A direct role for hepatitis B virus X protein in inducing mitochondrial membrane permeabilization

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Summary
Hepatitis B virus X protein (HBx) acts as a multifunctional protein that regulates intracellular signalling pathways during HBV infection. It has mainly been studied in terms of its interaction with cellular proteins. Here, we show that HBx induces membrane permeabilization independently of the mitochondrial permeability transition pore complex. We generated mitochondrial outer membrane-mimic liposomes to observe the direct effects of HBx on membranes. We found that HBx induced membrane permeabilization, and the region comprising the transmembrane domain and the mitochondrial-targeting sequence was sufficient for this process. Membrane permeabilization was inhibited by nonselective channel blockers or by N-(n-nonyl)deoxynojirimycin (NN-DNJ), a viroporin inhibitor. Moreover, NN-DNJ inhibited HBx-induced mitochondrial depolarization in Huh-7 cells. Based on the results of this study, we can postulate that the HBx protein itself is sufficient to induce mitochondrial membrane permeabilization. Our finding provides important information for a strategy of HBx targeting during HBV treatment.

Keywords
hepatitis B virus X protein, membrane permeabilization, viroporin

1 INTRODUCTION

Hepatitis B virus (HBV) is a major cause of hepatitis, and nearly 240 million people are infected worldwide.1 HBV is associated with hepatic fibrosis, cirrhosis and hepatocellular carcinoma.2,3 HBV is a DNA virus belonging to the Hepadnaviridae family and encodes 7 proteins: 5 structural and 2 nonstructural proteins. The structural proteins include 3 envelope proteins (small, middle and large surface proteins), capsid protein and polymerase. The nonstructural proteins include HBeAg (secreted e antigen) and HBx (hepatitis B virus X protein).4

HBx is a nonstructural protein with a molecular weight of 17.5 kDa. HBx functions as a multifunctional protein that interacts with various transcription factors and cell cycle-related regulatory proteins, adjusting the intracellular environment to optimal conditions for viral production.5,7 Interestingly, its intracellular distribution varies depending on its expression level in cells. For example, HBx was detected in the nucleus at a low expression level, while it was distributed throughout the cytoplasm at a high expression level.6 HBx has been reported to target the mitochondrial outer membrane, but not the endoplasmic reticulum.
2.2 | GUV preparation

Ten μL of a mitochondrial outer membrane-mimic lipid mixture (egg PC: egg PE: liver PI: brain PI: heart CL = 54: 13: 2: 1: 1 [weight ratio]) dissolved in chloroform: methanol = 4:1 (2 mg/mL) was dried on indium tin oxide (ITO)-coated glass at 50°C, and the chamber was filled with 200 mmol L⁻¹ sorbitol. Giant unilamellar vesicles (GUVs) were produced by the application of 2 V peak-to-peak and 8 Hz for 2 hours at 36°C using Vesicle Prep Pro (Nanion Technologies GmbH, Munich, Germany). For HBx targeting of GUVs, GUVs were prepared with 0.5% ATTO 390-DOPE.
challenging to evaluate in cells, we attempted to investigate HBx function using a recombinant HBx protein and an artificial membrane system. We synthesized recombinant HBx protein in E. coli and recovered its biological activity through a rational refolding method, as previously reported. The purity of the refolded recombinant HBx protein was examined via Coomassie blue staining (>95%), and it was further confirmed by anti-HBx and anti-His antibodies (Figure 1A,B). As HBx was shown to be inserted into the mitochondrial outer membrane via its transmembrane domain (residues 53-70), we generated liposomes with a rat liver mitochondrial outer membrane composition, as previously reported. To observe the HBx localization in liposomes, we prepared mitochondrial outer membrane-mimic giant unilamellar vesicles (MOM-GUVs) including ATTO 390-DOPE. We treated the recombinant HBx protein for 2 minutes, and HBx-targeted MOM-GUVs were then stained with nickel-nitrilotriacetate conjugated to ATTO 488 fluorescent dye (NTA-ATTO 488) for the detection of poly-His-tagged HBx by fluorescence microscopy. As expected, the recombinant HBx protein was shown to target the MOM-GUVs (Figure 1C).

