Journal of Steroid Biochemistry & Molecular Biology 164 (2016) 374–378

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Review

Comparison of the biological effects of exogenous and endogenous 1,25-dihydroxyvitamin D_3 on the mature osteoblast cell line MLO-A5

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 29 February 2016
Accepted 1 March 2016
Available online 4 March 2016

Keywords:
Osteogenic differentiation
Mineralisation
25-hydroxyvitamin D_3
Endogenous/exogenous 1,25$(OH)_2$-vitamin-D3
Cyp27b1
MLO-A5

ABSTRACT

Clinical and animal data indicate that serum 25-hydroxyvitamin D_3 (25D) exerts an anabolic effect on bone while serum 1α,25-dihydroxyvitamin D_3 (1,25D) stimulates bone mineral loss, although the mechanism responsible for these divergent actions is unknown. Biological effects of 25D on bone cells are dependent on the local conversion to 1,25D by the 25-hydroxyvitamin D-1α-hydroxylase enzyme, CYP27B1. Therefore, identification of possible differential activities of locally produced and exogenously supplied 1,25D in bone is likely to be informative for guiding optimal administration of vitamin D supplements for bone health. The mature osteoblastic cell line MLO-A5 expresses both the vitamin D receptor (*Vdr*) and *Cyp27b1*, and therefore is a suitable model for comparing the activities of 1,25D arising from these sources. Biologically, exogenous and endogenous sources of 1,25D have similar effects on proliferation, mineralisation and induction of a range of genes by MLO-A5 osteoblasts under osteogenic conditions although endogenous 1,25D levels are markedly lower than exogenous levels. Significant differences of pharmacokinetics and pharmacodynamics of 1,25D are evident between these two sources particularly in terms of modulating gene expression for *Cyp24a1* and other genes largely expressed by embedded osteoblasts/osteocytes suggesting that endogenously synthesised 1,25D is more efficiently utilised by the differentiating osteoblast.

Contents

1. Introduction

Vitamin D supplementation has been utilised for many years to increase blood 25-hydroxyvitamin D_3 (25D) levels with the aim of optimising bone health and reducing the risk of fracture in susceptible populations although the benefits from this strategy

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http://dx.doi.org/10.1016/j.jsbmb.2016.03.004
0960-0760/

remain controversial [1,2]. Data from both human and animal trials indicate that serum 25D exerts an anabolic effect on bone while serum 1α,25-dihydroxyvitamin D_3 (1,25D) is associated with bone loss [3,4]. However, the mechanism for these differential actions is yet to be elucidated. Many cell types, including osteoblasts [5,6], express the gene coding for the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1), required for the conversion of 25D to 1,25D. The mature osteoblastic cell line MLO-A5 lays down a lamellar bone-like, mineralised matrix, [7,8] expresses genes for both the vitamin D receptor (VDR) and CYP27B1, and is capable of metabolising 25D into measurable 1,25D [9], and is therefore a suitable model for comparing the activities of 1,25D arising from either source [9,10]. In this study, we examined the effects of 25D and 1,25D on media 1,25D levels, the induction of 25-hydroxyvitamin D 24-hydroxylase (*Cyp24a1*) mRNA, cell proliferation, osteogenic differentiation, including induction of a range of osteoblast genes, and mineral deposition by MLO-A5 cells.

2. Materials and methods

The MLO-A5 cell line was kindly provided by Professor Lynda Bonewald (University of Missouri-Kansas City, MO, USA). The cells were maintained and seeded for osteogenic assays as previously described [10]. The treatments of vehicle (0.1% v/v ethanol), 25D (100 nM; Wako Pure Chemicals, Osaka, Japan) or 1,25D (1 nM; Wako Pure Chemicals) were incorporated with media and supplied to cells at confluence. Fresh media including vitamin D metabolites were replenished every 3 days. The levels of 1,25D in media supernatants were measured by the iSYS-1,25-dihydroxyvitamin D immunoassay (ImmunoDiagnostic Systems, Boldon, UK) in triplicate for each treatment group. *In vitro* mineralisation was assessed using Alizarin Red staining and quantified by measuring the light absorption at 405 nm following acetic acid dissolution of the Alizarin Red/calcium precipitate [11]. Cell proliferation was determined by measuring the distribution of a pre-incorporated fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), during each cell cycle [5]. At the time points indicated, total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and cDNA against the total RNA template was synthesised by the Superscript-III kit (Life Technologies) following the manufacturer's instructions. The mRNA levels of genes of interest were measured by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA), as per the manufacturer's instructions. The sequences of mouse species-specific primer sets for carbonic anhydrase II (*Car2*) were: forward: 5′-gagcttcacttggttcactgg-3′, reverse: 5′-tgtgaggcaggtccaatcttc-3′, and for matrix metallopeptidase 13 (*Mmp13*) were: forward: 5′-agaccttgtgtttgcagagc-3′, reverse: 5′-attgcatttctcggagcctg-3′. The primer sequences for other genes examined were described previously [10,12]. The specificity of amplification was validated by melt-curve analysis after each PCR reaction. For quantification, gene expression was normalised to that of the housekeeping gene β-*Actin*. Two-tailed t-tests were performed to analyse the media 1,25D levels at 24 and 72 h as well as the effects of both vitamin D metabolites on cell proliferation and mineralisation. Two-way ANOVA tests were performed to analyse the effects of 25D or 1,25D

