decreasing mtROS production to relieve oxidative stress is very important for tumor survival and progression.

Manganese superoxide dismutase (MnSOD), a key antioxidant enzyme, is responsible for scavenging superoxide anion. Liver malignant tumors have been shown to express higher protein level and activity of MnSOD than their benign counterparts^[14]. Aggressive tumors possessing invasive phenotype also have a high level of MnSOD, which can facilitate them to reach distant organs^[15]. Therefore, increased MnSOD expression and activity may protect cells against apoptosis and offer a growth advantage, thereby acquiring a more aggressive phenotype.

The expression of MnSOD can be modulated by many molecular factors at transcription, translation and posttranslational modifications levels, for example, p53, Sp1, and NF- κ B^[16-18]. AMP-activated protein kinase (AMPK) is also reported to act as a new regulator of MnSOD expression in endothelial cells^[19]. Moreover, AMPK activation is associated with protection of hepatocytes against oxidative stress^[20].

Based on the aforementioned studies, we investigated the effect of HBV on the growth and survival of HepG2 cells, and explored the underlying molecular mechanisms. Herein, we demonstrated that HBV protected HepG2 cells from growth suppression and apoptosis in the condition of serum deprivation. Furthermore, AMPK activation-induced up-regulation of MnSOD contributed to the resistance of HBV-integrated HepG2 cells to apoptosis caused by superoxide, which could explain in part HBV-induced hepatocellular cell malignant transformation in the context of growth factor withdrawal.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line HepG2 was obtained from Cell Bank of Chinese Academy of Sciences where it was authenticated. HepG2.215 cell line, which was derived from HepG2 cells by integrating HBV genome and persistently produced HBV, was kindly provided by Prof. Erwei Song (Sun Yat-sen Memorial Hospital of Sun Yat-sen University, China). All of the cell lines were maintained in DMEM (Gibco, Gaithersburg, MD, United States) supplied with 10% fetal bovine serum and 1% penicillin/streptomycin, and incubated at 37 °C in a humidified incubator with 5% CO₂.

Reagents

The HiPerFect transfection reagent was obtained from QIAGEN (QIAGEN, Carson City, CA). Antibodies of AMPK α and phospho-AMPK α (Thr¹⁷²) were purchased from Cell Signaling (Cell Signaling Technology, MA). Anti-MnSOD antibody was from BD (BD Pharmingen, San Diego, CA, United States). Antibody of HBx (anti-HBx) was obtained from Abcam (Abcam, Cambridge, UK). AICAR, Compound C and anti- β -actin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States).

Cell viability assay

Cells were seeded in 24-well plates in quadruplicate. After indicated treatments, cells viability was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) (Sigma, MO) following the manufacturer's protocol. Absorbance was measured at a wavelength of 570 nm.

Cell apoptosis assay

Cells were prepared as described elsewhere^[21]. AnnexinV and propidium iodide (KeyGEN BioTECH, Nanjing, China) were added for incubation in the dark for 15 min at 4 °C, and then cells were analyzed with a flow cytometer (Gallios, Beckman).

Mitochondrial superoxide anion detection

Measurements of mitochondrial superoxide anion formation in cells were performed as previously described^[22]. In brief, HepG2 and HepG2.215 cells were incubated with 5 μ mol/L MitoSOX (Invitrogen, Carlsbad, CA) for 20 min at 37 °C. Cells were digested using EDTA (Invitrogen), and then washed three times using HBSS with Ca/Mg (Invitrogen). Mean fluorescent intensity was measured by flow cytometry (Gallios, Beckman).

MnSOD activity measurement

MnSOD activity was measured with a commercial SOD kit (Cayman Chemical) according to the manufacturer's protocol. Briefly, 1 mmol/L potassium cyanide was added in order to inhibit Cu/Zn-SOD and extracellular SOD, thus only MnSOD activity was detected. O₂⁻ was generated by adding hypoxanthine/xanthine oxidase and detected with tetrazolium salt through reading the absorbance at 450 nm.

RNA interference

The siRNAs for silencing AMPK, MnSOD and HBx genes as well as scrambled siRNA were purchased from Ribobio (Guangzhou, China). Transfection with synthetic siRNAs was performed with HiPerFect (QIAGEN, Carson City, CA) according to the manufacturer's instructions. The sense sequences of double-strand siRNA were as follows: siAMPK, 5'-UGCCUACCAUCUCAUAAUATT-3'; siMnSOD, 5'-GGAGAAUGUAACUGAAAGATT-3'; siHBx-1, 5'-CCGACCUUGAGGCAUACUUDtT-3'; siHBx-2,

5'-UGUGCACUUCGCUUCACCUUTT-3'.

