# Hepatitis B virus X protein modulates peroxisome proliferator-activated receptor γ through protein–protein interaction

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Received 8 August 2003; revised 19 November 2003; accepted 23 November 2003

First published online 24 December 2003

Edited by Felix Wieland

**Abstract Ligand activation of peroxisome proliferator-activated receptor γ (PPARγ) has been reported to induce growth inhibition and apoptosis in various cancers including hepatocellular carcinoma (HCC). However, the effect of hepatitis B virus X protein (HBx) on PPARγ activation has not been characterized in hepatitis B virus (HBV)-associated HCC. Herein, we demonstrated that HBx counteracted growth inhibition caused by PPARγ ligand in HBx-associated HCC cells. We found that HBx bound to DNA binding domain of PPARγ and HBx/PPARγ interaction blocked nuclear localization and binding to recognition site of PPARγ. HBx significantly suppressed a PPARγ-mediated transactivation. These results suggest that HBx modulates PPARγ function through protein–protein interaction.**
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*Key words:* Hepatitis B virus X protein; Peroxisome proliferator-activated receptor γ; Transactivation; Protein–protein interaction

## 1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the steroid hormone receptor superfamily and regulates cell growth, differentiation, insulin sensitization, and glucose homeostasis [1]. Upon receptor ligation, PPARγ forms heterodimers with the retinoid X receptor (RXR) [2], which bind to specific DNA regions, called peroxisome proliferator response elements (PPRE), in the 5′ flanking region of target genes [3], and form complexes with coactivators and corepressors involved in PPAR/RXR-mediated transactivation [1].

Recently, it has been reported that the ligand-induced activation of PPARγ shows anti-tumor effect by inducing growth inhibition, terminal differentiation, and apoptosis in various human cancer cells, including colon, prostate and breast cancer, and liposarcoma [4–8]. It was also reported that the ligand activation of PPARγ induced growth inhibition and apoptosis in hepatocellular carcinoma (HCC) cells [9–11], however, its effect on virus-associated HCC has not been clarified.

Hepatitis B virus (HBV) is considered to be the major cause of hepatitis, cirrhosis, and HCC. Among the HBV proteins, hepatitis B virus X protein (HBx) is considered to contribute significantly to the mediation of pathological events and to the dysregulation of gene expression through protein–protein interactions with endogenous cellular proteins and transcriptional factors. The transactivatory role of HBx is thought to be mediated by its interaction with transcription factors, such as RPB5 of RNA polymerase [12], TATA binding protein [13], transcription factor IIB [14], bZIP protein [15], and RXR [16]. It has been also reported that HBx interferes with p53 by direct binding and by sequestering p53 in the cytoplasm, leading to the abrogation of p53-mediated cellular processes [17–19]. Wang et al. [20] reported that HBx inhibits sequence-specific DNA binding and thus blocks the transcriptional activity of p53. HBx is known to promote cellular transformation, as demonstrated by the immortalization of primary mouse hepatocytes [21] and by the development of hepatoma in HBx transgenic (HBx-TG) mice [22–24]. HBx is involved in the activation of signal transduction pathways, such as Ras-Raf-MAP kinase, PI3 kinase, protein kinase C, Src kinase, nuclear factor (NF)-κB, and Jak1-STAT signal pathways, which lead to the activation and regulation of various genes that contribute to cellular proliferation and carcinogenesis [25].

In this study, we examined the effect of HBx on the growth inhibition induced by PPARγ ligand in HCC cells. We also described that HBx bound to PPARγ and HBx/PPARγ interaction in the cytoplasm, and that this interfered with the nuclear localization and DNA binding of PPARγ, leading to the

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*Abbreviations:* PPARγ, peroxisome proliferator-activated receptor γ; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; HBV, hepatitis B virus; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; HBx-TG, hepatitis B virus X protein transgenic; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; NE, nuclear extract; DBD, DNA binding domain; LBD, ligand binding domain; NLS, nuclear localization signal; IFA, immunofluorescence assay; rHBx, recombinant hepatitis B virus X protein

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doi:10.1016/S0014-5793(03)01449-2

suppression of the transcriptional activity of PPARγ. Thus, our data show a novel association between HBx and PPARγ, which may provide another important mechanism in the pathogenesis of HBV-associated HCC.

## 2. Materials and methods

### 2.1. Cell lines and culture

Chang liver (ATCC CCL-13), HepG2 (ATCC HB-8065) and HEK293 (ATCC CRL-1573) were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Chang, HEK293, HepG2 and HepG2.2.15 cells [26] were maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS). Chang X-34 cells are Chang liver cells expressing HBx under the control of tetracycline inducible promoter [19]. HBx was induced with 2 μg/ml doxycycline (Sigma, St. Louis, MO, USA) in Chang X-34 cells.

### 2.2. Plasmids and plasmid construction

A series of green fluorescent protein (GFP)–PPARγ fusion constructs (pEGFP-PPARγ F, D, DL, L) shown in Fig. 2B were generated by polymerase chain reaction (PCR) amplification of the PPARγ gene with specific primer sets, corresponding to the PPARγ full length (F; residues 1–475), PPARγ DNA binding domain (DBD) (D; residues 94–181), PPARγ DNA and ligand binding domain (LBD) (DL; residues 94–475), and PPARγ LBD (L; residues 281–475). These were then subcloned into the *Sac*I–*Sal*I site of pEGFP vector (Clontech, Palo Alto, CA, USA). To generate His fusion recombinant HBx (rHBx) protein, an amplified HBx fragment was cloned into *Eco*RI–*Hin*dIII sites of pRSET A (Invitrogen, Carlsbad, CA, USA). $PPRE_3$-thymidine kinase (tk)-LUC and pCMX-PPARγ [27] were kindly provided by Dr. R. Evans. The HBx DNA construct, pSVX, and its frameshift mutant, pSVXκB [28], were used for the transfection experiments. To express HBx with a hemagglutinin (HA) epitope tag at the N-terminal (pCMV-HA–HBx), the HBx fragment was amplified by PCR and subcloned into the *Eco*RI–*Kpn*I site of pCMV-HA plasmid (Invitrogen).

