# Cooperative effects of hepatitis B virus and TNF may play important roles in the activation of metabolic pathways through the activation of NF-κB

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**Abstract.** Elevated levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β are often observed in the sera of hepatitis B virus (HBV)-infected patients. It is well known that these cytokines activate nuclear factor-κB (NF-κB)-signaling, and are associated with endoplasmic reticulum (ER) stress. We investigated whether HBV or HBV X protein (HBx) enhanced the activation of NF-κB in the presence of TNF and/or IL-1β, and their effects on the expression of metabolic pathway-associated genes. We examined whether HBV or HBx enhanced cytokine-induced activation of NF-κB in hepatocytes, using a reporter assay, in the presence or absence of TNF and/or IL-1β. The expression of insulin-like growth factor binding protein 1 (IGFBP1), one of the NF-κB target genes was also examined. The expression of metabolic pathway-associated genes in HepG2 and HepG2.2.15 cells in the presence or absence of TNF was evaluated by RT-qPCR. Human hepatocytes expressed TNF receptors and IL-1 receptors. NF-κB was activated by cooperation between HBx and TNF in human hepatocytes. We observed IGFBP1 expression in HBV infection and that a number of metabolic pathway-associated genes were upregulated in HepG2.2.15 cells, compared with HepG2 cells with or without TNF treatment. We observed the cooperative effects of HBV and TNF which enhanced the activation of NF-κB as well as upregulated the expression of metabolic pathway-associated genes in hepatocytes. These effects may be important in the development of HBV-associated metabolic syndrome.

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## Introduction

Hepatitis B virus (HBV) infection is one of the major causes of hepatocellular carcinoma (HCC) (1,2). Approximately 1.25 million individuals in the United States are chronically infected with HBV and approximately 43,000 new infections occur annually (3). Thus, HBV infection is a major health issues and understanding the pathogenesis of HBV as well as developing drugs for the management of HBV are of major importance (4).

In chronic hepatitis B infection, the production of tumor necrosis factor-α (TNF-α) by peripheral blood mononuclear cells (PBMCs) and the liver is increased (5,6). Serum levels of interleukin (IL)-1β, IL-6 and TNF-α are higher in patients with acute hepatitis B (7). These findings indicated that these cytokines may contribute to the elimination of HBV (8).

The higher expression of these pro-inflammatory cytokines is observed in adipose tissue and this expression is also associated with systemic inflammation and accompanying insulin resistance (9). In agreement with an association between inflammatory pathways and insulin resistance, a recent study of experimental endotoxemia demonstrated that acute inflammation induced by endotoxin administration induced systemic insulin resistance, suppressed insulin receptor substrate 1 (IRS1) and markedly induced suppressor of cytokine signaling proteins in adipose tissue, which was accompanied by the increased tissue expression of pro-inflammatory cytokines (10).

Insulin and insulin-like growth factor-1 (IGF-1) act as growth factors to promote cell proliferation and inhibit apoptosis (11). IGF-1 is involved in hepatocarcinogenesis (11,12). IGF-binding protein 1 (IGFBP1) is one of the soluble proteins that regulates the activity of IGFs (13). The liver is the major source of IGFBP1 in non-pregnant humans (13). Higher serum levels of IGFBP1 are observed in liver cirrhosis and other liver diseases (13). IGFBP1 is also involved in human carcinogenesis (14,15). Thus, insulin and IGFBP1 may be involved in the pathophysiology of liver diseases.

The activation of nuclear factor-κB (NF-κB) in the cytoplasm results in translocation to the nucleus. This induces production of pro-inflammatory cytokines such as TNF-α and IL-1β (16). In the present study, we examined whether HBV activates NF-κB in the presence of TNF-α and/or IL-1β. We

also examined the effects of HBV with or without TNF on human genes involved in metabolic pathways.

## Materials and methods

*Plasmids*. The plasmid pCXN2 was kindly provided by Professor J. Miyazaki (17). The plasmid pCXN2-HBx was prepared as previously described (18). The plasmids pCMVHBV and pHBV were gratefully received from Professor A. McLachlan (19). The reporter plasmids pIGFBP1-luc and pNF-κB-luc were purchased from Abcam (Tokyo, Japan) and Agilent Technologies (Tokyo, Japan), respectively.

