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Ethanol potentiates hepatitis B virus replication through oxidative stress-dependent and -independent transcriptional activation

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ABSTRACT

Excessive alcohol intake accelerates disease progression in chronic hepatitis B virus (HBV) infection, but the mechanisms by which ethanol worsens the prognosis of chronic hepatitis B (CHB) are not fully understood. The aim of this study was to investigate whether HBV replication is augmented by alcohol or alcohol-induced cytochrome p450 2E1 (CYP2E1), and if so, whether oxidative stress is involved in the process. Ethanol treatment promoted HBV replication in HepAD38 cells that permit the conditional viral replication. Luciferase reporter assays confirmed that HBV core, preS1 and preS2/S promoter activities were augmented by ethanol. Ethanol did not induce oxidative stress in HepAD38 cells with minimal expression of CYP2E1. However, over-expression of CYP2E1 induced oxidative stress and amplified transcriptional activation of HBV by ethanol. Antioxidant glutathione treatment attenuated CYP2E1-mediated augmentation of HBV replication in ethanol-treated cells. In conclusion, ethanol enhances transcriptional activity of HBV promoters in human hepatoma cells in an oxidative stress-independent manner; and CYP2E1-mediated oxidative stress potentiates the ethanol-induced transactivation of HBV.

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1. Introduction

Alcohol is a global risk factor for chronic liver disease, which ranges from simple steatosis to more severe steatohepatitis, liver cirrhosis and hepatocellular carcinoma [1]. Several comorbidities have been identified that increase the risk of the development and progression of alcoholic liver disease such as obesity, malnutrition and chronic viral hepatitis [1,2]. Chronic hepatitis B virus (HBV) infection is one of the leading causes of hepatic decompensation and hepatocellular carcinoma worldwide: Approximately 400 million people are infected with HBV [3]. Because of the high prevalence of drinking in the general population, a significant portion of chronic hepatitis B (CHB) patients are believed to have concomitant alcoholic liver disease. Previous reports showed that excessive alcohol consumption worsens the natural course of CHB. Heavy alcohol intake in CHB patients is associated with a higher risk for developing liver cirrhosis [4–6], hepatocellular carcinoma [7–11] and liver-related mortality [12,13]. Although these effects of alcohol on accelerated liver injury may be explained by aggravated hepatic inflammation by alcohol [14,15], it can be also speculated that ethanol may enhance HBV replication. However, it is still unclear whether ethanol directly augments HBV replication because previous experimental studies gave contradictory results [16–18].

Cytochrome P450 2E1 (CYP2E1) is an inducible ethanol-metabolizing enzyme. CYP2E1 is especially active during excessive ethanol exposure, and it plays a major role in the pathogenesis of alcoholic liver disease by inducing oxidative stress [19,20]. However, it is unknown whether CYP2E1-mediated ethanol metabolism modulates HBV replication.

In this study, we aimed to investigate (1) the effect of alcohol on HBV replication at a molecular level and (2) the role of CYP2E1 on the interaction between alcohol and HBV using a cell model in which HBV RNA is transcribed from HBV covalently closed circular DNA (cccDNA), which is the natural template for HBV transcription in CHB [21].

Abbreviations: ADH, acetaldehyde; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CYP2E1, cytochrome p450 2E1; DCFDA, dichlorofluoresceindiacetate; FBS, fetal bovine serum; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH–EE, glutathione reduced ethyl ester; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HNF4α, hepatocyte nuclear factor 4α; MDA, malondialdehyde; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1-α; pgRNA, pregenomic RNA; PPARα, peroxisome proliferator-activated receptor α; rcDNA, relaxed circular DNA; ROS, reactive oxygen species; ssDNA, single-strand DNA.

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2. Materials and methods

2.1. Cell culture

Ethanol, acetaldehyde, 2',7'-dichlorofluorescindiacetate, *tert*-butyl hydroperoxide, and glutathione reduced ethyl ester (GSH–EE) were purchased from Sigma–Aldrich. HepG2 cells were maintained in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum (FBS), 50 μg/ml penicillin and 50 μg/ml streptomycin. HepAD38 cells, which permit HBV replication under the control of a tetracycline-responsive CMV-IE promoter (a generous gift from professor C. Seeger; Fox Chase Cancer Center, Philadelphia, PA) [21], were grown in the media for HepG2 cells and supplemented with 0.3 μg/ml tetracycline and 400 μg/ml G418 (Invitrogen, Carlsbad, CA). To induce HBV replication in these cells, tetracycline was omitted from the medium for 10 days before ethanol treatment. The cells were treated with ethanol in a closed chamber system as previously reported [22], with minor modifications (Supplementary materials and methods).

