COMMENTARY

1 α -Hydroxylase and the action of vitamin D

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ABSTRACT

The active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), is a pleiotropic hormone whose actions include the regulation of calcium homeostasis, control of bone cell differentiation and modification of immune responses. Synthesis of 1,25(OH)₂D₃ from the major circulating metabolite, 25-hydroxyvitamin D₃ (25(OH)D₃), is catalysed by a mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D-1 α -hydroxylase (1 α -OHase). Although 1 α -OHase is expressed predominantly in the kidney, extra-renal production of 1,25(OH)₂D₃ has also been demonstrated in tissues such as lymph nodes and skin. The tight regulation of 1 α -OHase which occurs in both renal and peripheral tissues has made studies of the expression and regulation of this enzyme remarkably difficult. However, the recent cloning of mouse, rat and human cDNAs for 1 α -OHase (CYP1 α /Cyp1 α) has enabled a more thorough characterization of this enzyme. In particular, analysis of the CYP1 α gene has identified mutations causing the inherited disorder vitamin D-dependent rickets type 1, also known as

pseudo-vitamin D deficiency rickets. Studies from our own group have focused on the distribution of 1 α -OHase in both renal and extra-renal tissues. Data indicate that the enzyme is expressed throughout the nephron, suggesting discrete endocrine and paracrine/autocrine functions. Further immunohistochemical analyses have shown that the enzyme is widely distributed in extra-renal tissues, and this appears to be due to the same gene product as the kidney. Collectively, these observations have raised important new questions concerning the role of 1 α -OHase in vitamin D signalling at a local level. The relationship between expression of protein for 1 α -OHase and enzyme activity has yet to be fully characterized and may be dependent on membrane proteins such as megalin. Similarly, elucidation of the mechanisms involved in differential regulation of renal and extra-renal 1,25(OH)₂D₃ production will be essential to our understanding of the tissue-specific functions of 1 α -OHase. These and other issues are discussed in the current review. *Journal of Molecular Endocrinology* (2000) **25**, 141–148

INTRODUCTION

Vitamin D is a seco-steroid whose actions are dependent on selective metabolic steps catalysed by cytochrome P450 enzymes. The first of these steps occurs in the liver and involves the enzyme 25-hydroxylase, which catalyses synthesis of 25-hydroxyvitamin D₃ (25(OH)D₃), the major circulating form of vitamin D. The 25(OH)D₃ molecule then acts as the substrate for other

hydroxylase enzymes, the most important of these being 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α -hydroxylase, 1 α -OHase) and vitamin D₃-24-hydroxylase (24-OHase). Although both of these enzymes are strongly expressed in the kidney they have also been detected in a variety of extra-renal tissues. The function of 24-OHase has yet to be fully determined. On the one hand, the enzyme may function as a negative regulatory enzyme by synthesizing relatively inactive vitamin D metabolites such as 1,24,25-trihydroxyvitamin D₃ (1,24,25(OH)₃D₃). Alternatively, recent studies have suggested that, in specific tissues, 24-OHase plays a more active role by generating local concentrations

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of the metabolite 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) (St-Arnaud & Glorieux 1998). The function of 1 α -OHase is more specific in that the enzyme catalyses the production of active, hormonal 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) (Henry 1997). Classically, 1,25(OH)₂D₃ plays a pivotal role in maintaining serum calcium homeostasis by modulating calcium/phosphate absorption, and parathyroid hormone (PTH) secretion (Reichel *et al.* 1989). More recently, 1,25(OH)₂D₃ has also been shown to act as a potent stimulator of cell differentiation, influencing the development of bone and immune cell lineages (Hewison & O'Riordan 1997). The actions of 1,25(OH)₂D₃ are mediated primarily through interaction with the intracellular vitamin D receptor (VDR) (Haussler *et al.* 1998), although several recent reports have highlighted rapid non-genomic effects of 1,25(OH)₂D₃, particularly in cells such as growth-plate chondrocytes (Sylvia *et al.* 1998). Much attention has focused on the role of membrane and nuclear receptors as mediators of both classical and non-classical actions of 1,25(OH)₂D₃. However, it is important to recognize that the efficacy of these signalling pathways is largely dependent on the availability of active 1,25(OH)₂D₃ and its subsequent metabolism. In particular, characterization of the expression and regulation of 1 α -OHase at both renal and extra-renal sites has provided new insights into the function of 1,25(OH)₂D₃.

