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Bone CYP27B1 gene expression is increased with high dietary calcium and in mineralising osteoblasts☆

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ABSTRACT

Although the regulation of renal 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1) is reasonably well understood, the same cannot be said about the regulation of bone CYP27B1 expression. We have compared the regulation of kidney and bone CYP27B1 expression with modulation of dietary vitamin D and calcium levels. Vitamin D-deplete and vitamin D-replete female Sprague–Dawley rats were fed either 1% Ca (HC) or 0.1% Ca (LC) diets from 6 months of age. At 9 months of age, animals were killed for mRNA analyses from kidney and bone by real-time RT-PCR. Additionally, primary bone cells were cultured from pCYP27B1-Luc reporter mice in pro-osteogenic media over 15 days and analysed for mRNA for CYP27B1 and other osteogenic markers. *In vivo* expression of bone CYP27B1 mRNA was independent of changes to kidney CYP27B1 levels with both serum 1,25D and PTH as negative determinants of bone CYP27B1 mRNA levels. Bone cells in pro-mineralising conditions significantly increased CYP27B1 promoter activity over 15 days ($P < 0.001$) which preceded marked increases in alkaline phosphatase, osteocalcin and vitamin D receptor mRNA expression and mineral deposition. These findings confirm that the regulation of bone CYP27B1 is unique from that in the kidney, and may play an important role in bone formation.

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1. Introduction

The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25D), is an important regulator of calcium and phosphorus homeostasis. In this context, 1,25D contributes to the health of the skeleton by directly stimulating the absorption of calcium in the small intestine. The final and rate limiting step in the vitamin D synthetic pathway is the conversion of 25-dihydroxyvitamin D_3 (25D) to 1,25D and is catalysed by the mitochondrial enzyme 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1). While circulating 1,25D has been shown to arise almost exclusively from renal CYP27B1 activity [1], CYP27B1 expression has also been identified in numerous other tissues [2,3]. The expression of CYP27B1 in tissues such as bone, skin, mammary and prostate is hypothesized to produce 1,25D that acts in an autocrine or paracrine manner to regulate cellular proliferation and differentiation. Recently, osteoblasts cultured in the presence of 25D have been demonstrated to generate sufficient 1,25D to regulate osteoblast cell activity [4–6]. Furthermore, we have previously reported a potential *in vivo* autocrine or paracrine activity of 1,25D produced by bone cells with the observation that increased expression of CYP27B1 mRNA in bone cells was strongly associated with increased bone cell expression of CYP24, a gene primarily regulated by 1,25D [7]. Therefore there is a strong suggestion that CYP27B1 activity in bone may serve separate biological roles to 1,25D in the circulation derived from the kidney. While the regulation of CYP27B1 gene expression in osteoblasts appears to differ from its regulation in renal cells [8], it is poorly understood particularly at the *in vivo* level. This study provides evidence for the specific regulation of bone CYP27B1 expression by dietary calcium. Furthermore, the regulation of osteoblastic CYP27B1 gene expression is shown to be strongly associated with the expression of genes associated with osteoblast maturation, as well as increased mineralisation.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats ($n = 24$) were allocated to either vitamin D-replete or vitamin D-deplete dietary treatment groups. Vitamin D-deplete (D−) animals were bred from dams fed a vitamin D-deficient diet and housed in an incandescent light environment. The D+ animals were maintained on 1% calcium (HCa) supple-

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mented semi-synthetic diet (AIN-93-VX, ICN, CA, USA) containing 1000 IU/kg diet of vitamin D. The D− animals were maintained on a 1% calcium semi-synthetic diet deficient in vitamin D (AIN Special vitamin D-deficient mixture, ICN, CA, USA) [9]. All animals were maintained on their assigned diets until 6 months of age at which time one half of each D+ animals or and D− animals were either maintained on the HCa diets or transferred to a 0.1% calcium (LCa) semi-synthetic diet. These animals were fed their assigned diets for a further 3 months, at which point they were killed. All animal procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

2.2. Biochemical analyses

Non-fasting blood samples were collected at time of death for analyses. A chemistry analyser was used to measure serum calcium (Cobas Bio, Roche, IN, USA). Serum 1,25D and 25D were measured by RIA (Immunodiagnostic Systems Ltd., Bolden, UK). Serum PTH was measured using rat-specific, two-site immunoradiometric assays (Immutopics, Inc., San Clemente, CA, USA).

