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Calcineurin Inhibitors Stimulate and Mycophenolic Acid Inhibits Replication of Hepatitis E Virus

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BACKGROUND & AIMS: Many recipients of organ transplants develop chronic hepatitis, due to infection with the hepatitis E virus (HEV). Although chronic HEV infection is generally associated with immunosuppressive therapies, little is known about how different immunosuppressants affect HEV infection. **METHODS:** A subgenomic HEV replication model, in which expression of a luciferase reporter gene is measured, and a full-length infection model were used. We studied the effects of different immunosuppressants, including steroids, calcineurin inhibitors (tacrolimus [FK506] and cyclosporin A), and mycophenolic acid (MPA, an inhibitor of inosine monophosphate dehydrogenase) on HEV replication in human hepatoma cell line Huh7. Expression of cyclophilins A and B (the targets of cyclosporin A) were knocked down using small hairpin RNAs. **RESULTS:** Steroids had no significant effect on HEV replication. Cyclosporin A promoted replication of HEV in the subgenomic and infectious models. Knockdown of cyclophilin A and B increased levels of HEV genomic RNA by 4.0- $\pm$ 0.6-fold and 7.2- $\pm$ 1.9-fold, respectively ($n = 6$; $P < .05$). A high dose of FK506 promoted infection of liver cells with HEV. In contrast, MPA inhibited HEV replication. Incubation of cells with guanosine blocked the antiviral activity of MPA, indicating that the antiviral effects of this drug involve nucleotide depletion. The combination of MPA and ribavirin had a greater ability to inhibit HEV replication than MPA or ribavirin alone. **CONCLUSIONS:** Cyclophilins A and B inhibit replication of HEV; this might explain the ability of cyclosporin A to promote HEV infection. On the other hand, the immunosuppressant MPA inhibits HEV replication. These findings should be considered when physicians select immunosuppressive therapies for recipients of organ transplants who are infected with HEV.

Keywords: Cell Culture Model; Liver Disease; Immunity; Transplantation.

Hepatitis E virus (HEV) is one of the most common causes of acute hepatitis worldwide. It is a single-stranded positive-sense RNA virus that mainly infects the liver hepatocytes. Although only a single HEV serotype is recognized, at least 4 different genotypes of human HEV exist.[1] Genotypes 1 and 2 are found mainly in developing countries and are transmitted via contaminated water sources. In contrast, genotypes 3 and 4 are prevalent in industrialized countries and are zoonotic nature and spread mainly through eating undercooked pork or game products.[2] In general, HEV infection is a self-limiting disease and is associated with low mortality, but fulminant hepatitis and high mortality have been described, reaching 25% in cases of pregnant women infected with genotype 1 in developing countries.[3] In the Western world, the main clinical challenge is posed by HEV genotype 3 infection in patients receiving orthotopic organ transplantation.[4] More than 60% of organ recipients infected with HEV will develop chronic hepatitis with rapid progression to cirrhosis.[5,6] Which factors that determine outcomes in these patients remains obscure at best, hampering efforts to develop rational therapy and to address the increasing challenge of HEV infection in organ transplantation recipients.

Organ transplant patients take immunosuppressants for life to prevent graft rejection. The resulting immunosuppression, however, also affects host immunity against viral challenges, and the use of immunosuppressive drugs has been proposed to be a key factor for developing chronic hepatitis after HEV infection.[4] Consequently, dose reduction of immunosuppression is often used as the first intervention strategy to achieve viral clearance in HEV-infected organ recipients.[7] Interestingly, however, clinical evidence suggests that different immunosuppressive regimens can differentially affect the infection course of HEV. The calcineurin inhibitor tacrolimus, but not cyclosporin A (CsA), has been found to be more frequently associated with persistent infection,[6] and mycophenolate mofetil, the pre-drug form of mycophenolic acid (MPA) can help to clear the virus.[8] However, the current clinical studies are not able to conclusively address the impact of different immunosuppressants because of limited patient numbers and lack of mechanistic insight as to how differences

Abbreviations used in this paper: **CsA, cyclosporin A; CypA/B, cyclophilin A/B; Dex, dexamethasone; HCV, hepatitis C virus; HEV, hepatitis E virus; IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid; Pred, prednisolone; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short-hairpin RNA.**