*Cell culture*. The human hepatoma cell lines HepG2, HepG2.2.15 and Huh7, which have been used in our laboratory (16,20), and the immortalized human hepatocyte (IHH) cell line (kindly provided by Professor R. Ray) (21) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) at 37˚C and 5% $CO_2$. PXB cells, fresh hepatocyte cells from chimeric mice with transplanted human hepatocytes, were purchased from PhoenixBio Co., Ltd. (Higashihiroshima, Japan) (22). PXB cells were infected with HBV genotype C strain as previously described (22).

*RNA extraction, cDNA synthesis and PCR array*. HepG2 and HepG2.2.15. cells with or without 0.1 μg/ml TNF were seeded into 6-well plates. After 48 h, total cellular RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples were eluted in 60 μl elution buffer and quantified using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA), and were then stored at -80˚C until use. cDNA was synthesized from 25 ng total RNA using the SABiosciences $RT^2$ First Strand kit (Qiagen) according to the manufacturer's instructions. Following a denaturation step at 42˚C for 5 min, RNA was reverse transcribed to single-stranded cDNA using RT Enzyme Mix provided by the $RT^2$ First Strand kit (Qiagen). The reverse transcription reaction was performed in a total volume of 20 μl at 42˚C for 15 min and 95˚C for 5 min. For the purpose of determining the expression of mRNAs associated with metabolic-related gene expression, quantitative PCR was performed using $RT^2$ SYBR-Green/ROX qPCR Master mix (PAHS-157Z, $RT^2$ Profiler™ PCR Array Human Fatty Liver, Qiagen). The PCR array combines the quantitative performance of SYBR-Green-based real-time PCR with the multiple gene profiling capabilities of a microarray. Ninety-six-well plates containing gene-specific primer sets for 84 metabolic-related genes, 5 housekeeping genes and 2 negative controls were used. After performing thermal cycling, amplification data were gathered using the ABI 7300 instrument for RT2 Profiler™ PCR arrays (Qiagen). Gene expression was normalized to internal controls (housekeeping genes) to determine the fold change in gene expression between the test sample (HepG2.2.15 with or without TNF) and the control sample (HepG2 with or without TNF) using the ΔΔCT (comparative cycle threshold) method (23).

*Transfection and reporter assay*. Approximately $1.0x10^5$ cells were seeded into 6-well plates (AGC Techno Glass, Shizuoka, Japan) 24 h prior to transfection. For the purpose of detecting the relative activity of NF-κB in Huh7 and IHH cells, the cells were co-transfected with 0.3 μg pHBV/pCXN2-HBx and 0.1 μg pNF-κB-luc, respectively in Effectene transfection reagent (Qiagen). The cells were treated for 24 h with 10 ng/ml TNF-α and/or 10 ng/ml IL-1β, with or without 1 μM MG132 (Z-Leu-Leu-Leu-al; Sigma-Aldrich) after transfection. For detecting the relative activity of IGFBP1-promoter in HepG2 cells, the cells were cotransfected with 0.2 μg pCXN2, pCXN2-HBX, pCMVHBV or pHBV and 0.1 μg pIGFBP1-luc in Effectene transfection reagent (Qiagen). At 30 h post-transfection, the cells were lysed with reporter lysis buffer (Promega, Madison, WI, USA) and the luciferase activities were determined using a luminometer (Luminescencer-JNR II AB-2300; ATTO Bio Instrument, Tokyo, Japan) as described previously (23).

*Antibodies and western blot analysis*. IL-1R2 (ab97388) was purchased from Abcam. TNF-R1 (H-5) (sc-8436), TNF-R2 (D-2) (sc-8041), IL-1R1 (N-20) (sc-688), XBP1 (M-186) (sc-7160), IGFBP1 (H-120) (sc-13097), GAPDH (4G5) (sc-51906) were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

The cells were seeded into 6-well plates and cell lysates were prepared after 48 h using 50 μl sodium dodecyl sulfate sample buffer. After sonication, the lysed proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5-20% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (ATTO Bio Instrument) for western blot analysis. The membranes were incubated with primary specific antibodies. After washing, the membranes were incubated with secondary horse-radish peroxidase-conjugated antibodies. Signals were detected by means of enhanced chemiluminescence (GE Healthcare, Tokyo, Japan) and scanned using an image analyzer (LAS-4000; FujiFilm, Tokyo, Japan).

