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1,25-Dihydroxyvitamin D_3 and extracellular calcium promote mineral deposition via NPP1 activity in a mature osteoblast cell line MLO-A5

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ABSTRACT

While vitamin D supplementation is common, the anabolic mechanisms that improve bone status are poorly understood. Under standard mineralising conditions including media ionised calcium of 1.1 mM, 1,25-dihydroxyvitamin D_3 (1,25D) enhanced differentiation and mineral deposition by the mature osteoblast/pre-osteocyte cell line, MLO-A5. This effect was markedly increased with a higher ionised calcium level (1.5 mM). Gene expression analyses revealed that 1,25D-induced mineral deposition was associated with induction of *Enpp1* mRNA, coding for nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) and NPP1 protein levels. Since MLO-A5 cells express abundant alkaline phosphatase that was not further modified by 1,25D treatment or exposure to increased calcium, this finding suggested that the NPP1 production of pyrophosphate (PPi) may provide alkaline phosphatase with substrate for the generation of inorganic phosphate (Pi). Consistent with this, co-treatment with *Enpp1* siRNA or a NPP1 inhibitor, PPADS, abrogated 1,25D-induced mineral deposition. These data demonstrate that 1,25D stimulates osteoblast differentiation and mineral deposition, and interacts with the extracellular calcium concentration. 1,25D regulates *Enpp1* expression, which presumably, in the context of adequate tissue non-specific alkaline phosphatase activity, provides Pi to stimulate mineralisation. Our findings suggest a mechanism by which vitamin D with adequate dietary calcium can improve bone mineral status.

1. Introduction

Combined vitamin D and calcium supplementation is a common approach in the elderly to treat age-related bone loss and reduce the risk of fracture (Bischoff-Ferrari et al., 2012). The physiological mechanism, by which this treatment achieves this outcome, is unclear and controversial. Importantly, in both human clinical trials (Bischoff-Ferrari et al., 2004) and rodent pre-clinical studies (Anderson et al., 2008), it is the circulating level of the pro-hormone form, 25-hydroxyvitamin D_3 (25D) that is associated with the increase in bone mineral density (BMD) and reduction in fracture risk rather than that of the active hormone, 1,25-dihydroxyvitamin D_3 (1,25D). The effects of 25D on bone metabolism are dependent on conversion to 1,25D locally in bone (Atkins et al., 2007) and the regulation of local 1,25D production in bone is independent from the mechanisms controlling circulating 1,25D metabolism (H.C. Anderson et al., 2005; P.H. Anderson et al., 2005).

Osteoblastogenesis is essential for bone formation, requiring a sequential process of cell proliferation and maturation. Multiple cellular and animal model studies have reported 1,25D exerting both inhibitory and enhancing effects on osteoblast differentiation and mineral deposition (Chen et al., 2012; Khanna-Jain et al., 2010; Matsumoto et al., 1991; Owen et al., 1991; Woeckel et al., 2010; Yamaguchi and Weitzmann, 2012; Yamamoto et al., 2013). Regulation of such effects remains unclear. One contributory factor is the stage of osteoblast maturation, such that 1,25D stimulates a catabolic action on bone in immature osteoblast-like cells as a result of induction of the osteoclastogenic cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) but not in more mature cells (Atkins et al., 2003; Yang et al., 2013). Another is extracellular calcium, which promotes osteogenic differentiation in human primary osteoblasts (Welldon et al., 2013).

In the current study, we hypothesised that mature osteoblasts/early osteocytes will respond to 1,25D to increase differentiation and enhance mineral deposition at higher levels of extracellular calcium. To study this, the late osteoblast/early osteocyte cell line MLO-A5

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was used, since this has been shown to deposit a lamellar bone-like mineralised matrix (Barragan-Adjemian et al., 2006) and 1,25D does not stimulate expression of *Rankl* in these cells (Yang et al., 2013). Two levels of total calcium concentration in the culture media were used: 1.8 mM, the basal level present in α-MEM media, and an elevated level of 2.8 mM. We examined the expression of genes including ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (*Enpp1*), ankylosis protein (*Ank*) and tissue non-specific alkaline phosphatase (*Tnap*), that are responsible for the metabolism, transport and catabolism, respectively, of inorganic pyrophosphate (PPi) to regulate mineralisation (Addison et al., 2007; Terkeltaub, 2006) in order to identify a molecular mechanism for vitamin D-induction of mineralisation.

2. Materials and methods

2.1. Cell culture

The MLO-A5 cell line was provided by Professor Lynda Bonewald (University of Missouri-Kansas City, MO, USA) and cultured as previously described (Kato et al., 2001). For differentiation experiments, cells were cultured in osteogenic differentiation media consisting of 2% v/v foetal bovine serum (Thermo Fisher Scientific, Scoresby, VIC, Australia), 10 mM β-glycerol-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 100 μM ascorbate-2-phosphate (Sigma-Aldrich) and α-MEM (Life Technologies, Grand Island, NY, USA) with and without the treatments of 1,25D (0.1 and 1 nM; Wako Pure Chemicals, Osaka, Japan) or vehicle (0.1% v/v ethanol) as indicated. Culture medium containing total calcium concentration at 2.8 mM was prepared by adding 0.1% v/v of 1 M calcium chloride (Sigma-Aldrich) to α-MEM media.

