

Hepatitis B virus induces hypoxia-inducible factor-2 α expression through hepatitis B virus X protein

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Abstract. A growing number of studies suggest that the hepatitis B virus X protein (HBx) enhances the protein stability of the hypoxia-inducible factor-1 α (HIF-1 α). However, the relationship between hepatitis B virus (HBV), HBx and hypoxia-inducible factor-2 α (HIF-2 α) has not yet been fully elucidated. Immunohistochemical analysis was employed to detect the expression of HIF-2 α in normal liver, HBV-related chronic hepatitis, and HBV-related and non-HBV-related hepatocellular carcinoma (HCC) tissues. Quantitative real-time PCR (qPCR) and western blotting were used to investigate the impact of HBV and HBx on the expression of HIF-2 α . Immunoprecipitation and immunofluorescence were applied to explore the underlying mechanisms. The HIF-2 α expression was found to be higher in HBV-related chronic hepatitis tissues than in normal liver tissues. Furthermore, it was higher in HBV-related HCC tissues and HBV-integrated HepG2 cells than in the corresponding non-HBV-related HCC tissues and HepG2 cells. Both HBV and HBx enhanced the protein stability of HIF-2 α . HBx-mediated upregulation of HIF-2 α resulted mainly from an inhibition of the degradation of HIF-2 α due to the binding of HBx to the von Hippel-Lindau protein (pVHL). In addition, HBx upregulated the expression of HIF-2 α by activating the NF- κ B signaling pathway. Thus, the present study identified that HBV induces the HIF-2 α expression through its encoded protein HBx. This upregulates the HIF-2 α expression by binding to the pVHL activating the NF- κ B signaling pathway.

Introduction

Hypoxia-inducible factor-1 (HIF-1) and HIF-2 present two important transcription factors in hypoxic conditions. Both factors are composed of a hypoxia-regulated α (HIF- α) and an oxygen-insensitive β subunit (1,2). Under normoxic conditions, HIF- α subunits are rapidly hydroxylated by prolyl hydroxylases (PHDs) and bind to the von Hippel-Lindau protein (pVHL), which results in the rapid ubiquitination of HIF- α subunits and the subsequent proteasome degradation. Under hypoxic conditions, the low availability of oxygen inhibits the activity of hydroxylase, which stabilizes the HIF- α subunits (1). Besides the hypoxic microenvironment, HIF-1 α and HIF-2 α are also affected by many other factors, such as viral hepatitis infections, the glucose metabolism, proto-oncogenes, and mutated tumor suppressor genes (3-6). It has been reported that both hepatitis B and C viruses can stabilize the HIF-1 α protein and promote a pseudohypoxic state (1,3,7).

HBV-encoded protein X (HBx) is a multifunctional regulator that modulates transcription, signal transduction, cell cycle progress, protein degradation, apoptosis and genetic stability by directly or indirectly interacting with host factors (8-10). The potential relationship between HBx and HIF- α has prompted widespread concerns. Previous studies have indicated that wild-type HBx can increase the level of HIF-1 α via two mechanisms. It has been shown that HBx can directly bind to the basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) domain of HIF-1 α to inhibit the interaction between pVHL and HIF-1 α , thus preventing degradation of the HIF-1 α protein (8,16). At the same time, HBx upregulates the HIF-1 α expression by stimulating the metastasis-associated protein 1, the histone deacetylase and the mitogen-activated protein kinase (MAPK) pathway (8,9). Although a growing body of evidence suggests that HBx stabilizes HIF-1 α , the majority of studies has been performed with ectopic protein expression instead of infectious virus, reflecting the limited amount of full-length HBV genomes *in vitro*. Furthermore, only a few studies investigated the relationship between HBV or HBx and HIF-2 α (1). The aim of the present study was to investigate the relationship between HBV, HBx and HIF-2 α .