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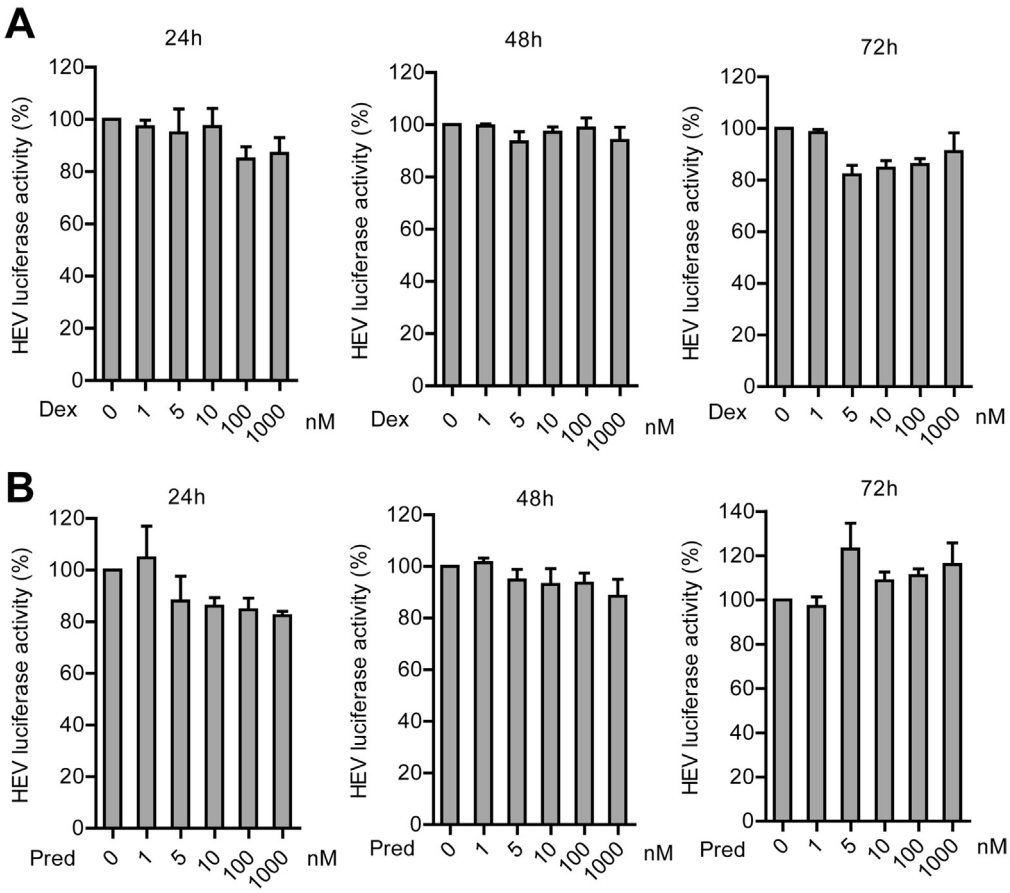

Figure 1. The effects of steroids on HEV replication in subgenomic cell culture model. Huh7 cell-based subgenomic HEV replicon containing the luciferase reporter gene was treated for 24, 48, and 72 hours with a dose-range of Dex and Pred. (*A*) Dex and (*B*) Pred did not significantly affect luciferase activity. Data presented as mean ± SD of 3 independent experiments.

in immunosuppressive medication might be linked with an altered clinical course of HEV infection.

The observation that different immunosuppressive medication seems to have specific effects on the outcomes of HEV infection suggests that such medication can have direct effects on viral replication, apart from influencing antiviral immunity. This consideration prompted us to test whether different immunosuppressive medication affects HEV replication in hepatocytes directly. The recent development of a genotype 3–based cell culture system[9,10] makes it possible to study such questions in a highly detailed fashion. We show that different commonly used immunosuppressants have very specific effects on viral replication and that especially calcineurin inhibitors strongly facilitate HEV replication, and MPA suppresses viral replication. These results will serve as an important reference about the choice of particular immunosuppressive medication for HEV-infected orthotopic organ transplant recipients.

Materials and Methods

Immunosuppressants

CsA and tacrolimus (FK506) were purchased from Abcam (Cambridge, MA). Dexamethasone (Dex), prednisolone (Pred) and MPA were purchased from Sigma (St Louis, MO). All the reagents were dissolved in dimethylsulfoxide, except MPA, which was dissolved in methanol. The effects of these immunosuppressants on host cell viability were determined by MTT assay (Supplementary Figure 1).

Cell Culture

Human hepatoma cell line Huh7 and human embryonic kidney epithelial cell line 293T cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

Hepatitis E Virus Cell Culture Models

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) and a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc) were used to generate HEV genomic RNA by using the Ambion mMESSAGE mMACHINE in vitro RNA transcription Kit (Life Technologies Corporation, Carlsbad, CA).[9,10] Huh7 cells were electroporated with p6 full-length HEV RNA or p6-Luc subgenomic RNA to generate infectious or replication models, respectively.[10]

Quantification of Hepatitis E Virus Infection

For the HEV replication model (p6-Luc), the activity of secreted gaussia luciferase in the cell culture medium was