Western blot analysis

Western blot analysis was performed as described previously^[23]. Antibodies for MnSOD, AMPK, Phospho-AMPK and HBx were used at 1:1000 dilution. Antibody for β -actin was used at 1:10000 dilution. Bound antibody was visualized using HRP-conjugated secondary antibodies.

Statistical analysis

All data are expressed as mean \pm standard deviation (SD). SPSS 13.0 software was used for one-way analysis of variance (ANOVA) and *t*-test in all statistical analyses (SPSS, Chicago, IL, United States). A *P* value less than 0.05 was considered statistically significant.

RESULTS

HepG2.215 is more tolerant to serum-deprivation environment

In order to assess the effect of HBV on the proliferation of HepG2 cells, we employed the HepG2.215 cell line which was derived from the HepG2 cell line and persistently produced HBV. We found that the HepG2.215 cell line showed faster growth kinetics compared with the HepG2 cell line on days 4 and 6 after serum depletion (Figure 1A). Moreover, the number of apoptotic HepG2 cells was significantly increased on days 4 and 6 compared with that of HepG2.215 cells. In contrast, the number of apoptotic HepG2.215 cells stayed at a much lower level at all testing time points (Figure 1B and C). These data suggest that HBV proteins may protect HepG2.215 cells against apoptosis induced by serum depletion.

Decreased mitochondrial superoxide level may be due to increased MnSOD expression and activity

To explain the different anti-apoptotic ability of the two cell lines, we investigated the production of mitochondrial superoxide which is a well-known killer of cells^[10]. Decreased mitochondrial superoxide level was found in the HepG2.215 cell line (Figure 2A). Since MnSOD is the regulator of mitochondrial superoxide, we therefore detected the expression and activity of MnSOD in the two cell lines. As shown in Figure 2B and C, both the expression and activity of MnSOD in HepG2.215 cells were higher than those of HepG2 cells.

MnSOD mediates the apoptotic resistance of HepG2.215 cells

To further verify the role of MnSOD in the apoptotic resistance of HepG2.215 cells, the MnSOD siRNA was synthesized. Western blot analysis revealed that MnSOD siRNA specifically knocked down MnSOD in HepG2.215 cells (Figure 3A). Knockdown of MnSOD decreased cell viability and increased mitochondrial

