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1,25-Dihydroxyvitamin D_3 suppresses inflammation-induced expression of plasminogen activator inhibitor-1 by blocking nuclear factor-κB activation

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ABSTRACT

Plasminogen activator inhibitor (PAI)-1 is a major fibrinolytic inhibitor. High PAI-1 is associated with increased renal and cardiovascular disease risk. Previous studies demonstrated PAI-1 down-regulation by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), but the molecular mechanism remains unknown. Here we show that exposure of mouse embryonic fibroblasts to TNFα or LPS led to a marked induction of PAI-1, which was blunted by $1,25(OH)_2D_3$, NF-κB inhibitor or p65 siRNA, suggesting the involvement of NF-κB in $1,25(OH)_2D_3$-induced repression. In mouse *Pai-1* promoter a putative *cis*-κB element was identified at −299. EMSA and ChIP assays showed that TNF-α increased p50/p65 binding to this κB site, which was disrupted by $1,25(OH)_2D_3$. Luciferase reporter assays showed that PAI-1 promoter activity was induced by TNFα or LPS, and the induction was blocked by $1,25(OH)_2D_3$. Mutation of the κB site blunted TNFα, LPS or $1,25(OH)_2D_3$ effects. $1,25(OH)_2D_3$ blocked IκBα degradation and arrested p50/p65 nuclear translocation. In mice LPS stimulated PAI-1 expression in the heart and macrophages, and the stimulation was blunted by pre-treatment with a vitamin D analog. Together these data demonstrate that $1,25(OH)_2D_3$ down-regulates PAI-1 by blocking NF-κB activation. Inhibition of PAI-1 production may contribute to the reno- and cardio-protective effects of vitamin D.

Introduction

Plasminogen activator inhibitor-1 (PAI-1), a 50 kDa glycoprotein, is the principal inhibitor of tissue-type plasminogen activators (t-PAs) and urokinase-type plasminogen activators (u-PAs), which convert inactive plasminogen to active plasmin. Plasmin is a serine protease and involved in the degradation of clot fibrin and extracellular matrix. Elevated plasma PAI-1 levels are associated with and contribute to thrombotic diseases including hyperthrombosis and myocardial infarction as well as fibrotic disorders such as atherosclerosis, pulmonary fibrosis and nephropathy [1,2], thus PAI-1 has long been considered an important therapeutic target. PAI-1 is produced by a broad range of cell types, including hepatocytes, glomerular mesangial cells, tubular epithelial cells, fibroblasts, vascular endothelial cells, smooth muscle cells, macrophages and adipocytes. Under normal conditions, PAI-1 is present in plasma at very low concentrations. High levels of PAI-1 are caused by a number of mechanisms, including stimulation by pro-fibrotic factors such as transforming growth factor β (TGFβ) [3] and inflammatory factors such as tumor necrosis factor α (TNFα), interleukin-1 β (IL-1β) and lipopolysaccharide (LPS) [4–8]. PAI-1 is thus considered as an inflammatory response gene. Given the important contribution of inflammation to the development of chronic renal and cardiovascular diseases [9–14], inflammatory stimulation of PAI-1 has important pathological implications. The mechanism underlying the inflammatory regulation of PAI-1, however, remains poorly defined.

1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the hormonal metabolite of vitamin D, is a pleiotropic hormone that exerts its actions by activating the vitamin D receptor (VDR[1]), a member of the nuclear receptor superfamily [15]. Vitamin D-deficiency

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[1] *Abbreviations used:* VDR, vitamin D receptor; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

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is now recognized as a global health issue with adverse consequences [16]. Accumulating epidemiological and clinical evidence has demonstrated an association of vitamin D-deficiency with increased risk of renal and cardiovascular diseases. For example, in patients with chronic kidney disease (CKD), vitamin D-deficiency is an independent risk factor for cardiovascular disease [17]. In hypertensive patients, low serum vitamin D levels markedly increase the risk of cardiovascular disease [18]. Vitamin D therapy reduces mortality in CKD patients including cardiovascular mortality and infectious mortality [19,20]. The molecular basis of these observations remains to be defined, but given the crucial role of PAI-1 in the development of renal and cardiovascular disorders, there is a good possibility for vitamin D to target PAI-1 in its renal and cardiovascular protection. Indeed, several previous studies have reported regulatory effects of vitamin D on PAI-1 production in a number of cell types. For example, $1,25(OH)_2D_3$ was shown to enhance plasminogen activator activity and decrease PAI-1 production in rat calvarial osteoblast-like cells and osteogenic sarcoma cells [21]. Activated vitamin D analogs were reported to suppress PAI-1 in human coronary artery smooth muscle cells [22,23]. While these observations appear to be important, the mechanism underlying vitamin D repression of PAI-1 remains to be defined.

In this study, we investigated the mechanism whereby $1,25(OH)_2D_3$ counters the induction of PAI-1 by TNFα and LPS in mouse embryonic fibroblasts (MEFs). We identified a functional *cis*-κB element in the proximal promoter of the mouse *pai-1* gene that mediates the up-regulation of PAI-1 by TNFα and LPS. We further demonstrated that $1,25(OH)_2D_3$ down-regulates PAI-1 expression via targeting the NF-κB activation pathway.

