Pro-inflammatory signaling by 24,25-dihydroxyvitamin D_3 in HepG2 cells

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Abstract

The vitamin D metabolite 24,25-dihydroxyvitamin D_3 (24, 25$[OH]_2D_3$) was shown to induce nongenomic signaling pathways in resting zone chondrocytes and other cells involved in bone remodeling. Recently, our laboratory demonstrated that 24,25-$[OH]_2D_3$ but not 25-hydroxyvitamin D_3, suppresses apolipoprotein A-I (apo A-I) gene expression and high-density lipoprotein (HDL) secretion in hepatocytes. Since 24,25-$[OH]_2D_3$ has low affinity for the vitamin D receptor (VDR) and little is known with regard to how 24,25-$[OH]_2D_3$ modulates nongenomic signaling in hepatocytes, we investigated the capacity of 24,25-$[OH]_2D_3$ to activate various signaling pathways relevant to apo A-I synthesis in HepG2 cells. Treatment with 24,25-$[OH]_2D_3$ resulted in decreased peroxisome proliferator-activated receptor alpha (PPARα) expression and retinoid-X-receptor alpha (RXRα) expression. Similarly, treatment of hepatocytes with 50 nM 24,25-$[OH]_2D_3$ for 1–3 h induced PKCα activation as well as c-*jun*-N-terminal kinase 1 (JNK1) activity and extracellular-regulated kinase 1/2 (ERK1/2) activity. These changes in kinase activity correlated with changes in c-*jun* phosphorylation, an increase in AP-1-dependent transcriptional activity, as well as repression of apo A-I promoter activity. Furthermore, treatment with 24,25-$[OH]_2D_3$ increased IL-1β, IL-6, and IL-8 expression by HepG2 cells. These observations suggest that 24,25-$[OH]_2D_3$ elicits several novel rapid nongenomic-mediated pro-inflammatory protein kinases targeting AP1 activity, increasing pro-inflammatory cytokine expression, potentially impacting lipid metabolism and hepatic function.

Key Words
- activator protein 1
- apolipoprotein A-I
- peroxisome proliferator-activated receptor α
- cardiovascular disease
- vitamin D

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Introduction

The vitamin D metabolite 24,25-$[OH]_2D_3$ is generated from 25-hydroxyvitamin D_3 (25-OHD_3) and 1,25-dihydroxyvitamin D_3 (1,25-$[OH]_2D_3$) by the activity of the cytochrome P450 24-hydroxylase (24A2) that is expressed in many tissues, including the liver (Chen *et al.* 1993). Once produced in peripheral tissues, 24,25-$[OH]_2D_3$ circulates in plasma and is taken up by the liver where it accumulates until it subjected to further hydroxylation and excretion in the bile and the feces. Though it was once thought to be inactive, studies during the past 20 years have shown that 24,25-$[OH]_2D_3$ has biological activity, stimulating the resting zone chondrocytes and promoting

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the maturation of cartilage to bone (Dickson & Maher 1985, Wientroub *et al.* 1987, Miyahara *et al.* 1994). Recently, our laboratory demonstrated that 24,25-$[OH]_2D_3$ is a potent inhibitor of apo A-I synthesis and HDL secretion by hepatocytes (Wehmeier *et al.* 2011). This suggests that this compound may have other unanticipated biologic effects that remain to be elucidated.

Many of the cellular effects of vitamin D are mediated by the vitamin D receptor (VDR), a member of the steroid hormone superfamily of nuclear receptors. After ligand binding, VDR and its partner retinoid x receptor (RXR) mediate the transcriptional effects of the hormone on target genes (Kimmel-Jehan *et al.* 1997). However, recent studies have shown that vitamin D exhibits nongenomic signaling activity including activation of ion channels and activation of various mitogen-activated protein (MAP) kinases and pp60c-*src* (Chappel *et al.* 1997, Gniadecki 1998, Zhang *et al.* 2007, Buitrago *et al.* 2013). Unlike 1,25-$[OH]_2D_3$, which has high affinity for the VDR (Makin *et al.* 1989), 24,25-$[OH]_2D_3$ binds the VDR poorly if at all (Uchida *et al.* 1997) but in resting zone chondrocytes and other cells, stimulates various intracellular protein kinases including pp60c-*src*, PKCα, and MAP kinase, and phospholipase A2 activity (van Leeuwen *et al.* 2001). Since several of these proteins are involved in signaling pathways shown to mediate TNF α-related suppression of apo A-I expression (Haas *et al.* 2003, Beers *et al.* 2006, Parseghian *et al.* 2014) and since 24,25-$[OH]_2D_3$ is a known and potent inhibitor of apo A-I synthesis (Wehmeier *et al.* 2011), we investigated whether some of these pathways are modulated by 24,25-$[OH]_2D_3$ in hepatocytes. The amount of 24,25-$[OH]_2D_3$ required for repressing apo A-I gene expression (Wehmeier *et al.* 2011) was significantly lower than media concentrations required for the activation of various PPARs or the bile acid receptor farnesoid-X receptor (FXR) (Vu-Dac *et al.* 1998, Sakamoto *et al.* 2000, Claudel *et al.* 2002). However, it is possible that 24,25-$[OH]_2D_3$ down-regulates the expression of various nuclear receptors that regulate lipid and cholesterol metabolism. Therefore, we investigated the effect of 24,25-$[OH]_2D_3$ on kinase signaling, nuclear receptor expression, and proinflammatory cytokine expression in HepG2 cells.

