Biochemical and Biophysical Research Communications 377 (2008) 674–678

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

1,25(OH)$_2$D$_3$ regulates collagen quality in an osteoblastic cell culture system

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ARTICLE INFO

Article history:
Received 3 October 2008
Available online 18 October 2008

Keywords:
Collagen post-translational modification
Vitamin D
Collagen cross-link
MC3T3-E1

ABSTRACT

The active form of vitamin D, 1,25(OH)$_2$D$_3$, has a broad range of effects on bone, however, its role in the quality of bone matrix is not well understood. In this study, using an osteoblastic cell (MC3T3-E1) culture system, the effects of 1,25(OH)$_2$D$_3$ on collagen cross-linking and related enzymes, i.e., lysyl hydroxylases (LH1-3) and lysyl oxidases (LOX, LOXL1-4), were examined and compared to controls where cells were treated with cholecalciferol or ethanol. When compared to the controls, gene expressions of LH1, LH2b and LOXL2 were significantly upregulated by 1,25(OH)$_2$D$_3$ up to 72 h of culture. In addition, hydroxylysine (Hyl), Hyl aldehyde (Hyl^{ald}), Hyl^{ald}-derived cross-links and a total number of cross-links of collagen were significantly higher and the cross-link maturation was accelerated in the 1,25(OH)$_2$D$_3$ treated group. These results demonstrate that 1,25(OH)$_2$D$_3$ directly regulates collagen cross-linking in this culture system likely by upregulating gene expression of specific LH and LOX enzymes.

The biologically most active hormonal form of vitamin D$_3$, 1,25(OH)$_2$D$_3$, which is formed after two sequential hydroxylations in the liver and kidney of cholecalciferol (VD$_3$), has a broad range of physiological effects including calcium homeostasis, bone metabolism, immune system and cancer [1]. These functions are mediated through a single vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily [2]. The overall positive effect of vitamin D on bone has been known for a century since it prevents and cures rickets and osteomalasia [3]. However, the direct role of 1,25(OH)$_2$D$_3$ in bone homeostasis is still not evident and the results from previous studies are not always consistent [4] possibly due to an involvement of many modulators of the vitamin D$_3$-induced signaling at multiple levels [5–7].

Fibrillar type I collagen, the predominant organic matrix component in bone, functions as a three-dimensional template to control the spatial aspect of mineralization [8]. The covalent intermolecular cross-linking is the final collagen post-translational modification crucial for the stability of the fibrils. The type and quantity of cross-links are primarily determined by the extent of hydroxylation and oxidative deamination of the specific lysine residues in collagen catalyzed by lysyl hydroxylases (LHs) and lysyl oxidase(s) (LOX), respectively. The specific cross-linking pattern appears to be vitally important for bone mineralization as indicated by in vitro studies [9,10], and its association with Bruck syndrome, a rare form of osteogenesis imperfecta with congenital contractures [11]. Though type I collagen gene is considered as one of the 1,25(OH)$_2$D$_3$ responsive genes in osteoblasts derived from various species [12–17], at present, little is known about its effect on collagen post-translational modifications. The objectives of this study were to investigate the effects of 1,25(OH)$_2$D$_3$ on gene expression of LHs (LH1, 2a/b, 3) and LOX/LOX-like proteins (LOXL1-4), lysine hydroxylation and cross-linking of collagen using an osteoblastic cell culture system.

Materials and methods

Cell culture. MC3T3-E1 cells (subclone 4) were purchased from American Type Culture Collection (CRL-2593). Cells were maintained in α-minimum essential medium (α-MEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Atlanta, Lawrenceville, GA, USA), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in a 5% CO_2 atmosphere at 37 °C. The medium was changed twice a week.

Cell growth. MC3T3-E1 cells were plated onto a 24-well plate in triplicate at a density of 2×10^4 cells/well, cultured overnight and treated with ethanol (Et-OH), 10^{-8} M VD$_3$ or 10^{-8} M 1,25(OH)$_2$D$_3$ for up to 14 days. The former two served as controls. At days 2, 5, 7, 10, and 14 of culture, cell number was determined by cell counting.