2.3. Messenger RNA analyses

The isolation of total RNA from whole rat kidney and femora was performed using a modified phenol/chloroform extraction method [10,11]. First strand cDNA synthesis was performed as previously described [12]. Real-time RT-PCR was performed using specific primers (Geneworks, SA, Australia) and Taqman® fluorogenic probes (PE Applied Biosystems, CA, USA). The sequence of the primers and fluorogenic probes for CYP27B1 (Acc. No.: NM053763), CYP24 (Acc. No.: X59506), VDR (Acc. No.: NM017058) and GAPDH (Acc. No.: NM017008) were designed as previously described [12]. Levels of CYP27B1, CYP24 and VDR mRNA were expressed as a ratio to the levels of GAPDH mRNA.

2.4. Primary bone cell cultures

Primary osteoblasts were cultured from femoral and tibial bone marrow isolated from pCYP27B1 (−1501 bp)-Luc transgenic mice [13–15] and seeded in αMEM (Sigma) containing 10% FBS and antibiotics. After 3 days of proliferation designated experimental day 0, media were replaced, and supplemented with 100 μM L-ascorbate-2-phosphate, 10 nM dexamethasone and 1.8 mM KH_2PO_4. The media were changed every 2nd day until day 15. At 3-day intervals, luciferase activity was measured following lysis of independent cultures (Promega), and additional dishes were stained with Alizarin Red S (Sigma). At each time point, total RNA was extracted from cultures and complementary DNA prepared. Target mRNA expression was quantified by qRT-PCR.

2.5. Data expression and statistical analyses

The effects of dietary treatment on biochemical markers and mRNA levels were statistically analysed with multivariate analysis of variance and Tukey's post hoc test analysis. Multiple linear regression analyses were used to determine the relationship between mRNA levels of specific target genes and biochemical markers.

3. Results

At time of death, mean body weights for each dietary group were not significantly different from each other (data not shown). Femur length was marginally shorter in the vitamin D-deficient animals (37.9 ± 0.3 mm) when compared to vitamin D-replete animals (39.2 ± 0.1 mm), independent of changes to dietary calcium levels

Table 1
Serum biochemistry of 25D, 1,25D, calcium, phosphate and PTH in animals from each dietary treatment group.

		LCa	HCa
D+	25D (nmol/L)	134.3(3.1)	96.9 (7.2)#
	1,25D (pmol/L)	405.5 (50.2)	19.6(3.9)#
	Calcium (mmol/L)	2.48 (0.02)	2.69 (0.05)#
	PTH (pmol/L)	23.5 (2.9)	2.07 (0.7)#
D−	25D (nmol/L)	14.0(1.5)*	14.3(1.7)*
	1,25D (pmol/L)	33.8 (0.3)*	13.2(2.9)*,#
	Calcium (mmol/L)	1.99(0.12)*	2.32 (0.03)*,#
	PTH (pmol/L)	167.0(28.8)*	32.2(3.1)*,#

Values are mean (SEM), $n = 5$. 25D, 25-hydroxyvitamin D3; 1,25D, 1,25 dihydroxyvitamin D3; PTH, parathyroid hormone.
* $P < 0.05$ significant difference between D− and D+.
# $P < 0.05$ significant difference between LCa and HCa.