*Statistical analysis*. Results are expressed as the means ± SD. Comparisons were performed using the Student's t-test. All P-values were two-tailed, and a P-value $<0.05$ was considered to indicate a statistically significant difference.

## Results

*Human hepatocytes express TNF receptors and IL-1β receptors*. Firstly, we examined the expression of TNF receptors [TNF receptor (TNF-R)1 and TNF-R2] and IL-1β receptors [IL-1 receptor (IL-1R)2 and IL-1R2] at the protein level in human hepatocytes (Fig. 1). Human hepatoma HepG2, HepG2.2.15 and Huh7 cells and IHH cells expressed both TNF receptors and IL-1β receptors.

*Activation of NF-κB by cooperation between HBV and TNF in human hepatocytes*. It is well known that HBV infection may upregulate the production of inflammatory cytokines (5-7). However, the effect of HBV and TNF and/or TNF/IL-1β on NF-κB activation in hepatocytes remains unclear. Thus, we next examined whether HBV enhanced NF-κB activation in the presence of TNF/IL-1β in human hepatoma Huh7 cells (Fig. 2A) and in IHH cells (Fig. 2B). The reporter gene pNF-κB-luc, containing a luciferase gene linked to NF-κB

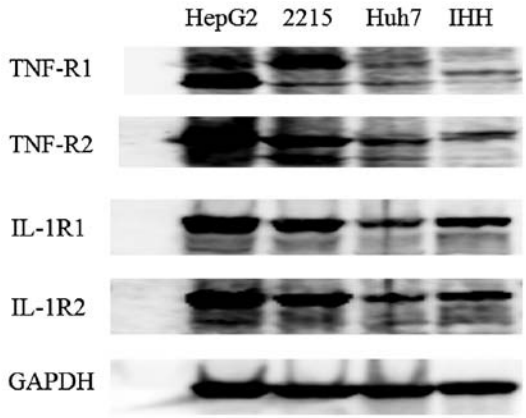


Figure 1. Tumor necrosis factor (TNF) receptor and interleukin-1β (IL-1β) receptor expression in human hepatocytes. TNF receptor (TNF-R)1, TNF-R2, IL-1 receptor (IL-1R)1, IL-1R2 and GAPDH protein expression were examined. Total cell lysates (5 $\mu$g) of HepG2, HepG2.2.15 (2215), Huh7 and IHH were subjected to 5-20% SDS-PAGE for western blot analysis with specific antibodies against TNF-R1, TNF-R2, IL-1R1, IL-1R2 and GAPDH.

response elements, was transfected into Huh7 or IHH cells, together with pHBV. After 24 h, the cells were treated with 10 ng/ml TNF-α and/or 10 ng/ml IL-1β, or left untreated. Reporter assays were performed 6 h later. In the Huh7 cells, reporter assays revealed that the treatment with TNF-α and/or IL-1β increased reporter activity compared with the untreated control (Fig. 2A), and that reporter activities were higher in the presence of HBV than in its absence (Fig. 2A). In the IHH cells, reporter assays revealed that the treatment with TNF-α, with or without IL-1β, increased reporter activity compared with the untreated control (Fig. 2B), and that reporter activities were higher in the presence of HBV than in its absence (Fig. 2B).

*Activation of NF-κB by cooperation between HBV X protein (HBx) and TNF in human hepatocytes.* HBx protein may activate a cellular transcription factor in hepatocytes (18). However, the effect of HBx and TNF and/or TNF/IL-1β on NF-κB activation in hepatocytes remains unclear. We therefore examined whether HBx enhanced NF-κB activation in the presence of TNF/IL-1β in human hepatoma Huh7 cells (Fig. 3A) and in IHH cells (Fig. 3B). The reporter gene pNF-κB-luc, containing a luciferase gene linked to NF-κB response elements was transfected into Huh7 or IHH cells, together with pCXN2-HBx. After 24 h, the cells were treated with 10 ng/ml TNF and/or 10 ng/ml IL-1β, or left untreated. Reporter assays were performed 6 h later. In the Huh7 cells, reporter assays revealed that treatment with TNF and/or IL-1β increased reporter activity compared with the untreated control (Fig. 3A), and that reporter activities were higher in the presence of HBx than those in the absence of HBx (Fig. 3A). In the IHH cells, reporter assays revealed that treatment with TNF with or without IL-1β, increased reporter activity compared with the untreated control (Fig. 3B), and that reporter activities were higher in the presence of HBx than in its absence (Fig. 3B).