2.2. Quantitative real-time RT PCR

HepAD38 cells were treated with lysis buffer (50 mM Tris–HCl [pH 8.0], 1 mM EDTA, 0.2% NP-40, and 150 mM NaCl), and the homogenate was centrifuged to separate nuclear and cytoplasmic fractions. Total RNA was extracted from the cytoplasmic fraction using Trizol (Invitrogen). HBV pre-genomic RNA (pgRNA) was quantified by transcript-specific quantitative real-time RT–PCR using the previously reported primer pairs (Table 1) [23]. The RNA levels were compared using the relative comparative threshold (Ct) method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Liver-enriched transcription factors and nuclear receptors that have been reported to bind to the HBV promoter/enhancer sequences were also measured by real-time RT–PCR (Table 1).

2.3. Southern and Northern blotting of HBV

HBV relaxed circular DNA (rcDNA) was isolated from the cytoplasmic fraction of HepAD38 cells and subjected to Southern blotting, as previously reported (Supplementary materials and methods) [24].

2.4. Determination of the half-life of HBV pgRNA

RNA stability was assessed by measuring HBV pgRNA at 1, 3 and 6 h after blocking transcription by adding actinomycin D to the culture medium at a final concentration of 10 μg/mL.

2.5. Luciferase reporter assay

The HepG2 cells were transfected with the HBV promoter reporter vectors and pRL–TK vector using Lipofectamine 2000 (Invitrogen) (Supplementary materials and methods). The cells were harvested after an additional incubation for 48 h. The effect of alcohol on HBV promoter activity was assessed using a Luciferase Assay Reagent (Promega) according to the vendor's recommendation. The firefly luciferase activity was normalized to *Renilla* luciferase activity to adjust for transfection efficiency.

Table 1
Primers for quantitative real-time RT-PCR.

Primer	Sequence (5′-3′)
GAPDH	F:GCACCGTCAAGGCTGAGAAC-R:ATGGTGGTGAAGACGCCAGT
HBV pgRNA	F:CACCTCTGCCTAATCATC R:GGAAAGAAGTCAGAAGGCAA
HNF-4α	F:CAACCTCATCCTCCTTCTT R:CATCTCACCTGCTCTACC
PPARα	F:GGCGAGGATAGTTCTGGAAGC R:CACAGGATAAGTCACCGAGGAG
FXRα	F:CGACAAGTGACCTCGACAAC R:TCAACCGCAGACCCTTTCAG
PGC-1α	F:GATGACAGCGAAGATGAA R:GAAGAACAAGAAGGAGACA
CYP450 2E1	F:GACTGTGGCCGACCTGTT R:ACTACGACTGTGCCCTTGG

2.6. Induction of human CYP2E1 expression

Total RNA was extracted from a human liver resection specimen with Trizol reagent (Invitrogen). The human CYP2E1 cDNA fragment (from +31 to +1585; NM_000773) was amplified by PCR using the following primers with restriction sites: 5′-AGCGGTACCATGTCTGCCCTCGGAGTCA-3′ (*Kpn*I) and 5′-TCAGCTCGAGAAATCCTGACCTCAAACAA-3′ (*Xho*I). The cDNA fragment was inserted into the pcDNA3.1 mammalian expression vector (Invitrogen) to generate pcDNA3.1-CYP2E1. The insertion and correct orientation of the cDNA fragment were confirmed by direct sequencing. The HepAD38 cells were transfected with the pcDNA3.1-CYP2E1 plasmid using Lipofectamine 2000 and the expression of CYP2E1 was measured by real-time quantitative RT–PCR [25].