The 1 α -OHase gene

Studies of the mechanisms involved in regulating 1,25(OH)₂D₃ availability at both a local and a systemic level have been greatly facilitated by the recent cloning of mouse, rat and human cDNA and genomic clones for 1 α -OHase (CYP1 α or Cyp1 α). The first of these was reported by Takeyama *et al.* (1997), who used a VDR knockout mouse model to isolate a candidate cDNA. The absence of functional VDR in the knockout mouse results in the loss of feedback control of renal 1,25(OH)₂D₃ production, and leads to constitutive over-expression of 1 α -OHase. Using this model, a candidate 2.5 kb cDNA was isolated which corresponded to a 507-amino-acid cytochrome P450-like protein, with a predicted size of 55 kDa. The mouse 1 α -OHase protein was shown to be homologous to other members of the cytochrome P450 family and, as such, has several key regions of amino acids. The mitochondrial target sequence (amino acids 13–18) showed 41.7% homology with rat 25-hydroxylase (Cyp27) and 31.6% homology with mouse 24-OHase (Cyp24). The sterol-binding domain (amino acids 367–382) was 93% homologous with rat Cyp27

and 60% with mouse Cyp24. However, the region with greatest overall homology was the haem-binding domain (70% with rat, 80% with mouse). Subsequent cloning of a human CYP1 α cDNA was achieved using mRNA from a non-classical source of the enzyme. Fu *et al.* (1997) used cultured human keratinocytes, which have previously been shown to synthesize 1,25(OH)₂D₃ (Pillai *et al.* 1988). Cloning of a candidate CYP1 α cDNA was achieved using PCR degenerate primers corresponding to the haem-binding domains of human CYP24 and CYP27. A full-length cDNA of 2.4 kb was shown to encode a 508-amino-acid protein with a predicted size of 56 kDa. Overall sequence identity to related human cytochrome P450 proteins ranged from 39% (CYP27) to 33% (11 β -hydroxylase). Characterization of the CYP1 α gene, which spans approximately 6 kb, consists of 9 exons and has approximately 500 bp of 5' untranslated mRNA, has also highlighted the homology between CYP1 α and its CYP27 counterpart (Monkawa *et al.* 1997). Both of these genes have the same number of exons and also have identical intronic insertions.

In parallel with the original cloning of the human gene, St-Arnaud *et al.* (1997) isolated the cDNA for rat Cyp1 α , which has 82.5% identity to the human cDNA. This report also confirmed the location of the human CYP1 α gene on chromosome 12q13.1-q13.3, providing further evidence that abnormal CYP1 α gene expression is the cause of hereditary pseudovitamin D-deficiency rickets (PDDR). Also known as vitamin D-dependent rickets type 1 (VDDR 1), PDDR is an autosomal recessive disorder characterized by low serum calcium, secondary hyperparathyroidism and low circulating levels of 1,25(OH)₂D₃. The disease locus for PDDR had been mapped previously to 12q13-q14, but the characterization of a specific genetic defect associated with the disorder only became possible following the cloning of the CYP1 α gene. The first description of a mutation in the CYP1 α gene associated with defective CYP1 α activity was carried out using mRNA isolated using cultured keratinocytes from a PDDR patient (Fu *et al.* 1997). The absence of CYP1 α activity in these cells was associated with deletion/frameshift mutations at codons 211 or 231, indicating that the patient was a compound heterozygote for two null mutations. Subsequent to this study, several other reports have been published which have documented families with mutations in the CYP1 α gene (Kitanaka *et al.* 1998, Wang *et al.* 1998, Yoshida *et al.* 1998).

