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iNOS promotes HBx-induced hepatocellular carcinoma via upregulation of JNK activation

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ABSTRACT

Inducible nitric oxide (iNOS) is closely correlated with chronic inflammation in hepatitis B virus X protein (HBx)-induced hepatocellular carcinoma (HCC). However, the molecular mechanisms through which iNOS contribute to hepatocarcinogenesis remain poorly understood. Therefore, we investigated the role of iNOS in signaling pathways underlying HBx-induced liver tumorigenesis. iNOS deletion showed a marked decrease in the hepatic tumor size and stage of *HBx* transgenic (Tg) mice, indicating a strong contribution of iNOS signaling pathways to hepatocarcinogenesis. In addition, we found that nitric oxide (NO) increased HBx mRNA by recruiting CREB to the CRE site of HBV enhancer in HepG2 cells, suggesting a positive feedback loop between HBx and iNOS signaling pathway. Moreover, iNOS-modulated JNK activation was associated with sustained upregulation of Cyclin D1 in *HBx*Tg mice and HepG2-*HBx* cells. These results imply that iNOS may play a key role in HBx-associated HCC development. Taken together, our findings demonstrate that iNOS aligns with HBx to promote tumor progression. These findings provide a better understating of the mechanism involving HBx-mediated hepatic tumorigenesis and selective inhibition of iNOS may have therapeutic applications in HBx-associated HCC.

1. Introduction

Hepatitis B virus (HBV) has been identified as a major risk factor for liver-associated diseases such as hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1,2]. We and others have previously shown that Hepatitis B virus X protein (HBx) plays a role in chronic inflammatory response and HCC development according to the HBV infection [3–5]. Although many researchers suggest that HBx is an important target for HBV-induced HCC, the underlying mechanisms by which HBx induces hepatocarcinogenesis are complicated and remain to be fully understood.

HBx was known to modulate a variety of cellular processes, including cell proliferation, cell cycle progression, DNA damage repair, and apoptosis [6,7]. It has been shown that HBx activates signaling of NF-κB, a transcription factor that can stimulate the expression of inflammatory target genes including inducible nitric oxide synthase (iNOS) [8]. Specifically, iNOS can be induced by various stimuli, including lipopolysaccharide (LPS), interferon (IFN)-γ, interleukin (IL)-1β or tumor necrosis factor (TNF)-α in macrophages and hepatocytes [9,10]. iNOS generates sustainable amounts of nitric oxide (NO) that can lead to cell damage and participates in processes leading to inflammation and tumorigenesis [11]. Previous studies show that overexpression of iNOS has been associated with chronic liver disorders such as hepatitis, steatosis, and HCC [12–14]. Interestingly, iNOS expression was also found to correlate with the histopathological grading and staging in HBV patients [15,16]. In addition, our previous study demonstrated that iNOS impairs hepatic insulin signaling by iNOS-induced JNK activation in the liver of *HBx* transgenic (Tg) mice and reciprocal activation between HBx and iNOS in HepG2 cells and *HBx*Tg mice [17]. Nonetheless, it remains to be determined how activation of iNOS signaling pathways induces HBx-driven tumorigenesis.

To address the question of how iNOS signaling is implicated in HBx-induced hepactocellular carcinoma, we examined the phenotypes of *HBx*Tg, and *HBx*Tg/*iNOS*$^{-/-}$ mice and the associated signaling pathway in HCC cell lines. In the present study, we

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demonstrated that iNOS plays an important role, along with HBx, to activate JNK-dependent pathway in HBx-mediated HCC development and progression.

2. Materials and methods

2.1. Animal model

Generation of *HBx*Tg and *HBx*Tg/*iNOS*$^{-/-}$ mice was previously described [17]. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, South Korea).

2.2. Cell culture and reagents

Human hepatoma cell lines (HepG2 and SNU449) were cultured in Dulbecco's modified Eagle medium (DMEM, Hyclone) or RPMI 1640 medium (Hyclone), supplemented with 200 unit/ml penicillin-G-sodium, 100 μg/ml streptomycin, 4 mM L-glutamine, and 10% fetal bovine serum (FBS, Hyclone). Cells were stimulated with the nitric oxide donor sodium nitroprusside (SNP, Sigma) and/or a cytokine mixture consisting of human TNF-α (R&D Systems), IL-1β (R&D Systems), and human INF-γ (R&D Systems). The specific JNK inhibitor SP600125 was purchased from Calbiochem.