### 2.3. Generation and infection of recombinant adenoviruses

The construction of adenoviral vectors was performed as described previously [29]. The adenoviral plasmids (pAdEasy-1, -2) and the shuttle vectors (pShuttle, pShuttle-CMV, pAdTrack, and pAdTrack-CMV) were constructed by subcloning the PCR product of HBx. Approximately $3\times10^6$ HEK293 cells were plated in 25 $cm^2$ flasks 24 h before transfection, by which time they had reached 50–70% confluency. 4 μg of recombinant adenoviral vector DNA, digested with *Pac*I, was then transfected using 20 μl LipofectAMINE (Gibco BRL). The transfected cells were monitored for GFP expression and harvested 7 days after transfection. Cells were collected and lysed by three cycles of freezing/thawing. A concentrated adenovirus fraction was prepared by CsCl gradient centrifugation and stored with 2× storage buffer (10 mM Tris, pH 8.0, 100 mM NaCl, and 0.1% bovine serum albumin (BSA), and 50% glycerol) at −20°C. 1 μl of virus stock was used to infect $3–5\times10^6$ cells. The efficiencies of the infections were monitored by GFP expression.

### 2.4. rHBx protein expression

His fusion rHBx protein was expressed in *Escherichia coli* strain BL21(DE3) and purified on nickel agarose bead. Protein expression, preparation of inclusion body protein, and refolding of denatured protein were performed as described previously [30].

### 2.5. HBx-TG mice

HBx-TG mice were generated [24] and maintained as described previously [31]. We used HBx homozygote transgenic mice and age-matched wild-type controls. The protocol for mice maintenance was approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University, according to the Guide for Animal Experiments edited by the Korean Academy for Medical Association.

### 2.6. Antibodies and reagents

Antibodies against PPARγ (sc-7273), HA (sc-805, sc-7392), and GFP (sc-9996, sc-8334) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiserum against HBx was prepared by intraperitoneal immunization to BALB/c mice with rHBx emulsified with complete and sequentially incomplete Freund's adjuvant and its reactivity was screened by Western blot and enzyme-linked immunosorbent assay (ELISA). The antibodies against α-tubulin and histone were from Oncogene (Darmstadt, Germany) and Chemicon International Inc. (Temecula, CA, USA), respectively. Protein G/A was purchased from Gibco, and protein assay reagent and enhanced chemiluminescence reagent were from Pierce (Rockford, IL, USA). Troglitazone, provided by SanKyo (Tokyo, Japan), was prepared as previously described [32].

### 2.7. Cell proliferation assay

Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [33]. Briefly, $1\times10^3$ cells were seeded into 96-well plates and treated with troglitazone and incubated for different time periods. After treatment, 50 μl MTT solution (1 mg/ml in phosphate-buffered saline (PBS)) was added to each well, and incubated for 4 h at 37°C. The plate was then centrifuged at 3000 rpm for 10 min, the supernatant was discarded, and the remaining adhered cells were dissolved in 50 μl dimethyl sulfoxide (DMSO) (Sigma). The optical density (OD) of each well was measured at 570 nm using an ELISA reader.

### 2.8. Reverse transcriptase (RT)-PCR

Total RNA was prepared using RNeasy Kit (Qiagen Inc, Chatsworth, CA, USA). Reverse transcription for cDNA was performed from 5 μg total RNA using 2 μg random hexamer (Amersham Pharmacia Biotech Inc., Uppsala, Sweden), 1.25 mM deoxyribonucleoside triphosphate (dNTP) (Boehringer-Mannheim, Mannheim, Germany) and with or without addition of 200 U M-MLV RT (Gibco BRL). PCR was performed using 0.25 mM dNTP, 0.25 U Taq polymerase (Perkin Elmer, Norwalk, CT, USA), 10 pmol primer pair and 3 μl cDNA with a thermal cycler. The primer sequences were as follows: HBx, 5′-CGGAATTCCGATGGCTGCTAGGAT-3′ (sense) and 5′-CGGGGTACCCCGT-TAGGCAGAGGT-3′ (antisense); β-actin, 5′-TTGTAACCAACTGGGACGATTGG-3′ (sense) and 5′-GATCTT-GATCTTCATGG-TGCTAGG-3′ (antisense). PCR cycling conditions were as follows: denaturation at 92°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min.

