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Vitamin D ameliorates stress ligand expression elicited by free fatty acids in the hepatic stellate cell line LX-2

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Background/aims: *Hepatic stellate cells play an important role as the major source of fibrillar and non-fibrillar matrix proteins in the process of liver fibrosis. Natural killer cells have an anti-fibrotic effect through the killing of activated hepatic stellate cells. Major histocompatibility complex class I-related molecules, MICA and MICB, function as ligands for the NKG2D receptor and play an important role in hepatic stellate cells susceptibility to natural killer cells during hepatic inflammation. The aim of this study was therefore to investigate the effect of vitamin D_2 and free fatty acids on stress ligands and pro-fibrotic activity in LX-2 cells and human primary hepatic stellate cells.* ***Methods:*** *LX-2 cells and primary human hepatic stellate cells were treated with vitamin D_2 (10^{-6} M) and free fatty acids at different concentrations (0.25 mM, 0.5 mM, and 1 mM) for 24 hours, and expressions of the stress ligands MICA/B as well as of transforming growth factor-β, α-smooth muscle actin and collagen 1α were assessed by quantitative real time-polymerase chain reaction.* ***Results:*** *Treatment of cells with 0.5 mM and 1 mM free fatty acids induced α-smooth muscle actin and transforming growth factor-β expression in LX-2 cells. Moreover, 1 mM free fatty acids resulted in increased expression of MICA. Surprisingly, collagen 1α expression was reduced after addition of free fatty acids. MICA/B expression in primary hepatic stellate cells was not affected by free fatty acids treatment. Vitamin D_2 treatment significantly downregulated the free fatty acids-induced expression of transforming growth factor-β and α-smooth muscle actin in LX-2 cells. Further, in hepatic stellate cells, a significant decrease in MICA/B mRNA with vitamin D_2, independent of free fatty acids treatment, was detectable.* ***Conclusions:*** *These results indicate that vitamin D2 may reduce inflammatory and pro-fibrogenic activity of stellate cells in vitro.*

Key words: Hepatic stellate cells, NKG2D ligand, MICA/B, vitamin D_2

D vitamini hepatik stellat hücrelerdeki serbest yağ asiti aracılı stres ligand ekspresyonunu azaltmaktadır

Amaç: *Hepatik stellat hücreleri karaciğer fibrozisinin ilerlemesinde fibriller ve fibril olmayan matriks proteinlerin major kaynağı olarak önemli rol oynamaktadır. Naturel killer hücreler hepatik stellat hücrelerin yok edilmesinde anti fibrotik bir etkiye sahiptir. Major histokompatibilite sınıf I zincir bağlantılı molekül, MICA ve MICB, NKG2D'nin reseptörünün ligandı olarak fonksiyon görmektedir ve hepatik inflamasyon sırasında natürel killer hücrelerinin hepatik stellat hücre duyarlılığında önemli bir rol oynamaktadır. Bu çalışmanın amacı LX-2 hücrelerinde ve insan primer hepatik stellat hücrelerinde vitamin D_2 ve serbest yağ asidinin stres ligandları ve profibrotik aktivite üzerine olan etkilerini araştırmaktır.****Yöntem:*** *LX-2 ve primer insan hepatik stellat hücreleri 24 saat boyunca farklı konsantrasyonlarda (0,25 mM, 0,5 mM ve 1 mM) serbest yağ asidi ve vitamin D ile tedavi edildi ve kantitatif Real Time PCR cihazı ile stres liganları (MICA ve MICB) ve TGF-β, α-SMA, COL1α'nin ekspresyonu değerlendirildi.* ***Bulgular:*** *LX-2 hücrelerinde 0.5 mM ve 1 mM serbest yağ asidi ile hücrelerin tedavisi sonucunda α-SMA ve TGF-β'nın ekspresyonunun indüklendiği, ilginç olarak, serbest yağ asidinin eklenmesinden sonra COL1α'nın ekspresyonunun azaldığı, primer hepatik stlellat hücrelerde MICA/B'nin ekspresyonunun serbest yağ asidi tedavisinden etkilenmediği bulunmuştur. LX-2 hücrelerinde vitamin D_2 tedavisi serbest yağ asidi ile indüklenen TGF-α ve α-SMA'nın ekspresyonunu downregüle etmiştir. Yani, hepatik stellat hücrelerinde serbest yağ asidi tedavisinden bağımsız olarak vitamin D_2 tarafından MICA/B'nin mRNA'sının önemli düzeyde azaldığı saptanmıştır.* ***Sonuç:*** *Bu sonuçlar in vitro ortamda vitamin D_2'nin hepatik stellat hücrelerinin inflamatuvar ve profibrogenik aktivitesini azaltabildiğini göstermektedir.*