2.3. HCC tissue samples

Liver specimens were obtained from primary HCC patients with HBV infection who received surgical resection in Yonsei University Hospital. All patients participating in this study gave informed consent before surgery.

2.4. Plasmid construction and transfection

The expression vector, pcDNA3-HA-HBx, was constructed as follows. A 475 bp HBV subtype *adr* CDS fragment was subcloned into an EcoRI/XhoI site of a pcDNA3-HA tagged vector, and then CMV promoter DNA in the vector was replaced with an HBV X-gene authentic promoter DNA derived from a pHEX1 vector. EGFP expression from the HBV-CRE promoters was compared to the expression driven by the HBV-mCRE promoter. The intact and mutated CRE region of the HBx enhancer was cloned into the BglII/HindIII sites of vector pEGFP-1 (Clontech). HepG2 cells were plated in 6-well culture plates for 24 h prior to transfection. Cells were transfected with 3 μg of pcDNA3-HA-HBx, HBV(E) CRE/GFP, and HBV(E) mCRE/GFP constructs using a Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.5. Flow cytometry analysis

Approximately 10^5 cells were grown in 6-well dishes and stimulated with the indicated SNP concentration for 48 h. After harvesting by centrifugation, the cells were resuspended in a small volume (250–400 μl) of phosphate-buffered saline (PBS). The GFP positive sorted cells were detected in the FL1 emission channel (535/30 nm) and subjected to FACS analysis on a FACScalibur (Becton Dickinson) and the data processed with the WinMDI 2.8 program.

2.6. Quantitative real-time PCR analysis

Total RNA was prepared from HepG2 cells or mouse tissue using TRIzol (Molecular Research Center) according to the manufacturer's protocol. cDNA synthesized with oligo (dT) primers with the first-strand cDNA Synthesis kit (Fermentas) was mixed with the SYBR Premix Ex Taq (TaKaRa) and sets of gene-specific primers. The resulting mixture was subjected to real-time RT-PCR quantification with the ExicyclerTM 96 detection system (Bioneer Corp., Korea). Primers for PCR were designed using the Primer3 software program. The specificity of primers was verified by BLAST analysis of the mouse and human genome, visualized by RT-PCR products after agarose gel electrophoresis, and analyzed by the melting point of the PCR products. Sequences for primers used for this study are provided as supplemental information (Table S1).

2.7. Western blot analysis

Homogenized liver tissues and cells harvested after treatment were lysed in RIPA buffer and subjected to Western blot analysis as described previously [17]. Antibodies were obtained from Cyclin D1 (Cell Signaling); JNK (Cell Signaling); phospho-JNK (Thr183/Thr185, Cell Signaling); p21 (Cell Signaling); PCNA (Dako); HA (Roche) and GAPDH (LabFrontier).

2.8. Histology staining

Liver tissues from mice were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned with 4 μm in thickness, and stained with hematoxylin-eosin.

2.9. Mri

In vivo MRI was performed on a 4.7-T animal MRI instrument. T1WI was obtained at 10 min after the injection of the Gd-DTPA (12 μmol of Gd/kg of body weight) in mice. All the animals were examined by contrast-enhanced T1-weighted MRI using the following imaging parameters: TR = 300 ms, TE = 10 ms, FOV = 25.6 × 25.6 mm, slice thickness = 1 mm, pixel resolution = 100 × 100 μm, in the 4.7-T instrument.

2.10. Statistical analysis

Data were analyzed using SigmaPlot 12.3 software. Quantitative data are presented as the mean ± the standard error of the mean (SEM) from at least three independent experiments. Comparisons between groups were analyzed by unpaired two-tailed Student *t*-tests, or one-way or two-way ANOVA with post hoc analyses, when appropriate, as indicated.