### 2.9. Immunoprecipitation and Western blot analysis

For the HBx and PPARγ co-immunoprecipitation assay, Chang X-34 cells were treated with 2 μg/ml doxycycline, and then lysed with radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–Cl, pH 7.4, 100 mM NaCl, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.2% NP-40 and 2 mM $MgCl_2$ supplemented with protease inhibitor cocktail (Sigma). To detect the intracellular interactions of HBx with the deletion mutants of PPARγ, HEK293 cells were cotransfected with pCMV-HA–HBx and pEGFP-PPARγ F, D, DL, or L, respectively. Cell lysates (500 μg) were then incubated with 1 μg anti-HA monoclonal antibody and incubated with protein G/A, and Western blotting was performed using polyclonal anti-HA, anti-PPARγ, and anti-GFP antibodies. To detect the HBx and PPARγ protein expression in the mouse liver tissues, tissues were fractionated into cytosolic, and nuclear extracts [32] and analyzed by quantitative Western blot analysis using HBx antiserum and anti-PPARγ antibody.

### 2.10. Immunofluorescence assay (IFA)

IFA was used to investigate subcellular localization of the HBx and PPARγ. Briefly, Chang liver cells were seeded into Lab-Tek two-chamber glass slides (Nalge Nunc, Naperville, IL, USA) and transiently transfected with pEGFP-PPARγ and pCMV-HA–HBx. Cells were incubated with 20 μM troglitazone for 24 h, washed and fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% saponin (Sigma) in PBS. Cells were then incubated with anti-HA antibody and then with Cy3-conjugated IgG (Zymed Laboratories Inc., San Francisco, CA, USA). After several washes, cells were mounted using mounting medium containing 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and visualized under a confocal microscope, LSM 510 (Carl Zeiss, Jena, Germany), or fluorescence microscope (Olympus America Inc., Melville, NY, USA) with MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA).

*2.11. Electrophoretic mobility shift assay (EMSA)*

Preparations of nuclear extract (NE) and EMSA were carried out as previously described [32]. For the binding reaction, 5 μg NE was incubated with the $^{32}$P-labeled probe in binding buffer containing 10 mM Tris, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml BSA, and 5% glycerol at room temperature for 30 min. For the competition experiments, a 100 molar excess cold probe was added to the reaction mixture. To examine the effect of HBx on the DNA binding activity of PPARγ, rHBx was added to the reaction mixture for 30 min, and then the probes were added. For SP-1 binding, 2 μg NE was incubated with the labeled probe. The sequence of the oligonucleotides used as probes was as follows: CYP4A-PPRE, 5′-TGAAACTAGGGTAAAGTTCA-3′; SP-1, 5′-GATCGATCGGGGCGGGGCGAG-3′.

*2.12. Luciferase reporter assay*

Chang liver cells ($2\times10^5$) were seeded onto 12-well culture plates and transfection was carried out using LipofectAMINE (Gibco BRL), according to the manufacturer's instruction. The total amount of DNA was adjusted to 1 μg; 500 ng $PPRE_3$-tk-LUC, 200 ng pCMX-PPARγ, 200 ng pSVX or pSVXκB, 100 ng pCMV-β-gal,