Materials and methods

Materials

Phorbol-12-myristate-13-acetate (PMA), gemfibrozil, and the vitamin D metabolite 24,25-$[OH]_2D_3$ was purchased from Sigma Chemical Company. Immobilon-P for Western blotting was purchased from Millipore and enhanced chemiluminescence materials were purchased from Pierce Biotechnology. Thin layer chromatography plates and antibodies to PKCα and phosphorylated PKCα were obtained from Millipore. Antibodies to phosphorylated and unphosphorylated forms of P38 MAP kinase, JNK, ERK1/2, and c-*jun* were purchased from Cell Signaling, Inc., as was an antibody to β-actin. Antibodies specific for human apo A-I and albumin were purchased from Thermo Fisher Scientific. Control and c-*jun*-specific siRNA and antibodies to PPARα and RXRα were purchased from Santa Cruz Biotechnology. Secondary antibodies were purchased from Southern Biotech (Birmingham, AL, USA). ^{14}C-chloramphenicol was purchased from Perkin-Elmer and reagents for luciferase assays were purchased from Promega. Enzyme-linked immunosorbent assay (ELISA) kits (DuoSets) for measuring interleukin 1β (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor α (TNF α) were purchased from R&D Systems. Lipopolysaccharide (*E. coli* O127:B8) (LPS) was purchased from Thermo Fisher Scientific. All other reagents were from Sigma Chemical Company or Thermo Fischer Scientific.

Western blotting

HepG2 cells were treated as described in each figure and protein samples were prepared by lysing the cells in buffer containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-Cl) (pH 7.4), 1% sodium dodecylsulfate (SDS), and 1 mM ethylenediaminetetraacetic acid (EDTA). For measurement of apo A-I and albumin levels, conditioned medium was obtained from the cells after 24-h of treatment. Protein content was measured using the bicinchoninic acid assay with a kit from Pierce Biotechnology. Fifty microgram of protein was fractionated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to nitrocellulose as described (Towbin *et al.* 1979). The nitrocellulose was blocked by shaking in 5% nonfat dry milk in phosphate-buffered saline (PBS) (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 for 2 h at room temperature, before addition of the primary antibody (diluted in 1% nonfat dry milk in PBS) diluted according to the manufacturer's recommendations, and incubated overnight at 4°C. After 4–5 min washes in PBS-T, the membranes were incubated with either a goat-anti-mouse or a goat-anti-rabbit secondary antibody (diluted 1:5000 in PBS). After 4–5 min washes, the binding was visualized by enhanced

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chemiluminescence and autoradiography. The signal for each band was quantified using NIH Image J software.

Transient transfection

Apo A-I promoter activity was measured in HepG2 cells transfected with pAI.474.CAT, which contains the apo A-I gene promoter fused to the chloramphenicol acetyltransferase (CAT) gene (Gorman *et al.* 1982). Collagenase promoter activity was measured in HepG2 cells transfected with phu.collase-73/+63-CAT, which contains the AP-1-responsive collagenase promoter fused to CAT (Mitchell & Cheung 1991), while the plasmid pJ6 TK pG13, containing six copies of a PPARα response element driving luciferase expression, was obtained from Dr. Bart Staels (Institut Pasteur de Lille, Lille, France). The plasmid pCMV.SPORT.β-gal, which contains the β-galactosidase gene fused to the cytomegalovirus promoter, was used to normalize transfection efficiency (Herbomel *et al.* 1984). Cells were transfected with 1 µg of each plasmid using Lipofectamine and 24 h later treated as indicated in each figure for 24 h.

Inhibition of c-*jun* expression via siRNA

HepG2 cells were transfected with either control or c-*jun*-specific inhibitory RNA (siRNA) using lipofectamine in the presence of various plasmids as described below. Previous studies in our laboratory have shown that this methodology knocks down c-*jun* expression over 80% (Parseghian *et al.* 2014).

Proinflammatory cytokine measurement by ELISA

IL-1β, IL-2, IL-6, IL-8, and TNF α levels were measured in 100 µL of conditioned medium from cells treated with solvent, 50 nM 24,25-$[OH]_2D_3$, 50 nM PMA, or 10 ng/mL LPS for 24-h. The immunoassays were performed essentially as described by the manufacturer's general ELISA protocol though two changes were incorporated. First, the samples and standards were allowed to bind the capture antibody by incubating the plates at 4°C overnight. Second, detection antibody binding was also at 4°C overnight. Optical densities at 450 nm (signal) and 570 nm (background) were measured with an ELx800 microplate spectrophotometer (BioTek Instruments) and exported to Microsoft Excel. Cytokine levels were calculated from the linear region of the standard curves using Microsoft Excel.

Statistics

Measurements are reported as the mean ± S.D. Analysis of variance (ANOVA) and the Students *t*-test for independent variables were performed with Statistica for Windows (Statsoft Inc, Tulsa, OK, USA). Statistical significance was defined as a two-tailed $P<0.05$.