($P < 0.01$). Serum 25D levels in the rats fed the vitamin D-deficient diet were reduced to approximately 14 nmol/L compared to 134.3 (±3.1) nmol/L achieved on the D+/LCa and 96.9 (±7.2) nmol/L on the D+/HCa diets respectively (Table 1). Serum calcium levels in the D−/LCa rats were low compared to all other animals ($P < 0.05$). This hypocalcemia was associated with markedly elevated serum PTH levels ($P < 0.01$). In the D−/HCa animals, the high calcium diet was sufficient to normalise serum calcium levels. The D+/HCa animals had the highest serum calcium levels and lowest serum PTH levels ($P < 0.05$).

Kidney levels of CYP27B1 mRNA in animals fed the D−/LCa diet were 100-fold higher than those found in the D−/HCa animals ($P < 0.0001$) (Fig. 1A). In contrast, bone CYP27B1 mRNA levels were more than 2-fold lower in D−/LCa animals when compared with D−/HCa animals ($P < 0.05$). A similar inverse relationship in CYP27B1 mRNA levels between kidney and bone occurred in the D+ animals. Kidney CYP24 mRNA levels in the D− animals were

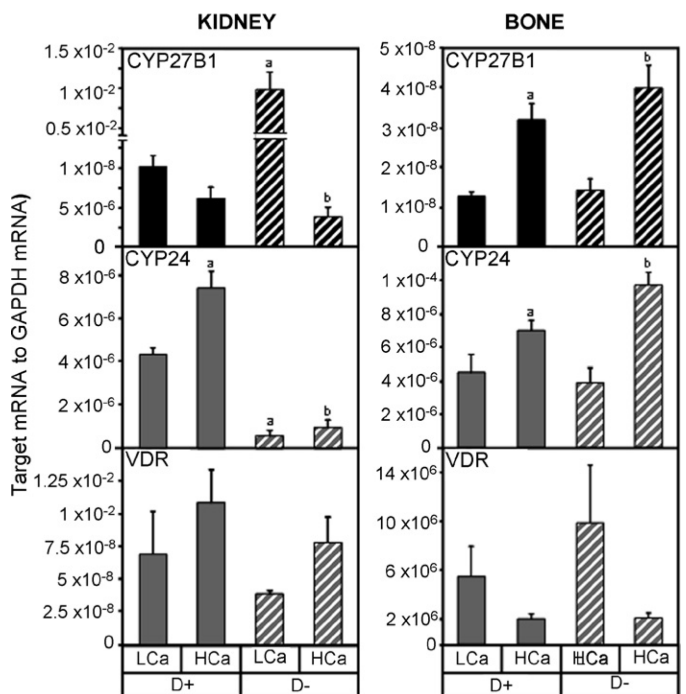

Fig. 1. Levels of CYP27B1, CYP24 and VDR mRNA (relative to GAPDH mRNA) in kidney (A) and bone (B) tissue from each dietary treatment group. Values are mean ± SEM ($n = 5$, except D+/LCa where $n = 4$). [a]$P < 0.05$ vs D+/LCa; [b]$P < 0.01$ vs D−/LCa. D+/LCa, vitamin D-replete fed 0.1% calcium; D+/HCa, vitamin D-replete fed 1% calcium; D−/LCa, vitamin D-deplete fed 0.1% calcium; D−/HCa, vitamin D-deplete fed 1% calcium; CYP27B1, 25-hydroxyvitamin D 1α-hydroxylase; CYP24, 25-hydroxyvitamin D 24-hydroxylase, VDR, vitamin D receptor.

