Vitamin D: An Innate Antiviral Agent Suppressing Hepatitis C Virus in Human Hepatocytes

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Vitamin D supplementation was reported to improve the probability of achieving a sustained virological response when combined with antiviral treatment against hepatitis C virus (HCV). Our aim was to determine the *in vitro* potential of vitamin D to inhibit HCV infectious virus production and explore the mechanism(s) of inhibition. Here we show that vitamin D_3 remarkably inhibits HCV production in Huh7.5 hepatoma cells. These cells express CYP27B1, the gene encoding for the enzyme responsible for the synthesis of the vitamin D hormonally active metabolite, calcitriol. Treatment with vitamin D_3 resulted in calcitriol production and induction of calcitriol target gene CYP24A1, indicating that these cells contain the full machinery for vitamin D metabolism and activity. Notably, treatment with calcitriol resulted in HCV inhibition. Collectively, these findings suggest that vitamin D_3 has an antiviral activity which is mediated by its active metabolite. This antiviral activity involves the induction of the interferon signaling pathway, resulting in expression of interferon-β and the interferon-stimulated gene, MxA. Intriguingly, HCV infection increased calcitriol production by inhibiting CYP24A1 induction, the enzyme responsible for the first step in calcitriol catabolism. Importantly, the combination of vitamin D_3 or calcitriol and interferon-α synergistically inhibited viral production. *Conclusion:* This study demonstrates for the first time a direct antiviral effect of vitamin D in an *in vitro* infectious virus production system. It proposes an interplay between the hepatic vitamin D endocrine system and HCV, suggesting that vitamin D has a role as a natural antiviral mediator. Importantly, our study implies that vitamin D might have an interferon-sparing effect, thus improving antiviral treatment of HCV-infected patients. (HEPATOLOGY 2011;54:1570-1579)

Hepatitis C virus (HCV) is a major cause of chronic hepatitis and the leading cause of endstage liver disease including liver cirrhosis and hepatocellular carcinoma.[1] It is a major global health challenge affecting an estimated 2.7 million people worldwide.[2] HCV is a small enveloped positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family.[3] It is characterized by a high genetic variability that reflects the low-fidelity rate together with the lack of a proofreading function of the viral RNA-dependant RNA polymerase.[1,3] HCV variability, which facilitates rapid development of antiviral resistance, provides a strong rationale for the development and implementation of antiviral combination therapies.[3]

The best available HCV antiviral therapy is a combination of pegylated interferon-α (IFNα) and ribavirin-based therapy.[4] This treatment is aimed to obtain a sustained viral response (SVR), which is defined as undetectable serum HCV RNA 24 weeks posttherapy.

Abbreviations: 1α-hydroxylase, 25-hydroxyvitamin-D 1α-hydroxylase; 1α,25(OH)2D, 1α,25-dihydroxyvitamin D; 24-hydroxylase, 25-hydroxyvitamin-D 24-hydroxylase; 25(OH)D, 25-hydroxyvitamin D; HCV, hepatitis C virus; IFNα, interferon-α; ISG, interferon-stimulated gene; SVR, sustained viral response; TLR3, Toll-like receptor 3; RIG-I, retinoic acid-inducible gene I; VDR, vitamin D receptor.

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Received May 8, 2011; accepted July 13, 2011.

Supported in part by the Caesarea Edmond Benjamin De Rothschild Foundation, Israel (RTK, RZ), and by the Cesarman Chair for Research in Liver Diseases, Tel Aviv University incumbent, Ran Tur-Kaspa. M.G-T. is the recipient of a returning researchers grant, the Science Absorption Center, Ministry of Immigration, Israel.

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However, this treatment has high toxicity and limited SVR rates,[1] which points to the need of discovering improved HCV treatments.

Vitamin D plays a central role in calcium and phosphate homeostasis and is essential for the proper development and maintenance of bone.[5] It is also known to be involved in cell proliferation, differentiation, and immunomodulation.[6] The active metabolite of vitamin D is obtained through two successive hydroxylations. The first hydroxylation occurs in the liver, where vitamin D, generated in the skin or obtained by diet, is hydroxylated to form the intermediate metabolite 25-hydroxyvitamin D [25(OH)D]. 25(OH)D, the major circulating form of vitamin D, is transported to the kidney, where it undergoes a second hydroxylation into the active form of the hormone, 1α,25-dihydroxyvitamin D [1α,25(OH)2D or calcitriol] by 25-hydroxyvitamin-D 1α-hydroxylase (1α-hydroxylase).[7,8] The systemic levels of calcitriol are mainly determined by the renal enzyme, although the local production of calcitriol from 25(OH)D has now been demonstrated in many extrarenal cells and tissues.[9-12] Most biological effects of calcitriol are mediated through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of ligand-activated transcription factors.[6] Calcitriol activates its own breakdown by up-regulating 25-hydroxyvitamin-D 24-hydroxylase (24-hydroxylase) expression, the enzyme responsible for its catabolism.[13,14] At the same time, it also down-regulates 1α-hydroxylase expression in the kidney, leading to decreased production of calcitriol.[14]

Vitamin D deficiency is associated with many pathological conditions, including cancer, autoimmune diseases, cardiovascular disease, and diabetes.[15,16] Moreover, the association between circulating vitamin D levels and morbidity related to infectious disorders has been recognized for more than a century.[17] Epidemiological studies provide evidence that vitamin D deficiency may confer increased risk of viral infections such as influenza, respiratory tract infections, and human immunodeficiency virus (HIV).[18] An association between vitamin D status and chronic liver diseases was also described.[19,20] Recently, an association between vitamin D status at the time of starting HCV antiviral therapy and achievement of SVR following treatment of chronic or recurrent HCV was reported.[21,22] It was shown that patients with severe vitamin D deficiency almost never achieved SVR, while those with near-normal or normal vitamin D obtained an SVR rate in about half the cases.[21,22] The recent report that vitamin D supplementation improved the probability of achieving an SVR following antiviral treatment indicates the causal relationship between vitamin D and HCV infection.[22]