2.7. Lipid peroxidation, glutathione and reactive oxygen species activity assay

Malondialdehyde (MDA)-bound protein adduct, a bi-product of lipid peroxidation, was quantified by ELISA using an OxiSelect MDA Adduct ELISA Kit (Cell Biolabs, CA) according to the recommended instructions. Cellular glutathione (GSH) is a systemic protectant against reactive oxygen species (ROS), and the depletion of GSH content reflects ethanol-induced oxidative stress in HepG2 cells [26]. Cellular GSH content was measured using a Glutathione Assay Kit (cat. no CS0260, Sigma–Aldrich) according to the manufacturer's instructions. The activity of the reactive oxygen species was also assessed by measuring 2′,7′-dichlorofluorescin as previously reported, with minor modifications (Supplementary materials and methods) [27].

3. Results and discussion

3.1. Ethanol promotes HBV replication at the transcription level in human hepatoma cells

Southern blotting analysis showed that HBV relaxed circular DNA (rcDNA) and single-strand DNA (ssDNA) were significantly over-expressed in ethanol-treated cells compared to untreated cells (Fig. 1A). The effect of ethanol was dose dependent, with a marked increase in HBV DNA expression at 200 mM ethanol. Although various concentrations of ethanol have been used in *in vitro* studies, 100 mM was used in subsequent experiments because a previous *in vitro* study in the HepG2/HBV model used 50–200 mM ethanol [16], and this concentration had no cytotoxic effect on cell culture [28].

Real-time RT–PCR assays showed that 100 mM ethanol increased cytoplasmic HBV pgRNA 2.5-fold compared to untreated controls (Fig. 1B). Northern blotting analysis confirmed the increased HBV pgRNA (3.5 kb) by ethanol (Fig. 1C). Of note, ethanol also increased levels of the 2.4 kb sub-genomic RNA as well as the HBV pgRNA.

To exclude the possibility that ethanol might suppress the decay of viral RNA, the stability of HBV pgRNA was assessed by quantitative real-time RT PCR. The RNA decay curves revealed that the half-life of HBV pgRNA was 3.2 and 2.6 h in control and ethanol-treated cells, respectively (Fig. 1D). Therefore, it is evident that

