Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Skeletal Biology and Medicine*

Vitamin D action

Lessons learned from genetic mouse models

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Mouse models with targeted deletion of the genes encoding the enzyme 25-hydroxyvitamin D 1alpha-hydroxylase [1α(OH)ase], and the vitamin D receptor (VDR), have provided considerable insight into the regulation of mineral and skeletal physiology by 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. Dietary manipulation induced different phenotypic changes and demonstrated that parathyroid gland function is coordinately regulated by calcium and 1,25$(OH)_2$D, but that mineralization of bone reflects ambient calcium (and phosphorus) levels rather than direct actions of the 1,25$(OH)_2$D/VDR system. In contrast, increased calcium absorption and optimal osteoblastogenesis and bone formation is modulated by the 1,25$(OH)_2$D/VDR system. Similar models have also been employed to study extraskeletal vitamin D actions. For example, increased blood pressure, activation of the renin/angiotensin system, myocardial hypertrophy, and cardiac dysfunction were observed in 1αOHase$^{-/-}$ mice, and could be prevented by 1,25$(OH)_2D_3$ administration. These models allow controlled examination of the pathophysiology associated with1,25$(OH)_2$D deficiency and intervention to prevent and treat these disorders.

Keywords: vitamin D; genetic mouse models; bone; calcium; extraskeletal vitamin D action

Introduction

The enzyme 25-hydroxyvitamin D-1alpha-hydroxylase (1αOHase) catalyzes the 1α-hydroxylation of the vitamin D metabolite, 25-hydroxyvitamin D (25OHD) and, as a result, is critical to the production of the active form of vitamin D, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D].[1] The 1αOHase is a mitochondrial enzyme comprising a cytochrome P-450 (which provides the specificity of the 25(OH)D metabolite for the 1αOHase), a ferredoxin, and a ferredoxin reductase, and the renal enzyme is the major source of the circulating concentration of 1,25$(OH)_2$D. Parathyroid hormone (PTH), acting through the PTH receptor by cyclic AMP signal transduction,[2] has been shown to be a major regulator of the renal enzyme by enhancing transcription of the gene encoding the cytochrome P-450, *CYP27B1*. Independently, low calcium and phosphorus also upregulate the *CYP27B1* gene. 1,25$(OH)_2$D per se exhibits negative feedback regulation of the renal enzyme, leading to reduced 1,25$(OH)_2$D synthesis, by interacting through its ligand-bound receptor with upstream elements in the promoter of the *CYP27B1* gene and downregulating transcription. Recently, the osteoblast/osteocyte-derived peptide, fibroblast growth factor 23 (FGF23), has also been demonstrated to be a potent inhibitor of renal 1α(OH)ase,[3] at least in part by transcriptional inhibition. Extrarenal sites of 1αOHase expression have been reported, but the best documented of these sites functionally include macrophages, placenta, and skin. Nevertheless, extrarenal 1αOHases have been implicated in the local generation of 1,25$(OH)_2$D from circulating 25OHD in a multitude of other cells and tissues, which may then act in a paracrine/autocrine or intracrine mode. The extrarenal 1αOHases appear to be regulated differently from the renal enzyme; for example, cytokines, and not PTH, upregulate expression of the 1αOHase in the macrophage.[4]

After being taken up by target cells and binding to intracellular shuttle proteins, 1,25$(OH)_2$D

doi: 10.1111/j.1749-6632.2009.05226.x

binds to its cognate receptor, the vitamin D receptor (VDR), a member of the nuclear receptor superfamily.[5] The ligand-activated VDR heterodimerizes with the retinoid X receptor in the nucleus of target cells and the dimer binds to vitamin D response elements (VDREs) on target genes.[6] Coregulators are recruited to link the dimer to the basal transcriptional machinery and thereby modulate gene transcription, and differences in gene-specific, tissue-specific, and differentiation-stage-specific coregulators present in vitamin D target cells modulate the wide array of genes that are regulated by this vitamin in each tissue at any given time. In addition, whether $1,25(OH)_2D$ causes upregulation or downregulation may be gene specific. The widespread distribution of the VDR, which is expressed in many cells throughout the body, has lent support to the concept that vitamin D may have actions beyond the calcium/skeletal homeostatic system.