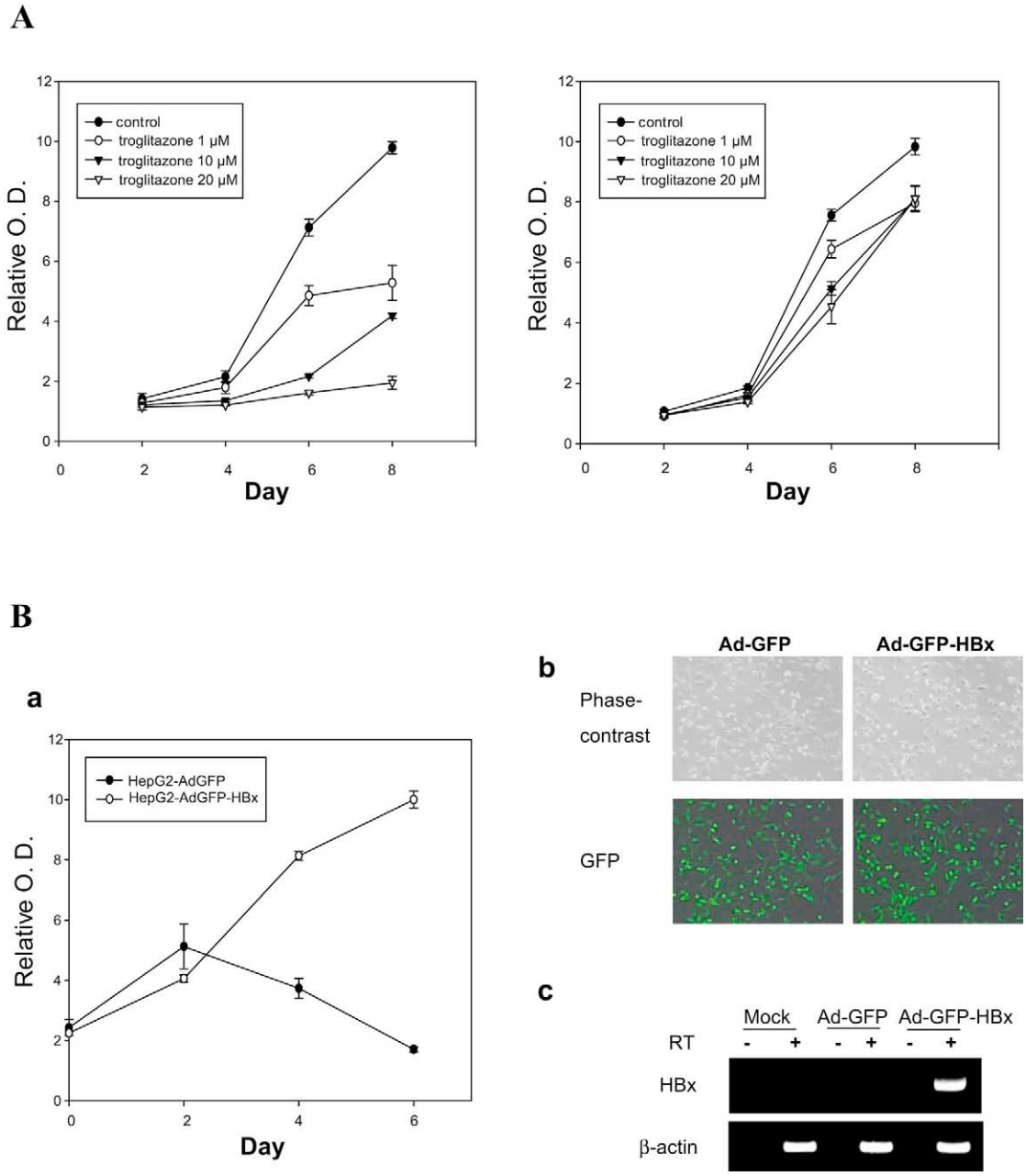


Fig. 1. HBx abolishes the inhibitory effect of troglitazone on cellular proliferation. A: HepG2 (left panel) and HepG2.2.15 (right panel) cells were treated with 1.0–20 μM troglitazone for the indicated times, and cell proliferation was determined by MTT assays. B: (a) HepG2 cells transduced with recombinant adenovirus carrying HBx (Ad-GFP–HBx) or control virus (Ad-GFP) were treated with 20 μM troglitazone for the indicated times and cell proliferation was determined by MTT assays. Results are represented as relative OD compared with the control and normalized values are shown as means ± S.D. Assays were performed at least three times using quadruplicate samples, and the data of a representative experiment are shown. (b) Transduction efficiency of Ad-GFP and Ad-GFP–HBx into HepG2 cells. GFP microscopic finding showed that more than 95% HepG2 cells were transduced in all the fields observed. (c) HBx expression after transduction of Ad-GFP–HBx. After transduction of Ad-GFP and Ad-GFP–HBx, total RNA was extracted from transduced HepG2 cells and treated with RNase-free DNase and then analyzed by RT-PCR with (+) or without (−) RT.

and empty vectors. After 3 h of transfection, the cells were washed and incubated in a complete medium containing 20 μM troglitazone for 24 h. Cells were then washed and lysed using 1× lysis buffer (Promega, Madison, WI, USA). Lysates were measured for luciferase activity using luciferin (Promega), and β-galactosidase activity was measured as an internal control.

## 3. Results

### 3.1. HBx suppresses PPARγ-induced growth inhibition in HCC cells

HepG2 cells express PPARγ, and their growth is inhibited by troglitazone [10,11]. However, in this study, we found that HepG2.2.15 cells, which had been established by transfecting four tandem copies of the HBV genome into HepG2 cells [26], were resistant to troglitazone. When cells were treated with troglitazone for different times, the proliferation of HepG2 cells significantly decreased in a dose-dependent manner. However, HepG2.2.15 cells appeared to be more resistant to troglitazone-induced growth inhibition than HepG2 cells (Fig. 1A). To exclude the possibility that the difference of sensitivity to troglitazone was due to different sublines used, we examined HepG2 infected with recombinant adenovirus carrying HBx (HepG2-Ad-HBx). As shown in Fig. 1B, the growth rate of HepG2-Ad-HBx cells was significantly higher than that of HepG2 cells infected with Ad-GFP empty vector. Thus, these results demonstrate that HBx significantly suppressed troglitazone-induced growth inhibition in HCC cells.

### 3.2. HBx binds to PPARγ in vivo

It was reported that HBx mediates its biological activity through protein–protein interactions with transcription factors [12–16]. To determine whether HBx interacts with PPARγ, an immunoprecipitation assay was performed. Chang X-34 cells, which express HBx-HA under the control of tetracycline inducible promoter, were incubated with doxycycline for various times. Cell lysates were immunoprecipitated with an anti-HA monoclonal antibody, and then analyzed using an anti-PPARγ antibody. The HBx–PPARγ complex was found to increase in proportion to the amount of HBx expressed by doxycycline, in a time-dependent manner, although the endogenous PPARγ protein level was unaffected by HBx (Fig. 2A). To determine which domain of PPARγ is involved in this binding, HEK293 cells were transfected with pCMV-HA–HBx and a series of GFP–PPARγ fusion constructs (Fig. 2B). Cell lysates were then immunoprecipitated with an anti-HA antibody, and analyzed with an anti-GFP antibody. As shown in Fig. 2C, upper panel, PPARγ F, D and DL were found to be associated with HBx. This finding demonstrates that HBx binds to PPARγ, and that the DBD–hinge region of PPARγ is critical for HBx binding, because GFP–PPARγ D is composed of DBD and hinge region.