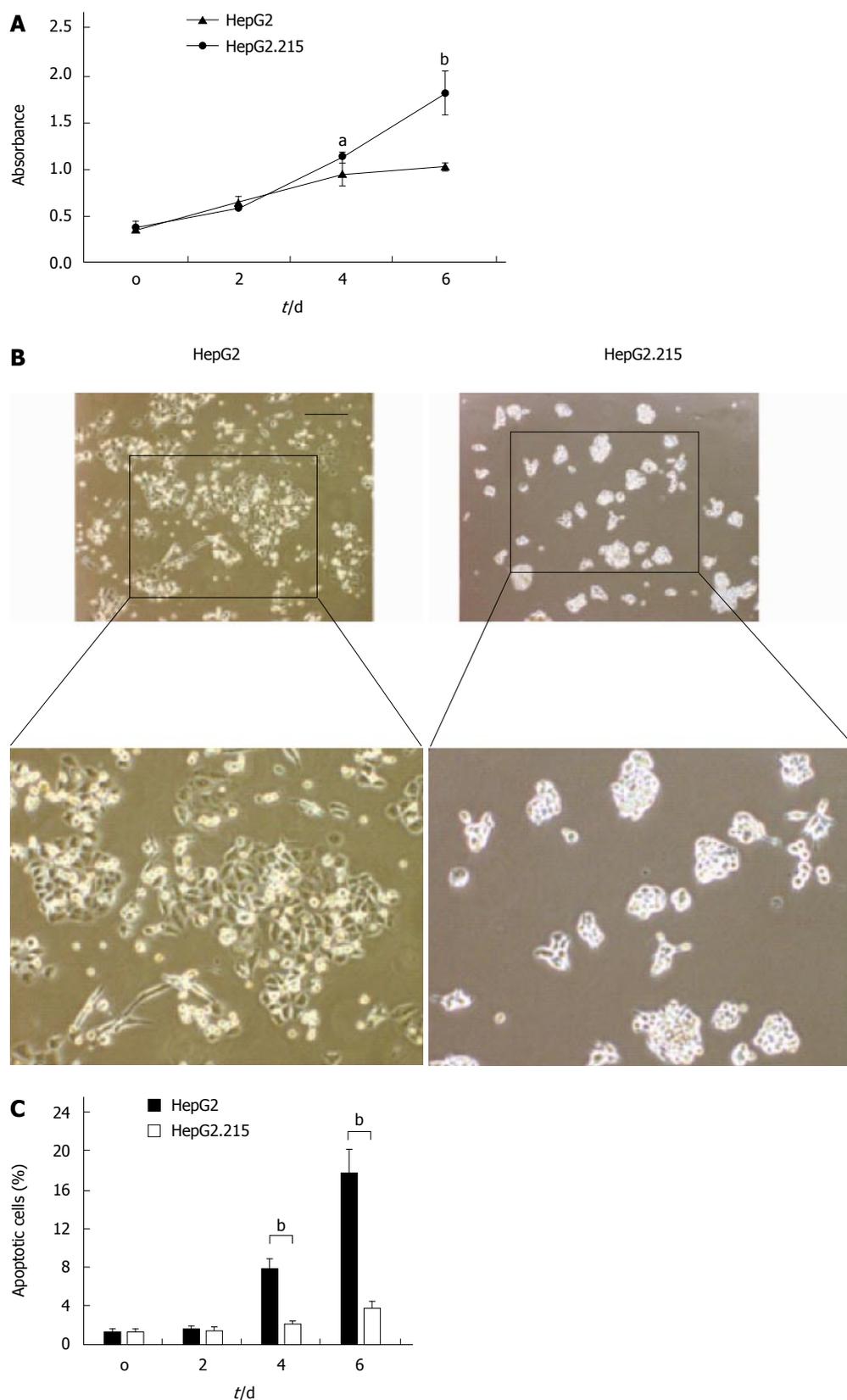


Figure 1 Resistance of HepG2.215 cells to apoptosis. A: Typical photographs of HepG2 and HepG2.215 cells cultured for 6 d after serum depletion. The data are representative of three independent experiments; B: MTT assay for the viability of cells cultured for 2, 4 and 6 d after serum depletion. Data represent absorbance at 570 nm and are shown as mean \pm SD of quadruplicates. ^a $P < 0.05$ and ^b $P < 0.01$ vs HepG2 cells group. The data are representative of three independent experiments; C: In parallel experiments, samples were subjected to FITC-Annexin V/propidium iodide staining and the quantitative analysis of apoptotic cells was performed using flow cytometry. Quantification of apoptotic cells is shown as mean \pm SD of triplicates.