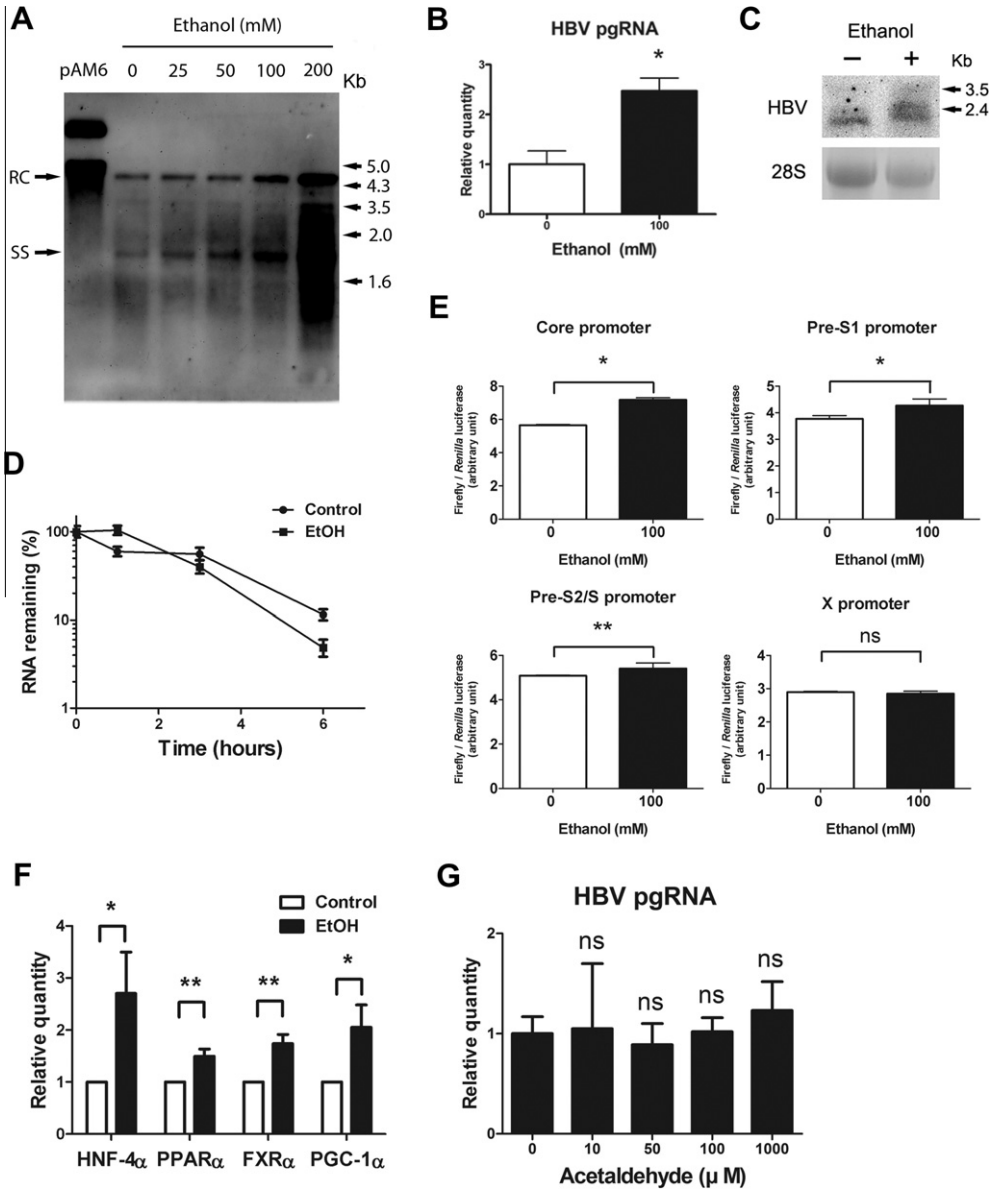

Fig. 1. Effect of ethanol on HBV replication in human hepatoma cells. Southern blot (A), transcript-specific real-time RT–PCR (B) and Northern blot analysis (C) of HBV in ethanol-treated HepAD38 cells pAM6, positive control plasmid. The positions of HBV pregenomic RNA (pgRNA) (3.5 kb) and pre-S1 RNA (2.4 kb) are indicated, and 28S ribosomal RNA was shown as the loading control (C). HBV pgRNA decay was assessed by real-time RT–PCR analysis (D). HBV promoter activities were assessed by luciferase reporter assay (E). Hepatocyte-enriched transcription factors were quantified by real-time RT–PCR (F). HBV pgRNA was quantified by real-time RT–PCR in HepAD38 cells treated with acetaldehyde (G). The values represent the mean ± SD ($^{*}p < 0.01$; $^{**}p < 0.05$; ns, not significant). RC, relaxed circular DNA; SS, single-strand DNA; HNF4α, hepatocyte nuclear factor 4α; PPARα, peroxisome proliferator-activated receptor α; FXR, farnesoid X receptor; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1-α.

ethanol increases HBV pgRNA by increasing transcription from HBV to cccDNA rather than inhibiting RNA decay.

Next, the effect of ethanol on the activity of HBV promoters was investigated using luciferase reporter assays. As shown in Fig. 1E, the HBV core, preS1 and preS2/S promoter activities were significantly augmented in ethanol-treated cells compared to untreated cells. This finding is in line with the Northern data, indicating that ethanol increases major HBV RNA transcripts by enhancing promoter activities.

HNF-4α, PPARα, FXRα and PGC-1α are liver-enriched nuclear receptors and co-activators of nuclear receptors that are also positive regulators of HBV pgRNA transcription [29,30]. Real-time RT–PCR showed that mRNA of these factors was significantly over-expressed in ethanol-treated cells compared to untreated cells (Fig. 1F). These results suggest that ethanol treatment increases the expression of liver-enriched transcription factors, which in turn activates the transcription of HBV RNA in hepatoma cells.

Acetaldehyde is the first metabolite of ethanol and is considered to play a major role in alcohol-induced liver damage [31]. Real-time RT–PCR assays showed that HBV pgRNA was not over-expressed by acetaldehyde in HepAD38 cells, indicating that the ethanol-mediated over-expression of HBV is a direct effect of alcohol itself rather than its metabolites (Fig. 1G).