*MG132 blocks the activation of NF-κB by HBx.* The proteasome inhibitor MG132, which blocks the degradation of IκB, may block NF-κB activation. We found that 1 $\mu$M MG132 blocked NF-κB activation by HBx with or without TNF in the Huh7 cells (Fig. 4A), and that 1 $\mu$M MG132 blocked NF-κB activation through the cooperation between HBx and TNF in Huh7 cells (Fig. 4B). These results suggested that proteasome pathways were involved in the HBx-mediated activation of NF-κB by TNF.

*HBV enhances IGFBP1 expression and HBx enhances IGFBP1 promoter activity.* It has been reported that TNF-α increased IGFBP1 production in HepG2 cells and that pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB activation, reduced IGFBP1 production induced by cytokines (24). We examined the effect of HBV on IGFBP1 expression. We extracted cell lysates from PXB cells infected with or without HBV genotype C strain at day 27 post-infection because both HBV DNA and HBsAg were positive at this point (22). PXB cells may

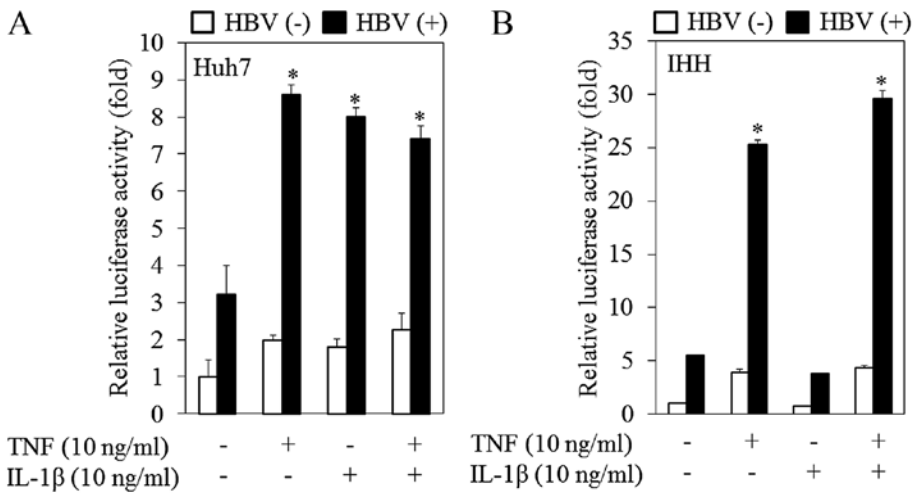


Figure 2. Nuclear factor-κB (NF-κB) activity is enhanced by cooperation between hepatitis B virus (HBV) and tumor necrosis factor (TNF)/interleukin (IL)-1β in hepatocytes. (A) Huh7 cells and (B) IHH cells. Luciferase activity was measured, and it is shown as the mean value from 3 independent experiments (means ± SD). Relative luciferase activity of untreated control was set at 1. Black columns and white columns indicate the results from transfection with and without pHBV, respectively. *P<0.05 vs. control.