Results

The effect of 24,25-$[OH]_2D_3$ on PPARα and RXRα expression

Previous studies suggested that 10–100 nM 24,25-$[OH]_2D_3$ were sufficient to maximally suppress hepatic apo A-I gene expression (Wehmeier *et al.* 2011). Therefore, in the following experiments, we chose to use 50 nM 24,25-$[OH]_2D_3$. To assess whether or not 24,25-$[OH]_2D_3$ affects expression of PPARα and RXRα, HepG2 cells were treated with 50 nM 24,25-$[OH]_2D_3$ for 0, 1, 2, 6, and 24 h. PPARα and RXRα were measured by Western blot (Fig. 1A) and normalized to β-actin levels (Fig. 1B and C). Treatment with 24,25-$[OH]_2D_3$ suppressed both PPARα and RXRα expression in a time-dependent fashion, though PPARα levels were much lower than RXRα levels by 24 h. In contrast, β-actin levels did not change in 24,25-$[OH]_2D_3$-treated cells. Since PPARα and RXRα regulate apo A-I gene expression (Zhang *et al.* 1992, Vu-Dac *et al.* 1998, Sakamoto *et al.* 2000), these results suggest that 24,25-$[OH]_2D_3$ may suppress apo A-I gene expression by inhibiting expression of these nuclear receptors.

The effect of 24,25-$[OH]_2D_3$ on PPARα activity

HepG2 cells were transfected with the plasmids pJ6 TK pG13, containing six consensus PPARα binding sites, and pCMV.SPORT.β-gal and treated with the PPARα ligand gemfibrozil (50 µM) or 50 nM 24,25-$[OH]_2D_3$, or both gemfibrozil and 24,25-$[OH]_2D_3$ for 24 h and luciferase activity was measured and normalized to β-galactosidase activity (Fig. 2). Treatment with gemfibrozil increased PPARα-dependent luciferase activity 2.6-fold ($P<0.0008$), while treatment with 24,25-$[OH]_2D_3$ suppressed PPARα-dependent luciferase activity 79.0% ($P<0.0007$). Furthermore, treatment with gemfibrozil was unable to reverse the effects of 24,25-$[OH]_2D_3$ on luciferase activity; luciferase activity decreased 76.9% relative to control cells ($P<0.0001$) in cells treated with gemfibrozil and 24,25-$[OH]_2D_3$. These results suggest that treatment with 24,25-$[OH]_2D_3$ may suppress PPARα-dependent gene expression in hepatocytes.

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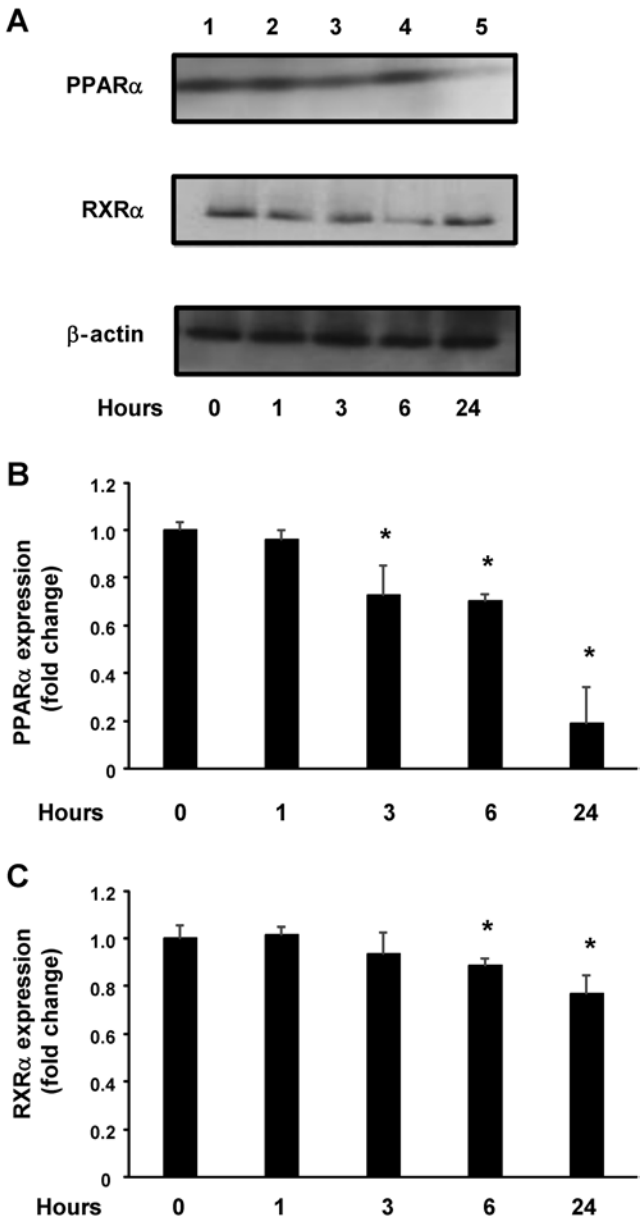

Figure 1
The effect of 24,25-$[OH]_2D_3$ on PPARα and RXRα expression. HepG2 cells were treated with 24,25-$[OH]_2D_3$ for 0, 1, 3, 6, and 24 h, and PPARα, RXRα, and β-actin expression were measured by Western blot (A) and normalized to β-actin levels (B, C). Treatment with 24,25-$[OH]_2D_3$ suppressed both PPARα and RXRα expression. (B) $N=6$; $^*P<0.008$, $P<0.0003$, and $P<0.0001$, respectively, relative to time-zero. (C) $N=6$; $^*P<0.03$ and $P<0.009$, respectively, relative to time-zero.