Anahtar kelimeler: Hepatik stellat hücreleri, NKG2D ligandı, MICA/B, vitamin D_2

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INTRODUCTION

Hepatic stellate cells (HSCs) play a critical role in apoptosis and inflammation (1). Activated HSCs secrete pro-fibrogenic cytokines, produce α-smooth muscle actin (SMA) and pro-collagen I, and are known to be a major source of collagens and inhibitors of matrix-degrading enzymes (tissue inhibitor of matrix metalloproteinases, TIMP) that are secreted during fibrosis (2). Non-alcoholic steatohepatitis (NASH) is a progressive liver disease that eventually leads to cirrhosis, liver failure and hepatocellular carcinoma (HCC) (3). Hepatocyte apoptosis is a key event of liver injury in the pathogenesis of NASH (4), which in turn activates HSCs.

Interestingly, vitamin D (VD) deficiency has been associated with several pathological conditions, including autoimmune diseases, chronic liver diseases, insulin resistance (IR), and diabetes (5-7). In patients with chronic liver disease, VD deficiency is associated with the progression of hepatic fibrosis (8). In contrast, apoptosis of activated HSCs is a prominent feature in the regression of liver fibrosis, and natural killer (NK) cells possess an anti-fibrotic activity through direct HSC killing (9). NK cell activity has been shown to decrease in patients with chronic liver disease as well as in HCC. Activation of NK cells is regulated by the balance of activating and inhibitory signalling (10). NKG2D is an immunoreceptor that is expressed on most NK cells, CD αβT cells and γδT cells. Major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) function as ligands for the NKG2D receptor. The interaction of MICA/B and NKG2D strongly activates NK cells, enhancing their cytolytic activity and cytokine production (11-13). In contrast to classic MHC class I molecules, MICA/B are minimally expressed on normal cells but frequently on tumor cells. MICA/B are cleaved proteolytically from tumor cells and appear as soluble forms in the sera of patients with various malignancies, including HCC. Recent studies have demonstrated that MICA/B are also elevated in the sera of patients with autoimmune and cholestatic liver diseases, as well as in NASH (13-16).

Therefore, we aimed to analyze whether these stress ligands are specifically upregulated in human HSCs in a cell culture model of fatty liver disease. Moreover, we investigated if VD treatment modulates the effects of fatty acids on HSCs.

MATERIALS AND METHODS

Isolation of Primary Human HSCs

For isolation of primary HSCs, explanted liver grafts or partially resected liver segments were perfused with HBSS to wash out remaining blood and HBSS with 440-450 U/ml collagenase to digest the connective tissues. The obtained cell suspension was filtered through a 4 µm mesh and washed 3 times in HBSS. The supernatant was collected and subjected to density gradient centrifugation, prepared as 12.5% iodixanol solution (Optiprep, Axis-Shield, Wädenswil, Austria) overlayed with a 9% iodixanol solution, overlayed with GBSS (Sigma, Steinheim, Germany). The upper layer between GBSS and 9% density was carefully transferred into MACS buffer and washed twice. The cell suspension was incubated with CD133 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and $CD133^+$ HSCs were separated by MACS. HSCs were cultured in DMEM (with 20% fetal calf serum [FCS], 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) at a density of approximately 1 mio. cells/1 cm^2 and kept for 2 weeks before the start of the experiments to reach a status of activation. Pre-treatment of HSCs was done with 0.25 mM free fatty acids (FFA) (2:1 oleic acid: palmitoleic acid in phosphate buffered saline [PBS] with 1% fat free bovine serum albumin [BSA], Sigma) for 24 hours (h). Cells were subsequently treated with VD_2 10^{-6} M (Biomol, Hamburg, Germany) or vehicle (ethanol) in fresh medium (without FFA) for another 24 h.

Cell Culture

The human immortalized HSC line LX-2 was cultured at 37° C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with a high glucose concentration (4.5 g/L) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% glutamine, and 1% heat-inactivated fetal bovine serum (FBS). Isolated primary human HSCs were grown in DMEM medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% glutamine, and 10% FBS.