3. Results

3.1. Deficiency of iNOS inhibits HBx-induced HCC development and progression

HBx activities contribute to the development of HBV-associated HCC, but studies on the topic do not fully account for plausible mechanisms that interact between HBx and HCC-related signaling events. Interestingly, iNOS has been implicated in hepatic pathology [18]. In addition, our previous study described that both iNOS and HBx synergistically induced gluconeogenic gene expression and regulated hepatic glucose homeostasis [17]. However, the molecular relationships between iNOS and HBx expression have not been evaluated in HCC development. To evaluate the association of iNOS with HBx-associated HCC risk, we produced *HBx*Tg and *HBx*Tg/*iNOS*$^{-/-}$ mice (Fig. 1A). Deletion of iNOS decreased the incidence and tumor size of HCC in *HBx*Tg mice (Fig. 1B and Table S2). Consistently, histological analysis revealed that HBx-mediated tumorigenesis was significantly decreased by iNOS deletion (Fig. 1C). Indeed, MRI data revealed that hepatic tumor size were more significantly reduced in *HBx*Tg/*iNOS*$^{-/-}$ mice than in

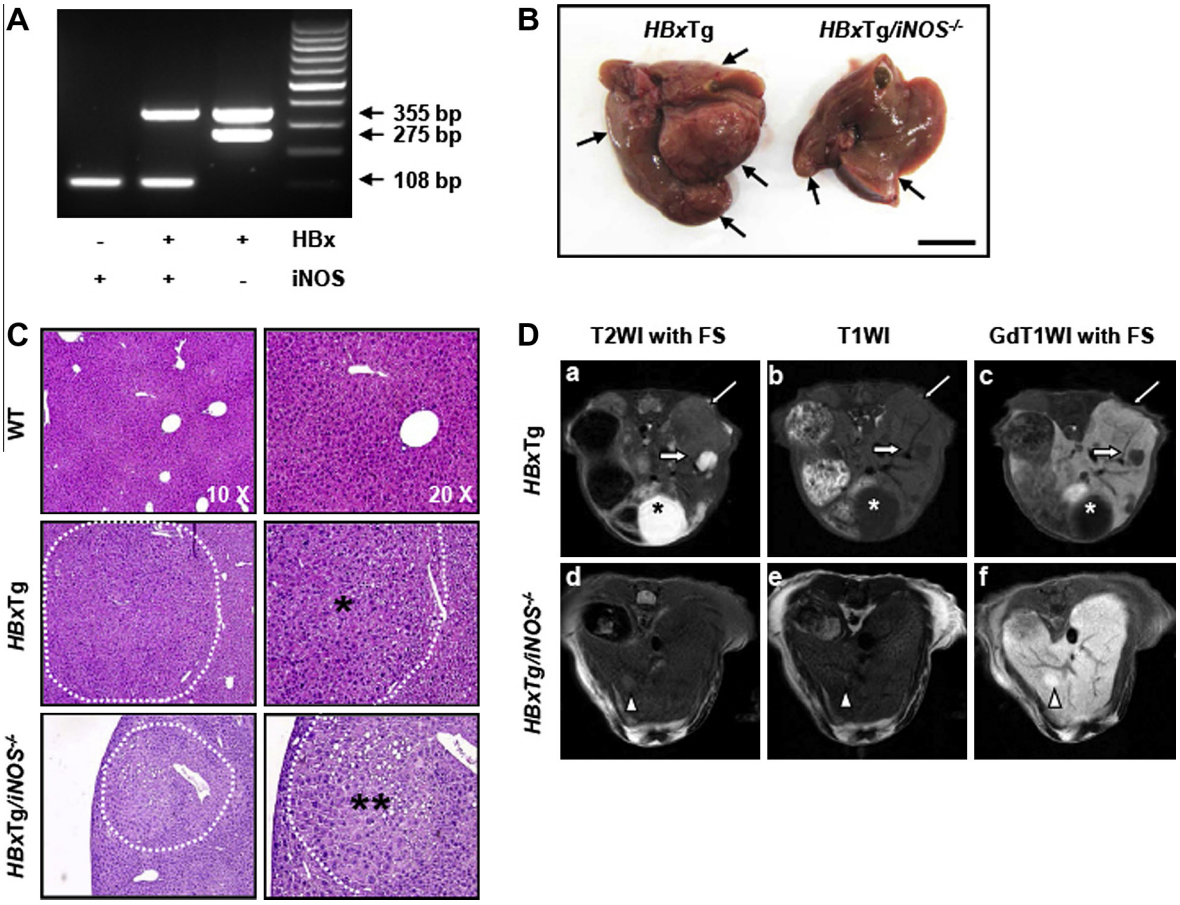

Fig. 1. iNOS reduced HBx-mediated hepatocarcinogenesis. (A) PCR analysis of genomic DNA from mouse tails (HBx allele, 355 bp; iNOS wild allele, 108 bp; iNOS mutant allele, 275 bp). (B) A broad view of representative HCC observed in *HBx*Tg and *HBx*Tg/*iNOS*$^{-/-}$ mice at 16 months of age. The scale bar indicates 1 cm. Black arrows indicate tumor regions. (C) Hematoxylin and eosin (H&E) stained paraffin-embedded liver sections of WT, *HBx*Tg and *HBx*Tg/*iNOS*$^{-/-}$ mice at 16 months of age (*HCC region, **Dysplastic nodule region). (D) MRI data of hepatic tumor in the *HBx*Tg and *HBx*Tg/*iNOS*$^{-/-}$ mice at 16 months of age. (a and d) Turbo RARE T2-weighted image with fat saturation (repetition time msec/echo time msec 3500/38) shows a large mass in figure a (arrows) and a smaller nodule in figure b (arrowhead) with ill-defined margins. These tumors are hyperintense to liver and hypointense to hepatic cysts (asterisk). (b and e) Unenhanced fat-saturated T1-weights image (361.5/11) shows that those tumors (arrows in b, arrowhead in e) are mildly hypointensive to the adjacent liver. (c and f) Contrast-enhanced fat-saturated T1-weighted image (361.5/11) obtained during the hepatocyte selective phase demonstrates heterogeneous enhancement of the tumor (arrows) in figure (c) and homogenous enhancement of the tumor (arrowhead) in figure (f).

*HBx*Tg mice (Fig. 1D). These results are suggestive of a possible role of iNOS in HBx-induced hepatocarcinogenesis.