Experiments of nature have confirmed the special importance of vitamin D in calcium and skeletal homeostasis, despite the potential significance of any extraskeletal actions of vitamin D. Thus loss-of-function mutations in the 1αOHase gene in humans cause the autosomal recessive disorder vitamin D-dependent rickets type 1 (VDDR1), also known as pseudo-vitamin D-deficiency rickets.[7] The disease is caused by homozygosity for a single abnormal *CYP27B1* gene or by compound heterozygosity of abnormal *CYP27B1* genes, leading to 1α(OH)ase deficiency, and is characterized by low serum calcium and phosphate, secondary hyperparathyroidism, and low circulating levels of $1,25(OH)_2D$. Loss-of-function mutations in the VDR in humans cause the autosomal recessive disorder vitamin D-dependent rickets type 2, also known as hereditary vitamin D-resistant rickets.[8] This syndrome, although presenting clinically with rachitic changes, can be distinguished from VDDR1 by the elevated circulating levels of $1,25(OH)_2D$ and by the lack of responsiveness to vitamin D treatment. Consequently, in human disorders characterized by either loss of the capacity to synthesize the active form of vitamin D, or by loss of the capacity of vitamin D to act, skeletal and mineral abnormalities are the major phenotypic presentation, thus pointing to the critical role of vitamin D for this function.

Actions of $1,25(OH)_2D$ on skeletal and mineral homeostasis

We and others have genetically engineered *Cyp27b1*[9,10] and VDR knockout mice,[11–14] which has permitted more controlled and extensive examination of the phenotypes than are possible by examination of the corresponding human disorders VDDR1 and VDDR2, respectively. These mouse phenocopies have provided important insights into both the skeletal and extraskeletal actions of the vitamin D system.

We generated a 1αOHase null mouse by deleting exons encoding both the hormone-binding domain, and the heme-binding domain of *Cyp27b1*.[9] The null mutant mice [$1(OH)ase^{-/-}$] appeared grossly normal from birth until weaning but then displayed marked growth retardation after weaning. Examination of known $1,25(OH)_2D$ target genes revealed that the expression of the mRNA encoding the 25-hydroxyvitamin D-24 hydroxylase enzyme [24(OH)ase], which catalyzes the first step in the degradative pathway, was almost completely ablated in the null mutant mice. Circulating concentrations of $1,25(OH)_2D$ were undetectable in the homozygous null mice, but serum 25(OH)D concentrations were elevated, likely reflecting its accumulation in the absence of 1- and 24-hydroxylating enzymes. Serum calcium and phosphate concentrations were reduced and serum PTH and urinary phosphate concentrations were markedly elevated in the homozygous null mice. Typical histologic features of advanced rickets were observed, including widening of the epiphyseal growth plates, mainly because of a widened and disorganized hypertrophic zone, inadequate mineralization of cartilage, of the primary spongiosa, and of cortical bone, and an increase in osteoid in both trabecular and cortical bone. Osteoblasts lining bone surfaces were increased and trabecular bone in the primary spongiosa was augmented.

We compared the phenotype of the $1\alpha(OH)ase^{-/-}$ with that of the VDR null mouse.[12] Expression of renal 1α(OH)ase mRNA is elevated and 24(OH)ase mRNA is suppressed in $VDR^{-/-}$ mice. Thus in the absence of VDR, 1α(OH)ase could not be suppressed or 24(OH)ase stimulated by endogenous $1,25(OH)_2D$, resulting in high circulating $1,25(OH)_2D$ concentrations. We

also determined the consequences of 1α(OH)ase deficiency on the $VDR^{-/-}$ phenotype by crossbreeding $1\alpha(OH)ase^{+/-}$ and $VDR^{+/-}$ mice to obtain the compound mutants, $1\alpha(OH)ase^{-/-}VDR^{-/-}$. These three genetically diverse mutants were analyzed after exposure to different environments (i.e., diets with differing calcium intakes and after administering exogenous $1,25(OH)_2D_3$).[15] Thus, after weaning, mice received either a high calcium intake on which they remained hypocalcemic, a high calcium intake with injections of $1,25(OH)_2D_3$ intraperitoneally three times per week, or a "rescue" diet containing high calcium, high phosphorus, and 20% lactose. This diet appears to increase calcium transport, at least in the rodent intestine, independent of the $1,25(OH)_2D$/VDR system and can normalize serum calcium in vitamin D deficiency, possibly by enhancing passive transport of calcium via a paracellular route.