For experimental studies, cells were seeded in 6-well plates at a cell count of $1x10^5$ cells/well in DMEM medium with 1% FBS overnight. On the following day, the medium was removed and replaced with medium containing either 0.25 mM, 0.5 mM, or 1 mM FFA, or vehicle. After 24 h incubation with FFA or vehicle, fresh medium was added,

containing either VD_2 (10^{-6} M, Biomol, Hamburg, Germany) or vehicle (ethanol) for 24 h. After the incubation, cells were washed with DPBS and harvested in RLT-Buffer (Qiagen, Hilden, Germany) for RNA isolation.

RNA Isolation and Reverse Transcription

Total RNA was isolated from cell lines using RNeasy Mini Kit (Qiagen). RNA concentrations and purities were determined photometrically (Biophotometer, Eppendorf, Hamburg, Germany) at 260 nm and 280 nm. RNA samples were adjusted to final concentrations of 0.125 µg/µl by adding RNAse-free water and kept at -80° C until further use. The QuantiTect Reverse Transcription Kit (Qiagen) was applied according to the manufacturer's instructions with 2 minute (min) pre-incubation at 42° C, reverse transcription for 20 min at 42° C and enzyme inactivation at 95° C for 3 min. The resulting cDNAs were dissolved in 30 µl *Aqua Bidest* and kept at -20° C until further use, as described previously by Bechmann et al. (17).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The mRNA levels of MICA/B, transforming growth factor (TGF)-ß, SMA, and collagen (COL)1α genes were assessed by qRT-PCR using hypoxanthine-phosphoribosyltransferase 1 primers as a housekeeping gene. The oligonucleotides utilized for PCR are given in Table 1. qRT-PCR was performed using the iCycler IQ thermocycler (BioRad, Munich, Germany). A single reaction consisted of 15 µl Quanti Tect SYBR Green master mix (Qiagen), 2 µl cDNA, 1 µl forward primer, 1 µl reverse primer (at 10 pmol/µl each), and 11 µl *Aqua Dest* per well of 96-well qRT-PCR reaction plates (Nerbe Plus, Winsen/Luhe, Germany). Amplification was performed for 15 min at 95° C, followed by 40 cycles of 30 seconds (s) at 95° C, 30 s at 55° C, and 30 s at 72° C. Melting-curve data were collected from 95° C to 55° C, at -0.5° C steps for 10 s each. qRT-PCR data were recorded and analyzed with iCycler iQ Optical System Software 3.1a and subsequently analyzed with the Gene Expression Analysis for iCycler iQ v1.10 Excel Macros (both by BioRad).

Nile Red Staining for FFA Uptake

Cultured LX-2 cells were fixed with a cold methanol-acetone-dilution for 10 min and washed 3 times with PBS. Fixed cells were incubated in PBS with 20 µg/ml Nile red for 1 h. Counterstaining was performed with DAPI. Lipid drops were detected and photographed on an Axiovert 40 LSM microscope (Zeiss, Jena, Germany).

Statistical Analysis

Data shown are means ± SEM, if not stated otherwise, and were evaluated using MS Excel 2003 (Microsoft) and GraphPad-Prism (GraphPad Software, La Jolla, CA, USA). Differences between experimental conditions were detected using the unpaired Student (two-tailed) *t*-test and subsequently controlled with the F-test for excluding non-uniform variances. Statistical significance was assumed at $p<0.05$.

RESULTS

Does FFA or VD_2 Treatment Alter LX-2 Cell Morphology?

To determine FFA uptake in LX-2 cells, Nile red staining was used. As shown in Figure 1, uptake of FFAs by cells after treatment with 0.5 mM FFAs for 24 h was evident. After incubation with 0.5 mM FFA, lipid droplet formation was observed in the cytoplasm of LX-2 cells, while treatment with 1 mM FFA resulted in more pronounced lipid droplet formation within the cells. Additionally, LX-2 cell morphology was altered and the cell count was diminished. LX-2 cells were treated with

Table 1. Oligonucleotides utilized as primers for quantitative real time-polymerase chain reaction