### 3.3. HBx interferes with the nuclear localization of PPARγ

DBD of PPARγ is involved in the binding to PPRE, and the hinge region participates in nuclear localization and the formation of heterodimers [34,35]. Having observed that HBx binds to the DBD–hinge region of PPARγ, we investigated whether the HBx–PPARγ interaction affects the nuclear localization of PPARγ. Chang liver cells were cotransfected with pEGFP-PPARγ and pCMV-HA–HBx, and IFA was then performed. In the absence of HBx, PPARγ was found to be localized mainly in the nucleus, regardless of troglitazone

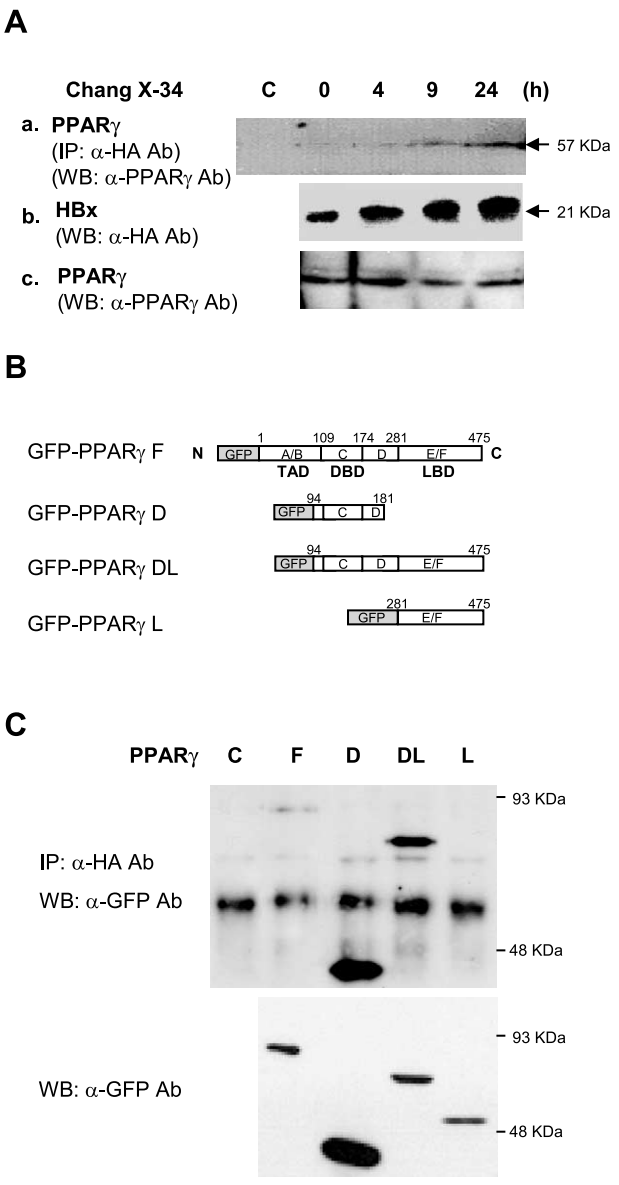


Fig. 2. Interaction of PPARγ with HBx in vivo. A: After treating Chang X-34 cells with 2 μg/ml doxycycline, (a) the cell lysate was immunoprecipitated with anti-HA monoclonal antibody and analyzed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Western blot was performed with anti-PPARγ antibody. C, immunoprecipitation with normal mouse immunoglobulin. (b) Western blot with anti-HA antibody. (c) Western blot with anti-PPARγ, showing that an equal amount of PPARγ had been loaded. B: A schematic diagram of four GFP–PPARγ fusion constructs encoding the indicated domain. C: After transfection with the HA–HBx and GFP–PPARγ fusion constructs (PPARγ F, D, DL, or L), HEK293 cells were lysed and the cell lysates were immunoprecipitated with mouse anti-HA monoclonal antibody. Western blot was performed with rabbit anti-GFP polyclonal antibody after immunoprecipitation (upper panel). C, immunoprecipitation with normal mouse immunoglobulin. The same amount of cell lysates was analyzed by Western blot with mouse anti-GFP monoclonal antibody (lower panel).

treatment (Fig. 3A, upper panel). However, in the presence of HBx the localization of PPARγ changed from the nucleus to the cytoplasm, although some PPARγ remained in the nucleus (Fig. 3A, lower panel). As shown in Fig. 3B, this cytoplasmic PPARγ was found to be colocalized with HBx (merged; yellow color).