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Materials and methods

Patients and clinicopathologic information. Abnormal liver samples from 80 adult patients with HBV-related HCC (n=30), non-HBV-related HCC (n=30), HBV-related cirrhosis (n=10), and HBV-related chronic hepatitis (n=10) were sectioned for immunohistochemistry (IHC) in addition to normal liver samples from hepatic hemangioma of 10 healthy adults. The samples were collected from the Affiliated Hospital of Guiyang Medical College (Guiyang, China) between January 2007 and January 2011. All patients or their legal representatives gave signed informed consent for participation in the present study. All patients were tested positive for hepatitis B surface antigen (HBsAg) and negative for the hepatitis C virus (anti-HCV) and the human immunodeficiency virus (anti-HIV).

Reagents and cell lines. Mouse anti-HIF-2 α /EPAS1 monoclonal antibodies (190 b) and mouse anti-human NF- κ B p65 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DAPI was purchased from Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO, USA). The HA-tagged HIF-2 α and FLAG-tagged pVHL constructs were gifts from Drs L. Neckers and J.S. Isaacs (Center for Cancer Research, National Cancer Institute, Rockville, MD, USA). The HBx-shRNA plasmid was obtained from Dr Yu Chao (Department of Hepatobiliary Surgery, Affiliated Hospital of Guiyang Medical College, Guiyang, China) as a generous gift. The human HCC cell line HepG2 was purchased from American Type Culture Collection (Rockville, MD, USA). HepG2.2.15 cell line was kindly provided by Professor Mary Miu-Yee Waye (Department of Biochemistry, The Chinese University of Hong Kong).

Immunohistochemical analysis. Immunohistochemical analysis was performed as described previously (11). The HIF-2 α protein levels were scored according to the number of cells exhibiting cytoplasmic and nuclear staining, applying the same classification system as Sun *et al*: if <50% of the tumor cells exhibited nuclear or cytoplasm staining, the protein level was categorized as low expression, otherwise it was categorized as high expression (12).

Real-time PCR assay and western blot analysis. RNA extraction, cDNA synthesis, quantitative real-time PCR (qRT-PCR) reactions and western blots were performed as previously reported (13). The primer sequences were: human HIF-2 α , forward 5'-TCATGCGACTGGCAATCAGC-3' and reverse 5'-GTCACCACGGCAATGAAACC-3' (GenBank NM_001430.4), human β -actin, forward 5'-GTCCACCGCAAATGCTTCTA-3' and reverse 5'-TGCTGTACCTTCACCGTTC-3' (GenBank NM_001101); HBx, 5'-CGTCCTTTGTCTACGTC-3' and 5'-AAGTTGCATGGTGCTGGTGA-3' (GenBank U95551).

Immunoprecipitation. Proteins extracted from the cells with RIPA buffer were separated with 8-15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Blocking was performed in 5% (w/v) non-fat dried milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20. Subsequently, the proteins

were incubated with specific antibodies against FLAG (Sigma-Aldrich), HIF-2 α , pVHL, HBx, and β -actin (Santa Cruz Biotechnology). Secondary antibodies conjugated with horseradish peroxidase were employed, and immunoreactive proteins were detected via ECL (Thermo Fisher Scientific, Rockford, IL, USA). For the immunoprecipitation, 500 μ g of whole cell lysates were incubated with 2 μ g of the indicated antibodies, and the resulting immunocomplex was precipitated by adding 20 μ l of protein A/G agarose slurry (Santa Cruz Biotechnology). The immunocomplex was then washed three times with lysis buffer, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. These membranes were probed with the indicated antibodies.