Most notable amongst these studies is a report by Mawer and colleagues, who used activated monocytes from PDDR patients to characterize abnormalities in 1,25(OH)₂D₃ production (Smith

et al. 1999). This study identified a further two unrelated families with the 7 bp insertion in exon 8. In particular, the authors noted that synthesis of 1,25(OH) $_2$ D $_3$ in activated macrophages from PDDR patients was lower than that in controls, and the CYP1 α mutations were detectable using cDNA from these cells. These data provide further evidence of a common genetic origin for 1 α -OHase activity at both renal and extra-renal sites.

Renal distribution of 1 α -OHase

The availability of sequence information for CYP1 α has also facilitated the development of new molecular tools for further analysis of the expression and regulation of 1,25(OH) $_2$ D $_3$ synthesis by both renal and non-renal cells. Specifically, we have developed polyclonal antisera to both human and mouse 1 α -OHase which have allowed us to document, for the first time, the precise distribution of this enzyme in the kidney and other tissues. Data using normal human kidneys confirmed the expression of mRNA and protein for 1 α -OHase in proximal tubules (Zehnder *et al.* 1999). However, protein and mRNA were also expressed in distal tubules and in collecting ducts. The specificity of 1 α -OHase expression in the kidney was emphasized by stringent controls for both the *in situ* hybridization and the immunohistochemistry analyses. The other key sites of 1 α -OHase expression along the nephron were the medullary collecting ducts and papillary epithelium. RT-PCR, Western blots and enzyme-activity studies using primary cell culture confirmed the presence of 1 α -OHase in human cortical and medullary renal tissue, with mRNA and protein corresponding to the size of previously reported species for the enzyme.

Although previous studies of the renal function of vitamin D have focused primarily on the production and function of 1,25(OH) $_2$ D $_3$ in proximal tubules, there is increasing evidence of a role for this hormone in more distal parts of the nephron. In view of studies with vitamin D deficient animals, it seems likely that production of 1,25(OH) $_2$ D $_3$ in the proximal tubules acts in an endocrine fashion to support circulating levels of 1,25(OH) $_2$ D $_3$, whereas more distal areas of the nephron fulfil an autocrine or paracrine function. Previous studies have shown that 1,25(OH) $_2$ D $_3$, as well as calcitonin and PTH, stimulate calcium absorption in the distal nephron (Bouhtiauy *et al.* 1993, Friedman & Gesek 1993, You *et al.* 1997, Hoenderop *et al.* 1999). It is also important to recognize that in some cases the impact of 1,25(OH) $_2$ D $_3$ on renal function may occur through indirect mechanisms. In particular, the observation that the calcium-sensing receptor is

primarily regulated by 1,25(OH) $_2$ D $_3$, and not PTH or calcium, suggests that this may be the key target for local 1 α -OHase activity in the distal nephron (Brown *et al.* 1996).