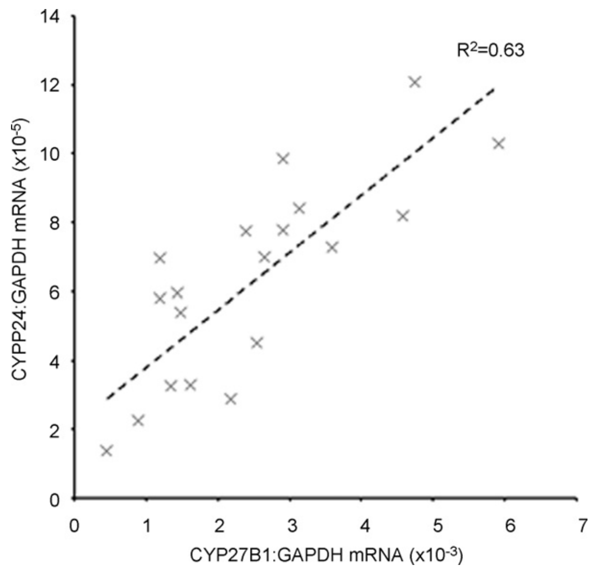

Fig. 2. Relationship between bone CYP27B1 mRNA and bone CYP24 mRNA. The coefficient of determination (R^2) is shown for the line-of-best-fit. CYP27B1, 25-hydroxyvitamin D 1α-hydroxylase; CYP24, 25-hydroxyvitamin D-24-hydroxylase.

markedly reduced when compared to the D+ animals ($P < 0.001$) with the highest levels of CYP24 mRNA in the D+/HCa animals ($P < 0.01$). Interestingly, bone CYP24 mRNA levels were not reduced as a result of vitamin D-depletion and did not correlate with serum 1,25D levels (data not shown). However, bone CYP24 mRNA levels were strongly and positively associated with bone CYP27B1 RNA levels ($R^2 = 0.63$, $P < 0.001$) (Fig. 2).

Serum 1,25D levels were positively correlated with kidney CYP27B1 mRNA levels and negatively correlated with kidney CYP24 mRNA levels in the D+ animals (data not shown). Multiple linear regression analysis indicated that the combination of CYP27B1 and CYP24 mRNA levels in the kidney accounted for 85% variance of serum 1,25D levels, although CYP27B1 mRNA did not achieve statistical significance (data not shown). In the D− animals, the markedly elevated serum PTH levels in the LCa fed animals was responsible for the strong increase in kidney CYP27B1 mRNA ($R^2 = 0.92$, $P < 0.0001$) (data not shown). In contrast, the levels of bone CYP27B1 mRNA were negatively correlated with serum 1,25D ($R^2 = 0.53$, $P < 0.05$) and while PTH was not an individual determinant of bone CYP27B1 mRNA levels, in a multiple regression including 1,25D, PTH was a significant negative determinant ($P < 0.04$) (Table 2).

In the time course experiments with pCYP27B1-Luc mouse bone marrow cultures, the alizarin red staining showed increasing mineral content in bone cell cultures after day 6 (Fig. 3A). Luciferase measurements showed activation of the CYP27B1 promoter occurring after day 3, with significant increases in expression until day 12 (Fig. 3B). OCN mRNA levels were only detected after 9 days of culture with the highest levels observed at day 15 of culture (Fig. 3C). ALP, Type 1 Collagen and VDR mRNA all increased as a function of mineralisation.

4. Discussion

Despite the fact that the regulation of CYP27B1 gene expression in the kidney has been well described, much less is known about the regulation of CYP27B1 activity in bone cells. It is evident that CYP27B1 activity in bone is biologically relevant as we have previously demonstrated that CYP27B1 expression in mature osteoblasts is essential for 1,25D synthesis and down-stream gene regulation of a number of vitamin D responsive genes including osteocalcin and CYP24 [6]. Furthermore, intracrine activities of CYP27B1 in chondrocytes [16] and osteoclasts ([17], this issue) play important roles in the regulation of cellular activity and development.