The association between vitamin D and infectious disorders has been suggested to be linked to its ability to modulate both innate and adaptive immune responses.[17] The finding that vitamin D induces the expression of the antimicrobial peptide, cathelicidin, led to the suggestion that it increases the antimicrobial aspect of innate immunity. On the other hand, vitamin D is known for its antiinflammatory action in cutaneous and mucosal inflammatory disorders.[23,24]

In this study we demonstrate for the first time that vitamin D can be metabolized to its active form calcitriol in hepatoma Huh7.5 cells, which in turn induces its target gene CYP24A1. We demonstrate that both vitamin D and its active metabolite inhibit HCV production in infected cells and acts in a synergistic fashion with interferon-α treatment. Interferon-β and the interferon-stimulated gene (ISG), MxA, are up-regulated upon treatment with vitamin D alone, suggesting the involvement of the interferon signaling pathway in the antiviral activity of vitamin D.

Materials and Methods

Reagents. Vitamin D_3 was purchased from Sigma Chemical (St. Louis, MO). Calcitriol was obtained from Teva Pharmaceutical Industries (Israel).

Virus and Cell Lines. Virus assays were carried out with the intergenotypic HJ3-5 chimeric HCV virus.[25] Virus stocks were produced in FT3-7 cells.[26] Huh-7.5 cells were used for all assays and were cultured as described.[25]

RNA Isolation and Complementary DNA (cDNA) Synthesis. Total RNA was extracted from cells using the guanidine isothiocyanate method.[27] RNA samples were treated with DNaseI (Ambion, Cambridgeshire, UK). Total RNA (1 μg) was subjected to reverse transcription (RT) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Real-time RT-PCR assays were performed in the ABI 7000

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24575

Potential conflict of interest: Nothing to report.

sequence device (Applied Biosystems), using TaqMan gene expression assays essentially as described.[28] For measurement of HCV expression, 20-μL reactions were prepared in a 96-well format, using 1 μL of the template cDNA, 10 μL of 2× TaqMan Universal PCR Master (Applied Biosystems), 10 pmol of forward primer HCV-F (nucleotides [nt] 130-146), and reverse primer HCV-R (nt 272-290) (AF139594) for the HCV nontranslated region, and 5 pmol of an HCV-specific TaqMan probe. Assay-on-Demand Gene Expression Products (Applied Biosystems) were used for the measurement of IFN-β (Hs02621180_s1), GAPDH (Hs99999905_m1) CYP27B1 (Hs00168017_m1), CYP24A1 (Hs001679999_m1), and VDR (Hs00172113_m1). Results are expressed as the ratio of the target gene messenger RNA (mRNA) and GAPDH mRNA threshold values. Analysis of the ISG MxA expression level was performed by SYBR Green I dye (Applied Biosystems) using MxA-specific primers as published[29] and GAPDH as the internal control gene (primers: GAPDHS 5′-GAAGGTGAAGGTCGGA GTC-3′ and GAPDHAS 5′-GAAGATGGTGATGG GATTTC-3′) using the ABI 7000 (Applied Biosystems) detection system.

Calcitriol Determination. The level of calcitriol in the supernatants was determined by specific 1α,25-dihydroxyvitamin D enzyme-linked immunosorbent assay (ELISA) kit following extraction with monoclonal anti-1α,25-dihydroxyvitamin D antibody according to the manufacturer's instructions (Immunodiagnostic Systems, Boldon, UK).

Inhibition of Infectious Virus Production. Huh-7.5 cells were pretreated with vitamin D_3, calcitriol, or the vehicle ethanol (ethanol concentration did not exceed 0.015%) for 3 hours before infection with the HJ3-5 virus at a multiplicity of infection (moi) of 0.1-0.01. The medium was replaced with fresh medium containing vitamin D_3 or calcitriol after 24 hours and with fresh medium not containing vitamin D_3 or calcitriol after 48 hours. Supernatant fluids were collected from the cell cultures and the titer of infectious virus was determined by the focus-forming unit (FFU) assay, essentially as described.[25] For combination treatment of vitamin D_3/calcitriol with IFN-α, inhibition assays were carried out as described above with the addition of 0 and 0.025 ng/mL IFN-α to each concentration of vitamin D_3 or calcitriol. The titer of infectious virus was determined by FFU assay.

Cytotoxicity Assay. Relative cell number in culture was assessed by staining with crystal violet (CV) as described.[30] In brief, cells were stained for 30 minutes with a 0.1% CV solution in 20% ethanol. The dye was rinsed with water and extracted with 70% ethanol and its absorbency was determined at 550 nm using a microplate reader.

Immunoblot Analysis. Cells were washed with phosphate-buffered saline (PBS), scraped and lysed in sodium dodecyl sulfate (SDS) sample buffer. For immunoblotting, protein samples were separated on 15% SDS/polyacrylamide gel, transferred to nitrocellulose, and detected using mouse polyclonal anti-MxA1 antibody (Abnova, Taipei, Taiwan), mouse monoclonal antibody C7-50 to core protein (1:300) (ABR; Affinity Bioreagents, Golden, CO), and mouse monoclonal anti-β actin (Abcam, UK) for loading control, followed by goat antimouse antibodies (LI-COR Biosciences, Lincoln, NE). Western blots were analyzed with the Odyssey infrared imaging system (LI-COR Biosciences). The images were scanned on the Odyssey system and signal intensities were quantified.