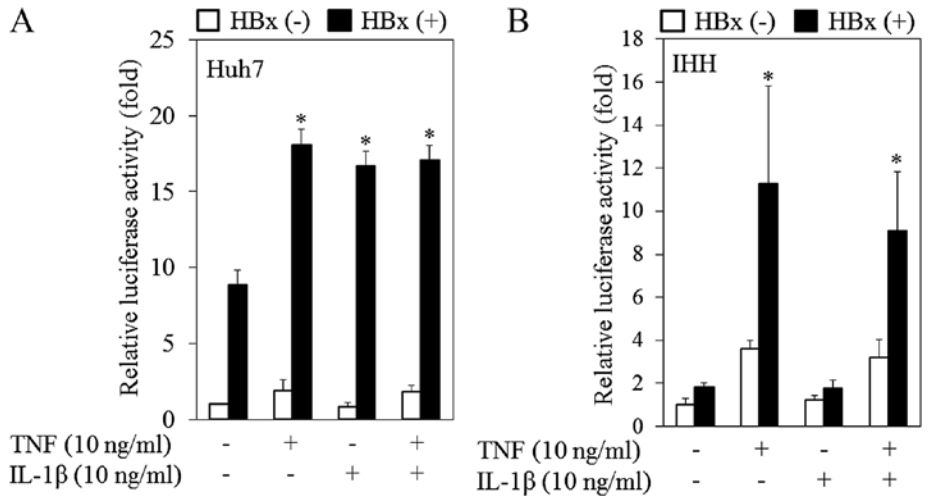


Figure 3. Nuclear factor-κB (NF-κB) activity is enhanced by the cooperation between HBV X protein (HBx) and tumor necrosis factor (TNF)/interleukin (IL)-1β in hepatocytes. (A) Huh7 cells and (B) IHH cells. Luciferase activity was measured, and it is shown as the mean value from 3 independent experiments (means ± SD). Relative luciferase activity of untreated control was set at 1. Black columns and white columns indicate the results from transfection with pCXN2-HBx or pCXN2, respectively. *P<0.05 vs. control.

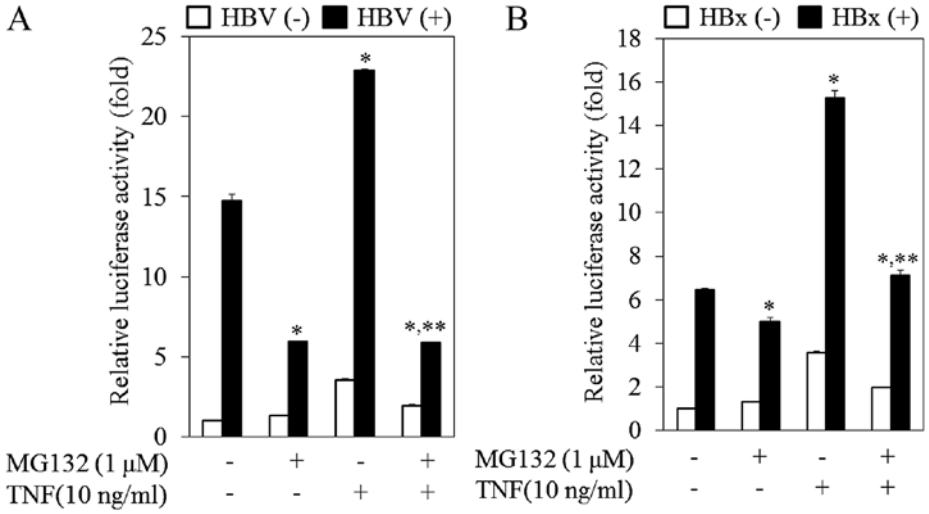


Figure 4. MG132 inhibits the activation of nuclear factor-κB (NF-κB) activity enhanced by cooperation between hepatitis B virus (HBV)/ HBV X protein (HBx) and tumor necrosis factor (TNF) in Huh7 cells. (A) HBV. Black columns and white columns indicate the results from transfection with and without pHBV, respectively. (B) HBx. Black columns and white columns indicate the results from transfection with pCXN2-HBx and pCXN2, respectively. Luciferase activity was measured, and it is shown as the mean value from 3 independent experiments (means ± SD). Relative luciferase activity of untreated control was set at 1. *P<0.05 vs. control; **P<0.05 vs. TNF.

support HBV replication (22). Total cell lysates were subjected to SDS-PAGE for western blot analysis with specific antibodies against IGFBP1 and tubulin. We observed that IGFBP1 expression was enhanced in the HBV-infected PXB cells, compared with the uninfected PXB cells (Fig. 5A). Reporter assays revealed that HBx enhanced IGFBP1 promoter activity in HepG2 cells (Fig. 5B). This enhancement of IGFBP1 promoter activity induced by HBx was increased ~1.7-fold by the addition of TNF (data not shown). These results suggest that HBV infection results in the activation of IGFBP1, one of the important genes involved in the insulin signaling pathway.

*Expression of metabolic pathway-associated genes is modulated in HBV-infected hepatocytes*. Thus, IGFBP1, which is a NF-κB-dependent metabolic pathway-associated gene, was upregulated by HBV. We further examined the effects of HBV on the expression of metabolic pathway-associated genes. To gain mechanistic insights into the effects of HBV on metabolic pathway-associated genes, we used a pathway-specific gene array to identify HBV target genes in HepG2.2.15 cells compared with HepG2 control cells, in the presence or absence of TNF (Fig. 6). HepG2.2.15 cells may produce infectious HBV virions and HepG2 is its parental cell line (16). We extracted total RNA from HepG2 and HepG2.2.15 cells in order to study the effect of HBV on metabolic pathway-associated gene expression using a human fatty liver PCR array.