Gene		Sequence
Hypoxanthine-phosphoribosyltransferase 1	forward	5'-GAC-CAG-TCA-ACA-GGG-GAC-AT-3'
	reverse	5'-CTT-GCG-ACC-TTG-ACC-ATC-TT-3'
MICA	forward	5'-GTA-TTG-GGA-CCG-GAA-CAC-AC-3'
	reverse	5'-ATG-CTC-TGG-AGG-GTG-TGAGA-3'
MICB	forward	5'-TGC-CAT-GAA-GAC-CAAGAC-AC-3'
	reverse	5'-GGG-GCA-CTG-TTCTCC-TGA-T-3'
Collagen 1α	forward	5'-AAC-AGC-CGC-TTC-ACC-TAC-AG-3'
	reverse	5'-GGA-GGT-CTT-GGT-GGT-TTG-GT-3'
α-Smooth muscle actin	forward	5'-TTC-GTT-ACT-ACT-GCT-GAG-CGT-GAG-A-3'
	reverse	5'-AAG-GAT-GGC-TGG-AAC-AGG-GTC-3'

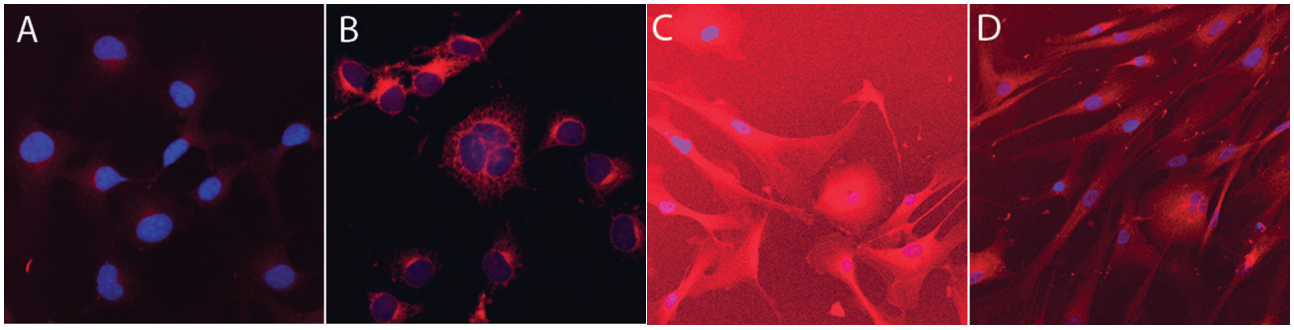

Figure 1. Fatty acid uptake by LX-2 cells and hepatic stellate cells. LX-2 cells (A, B) were incubated in the absence (control; A) or presence (B) of 0.5 mM of free fatty acids (FFA) for 24 h. Nile red staining was conducted to visualize uptake of FFA using fluorescent microscopy. In cells with vehicle, no lipid drops were detected in the cytoplasm of LX-2 cells, but in FFA-incubated cells, strong accumulation of lipid droplets was observed. Primary human hepatic stellate cells were incubated without FFA (C) or treated with 0.25 mM FFAs (D). No generation of lipid droplets or accumulation of fat in the cells was detectable.

VD_2 in the absence or presence of different FFA concentrations for 24 h. Morphological changes were not observed in LX-2 cells when treated with VD_2 alone. After VD_2 treatment, LX-2 cells pre-incubated with FFAs exhibited equal morphological changes as cells with FFA alone (Figure 2).

Does FFA Uptake Induce Stress Ligand Upregulation in LX-2 cells?

MICA/B mRNA expression was measured by quantitative RT-PCR. Treatment of LX-2 cells with 1 mM FFAs resulted in a significant upregulation of MICA after 24 h ($p<0.005$; Figure 3A), while MICB mRNA expression was not altered (Figure 3B). However, treatment with 0.5 mM FFA did not alter MICA or MICB mRNA expression. Addition of VD_2 to 1 mM FFA pre-incubated cells significantly reduced MICA mRNA expression as compared to FFA-stimulated cells ($p<0.005$). However, VD_2 did not induce changes in MICB mRNA expression in 1 mM FFA pre-stimulated cells versus FFA-treated cells.