Immunofluorescence. Six groups of cells, the L02-pcDNA3.1 cell group (vector control group), the untreated L02-HBx cell group, and four L02-HBx cell groups were treated with one of four concentrations of PDTC (12.5, 25, 50 or 100 μ M) for 24 h. PDTC was added after the cells became adherent and was removed 24 h later by washing three times with PBS. The cells were then fixed with acetone for 10 min, washed three times with PBS, and treated with 0.25% Triton X-100 for 10 min. Then they were treated with normal goat serum for 30 min, incubated with NF- κ B p65 monoclonal antibody (1:100) at 4°C overnight, incubated with goat anti-mouse FITC-IgG antibody (1:500) at room temperature for 30 min, and stained with propidium iodide for 10 min to visualize nuclear condensation. Double labeling was observed using a Nikon Digital Eclipse CI confocal microscope (Tokyo, Japan) with a nitrogen-argon laser, operating at a 488/543 nm excitation/emission wavelength with an excitation energy of 10%.

Statistical analysis. Statistical analyses were performed using the SPSS computer software (version 20.0, SPSS Inc., Chicago, IL, USA). Paired t-test, Mann-Whitney U test, or one-way ANOVA were applied for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference. Results are expressed as mean \pm SD of three independent and representative experiments.

Results

HBV enhances the expression of HIF-2 α . To investigate the effect of HBV on the expression of the HIF-2 α protein, we compared the HIF-2 α expression of normal liver tissues and HBV-related hepatitis tissues, as well as the HIF-2 α expression of HBV-related and non-HBV-related HCC tissues. The results showed that the expression of the HIF-2 α protein was higher in HBV-related hepatitis tissues than in normal liver tissues, and that it was higher in HBV-related HCC tissues than in non-HBV-related HCC tissues (Fig. 1A and B). The expression of the HIF-2 α protein was detected in HBV-related hepatitis tissues, HBV-related HCC tissues, and non-HBV-related HCC tissues at 30.0 (3/10), 40 (12/30) and 33.3% (10/30) of the samples, respectively. Immunohistochemical staining revealed a weak HIF-2 α expression with a yellowish staining in hepatitis tissues (Fig. 1A). A moderate or strongly positive expression with diffuse granular staining was observed in HBV-related HCC tissues. In contrast, all normal liver tissue samples