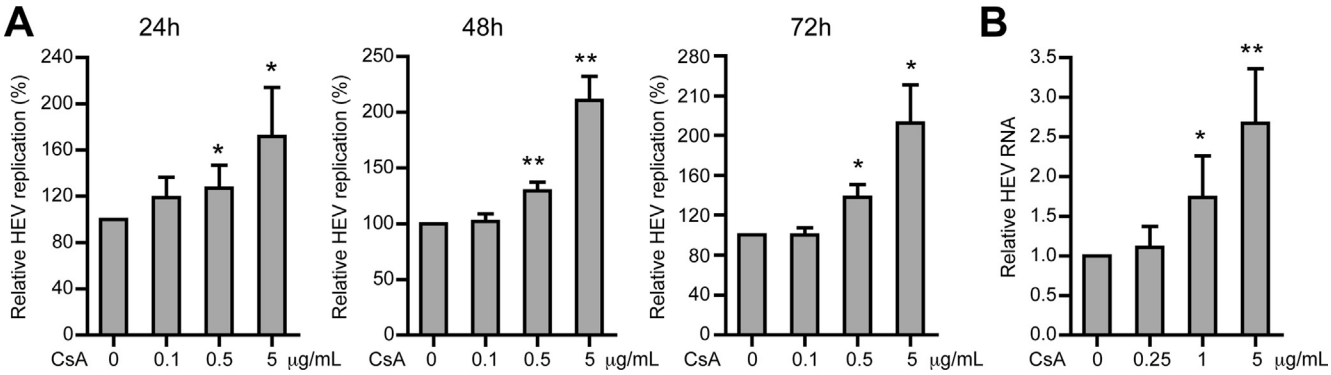

Figure 2. CsA promoted HEV infection. (*A*) The subgenomic HEV replicon containing the luciferase reporter gene was treated for 24 hours (n = 5), 48 hours (n = 7), and 72 hours (n = 7) with different doses of CsA. Treatment with CsA (0.5 or 5 μg/mL) significantly increased HEV luciferase activity. (*B*) The Huh7 cells-based infectious HEV model was treated with CsA for 48 hours. CsA significantly increased HEV RNA at 0.5 and 5 μg/mL concentrations (n = 5). Data presented as mean ± SD of multiple experiments. *$P < .05$; **$P < .01$

measured using BioLux *Gaussia* Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA), as quantification of viral replication. To further determine the specific effects on viral replication-related luciferase activity, Huh7 cells constitutively expressing the firefly luciferase reporter gene driven by the human PGK promoter were used as household luciferase activity for normalization.[11] For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 minutes at 37°C. Both gaussia and firefly luciferase activity were quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

For the p6 infectious HEV model, SYBR Green–based quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify genomic RNA. The HEV primer sequences were 5′-ATTGGCCAGAAGTTGGTTTTCAC-3′ (sense) and 5′-CCGTGGCTATAATTGTGGTCT-3′ (antisense), and the primers of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were 5′-TGTCCCCACCCCCAATGTATC-3′ (sense) and 5′-CTCCGATGCCTGCTTCACTACCTT-3′ (antisense).

Gene Knockdown by Lentiviral Vector Delivered Short-Hairpin RNA

Lentiviral vectors, targeting cyclophilin A (CypA), cyclophilin B (CypB) or green fluorescent protein, were produced in 293T cells as described previously.[12] After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. The shRNA sequences were: CypA, 5′-CCGGTGGT GACTTCACACGCCATAACTCGAGTTATGGCGTGTGAAGTCACCAT TTTTG-3′, and CypB, 5′-CCGGGCCTTAGCTACAGGAGAGAAAC TCGAGTTTCTCTCCTGTAGCTAAGGCTTTTTG-3′.

To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Because the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 μg/mL puromycin (Sigma) in the cell culture medium. For the infectious model, HEV particles were incubated with knockdown and control Huh7 cells. For the subgenomic model, p6-Luc cells were directly transduced with lentiviral shRNA vectors and selected by adding 2.5 μg/mL puromycin.

Western Blot

For Western blot, commercial antibodies against CypA and CypB (rabbit polyclonal; Abcam) were used. Proteins in cell lysates were heated 5 minutes at 95°C, followed by loading onto a 15% sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. After 90 minutes running in 115-V voltage, proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Invitrogen) for 1.5 hours with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 mL blocking buffer and 2.5 mL phosphate-buffered saline containing 0.05% Tween 20. It was followed by incubation with rabbit anti-CypA (1:5000) or anti-CypB (1:7500) antibody overnight at − 4°C. Membrane was washed 3 times followed by incubation for 1.5 hours with an anti-rabbit peroxidase–conjugated secondary antibody (1:5000). After washing 3 times, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

Statistical Analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). *P* values <.05 were considered statistically significant.

Results

Glucocorticosteroids Did Not Affect Hepatitis E Virus Replication

Pred and its close analogue Dex remain important drugs in the clinical management of patients receiving orthotopic organ transplantation.[13] To study the possible effects of these drugs on HEV replication, we used a model in which cells were transfected with a subgenomic construct of HEV coding sequence in which the 5′ portion of ORF2 was replaced with the in-frame secreted form of luciferase derived from the marine copepod Gaussia princeps. Accumulation of luciferase serves as reporter for HEV RNA synthesis (p6-luc), and the loss of the capsid protein precludes the formation of novel viral particles. In parallel, Huh7 cells constitutively expressing a nonsecreted firefly