In the absence of TNF, a total of 22 genes were significantly upregulated in HepG2.2.15 cells (P<0.05). In the presence of TNF, a total of 40 genes were significantly upregulated in HepG2.2.15 cells (P<0.05). Among them, there were 17

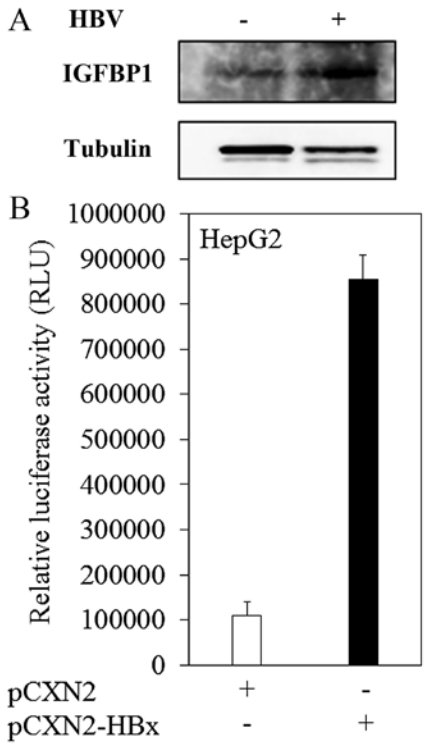


Figure 5. Hepatitis B virus (HBV) enhances insulin-like growth factor binding protein-1 (IGFBP1) expression in human hepatocytes. (A) IGFBP1 expression in PXB cells infected with or without HBV genotype C. Total cell lysates (5 $\mu$g) were subjected to 5-20% SDS-PAGE for western blot analysis with specific antibodies against IGFBP1 and tubulin. (B) HBx enhanced IGFBP1 promoter activity in HepG2 cells in the presence of HBx (black column) or in its absence (white column).

shared genes identified in the absence and presence of TNF [apolipoprotein B (APOB); apolipoprotein A-I (APOA1); solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2); pyruvate kinase, liver and RBC (PKLR); acyl-CoA synthetase long-chain family member 5 (ACSL5); apolipoprotein C-III (APOC3); nuclear receptor subfamily 1, group H, member 4 (NR1H4); retinol binding protein 4, plasma (RBP4); apolipoprotein E (APOE); insulin-like growth factor binding protein 1 (IGFBP1); phosphoinositide-3-kinase, regulatory subunit 1 (PIK3R1); glycerol kinase (GK); diacylglycerol O-acyltransferase 2 (DGAT2); X-box binding protein 1 (XBP1); serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1); nuclear receptor subfamily 1, group H, member 4 (NR1H3); peroxisome proliferator-activated receptor alpha (PPARA)]. Several genes associated with insulin and adipokine signaling pathways, such as PPARA, IGFBP1 and PIK3R1, were activated. The endoplasmic reticulum stress gene XBP1 was also upregulated. Notably, we observed upregulation of other metabolic pathway-associated genes in the HepG2.2.15 cells. On the other hand, none were significantly downregulated in HepG2.2.15 cells in the absence of TNF, and 6 genes [CCAAT/enhancer binding protein (C/EBP), beta (CEBPB); suppressor of cytokine signaling 3 (SOCS3); sterol regulatory element binding transcription factor (SREBF)2; fatty acid binding protein 3, muscle and heart (FABP3); SREBF1; and IL-6] were significantly downregulated in HepG2.2.15 cells treated with TNF (data not shown).

## Discussion

In the present study, we demonstrated the cooperative effects of HBV and TNF on NF-κB activation in human hepatocytes. HBx appears to be involved in the activation of NF-κB. The activation of NF-κB was inhibited by the proteasome inhibitor MG132, suggesting that proteasome pathways were involved in the HBV/HBx-mediated activation of NF-κB. We also observed that IGFBP1 expression was enhanced in human hepatocytes infected with HBV and that HBV enhanced the expression of metabolic pathway-associated genes in the presence or absence of TNF.