Regulation of intestinal calcium absorption

The presence of hypocalcemia after weaning onto the high calcium intake in both the $1\alpha(OH)ase^{-/-}$ mice with intact VDR but deficient $1,25(OH)_2D$ production and in the $VDR^{-/-}$ mice with elevated endogenous levels of $1,25(OH)_2D$ but deficient VDR, and the failure of treatment with exogenous $1,25(OH)_2D_3$ to normalize the serum calcium in $VDR^{-/-}$ mice on the high calcium intake, strongly indicates that both $1,25(OH)_2D$ and the VDR are necessary for optimal intestinal absorption of calcium. In contrast, $1\alpha(OH)ase^{-/-}$ mice on the same diet, when treated with the same dose of exogenous $1,25(OH)_2D_3$ did normalize serum calcium both in our studies and in those of others.[16] Intestinal calcium absorption, therefore, appears to require both $1,25(OH)_2D$ and the VDR.

Regulation of the 1α(OH)ase and of the 24(OH)ase enzymes

Although both $1,25(OH)_2D$ and the VDR are required for downregulation of gene expression of the 1α(OH)ase and upregulation of 24(OH)ase gene expression *in vivo*,[1,17,18] elimination of hypocalcemia alone, using a rescue diet, also normalized 24(OH)ase in $1\alpha(OH)ase^{-/-}$ mice and both 1α(OH)ase levels and 24(OH)ase in $VDR^{-/-}$ mice. This, therefore, demonstrates a calcium effect on gene expression of these enzymes independent of the $1,25(OH)_2D$/VDR system. Whether this effect of calcium is entirely indirect, by suppressing ambient PTH concentrations,[1,17,18] or is partly direct, remains to be determined.

Regulation of parathyroid gland function

Calcium can inhibit PTH secretion via the calcium-sensing receptor (CaSR),[19] increase intracellular proteolysis of PTH to inactive fragments,[20] and decrease PTH mRNA translation.[21] Calcium can also decrease parathyroid cell growth.[22] $1,25(OH)_2D$ has been reported to inhibit PTH gene transcription[23] and parathyroid cell growth *in vitro*.[22] On a normal or high calcium diet, when hypocalcemia is present, increased circulating PTH concentrations and enlarged parathyroid glands occur in both the $1\alpha(OH)ase^{-/-}$,[9,10] and the $VDR^{-/-}$[12,15] mutant mice. On a rescue diet, serum PTH concentrations fell in both mutants, indicating that raising the ambient calcium could alone normalize PTH secretion. Parathyroid gland size was reduced, but remained moderately enlarged in $1\alpha(OH)ase^{-/-}$ mice on a rescue diet. Treatment of $1\alpha(OH)ase^{-/-}$ mice with exogenous $1,25(OH)_2D_3$ normalized serum calcium and also normalized parathyroid gland size. Consequently both calcium and $1,25(OH)_2D$ appear to act cooperatively to diminish PTH production and parathyroid gland size (Fig. 1).

Bone and cartilage remodeling

Osteoblast numbers, bone formation, and bone volume are markedly increased in all hypocalcemic knockout models of the vitamin D/VDR system (i.e., $1\alpha(OH)ase^{-/-}$ mice, $VDR^{-/-}$ mice, and $1\alpha(OH)ase^{-/-}VDR^{-/-}$ double mutants) on either a lactose-free, normal, or high-calcium diet.[9,10,12,15] This appears to be due to the "anabolic" effect of PTH, which is markedly elevated in association with the severe secondary hyperparathyroidism in these animals. The increased bone volume is largely due to increased unmineralized osteoid. Although a sustained elevation of PTH is generally associated with increased osteoclastic bone resorption as well as increased bone formation, the osteoclast number and resorbing surface are generally not elevated in these models. This suggests, therefore, that there is uncoupling of bone turnover in the presence of a defective $1,25(OH)_2D$/VDR system, and an intact $1,25(OH)_2D$/VDR system appears to be required