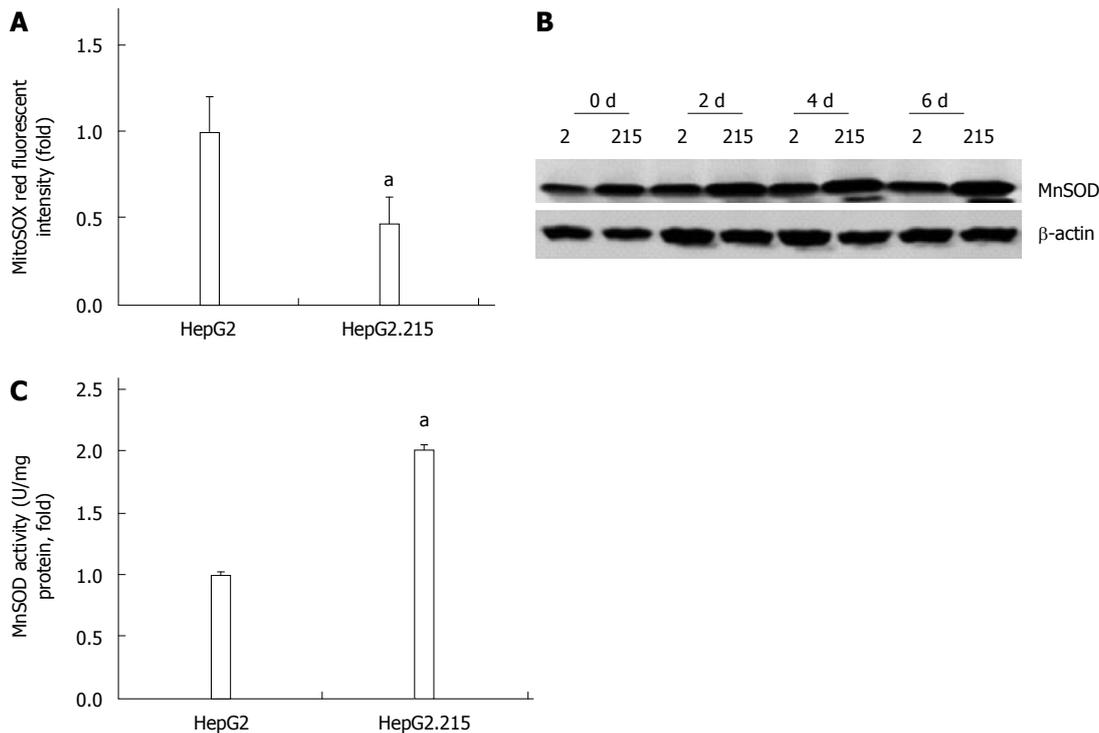


Figure 2 Decreased mitochondrial superoxide production is accompanied by increased MnSOD expression and activity in HepG2.215 cells. A: Mitochondrial superoxide level was decreased in HepG2.215 cells. Cells were cultured in serum-free medium for 6 d. Mitochondrial superoxide anion formation was measured by flow cytometry and MitoSOX. Quantification of mitochondrial superoxide anion is shown as mean \pm SD of triplicates. ^a $P < 0.05$ vs HepG2 cell group; B: Cell lysates were subjected to Western blot analysis. The protein level of MnSOD was detected; C: MnSOD activity was measured with a commercial SOD Assay Kit. Quantification of MnSOD activity is shown as mean \pm SD of triplicates. ^a $P < 0.05$ vs HepG2 cells group.

superoxide formation and the number of apoptotic HepG2.215 cells (Figure 3B-D), which suggests that MnSOD plays a critical role in apoptotic resistance of HepG2.215 cells.

AMPK activation contributes to up-regulation of MnSOD in HepG2.215 cells

To figure out the upstream factor involving the modulation of MnSOD, AMPK was investigated. We showed the protein levels of p-AMPK and AMPK were increased in HepG2.215 cells (Figure 4A). Both knockdown of AMPK and treatment with AMPK inhibitor Compound C reduced the expression of MnSOD (Figure 4B and C). Conversely, AMPK activator AICAR increased the expression of MnSOD (Figure 4C). Furthermore, the expression of p-AMPK, AMPK and MnSOD was inhibited by HBx knockdown (Figure 4D). These results suggest that HBV up-regulates MnSOD via AMPK.

DISCUSSION

As a major cause for HCC development, HBV can promote HCC in many ways, including enhancing host chromosomal stability, inducing inflammation-mediated immune escape, regulating epigenetic modification or altering the expression of oncogenes and tumor-suppressor genes^[24]. Due to these internal changes, hepatoma cells acquire the capacity of fast

growth, anti-apoptosis and metastasis^[25,26]. In this study, we confirmed that HBV-integrated HepG2 cells exerted survival benefit compared with its parent cell line HepG2 in the serum-deprivation condition which can to some extent mimic the adaptation of tumor cells to adverse growth conditions. In line with previous studies, we also found that HBV conferred HepG2 cells resistance to apoptosis^[26,27]. Our data suggest that HBV apparently acts to promote the growth and viability of hepatoma cells in growth factor-restricted conditions.

An increased level of ROS by creating a potentially toxic environment to the cells represents a critical mechanism underlying cell death^[28]. Superoxide anion is the precursor of other ROS such as H₂O₂ and peroxynitrite, and because of that the organelles most vulnerable to oxidative stress are the mitochondria^[29]. MnSOD is an essential antioxidant enzyme in the mitochondrion that acts on superoxide anion^[30]. Here, we showed that HBV reduced the level of superoxide anion. Consistently, the expression and activity of MnSOD were up-regulated in HBV-integrated HepG2 cells. This result was supported by the finding of a previous study that in patients with HBV infection, there was an average 5-fold rise of serum MnSOD^[31].