3.2 | HBx induces membrane permeabilization

HBx triggers membrane depolarization, probably through interaction with the mitochondrial permeability transition pore. In this study, we investigated HBx-induced membrane permeabilization using MOM-GUVs without any mitochondrial proteins. First, we created MOM-GUVs with 200 mmol L\(^{-1}\) sorbitol solution, and they were added to the external buffer containing 100 mmol L\(^{-1}\) KCl and 10 mmol L\(^{-1}\) HEPES/KOH (pH 7.4). Using phase contrast microscopy, we found that MOM-GUVs became transparent after treatment with HBx in a time-dependent or concentration-dependent manner (Figure 2A,B). Loss of contrast around MOM-GUVs was further analysed by line profiling (Figure 2C). Because MOM-GUVs were prepared with 200 mmol L\(^{-1}\) sorbitol solution and added to the external buffer containing 100 mmol L\(^{-1}\) KCl and 10 mmol L\(^{-1}\) HEPES/KOH (pH 7.4), this transparency phenomenon implies that the refractive index of the internal solution of MOM-GUVs became equal to that of the external buffer. This indicates that the internal and external solutions of GUVs were equilibrated through the membrane.

To quantify the HBx-induced membrane permeabilization, we performed a liposome permeabilization assay; these are generally used in viroporin studies to evaluate pore-forming activity. As CF is self-quenched at high concentrations and becomes fluorescent at low concentrations, we made CF-LUVs with the lipid composition of the mitochondrial outer membrane. Recombinant HBx protein added to CF-LUVs caused liposome permeabilization in a time-dependent or concentration-dependent manner, similar to its activity on GUVs (Figure 3A,B). We also tested the HCV p7 protein (a known cause of membrane permeabilization) as a positive control. These results indicated that HBx directly induces membrane permeabilization, independently of other cellular proteins.

3.3 | HBx-induced membrane permeabilization is inhibited by nonselective channel blockers and a viroporin inhibitor

We hypothesized that the liposome permeabilization induced by HBx could be nonselective permeabilization by protein-lipid pore formation, similar to the activity of some viroporins (eg the severe acute respiratory syndrome coronavirus E protein). In most cases, viroporin-induced pore formation is nonselective or weakly selective for cations. Therefore, we examined the inhibitory effect of gadolinium ion (Gd\(^{3+}\)) and lanthanum ion (La\(^{3+}\)), both known as nonselective channel blockers. Interestingly, Gd\(^{3+}\) and La\(^{3+}\) effectively inhibited HBx-induced liposome permeabilization, but Ca\(^{2+}\) and Mg\(^{2+}\) ions did not (Figure 4A,B). The inhibitory effect of Gd\(^{3+}\) (Figure 4C) and La\(^{3+}\) (Figure 4D) was observed in a concentration-dependent manner.

We next examined whether the liposome permeabilization caused by HBx was inhibited by well-known viroporin inhibitors: amantadine, rimantadine and NN-DNJ. Interestingly, amantadine and rimantadine showed no significant inhibitory effects, while NN-DNJ efficiently inhibited HBx-induced liposome permeabilization (Figure 4E). The inhibitory effect of NN-DNJ was observed in a concentration-dependent manner (Figure 4F). Taken together, these results indicate that HBx-induced (nonselective) permeabilization can be inhibited by nonselective channel blockers or by a viroporin inhibitor.
3.4 | **NN-DNJ inhibits HBx-induced mitochondrial depolarization in Huh-7 cells**

In this study, we showed that NN-DNJ inhibited HBx-induced liposome permeabilization. Next, we attempted to examine the NN-DNJ effect on HBx-induced mitochondrial depolarization in Huh-7 cells. We transfected HBx into Huh-7 cells for 6 hours and treated cells with NN-DNJ for an additional 18 hours (Figure 5A). Mitochondrial membrane potential was quantified using TMRE, a mitochondrial membrane potential indicator. We found that HBx-induced mitochondrial depolarization was inhibited by NN-DNJ (Figure 5B,C), and NN-DNJ did not change the expression level of HBx (Figure 5D). As demonstrated in Figure 4E, amantadine and rimantadine showed no significant inhibitory effects (Figure 5E). These results indicate that HBx-induced mitochondrial depolarization could be inhibited by NN-DNJ, and this may be related to the membrane permeabilization induced by HBx.