In a separate set of experiments, cells were plated onto a 96-well plate in triplicate at a density of 1×10^4 cells/well, treated and subjected to MTS cell proliferation assay (CellTier 96; Promega, Madison, WI, USA) at days 2, 5, and 7 of culture as reported previously [18].

Quantitative real-time polymerase chain reaction (PCR). Cells are plated onto 35 mm dishes at a density of 2×10^5 cells/dish. After reaching confluence, the medium was replaced with that

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doi:10.1016/j.bbrc.2008.10.036

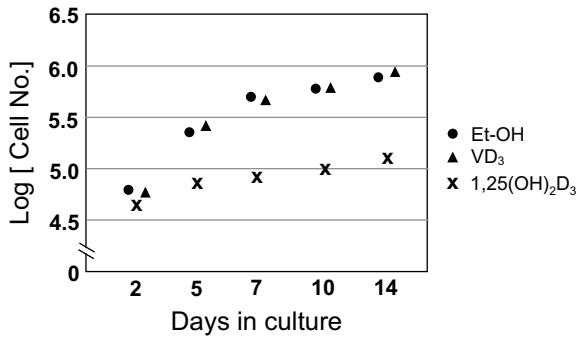

Fig. 1. Effect of $1,25(OH)_2D_3$ on cell growth. The cell number of each group is shown in a logarithmic scale at each time point indicated. Et-OH: ethanol, VD_3: cholecalciferol.

containing Et-OH, VD_3 or $1,25(OH)_2D_3$. At 12, 24, 48 and 72 h of culture, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the first-strand cDNA was synthesized using an Omniscript Reverse Transcriptase kit (Qiagen, Valencia, CA, USA). Real time PCR was performed in triplicate using the specific primers-probe for Cbfa1/Runx2 (Applied Biosystems, ABI assay number: Mm00501578_m1), Col1a2 (Mm00483888_m1), LH1 (Mm 00599925_m1), LH2 (Mm 00478767_m1), LH3 (Mm 00478798_m1), LOX (Mm 00495386_m1), LOXL1 (174595), LOXL2 (Mm 00804740_m1), LOXL3 (Mm 00442953_m1) and LOXL4 (Mm 00446385_m1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ABI assay number: 4308313), and analyzed by the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). The mean fold changes in gene expression relative to GAPDH were calculated using the values obtained from Et-OH treated MC3T3-E1 cells as a calibrator by means of $2^{-\Delta\Delta C}{}_T$ method at each time point [19]. All analyses were done in triplicate and the results were confirmed by three independent experiments.

Determination of LH2 variants by reverse transcription (RT)-PCR. The expressions of LH2 variants (LH2a and b) were analyzed using specific primers as described previously [9]. This set of primers amplifies LH2a (without an insert of 63 bp exon 13A) as a 158 bp fragment and LH2b (with the splice insert) a 221 bp fragment, respectively [20]. A set of GAPDH primers; forward, 5′ACC ACAGTCCATGCCATCAC3′, reverse, 5′TCCACCACCCTGTTGCTGTA3′ were also designed to use as an internal control. MC3T3-E1 cells were cultured and treated with Et-OH, VD_3 or $1,25(OH)_2D_3$ for 48 h as described above, and RT-PCR was performed by HotStarTaq DNA polytmerase (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The optimal numbers of PCR cycles selected were 35 cycles for LH2a and GAPDH, and 22 cycles for LH2b. The PCR products were then resolved by 12% polyacrylamide gel electrophoresis and analyzed. Three independent experiments were performed to confirm the results.

Amino acid analysis. MC3T3-E1 cells were maintained in the same manner as described, cultured in the medium containing 50 μg/ml ascorbic acid and treated with Et-OH, VD_3 or $1,25(OH)_2D_3$. After 14 days of culture, cells/matrices were scraped, washed with phosphate buffered saline (PBS) and lyophilized. Aliquots of dried samples (~2 mg) were reduced with standardized NaB^3H_4, hydrolyzed with 6N HCl and an aliquot of each hydrolysate was subjected to amino acid analysis on a Varian high-performance liquid chromatography (HPLC) system (Prostar 240/310, Varian, Walnut Creek, CA, USA) with a strong cation exchange column (AA-911,