TNF-α is at present considered as the pro-inflammatory cytokine that introduced the link between inflammation, obesity and insulin resistance (9,10,25,26). During the search for the underlying mechanisms, researchers studied various signaling pathways and transcription factors and identified the IKKβ pathway as a target for TNF-α-induced insulin resistance (27). Two other studies have also demonstrated the association between IKKβ expression in the liver and insulin resistance (28,29). In terms of these studies, our findings suggested that the cooperative effects of HBV and TNF on NF-κB activation may have induced insulin resistance by upregulating IGFBP1. On the other hand, considerable evidence has been generated regarding the roles of the IL-1 family of cytokines in metabolic inflammation (30,31). IL-1β, similar to other pro-inflammatory stimuli, is capable of reducing IRS1 expression at a transcriptional level through an ERK-dependent mechanism (32). Both IL-1α- and IL-1β-deficient mice are almost entirely protected from liver inflammation following diet-induced steatosis suggesting that these pro-inflammatory IL-1 cytokines may be crucially involved in the development of liver inflammation (33). Lang *et al* have demonstrated that, in human hepatoma cells, IL-1 and TNF-α produce dose-dependent increases in IGFBP1 protein release and IGFBP1 mRNA expression and that the increased rate of IGFBP1 synthesis is due primarily to the stimulation of transcription (24).

HCC develops from a background of chronic liver diseases. Common causes include viral hepatitis [HBV or hepatitis C virus (HCV)], alcoholic liver diseases and autoimmune liver diseases. Emerging evidence suggests an association between metabolic factors and the risk of HCC in both developing and developed countries (34). Experimental evidence suggests that HBx promotes cell growth and HCC by upregulating lipogenic gene expression (35-37). Na *et al* has used transgenic mice and cell lines to show that HBx enhances the expression of liver X receptor (LXR) and its lipogenic target genes, such as sterol regulatory element-binding protein 1 (SREBP1), FAS and PPAR, which are accompanied by lipid droplet accumulation and eventually lead to HCC (35). Another study has shown that HBx induced the activation of SREBP1 through the PI3K-Akt pathway (38). The tumorigenic effects of insulin resistance may be mediated directly by insulin signaling, or indirectly through changes in endogenous hormone metabolism. In particular, metabolic pathway-associated genes such as IGF1, which undergo bioactive modulation by IGFBP1, may be involved. To the best of our knowledge, there do not appear to have been any metabolic studies comparing HBV-positive and HBV-negative subjects and furthermore, there are no specific