Results

Vitamin D Inhibits HCV Production. To evaluate the potential of vitamin D to inhibit production of infectious HCV in cell culture, we used the intergenotypic HJ3-5 chimeric virus.[25] Huh7.5 cells were treated with various concentrations of vitamin D_3 or vehicle and 3 hours later were infected with the virus. The titer of infectious virus was determined by FFU assay 3 days posttreatment (see Materials and Methods). To prevent vitamin D interfering with the FFU reduction assay, the medium was replaced with fresh vitamin D-free medium 24 hours before assaying. The results in Fig. 1A show that vitamin D_3 inhibited infectious virus production in a dose-dependent manner. Efficient inhibition was observed at the highest vitamin D_3 concentration (5 μM), reaching up to 70%-80%. To ensure that the observed inhibitory effect of vitamin D is not due to a cytotoxic effect, we tested Huh7.5 cell viability. The results (Fig. 1B) show that treatment with vitamin D_3 in the concentration needed to exert its antiviral activity does not affect cell viability.

Full Machinery That Converts Vitamin D to Calcitriol Is Present in Hepatoma Huh7.5 Cells. Vitamin D itself is biologically inert and has to be metabolized to the active hormone calcitriol in order to exert its effect by way of the VDR.[9] There are no reports of calcitriol production or VDR transcriptional activation by active vitamin D derivatives in liver cells. Because vitamin D was biologically active in our system we assessed whether vitamin D is converted to calcitriol in hepatoma Huh7.5 cells. To evaluate the potential of

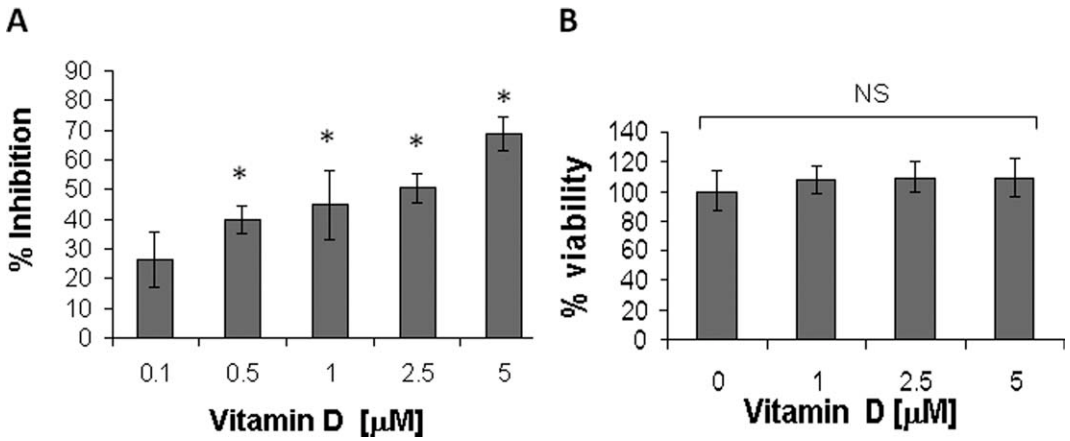

Fig. 1. Specific inhibition of infectious HCV production by vitamin D_3. (A) Inhibition of HJ3-5 virus production, as determined by FFU assay of virus released into media following infection and treatment with 0.1-5 μM vitamin D_3. Percent of inhibition was determined compared with virus released by nontreated cells set as 100%. (B) Cell number in HCV-infected cultures treated with 0-5 μM vitamin D_3 was assessed by staining with crystal violet. Percent of viable cells was determined compared with nontreated cells set as 100%. Statistical significance was calculated by two-tailed Student's *t* test and is indicated as follows: $*P < 0.001$; NS, nonsignificant.

Huh7.5 cells to produce calcitriol, we tested the expression level of the gene CYP27B1 encoding for 1α-hydroxylase, the enzyme responsible for the synthesis of calcitriol. Real-time RT-PCR analysis showed that these cells express CYP27B1, the level of which increased following 24 hours incubation with vitamin D_3 (Fig. 2A).

Calcitriol binds to VDR to induce the expression of the first enzyme in the pathway leading to its catabolism, 24-hydroxylase, in most of its target cells. In fact, induction of this enzyme serves as an indicator of the transcriptional activation of VDR. Therefore, we tested the expression level of the VDR-regulated gene CYP24A1, encoding for 24-hydroxylase, in response to vitamin D by real-time RT-PCR. Treatment with vitamin D_3 (5 μM) markedly up-regulated the CYP24A1 expression level (Fig. 2B). These data demonstrate that treatment with vitamin D_3 leads to transactivation of VDR, presumably as a consequence of its conversion to the VDR ligand, calcitriol. Furthermore, the addition of vitamin D_3 up-regulated the mRNA level of VDR (Fig. 2C), which may increase the responsiveness to calcitriol in these cells.

Next we directly examined the potential of Huh7.5 cells to convert vitamin D into its active form, calcitriol. The cells were treated with vitamin D_3 (5 μM) and the level of calcitriol in the supernatant at 5 and 24 hours posttreatment was measured by specific ELISA. The results presented in Fig. 2D demonstrate that Huh7.5 cells convert vitamin D_3 into calcitriol. Production of calcitriol was detected as early as 5 hours after treatment and was markedly higher at 24 hours posttreatment. Treatment for 48 hours did not further increase calcitriol production (data not shown). Thus, calcitriol can be constitutively produced in these cells. Taken together, these results indicate that the hepatoma Huh7.5 cell system contains: the complete enzymatic machinery needed for the conversion of the parent compound, vitamin D, to its hormonal metabolite, a functional vitamin D response system, and the enzyme responsible for the first step of calcitriol catabolism.