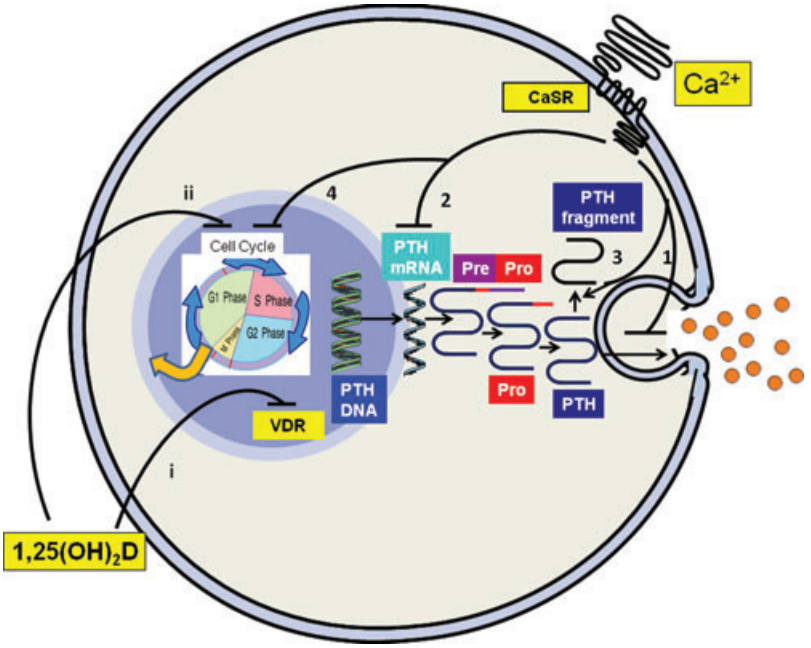

Figure 1. **Interdependent regulation of parathyroid cell function by calcium (Ca^{2+}) and $1,25(OH)_2D$. Calcium, acting via the calcium-sensing receptor (CaSR), can inhibit secretion of intact PTH (1). Calcium can also inhibit PTH mRNA translation, (2) thereby reducing production of the biosynthetic precursor PrePrePTH, and can increase intracellular proteolysis of PTH to inactive fragments (3). The sterol $1,25(OH)_2D$ can inhibit PTHrP gene transcription via the vitamin D receptor(VDR) (i). Calcium and $1,25(OH)_2D$ can each inhibit cell cycle progression (4 and ii, respectively) and reduce parathyroid cell proliferation.**

for an appropriate osteoclastic response to increased PTH.

In view of the fact that osteoclast/chondroclast production at the chondro–osseous junction may also be defective, diminished removal of hypertrophic chondrocytes may occur in this region, leading to altered cartilage growth plate remodeling. Therefore the enlargement of the cartilaginous growth plate, and notably the hypertrophic zone, may also be in part due to reduced activity of the $1,25(OH)_2D$/VDR system on the chondroclast/osteoclast system.[24]

Mineralization of bone

On a rescue diet, mineralization of bone normalized, and osteoid accumulation returned to wild-type levels in $1\alpha(OH)ase^{-/-}$ mice, $VDR^{-/-}$ mice, and $1\alpha(OH)ase^{-/-}VDR^{-/-}$ compound mutants.[15,22–25] Consequently mineralization of bone appears to be determined by ambient calcium and phosphate levels rather through the direct participation of the $1,25(OH)_2D$/VDR system.

Effects of $1,25(OH)_2D$ on bone volume

$1,25(OH)_2D_3$ has been shown to be a potent stimulator of osteoclastogenesis *in vitro*, and administration of high doses of $1,25(OH)_2D_3$ can exert an osteoclastogenic and bone-resorbing effect in wild-type animals *in vivo*.[29] However, in 4-month-old $1\alpha(OH)ase^{-/-}$, $VDR^{-/-}$, and $1\alpha(OH)ase^{-/-}VDR^{-/-}$ mutant mice, when hypocalcemia and secondary hyperparathyroidism were prevented by a rescue diet, osteoblast numbers, the mineral apposition rate, and bone volume were suppressed below levels seen in wild-type mice.[15] This suggested that the $1,25(OH)_2D$/VDR system may exert a skeletal "anabolic" effect, which is necessary to sustain basal bone-forming activity and which is unmasked when the defective $1,25(OH)_2D$/VDR system exists in the presence of normal PTH (Fig. 2). Previous studies in other

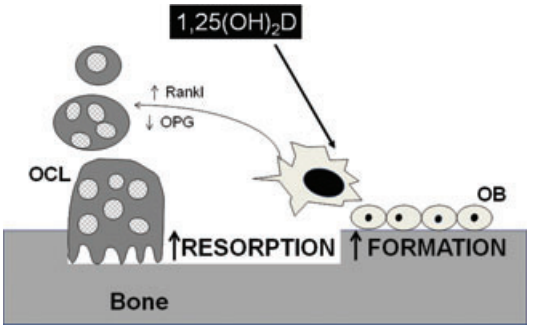

Figure 2. Effects of $1,25(OH)_2D$ on bone turnover. $1,25(OH)_2D$ can increase osteoblast differentiation and bone matrix production. $1,25(OH)_2D$ can also increase RANKL release and decrease OPG release from osteoblastic cells and stimulate osteoclastogenesis, resulting in bone resorption.