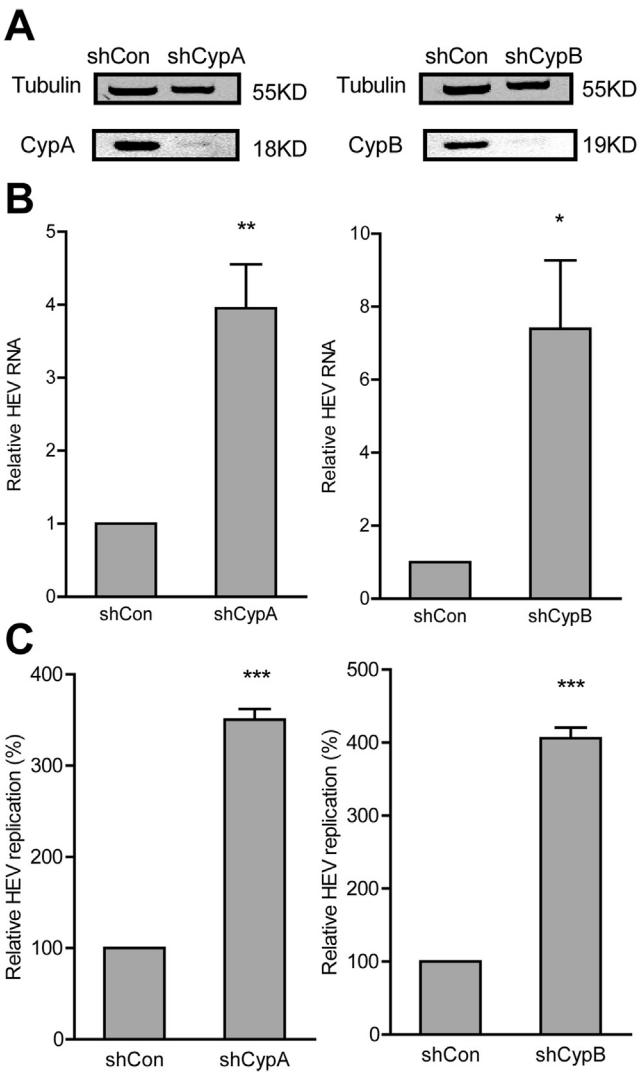

Figure 3. Gene silencing of CypA or CypB facilitated HEV infection. (*A*) Western blotting showed dramatic down-regulation of CypA and CypB protein by lentiviral RNA interference–mediated gene knockdown. (*B*) Silencing of CypA or CypB resulted in a significant increase of cellular HEV RNA. Data presented as mean ± SEM of 6 independent experiments. *$P < .05$; **$P < 01$. (*C*) Silencing of CypA or CypB significantly increased viral replication-related luciferase activity in the HEV subgenomic model (mean ± SEM, n = 12 replicates of 3 experiments in total). ***$P < .001$.

luciferase were used for normalization of nonspecific effects on luciferase signals. However, as shown in Figure 1 neither Pred nor Dex significantly affected HEV replication. We conclude that steroids have no direct effects on HEV replication.

Cyclosporin A Dose-Dependently Enhanced Hepatitis E Virus Replication

CsA, a calcineurin inhibitor, is an important drug for prevention of graft rejection. To examine the effects of CsA on HEV replication, we tested the effects of 0.1, 0.5, and 5 μg/mL CsA on viral replication using the subgenomic p6-Luc model as a read-out. It appeared that CsA dose-dependently increased HEV replication-related luciferase activity (Figure 2*A*). Consistently, CsA also dose-dependently increased HEV infection in the full-length (p6) infectious model (Figure 2*B*). Forty-eight hours of treatment with CsA (5 μg/mL) resulted in a mean ± SD of 2.67 ± 0.7-fold (n = 5; $P < 0.01$) increase of HEV genomic RNA level (determined by qRT-PCR), compared with the control (Figure 2*B*). CsA directly promotes viral replication in a hepatocyte-like cells and experimentation was initiated to establish the molecular basis of this effect.

Silencing the Cellular Targets of Cyclosporin A, Cyclophilin A and B, Enhanced Hepatitis E Virus Replication

CsA acts through binding and inhibition of the CypA/B complex. The effects of CsA on HEV replication could be potentially mediated through cyclophilins. CypA[14] and CypB[15] have been implicated in the anti–hepatitis C virus (HCV) mechanism of CsA. Therefore, lentiviral-mediated RNA interference was used for knockdown of these 2 genes, as to allow investigation of their potential function in the effects of CsA on HEV replication. To this end, Huh7 cells were transduced with integrating lentiviral vectors expressing both shRNA and puromycin. Cells stably transduced with the vector were selected and expanded by adding puromycin to the relevant cell cultures. The shRNA clones with most potent efficacy of CypA and CypB knockdown were selected for follow-up experimentation (Figure 3*A*). Cells stably integrated with shRNA targeting GFP (as control), CypA or CypB were inoculated with infectious HEV viruses (p6). The level of infection was quantified by qRT-PCR of genomic viral RNA in the cells 3 days post inoculation. As shown in Figure 3*B*, knockdown of CypA resulted in a 4.0 ± 0.6-fold (n = 6; $P < .01$) increase of HEV RNA; and knockdown of CypB has resulted in a 7.4 ± 1.9-fold (n = 6; $P < .05$) increase of viral genomic RNA. Consistently, silencing of CypA and CypB in HEV subgenomic model significantly increased viral replication-related luciferase activity by a mean ± SEM of 350.4% ± 11.7% (n = 12; $P < .0001$) and 406% ± 14.5% (n = 12; $P < .0001$), respectively (Figure 3*C*). The most straightforward explanation of these results is that CsA through cyclophilin binding and inhibition facilitates HEV infection (Figure 2).

High Dose of FK506 Promoted Hepatitis E Virus Replication

FK506 is another type of calcineurin inhibitor that binds to FK binding proteins. To determine the effects of FK506 on HEV replication, p6-Luc cells were treated with FK506 at concentrations of 0.1, 0.5, and 5 μg/mL. As shown in Figure 4*A*, only high does (5 μg/mL) of FK506 significantly increased HEV replication, seen at 24, 48, and 72 hours post-treatment. This was also further confirmed in the p6 infectious model that HEV genomic RNA was increased by a mean ± SD of 35% ± 9.6% (n = 4; $P < 0.01$) by treatment with 5 μg/mL FK506 for 48 hours (Figure 4*B*).