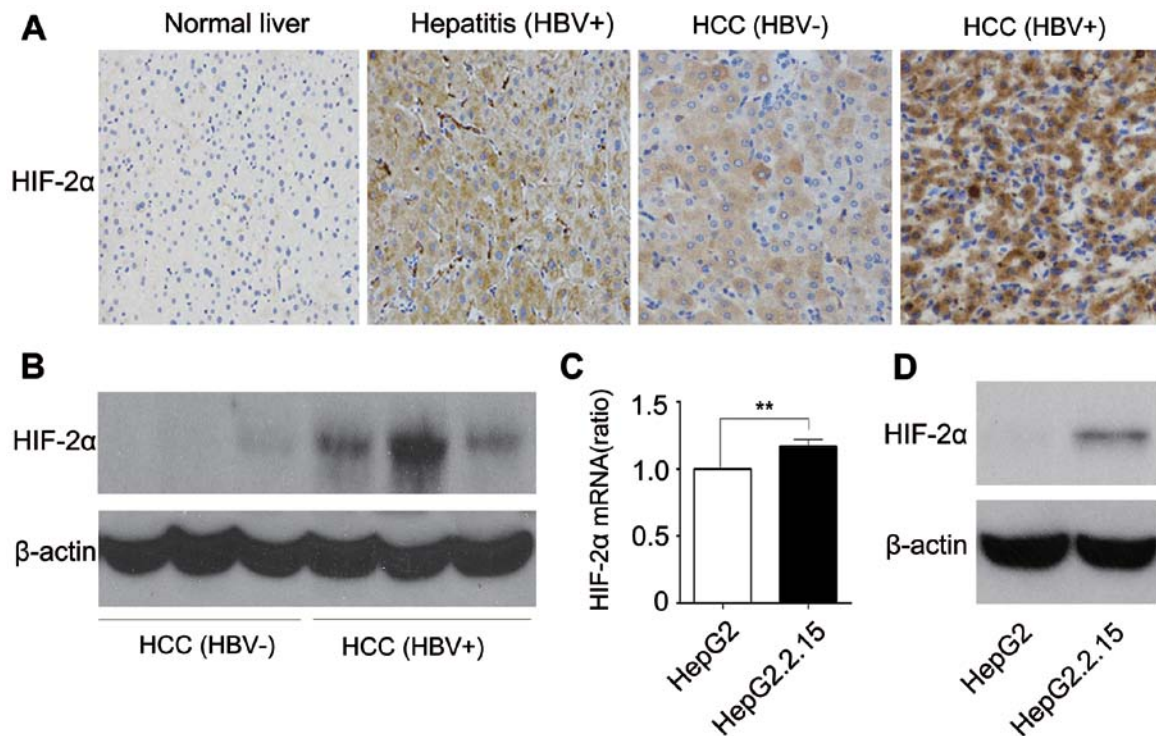


Figure 1. Comparison of HIF-2α levels of HBV-related and non-HBV related tissues and cells. (A) Immunohistochemical analysis of HIF-2α protein expression in normal liver tissues, HBV-related chronic hepatitis tissues, and HBV-related and non-HBV-related HCC tissues (magnification, x200). (B) Western blot analysis of HIF-2α protein expression in HBV-related HCC and non-HBV-related HCC tissues. (C) Real-time PCR analysis of HIF-2α expression in HepG2 cells and HepG2.2.15 cells. The HIF-2α expression level is indicated relative to the mRNA expression level as the HIF-2α/mRNA ratio and normalized to β-actin (**P<0.01). Results are expressed as the mean ± SD of three independent experiments. (D) Western blot analysis of HIF-2α expression in HepG2 cells and HepG2.2.15 cells.

(10/10) showed a negative HIF-2α expression (Fig. 1A). We also compared the expression of HIF-2α at both mRNA and protein levels of HepG2 cells (negative for HBV) and HepG2.2.15 cells, which were stably transfected with a complete HBV genome. This revealed that HIF-2α mRNA was slightly but still significantly higher in HepG2.2.15 cells than in HepG2 cells. The HIF-2α protein was hardly detectable in HepG2 cells but obvious in HepG2.2.15 cells (Fig. 1C and D). Therefore, we hypothesize that HBV may be a positive regulator of HIF-2α.

HBx is involved in HBV-induced increase of HIF-2α. To investigate the mechanism of the HBV-mediated upregulation of HIF-2α further, we examined the effect of HBx on the expression of HIF-2α. We found that L02 and HepG2 cells transfected with HBx plasmids expressed higher protein levels of HIF-2α and its target gene Oct4 (Fig. 2A and B). Furthermore, HBx-shRNA plasmids were transfected into HepG2.2.15 cells to silence HBx. This decreased the levels of HIF-2α and Oct4 (Fig. 2C and D), as expected. Therefore, we assume that HBx may be involved in the HBV-induced increase of HIF-2α.

HBx up-regulates the expression of HIF-2α by preventing it from binding to pVHL. To explore the mechanism responsible for the HBx-mediated expression of the HIF-2α protein, we first investigated the impact of pVHL on the expression of the HIF-2α protein. We found that the transfection of the pVHL plasmid decreased the HIF-2α level sharply and, further-

more, that HBx could prevent this decrease (Fig. 3A). This is surprising, as HBx had no direct effect on the expression of the pVHL protein. Our immunoprecipitation analysis further demonstrated that HBx significantly inhibited the binding of pVHL to HIF-2α (Fig. 3B). This inhibition appeared to be mediated through competitive binding of HBx to pVHL rather than directly to HIF-2α as HBx co-precipitated with pVHL, but not with HIF-2α. These findings indicate that HBx enhances the HIF-2α level by inhibiting HIF-2α to bind to its degrading molecule, pVHL, which may thus stabilize the HIF-2α protein.