3.2. CYP2E1 over-expression potentiates the ethanol-induced transactivation of HBV

CYP2E1 is minimally, if at all, expressed in HepG2 cells [32,33]. Because HepAD38 cells are derived from HepG2 cells, we sought to investigate whether induction of CYP2E1expression has any effect

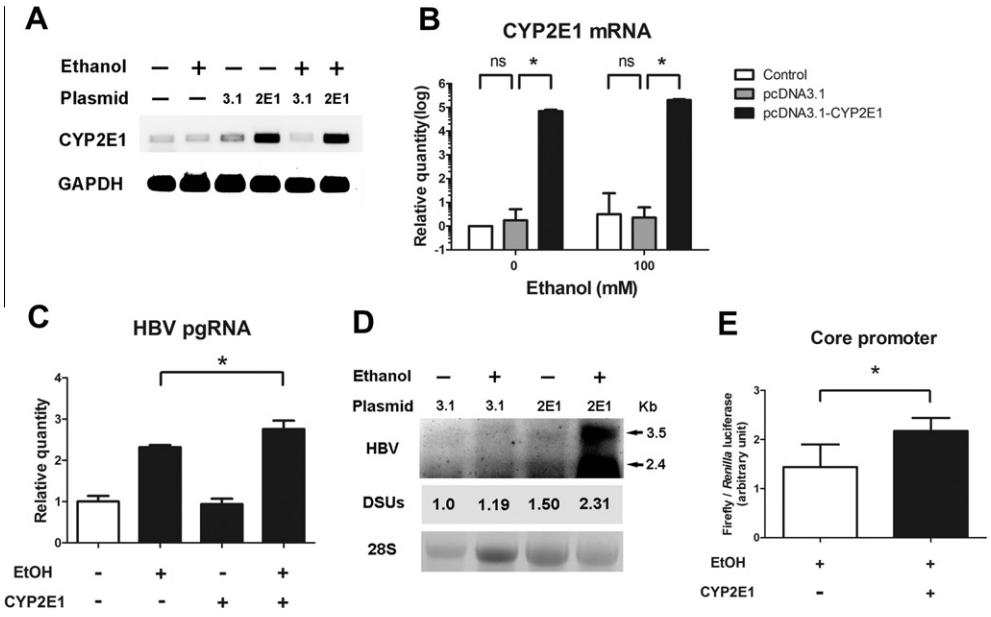

Fig. 2. Effect of CYP2E1 expression on HBV replication in ethanol-treated HepAD38 cells. Expression of CYP2E1 was assessed by RT–PCR (A) and real-time RT–PCR (B) analysis in ethanol-treated HepAD38 cells. HBV pgRNA was quantified by real-time RT–PCR (C) and Northern blot analysis (D) in CYP2E1-expressing HepAD38 cells. 28S ribosomal RNA is shown as the loading control. HBV core promoter activity was assessed by luciferase reporter assay in ethanol-treated HepAD38 cells with or without CYP2E1 expression (E). The values represent the mean ± SD of three independent experiments. All variables in each experiment were tested in triplicate (*$p < 0.05$; ns, not significant) 3.1, pcDNA3.1; 2E1, pcDNA3.1-CYP2E1; DSUs, densitometry scanning units.

on ethanol-induced HBV replication. Indeed, HepAD38 cells minimally expressed CYP2E1 regardless of ethanol treatment. In contrast, the transfection of pcDNA3.1-CYP2E1 induced CYP2E1 over-expression of by more than five orders of magnitude regardless of ethanol treatment (Fig. 2A and B). Interestingly, the over-expression of CYP2E1 significantly exaggerated the ethanol-induced augmentation of HBV replication (Fig. 2C and D). The luciferase reporter assay showed that the core promoter activity was also significantly augmented by CYP2E1-overexpression in ethanol-treated cells (Fig. 2E).