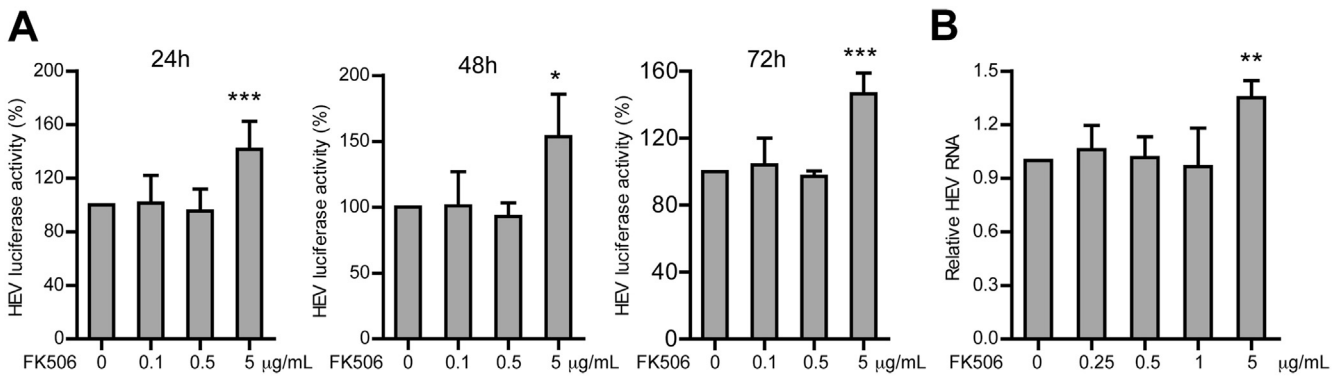

Figure 4. High dose of FK506 enhanced HEV infection. (*A*) Treatment with 5 μg/mL (but not 0.5 and 1 μg/mL) resulted in significant increase of luciferase activity in the HEV subgenomic model (mean ± SD; n = 5−8) and (*B*) significant increase of HEV RNA in the infectious model (mean ± SD, n = 4). *$P < .05$; **$P < 01$; ***$P < 001$.

Because the immunosuppressive mechanisms of calcineurin inhibitors are mediated via the Ca^{2+}-NFAT signal transduction, we tested the effects of N,N-dimethyl-D-erythro-sphingosine, a compound that can efficiently increase cellular Ca^{2+} levels,[16] on HEV infection. As shown in Supplementary Figure 2*A*, N,N-dimethyl-D-erythro-sphingosine (1−4 μg/mL) triggered clear induction of Ca^{2+} levels in Huh7 cells visualized with a fluorescent dye, Fluo-4/AM. However, no clear effects were observed on HEV infection in either the subgenomic (Supplementary Figure 2*B*) or the infectious (Supplementary Figure 2*B*) model. Therefore, the proviral effects of calcineurin inhibitors on HEV infection appear to be independent of Ca^{2+} levels.

Mycophenolic Acid Inhibited Hepatitis E Virus Replication by Depletion of Cellular Nucleotide Pool

MPA, an inhibitor of inosine monophosphate dehydrogenase (IMPDH) (the biosynthesis of guanine), is an immunosuppressive drug often used in organ transplantation, but also has a broad antiviral activity against a spectrum of viruses.[17] We investigated whether MPA could also be able to inhibit HEV infection. Treatment with MPA (0.1−10 μg/mL) has resulted in a significant reduction of HEV replication-related luciferase activity in the subgenomic replicon. For example, with 10 μg/mL MPA treatment, the luciferase activity were 42.8% ± 2.3% (mean ± SEM) (n = 9; $P < .001$), 32.8% ± 5.3% (n = 10; $P < .001$), and 39.5% ± 4.6% (n = 12; $P < .001$) of the control group at days 1, 2, and 3, respectively (Figure 5*A*). Consistently, MPA also dose-dependently inhibited cellular viral RNA in the infectious HEV model. Forty-eight hours of treatment with MPA (10 μg/mL) resulted in 65% ± 9% (n = 5; $P < .01$) inhibition of HEV genomic RNA level (determined by qRT-PCR) compared with the control (Figure 5*B*).

To further investigate whether the effects of MPA are via depletion of cellular nucleotides, additional guanosine was added to the MPA treatment. As shown in Figures 6, supplement of exogenous guanosine completely abrogated the antiviral activity of MPA in both subgenomic and infectious HEV models, suggesting that the action of MPA is exclusively via nucleotide depletion. Immunosuppressive drugs have highly diverse effects on HEV replication, calcineurin inhibitors stimulating viral replication, but MPA exerting direct inhibition of HEV replication.