Extra-renal expression of 1 α -OHase

The original description of extra-renal 1 α -OHase expression was based on studies of the granulomatous disease sarcoidosis, which frequently presents with associated hypercalcaemia (Papapoulos *et al.* 1979, Barbour *et al.* 1981). Enzyme-activity analyses using lymph-node homogenates and pulmonary alveolar macrophages from patients with sarcoidosis showed high levels of 1 α -OHase activity (Adams *et al.* 1983, Adams & Gacad 1985, Reichel *et al.* 1987). Furthermore, addition of exogenous 1,25(OH) $_2$ D $_3$ did not appear to inhibit macrophage 1 α -OHase as is classically observed with its renal counterpart. This would explain the apparently unregulated synthesis of 1,25(OH) $_2$ D $_3$ which is characteristic of the more severe forms of this disease, but also suggested that the expression and regulation of 1 α -OHase in extra-renal tissues was different from that observed with the kidney enzyme. Prior to the cloning of the 1 α -OHase gene it seemed likely that extra-renal production of 1,25(OH) $_2$ D $_3$ was due to a separate enzyme. However, as illustrated above, it now appears that renal and extra-renal 1 α -OHase activity is due to a single gene product. On the basis of these observations, we have used immunohistochemistry and Western analyses with renal 1 α -OHase antisera to characterize the extra-renal distribution of the enzyme in human tissues (Zehnder *et al.* 2000). As would be predicted from previous *in vitro* studies, 1 α -OHase was detectable in tissues such as normal skin (stratum basalis) and sarcoid lymph nodes. In the latter, staining for 1 α -OHase was coincident with expression of the cell-surface antigen CD68, which is a macrophage marker. 1 α -OHase was also highly expressed in skin from sarcoid patients. In this case, the enzyme was observed predominantly in inflammatory infiltrates, with expression in the stratum basalis remaining normal. In contrast, analysis of psoriatic skin indicated that 1 α -OHase was expressed in keratinocytes throughout the dysregulated stratum spinosum. This observation is somewhat paradoxical in view of the fact that current therapy for psoriasis includes the use of 1,25(OH) $_2$ D $_3$ analogues as antiproliferative agents (Bikle 1995). Immunohistochemistry also confirmed previous enzyme-activity studies which indicated that 1 α -OHase is expressed in decidual cells (Weisman *et al.* 1979, Glorieux *et al.* 1995). However, the enzyme was also detectable in

FIGURE 1. Schematic representation of the possible mechanisms involved in the regulation of 1 α -OHase activity. Membrane receptors include the following: parathyroid hormone (PTH) receptors 1, 2 and 3; the calcitonin (CT) receptor; the calcium (Ca²⁺)-sensing receptor; the toll-like receptor (TLR); the lipopolysaccharide receptor (CD14); the tumour necrosis factor- α (TNF α) receptor. Associated kinase activity includes phospholipase C (PLC), phospholipase A (PLA) and phosphokinase A (PKA). Second messengers include cyclic AMP (cAMP), calcium (Ca²⁺) and nuclear factor κ B (NF κ B).

trophoblasts and syncytiotrophoblasts, suggesting potentially diverse functions for 1 α -OHase in placentation and feto-placental calcium homeostasis (Kovacs & Kronenberg 1997). Novel sites for 1 α -OHase expression included the parathyroids, pancreas, adrenal medulla, colon and cerebellum, with negative tissues including the heart, liver and adrenal cortex. In all cases, Western blot analyses suggested that expression of 1 α -OHase was due to the reported protein species.

The regulation of vitamin D metabolism

The apparent widespread distribution of protein and mRNA for 1 α -OHase in both renal and extra-renal tissues has raised important questions

concerning the local enzyme activity at these sites. The relationship between expression of 1 α -OHase and actual synthesis of 1,25(OH)₂D₃ in a particular tissue probably involves two specific mechanisms, the first of these being substrate access, and the second being auto-regulation of 1 α -OHase activity by 1,25(OH)₂D₃ itself. The former questions the assumption that, in common with other steroid hormones, 1,25(OH)₂D₃ enters cells by a passive mechanism by virtue of its lipophilic nature. The latter raises the possibility that local 1 α -OHase activity in extra-renal tissues is under even tighter control than that observed with the endocrine enzyme and, thus, local production of 1,25(OH)₂D₃ *in vivo* may be difficult to detect.