3.5 | **The region of HBx with residues 53-117 is responsible for membrane permeabilization**

We investigated the specific region of HBx that accounted for liposome permeabilization. We hypothesized that HBx-induced membrane permeabilization was not dependent on the transactivation region. Considering that the hydrophobic region of cytochrome c has been shown to insert into membranes to form lipidic pores, we synthesized an HBx deletion mutant (53-117) without the transactivation domain but with the transmembrane domain (Figure 6A). As expected, the 53-117 mutant of HBx induced liposome permeabilization-like wild-type HBx (Figure 6B). Nonselective channel inhibitors (Gd$^{3+}$ and La$^{3+}$) (Figure 6C) and NN-DNJ (Figure 6D) showed inhibitory effects on the membrane permeabilization induced by the 53-117 mutant of HBx, but they only partially blocked liposome permeabilization (in contrast to the more robust inhibition of permeabilization seen with wild-type HBx). These results indicate that residues 53-117 of HBx are...
brane permeabilization is independent of its transactivation function. Viruses alter the function of cellular organelles to enhance viral replication and escape from host cells. One of these organelles is the mitochondrion, and the modulation of this organelle is indispensable for the viral life cycle.26 Mitochondria have a diverse set of functions including energy production, apoptosis and metabolite supply.27 One of the main events during apoptosis is mitochondrial depolarization, which is caused by the opening of a permeability transition (PT) pore.20 The PT pore includes adenine nucleotide translocator (ANT) protein, voltage-dependent anion channel (VDAC) protein and matrix cyclophilin D protein.28 A variety of viruses control the PT pore to trigger apoptosis. The influenza A virus PB1-F2 protein interacts with VDAC1 and ANT3 to decrease mitochondrial membrane permeability ( MMP), inducing apoptosis by the release of pro-apoptotic proteins.29 The human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr), the Walleye dermal sarcoma virus Orf C protein and the human T-lymphotropic virus type 1 (HTLV-1) p13II protein have all been reported to localize in the mitochondria and modulate mitochondrial proteins to induce apoptosis.30-32 In contrast, some viruses directly induce mitochondrial depolarization through viral-encoded viroporins. HCV p7, human T-cell leukaemia virus type 1 (HTLV-1) p13 and picornavirus 2B proteins detected in mitochondria all induce mitochondrial depolarization.33,34 In the case of HCV p7, the protein forms hexameric complexes that act as ion channels permeable to cations to enhance the entry and escape of viral particles.19,35,36 HCV p7 was used for a positive control required for membrane permeabilization and that HBx-induced membrane permeabilization is independent of its transactivation function.