3.3. Ethanol induce HBV replication by oxidative stress-independent and -dependent mechanisms

Oxidative stress and lipid peroxidation are the major mechanisms underlying the pathogenesis of alcoholic liver disease [34–36]. It may be also speculated that oxidative stress plays a role in ethanol-induced HBV replication in our model. To test this hypothesis, parameters of oxidative stress were measured in HBV-replicating HepAD38 cells with or without 100 mM ethanol treatment for 2 days. As shown in Fig. 3A, MDA adduct, DCFDA fluorescence and GSH levels were not significantly different between ethanol-treated and untreated cells. This result is in line with the previous report which showed that treatment with alcohol or acetaldehyde does not increase oxidative stress in HepG2 cells [26]. Therefore, it is evident that ethanol promotes HBV replication in an oxidative stress-independent manner in HepAD38 cells. However, this does not necessarily mean that oxidative stress has no effect on HBV replication. Inducible forms of MEOS, especially CYP2E1, is the most important sources of ROS [37,38]. In the presence of ethanol, over-expression of CYP2E1 significantly decreased cellular GSH levels (Fig. 3B). GSH–EE treatment attenuated increased HBV replication and core promoter activation by CYP2E1 (Fig. 3C and D). Moreover, the expression of HNF-4α, a major transcription factor for the HBV core promoter, was enhanced by CYP2E1 expression, and GSH treatment decreased the ethanol-induced enhanced HNF-4α expression (Fig. 3E). These results suggest that CYP2E1 over-expression results in increased oxidative stress, which in turn may contribute to the augmented HBV replication in HepAD38 cells treated with ethanol.

Fig. 4 depicts the proposed mechanisms of ethanol-induced viral replication in CHB based on our results. Ethanol *per se* stimulate transcription of HBV genome in an oxidative stress-independent manner, possibly by up-regulating liver-specific transcription factors/nuclear receptors. Transcription of HBV RNA is regulated by hepatocyte-enriched transcription factors and nuclear receptors such as HNF-4α, PPARα, FXRα and PGC-1α [29,30]. These transcription factors bind core promoter and up-regulate HBV pgRNA transcription [39–41]. Thus, increase in these transcription factors may explain the ethanol-induced activation of HBV transcription in this study. HNF-4α and PPARα also increase transcription from S promoter [29,30], and this fact is also in line with our observations.

Another novel finding in this study is that CYP2E1 over-expression augments ethanol-induced HBV replication Chronic alcohol intake induces CYP2E1 expression, and CYP2E1-mediated oxidative stress plays a major role in the pathogenesis of alcohol liver disease [42]. Induction of CYP2E1 by ethanol increases oxidative stress, which in turn activate HBV transcription in a way similar to oxidative stress-independent pathway. However, it may be also possible that alcohol-induced oxidative stress may affect other mechanisms of transcriptional control of HBV cccDNA such as innate immunity and epigenetic control [43–45].

The interaction of alcohol use and hepatitis B virus infection is clinically important because of the high prevalence of both conditions. Moreover, the prevalence of hepatitis B infection in alcoholic liver disease is reported to be higher than in healthy non-alcoholic controls [46–48]. In clinical settings, it can be speculated that chronic alcohol intake in CHB patients may induce CYP2E1 over-expression, and alcohol along with induced CYP2E1 may increase HBV replication. Because a high viral titer is associated with a poor prognosis for patients with CHB virus infection [49,50], this mechanism may explain the adverse outcome of CHB infection with excessive alcohol intake. Regarding the relationship between alcohol and HBV replication, there is only one small clinical study in which five HBV carriers did not show enhanced viral replication after one-week period of social drinking [51]. However, the impact