The expression and activity of MnSOD are not static in different tumorigenesis stages. For transformed phenotype, MnSOD levels were maintained at a low level and could directly potentiate mitochondrial

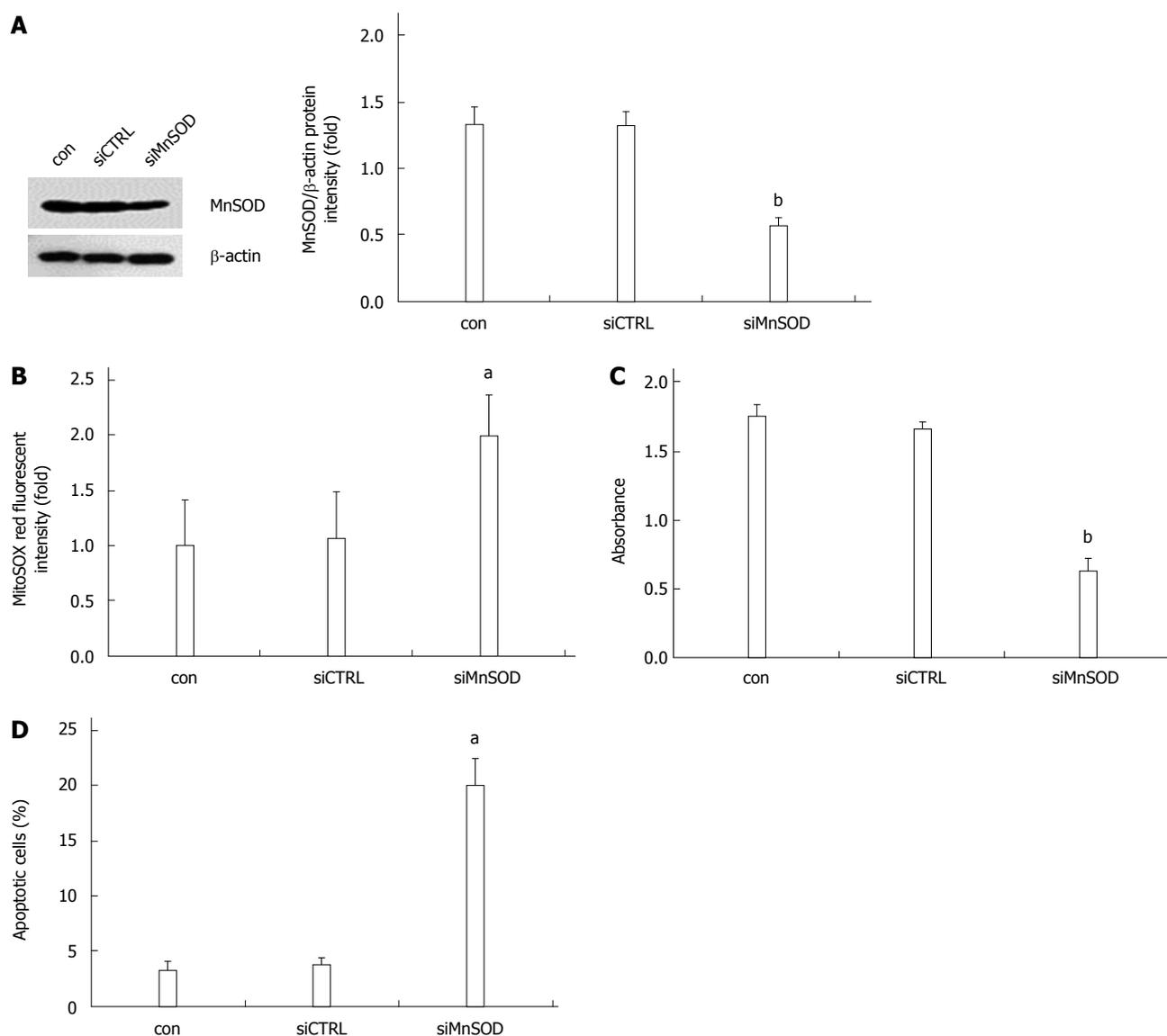


Figure 3 MnSOD contributes to decreased mitochondrial superoxide and apoptotic cells in HepG2.215 cells. A: After 6 d, the interference effect of MnSOD siRNA (siMnSOD) was analyzed by Western blot analysis. MnSOD siRNA (siMnSOD) or non-specific siRNA (siCTRL) was transfected into HepG2.215 cells for 12 h before serum depletion; B: After 6 d, cells were harvested for quantification of mitochondrial superoxide anion formation by flow cytometry. In parallel, (C) cell viability and (D) apoptotic cells were separately determined by MTT assay and flow cytometry. ^a $P < 0.05$ and ^b $P < 0.01$ vs siCTRL group.