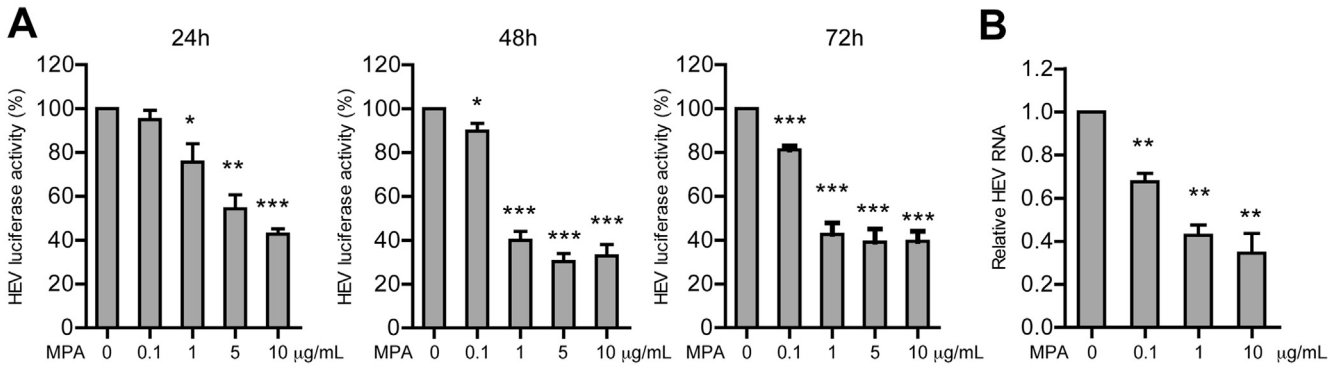

Figure 5. Potent anti-HEV activity of MPA. (*A*) Treatment of MPA for 24, 48, or 72 hours has resulted in significant reduction of HEV luciferase activity in the subgenomic model (mean ± SEM, n = 9−12). (*B*) In the infectious model, treatment with 0.1, 1, and 10 μg/mL of MPA for 48 hours has significantly inhibited HEV RNA by 32%, 57%, and 65%, respectively (mean ± SEM, n = 5). *$P < .05$; *$^{*}P < .01$; **$^{*}P < .001$.

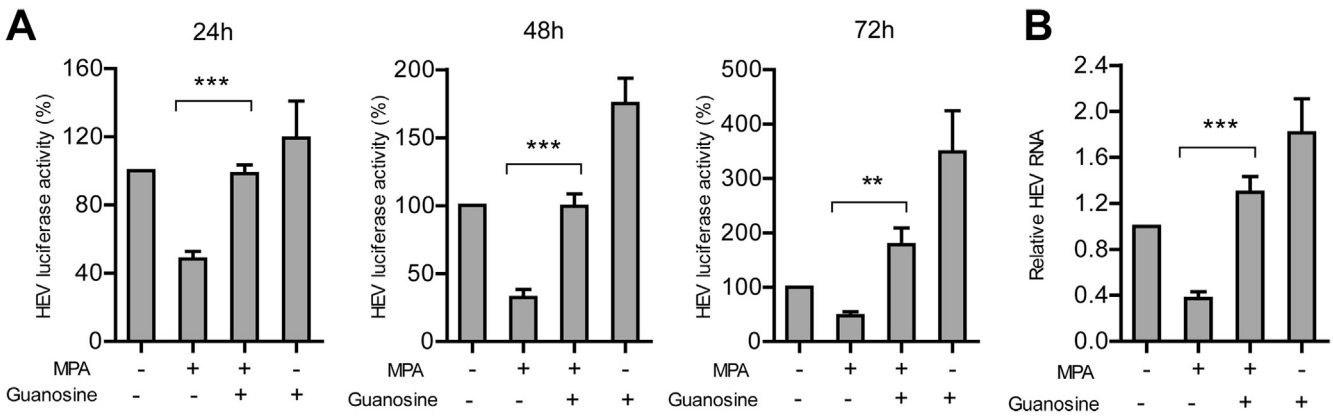

Figure 6. Supplement of exogenous guanosine completely abrogated the anti-HEV effects of MPA. (*A*) In the subgenomic HEV replicon, the antiviral effects by treatment of MPA at concentration of 10 μg/mL for 24 hours, 48 hours, and 72 hours were abrogated by adding exogenous guanosine (100 μg/mL) (mean $\pm$ SEM, n = 7–10). (*B*) Similarly, the antiviral effects by treatment of MPA at concentration of 10 μg/mL for 48 hours was also abrogated by adding 100 μg/mL exogenous guanosine in the infectious model (mean $\pm$ SEM, n = 8). $^{**}P < .01$; $^{***}P < .001$.

Combination of Mycophenolic Acid With Ribavirin Extended Their Antiviral Activity

Because the use of ribavirin monotherapy as off-label drug is gaining favor for treating hepatitis E,[18] we also investigated the antiviral effects of combining MPA with ribavirin. As shown in Figures 7, a serial of combination groups demonstrated a general beneficial effect and no negative drug–drug interference was observed. For instance, combining 1 μg/mL MPA with 25 μm ribavirin resulted in a mean $\pm$ SEM of 76% $\pm$ 1% inhibition of HEV luciferase, and MPA alone resulted in 60% $\pm$ 2% and ribavirin alone resulted in 17% $\pm$ 3% inhibition (n = 16; $P < .001$) after 72 hours treatment (Figure 7*A*). Therefore, a combination of ribavirin with MPA appears compatible against HEV infection and constitutes an attractive clinical option for preventing rejection in HEV-infected patients.