The effect of 24,25-$[OH]_2D_3$ on PKCα, JNK1, ERK1/2, and c-*jun* expression and phosphorylation

HepG2 cells were treated with 50 nM 24,25-$[OH]_2D_3$ for 0, 1, 3, 6, and 24 h and protein extracts were prepared as described above. PKCα activity, as assessed by phosphorylation on serine 3 (Fig. 3A and B), increased

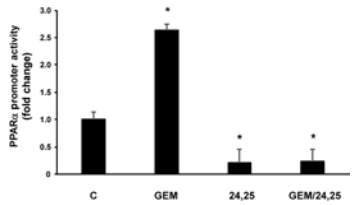

Figure 2
The effect of 24,25-$[OH]_2D_3$ on PPARα activity. HepG2 cells were transfected with the plasmids J6 TK pG13 and pCMV.SPORT.β-gal and treated with solvent, 50 μM gemfibrozil, 50 nM 24,25-$[OH]_2D_3$, or both gemfibrozil and 24,25-$[OH]_2D_3$ for 24 h and luciferase activity was measured and normalized to β-galactosidase activity. Treatment with gemfibrozil induced PPARα-driven luciferase activity while treatment with 24,25-$[OH]_2D_3$ suppressed activity. Luciferase activity in cells treated with both gemfibrozil and 24,25-$[OH]_2D_3$ was similar to luciferase activity in cells treated with 24,25-$[OH]_2D_3$ alone. $N=6$; $^*P<0.0008$, $P<0.0007$, and $P<0.0001$, respectively, relative to control cells.

by 3.5-fold ($P<0.0001$) at 1 h and this was essentially maintained throughout the 24 h. These results suggest that 24,25-$[OH]_2D_3$ induces PKCα-mediated signaling in hepatocytes. JNK1 phosphorylation (C and D) increased 2.1-fold at 3 h ($P<0.0004$ relative to phospho-JNK1 levels at time-zero) but decreased to control levels by 24 h. ERK1/2 phosphorylation (E and F) increased 1.8-fold at 3 h ($P<0.0006$ relative to phospho-ERK1/2 levels at time-zero) and decreased slightly from peak levels at 24 h. Phosphorylation of c-*jun* (G and H) reached a maximum at 1 h (2.9-fold) ($P<0.0005$ relative to phospho-c-*jun* levels at time-zero) and was sustained at 24 h. Total PKCα, JNK1, ERK1/2, and c-*jun* levels did not change with 24,25-$[OH]_2D_3$ treatment. These results suggest that 24,25-$[OH]_2D_3$ increases c-*jun* phosphorylation, correlating with changes in PKCα, JNK1, and ERK1/2 phosphorylation.

The effect of 24,25-$[OH]_2D_3$ on AP1 activity

Since the c-*jun* component of the transcription factor AP1 is a substrate for PKCα and the MAP kinases ERK1/2 and JNK1, and 24,25-$[OH]_2D_3$ induced PKCα, JNK1, and ERK1/2 activity, we investigated whether or not 24,25-$[OH]_2D_3$ induces AP1-dependent transcriptional activity. HepG2 cells were transfected with a plasmid containing the AP1-dependent collagenase promoter and treated with either 100 nM PMA or 50 nM 24,25-$[OH]_2D_3$ for 24-h (Fig. 4A). Treatment with PMA induced collagenase promoter activity 2.7-fold ($P<0.001$). Likewise, treatment with 24,25-$[OH]_2D_3$ induced collagenase promoter activity 2.2-fold ($P<0.008$). These results suggest that 24,25-$[OH]_2D_3$, like PMA, induces AP1 activity in HepG2 cells.