HCV Increases the Level of Calcitriol in Huh7.5 Cell Culture. Next we examined whether infection with HCV affects vitamin D metabolism in Huh7.5 cells. First, we evaluated the expression level of CYP27B1 and CYP24A1 genes in vitamin D_3-treated cells in response to HCV infection. Infection with the virus did not significantly affect the expression of CYP27B1 (Fig. 3A), while significantly reducing CYP24A1 expression (Fig. 3B). These findings may suggest that vitamin D conversion to calcitriol may not be enhanced by HCV but rather calcitriol catabolism may be decreased, which may result in higher levels of calciriol in the infected cells. This was confirmed by comparing the levels of calcitriol in the supernatant of infected and uninfected Huh7.5 cells after treatment with vitamin D_3 (5 μM) as measured by ELISA. The level of calcitriol was significantly higher in the supernatant of infected cells (Fig. 3C).

Calcitriol Inhibits HCV Production. We then addressed the question whether the inhibition of HCV production by vitamin D_3 could be mediated by its hormonal metabolite, calcitriol. First, we evaluated the potential of calcitriol to inhibit production of infectious HCV in cell culture. Huh7.5 cells were treated with various concentrations of calcitriol and 3 hours

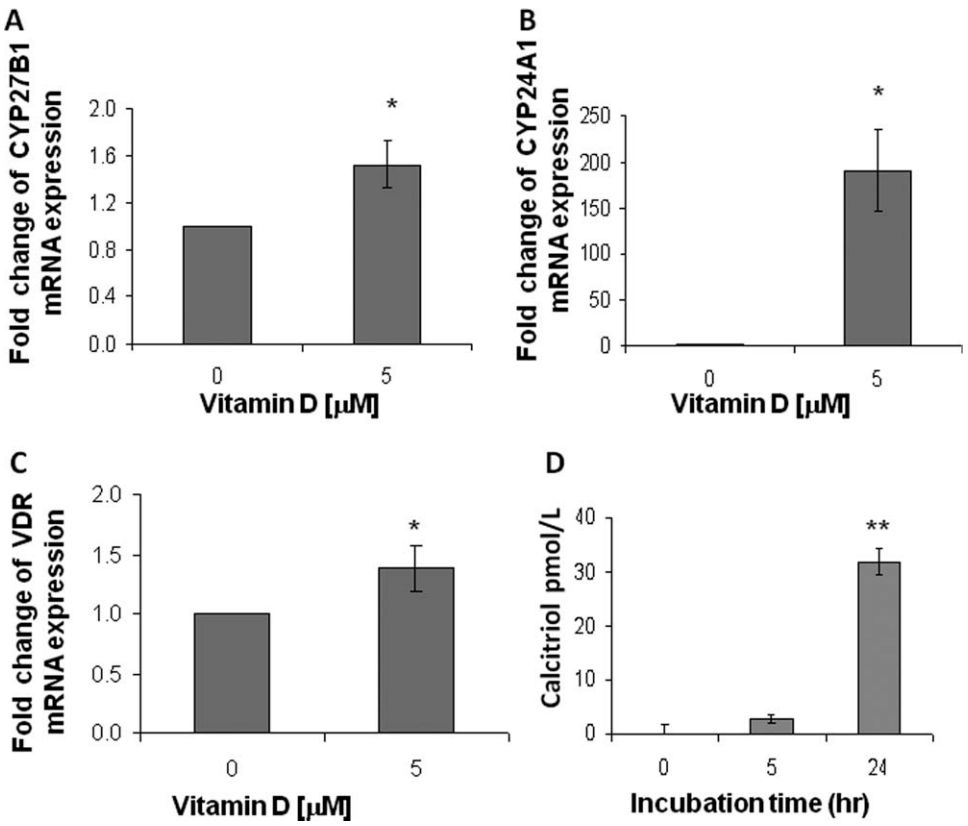

Fig. 2. Metabolism of vitamin D in Huh7.5 cells. Real-time PCR analysis of (A) CYP27B1, (B) CYP24A1, and (C) VDR RNA expression levels in Huh7.5 cells 24 hours posttreatment with vitamin D_3 (5 μM) compared with nontreated cells. All real-time PCR analyses are shown as relative quantity of the target gene normalized to GAPDH mRNA values. The control nontreated cells were assigned a value of 1 and results are normalized to control. (D) ELISA analysis of calcitriol levels produced by Huh7.5 cells 0, 5 and 24 hours posttreatment with vitamin D (5 μM). Statistical significance was calculated by two-tailed Student's *t* test and is indicated as follows: $*P \leq 0.005$; $**P \leq 0.001$.

later were infected with the virus and treated as described in Fig. 1 for vitamin D_3. The results obtained in the FFU assay demonstrate that calcitriol inhibited infectious virus production in a dose-dependent manner similar to the results obtained with vitamin D_3 (Fig. 4A). Marked inhibition (40%) was observed already at a concentration of 1 nM, attaining 70%-80% at 100 nM of calcitriol. Similar to the results obtained with vitamin D_3, the inhibitory effect of calcitriol is not due to cell cytotoxicity, as it did not affect cell viability at effective antiviral doses (Fig. 4B).

To further confirm these results we examined the impact of vitamin D_3 and calcitriol on HCV RNA replication and viral protein expression. We carried out a real-time RT-PCR analysis using primers that targeted the 5′ noncoding region of the HCV RNA. We found that the abundance of HCV RNA was markedly reduced in cells treated with vitamin D_3 or calcitriol (Fig. 4C). Immunoblot analysis shows efficient inhibition of HCV core protein expression in cells treated with vitamin D_3 (5 μM) or calcitriol (100 nM), (Fig. 4D).