Discussion

Immunosuppressive medication has been proposed to be a key factor for developing chronic hepatitis E in organ transplantation recipients[4] and is often solely attributed to diminished antiviral immunity. Clinical evidence, however, suggests that different immunosuppressive regimens can differentially affect the infection course of HEV.[6,8] By testing different immunosuppressants in 2 HEV replication models, we have consistently demonstrated that steroids (Pred and Dex) did not affect viral replication, calcineurin inhibitors (CsA and FK506) promoted HEV infection, and MPA suppressed viral infection in vitro. The concentrations of these immunosuppressants used in this study are in general covering the achievable blood concentrations in patients.[19–21] Of note, animal studies have indicated that certain immunosuppressants even accelerate in the liver and drug levels in hepatocytes will exceed those observed in serum.[22] Therefore, we propose that the results of this in vitro study will be a valuable reference regarding the choice of the particular immunosuppressant for orthotopic organ transplantation patients who are infected with HEV.

Steroids have been used since the early years of organ transplantation. Pred and its close analogue Dex are potent suppressors of the immune system, as they modulate cellular and inflammatory responses via stimulation or inhibition of gene transcription.[23] In the setting of liver transplantation for HCV patients, evidence suggested that steroid boluses used to treat acute rejection are associated with an increase in viral load and the severity of HCV recurrence.[24,25] Using subgenomic cell culture model of HCV replicon, a study demonstrated that both Pred and Dex have no stimulatory effect on viral RNA levels, but rather have minor inhibitory effects.[13] As to infectious HCV model, however, Pred was reported to promote HCV infection by enhancing virus entry, including up-regulation of 2 essential HCV entry factors: occludin and scavenger receptor class B type I.[26] In both subgenomic and infectious models of HEV, we did not observe clear effect on HEV infection by either Pred or Dex. Although limited studies have reported the impact of steroids in HEV patients, one case report has documented a good clinical and biochemical response to steroid therapy in a patient with acute hepatitis E with autoimmune hepatitis, who maintained health with low dose of steroids.[27]

The first in vitro evidence that CsA but not FK506 can inhibit HCV replication[28] sparked the clinical debate on the possible differential effects of these 2 drugs on HCV recurrence after liver transplantation.[29] Several follow-up studies have demonstrated that the targets of CsA, CypA, and CypB are host factors supporting HCV infection.[14,15] CsA exerts anti-HCV effects by inhibition of these cellular factors.[30] Interestingly, we observed a proviral effect of CsA in HEV cell culture models. Using RNA interference gene silencing approach, we further demonstrated that knockdown of either CypA or CypB enhanced HEV infection, suggesting that both factors could restrict HEV infection. This convincingly explained why CsA could facilitate HEV infection. Although a number of reports have demonstrated a supportive role of CypA in infections of HIV,[31] HCV,[14] or HBV,[32] recent studies also reported that CypA possesses a repressive effect on the replication of some viruses,