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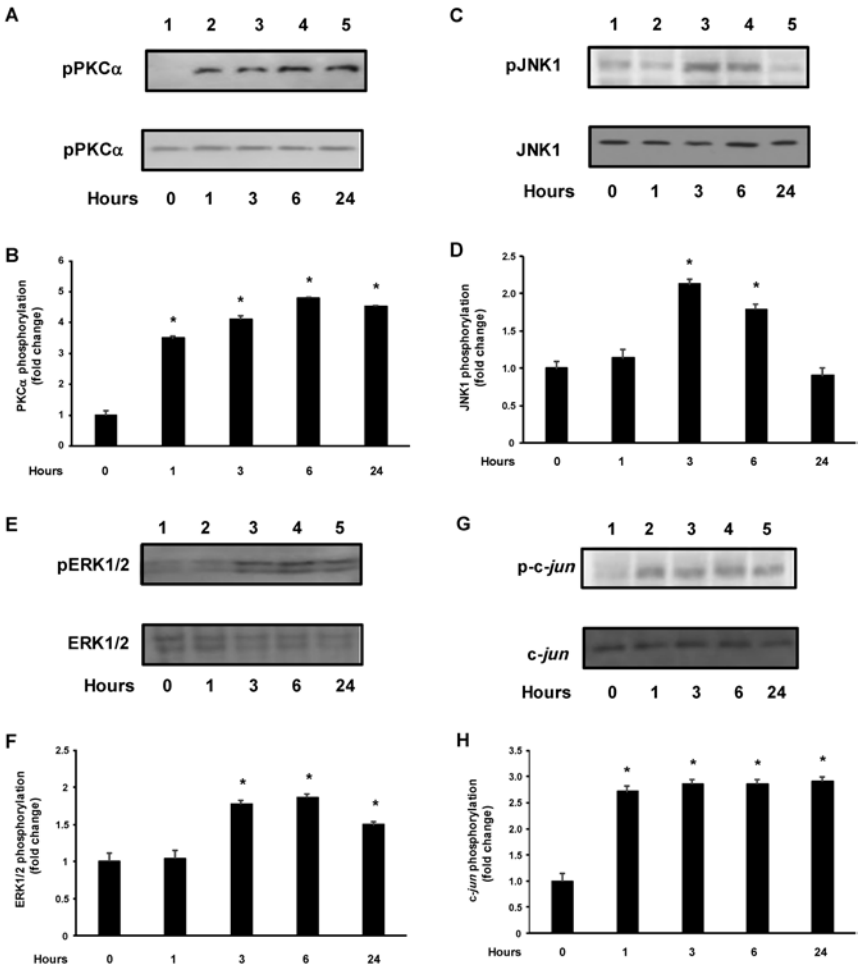

Figure 3
The effect of 24,25-$[OH]_2D_3$ on PKCα, JNK1, ERK1/2, and c-*jun* expression and phosphorylation. HepG2 cells were treated with 24,25-$[OH]_2D_3$ for 0, 1, 3, 6, and 24 h, and PKCα (A and B), JNK1 (C and D), ERK1/2 (E and F), and c-*jun* (G and H) phosphorylation and expression were measured by Western blot. Treatment with 24, 25-$[OH]_2D_3$ resulted in rapid PKCα phosphorylation (1 h) which correlated with changes in c-*jun* phosphorylation (also 1 h). Phosphorylation of both JNK1 and ERK1/2 was elevated only at 3 h post-treatment. (B) $N=6$; $^*P<0.0001$, $P<0.0001$, $P<0.0001$, and $P<0.0001$ respectively, relative to time-zero. (D) $N=6$; $^*P<0.0004$ and $P<0.001$, respectively, relative to time-zero. (F) $N=6$; $^*P<0.0006$, $P<0.0004$, and $P<0.0002$, respectively, relative to time-zero. (H) $N=6$; $^*P<0.0005$, $P<0.0003$, $P<0.0003$, and $P<0.0001$, respectively, relative to time-zero.

We next determined if the c-*jun* component of AP1 was required for PMA and 24,25-$[OH]_2D_3$ to induce collagenase promoter activity. HepG2 cells were transfected with the collagenase reporter plasmid in the presence of a control siRNA or a c-*jun*-specific siRNA. After 48-h, the cells were treated with 100 nM PMA or 50 nM 24,25-$[OH]_2D_3$ for 24 h and CAT activity was measured (Fig. 4B). In the presence of the control siRNA, PMA induced collagenase reporter gene expression 2.4-fold ($P<0.0001$) and 24,25-$[OH]_2D_3$ increased collagenase reporter gene expression 2.1-fold ($P<0.0005$). In contrast, in cells transfected with the c-*jun*-specific siRNA, both PMA and 24,25-$[OH]_2D_3$ induced collagenase reporter gene expression by only 1.2-fold (both NS relative to control cells). Furthermore, collagenase promoter activity was significantly lower in cells transfected with the c-*jun* siRNA and treated with PMA relative to cells transfected with the control siRNA and treated with PMA ($P<0.0001$). Likewise, collagenase promoter activity was significantly lower in cells transfected with the c-*jun*-specific siRNA and treated with 24,25-$[OH]_2D_3$ relative to cells transfected with the control siRNA and treated with 24,25-$[OH]_2D_3$ ($P<0.0008$). These results suggest that 24,25-$[OH]_2D_3$ induces AP1 activity by activating endogenous c-*jun*.

The effect of 24,25-$[OH]_2D_3$ on apo A-I promoter activity

HepG2 cells were transfected with the plasmid pAI.474. CAT and either a c-*jun*-specific siRNA or a control siRNA and treated with either 100 nM PMA or 50 nM 24,25-$[OH]_2D_3$ for 24 h (Fig. 5). In the presence of the control siRNA, treatment of HepG2 cells with PMA decreased apo A-I promoter activity by 41.2% ($P<0.002$), while treatment with 24,25-$[OH]_2D_3$ decreased apo A-I promoter activity by 38.7% ($P<0.002$). However, in cells depleted of c-*jun* via siRNA-mediated knockdown, neither PMA nor 24,25-$[OH]_2D_3$ suppressed apo A-I promoter activity significantly (only 5.9 and 7.7%, respectively, relative to control; both changes were not statistically significant). Furthermore, apo A-I promoter activity was significantly higher in cells transfected with the c-*jun* siRNA and treated with PMA relative to cells transfected with the