Vitamin D and Calcitriol Enhance IFN Signaling in HCV-Infected Cells. Cells respond to HCV infection mainly through the membrane-bound Toll-like receptor 3 (TLR3) and cytosolic retinoic acid-inducible gene I (RIG-I).[31] These signaling pathways lead to the synthesis of type I IFNs (IFNα/β), numerous ISGs, and proinflammatory cytokines that directly limit HCV replication. It is noteworthy that in Huh7.5 cells, the only highly permissive cell line for HCV production, neither TLR3 nor RIG-I pathways are functional.[32] Here we examined whether treatment with vitamin D affects this innate immune response in HCV-infected cells. To this end IFN-β induction in response to vitamin D_3 or calcitriol treatment was assessed in HCV-infected Huh7.5 cells. Cells were treated with vitamin D_3 or calcitriol and infected with HCV (as described above). At 72 hours postinfection IFN-β expression was determined by real-time RT-PCR. In HCV-infected cells minimal expression of IFN-β mRNA was observed (Fig. 5A,B). Vitamin D and calcitriol had minimal effect on IFN-β expression when applied to noninfected cells (data not shown). However, the addition of vitamin D_3 (5 μM) or calcitriol (100 nM) increased IFN-β gene expression in HCV-infected cells.

Interferon-β triggers the expression of ISGs that have diverse antiviral activities.[33,34] To validate that the increased IFN-β expression has functional consequences under these conditions, we examined a downstream effect of the cytokine, the induction of the ISG gene MxA. As shown in Fig. 5C,D, treatment of HCV-infected cells with vitamin D_3 or calcitriol increased the mRNA expression of MxA. These effects were confirmed at the

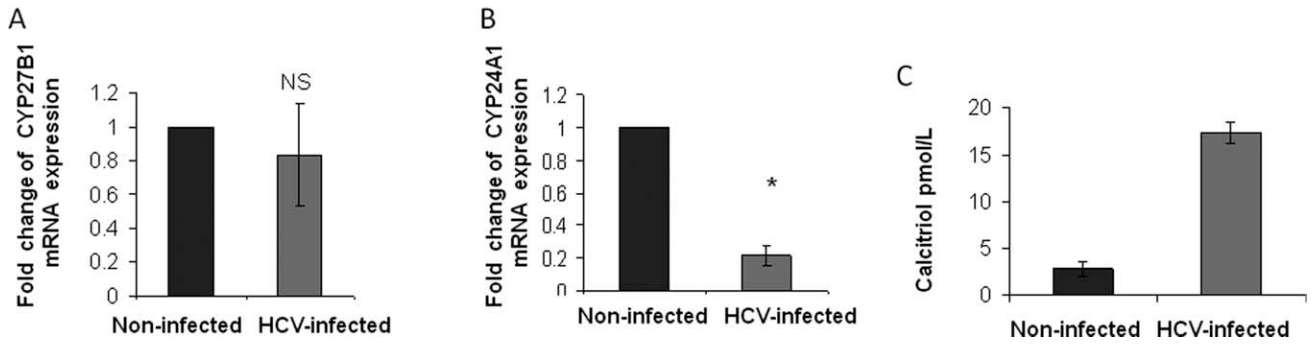

Fig. 3. Increase of vitamin D metabolism by HCV infection. Real-time PCR analysis of (A) 1α-hydroxylase (CYP27B1), (B) 24-hydroxylase (CYP24A1) mRNA expression level in Huh7.5 cells 5 hours postinfection with HCV and treatment with vitamin D_3 (5 μM) compared with noninfected cells treated with vitamin D_3. The control noninfected cells were assigned a value of 1 and results are normalized to control. Statistical significance was calculated by two-tailed Student's *t* test and is indicated as follows: NS, nonsignificant; *$P \leq 0.001$. (C) ELISA analysis of calcitriol produced by Huh7.5 cells 5 hours postinfection with HCV and treatment with vitamin D (5 μM). Each point represents the mean $\pm$SD of a set of data determined in two experiments.

protein level. As shown in Fig. 5E, both vitamin D_3 and calcitriol treatment increased MxA protein expression in HCV-infected cells in a dose-dependent manner. The results shown here indicate that vitamin D or calcitriol treatment induces antiviral IFN-mediated signaling pathways in HCV-infected cells.

Vitamin D and Calcitriol Synergize with IFN-α to Inhibit HCV Production. To simulate *in vitro* the *in vivo* combination therapy of IFN-α and vitamin D_3,[22] we treated HCV-infected cells with a combination of both agents. In a preliminary experiment we evaluated the effective concentration of IFN-α that achieves 50% inhibition of virus production (EC50) by the FFU reduction assay and found it to be 0.1 ng/mL (data not shown). For combination treatment, Huh7.5 cells were treated with various concentrations of vitamin D_3 or calcitriol in combination with a sub-EC50 concentration of IFN-α (0.025 ng/mL), which exert only minimal anti-HCV inhibitory effects. After 3 hours the cells were infected with the virus and treated as described above. Virus titer was determined by FFU assay 72 hours postinfection. As shown in Fig. 6A,B, calcitriol and vitamin D_3 as single agents inhibited virus production in a dose-dependent manner as described above, whereas only minimal (10%-20%) inhibition of viral production was observed by treatment with 0.025 ng/mL IFN-α alone. Treatment with low concentrations of vitamin D_3 (0.1 μM) or calcitriol (0.1 nM), which inhibited viral production by $\leq$10%, combined with a low concentration of IFN-α (0.025 ng/mL), resulted in a synergistic effect attaining 70%-80% inhibition. At moderate concentrations of vitamin D (0.5-1 μM) or calcitriol (1-10 nM), the addition of IFN increased the inhibition of virus production additively.

Discussion

To date, the association between circulating vitamin D levels and morbidity related to infectious disorders has been mainly based on epidemiological studies. These studies provide evidence that vitamin D deficiency may confer increased risk of viral infections such as influenza, respiratory tract infections, and HIV and suggested that vitamin D possesses antiviral activity. However, this notion is mainly based on the known ability of vitamin D to up-regulate antimicrobial peptides.[18,35] The recent findings that low vitamin D serum levels are related to low responsiveness to IFN-based therapy in chronic hepatitis C[21] and that supplementation of vitamin D significantly improved interferon therapy outcome in these patients[22,36] led us to surmise that vitamin D may have a direct antiviral effect.