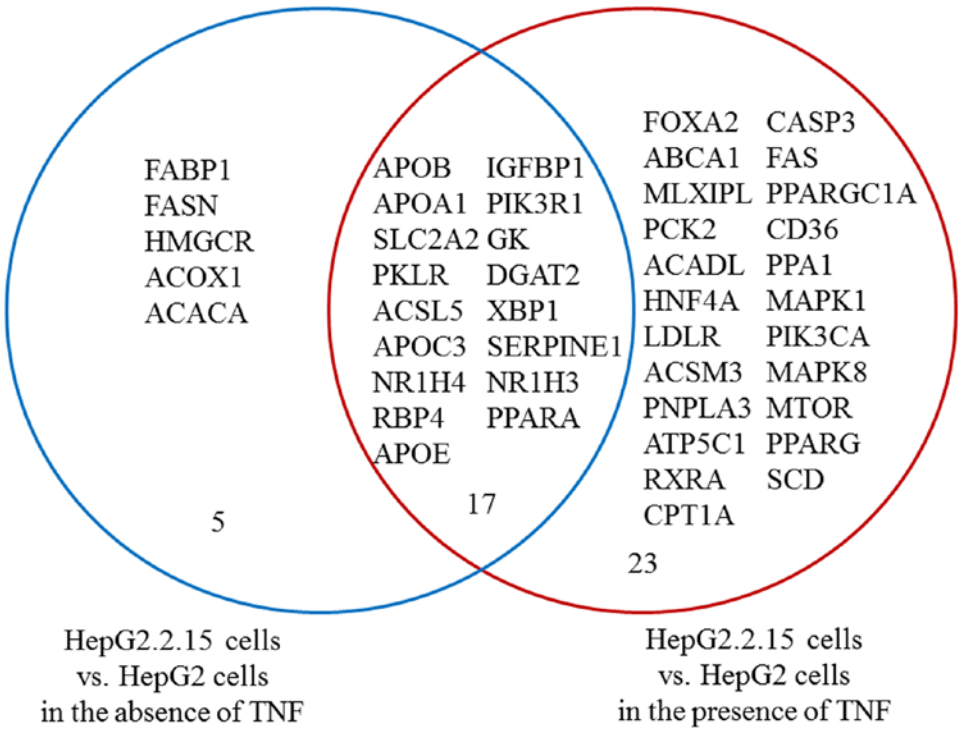


Figure 6. Venn diagram representing unique and shared metabolic pathway-associated genes differentially expressed in HepG2.2.15 (vs. HepG2) with or without tumor necrosis factor (TNF). Genes indicated as $P<0.05$. Total RNAs were extracted from HepG2 and HepG2.2.15 treated with or without TNF. Following the synthesis of cDNA, human fatty liver PCR arrays were performed. Data were analyzed with $RT^2$ profiler PCR array data analysis software. Fatty acid binding protein 1, liver (FABP1); fatty acid synthase (FASN); 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR); acyl-CoA oxidase 1, palmitoyl (ACOX1); acetyl-CoA carboxylase alpha (ACACA); apolipoprotein B (APOB); apolipoprotein A-I (APOA1); solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2); pyruvate kinase, liver and RBC (PKLR); acyl-CoA synthetase long-chain family member 5 (ACSL5); apolipoprotein C-III (APOC3); nuclear receptor subfamily 1, group H, member 4 (NR1H4); retinol binding protein 4, plasma (RBP4); apolipoprotein E (APOE); insulin-like growth factor binding protein 1 (IGFBP1); phosphoinositide-3-kinase, regulatory subunit 1 (PIK3R1); glycerol kinase (GK); diacylglycerol O-acyltransferase 2 (DGAT2); X-box binding protein 1 (XBP1); serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1); nuclear receptor subfamily 1, group H, member 3 (NR1H3); peroxisome proliferator-activated receptor alpha (PPARA); forkhead box A2 (FOXA2); ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1); MLX interacting protein-like (MLXIPL); phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2); acyl-CoA dehydrogenase, long chain (ACADL); hepatocyte nuclear factor 4, alpha (HNF4A); low density lipoprotein receptor (LDLR); acyl-CoA synthetase medium-chain family member 3 (ACSM3); patatin-like phospholipase domain containing 3 (PNPLA3); ATP synthase, $H^+$ transporting, mitochondrial F1 complex, γ polypeptide 1 (ATP5C1); retinoid X receptor alpha (RXRA); carnitine palmitoyltransferase 1A (liver) (CPT1A); caspase-3, apoptosis-related cysteine peptidase (CASP3); Fas cell surface death receptor (FAS); peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A); CD36 molecule (thrombospondin receptor) (CD36); pyrophosphatase (inorganic) 1 (PPA1); mitogen-activated protein kinase 1 (MAPK1); phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA); mitogen-activated protein kinase 8 (MAPK8); mechanistic target of rapamycin (serine/threonine kinase) (MTOR); peroxisome proliferator-activated receptor gamma (PPARG); stearoyl-CoA desaturase (delta-9-desaturase) (SCD).

biomarkers for predicting HCC development in HBV-positive patients with or without cirrhosis. Our results also indicated that the cooperative effect of HBV and TNF on the expression of metabolic pathway-associated genes, which potentially play important roles in the activation of metabolic pathways, may shed light on the metabolic studies in HBV-induced HCC.

The transcription factor PPARG is an important regulator of hepatic lipid metabolism. Degenhardt *et al* (39) have demonstrated that the mRNA expression of IGFBP1 is under the primary control of PPARG ligands by identifying PPAR response elements (PPREs) located within IGFBP1, in human HCC cells and in normal mouse liver. The results of our metabolic pathway-associated gene expression PCR array (Fig. 6) indicated that PPARG was significantly upregulated by HBV and TNF stimulation (2.36- vs. 1.90-fold without TNF), suggesting that HBV and TNF upregulated IGFBP1 expression through the upregulation of PPARG expression.

In conclusion, the cooperative effects of HBV and TNF may play important roles in the activation of NF-κB and the expression of metabolic pathway-associated genes including IGFBP1. These results suggest that metabolic factors may also modify HBV-associated diseases as well as HCV (40,41).

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