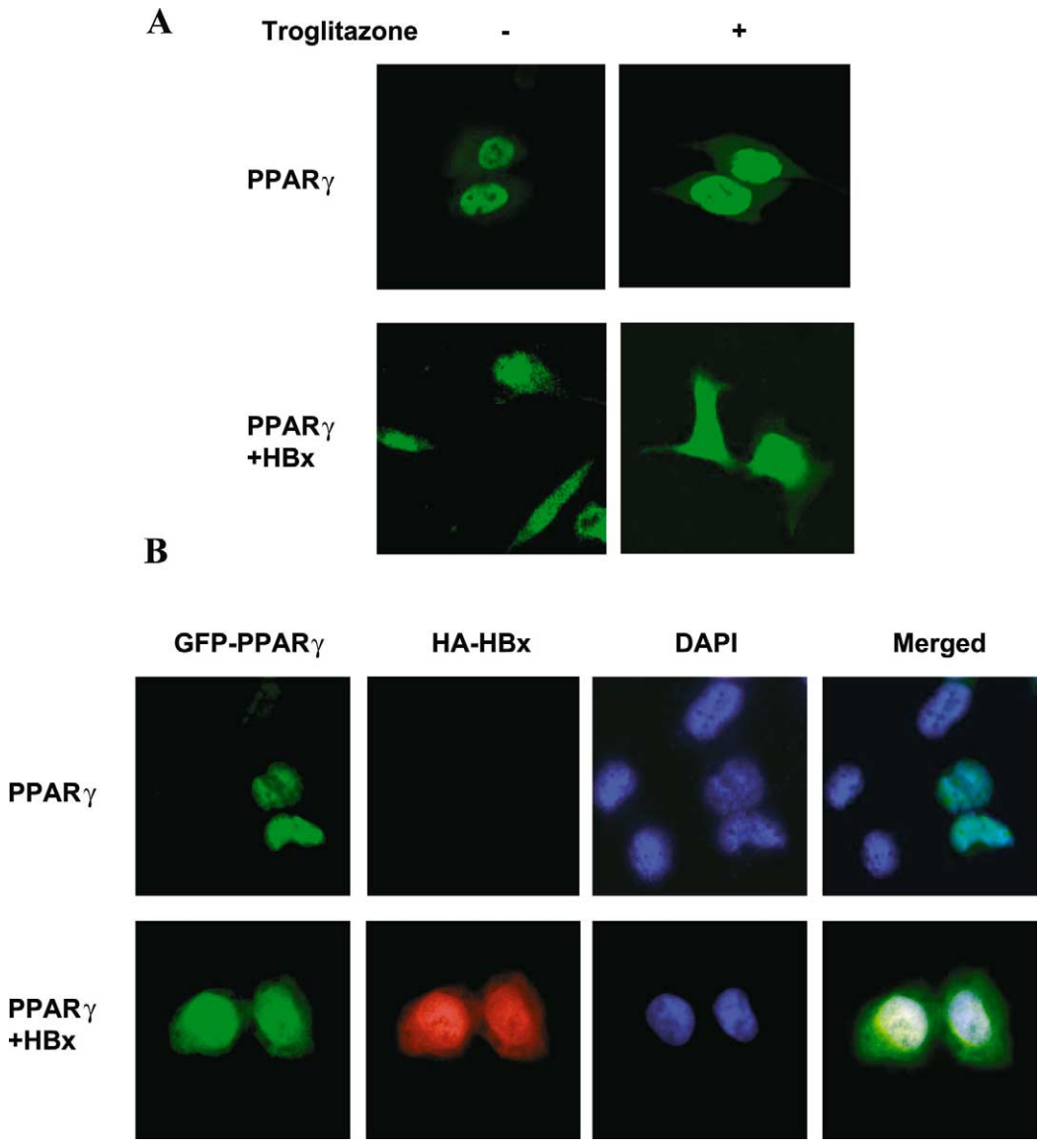


Fig. 3. Colocalization of PPARγ with HBx. A: pEGFP-PPARγ with or without pSVX-HBx was transfected into Chang liver cells and treated with or without 20 μM troglitazone for 20 h, cells were observed under a confocal microscope. The partial cytoplasmic sequestration of PPARγ was observed in the presence of HBx. Upper panel, pEGFP-PPARγ transfected Chang liver cells; lower panel, pEGFP-PPARγ and pSVX-HBx cotransfected Chang liver cells. B: pEGFP-PPARγ was transfected into Chang liver cells with or without pCMV-HA–HBx. After incubation with 20 μM troglitazone for 20 h, the cells were stained and analyzed under a confocal microscope. Cells were visualized with triple colors, i.e. GFP (PPARγ), and rabbit anti-HA antibody and Cy3-conjugated anti-rabbit IgG (HBx), and DAPI (nucleus), and merged.

### 3.4. *PPARγ expression is reduced in the nucleus of HBx-TG mouse liver*

To determine whether this interference with the nuclear localization of PPARγ by HBx occurs in vivo, we prepared the mouse liver tissue extracts from HBx-TG and age-matched control C57BL/6 which were fractionated into cytosolic, and nuclear extracts for the quantification, and then Western blot was performed using anti-PPARγ antibody and HBx antiserum. Both proteins were detected in total and cytosolic compartments of HBx-TG. No significant difference was found in the PPARγ expression in the total and cytosolic compartments between the HBx-TG and control C57BL/6 mouse liver tissues, however the expression of PPARγ in the nuclear extracts of the HBx-TG was significantly lower than in that of the control (Fig. 4).

### 3.5. *The DNA binding of PPARγ is reduced in HBx-TG mouse liver*

To determine whether reduced PPARγ expression in the nucleus of HBx-TG mouse liver is associated with the DNA binding activity of PPARγ, EMSA was performed with PPRE. NE was prepared from the livers of 13-month-old HBx-TG mice and age-matched C57BL/6 mice. As shown in Fig. 5A, the NE from the control C57BL/6 mouse livers showed significant DNA binding to PPRE, however the DNA binding of PPARγ was completely abrogated in the NE of HBx-TG mouse livers. Since HBx interacts with the PPARγ DBD–hinge region, we also investigated whether HBx inhibits the DNA binding activity of PPARγ by performing EMSA using NE from control C57BL/6 mouse liver, which was preincubated with rHBx protein. Fig. 5B shows that

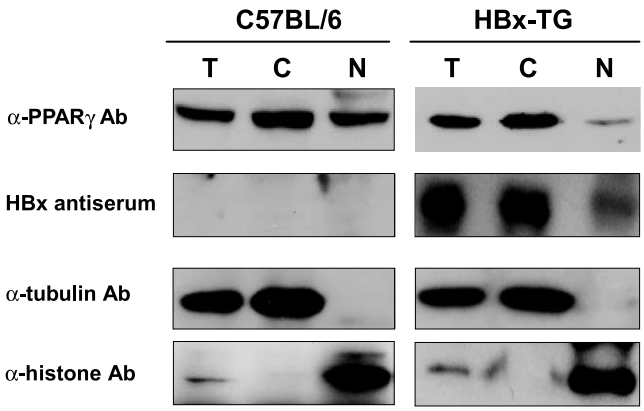


Fig. 4. Reduced PPARγ protein level in the nucleus of HBx-TG mouse liver. Total (T: 100 μg), cytosol (C: 100 μg) and nuclear (N: 25 μg) extracts were analyzed using anti-PPARγ antibody and antiserum against HBx. Western blotting with anti-α-tubulin and anti-histone antibodies were used to confirm the fractionation.

rHBx significantly reduced the DNA binding activity of PPARγ. Overall, it appears that binding of HBx to PPARγ interferes with the nuclear localization and the DNA binding activity of PPARγ.