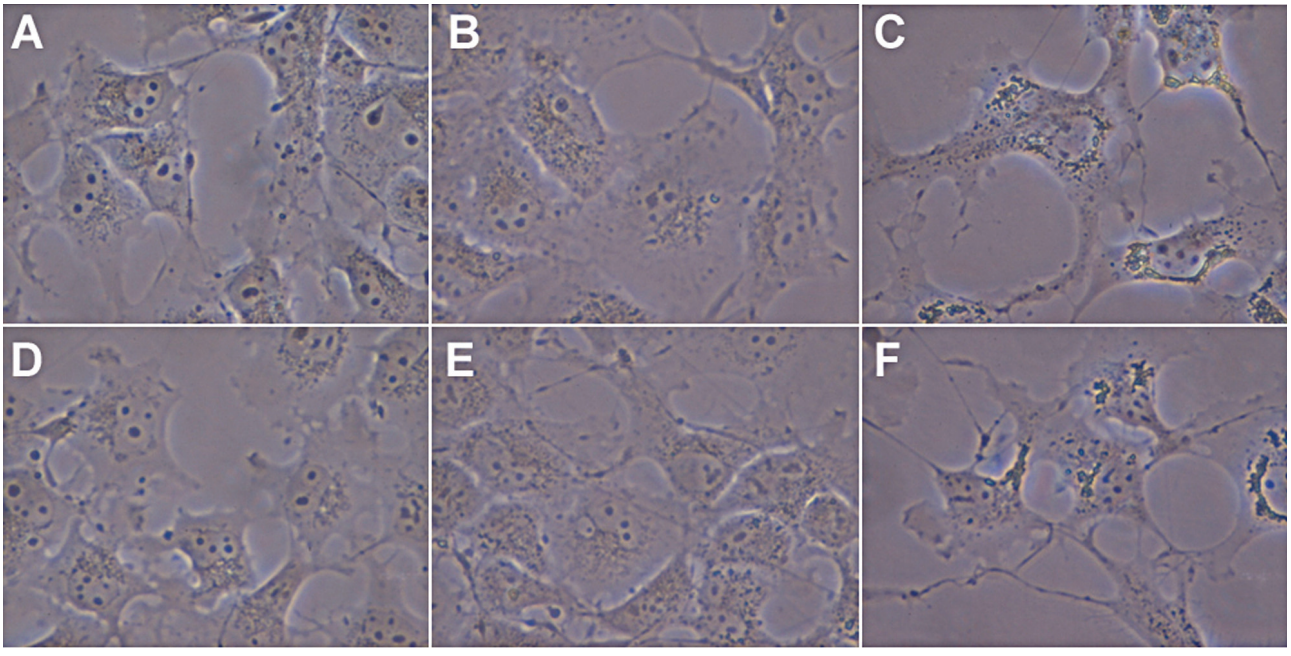

Figure 2. Cell morphology alteration in LX-2 cells by free fatty acid (FFA) treatment is not changed with vitamin D (VD_2) supplementation. LX-2 cells were treated with VD_2 (D, E, F) in the absence or presence of 0.5 mM (B, E) or 1 mM (C, F) FFAs for 24 h. After fixation, cells were imaged by light microscopy. Treatment with VD_2 alone (D), 0.5 mM FFA (B) or 0.5mM FFA and VD_2 (E) did not change cell morphology compared to untreated controls (A). Treatment with 1 mM FFA (C) led to the disruption of some cells and to the formation of lipid droplets in the cells, which was independent of VD_2 addition (F).