While there is limited understanding of CYP27B1 gene regulation in bone, these current data support the view that the expression of bone CYP27B1 mRNA is controlled by different factors to those well described for renal CYP27B1 gene regulation. In particular, serum PTH does not appear to stimulate bone CYP27B1 gene expression. This is consistent with our previous report that PTH did not up-regulate a reporter construct containing the full length (−1501 bp) of the 5′-flanking region of the human CYP27B1 gene in transiently transfected ROS 17/2.8 osteoblast cells [18]. Similarly, PTH has been shown to have no effect on the expression of CYP27B1 mRNA in pulmonary macrophages [19]. Our current data show a negative association between PTH and bone CYP27B1 mRNA levels suggesting that PTH may in fact inhibit CYP27B1 gene expression in bone. In addition, serum 1,25D levels were negatively correlated with bone CYP27B1 mRNA levels, which suggests that the control of bone CYP27B1 mRNA levels may be co-ordinated with changes in the renal production of 1,25D. Inhibition of CYP27B1 mRNA expression by 1,25D itself has been described before in a mouse kidney cell line [20]. This mechanism involves an E-box site in the 5′-flanking region of the CYP27B1 gene and a bHLH E47 homologue named VDIR, which can mediate the inhibitory actions of 1,25D. However, 1,25D does not repress transcriptional activity of the −1501 bp promoter region of the human CYP27B1 gene [18] and a role for VDIR-mediated regulation of CYP27B1 expression is yet to be established in any tissue or cells outside of the kidney.

Of considerable interest is the strong positive correlation found between bone CYP27B1 mRNA and bone CYP24 mRNA levels. This result, which is in contrast to the negative association between CYP27B1 and CYP24 mRNA levels in the kidney of D+ animals, confirms our previous report of co-expression of these synthetic and catabolic enzymes in the bone with changes in age [7]. Furthermore, the levels of bone CYP24 mRNA in the current study did not correlate with circulating levels of 1,25D, suggesting that bone CYP24 mRNA expression is not determined by renal synthesis of 1,25D. This is consistent with our previous findings in HOS osteoblast cells, using the highly specific method of RNAi gene silencing for CYP27B1. In that study, 1,25D synthesis and the expression of osteocalcin, RANKL, and CYP24 mRNA in response to 25D, were all shown to be dependent on bone cell CYP27B1 gene expression [4,6] suggesting that the expression of CYP24 mRNA in bone is not

Table 2
Serum 1,25D and PTH are negative determinants of bone CYP27B1 mRNA levels (corrected by GAPDH) in animals from all dietary vitamin D groups.

Independent variable	Equation	R^2	P-Value
1,25D	Bone CYP27B1 = -3.9×10^{-6}(1,25D) + 2.9×10^{-3}	0.4	0.05
PTH	Bone CYP27B1 = -1.56×10^{-5}(PTH) + 3×10^{-3}	0.2	0.05
1,25D	Bone CYP27B1 = -5.2×10^{-6}(1,25D)		0.003
+PTH	-1.4×10^{-5}(PTH)		0.003
	$+3.7 \times 10^{-3}$	Multiple $R^2 = 0.73$	

1,25D, 1,25 dihydroxyvitamin D_3; PTH, parathyroid hormone; CYP27B1, 25-hydroxyvitamin D 1α-hydroxylase.