### 3.6. HBx suppresses the transcriptional activity of PPARγ

To determine whether the interruption of PPARγ localization by HBx influences the transcriptional activity of PPARγ, a luciferase reporter assay was performed. Compared with Chang liver cells transfected with $PPRE_3$-tk-LUC alone, luciferase activity was found to be increased by PPARγ overexpression, and this activity was enhanced by troglitazone. However, luciferase activity was markedly reduced by HBx even in the presence of troglitazone, though this suppressive effect was not observed by the HBx mutant (Fig. 6A). In addition, this suppressive effect of HBx on transcriptional activity was dose-dependent (Fig. 6B). These results suggest that HBx inhibits the transcriptional activity of PPARγ.

## 4. Discussion

PPARγ is a nuclear hormone receptor with diverse functions, which are critical for maintaining cellular processes [36]. Several studies have demonstrated that the ligands of PPARγ may induce apoptosis and growth inhibition in HCC cells, which suggests that PPARγ has an anti-cancer effect [9–11]. In the present study, the proliferation of troglitazone-treated HepG2 cells decreased significantly in a dose-dependent manner, which agrees with previous reports [10,11]. Interestingly, HepG2.2.15 and HepG2 cells infected with Ad-GFP–HBx were resistant to troglitazone, suggesting that the introduction of the HBV genome or HBx appeared to convert troglitazone-susceptible cells to troglitazone-resistant cells.

PPARγ is composed of two zinc finger motifs, nuclear localization signals (NLS), a ligand binding pocket, and several activation functions (AF) [35]. DBD has two zinc finger motifs that can bind directly to DNA, and the hinge region contains NLS. Our immunoprecipitation assay with Chang X-34 cells demonstrated that the protein–protein interaction occurred between HBx and the PPARγ, and this interaction occurred through DBD–hinge region of PPARγ. Since the DBD–hinge region participates in the interaction with HBx, this interaction might modulate the nuclear localization and DNA binding of PPARγ. DBD is the most highly conserved region between other members of steroid hormone receptor superfamily. This fact leads to the possibility that HBx may interact with and affect other PPARγ isoforms or other steroid receptor members. Previously, it was reported that the DBD of several steroid hormone receptors could interact with DBD-associating proteins. Thioredoxin, one of the DBD-associating proteins, interacts with the DBD of glucocorticoid receptor [37], and the small nuclear ring finger protein SNURF directly binds to the DBD of androgen receptor [38]. In addition to these cellular proteins, viral proteins can also modulate steroid hormone receptors by direct protein–protein interaction. Hepatitis C virus core protein interacts with the DBD of RXRα [39] and HBx binds to the LBD of RXR [16]. Since PPARγ forms a complex by heterodimerizing with RXR and binding to other coactivators or corepressors, it is also possible that PPARγ indirectly binds to HBx through complex formation with other components. However, the strong dimerization of PPARγ with RXRα is mainly mediated by the LBD of PPARγ [40], and our data show an interaction between PPARγ and HBx via the DBD–hinge region of PPARγ, thus it is probable that HBx interacts with PPARγ by direct protein–protein interaction. Moreover, our native gel shift assay of the in vitro binding of HBx to PPARγ demonstrated a direct protein–protein interaction. To exclude the possibility that HBx binds to PPARγ indirectly via interaction with RXR, we performed an in vitro binding assay with rHBx and three His-PPARγ proteins corresponding to PPARγ D, DL, and L. A native gel shift assay showed shifted bands in mixtures of HBx with PPARγ D, or PPARγ DL, but not in mixture of HBx with PPARγ L (our unpublished observation). These results indicate that HBx binds directly to the DBD–hinge region of PPARγ, which plays important roles

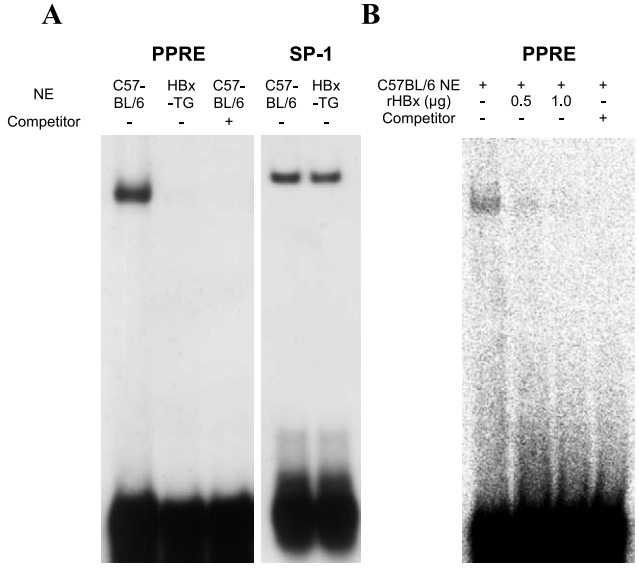


Fig. 5. The lower PPRE binding of NE from HBx-TG mouse liver tissues. A: NE (5 μg) from control C57BL/6 and from HBx-TG mouse liver were analyzed by EMSA. For the competition assay, a 100 molar excess of the cold probe was added to the reaction mixture. SP-1 binding was used as control. B: NE from control C57BL/6 mouse liver was preincubated with rHBx at room temperature for 30 min before the addition of the probe.