We here demonstrate for the first time that vitamin D has a direct inhibitory effect on viral production. This inhibition may be partially attributed to augmentation of the innate immune response, as treatment of HCV-infected cells with vitamin D or calcitriol up-regulated the expression of IFN-β, the immediate cellular response to viral infection.[32] Moreover, we observed the downstream induction of the IFN-stimulated gene MxA, which has been shown to directly inhibits viral production.[37] It is important to note that these observations were obtained using the hepatoma cell line Huh7.5, which is the only highly permissive cell line for HCV production.[26] They have nonfunctional RIG-I and TLR3 pathways resulting in impaired IFN responses.[32] Indeed, in our study infection with HCV resulted in only a minor expression of IFN-β mRNA. Similarly, the exposure to vitamin D_3 or calcitriol alone had minimal effect on IFN signaling. In

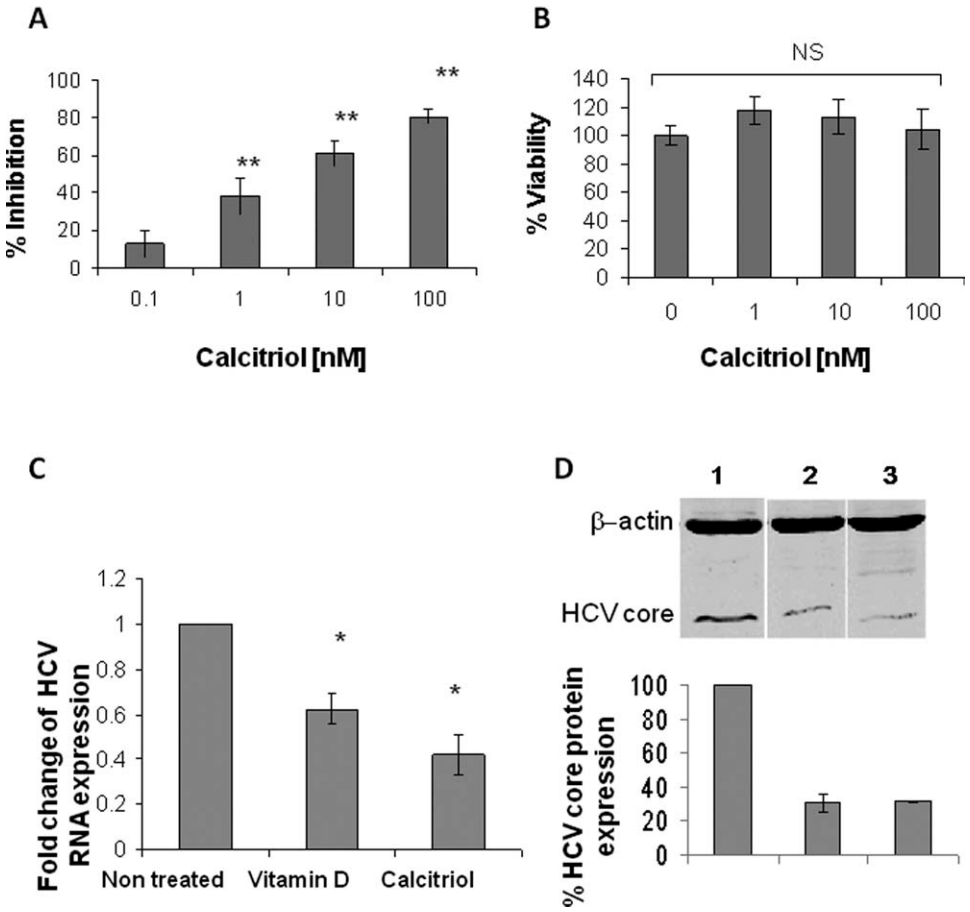

Fig. 4. Specific inhibition of infectious HCV production by calcitriol. (A) Inhibition of HJ3-5 virus production, as determined by FFU assay of virus released into media following infection and (B) cell number in HCV-infected cultures assessed by staining with crystal violet following treatment with 0.1-100 nM calcitriol. Percent of inhibition and percent of viable cells was determined compared with nontreated cells set as 100%. (C) Real-time PCR analysis of HCV RNA expression level in Huh7.5 cells 72 hours postinfection with HCV and treatment with vitamin D_3 (5 μM) or calcitriol (100 nM) compared with nontreated cells. The results are shown as the relative quantity of the HCV noncoding region normalized to GAPDH mRNA values; the control cells were assigned a value of 1 and results are normalized to control. (D) Immunoblot analysis of Huh7.5 cells 72 hours postinfection with HCV and treatment with (2) vitamin D_3 (5 μM) or (3) calcitriol (100 nM) compared with (1) nontreated cells. HCV core protein was detected by mouse monoclonal antibody C7-50 to core protein and mouse monoclonal anti-β-actin for loading control. Quantification of the immunoblot was performed by scanning image on the Odyssey infrared imaging system; HCV core protein expression is presented as percent of control. Statistical significance was calculated by two-tailed Student's t test and is indicated as follows: *$P \leq 0.01$; **$P \leq 0.005$; NS, nonsignificant.

contrast, treatment of HCV-infected cells with vitamin D or calcitriol significantly up-regulated the expression of IFN-β and of the ISG MxA. The mechanism by which vitamin D enhances the expression of INF-β signaling in these RIG-I and TLR3-deficient cells requires further investigation.