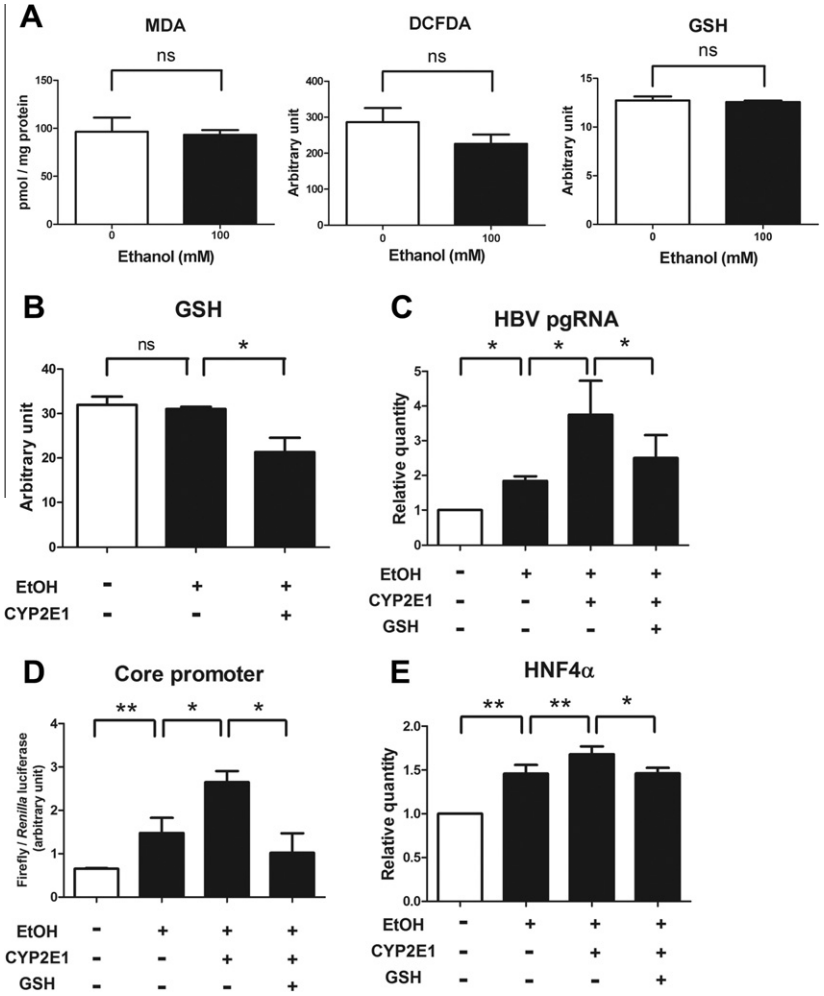

Fig. 3. Effect of oxidative stress on the ethanol-induced transactivation of HBV in CYP2E1-expressing HepAD38 cells. Oxidative stress levels were assessed by measuring the concentrations of MDA-bound protein adduct, DCFDA fluorogenic activity and cellular GSH in HepAD38 cells which were induced to replicate HBV with or without 100 mM ethanol for 2 days (A). Cellular GSH content was also measured in HepAD38 cells transfected with a control plasmid or pcDNA3.1-CYP2E1 (B). Levels of HBV pgRNA was compared by real-time RT–PCR in ethanol-treated HepAD38 cells in terms of CYP2E1 expression and GSH–EE treatment (C). HBV core promoter activity (D) and HNF4α mRNA expression (E) was compared in the same way. The values represent the mean ± SD of three independent experiments. All variables in each experiment were tested in triplicate ($^{*}p < 0.01$; $^{**}p < 0.05$; ns, not significant). MDA, malondialdehyde; DCFDA, dichlorofluoresceindiacetate; GSH, glutathione.

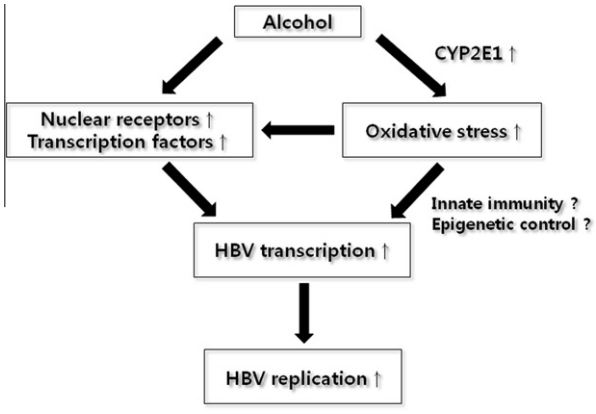

Fig. 4. Schematic summary of the model explaining the relationship between alcohol and HBV replication.

of repeated drinking, which is the case in a real-world situation, on HBV replication has not been established.

The limitation of our study is that the interaction between host immune cells and hepatocytes was not examined, so that the effect of ethanol on the immunologic milieu in HBV-replicating cells is still unknown. Prospective clinical studies are warranted to determine the effect of alcohol on HBV replication in CHB patients.

In summary, this is the first study to demonstrate that ethanol activates transcription of HBV promoters by increasing the expression of nuclear receptors and transcriptions factors in the absence of CYP2E1 expression. Moreover, CYP2E1-induced oxidative stress potentiates the ethanol-related transactivation of HBV. Our data may provide a mechanistic clue to the expedited disease progression in CHB patients with excessive alcohol intake, and also provide a rationale for alcohol abstinence in chronic HBV infection.

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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.2012.12.081.

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