3.2. iNOS regulates HBx expression levels through the CRE site in the HBx promoter

We revealed reciprocal regulation in expression between HBx and iNOS in induction of gluconeogenesis [17]. When we examined the expression level of HBx and iNOS in *HBx*Tg mice at different ages, the iNOS mRNA expression pattern was largely similar to that of the HBx gene (Fig. 2A). Indeed, HBx mRNA expression was also increased in HepG2-*HBx* cells after treatment with sodium nitroprusside (SNP), a nitric oxide (NO) donor (Fig. 2B and C). To investigate whether NO could regulate HBx gene expression, we performed promoter assays by FACS and qRT-PCR in HepG2 cells expressing intact HBV enhancer/GFP or CRE-mutated HBV enhancer/GFP. Because exogenous NO inhibits luciferase activity through decreasing luciferase mRNA stability [19], we examined CRE-HBV promoter effects using GFP reporter assays and qPCR by NO donor treatment. NO-mediated GFP levels were ~1.5–2.5-fold increased in HepG2 cells expressing intact HBV enhancer/GFP, but these effects were not seen in HepG2 cells expressing CRE-mutated HBV enhancer/GFP (Fig. 2D and E), suggesting that NO regulates HBx gene expression via its promoter CRE site. Combined with the previous findings that HBx induces the recruitment of CREB and ATF2 to the CRE-like sequence present in the HBV enhancer [20] and NO controls CREB-mediated gene expression [21], it is conceivable that HBx transcription is regulated by NO signaling in a feedback mechanism, that is produced by HBx-mediated iNOS activation. Taken together, these findings indicate that iNOS may promote HBx gene expression via transcriptional regulation in response to elevated NO level.

3.3. iNOS upregulates HBx-mediated JNK signaling pathway in hepatocarcinogenesis

To investigate whether iNOS regulates a signaling pathway leading to HBx induced tumorigenesis, we performed a western blot for the proteins associated with hepatic tumorigenesis. It was established that JNK plays a key role in HCC development [22]. We therefore examined the phosphorylation of JNK (pJNK) in liver tumor tissues of *HBx*Tg and *HBx*Tg/*iNOS*$^{-/-}$ mice at 16 months of age. We found that elevated pJNK levels in *HBx*Tg mice were significantly reduced by a deficiency of iNOS in *HBx*Tg/*iNOS*$^{-/-}$ mice (Fig. 3A and B). Furthermore, upregulated expression levels of Cyclin D1 and p21 that are associated with pJNK levels in *HBx*Tg mice were markedly decreased in *HBx*Tg/*iNOS*$^{-/-}$ mice. The result in mice was confirmed in HBV patients by activation of JNK signaling in tumors compared to non-tumors