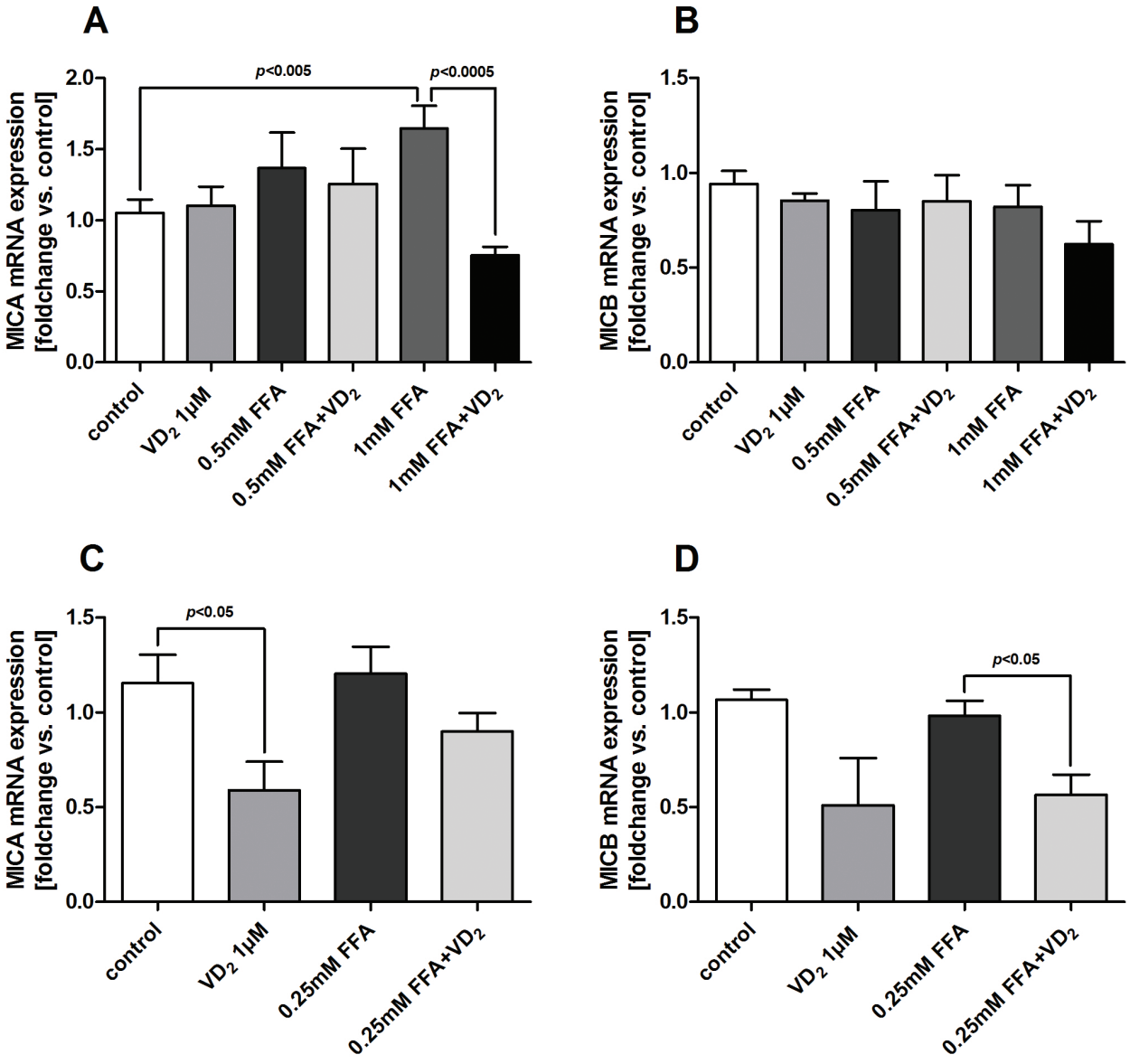

Figure 3. mRNA expression of stress-induced ligands MICA/B in LX-2 and primary hepatic stellate cells. MICA expression was increased in LX-2 cells by 1 mM free fatty acid (FFA) treatment, which was ameliorated by addition of vitamin D (VD_2) (A). In contrast, expression of MICB was not affected by FFA or VD_2 supplementation (B). Primary human hepatic stellate cells exhibited a downregulation of MICA (C) and MICB (D) mRNA with VD_2 treatment, independent of prestimulation with 0.25 mM FFA, which did not affect stress ligand expression as compared to untreated or vehicle controls. Data shown are means of 12 independent experiments ± SEM.

Does VD_2 Influence Stress Ligand Expression in Isolated Primary HSCs?

Primary HSCs were incubated for 24 h with VD_2 alone, 0.25 mM FFAs or 0.25 mM FFA pre-incubation and VD_2. As displayed in Figures 3C and 3D, VD2 significantly downregulated the mRNA expression of MICA and MICB versus controls ($p<0.05$). A similar downregulation was also observed for MICB mRNA by 0.25 mM FFAs as compared to the control group ($p<0.05$).

Does VD_2 Ameliorate Pro-Fibrogenic Genes in Activated LX-2 Cells Under Stress Conditions?

Incubation with 0.5 mM FFA or 1 mM FFA resulted in a significant upregulation of α-SMA and TGF-β mRNA expression, which was abrogated by addition of VD_2 (Figure 4). Surprisingly, expression of COL1α was reduced not only by VD treatment, but also by FFA incubation of LX-2 cells. No additional effect of VD_2 and FFA was detectable.