We also studied the effect of vitamin D in combination with IFN-α treatment on HCV production. Combined treatment of infected cells with low concentrations of IFN-α and vitamin D or calcitriol, which by themselves had almost no antiviral effect, resulted in a synergistic inhibition of viral production. These *in vitro* studies point to the fact that in the presence of vitamin D lower IFN-α concentrations are sufficient to achieve a vigorous antiviral effect. These results may underlie the recently published clinical studies of improved anti-HCV therapy with vitamin D supplementation to the standard Peg-IFN and ribavirin therapy.[21,22] Although further studies are needed to address the question of how relevant are our *in vitro* results to the *in vivo* setting, it seems possible that vitamin D will have an interferon-sparing effect, thus providing a therapy opportunity to patients who cannot tolerate the standard interferon regimen.

Vitamin D inhibited HCV production presumably through its active hormonal form calcitriol. The conversion to calcitriol, the second step in vitamin D bioactivation, occurs mainly in the kidney by the renal 1α-hydroxylase. However, there is substantial evidence for additional extrarenal sites of production of calcitriol, which primarily serves as an autocrine/paracrine factor with cell-specific functions.[9] 1α-Hydroxylase has been reported in many cells and tissues including the skin, prostate, brain, breast, colon, lung, pancreatic islets, lymph nodes, monocytes, parathyroid, placenta, colonic epithelial cells, and in adipose tissue.[9-12]

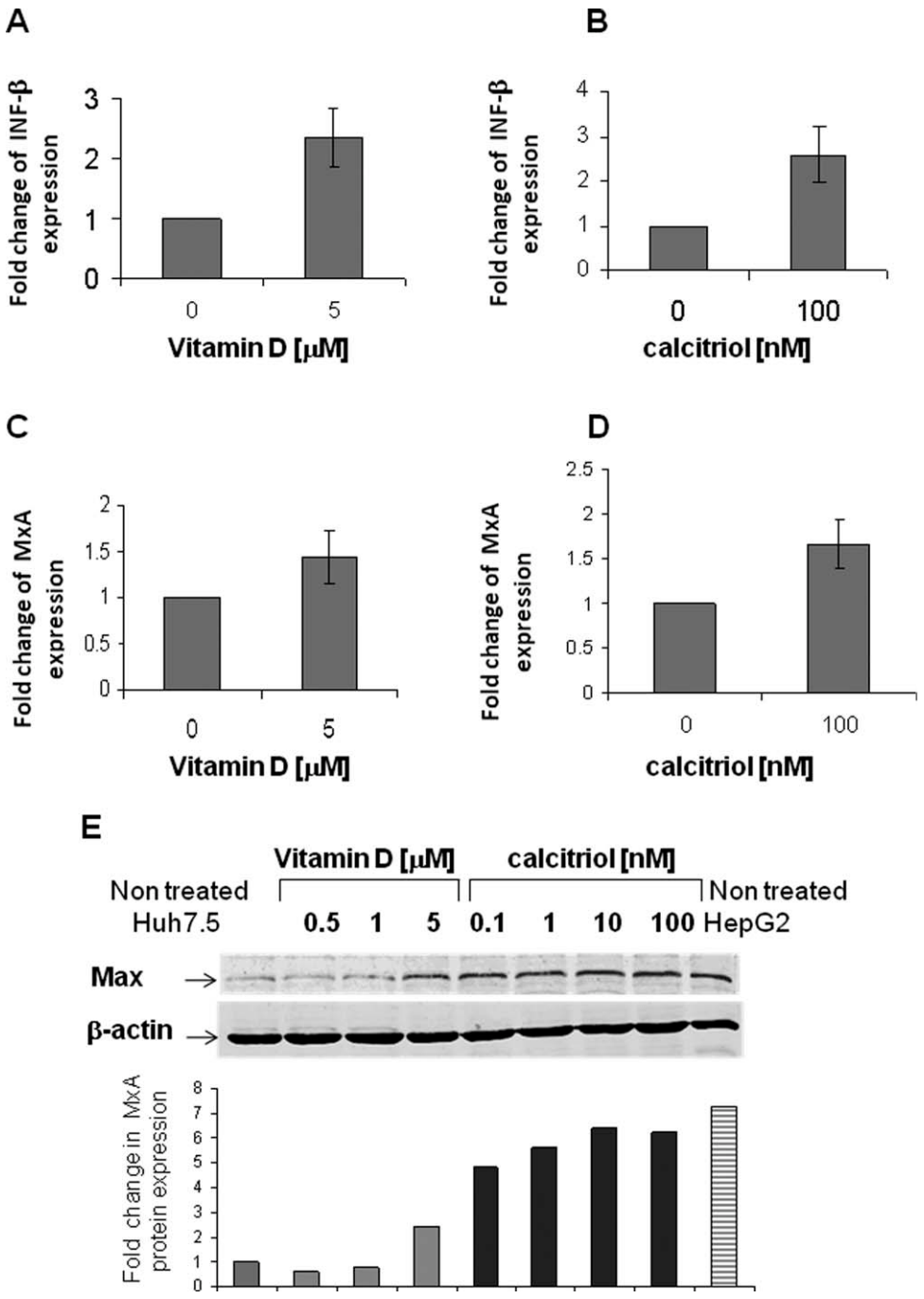

Fig. 5. Induction of interferon signaling by HCV infection in combination with vitamin D or calcitriol treatment. Real-time RT-PCR analysis of INF-β expression level in HCV infected Huh7.5 cells 72 hours posttreatment with (A) vitamin D_3 or (B) calcitriol. (C,D). Real-time RT-PCR analysis for MxA RNA expression level in Huh7.5 cells 72 hours posttreatment with vitamin D_3 (C) or calcitriol (D) compared with non treated cells. All real-time PCR analyses are shown as relative quantity of the target gene normalized to GAPDH mRNA values. The control nontreated cells were assigned a value of 1 and results are normalized to control. Statistical significance was calculated by two-tailed Student's *t* test and is indicated as follows: $*P \leq 0.05$. (E) Immunoblot analysis of Huh7.5 cells 72 hours postinfection with HCV and treatment with various concentrations of vitamin D_3 or calcitriol compared with nontreated Huh7.5 and HepG2 cells. MxA protein was detected by mouse polyclonal anti-MxA antibody and mouse monoclonal anti-β-actin for loading control, followed by goat antimouse antibodies.