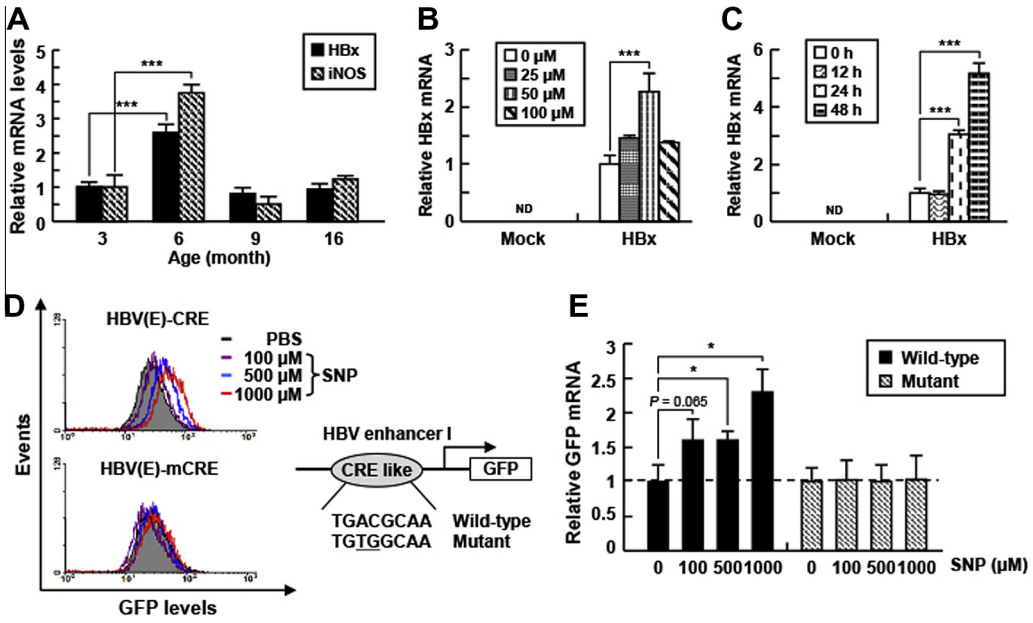

Fig. 2. iNOS upregulates HBx gene expression in HepG2 cells and *HBx*Tg mice. (A) mRNA levels of HBx and iNOS in *HBx*Tg mice (n = 6–8) at the indicated ages. $^{***}P < 0.001$. Error bars represent the mean ± SEM. (B and C) Relative HBx mRNA levels in cells at indicated doses and times. Cells were treated with SNP. (D and E) EGFP expression by the HBV-CRE promoters was compared to the expression driven by the HBV-mCRE promoter in HBV(E)-CRE and HBV(E)-mCRE stable cells treated with indicated concentrations of SNP for 48 h. The activity of each promoter was obtained with FACS and GFP mRNA levels. All the data were the mean of three independent experiments and indicated as means ± SEM ($^{*}P < 0.05$, $^{***}P < 0.001$. vs. 0 μM).

