

# Inhibitory effect of cyclosporine A on hepatitis B virus replication *in vitro* and its possible mechanisms

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**BACKGROUND:** Hepatitis B related end-stage liver disease is recently acknowledged as one of the main indications for orthotopic liver transplantation (OLT). However, the high recurrence rate of hepatitis B virus infection following transplantation is regarded as a major factor affecting the long-term survival of transplant recipients especially in China. Cyclosporine A (CsA), which is routinely used to prevent the allograft rejection, is reported to have the inhibitory activity on hepatitis B virus (HBV) replication *in vitro*. In this paper, we review the inhibitory effect and its possible mechanisms of CsA on HBV replication *in vitro*.

**DATA RESOURCES:** An English-language literature search was conducted using MEDLINE (1990-2004) on cyclosporine A, hepatitis B virus, mitochondria, calcium and other related reports and review articles.

**RESULTS:** Hepatitis B x protein (HBx) is essential to HBV replication. The cytosolic calcium signaling mediated by mitochondria and the Src kinase pathway were involved during HBx activation of HBV replication. CsA inhibits the HBV replication *in vitro* by its binding to mitochondrial cyclophilin D, then blocking the mitochondria-mediated cytosolic calcium signaling. The derivatives of CsA also have the HBV replication inhibitory effect *in vitro*.

**CONCLUSIONS:** By interacting with mitochondria, preventing the release of intramitochondrial calcium, and then blocking the cytosolic calcium signaling, CsA inhibits the HBV replication *in vitro*. The derivatives of CsA also have this activity.

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**KEY WORDS:** hepatitis B virus;  
cyclosporine A; calcium

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## Introduction

Hepatitis B virus (HBV) infects more than 300 million individuals worldwide, and hepatitis B related end-stage liver disease is responsible for approximately one million deaths annually. In the past, active viral hepatitis was generally considered as a relative contraindication for orthotopic liver transplantation (OLT) as the recurrence rate of hepatitis B virus infection was very high. Because of prophylactic antiviral measures, the high dose, long-term and combination therapy of hepatitis B immunoglobulin (HBIG) and novel drugs such as lamivudine and ribavirin, hepatitis B related end-stage liver disease has recently been acknowledged as one of the main indications for OLT.<sup>[1]</sup> However, the high recurrence rate of hepatitis B virus infection following transplantation is regarded as a major factor affecting the long-term survival of transplant recipients especially in China. Immunosuppressive therapy consists of cyclosporine A (CsA) or tacrolimus, mycophenolate mofetil (MMF) or azathioprine. Prednisone is routinely used to prevent the allograft rejection. In recent years, some immunosuppressants such as CsA and mycophenolic acid (MPA, the active form of MMF) have the inhibitory effect on HBV replication *in vitro*.<sup>[2]</sup> This review focuses on HBV replication, and the inhibitory effect of CsA on HBV replication *in vitro* and its possible mechanisms.

## Multifunctional HBx protein and HBV replication

HBV is the prototype of the hepadnavirus family. All genomes of mammalian hepadnaviruses contain four open reading frames (ORFs), and they encode four main viral proteins: hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), polymerase and hepatitis B x protein (HBx). Viral replication includes reverse transcription of pregenomic mRNA and synthesis of the viral dsDNA genome which is mediated by the virally encoded polymerase.<sup>[3]</sup>

Studies in the Woodchuck model system have

shown that expression of WHV HBx (WHx) is essential to viral replication during chronic infection.<sup>[4]</sup> Other studies *in vitro* showed that HBV replication is apparently inhibited by the hepatitis B virus X binding protein (XIP) or in the HBx(-)HBV transfected cell lines.<sup>[5-7]</sup> Hence it is recognized that HBV replication is significantly dependent on the HBx protein.

The HBx protein is distributed not only in the cytoplasm, but also in the nuclei of transfected cells. Thus it is thought to have dual functions. One is related to the cytoplasmic localization, which can activate the Ras/Faf/ERK and MEKK-1/JNK mediated signal transduction pathway.<sup>[8,9]</sup> The other is associated with its nuclear localization and is shown to transactivate a variety of viral and cellular promoters/enhancers through the cis-acting sites for AP-1,<sup>[8]</sup> NF- $\kappa$ B,<sup>[9]</sup> AP-2, and ATE/CREB.<sup>[10]</sup> It was also demonstrated that the calcium/calmodulin regulated dephosphorylation and nuclear translocation of nuclear factors of activated T cell (NFAT) are activated by HBx in a CsA-sensitive mechanism.<sup>[11,12]</sup> HBx interacts with and stimulates components of the cellular transcriptional machinery, but whether the HBx activation of transcription is involved during HBV replication remains uncertain. In an HBV transgenic mouse model, HBx dependent transcriptional activation was found to be important for viral replication.<sup>[13]</sup> But studies *in vitro* have not proven that transcriptional activation by HBx is essential to viral replication.