model systems have also pointed to an anabolic effect of $1,25(OH)_2D$.[26]

We next examined skeletal development in $1\alpha(OH)ase^{-/-}$ mice in the neonatal period and compared them to mice with targeted deletion of the PTH gene ($PTH^{-/-}$ mice) and to compound mutant $PTH^{-/-}1\alpha(OH)ase^{-/-}$ mice.[27] At 2 weeks of age, both mutants showed reduced osteoblastic bone formation. These results therefore showed that $1\alpha(OH)ase^{-/-}$ mice are osteopenic as early as 2 weeks of age and suggested that PTH plays a predominant role in appositional bone growth, whereas $1,25(OH)_2D$ acts predominantly, although not exclusively on endochondral bone formation.

To eliminate the potential confounding effects on the skeleton of endogenous PTH and of endogenous $1,25(OH)_2D$, double homozygous $PTH^{-/-}1\alpha(OH)ase^{-/-}$ mice were treated with $1,25(OH)_2D_3$, from the age of 4 days to age 14 days and compared with vehicle-treated animals.[28] Exogenous $1,25(OH)_2D_3$ increased both trabecular and cortical bone, augmented both osteoblast number and type I collagen deposition in bone matrix, and upregulated expression levels of the osteoblastic genes alkaline phosphatase, type I collagen, and osteocalcin. The results indicated that administered $1,25(OH)_2D_3$ can promote endochondral and appositional bone increases independent of endogenous PTH.

In summary these studies show the fundamental role of the vitamin D/VDR system on mineral and skeletal homeostasis and also point to discrete and interacting functions of calcium and the vitamin D system in modulating mineral and skeletal homeostasis.

Extraskeletal actions of $1,25(OH)_2D$

In view of the widespread distribution of the VDR[30] and the broad spectrum of vitamin D-dependent genes determined from gene chip arrays,[31] it seemed possible that vitamin D might subserve important functions other than the regulation of mineral and skeletal homeostasis. Indeed, in recent years, vitamin D action has been implicated in a variety of critical processes including immune function, cancer development, and cardiovascular disease.[32] The identification of circulating 25OHD as a more valuable clinical biomarker then circulating $1,25(OH)_2D$ for the many health-related effects in which vitamin D has been implicated[32] has also lent support to the importance of extrarenal $1\alpha(OH)ases$[33] in producing locally active $1,25(OH)_2D$, as representing at least one mechanism to explain why circulating 25OHD, the substrate of these extrarenal $1\alpha(OH)ases$, correlates better with extraskeletal health outcomes than does the circulating $1,25(OH)_2D$.

Epidemiologic studies have supported the broad importance of the extraskeletal actions of vitamin D. One such study emphasized the decrease in all-cause mortality (from 28.6 to 13.8% over 2 years) in hemodialysis patients supplemented with active vitamin D versus those not receiving vitamin D.[34] The study also showed a reduction in cardiovascular mortality, the major cause of death in hemodialysis patients, from 14.6 to 7.6% over 2 years. End-stage chronic kidney disease, however, represents an extreme case of deficiency of the active circulating metabolite of vitamin D. A less extreme scenario was evaluated in a meta-analysis of all-cause mortality in 18 randomized controlled trials in which vitamin D treatment was used, generally for osteoporosis and fracture prevention (in nondialysis patients).[35] Not unexpectedly this study demonstrated a more modest, but still significant mortality reduction of 7%.

The availability of mice with targeted deletion of the $1\alpha(OH)ase$ and the VDR genes has facilitated the examination, in a carefully controlled fashion, of the potential consequences of vitamin D