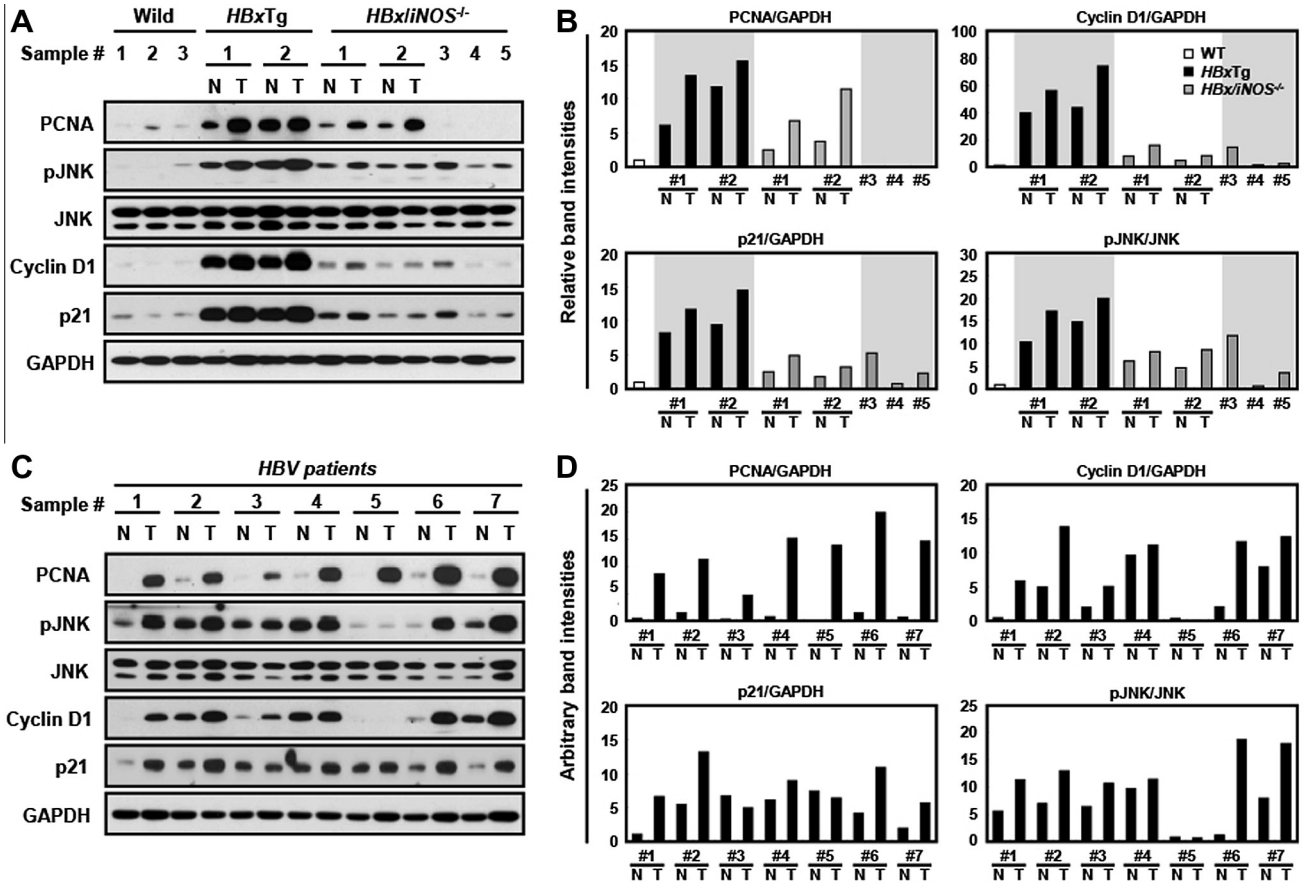

Fig. 3. iNOS deletion reduced HBx-mediated JNK signaling pathway. (A) Immunoblot analysis with specific antibodies in non-tumor (N) and tumor (T) tissues of livers of WT, *HBx*Tg, and *HBx*Tg/*iNOS*$^{-/-}$ mice at 16 months of age. (B) Band intensities of (A) were quantified using densitometric analysis and normalized to GAPDH or JNK levels and graphed. (C) Immunoblot analysis with specific antibodies in the liver lysates of non-tumor/tumor tissues in HBV patients. (D) Band intensities of (C) were quantified using densitometric analysis and normalized to GAPDH or JNK levels and graphed.

(Fig. 3C and D). Taken together, these data support a potential role for JNK-regulated expression of Cyclin D1 and p21 during HBx-induced HCC development. Collectively, these data suggest that iNOS plays a pivotal role in JNK activation during HBx-mediated HCC.

3.4. Nitric oxide mediated upregulation of JNK signaling pathway in HBx-expressing liver cancer cells

To assess the contribution of NO to JNK activation in HCC cells, we examined pJNK levels in HepG2-*HBx* cells in response to SNP. pJNK levels were increased in both HepG2-Mock and HepG2-*HBx* cells with treatment of SNP in a dose/time dependent manner. However, pJNK levels were significantly increased in HepG2-*HBx* cells compared to HepG2-Mock cells that were accompanied by increased p21 protein (Fig. 4A and B). However, it has been shown that exposure of tumor cells to exogenous NO such as S-nitroso-N-acetylpenicillamine (SNAP), SNP, and 8-pCPT-cGMP leads to a decrease in Cyclin D1 due to the attenuation of Cyclin D1 synthesis [23]. Thus, we have examined endogenously produced NO following stimulation with a cytokine mixture (CM) of TNF-α, IL-1β, and IFN-γ. As shown in Fig. 4C, CM is also responsible for activated JNK and elevated Cyclin D1 and p21 expression, implying that NO is involved in this JNK signaling pathway in HBx-mediated tumorigenesis. Next, HepG2-*HBx* cells were depleted of JNK through treatment of a specific inhibitor (SP600125) to assess the involvement of iNOS induced Cyclin D1 and p21 expression by JNK activation. To address this question, SP600125 treatment in HepG2-*HBx* cells was performed after the addition of the CM. Notably, after the suppression of pJNK in HepG2-*HBx* cells, Cyclin D1 and p21 expression were reduced compared to the control. Interestingly, iNOS and HBx expression were also reduced when inhibiting JNK activation. These results support that HBx leads to activation of the JNK/iNOS axis contributing to the HBx-induced hepatocarcinogenesis.