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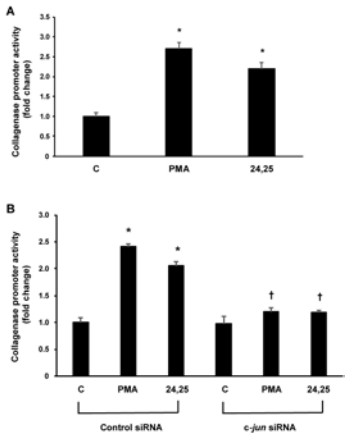

Figure 4
The effect of 24,25-$[OH]_2D_3$ on AP1 activity. HepG2 cells were transfected with the plasmids hu.collase −73/+63.CAT and pCMV.SPORT.β-gal with either the control siRNA or a siRNA-targeting c-*jun*. Forty-eight hours later, the cells were treated with solvent, 100 nM PMA, or 50 nM 24,25-$[OH]_2D_3$, and CAT and β-galactosidase activity were measured 24 h later. In the presence of the control siRNA, treatment with PMA and 24,25-$[OH]_2D_3$ induced reporter gene expression. In contrast, in cells transfected with the c-*jun*-specific siRNA, treatment with PMA and 24,25-$[OH]_2D_3$ had no effect on reporter gene expression. $N=6$; $^*P<0.0001$ and $P<0.0005$, respectively, relative to control cells transfected with the control siRNA. $^\dagger P<0.0001$ and $P<0.0008$, respectively, relative to cells transfected with the control siRNA and treated with PMA and 24,25-$[OH]_2D_3$, respectively.

control siRNA and treated with PMA ($P<0.001$). Likewise, apo A-I promoter activity was significantly higher in cells transfected with the c-*jun*-specific siRNA and treated with 24,25-$[OH]_2D_3$ relative to cells transfected with the control siRNA and treated with 24,25-$[OH]_2D_3$ ($P<0.002$). These results suggest that 24,25-$[OH]_2D_3$ suppresses apo A-I gene expression by inducing AP1 activity.

The effect of 24,25-$[OH]_2D_3$ on proinflammatory cytokine expression in HepG2 cells

HepG2 cells were treated as described in Materials and methods, and IL-1β (A), IL-2 (B), IL-6 (C), IL-8 (D), and TNF α levels were measured by ELISA and apo A-I and albumin levels were measured by Western blot of the conditioned medium (Fig. 6). TNF α levels were below the level of detection in all treatment groups. IL-1β, IL-6, and IL-8 levels were all increased in cells treated with 24,25-$[OH]_2D_3$, but not IL-2. Both PMA and LPS treatment induced IL-1β, Il-2, IL-6, and IL-8 secretion from HepG2 cells. Apo A-I levels, but not albumin levels, were significantly lower in cells treated with 24,25-$[OH]_2D_3$, PMA, and LPS as reported previously (Morishima *et al.* 2003, Wehmeier *et al.* 2011).

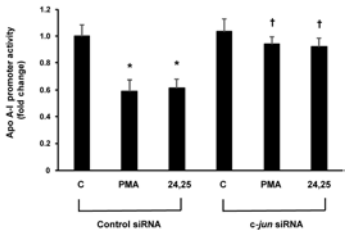

Figure 5
The effect of 24,25-$[OH]_2D_3$ on apo A-I promoter activity. HepG2 cells were transfected with the plasmids pAI.474.CAT and pCMV.SPORT.β-gal with either the control siRNA or a siRNA-targeting c-*jun*. Forty-eight hours later, the cells were treated with solvent, 100 nM PMA, or 50 nM 24,25-$[OH]_2D_3$, and CAT and β-galactosidase activity were measured 24 h later. In the presence of the control siRNA, treatment with PMA and 24,25-$[OH]_2D_3$ suppressed apo A-I promoter activity. In contrast, in the presence of the c-*jun*-specific siRNA, treatment with PMA and 24,25-$[OH]_2D_3$ had no effect on apo A-I promoter activity. $N=6$; $^*P<0.002$ and $P<0.002$, respectively, relative to control cells transfected with the control siRNA. $^\dagger P<0.001$ and $P<0.002$, respectively, relative to cells transfected with the control siRNA and treated with PMA and 24,25-$[OH]_2D_3$, respectively.

These results suggest that 24,25-$[OH]_2D_3$ has pro-inflammatory effects in HepG2 cells.

Discussion

Previous studies in our laboratory have shown that 1,25-$[OH]_2D_3$ inhibits apo A-I gene expression via a VDR-mediated mechanism (Wehmeier *et al.* 2005). In contrast, 24,25-$[OH]_2D_3$-mediated suppression of apo A-I did not require VDR expression since knockdown of VDR expression with siRNA had no effect on the efficacy of 24,25-$[OH]_2D_3$ to inhibit apo A-I gene expression (Wehmeier *et al.* 2011). Therefore, we examined the potential of 24,25-$[OH]_2D_3$ to induce various signaling pathways that are independent of VDR and account for its apo A-I suppressive effect.