defects, leading to gene mutations. For acquiring a more aggressive phenotype, enhanced MnSOD activity may protect cells against mitochondrial injury, thereby conferring a growth advantage to the cancer cells^[16]. The present study demonstrated that knockdown of MnSOD increased the production of superoxide anion and the apoptosis of HepG2.215 cells, which indicated that MnSOD protected hepatoma cells against apoptosis by detoxing superoxide anion, and conferred a growth advantage to those cells. However, since the function of MnSOD is to convert diffusion-restricted and mild-toxicant superoxide anion to freely diffuse and strong-toxicant H₂O₂, which means that increased MnSOD may enhance the production of more toxicant H₂O₂, the mechanism of modulation of tumor cell survival by MnSOD seems confusing. It has been reported that HBx expressing cell line showed

significantly reduced sensitivity to H₂O₂-induced cell death, and the level of intracellular ROS did not elevate in HBx expressing cell line after exposure to H₂O₂ in the medium^[32]. Based on these findings, we speculate that HBV-infected cells may express relatively high amounts of catalase, they would be able to counteract the cytotoxic effects of peroxide, and thus the outcome of increased MnSOD activity would more likely reflect the capacity of MnSOD to reduce levels of oxygen radicals. Unexpectedly, the level of catalase in HBV-related hepatocellular carcinoma specimens was lower than that of surrounding non-tumor tissues^[33]. Thus, further investigation is required to explain the tolerance of HBV-infected cells to H₂O₂-induced cell apoptosis, which will be helpful for understanding the mechanism of MnSOD-modulated tumor cell survival.

AMPK, a serine/threonine protein kinase, is well

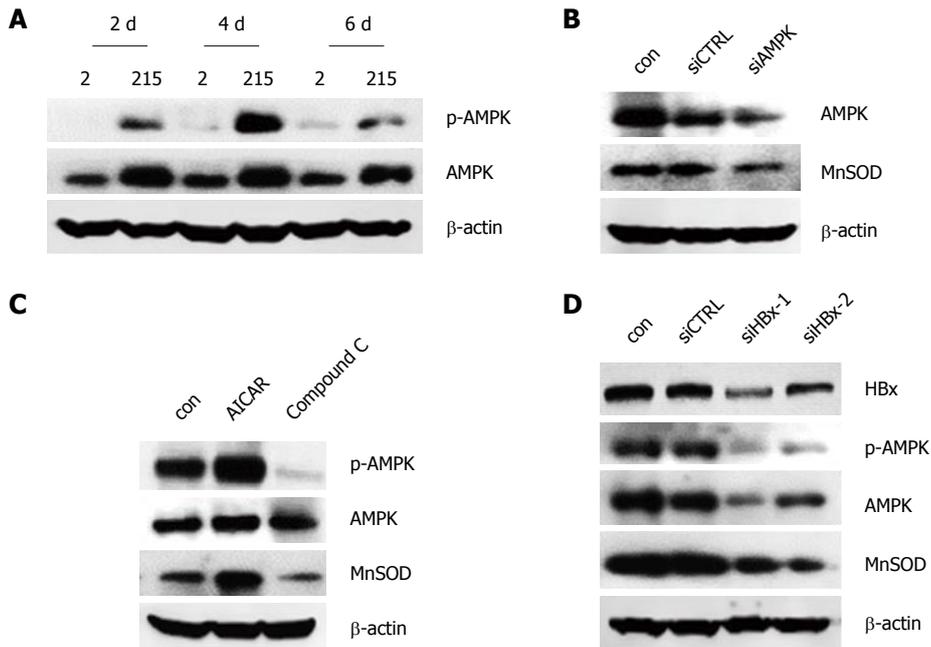


Figure 4 AMPK activation is required for MnSOD expression in HepG2.215 cells. A: Cell lysates were subjected to Western blot analysis. The protein levels of AMPK and p-AMPK were detected; B: Cells were transfected with AMPK siRNAs (siAMPK) or a nonspecific control siRNA (siCTRL) for 6 d. The protein levels of AMPK and MnSOD were detected by Western blot analysis; C: Cells were subjected to 1 mM AMPK activator AICAR or inhibitor compound C for 6 d. The protein levels of AMPK, p-AMPK and MnSOD were detected by Western blot analysis; D: Cells were transfected with HBx siRNAs (siHBx) or a nonspecific control siRNA (siCTRL) for 6 d. The protein levels of AMPK and MnSOD were detected by Western blot analysis.