4. Discussion

In the present study, we focused on the investigation of the function of iNOS in the development of HBx-induced HCC. It has been reported that iNOS-derived NO plays an important role in tumor development [11,14]. More greatly increased iNOS expression and/or activity was reported in HBV-related HCC tissues than in the tissues of a normal liver [16]. In addition, we reported that iNOS is responsible for HBx-driven hepatic metabolic disorder [17], which is associated with increased HCC risk in HBV-infected patients [24]. Our finding suggests that a possible interplay between iNOS and HBx gives some new clues to the role in HBV-associated HCC. However, to our knowledge, there is no evidence of the contribution of iNOS to the development and progression of HBx-induced HCC using genetically-engineered mouse models. To address whether there is significance of iNOS in the development of HBx-induced hepatocarcinogenesis, we crossed *HBx*Tg mice with

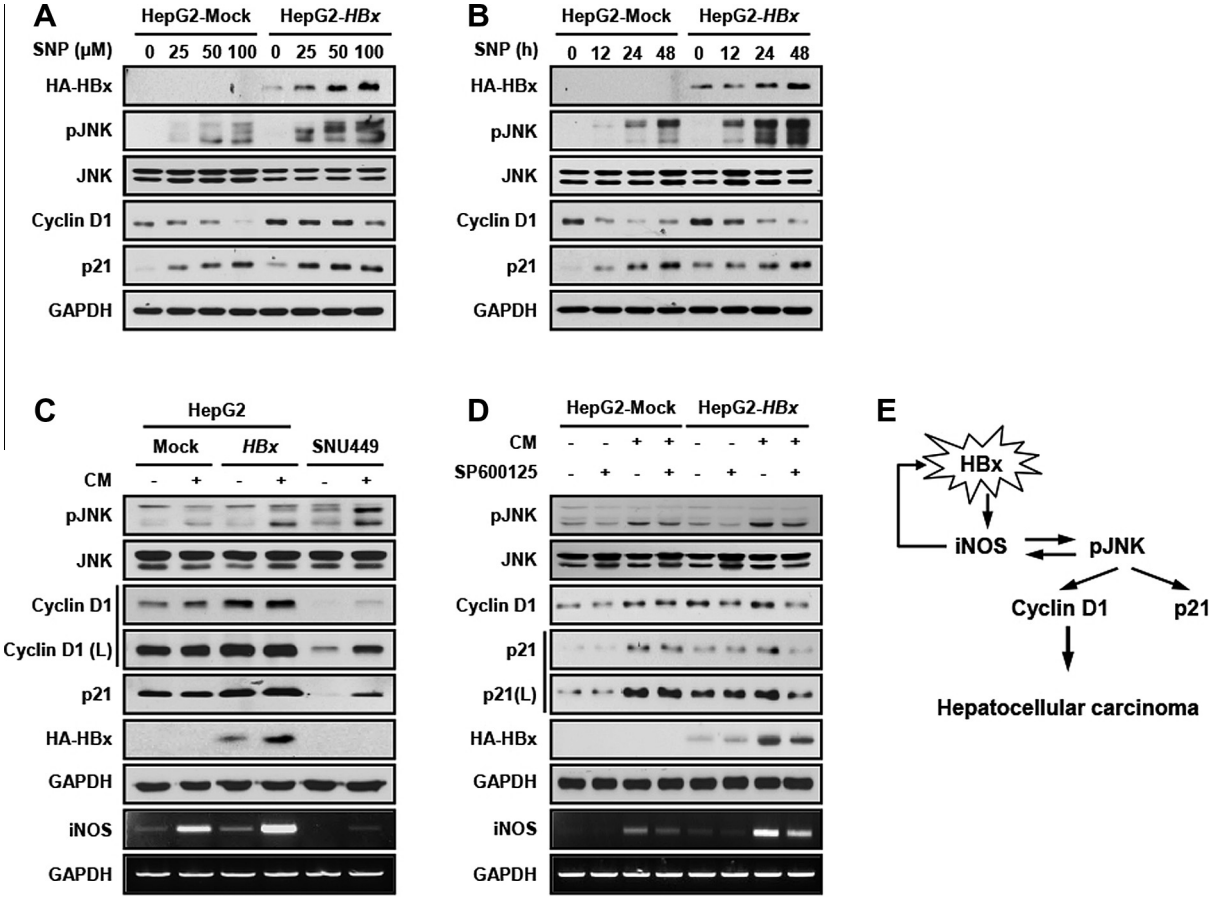

Fig. 4. Upregulation of JNK signaling pathway induced by nitric oxide in HepG2-*HBx* cells. (A and B) Immunoblot analysis with specific antibodies in the HepG2-Mock and *HBx* cells treated with SNP. Cells were treated at indicated doses and times. (C) Immunoblot analysis with specific antibodies in the SUN449, HepG2-Mock and *HBx* cells were incubated with or without cytokine mixture (TNF-α 10 ng/ml + IL-1β 10 ng/ml + IFN-γ 10 ng/ml) for 24 h and then harvested for protein or RNA extraction. The iNOS mRNA levels were also measured by semi-quantitative RT-PCR and the GAPDH mRNA levels were used as an internal control (bottom panel). (D) Immunoblot analysis with specific antibodies in the HepG2-Mock and *HBx* cells were incubated with or without SP600125 (20 μM) in the presence of either CM or vehicle (DMSO) for 1 h and then harvested for protein or RNA extraction. The iNOS mRNA levels were also measured by semi-quantitative RT-PCR and the GAPDH mRNA levels were used as an internal control (bottom panel). (L) Longer exposure. (E) Schematic representation depicting some possible molecular interplay between HBx, iNOS, and pJNK.

iNOS knockout mice. A histopathologic examination revealed only 28% of HCCs and 17% of dysplastic nodules in the livers of *HBx*Tg/*iNOS*$^{-/-}$ mice, respectively, compared to 60% and 65% in the livers of *HBx*Tg mice at 15 and 18 months (Fig. 1 and Table S2). These data clearly suggest that loss of iNOS function is the phenotypic determinant of tumorigenesis in HBx-associated HCC.