HBx upregulates the expression of HIF-2α by activation of the NF-κB signaling pathway. It has been reported that the activation of the NF-κB signaling pathway caused the increase of HIF-2α (14-16). Therefore, we questioned whether the NF-κB pathway was also involved in the HBx-mediated up-regulation of the HIF-2α expression. In L02-pcDNA3.1 cells, NF-κB p65 was mainly seen in the cytoplasm, whereas it was seen in the nucleus of L02-HBx cells. Since NF-κB p65 stains green and condensed nucleus stains red, the nuclei appear yellow as a result of the overlay of green and red. After a treatment with 12.5 μM PDTC for 24 h, the nuclear expression of NF-κB p65 was slightly reduced in the L02-HBx cells. Thereby, the increase of the PDTC concentration provoked a decrease of the NF-κB p65 expression in the nucleus. After reaching a PDTC concentration of 50 μM, the nuclear expression of NF-κB p65 was completely inhibited (Fig. 4A).

Compared with the non-HBx-transfected control cells (L02-pcDNA3.1), the HIF-2α expression was increased

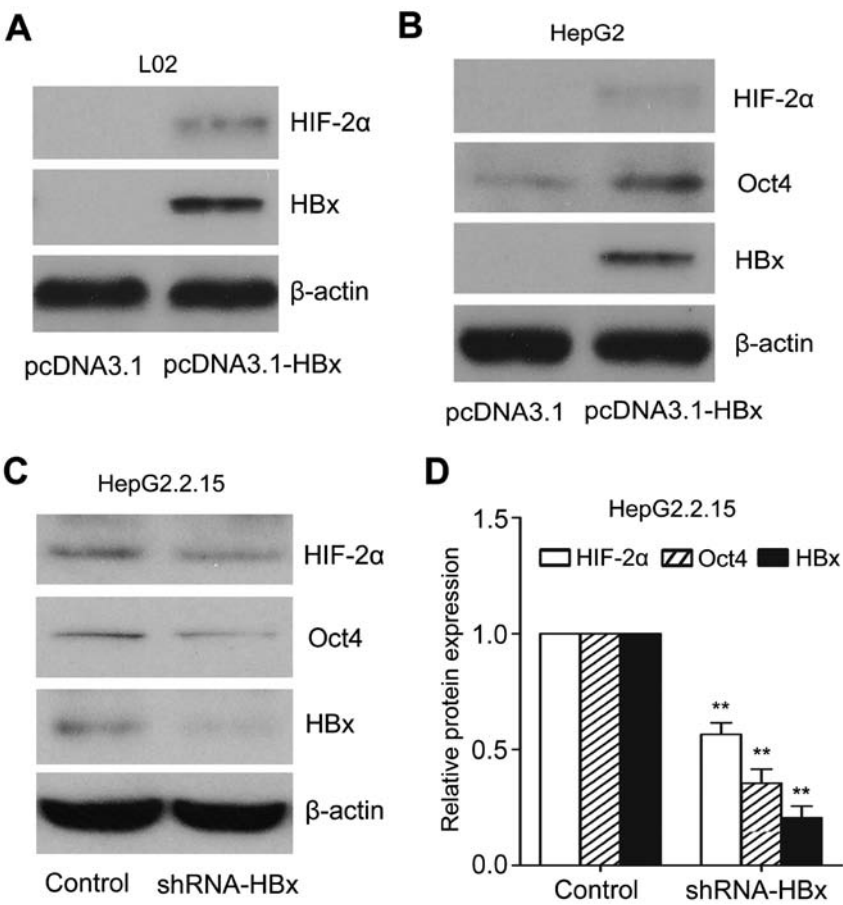


Figure 2. Effect of HBx on the HIF-2 α expression. (A and B) Western blot analysis of HIF-2 α in L02 and HepG2 cell lines after transient transfection with pcDNA3.1 with and without HBx. (C and D) Western blot analysis of HIF-2 α in HepG2.2.15 cell lines after transient transfection with HBx-shRNA and the corresponding controls (* P <0.05 and ** P <0.01). Results are expressed as the mean \pm SD of three independent experiments.