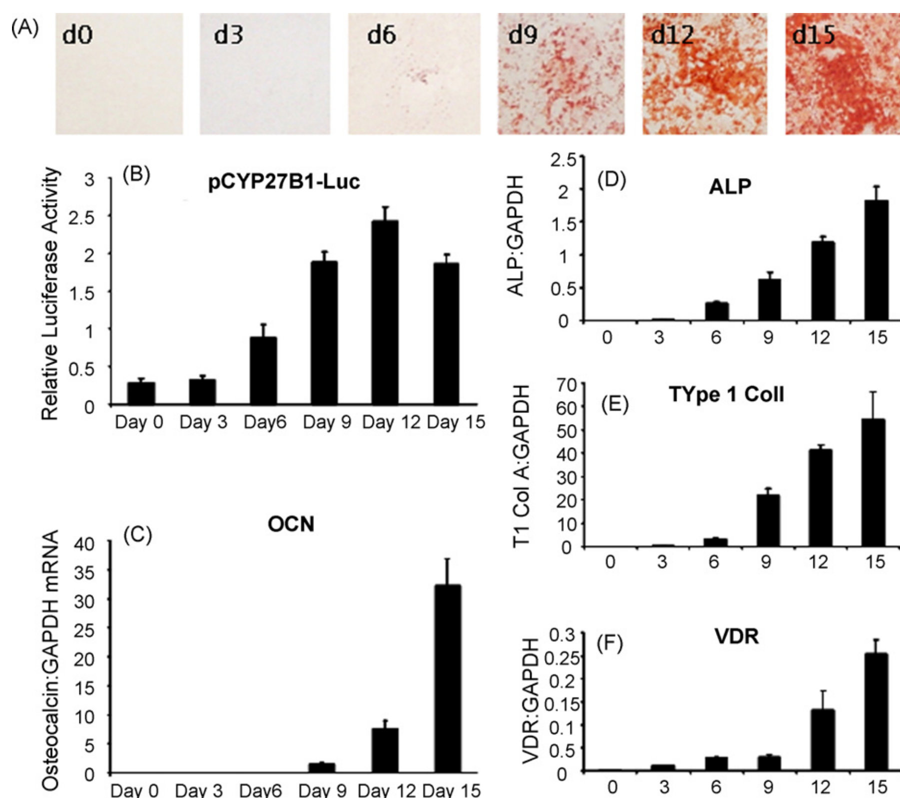

Fig. 3. (A) Alizarin red staining of mineral in pCYP27B1(−1501 bp)-Luc mice bone cell cultures from day 0 to 15. (B) CYP27B1 promoter driven luciferase activity in bone marrow cell cultures grown in pro-osteogenic media. Cells were derived from femur and tibia of pCYP27B1(−1501 bp)-Luc mice. Graph represents mean ± SD. (C–F) OCN, ALP, Type 1 Collagen and VDR mRNA expression as quantified by qRT-PCR from bone cell cultures. Data are normalised to GAPDH and represented as mean ± SD.

determined by circulating 1,25D levels but rather by the production of 1,25D in bone cells resulting from the local expression of CYP27B1.

It is not possible to identify which cells are responsible for increased bone CYP27B1 mRNA expression in response to high dietary calcium. However, such treatment has been shown to improve mineralisation when compared to rats fed low calcium diets [21,22]. This observation suggests that increased CYP27B1 activity in bone is associated with bone anabolic processes and is consistent with the phenotype of increased bone mineral observed in transgenic mice that over-express the vitamin D receptor specifically in mature osteoblasts [23]. Moreover in the present study, mouse primary osteoblast cells cultured under pro-osteogenic conditions have increased CYP27B1 promoter activity as assessed by luciferase activity in late maturing and early mineralising cultures. This increased CYP27B1 promoter activity was also associated with increased expression of key genes associated with osteoblast maturation and mineralisation.

In summary, we have demonstrated that expression of bone CYP27B1 mRNA expression is inversely related to the levels of CYP27B1 mRNA in the kidney. Thus, the supply of 1,25D to bone cells from endocrine and intracrine sources is more complex than hitherto has been generally recognised. One hypothesis is that the renal supply of 1,25D is necessary to maintain calcium and phosphate homeostasis, through regulation of intestinal calcium and phosphate absorption and stimulating bone resorption, which can override the bone cell production of 1,25D. The bone cell production of 1,25D may act to improve bone mineralisation when calcium and phosphate levels are adequate. The strong co-ordinated expression of bone CYP24 with bone CYP27B1 mRNA levels suggests that the production of 1,25D in bone is more active in bone cells than the renal supply. In osteoblasts, this activity of CYP27B1 is greatest in the mature osteoblasts, suggesting the local production of 1,25D is important for the mineralisation process. Such a hypothesis is yet to be tested directly and is currently being investigated.

Conflicts of interest

None.

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