Pro-inflammatory signaling by 24,25-dihydroxyvitamin D₃ in HepG2 cells

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Abstract

The vitamin D metabolite 24,25-dihydroxyvitamin D₃ (24, 25[OH]₂D₃) 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]₂D₃ but not 25-hydroxyvitamin D₃, suppresses apolipoprotein A-I (apo A-I) gene expression and high-density lipoprotein (HDL) secretion in hepatocytes. Since 24,25-[OH]₂D₃ has low affinity for the vitamin D receptor (VDR) and little is known with regard to how 24,25-[OH]₂D₃ modulates nongenomic signaling in hepatocytes, we investigated the capacity of 24,25-[OH]₂D₃ to activate various signaling pathways relevant to apo A-I synthesis in HepG2 cells. Treatment with 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃ increased IL-1β, IL-6, and IL-8 expression by HepG2 cells. These observations suggest that 24,25-[OH]₂D₃ 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]₂D₃ is generated from 25-hydroxyvitamin D₃ (25-OHD₃) and 1,25-dihydroxyvitamin D₃ (1,25-[OH]₂D₃) 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]₂D₃

circulates in plasma and is taken up by the liver where it accumulates until it is 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]₂D₃ has biological activity, stimulating the resting zone chondrocytes and promoting

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]₂D₃ 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]₂D₃, which has high affinity for the VDR (Makin *et al.* 1989), 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃ in hepatocytes. The amount of 24,25-[OH]₂D₃ 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]₂D₃ down-regulates the expression of various nuclear receptors that regulate lipid and cholesterol metabolism. Therefore, we investigated the effect of 24,25-[OH]₂D₃ 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]₂D₃ 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). ¹⁴C-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 50mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-Cl) (pH 7.4), 1% sodium dodecylsulfate (SDS), and 1mM 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) (50mM sodium phosphate, 150mM NaCl, pH 7.4) containing 0.05% Tween-20 for 2h 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 4–5min 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 4–5min washes, the binding was visualized by enhanced

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 PPAR α 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 μ g 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-1 β , IL-2, IL-6, IL-8, and TNF α levels were measured in 100 μ L of conditioned medium from cells treated with solvent, 50 nM 24,25-[OH]₂D₃, 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 $\pm$ 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]₂D₃ on PPAR α and RXR α expression

Previous studies suggested that 10–100 nM 24,25-[OH]₂D₃ 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]₂D₃. To assess whether or not 24,25-[OH]₂D₃ affects expression of PPAR α and RXR α , HepG2 cells were treated with 50 nM 24,25-[OH]₂D₃ for 0, 1, 2, 6, and 24 h. PPAR α and RXR α were measured by Western blot (Fig. 1A) and normalized to β -actin levels (Fig. 1B and C). Treatment with 24,25-[OH]₂D₃ suppressed both PPAR α and RXR α expression in a time-dependent fashion, though PPAR α levels were much lower than RXR α levels by 24 h. In contrast, β -actin levels did not change in 24,25-[OH]₂D₃-treated cells. Since PPAR α and RXR α 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]₂D₃ may suppress apo A-I gene expression by inhibiting expression of these nuclear receptors.

The effect of 24,25-[OH]₂D₃ on PPAR α activity

HepG2 cells were transfected with the plasmids pJ6 TK pG13, containing six consensus PPAR α binding sites, and pCMV.SPORT. β -gal and treated with the PPAR α ligand gemfibrozil (50 μ M) or 50 nM 24,25-[OH]₂D₃, or both gemfibrozil and 24,25-[OH]₂D₃ for 24 h and luciferase activity was measured and normalized to β -galactosidase activity (Fig. 2). Treatment with gemfibrozil increased PPAR α -dependent luciferase activity 2.6-fold (*P* < 0.0008), while treatment with 24,25-[OH]₂D₃ suppressed PPAR α -dependent luciferase activity 79.0% (*P* < 0.0007). Furthermore, treatment with gemfibrozil was unable to reverse the effects of 24,25-[OH]₂D₃ on luciferase activity; luciferase activity decreased 76.9% relative to control cells (*P* < 0.0001) in cells treated with gemfibrozil and 24,25-[OH]₂D₃. These results suggest that treatment with 24,25-[OH]₂D₃ may suppress PPAR α -dependent gene expression in hepatocytes.