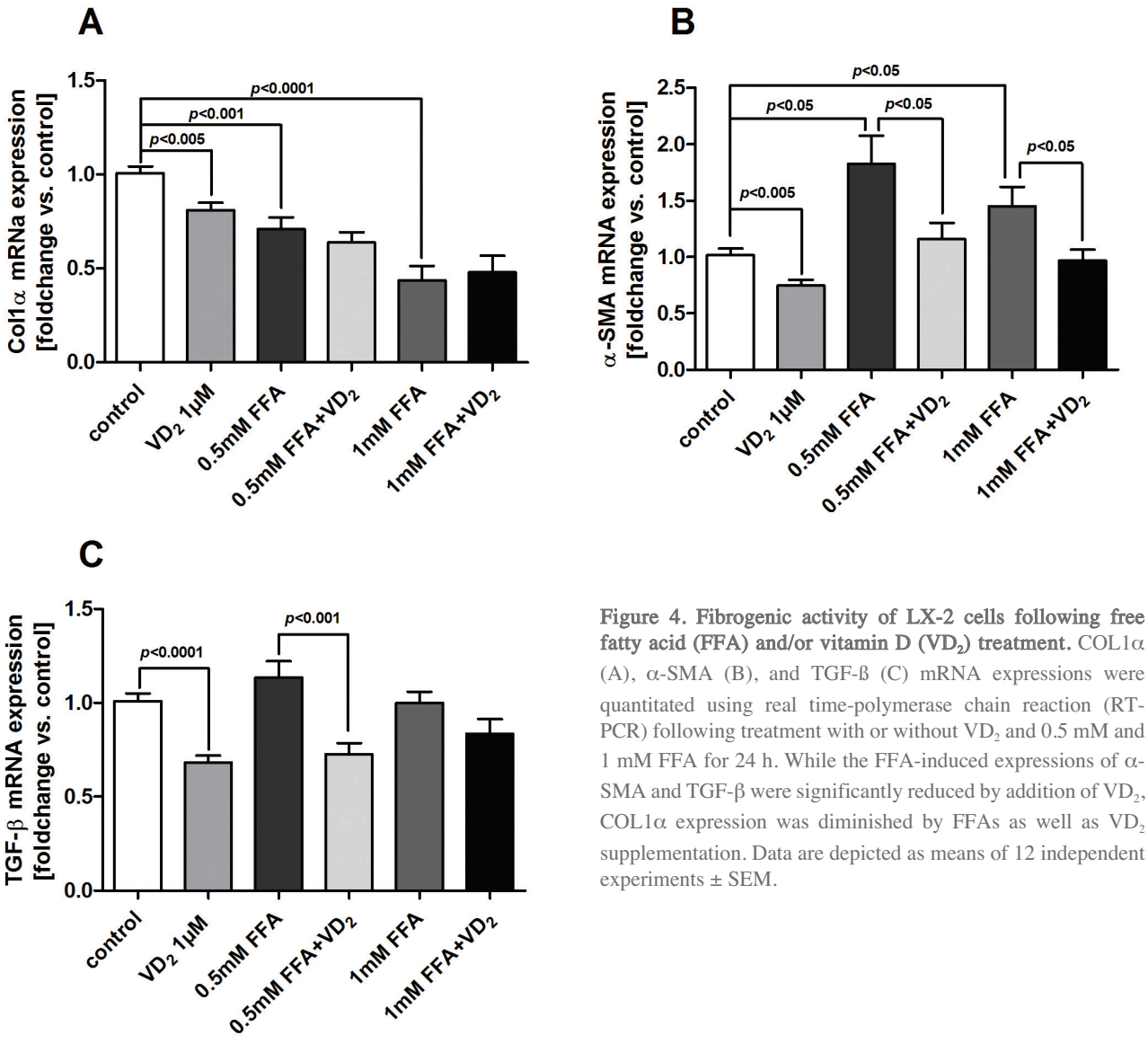

Figure 4. Fibrogenic activity of LX-2 cells following free fatty acid (FFA) and/or vitamin D (VD_2) treatment. COL1α (A), α-SMA (B), and TGF-ß (C) mRNA expressions were quantitated using real time-polymerase chain reaction (RT-PCR) following treatment with or without VD_2 and 0.5 mM and 1 mM FFA for 24 h. While the FFA-induced expressions of α-SMA and TGF-β were significantly reduced by addition of VD_2, COL1α expression was diminished by FFAs as well as VD_2 supplementation. Data are depicted as means of 12 independent experiments ± SEM.