Materials and methods

Cell culture

MEFs were isolated from 13.5-day old VDR(+/−) and VDR(−/−) mouse embryos as described previously [24]. VDR(+/−) and VDR(−/−) MEFs were cultured in DMEM supplemented with 10% FBS. For most experiments, the cells were pre-treated for 24 h with 20 nM $1,25(OH)_2D_3$ before being stimulated with either TNFα (20 ng/ml) or LPS (100 ng/ml). MEFs used in the experiments are VDR(+/−) unless otherwise stated. Detailed experimental conditions are described in figure legends.

RT-PCR

Total cellular RNAs were extracted using TRIzol reagent (Invitrogen). First-strand cDNAs were synthesized from total RNAs using MML-V reverse transcriptase (Invitrogen) and hexanucleotide random primers. The cDNAs were used as templates for PCR amplifications with the following primers. PAI-1: 5′TCATCCTGCCTAAGT TCTCTCT3′ (forward) and 5′GCTCTTGGTCGGAAAGACTT3′ (reverse); and p65: 5′CAGGCAGAGTGACTTCATGG3′ (forward) and 5′GCT CGTGAGAACTGCTGCTA3′ (reverse). The internal control for the PCR reaction was β2-microglobulin (B2M) with primers 5′ACC GGCCTGTATGCTATCCAGAAA3′ (forward) and 5′ATTTCAATGTGAG GCGGGTGGAAC3′ (reverse).

Western blotting

Proteins were separated by SDS–PAGE and transferred onto Immobilon membranes. Western blotting was carried out as previously described [25], using antibodies against PAI-1 or IκBα (Santa Cruz Biotechnologies).

Immunostaining

VDR(+/−) and VDR(−/−) MEFs (grown on cover slips) pre-treated with 20 nM $1,25(OH)_2D_3$ overnight were stimulated with TNF-α for 30 min. The cells were fixed with 4% paraformaldehyde for 30 min and stained with anti-p65 antibody as described [26]. Cell nuclei were stained with DAPI (4′-6-diamidino-2-phenylindol).

Northern Blot

Northern blot analyses were performed as described previously [27] with ^{32}P-labelled PAI-1 cDNA as hybridization probe.

PAI-1 promoter constructs and luciferase reporter assays

The 5′ upstream region from −362 to +10 in the mouse *pai-1* gene was amplified by PCR using primers 5′ATGGCTGTCTCCAAAA-AAAG3′ (forward) and 5′CGGACGCGTAGCCTGATCCAGCTGTGCT3′ (reverse), and cloned into pGL3 basic vector (Promega) to generate reporter plasmid pGL-Pai-Luc. Reporter plasmid pGL-Pai-m-Luc carrying mutations at the −299 κB site (mutated from 5′GGGAATTCCA3′ to 5′GGGcAcTCCA3′) was generated using the RapidChange mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing. Cells were transfected using Lipofectamine 2000 (Invitrogen) in serum free media with pGL-Pai-Luc, pGL-Pai-m-Luc or pNF-κB-Luc (Promega) as indicated in the experiment. Twenty-four hours after transfection the cells were exposed to 20 ng/ml TNFα or 100 ng/ml LPS in the presence or absence of 20 nM $1,25(OH)_2D_3$ for 24 h. The cells were lysed and luciferase activity determined using Luciferase Assay Systems (Promega) as reported previously [28].

Electrophoretic mobility shift assays (EMSA)

EMSA were performed as described previously [28]. Confluent MEFs were stimulated with 20 ng/ml TNFα for 2 h in the presence or absence of 20 nM $1,25(OH)_2D_3$ for 24 h. Nuclear extracts were prepared from MEFs following an established method described previously [28]. For EMSA, 5 μg of nuclear extracts were incubated with 5×10^6 cpm of ^{32}P-labeled −299 κB probe (5′AGGAA-GGGAATTCCAAACAC3′; underlined is the core NF-κB binding site) at room temperature for 20 min. The specificity of protein-DNA interaction was confirmed by competition with an excess amount of unlabeled probes of the same sequence, the canonical κB probe (designated as κBc, (5′AGTTGAGGGGCATTTCCCAGGC3′, Santa Cruz Biotechnology), or the mutated probe −299 κBmut (5′AGGAAGGGcAcTCCAAACAC3′). The presence of p65 or p50 in the DNA–protein complex was confirmed with antibody supershift assays using anti-p65 or anti-p50 antibody (Santa Cruz Biotechnology).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out as described previously [28] using a commercial kit from Upstate Biotechnology (Lake Placid, NY). Briefly, MEFs were pre-treated with or without 20 nM $1,25(OH)_2D_3$ in serum-free media for 24 h, and then stimulated with TNF-α for 30 min. After treatment with 1% formaldehyde to cross-link proteins to DNA, cells were lysed and sonicated to shear the chromatins. The sonicated chromatins were incubated with anti-p65 antibody or anti-p50 antibody and the chromatin-antibody complex was precipitated with protein-A-agarose beads. The DNA isolated from the complex was subjected to PCR amplification using the following primers flanking the −299 κB site in the *pai-1* gene promoter: 5′ATGGCTGTCTCCAAAAAAAG3′ (forward) and 5′GTGTGTGTACG TGTGAAAGG3′ (reverse). The PCR products were visualized by