Since nuclear receptors PPARα and RXRα have been shown to be important in regulating apo A-I gene expression (Beigneux *et al.* 2000, Wang & Wan 2008), we examined the effects of 24,25-$[OH]_2D_3$ on their expression and activity. Treatment with 24,25-$[OH]_2D_3$ suppressed both PPARα and RXRα expression in HepG2 cells, though PPARα levels were affected more so than RXRα. These changes in PPARα and RXRα expression affected the capacity of PPARα to stimulate a PPARα-responsive reporter gene, even in the presence of a potent PPARα ligand gemfibrozil. It is not clear how 24,25-$[OH]_2D_3$ reduces PPARα and RXRα levels, though the rapid kinetics suggests that it occur, protein degradation may be involved (Genini & Catapano 2006, Lefebvre *et al.* 2010). PPARα is phosphorylated by PCKα in PMA-treated

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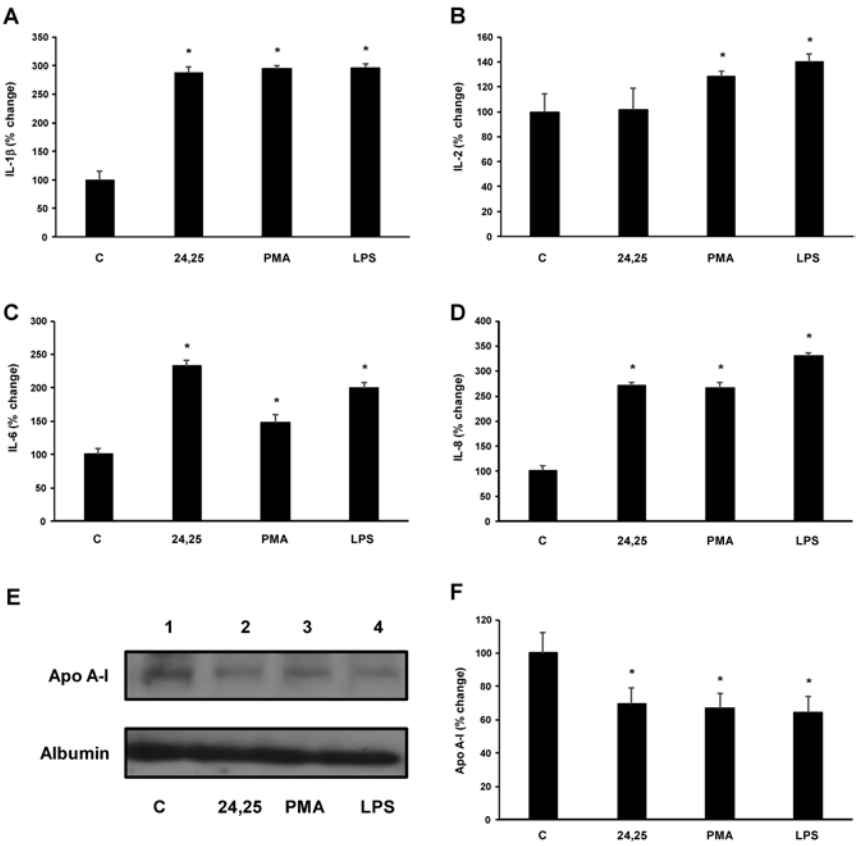

Figure 6
The effect of 24,25-$[OH]_2D_3$ on proinflammatory cytokine expression in HepG2 cells. HepG2 cells were treated with solvent (control cells, C), 50 nM 24,25-$[OH]_2D_3$, 50 nM PMA, or 10 ng/mL LPS for 24 h, and IL-1β (A), IL-2 (B), IL-6 (C), IL-8 (D), TNF α, apo A-I, and albumin levels were measured either by ELISA (IL-1β, IL-2, IL-6, IL-8, and TNF α) or Western blot (apo A-I and albumin) (E, F). TNF α levels were below the level of detection in all treatment groups. While PMA and LPS treatment induced expression of all the other proinflammatory cytokine levels, 24,25-$[OH]_2D_3$ increased only IL-1β, IL-6, and IL-8. (A) $N=3$; $^*P<0.0007$, $P<0.0002$, and $P<0.0002$, relative to control cells. (B) $N=3$; $^*P<0.03$ and $P<0.01$, relative to control cells. (C) $N=3$; $^*P<0.0003$, $P<0.02$, and $P<0.001$, relative to control cells. (D) $N=3$; $^*P<0.0001$, $P<0.0008$, and $P<0.0001$, relative to control cells. (E). Apo A-I and albumin expression in control cells and cells treated with 24,25-$[OH]_2D_3$, PMA, and LPS. (F) Apo A-I levels were quantified and normalized to albumin expression. $N=3$; $^*P<0.02$, $P<0.02$, and $P<0.01$, relative to control cells.

cells (Gray *et al.* 2005), though this post-translational modification resulted in increased transcriptional activity. Likewise, ERK1/2 has been shown to phosphorylate PPARα in insulin-treated cells, also enhancing its transcriptional activity (Shalev *et al.* 1996). In contrast, JNK1 has been shown to suppress PPARα activity in hepatocytes, though this effect was mediated by the corepressors nuclear corepressor 1 (NCoR1) and nuclear receptor interacting protein 1 (NRIP1), without changes in PPARα expression (Vernia *et al.* 2014). Additional studies will be required to determine the mechanism. However, these results suggest that 24,25-$[OH]_2D_3$ may suppress apo A-I gene expression in part by reducing the expression of these two key transcriptional regulators.