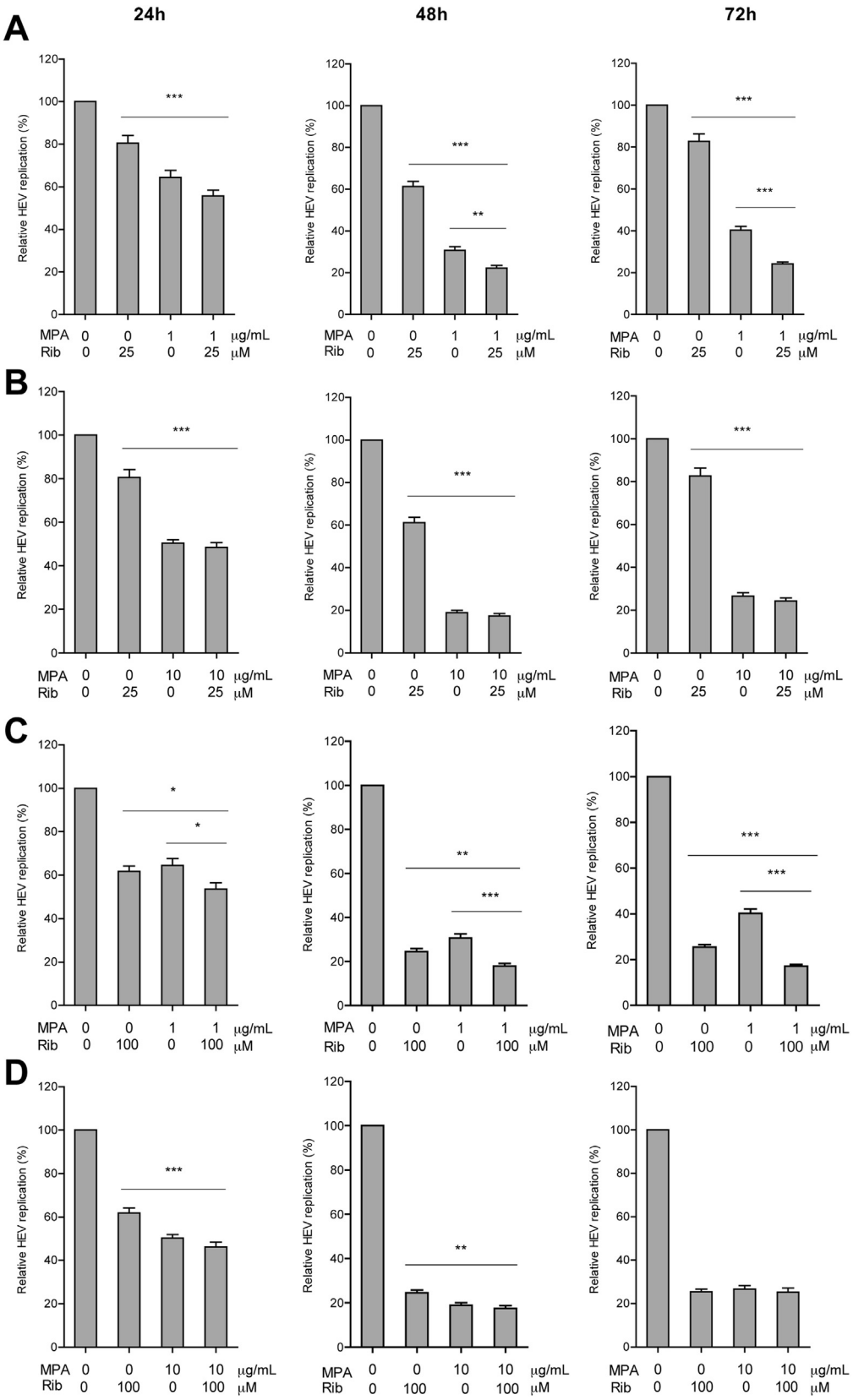

Figure 7. Combination of MPA with ribavirin extended their antiviral activity. Treatment with ribavirin alone has showed significant anti-HEV effects (mean ± SEM, n = 16 replicates in total) and a combination of MPA with ribavirin demonstrated an additional antiviral potency in particular combination groups; MPA doses: 1 µg/mL; 10 µg/mL; ribavirin doses: 25 µm; 100 µm. (*A*) 1 µg/ml MPA combined with 25 µm ribavirin. (*B*) 10 µg/ml MPA combined with 25 µm ribavirin. (*C*) 10 µg/mL MPA combined with 100 µm ribavirin. (*D*) 1 µg/mL MPA combined with 25 µm ribavirin. *$P < .05$; **$P < .01$; ***$P < .001$.

including influenza A virus[33] and rotavirus,[34] similar to what we have observed for HEV. Because the mechanistic insight is still largely missing for the antiviral action of cyclophilins, it deserves additional investigation. In addition, we also observed a proviral effects of FK506, but only at high dose. To our knowledge, there is no evidence of FK506

affecting HCV infection in cell culture.[28,35] In fact, compared with CsA, dose reduction of FK506 was assumed to be associated more with clearance of HEV in cases of renal transplantation with acute infection.[36] In a large retrospective study (although only 85 patients were included), the use of FK506 was the main predictive factor for chronic hepatitis E in organ recipients.[6] Our in vitro results have indicated that both FK506 and CsA can promote HEV infection. However, these data do not necessarily contradict to the clinical observation, because the number of patients currently investigated in the clinic is rather too small to draw solid conclusion. In addition, besides the direct effects we observed in cell culture, drugs can also have indirect influence on the infection.

The antiviral effects of MPA/mycophenolate mofetil have been demonstrated against a broad spectrum of viruses, including Dengue virus, West Nile, yellow fever virus, Chikungunya virus, HBV, and HCV.[36–39] This is consistent with our finding that MPA also potently inhibited HEV replication. For several viruses, MPA exerts antiviral effects by targeting IMPDH to deplete cellular nucleotide pools.[36] In the case of HCV, the IMPDH-dependent pathway only partially contributed to its antiviral activity.[11] In contrast, supplementation of exogenous guanosine completely abrogated the anti-HEV activity of MPA, suggesting a crucial role of IMPDH inhibition leading to depletion of cellular nucleotides. Interestingly, clearance of HEV after heart transplantation was found to be more frequent in patients with immunosuppressive medication containing MMF,[8] although this might be biased by a reduced dose of CsA or FK506 in these cases.

Despite a clear benefit to manipulating immunosuppressive regimens, a substantial proportion of patients are still not able to clear the virus and rapidly progresses toward chronic hepatitis.[6] Although no proven medication is available, the use of ribavirin monotherapy as off-label drug is gaining acceptance for treating hepatitis E.[18] An intriguing question is whether immunosuppressants can interfere with or promote the anti-HEV efficacy of ribavirin. In this study, we have finally demonstrated a beneficial effect of combining ribavirin with MPA (Figure 7). This does provide a proof of concept that it is important to choose the right immunosuppressive medication while under antiviral therapy of HEV in organ transplant recipients.

In conclusion, this study has profiled differential effects of different immunosuppressants on HEV infection in cell culture. Steroids did not affect genotype 3 HEV replication in vitro, but a high dose of FK506 promoted HEV infection. CsA dose-dependently facilitated HEV infection by targeting cellular factors CypA and CypB. In contract, MPA potently suppressed HEV infection by depletion of cellular nucleotide pools. In addition, a clear beneficial effect was observed when MPA combined with another antiviral regimen ribavirin. Although experimental research alone will not be able to clarify these complicated but important clinical issues, the knowledge gained from this study is certainly a valuable reference for the management of immunosuppression in organ transplant recipients infected with HEV. Hopefully, it will also promote the initiation of randomized controlled clinical studies to address these issues in the near future.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.02.036.

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Conflicts of interest
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