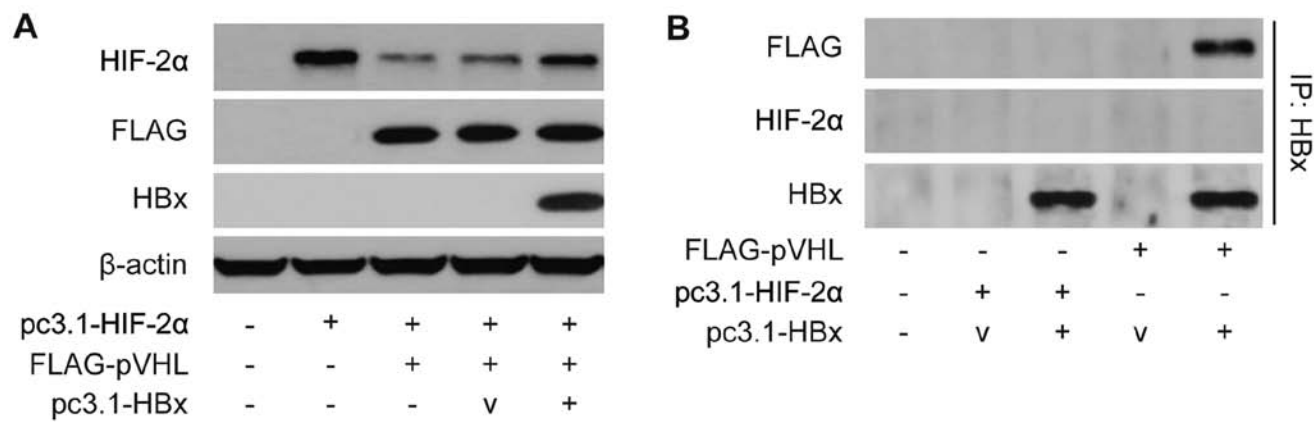


Figure 3. HBx upregulates the expression of HIF-2 α by preventing HIF-2 α binding to the HIF-2 α -degrading protein pVHL. (A) Western blot analysis of the proteins HIF-2 α , FLAG and HBx extracted from HepG2 cells that had been transfected with the plasmids HA-HIF-2 α , pc3.1-HBx, or FLAG-VHL (2 μ g of the respective plasmid for 24 h), as indicated (+/-, transfected/not transfected; V, vector; pc3.1, pcDNA 3.1). (B) Western blot analysis of the immunocomplexes that were obtained from the whole cell lysates (500 μ g) of HepG2 cells that had been transfected with the plasmids HA-HIF-2 α , pc3.1-HBx, or FLAG-VHL (2 μ g of the respective plasmid for 24 h; +/-, transfected/not transfected; V, vector; pc3.1, pcDNA 3.1) after immunoprecipitation (IP) with anti-HBx or anti-HIF-1 α antibodies, as indicated.

3.6 \pm 0.31-fold in L02 cells with HBx transfection (L02-HBx), but decreased gradually when the concentration of PDTC was increased. PDTC (50 μ M for 24 h) decreased the HIF-2 α level in L02-HBx cells \sim 45.3 \pm 2.8% of the initial 3.6 \pm 0.31-fold increase down to only a 1.97 \pm 0.28-fold increase with respect

to the control cells (Fig. 4B). These findings indicate that HBx is likely the major factor to upregulate the expression of HIF-2 α through the NF- κ B signaling pathway. We also noted that the HIF-2 α protein level was still higher in L02-HBx cells than in L02 cells after the NF- κ B signaling pathway