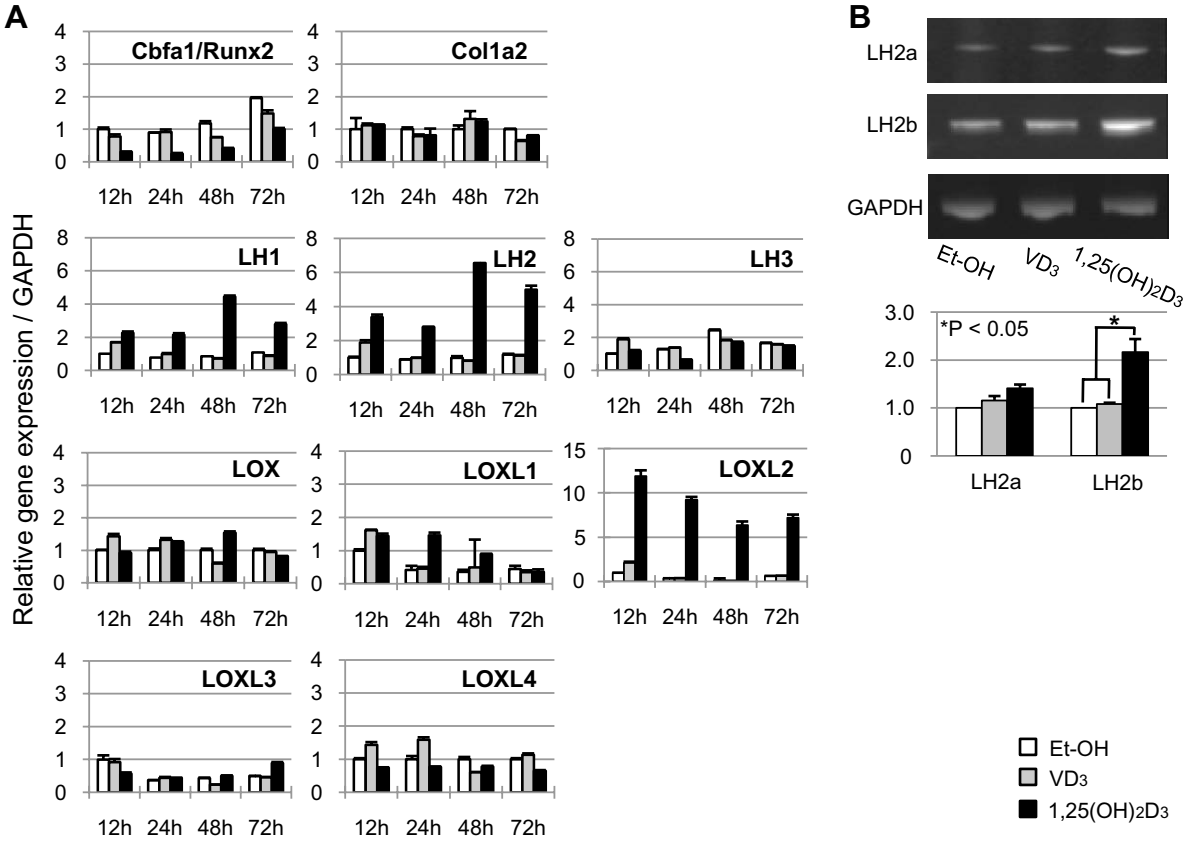

Fig. 2. (A) Effect of $1,25(OH)_2D_3$ on the gene expression of osteogenic markers and collagen modifying enzymes. After MC3T3-E1 cells were treated with Et-OH, VD_3 or $1,25(OH)_2D_3$, the gene expression at various time points (12–72 h) was analyzed by real-time PCR. Note that the gene expressions of LH1 and 2, and LOXL2 were significantly upregulated by $1,25(OH)_2D_3$ treatment. (B) The expression of LH2 splicing variants (LH2a and b) analyzed by RT-PCR at 48 h after treatments (upper panel). The expression levels were measured by Scion Image and are shown in the lower panel. Et-OH: ethanol, VD_3: cholecalciferol. *Indicates significant difference ($p < 0.05$) ($n = 3$).