Furthermore, expression of HBx was closely related with iNOS (Fig. 2A). Our laboratory previously found that the expression levels of HBx were increased by treatment with SNP in HepG2-*HBx* cells [17]. Although HBx was responsible for the upregulation of iNOS expression via NF-κB activation [8], the mechanism of upregulation of HBx by iNOS remains unclear. It has been reported that the enhancer I region of HBx contains a CRE-like element [20]. Here, we found that iNOS is able to activate the HBx enhancer by depending on CREB binding sites (Fig. 2D and E). Thereby, our data suggest that iNOS is able to upregulate HBx through a positive feedback loop between HBx and iNOS signaling.

HBx activates several intracellular signal pathways that can lead to HBV-related tumor progression. Previous studies have suggested a link between HBx and JNK signaling pathway as well as Cyclin D1, which contribute to the sustained promotion of tumor development [25,26]. Enhanced Cyclin D1 expression emerged as an important target for the JNK pathway in liver proliferation [22,27]. In addition, HBx-induced p21 overexpression positively regulates cell proliferation in the hepatocarcinogenesis [28], indicating that upregulation of p21 was positively correlated with the expression of Cyclin D1 in HBx-expressing liver cancer cells. Consistent with this, our study also revealed that pJNK, Cyclin D1, and p21 were highly expressed in clinical HBV-associated HCC tumor tissues, suggesting that these molecules may play a role in tumorigenesis in HBx-associated HCC (Fig. 3C and D). Interestingly, loss of iNOS expression markedly reduced the expression of Cyclin D1, p21, and pJNK in liver tumors of *HBx*Tg mice (Fig. 3A and B). In contrast, there was also a remarkable rise in expression of Cyclin D1, p21, and pJNK related to iNOS induction by CM in HBx-expressing cells (Fig. 4C). Additionally, suppression of pJNK signaling by SP600125 triggered a decrease in pJNK that was associated with decreased expression of HBx and iNOS, supporting iNOS targeting by pJNK (Fig. 4D and E).

In conclusion, our present data strongly support that alignment between iNOS and HBx enhances the JNK signaling pathway and promotes HBx-induced HCC. Our findings provide insight into the mechanism of iNOS in the development of HBx-associated HCC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.071.

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