DISCUSSION

Activation of hepatic HSCs is an important step in the development of liver fibrosis. In response to liver injury, these cells lose their intracellular vitamin A stores, express α-SMA, and produce large amounts of extracellular matrix proteins, such as collagen, resulting in liver fibrosis. In addition, development of fibrosis is characterized by a diminution of matrix degrading metalloproteinases (MMPs) activity and enhanced activity of TIMPs, which are also produced by activated HSCs (2,18). Understanding the mechanisms linking chronic liver injury to fibrogenesis is essential to develop therapies countering this effect (1).

Vitamin D (VD) deficiency has been linked to many different diseases, including cancer, chronic liver diseases, diseases mediated by the immune system, IR, and also diabetes. Moreover, VD deficiency in patients with chronic liver disease can lead to the progression of hepatic fibrosis (5-7). Some studies have found low serum 25 (OH) VD levels in patients with chronic hepatitis and cirrhosis of different origins. A recent study by Targher et al. (19) demonstrated a decrease of serum 25 (OH) VD concentrations in non-alcoholic fatty liver disease (NAFLD) patients, which was associated with the histopathological features of NAFLD. However, several experimental studies reported that VD, via interaction with the VD receptor, protects against oxidative stress, and can influence migration, proliferation and gene expression of fibroblasts and reduce the inflammatory and fibrogenic activity of HSCs (5,20,21).

Natural killer (NK) cells have an anti-fibrotic activity through killing of activated HSCs and may play an important role in the resolution of liver fibrosis in humans. NK cell activity has been demonstrated to be decreased in patients with chronic liver diseases as well as HCC. NKG2D ligands (MICA/B) are known to be sensors of cellular stress. These ligands are rarely expressed on normal cells but frequently on tumor cells, including HCC, colon cancer, and prostate and brain tumors. It has been shown recently that soluble MICA/B levels were significantly increased in the sera of patients with chronic liver disease and HCC. Indeed, Jinushi et al. (16) found that MICA/B were expressed in a subset of human HCC tissues as well as hepatoma cell lines, but not in the surrounding non-tumor tissue. They also demonstrated that these stress ligands play an important role in NK cytolysis against hepatoma cells. Furthermore, they could demonstrate that retinoic acid upregulated the expression of MICA/B in Huh7 and HepG2 cells.

We previously investigated the role of MICA/B on liver injury, apoptosis and fibrosis in patients with NASH undergoing bariatric surgery for obesity. We found that hepatic NK cell number was elevated and NKG2D as well as MICA/B transcripts were increased in NASH. Moreover, we could identify a significant positive correlation between MICA/B proteins and markers of disease severity in patients with NASH. Therefore, measurement of MICA/B serum levels may be utilized as a novel marker to assess liver injury in NASH in a non-invasive fashion. Importantly, our data indicated that MICA/B are expressed on hepatocytes in patients with NASH and their expression may play an important role in their susceptibility to NK cells during hepatic inflammation as mediated by fatty infiltration of the liver (22).

However, the role of stress ligands in HSCs is yet unknown. Therefore, in the present study, using LX-2 cells and primary human HSCs, we investigated the effects of FFAs and VD_2 treatment on MICA/B as well as pro-fibrogenic genes. Our results demonstrated that (1) FFAs activated stellate cells; (2) VD_2 downregulates the expression of MICA/B genes; and (3) VD_2 reduces the expression of TGF-ß and α-SMA as markers of hepatic fibrogenesis. Interestingly, COL1α expression was not only reduced by VD_2, but also by FFA treatment.

Free fatty acids (FFAs) promote the process of liver fibrogenesis as they induce hepatocyte apoptosis (23,24). We previously examined the combined effects of FFAs and resveratrol on HSCs, as it was proposed that resveratrol may have a protective effect in fatty acid-induced liver injury. In contrast, we found that resveratrol amplified the profibrogenic effects of FFA on LX-2 cells (25).

Taken together, our results demonstrate that FFAs activate LX-2 cells *in vitro* to produce profibrogenic factors. VD_2 ameliorates this effect and also reduces MICA/B expression in primary HSCs, probably due to a protective effect of VD_2 on NK cell killing of HSCs. In a setting of fibrogenic activity, this may not be a reasonable therapeutic option. Therefore, more detailed studies investigating the effects of FFA and VD_2 treatment on the interaction of HSCs and NK cells could help to clarify the contribution of HSCs in NASH-associated fibrogenesis.

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