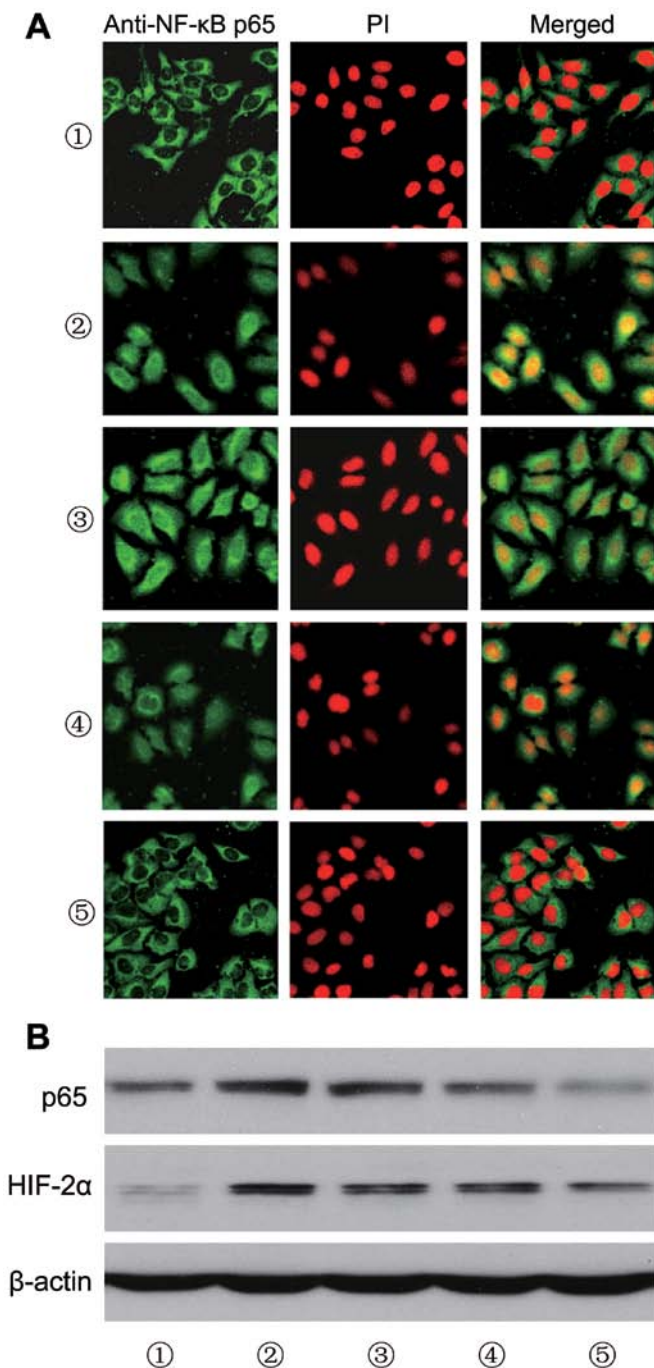


Figure 4. HBx upregulates the expression of HIF-2 α by activating the NF- κ B signaling pathway. Immunofluorescence and western blot analysis was used to detect the expression of NF- κ Bp65 (A and B) and HIF-2 α (B) in the L02-pcDNA3.1 cell group, the L02-HBx cell group and the PDTC treated group. Lane 1, L02-pcDNA3.1 cell group; lane 2, L02-HBx cell group; lane 3, L02-HBx cells treated with 12.5 μ M PDTC for 24 h; lane 4, L02-HBx cells treated with 25 μ M PDTC for 24 h and lane 5, L02-HBx cells treated with 50 μ M PDTC for 24 h; magnification, \times 200).

was totally blocked, suggesting that factors other than NF- κ B probably also contribute to the HBx-mediated upregulation of the HIF-2 α expression in liver cells.