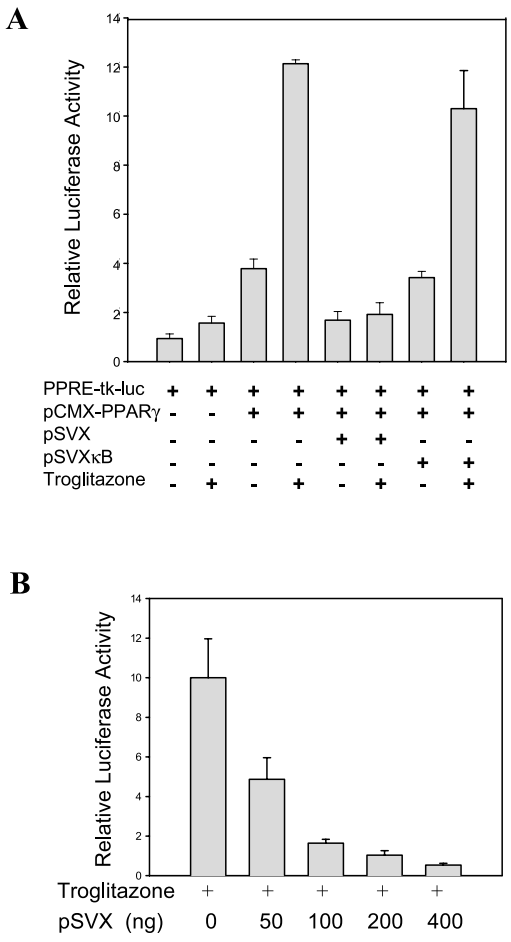


Fig. 6. Inhibition of PPARγ transcriptional activity by HBx. Transient transfection in Chang liver cells and the luciferase reporter assay were used to examine the effect of HBx on PPAR transcriptional activity, as described in Section 2. A: Luciferase activity was found to be inhibited by the overexpression of HBx, but not by mutated HBx. Each bar was normalized versus β-galactosidase activity. Luciferase activities are represented as fold inductions relative to the basal activity of the empty vector in the absence of troglitazone. B: Dose-dependent effect of HBx on luciferase activity. Normalized values are shown as means ± S.D. of independent experiments performed in triplicate and repeated at least three times.

in both the nuclear translocation and the DNA binding of PPARγ.

IFA data demonstrated that PPARγ was partially sequestered and colocalized with HBx in the cytoplasm. These results might be due to the effect of HBx on DBD and NLS at the hinge region, leading to the interruption of the nuclear localization of PPARγ. It was observed that the amount of nuclear PPARγ and its DNA binding activity were reduced in the NE of HBx-TG mouse liver tissues. We also found that HBx displayed an inhibitory effect on the PPRE binding of PPARγ. The addition of rHBx into the NE of C57BL/6 reduced the DNA binding activity of PPARγ, suggesting that nuclear PPARγ cannot bind effectively to DNA in the presence of HBx. Overall, our results suggest that the partial cytoplasmic sequestration of PPARγ by HBx may sufficiently reduce the concentration of nuclear PPARγ to prevent DNA binding.

The physiological meaning of our findings needs further study, because the expression of HBx is largely confined to liver parenchymal cells, where the expression of PPARγ is low and where the function of PPARγ is unclear under normal physiologic conditions. However, our study shows that HBx is responsible for the suppression of the growth inhibitory effect of troglitazone in HCC cells containing HBx. It is conceivable that the inhibitory effect of HBx on PPARγ may be advantageous to HBx-containing hepatocytes, leading to survive the natural ligand of PPARγ. It is also possible that HBx plays a role in biological modulation in HBV-associated diseases by interacting with and suppressing PPARγ. According to previous reports, nuclear receptors, including PPARγ, enhance HBV replication [41]. HBV non-responsive mutants to PPARγ showed reduced viral replication and a lower expression level of viral antigen [42,43], however the reduced production of viral proteins may be favorable for the virus, giving it rise to evade the host immune system. These results imply that the modulation of PPARγ by HBx can effectively regulate the life cycle of HBV and its replication. Therefore, our data suggest the possibility that HBx has an immunological or biological modulatory role in HBV-associated liver diseases, by interacting with and suppressing PPARγ.

In the present investigation, a novel interaction between HBx and PPARγ was studied. The results obtained from this study show that HBx suppresses the transcriptional and proapoptotic activities of PPARγ. Recognizing the importance of PPARγ-mediated cellular events, our results may offer a mechanism involved in the pathological effects of HBx.

*Acknowledgements:* We thank Drs. Ronald M. Evans, David J. Mangelsdof, and Heonjoong Kang for providing us with $PPRE_3$-tk-LUC and pCMX-mPPARγ and Sankyo Co. Ltd for the troglitazone. This work was supported by a grant from the Korean National Cancer Control Program, Ministry of Health and Welfare, South Korea (02-1-2-0590).

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