However, to the best of our knowledge, no previous reports have shown either the expression of 1α-hydroxylase in hepatocytes or the synthesis of calcitriol in these cells. In our study we describe for the first time the expression of the 1α-hydroxylase gene, CYP27B1, in hepatoma cells. This expression is reflected in the production of calcitriol in cell cultures supplemented with vitamin D_3 (Fig. 2). Moreover, treatment of these cells with calcitriol or with vitamin D_3 resulted in upregulation of the 24-hydroxylase gene, CYP24A1, a vitamin D target gene which is transactivated by the vitamin D receptor.[38] Taken together, these results imply that Huh7.5 cells possess the molecular machinery to both metabolize and respond to vitamin D. Of special importance is the finding that HCV infection markedly increased the levels of calcitriol in cell cultures. This was not due to increased production of calcitriol, as the level of 1α-hydroxylase was not altered, but rather to the prevention of induction of 24 hydroxylase, the enzyme responsible for the first step in the catabolism of calcitriol. Thus, HCV increases the efficacy of the vitamin D endocrine system of the hepatocyte.

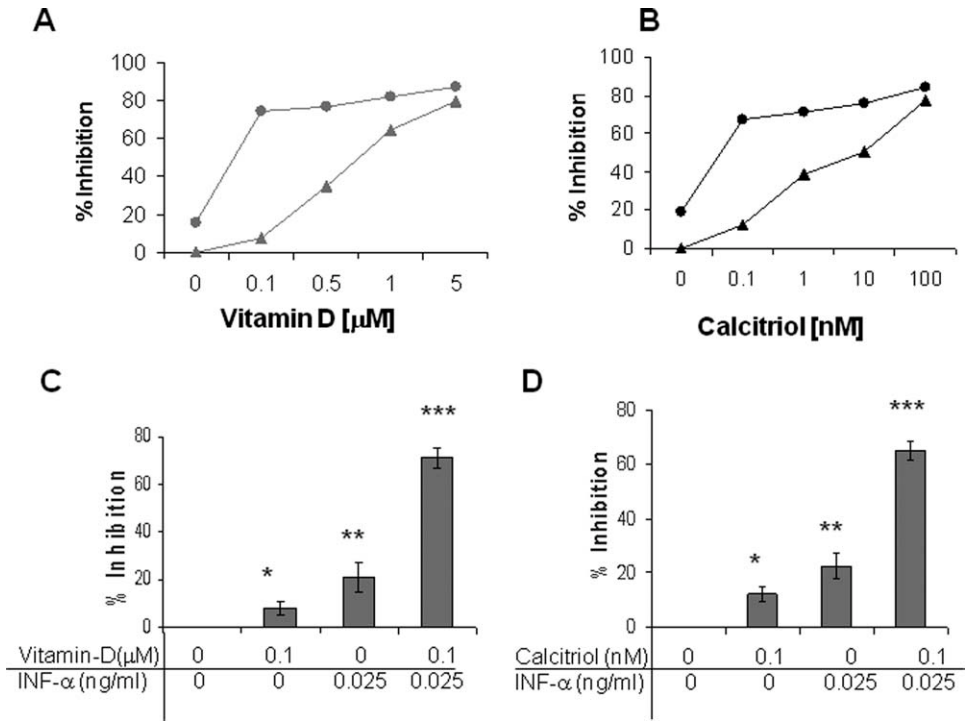

Fig. 6. Inhibition of infectious HCV production by combination treatment of interferon-α and vitamin D or calcitriol. Inhibition of HJ3-5 virus production was determined by FFU assay of virus released into media following infection and treatment with various concentrations of (A) vitamin D_3 or (B) calcitriol in combination with 0 (filled triangle) or 0.025 ng/mL (filled circles) interferon-α. Percent inhibition was determined compared with virus released by nontreated cells set as 100%. Presentation of the synergistic effect of vitamin D_3 (0.1 μM) (C) and calictriol (0.1 nM) (D) with 0.025 ng/mL interferon-α. Statistical significance was calculated by two-tailed Student's *t* test compared with nontreated cells (control) and is indicated as follows: $*P \leq 0.05$; $**P \leq 0.005$; $***P \leq 0.0001$.

It is by now established that vitamin D promotes innate immune responses associated with pathogen elimination such as macrophage phagocytic function, and TLR2/1, TLR4, and cathelicidin induction in various cell types.[39,40] Our findings that vitamin D induced interferon and synergized with it adds another facet to its activity as an enhancer of innate immunity.

Our study unravels an interplay between vitamin D and HCV: on the one hand, viral infection increases the production of the active metabolite of vitamin D and, on the other hand, this metabolite suppresses viral infection. This interplay, together with the finding that vitamin D employs the interferon system to combat HCV, suggests a physiological role for the hormone in the antiviral arm of hepatic innate immunity. It is maintained that HCV persistence is associated with its ability to evade innate immune defenses by suppressing the RIG-I and TLR3 pathways, thereby impairing interferon production in infected hepatocytes. As mentioned before, Huh7.5 cells have similar defects in the interferon pathway. Interestingly, treatment with vitamin D restored the ability of Huh7.5 cells to produce interferon. It seems plausible that vitamin D may have a similar effect in virus-infected normal hepatocytes, thus counteracting the disruption of the interferon pathway by the virus. It is therefore tempting to assign vitamin D a role in the ongoing coevolutionary arms race between the virus and the host.

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