Figure 1

The effect of 24,25-[OH]₂D₃ on PPARα and RXRα expression. HepG2 cells were treated with 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃ on PKCα, JNK1, ERK1/2, and c-jun expression and phosphorylation

HepG2 cells were treated with 50 nM 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃, or both gemfibrozil and 24,25-[OH]₂D₃ 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]₂D₃ suppressed activity. Luciferase activity in cells treated with both gemfibrozil and 24,25-[OH]₂D₃ was similar to luciferase activity in cells treated with 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃ treatment. These results suggest that 24,25-[OH]₂D₃ increases c-jun phosphorylation, correlating with changes in PKCα, JNK1, and ERK1/2 phosphorylation.

The effect of 24,25-[OH]₂D₃ 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]₂D₃ induced PKCα, JNK1, and ERK1/2 activity, we investigated whether or not 24,25-[OH]₂D₃ 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]₂D₃ for 24 h (Fig. 4A). Treatment with PMA induced collagenase promoter activity 2.7-fold ($P<0.001$). Likewise, treatment with 24,25-[OH]₂D₃ induced collagenase promoter activity 2.2-fold ($P<0.008$). These results suggest that 24,25-[OH]₂D₃, like PMA, induces AP1 activity in HepG2 cells.

Figure 3

The effect of 24,25-[OH]₂D₃ on PKCα, JNK1, ERK1/2, and *c-jun* expression and phosphorylation. HepG2 cells were treated with 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ relative to cells transfected with the control

siRNA and treated with 24,25-[OH]₂D₃ (*P*<0.0008). These results suggest that 24,25-[OH]₂D₃ induces AP1 activity by activating endogenous *c-jun*.

The effect of 24,25-[OH]₂D₃ 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 100nM PMA or 50nM 24,25-[OH]₂D₃ 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]₂D₃ 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]₂D₃ 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

Figure 4

The effect of 24,25-[OH]₂D₃ 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]₂D₃, 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]₂D₃ induced reporter gene expression. In contrast, in cells transfected with the *c-jun*-specific siRNA, treatment with PMA and 24,25-[OH]₂D₃ 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. †*P*<0.0001 and *P*<0.0008, respectively, relative to cells transfected with the control siRNA and treated with PMA and 24,25-[OH]₂D₃, 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]₂D₃ relative to cells transfected with the control siRNA and treated with 24,25-[OH]₂D₃ (*P*<0.002). These results suggest that 24,25-[OH]₂D₃ suppresses apo A-I gene expression by inducing AP1 activity.

The effect of 24,25-[OH]₂D₃ 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]₂D₃, 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]₂D₃, PMA, and LPS as reported previously (Morishima *et al.* 2003, Wehmeier *et al.* 2011).

Figure 5

The effect of 24,25-[OH]₂D₃ 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]₂D₃, 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]₂D₃ suppressed apo A-I promoter activity. In contrast, in the presence of the *c-jun*-specific siRNA, treatment with PMA and 24,25-[OH]₂D₃ 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. †*P*<0.001 and *P*<0.002, respectively, relative to cells transfected with the control siRNA and treated with PMA and 24,25-[OH]₂D₃, respectively.

These results suggest that 24,25-[OH]₂D₃ has pro-inflammatory effects in HepG2 cells.

Discussion

Previous studies in our laboratory have shown that 1,25-[OH]₂D₃ inhibits apo A-I gene expression via a VDR-mediated mechanism (Wehmeier *et al.* 2005). In contrast, 24,25-[OH]₂D₃-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]₂D₃ to inhibit apo A-I gene expression (Wehmeier *et al.* 2011). Therefore, we examined the potential of 24,25-[OH]₂D₃ 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]₂D₃ on their expression and activity. Treatment with 24,25-[OH]₂D₃ 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]₂D₃ 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

Figure 6

The effect of 24,25-[OH]₂D₃ on proinflammatory cytokine expression in HepG2 cells. HepG2 cells were treated with solvent (control cells, C), 50 nM 24,25-[OH]₂D₃, 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]₂D₃ 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]₂D₃, 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]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ 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]₂D₃ would inhibit apo A-I gene expression to a greater extent than observed with PMA treatment.

Finally, treatment with 24,25-[OH]₂D₃ increased IL-1β, IL-6, and IL-8 levels in the conditioned medium, similar to

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]₂D₃, increased IL-2 levels. Of the cytokines that were induced by 24,25-[OH]₂D₃, the most is known about IL-1 β inflammasome activity in the liver. Though liver immune cells such as Kupffer cells express most of the IL-1 β 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-1 β 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-1 β 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-1 β 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]₂D₃ may enhance hepatic inflammation in part by stimulating IL-1 β , IL-6, and IL-8 expression.

In conclusion, the results of our studies strongly suggest that the principle vitamin D metabolite 24,25-[OH]₂D₃ has proinflammatory effects in HepG2 cells. Serum 24,25-[OH]₂D₃ 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]₂D₃ 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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