Circulating vitamin D metabolites can bind to a variety of serum proteins, but by far the most important of these is the vitamin D-binding protein (DBP), which is synthesized in the liver. Previous studies *in vitro* have suggested that DBP-bound vitamin D metabolites have limited access to target cells and, as such, the free forms of vitamin D metabolites, with greater apparent accessibility to target cells, are more biologically active (Bikle & Gee 1989). However, analysis of the DBP null mouse indicated that these animals were less susceptible than the wild type to vitamin D-induced hypercalcaemia (Safadi *et al.* 1999). The DBP null mice also developed vitamin D deficiency much earlier than their normal litter-mates. Taken together, these findings suggest that, in addition to its function as a transport protein, DBP may play an active role in directing vitamin D responses. In particular, because of its relatively high capacity for binding 25(OH) $_2$ D $_3$, DBP is likely to be a key determinant of the availability of substrate to 1 α -OHase. Recent studies have shown that DBP and DBP-bound vitamin D metabolites are filtered through the glomerulus and reabsorbed by the luminal endocytic receptor megalin (gp330) in the proximal tubules (Nykjaer *et al.* 1999). Megalin belongs to the low-density-lipoprotein receptor gene family (Saito *et al.* 1994) and is expressed in a variety of tissues (Lundgren *et al.* 1997). The precise physiological role of this receptor has yet to be fully clarified, but, importantly, megalin-null mice have high urinary excretion of 25(OH) $_2$ D $_3$ and DBP with associated bone disease (Nykjaer *et al.* 1999). Megalin has also been shown to be located in the brush border membrane of the proximal tubules, whereas cells of the distal nephron appear to be megalin-negative (Lundgren *et al.* 1997). Thus, megalin-mediated endocytosis of DBP-bound 25(OH) $_2$ D $_3$ may act as an additional mechanism controlling tissue-specific synthesis of 1,25(OH) $_2$ D $_3$ by modulating the availability of substrate to the 1 α -OHase protein. This may provide a partial explanation for the discrepancy between widespread 1 α -OHase protein expression along the nephron and more discrete patterns of actual enzyme activity *in vivo*.

Previous studies have shown that, during vitamin D sufficiency, 1,25(OH) $_2$ D $_3$ production by the kidneys is very tightly regulated, but there is a striking up-regulation of 1 α -OHase activity in proximal tubule cells in vitamin D-deficient states (Brunette 1977, Kawashima *et al.* 1981). This response appears to be a function of several direct and indirect mechanisms, including changes in accessory proteins such as ferredoxin, or alterations in VDR or 24-OHase expression. Studies *in vivo*

suggest that the key activator of 1 α -OHase is a PTH and that this effect is mediated, at least in part, by target-cell induction of cAMP (Henry & Luntao 1989) (see Figure 1). More recent reports have highlighted potential cAMP response elements in downstream areas (–1.4 kb) which are PTH-responsive in promoter-reporter assays (Brenza *et al.* 1998, Kong *et al.* 1999). In both of these studies, the authors were unable to show any self-regulation of basal CYP1 α promoter activity, and no vitamin D response elements (VDREs) were identified in the 1.4 kb fragment. However, in each case 1,25(OH) $_2$ D $_3$ was able to suppress PTH-induced transactivation. This suggests either that the CYP1 α gene promoter has an atypical VDRE, or that 1,25(OH) $_2$ D $_3$ achieves its effects by an indirect mechanism. These reports contrast with analysis of the murine promoter, which demonstrated both positive (PTH) and negative (1,25(OH) $_2$ D $_3$) responsiveness in a region downstream of –0.9 kb (Murayama *et al.* 1998). In this study, calcitonin was shown to be a potent stimulator of 1 α -OHase expression, supporting previous reports in which calcitonin was shown to stimulate 1 α -OHase mRNA and activity under normocalcaemic conditions (Shinki *et al.* 1999). This suggests that calcitonin, acting via distal areas of the nephron, may play an important role in the ‘fine-tuning’ of serum 1,25(OH) $_2$ D $_3$ levels during vitamin D sufficiency.