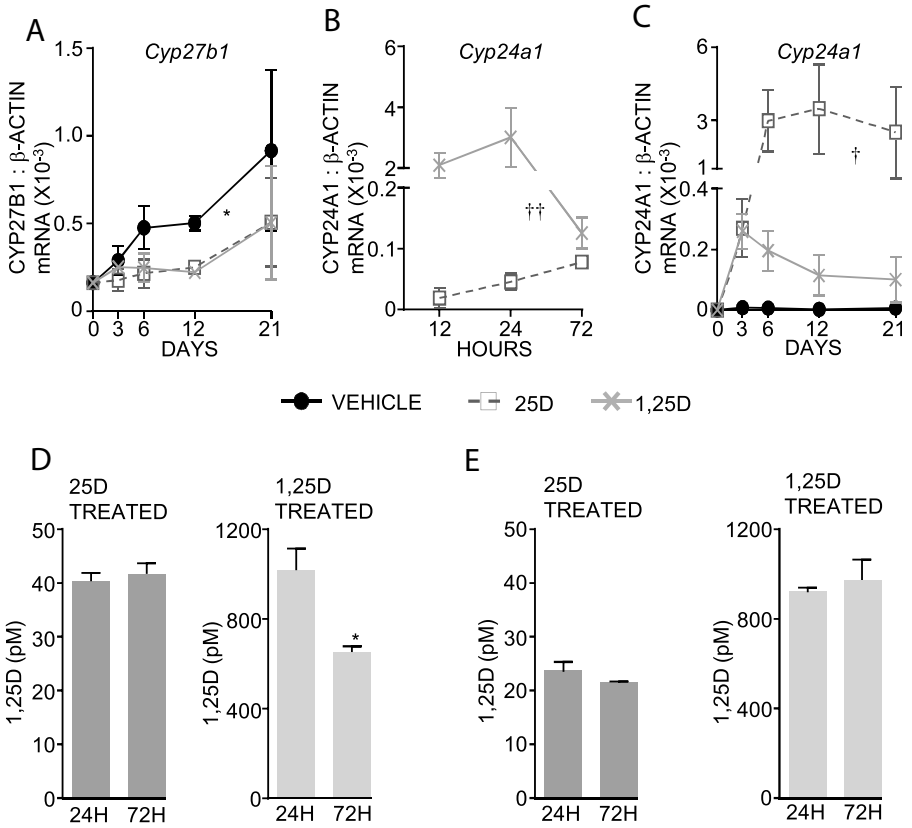

Fig. 1. The mRNA ratios of *Cyp27b1* (A) to *β-Actin* with vehicle (-•-), 25D (100 nM) (-▫-) and 1,25D (1 nM) (-×-) treatments for 21 days on MLO-A5 cultures; the mRNA ratios of *Cyp24a1* to *β-Actin* with these treatments within the first 72 h (B) and for 21 days (C) on MLO-A5 cultures. The locally produced 1,25D level from 25D (100 nM) treatment and remaining 1,25D level from 1,25D (1 nM) treatment, at the initial 24 and 72-h time points (D) and at 24 and 72-h time points following 18 days of culture (E). All data represent means ± standard errors of the mean (SEM) of three biological replicates. Statistical significance from the control group is indicated by $^*p < 0.05$ and † and †† indicate the effects of 25D treatment on *Cyp24a1* levels compared to 1,25D treated groups, with $p < 0.05$ and $p < 0.01$, respectively.