Discussion

Although increasing evidence indicates that HBx stabilizes the HIF-1 α protein (17-19), only a few studies have explored

the relationship between HBV or HBx and the protein HIF-2 α . In our study, we first compared the HIF-2 α expression of normal liver tissues and HBV-related chronic hepatitis tissues, as well as the HIF-2 α expression of HBV-related HCC tissues and non-HBV-related HCC tissues. In addition, we compared the HIF-2 α expression of HBV-negative HepG2 cells and HepG2.2.15 cells, which were stably transfected with a complete HBV genome. The results showed that the HIF-2 α expression was obviously higher in HBV-related hepatitis tissues than in normal liver tissues, and it was higher in HBV-related HCC tissues than in non-HBV-related HCC tissues. Consistent with the results obtained from liver tissues, the HIF-2 α level was significantly higher in HepG2.2.15 cells than in HepG2 cells. These results indicate that an HBV infection can enhance the expression of HIF-2 α . To determine whether HBx is the viral component that contributes to an HBV-mediated upregulation of HIF-2 α , we knocked down HBx with HBx-shRNA plasmids, and we enhanced the endogenous levels of HBx by using an HBx expression plasmid. We found that overexpression of HBx significantly increased the HIF-2 α levels, and that the HBx knockdown resulted in a significant reduction of the intracellular HIF-2 α levels. These results suggest that HBx is involved in the HBV-mediated upregulation of HIF-2 α .

The underlying mechanism by which HBx upregulates HIF-2 α was also explored in the present study. In the presence of oxygen, HIF-2 α was prolyl hydroxylated by PHDs (2). This modification can recruit pVHL, which subsequently targets HIF-2 α for proteasomal degradation (20). We revealed that HBx does not decrease the protein levels of pVHL, but binds directly to pVHL, which leads to a decreased interaction between pVHL and hydroxylated HIF-2 α and the subsequent reduction of the proteasomal degradation of HIF-2 α . As a tumor suppressor, pVHL is often inactivated due to mutations, which are known to correlate with the development of numerous tumors, such as clear cell renal carcinomas, hemangioblastomas, and pheochromocytomas (21,22). Our results revealed a novel mechanism for the inactivation of pVHL, in which HBx directly binds to pVHL to limit the negative effect of pVHL on the HIF-2 α protein in HBV-containing HCC cells. It has been reported before that HBx directly interacts with HIF-1 α (17). However, this may not be the case for HIF-2 α as we could not detect any direct interaction between HBx and HIF-2 α .

Furthermore, we found that the NF- κ B pathway is also involved in the HBx-mediated upregulation of HIF-2 α . NF- κ B is a nuclear transcription factor involved in the regulation of many cellular functions. When cells are quiescent, NF- κ B remains in the cytoplasm in a non-active form where it is complexed to its inhibitor protein I κ B (23,24). HBx activates IKK α and IKK β to phosphorylate I κ B, causing I κ B ubiquitination followed by the proteasome-mediated degradation of I κ B proteins. These translocate NF- κ B from the cytoplasm to the nucleus where the transcriptional activity of NF- κ B is renewed and the transcription and activation of the target gene occurs (23,24). Our study showed that NF- κ B p65 is in L02 cells mainly expressed in the cytoplasm. In contrast, HBx-containing L02-HBx cells exhibited NF- κ B p65 in both the nucleus and the cytoplasm. These findings indicate a positive effect of HBx on the activation of the NF- κ B pathway. The application of PDTC, a known inhibitor of the NF- κ B

activation (25,26), confirmed this result by revealing that the expression of the HIF-2 α protein increased after the transfection of HBx, but was inhibited by PDTC in a dose-dependent manner. It is worthwhile to note that the HIF-2 α level was still higher in L02-HBx cells than in L02 cells even after the NF- κ B signaling pathway was totally blocked. This suggests that factors other than NF- κ B contribute to the HBx-mediated upregulation of the HIF-2 α expression.

In conclusion, we investigated the possibility of crosstalk between HBV and HIF-2 α . We demonstrated for the first time that HBV enhances the stability of the HIF-2 α protein, probably through HBx-binding to pVHL and activating the NF- κ B signaling pathway, leading to an overexpression of HIF-2 target genes such as Oct4, which play an important role in hepatocarcinogenesis.

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