It is noteworthy that proinflammatory cytokines including tumor necrosis factor α inhibit apo A-I gene expression by down-regulating the expression of various steroid hormone nuclear receptors including PPARα and RXRα (Beigneux *et al.* 2000, Wang & Wan 2008). This effect is in part due to induction of ERK1/2 and JNK1 branches of the MAP kinase pathway, leading to the activation of c-*jun* and squelching of apo A-I promoter activity (Haas *et al.* 2003, Beers *et al.* 2006, Parseghian *et al.* 2014). Since 24,25-$[OH]_2D_3$ has been shown to stimulate MAP kinase activity in other tissues (van Leeuwen *et al.* 2001), we determined the response and kinetics of PKCα, JNK1, and ERK1/2 activation in HepG2 cells. Treatment with 24,25-$[OH]_2D_3$ rapidly stimulated (within 1 h) PKCα phosphorylation as well as c-*jun* phosphorylation, whereas JNK1 and ERK1/2 required longer (3 h) leading to the rapid activation of AP1 activity by stimulating serine 63 phosphorylation on c-*jun* (Davis 2000). Furthermore, treatment with 24,25-$[OH]_2D_3$ induced the AP1-responsive collagenase reporter gene expression to a similar extent as the phorbol ester PMA. However, this response was inhibited by the addition of a c-*jun* siRNA but not a control siRNA. Likewise, treatment with 24,25-$[OH]_2D_3$ inhibited apo A-I promoter activity as did the phorbol ester PMA, to a similar extent, which was inhibited by the c-*jun*-specific siRNA but not the control siRNA. This data suggests that of the two potential pathways involved in suppressing apo A-I gene expression (nuclear receptor expression or AP1-dependent inhibition), AP1-dependent inhibition is the most likely pathway involved. If both pathways were involved, 24,25-$[OH]_2D_3$ would inhibit apo A-I gene expression to a greater extent than observed with PMA treatment.

Finally, treatment with 24,25-$[OH]_2D_3$ increased IL-1β, IL-6, and IL-8 levels in the conditioned medium, similar to

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cells treated with PMA and LPS. While TNF α levels were undetectable under all treatment conditions, both PMA and LPS treatment, but not 24,25-$[OH]_2D_3$, increased IL-2 levels. Of the cytokines that were induced by 24,25-$[OH]_2D_3$, the most is known about IL-1β inflammasome activity in the liver. Though liver immune cells such as Kupffer cells express most of the IL-1β released by inflammasome activation (Petrasek *et al.* 2012), hepatocytes, including HepG2 cells, also express the cytokine (Palabiyik *et al.* 2016, Zhang *et al.* 2016). Once released, IL-1β has been shown to act in both autocrine and paracrine fashions to increase its own expression (Szabo & Petrasek 2015) as well as the expression of other proinflammatory cytokines, including TNF α (Mandrekar *et al.* 2011), though we did not observe the latter in our studies. IL-1β activates hepatic stellate cells leading to fibrosis (Miura *et al.* 2010) and increases triglyceride accumulation in hepatocytes (Miura *et al.* 2010, Petrasek *et al.* 2012). IL-6 is also expressed in HepG2 cells (Deng *et al.* 2014) and is important in promoting the acute phase response as well as liver regeneration and obesity and insulin resistance. IL-6 induces the expression of c-reactive protein, serum amyloid A, fibrinogen, and haptoglobin (Bode *et al.* 2012), while IL-6 knockout mice have impaired liver regeneration (Cressman *et al.* 1996). The effect of IL-6 on obesity and glucose metabolism is more complex. While IL-6 administration decreased insulin action in skeletal muscle and liver in hyperinsulinemic-euglycemic clamp studies (Kim *et al.* 2004), IL-6-deficient mice displayed hyperleptinemia and hyperinsulinemia, liver inflammation, and steatohepatitis (Wallenius *et al.* 2002, Matthews *et al.* 2010). Similar to IL-1β and IL-6, IL-8 is also expressed in HepG2 cells (Wang *et al.* 2016). IL-8 is an angiogenic factor for human microvascular cells (Heidemann *et al.* 2003) and has chemoattractant and neutrophil-activating activity (Stillie *et al.* 2009). These results suggest that 24,25-$[OH]_2D_3$ may enhance hepatic inflammation in part by stimulating IL-1β, IL-6, and IL-8 expression.

In conclusion, the results of our studies strongly suggest that the principle vitamin D metabolite 24,25-$[OH]_2D_3$ has proinflammatory effects in HepG2 cells. Serum 24,25-$[OH]_2D_3$ levels in healthy individuals has been reported to be 2.3 ng/mL (6.2 nM) (Tartarotti *et al.* 1984), and though it can rapidly concentrate in vitamin D-responsive tissues as well as the liver, to our knowledge, there have been no studies examining tissue levels in humans; nor have there been studies examining how changes in physiological state may lead to changes in tissue levels. These aspects, as well as additional studies examining the molecular mechanism by which 24,25-$[OH]_2D_3$ enhances inflammation remain for investigation both *in vitro* and *in vivo*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

All of the authors contributed significantly to the experimental design, carrying out the studies, and writing and editing the finished manuscript.

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