Reports have shown that cytosolic calcium signaling mediated by mitochondria and the Src kinase pathway are involved during the HBx activation of HBV replication.<sup>[3,5,6]</sup> In their studies, the HBx activation of HBV replication was blocked by inhibiting mitochondrial calcium channels or by Src kinase inhibitors. Moreover, reagents that increased cytosolic Ca<sup>2+</sup> were found to have the ability to substitute for HBx in HBV replication. And HBx was shown to stimulate viral polymerase activity through a Src kinase mediated pathway.<sup>[3]</sup> The absence of HBx, blocking of cytosolic calcium signaling and inhibition of the Src kinase pathway were all associated with a marked decrease of viral polymerase activity, which is essential to HBV replication.

### Mitochondria, calcium signaling and HBx protein

Mitochondria accumulates Ca<sup>2+</sup> up to 0.5 mmol/L, and is regarded as a Ca<sup>2+</sup> storage apart from the extracellular space and the endoplasmic reticulum (ER). It is accepted that there is an interplay between mitochondrial function and cytosolic calcium signaling.

The inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM) are important in equilibration of ions between the cytosol and the mi-

tochondrial matrix. Several models of mitochondrial membrane permeabilization especially the mitochondrial permeability transition (MPT) have become the focus of recent studies. The MPT refers to the massive swelling and depolarization of the mitochondria. The onset of the MPT is mediated by opening of a non-specific, high conductance mitochondrial permeability transition pore complex (MPTP).<sup>[14]</sup> The MPTP probably localizes at the contact site between the IMM and OMM.<sup>[15,16]</sup> It can be triggered by elevated matrix Ca<sup>2+</sup> and oxidative stress, then causing massive swelling of the mitochondria, rupture of the OMM, and release of intramitochondrial components smaller than 1500 daltons.<sup>[14,17]</sup> To date, the molecular structure of the MPTP has not yet been determined. One postulated model of the MPTP is formed by the voltage-dependent anion channel (VDAC) in the OMM, the cyclophilin D in the mitochondrial matrix, the adenine nucleotide translocator (ANT) in the IMM and several other proteins such as Bcl-2.<sup>[14,18,19]</sup>

The release of Ca<sup>2+</sup> from the mitochondrial matrix is accompanied by the activation of the MPTP.<sup>[20]</sup> It is proposed that the MPTP probably provides the mitochondria with a fast Ca<sup>2+</sup> release channel, thus contributing to intracellular Ca<sup>2+</sup> homeostasis and signaling.<sup>[20,21]</sup> The VDAC proteins, one of the key components of the MPTP, were reported highly permeable to Ca<sup>2+</sup>, and provided the pathway for Ca<sup>2+</sup> transport into and out of the mitochondria.<sup>[20]</sup> The opening of the MPTP can be blocked by inhibition of the VDAC activity<sup>[22,23]</sup> or in the VDAC-deficient mitochondria.<sup>[24,25]</sup> In humans, two isoforms of VDAC genes termed HVDAC1 and HVDAC2 respectively have been cloned.

HVDAC3 is another isoform identified as a target of HBx.<sup>[26]</sup> HBx interacts with HVDAC3 and causes depolarization of the mitochondria.<sup>[27]</sup> HBx induction has a stimulatory effect on Ca<sup>2+</sup> mobilization from intracellular calcium stores, perhaps mitochondria into the cytosol, and causes increase of cytosolic Ca<sup>2+</sup>.<sup>[5,28,29]</sup> So it is hypothesized that HBx acts on the MPTP by interacting with the HVDAC3, causes the depolarization of the mitochondria, perhaps opening of the MPTP, and results in outfluxing of Ca<sup>2+</sup> from the mitochondrial matrix to the cytosol. The increased cytosolic Ca<sup>2+</sup> activates the proline-rich tyrosine kinase-2 (Pyk2), a cytosolic calcium-dependent Src kinases activator, and then activates the Src kinase pathway, which in turn promotes the polymerase activity, enhance the synthesis of viral dsDNA, and activates HBV replication.<sup>[5,7]</sup>

### Cyclosporine A and its anti-viral function

Cyclosporine A (CsA) is a neutral lipophilic cyclic undecapeptide isolated from the fungus *Hypocladium inflatum*

gams. It has been widely used in the treatment of allograft rejection. Early biological studies revealed that CsA acts on the immune system by inhibiting the initial steps of T cell activation via blocking the transcription of cytokine genes including IL-2, IL-4, TNF- $\alpha$  and IFN- $\gamma$ , especially IL-2.<sup>[30]</sup> After entering T cells, CsA binds with a high affinity to cyclophilins, which are a family of molecular chaperones possessing peptidyl-proline cis-trans isomerase (PPIase) activity, an enzymatic activity mediating protein folding.<sup>[31]</sup> The cyclophilin-CsA complex can bind directly to another cytoplasmic protein named calcineurin, which can dephosphorylate the NFAT, and facilitate its translocation into the nucleus and activation of gene expression.<sup>[32]</sup> By inhibiting the phosphatase activity of calcineurin, preventing dephosphorylation and nuclear translocation of the NFAT, then inhibiting subsequent gene expression in activated T cells, CsA suppresses the T cell activation. In addition to this pathway, it has been identified that CsA blocks both JNK and p38 signaling pathways in its immunosuppressive action.<sup>[33]</sup>