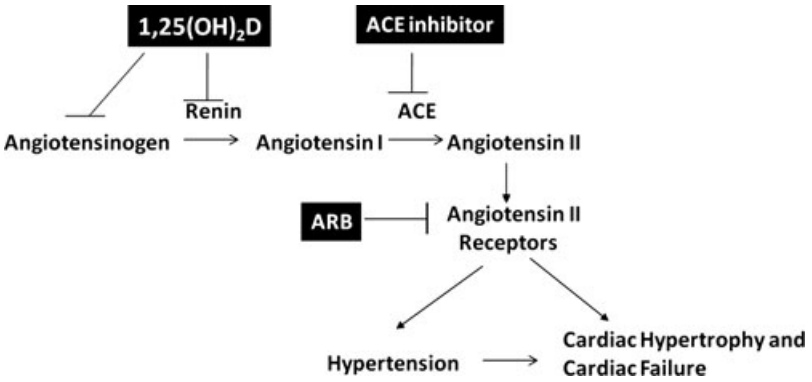

Figure 3. **Effects of vitamin D and inhibitors of the renin/angiotensin system in 1α(OH)ase$^{-/-}$ mice. 1,25(OH)$_2$D was compared with captropril (Cap), an inhibitor of angiotensin-converting enzyme (ACE), and losartan (Losa), an angiotensin II type-1 receptor blocker (ARB). 1,25(OH)$_2$D was found to inhibit angiotensinogen gene expression and to inhibit renin production. All three reduced blood pressure, prevented cardiac hypertrophy, and prevented a decrease in cardiac function.**

deficiency on a number of extraskeletal functions. These include immune function,[36] cancer development,[37] abnormal insulin secretion,[38] and blood pressure regulation.[39]

We examined the effect of the absence of 1,25(OH)$_2$D production on blood pressure regulation and cardiac structure and function in the 1α(OH)ase null mouse and the consequences of repleting the animals with active vitamin D or treating with antihypertensives.[40] In 1α(OH)ase$^{-/-}$ mice on a normal diet, systolic blood pressure was increased, associated with an increase in both renal angiotensinogen and renal renin expression, as well as increased circulating renin, increased circulating angiotensin II, and increased aldosterone. Myocardial structure was abnormal, with an increased heart-to-body ratio, increased myocyte diameter, increased interventricular septum thickness, increased left ventricular mass relative to body weight, and increased relative wall thickness. Myocardial function was reduced, as evidenced by a reduction in percent fractional shortening and a reduction in the systolic ejection fraction. On a rescue diet, the hypocalcemia, hypophosphatemia, and elevated PTH levels, each of which could potentially have an impact on the parameters measured and conceivably confound interpretation of the direct effects on vitamin D, were all normalized. In spite of this, the elevated blood pressure, activation of the renin/angiotensin system, altered myocardial structure and reduced myocardial function remained unchanged. The use of the 1α(OH)ase$^{-/-}$ model facilitated treatment with 1,25(OH)$_2$D$_3$ to assess effects on the cardiovascular phenotype. Such treatment was compared with the effects of the angiotensin-converting enzyme inhibitor captopril and with the angiotensin type 1 receptor antagonist losartan. Each agent prevented the elevation in blood pressure, prevented the development of myocardial hypertrophy, and prevented the reduction in cardiac function (Fig. 3). In this study cardiac angiotensinogen and renin were both elevated in untreated 1α(OH)ase$^{-/-}$ mice in addition to the corresponding renal proteins. Furthermore VDR and 1α(OH)ase were both detected in whole heart of wild-type mice, but the cardiac compartment in which these components of the vitamin D/VDR system were localized was not determined. As with many extraskeletal functions of vitamin D, it is uncertain whether a local system of 1,25(OH)$_2$D production and action is the physiologically more relevant one; however, in most cases, as in the example cited here, circulating 1,25(OH)$_2$D$_3$ can access the tissue VDR and modify function.

In summary, although the actions of 1,25(OH)$_2$D on skeletal and mineral homeostasis are the most prominent, 1,25(OH)$_2$D actions may occur well beyond the skeletal system and may be pleiotropic and of broad importance. Indeed, there appears to be no precedent for the sole action of a nuclear receptor activator on a single cell or organ system The pleiotropic effects of 1,25(OH)$_2$D may therefore be

analogous to the pleiotropic effects of most nuclear receptor agonists.

Conclusions

Careful analysis of genetically engineered mice with null mutations in the $1,25(OH)_2D$-synthesizing enzyme and in the VDR have illustrated the complex independent as well as interdependent actions of calcium, and the $1,25(OH)_2D$/VDR system, in regulating parathyroid function, cartilaginous growth plate development, matrix mineralization, and bone turnover. These models are also shedding important light on the extraskeletal actions of the $1,25(OH)_2D_3$/VDR system.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research to D.G. I thank Drs. N. Amizuka, D. Panda, R. Samadfam, Y. Xue, G. N. Hendy, A. Karaplis, and D. Miao for their invaluable contributions to this work. I also thank Dr. Marie Demay for having kindly provided the VDR null mice.

Conflicts of interest

The author declares no conflicts of interest.

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