known for its role in controlling energy metabolism. Recently, it comes into focus because of its potential roles in regulating other signaling pathways, such as regulating oxidative stress^[34]. Studies have reported that activation of AMPK by AICAR, or overexpression of constitutively activated AMPK suppressed O_2^- production in human neutrophils or HUVECs^[35,36]. A similar observation was also found in HepG2 cells, which showed that AA+ iron-induced reactive oxygen species generation was inhibited by isorhamnetin through AMPK activation^[20]. These studies indicate that AMPK appears to be the key factor for cellular function protection in the presence of oxidative stress. Emerging evidence suggests that AMPK inhibits oxidant production by decreasing the expression of NADPH oxidases or increasing the expression of UCP-2 as well as MnSOD^[19,35,36]. In the present study, HBV-integrated HepG2 cells displayed elevated AMPK protein level, which remains consistent with the expression of MnSOD. By utilizing a specific siRNA, or a selective agonist (AICAR) and antagonist (compound C) of AMPK, we observed that knockdown of AMPK and compound C resulted in the reduction of MnSOD protein level. Moreover, activation of AMPK by AICAR up-regulated the expression of MnSOD. Taken together, these findings demonstrate that AMPK is responsible for the up-regulation of MnSOD expression in HBV-integrated HepG2 cells.

Additionally, numerous studies have shown that HBx protein serves as a transactivator in the pathogenesis of HCC through regulating cell transformation,

apoptosis and cellular immune system^[37-39]. In our study, HBx was identified as the active ingredient of HBV proteins to promote the expression of AMPK and MnSOD. This is consistent with previous investigation reported by Severi *et al.*^[32] that HBx expressing cell line is more resistant to ROS-induced cell apoptosis than HBsAg expressing cell line. These data suggest that HBx may alleviate oxidative stress by up-regulating AMPK/MnSOD axis to maintain "normal" live cancer cell functions.

In summary, our current study demonstrates that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway in HBV-infected HepG2 cells. These findings may provide a novel mechanism involved in HBV-triggered carcinogenesis, and therefore might be useful in the design of new pharmacological approaches to prevent HCC.

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COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is one of the major risk factors for the development of hepatocellular carcinoma (HCC). However, the mechanisms

underlying HBV-induced HCC remain ambiguous. Recently, accumulated evidence has shown that escape of apoptosis may contribute to carcinogenesis.

Research frontiers

Previous experiments have revealed that liver malignant tumors and patients with HBV-infection express higher protein level of manganese superoxide dismutase (MnSOD) than their counterparts. Here, the authors showed that high expression of MnSOD protected hepatoma cells against apoptosis by detoxing superoxide anion, and conferred a growth advantage to those cells. These results explain how HBV offers a survival benefit to hepatoma cells.

Innovations and breakthroughs

This is the first study to demonstrate that HBV protects hepatoma cells against apoptosis via AMPK/MnSOD signaling pathway. HBV markedly protected the cells from growth suppression and cell death in the condition of serum deprivation. A decrease of superoxide anion production accompanied with an increase of MnSOD expression and activity was found in HepG2.215 cells. Moreover, AMPK activation contributed to the up-regulation of MnSOD. HBx protein was identified to promote the expression of AMPK and MnSOD. These results provide further evidence for the role of HBV as a major cause of HCC development via an anti-apoptosis mechanism involving activation of AMPK/MnSOD signaling pathway.

Applications

The present results suggest that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway, which may be useful in the design of new pharmacological approaches to prevent HCC.

Peer-review

In this study, Li *et al* aimed to investigate the anti-apoptotic capability of the hepatitis B virus in the HepG2 hepatoma cell line by suppressing mitochondrial superoxide levels. Generally, their findings seem to be interesting, anyway it should be validated in different cell lines, such as HepG2.117.

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