Besides the immunosuppressive action, the anti-viral action of CsA was reported.<sup>[34-38]</sup> CsA inhibits the replication of hepatitis C virus (HCV) probably by inhibiting the PPIase activity of cyclophilin, which may be crucial for the processing and maturation of viral proteins and viral replication.<sup>[34,35]</sup> A clinical controlled trial showed that combination therapy with IFN- $\alpha$  and CsA was more effective than IFN- $\alpha$  monotherapy in the treatment of chronic hepatitis C.<sup>[36]</sup> CsA also showed the anti-viral effects on human immunodeficiency virus-1 (HIV-1) through competing with gag protein for the same binding site on cyclophilin A,<sup>[37]</sup> which is packaged into HIV-1 virions and catalyzes cis-trans isomerization of viral capsid protein as a molecular chaperon.<sup>[38]</sup> In a word, the effect of CsA on calcineurin accounts for its immunosuppressive action; but this mechanism is not involved in its anti-viral action, and the inhibition of the PPIase activity of cyclophilins by CsA may be responsible for its anti-viral activity.

### CsA inhibits HBV replication by interaction with mitochondria

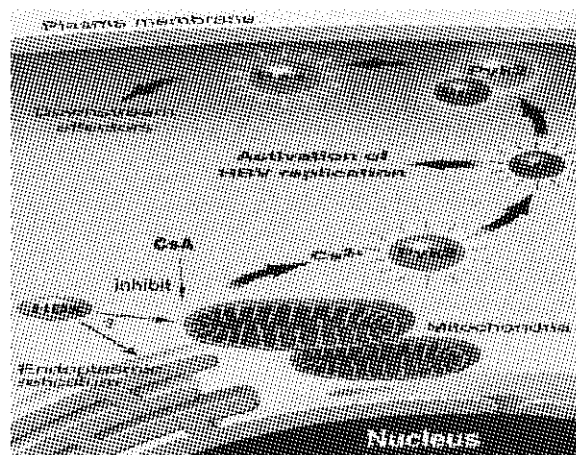
Cyclophilin D, also named mitochondrial +cyclophilin, plays a decisive role in MPT.<sup>[39]</sup> It is a unique cyclophilin and distinct from the well-known cytosolic cyclophilin A,<sup>[40,41]</sup> which is the most abundant cyclophilin in T cells and plays an essential role in the immunosuppressive action of CsA.<sup>[31,42]</sup> Mitochondrial matrix  $Ca^{2+}$  can bind to the intramitochondrial loops of ANT, and trigger the conformational change of the ANT, which is required to induce the activation of the MPTP. Cyclophilin D binds specifically to the ANT probably on Pro<sup>61</sup> on

loop 1. A cis-trans isomerization of the peptide bond adjacent to Pro<sup>61</sup> catalysed by cyclophilin D is thought to be involved in  $Ca^{2+}$  triggered conformational change of ANT. So the binding of cyclophilin D to the ANT facilitates and is required for the calcium-triggered activation of the MPTP.<sup>[14,43-45]</sup> During this process, the PPIase activity of cyclophilin D may play an important role.

Like cyclophilin A and other cyclophilin family members, cyclophilin D is targeted by CsA and its derivatives such as CsH, SDZ NIM811. Cyclophilin D confers the sensitivity of the MPTP to CsA and its derivatives.<sup>[43]</sup> Many studies have identified that the MPTP can be inhibited by CsA and its derivatives.<sup>[14,43,46]</sup>

CsA binds to cyclophilin D, inhibits its PPIase activity, which is necessary to calcium-triggered conformational change of ANT, then inhibits the activation of the MPTP, prevents the release of mitochondrial matrix  $Ca^{2+}$  and increase of cytosolic calcium, and blocks the cytosolic calcium signaling and Src kinases pathway, which are essential to HBx activated HBV replication. The viral polymerase activity is subsequently inhibited. As a result, HBV replication is inhibited by CsA and its derivatives (Fig.).

CsA especially its derivatives has inhibitive effect on HBV replication, which presents some insights on the treatment of hepatitis B as well as on immunosuppressive therapy and anti-viral therapy in the prevention of hepatitis B recurrence in transplant recipients with hepatitis B.



**Fig.** The calcium signaling and Src pathway involved in HBx activated HBV replication. HBx acts on either mitochondria or endoplasmic reticulum (or both) to trigger  $Ca^{2+}$  release into the cytosol. The increased cytosolic  $Ca^{2+}$  then triggers activation of Pyk2 kinase, which in turn binds and activates the Src kinase pathway and the downstream Ras pathway. But the Ras pathway is not involved in HBx activated HBV replication. CsA inhibits HBV replication by acting on the mitochondria and then blocking the calcium signaling, which is necessary to HBV replication.<sup>[3,5,7,47]</sup>

## Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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*Metabolomics ( metabonomics )*—Application of system-wide techniques ( normally based on nuclear magnetic resonance ) for metabolic profiling. Some use the term metabolomics to cover analyses in both simple ( cellular ) and complex ( tissue or whole body ) systems. Others distinguish between “metabolomics” studies in simple systems only and “metabonomics” in complex systems