4 | DISCUSSION

Viruses alter the function of cellular organelles to enhance viral replication and escape from host cells. One of these organelles is the mitochondrion, and the modulation of this organelle is indispensable for the viral life cycle.26 Mitochondria have a diverse set of functions including energy production, apoptosis and metabolite supply.27 One of the main events during apoptosis is mitochondrial depolarization, which is caused by the opening of a permeability transition (PT) pore.20 The PT pore includes adenine nucleotide translocator (ANT) protein, voltage-dependent anion channel (VDAC) protein and matrix cyclophilin D protein.28 A variety of viruses control the PT pore to trigger apoptosis. The influenza A virus PB1-F2 protein interacts with VDAC1 and ANT3 to decrease mitochondrial membrane permeability (MMP), inducing apoptosis by the release of pro-apoptotic proteins.29 The human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr), the Walleye dermal sarcoma virus Orf C protein and the human T-lymphotropic virus type 1 (HTLV-1) p13II protein have all been reported to localize in the mitochondria and modulate mitochondrial proteins to induce apoptosis.30-32 In contrast, some viruses directly induce mitochondrial depolarization through viral-encoded viroporins. HCV p7, human T-cell leukaemia virus type 1 (HTLV-1) p13 and picornavirus 2B proteins detected in mitochondria all induce mitochondrial depolarization.33,34 In the case of HCV p7, the protein forms hexameric complexes that act as ion channels permeable to cations to enhance the entry and escape of viral particles.19,35,36 HCV p7 was used for a positive control required for membrane permeabilization and that HBx-induced membrane permeabilization is independent of its transactivation function.
**FIGURE 4** Inhibitory effects of nonselective channel blockers and viroporin inhibitors on HBx-induced liposome permeabilization. A, CF-enclosed MOM-LUVs were incubated with the indicated ions (100 nmol L⁻¹) after exposure to recombinant HBx (20 nmol L⁻¹). B, HBx-induced liposome permeabilization was quantified at 20 min. Concentration-dependent inhibitory effects of GdCl₃ (C) or LaCl₃ (D) on HBx-induced liposome permeabilization were quantified at 20 min. E, CF-enclosed MOM-LUVs were incubated with the indicated viroporin inhibitors (1 µM) after exposure to recombinant HBx (20 nmol L⁻¹) for 20 min. F, Concentration-dependent inhibitory effects of NN-DNJ on HBx-induced liposome permeabilization were quantified at 20 min. **P ≤ 0.01, ***P ≤ 0.001 vs. control by two-way analysis of variance (ANOVA). N.S.; not significant, Aman., Amantadine; Riman., Rimantadine.

**FIGURE 5** Inhibitory effect of NN-DNJ on HBx-induced mitochondrial depolarization. A, Flag HBx was transfected for 6 hrs, and NN-DNJ was added for an additional 18 hrs. B, C, E, After transfection with vector or HBx for 6 hrs, the Huh-7 cells were treated with viroporin inhibitors for 18 hrs and then stained with TMRE. Decreased levels of ΔΨm were analysed by flow cytometry. D, Cell lysates were analysed by immunoblot with anti-Flag antibody. *P ≤ 0.05, **P ≤ 0.01 vs. control by two-way analysis of variance (ANOVA). N.S.; not significant, Cont., Control; Ama., Amantadine; Rim., Rimantadine.
In this study, we tested 3 viroporin inhibitors (amantadine, rimantadine and N,N-DNJ). Interestingly, HBx-induced membrane permeabilization was not inhibited by adamantane derivatives (amantadine and rimantadine), whereas N,N-DNJ inhibited HBx-induced membrane permeabilization independently of the mitochondrial permeability transition pore complex (Figure 4). Membrane permeabilization induced by the HBx protein was observed in the artificial membrane system, and it now needs to be confirmed in cells. However, it is difficult to confirm this finding in cells because the mechanism behind HBx-induced mitochondrial dysfunction is very complex and has not been well elucidated yet. Instead, we showed that N,N-DNJ inhibited HBx caused mitochondrial depolarization in Huh-7 cells (Figure 5). Extensive studies remain to fully explain the direct role of HBx in triggering mitochondrial depolarization.

NN-DNJ has low cytotoxicity and has potential to be a therapeutic agent for HBV.\textsuperscript{40,41} NN-DNJ has been used to inhibit HBV, and its inhibitory concentration 50 (IC\textsubscript{50}) is in the range of 1-10 μM. Similarly, our results showed that 10 μM of NN-DNJ was sufficient to inhibit HBx-induced mitochondrial depolarization. From the combined results, we can suggest that the NN-DNJ effect on HBV may be related to membrane permeabilization by HBx, and additional experiments to support this hypothesis are needed. In conclusion, we have demonstrated that HBx induces membrane permeabilization, and that this effect is inhibited by 1 viroporin inhibitor and by 2 triply-charged lanthanides. It is possible that other viroporin inhibitors or nonselective channel blockers also inhibit HBx activity. In the present study, we identified a novel function for HBx, demonstrating its direct role in inducing mitochondrial membrane permeabilization. Based on the results obtained in this study, we propose that HBx-induced membrane permeabilization may be an attractive therapeutic target for HBV treatment.

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