2.2. Proliferation assay

Cells for the proliferation assay were pre-labelled with the cell permeable fluorescent dye carboxyfluoresceinsuccinimidyl ester (CFSE), which provides an accurate measure of the percentage of divided cells (Lyons and Parish, 1994), as described previously (Atkins et al., 2007; Vincent et al., 2009). Cells were seeded at the density of 3×10^4 cells per well in 1 ml osteogenic differentiation media, as described earlier, in 12-well plates for 5 days. On day 5, cells were harvested using 0.1% w/v trypsin for flow cytometric analysis of CFSE intensity (Atkins et al., 2007). The cell cycle generations were calculated using ModFit LT 3 software (Verity Software House, ME, USA).

2.3. Differentiation/mineralisation assay

Cells for differentiation/mineralisation assays were seeded at 3×10^4 cells per well in 0.5 ml media in 24-well plates for mRNA analysis and Alizarin Red staining, or 1×10^4 cells per well in 0.25 ml media in 8-well chamber slides (Ibidi GmbH, Planegg, Martinsried, Germany). Cells were cultured to 100% confluence and then differentiation media were added, containing combinations of 1,25D (1 nM) or vehicle (0.1% v/v ethanol) at two concentrations of total calcium (1.8 and 2.8 mM). Media were replenished every 3 days until day 21. Measurement of the ionised calcium concentrations in media preparations (Siemens RAPIDLAB 1265 system, Munich, Germany) revealed corresponding levels of 1.1 and 1.5 mM, respectively. On days 3, 6, 12 and 21 total RNA was extracted from triplicate culture wells of 24-well plates with TRIZOL reagent (Life Technologies) according to the manufacturer's instructions. cDNA against the total RNA template was synthesised using the Superscript-III kit (Life Technologies). The mRNA expression of genes of interest was measured by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA), as per the manufacturer's instructions. Oligonucleotide primer sets targeting *Cyp24a1*, *Gja1*, *Dmp1*, *Ank*, *Enpp1*, *E11* and *Phex* gene sequences are listed in Table 1. Primer sets for other genes were described previously (Yang et al., 2013). The specificity of primer binding was confirmed by melt-curve analysis after the PCR reactions. For quantification, gene expression was normalised to that of the housekeeping gene β-actin.

Table 1
Primer sets used for real-time PCR.

Gene	Sequence (5′–3′)	Gene bank accession number	Amplicon (bp)
Cyp24a1	Forward: ttgaaagcatctgccttgtgt Reverse: gtcaccatcatcttcccaaat	NM_009996.3	130
Gja1	Forward: aagtgaaagagaggtgccca Reverse: gtggagtaggcttggacctt	NM_010288.3	79
Dmp1	Forward: gaaagctctgaagagaggacggg Reverse: tgtccgtgtggtcactatttgcct	NM_016779.2	121
Ank	Forward: tcgctgacgctctgttttgt Reverse: ggcaaagtccactccaatgatat	NM_020332.4	84
Enpp1	Forward: aagcgcttacacttcgctaaaag Reverse: tgatggattcaacgcaagttg	NM_008813.3	87
E11	Forward: aaacgcagacaacagataagaaagat Reverse: gttctgtttagctctttagggcga	NM_010329.2	158
Phex	Forward: gaaaagctgttcccaaaacagag Reverse: tagcaccataactcagggatcg	NM_011077.2	156

In vitro mineral deposition was visualised by Alizarin Red staining of cultures on days 3, 6, 12 and 21. The Alizarin Red-calcium complexes were dissolved in 10% v/v acetic acid, neutralised to pH 4.2 by 10% v/v ammonium hydroxide and quantified by measuring light absorption at 405_{nm} (Gregory et al., 2004). The co-localisation of alkaline phosphatase activity and mineral deposition *in situ* was confirmed by adding calcein (4 μg/ml; Sigma-Aldrich) to the differentiation media at the final media change. Cultures were then fixed in 4% paraformaldehyde in PBS and then supplied with the alkaline phosphatase substrate, StayRed/AP Plus (Abcam, MA, USA) for 10 min. The chemical composition of the mineral deposited by these cultures was investigated by analysing the ratio of elemental calcium to phosphorus using energy dispersive spectroscopy (EDS) analysis, as described previously (Kumarasinghe et al., 2012).

2.4. NPP1 western blotting

Cell lysates were harvested and 50 μg of total protein from each lysate sample was subjected to Western blotting as described (Atkins et al., 2011). NPP1 was detected using rabbit anti-NPP1 antibody (Cat# PA5-17097, Thermo-Fisher, Waltham MA, USA) and goat-anti-rabbit IgG-AP (Thermo-Fisher). Protein loading was measured by the expression of GAPDH using directly conjugated mouse anti-GAPDH-HRP (Sigma-Aldrich) and detected with the enhanced chemiluminescence detection system (GE, Buckinghamshire, UK).

2.5. Inhibition of NPP1 activity

The effects of inhibiting *Enpp1* mRNA expression were examined by transfecting 18 nM of *Enpp1* specific siRNA (EsiRNA, Sigma-Aldrich) to cells in comparison to the same concentration of scrambled control siRNA transfected cultures for 24 hours, using Lipofectamine 3000 (Life Technologies), according to the manufacturer's instructions. *Enpp1* mRNA levels and mineral deposition under 1,25D and/or calcium treatments at 72 hours were then quantified as earlier. The effects of inhibiting the enzyme activity of NPP1 on mineral deposition were examined using pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) (Sigma-Aldrich) at 25 and 50 μM. Mineral deposition at day 6 was quantified.