Amongst the most prominent inhibitors of 1 α -OHase are calcium, phosphate and 1,25(OH) $_2$ D $_3$ itself, the latter also stimulating an increase in 24-OHase activity (Murayama *et al.* 1999). It seems likely that many of these effects are mediated indirectly through modulation of PTH production and secretion. However, as a consequence of the tight regulation of 1,25(OH) $_2$ D $_3$ production, analysis of the precise mechanisms involved in controlling 1 α -OHase has proved difficult. In recent studies using a transformed human proximal tubule cell line, HKC-8, we confirmed the cAMP-mediated up-regulation of 1 α -OHase expression as well as inhibition of expression by 1,25(OH) $_2$ D $_3$ (Bland *et al.* 1999). However, we also noted that the most potent and rapid modulation of 1 α -OHase expression and activity occurred following changes in extra-cellular calcium. Relatively high levels of calcium (2 mM versus 1 mM) reduced the synthesis of 1,25(OH) $_2$ D $_3$, whereas relatively low levels (0.5 mM versus 1 mM) increased the enzyme activity. These responses occurred within 4 h but were transient, with activities returning to normal at 24 h. These observations, coupled with the widespread expression of calcium-sensing receptors along the nephron, suggest that changes in

local calcium sensing may act as a major determinant of tissue-specific 1,25(OH) $_2$ D $_3$ production (Figure 1).

Another approach to the *in vitro* analysis of 1 α -OHase has been to use preparations of keratinocytes or activate macrophages as a source of 1 α -OHase activity. The main difficulty associated with these model systems is that current evidence suggests that there are substantial differences between the regulation of 1 α -OHase in the kidney and that in extra-renal sites. For example, the synthesis of 1,25(OH) $_2$ D $_3$ by activated macrophages is not inhibited by 1,25(OH) $_2$ D $_3$, and this appears to be the basis for the unregulated 1 α -OHase activity associated with granulomatous diseases such as sarcoidosis, and which frequently leads to hypercalcaemia in these patients (Papapoulos *et al.* 1979, Barbour *et al.* 1981, Adams *et al.* 1983, Adams & Gacad 1985, Reichel *et al.* 1987). This is difficult to explain, particularly in view of recent studies which suggest that renal and extra-renal 1 α -OHase are due to the same gene product. Macrophage-like cells are known to express VDR, and several groups (including ourselves) have demonstrated functional responses to 1,25(OH) $_2$ D $_3$ in these cells (Hewison & O'Riordan 1997). Therefore, the most likely explanation is that induction of extra-renal 1 α -OHase involves regulatory pathways that differ from renal, cAMP-mediated mechanisms and are less sensitive to autoregulation by 1,25(OH) $_2$ D $_3$. Induction of extra-renal 1 α -OHase frequently involves antigenic activators such as lipopolysaccharide or inflammatory mediators such as interferon- γ . Because these agents signal via nuclear factor κ B we can postulate that this pathway activates 1 α -OHase in a manner unlike that of calciotropic factors and, as a consequence, shows differential sensitivity to feedback control by 1,25(OH) $_2$ D $_3$. Further analysis of the signal-transduction pathways involved in regulating 1 α -OHase will be crucial to our understanding of the way in which 1,25(OH) $_2$ D $_3$ functions in extra-renal tissues (see Figure 1).

Summary

In recent years, our understanding of the molecular endocrinology of vitamin D metabolism has increased dramatically. Mutations in the CYP1 α gene have been shown to be associated with rare inherited disorders of 1,25(OH) $_2$ D $_3$ production; current data suggest that a single gene product is responsible for renal and extra-renal 1 α -OHase expression. Analysis of the renal and extra-renal expression of 1 α -OHase has provided the first definitive picture of the tissue distribution of 1 α -OHase protein. Data indicate that the enzyme is

expressed throughout the kidney, suggesting novel roles for local synthesis of 1,25(OH) $_2$ D $_3$ (particularly in the distal nephron). Extra-renal studies have confirmed the presence of 1 α -OHase in tissues such as the skin and lymph nodes but also highlight novel sites such as the pancreas and the colon. Future analysis of the mechanisms involved in the production of 1,25(OH) $_2$ D $_3$ will provide a clearer picture of the importance of 1 α -OHase as a modulator of both renal and extra-renal tissue function.

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