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EFFECT OF THREE A-RING ANALOGS OF 1α,25-DIHYDROXYVITAMIN D_3 ON 25-OH-D_3-1α-HYDROXYLASE IN ISOLATED MITOCHONDRIA AND ON 25-HYDROXYVITAMIN D_3 METABOLISM IN CULTURED KIDNEY CELLS

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Summary—Three A-ring analogs of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$)—2-nor-1,3-seco-1,25$(OH)_2D_3$ (2-nor analog), 2-oxa-3-deoxy-25-OH-D_3 (2-oxa analog), and A-homo-3-deoxy-3,3-dimethyl-2,4-dioxa-25-OH-D_3 (A-homo analog)—were tested for their ability to inhibit 25-OH-D_3-1α-hydroxylase (1α-hydroxylase) in isolated mitochondria and to alter 25-OH-D_3 metabolism in cultured chick kidney cells. The 2-nor and 2-oxa analogs were relatively potent (K_is of 60 and 30 nM, respectively, compared with 170 nM for 1,25$(OH)_2D_3$), whereas the A-homo analog was completely ineffective in inhibiting 1α-hydroxylase activity. In contrast, all three analogs were able to repress 1α-hydroxylase and induce 24-hydroxylase activity in cultured chick kidney cells, suggesting that this process is not one of direct action in the mitochondria, but is more likely to be a receptor-mediated one.

INTRODUCTION

The final and rate-limiting step in the biosynthesis of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the potent calcitropic steroid hormone, is the 1α-hydroxylation of 25-OH-D_3, the major circulating derivative of vitamin D_3. This hydroxylation is carried out by a kidney mitochondrial cytochrome *P*450 dependent mixed function oxidase. 25-OH-D_3-1α-hydroxylase (1α-hydroxylase) can easily be measured in mitochondria isolated from kidney tissue from vitamin D deficient chicks [1]. In addition both the 1α- and 24*R*-hydroxylation of 25-OH-D_3 can be assessed in primary cultures of chick kidney cells [2]. In this case the relative activities of the two hydroxylases are dependent on the exposure of the cells to 1,25$(OH)_2D_3$, or active analogs of the steroid hormone, which repress the 1α-hydroxylase and induce the 24*R*-hydroxylase. The mechanism of these effects have not been elucidated on a molecular level but the induction of the 24-hydroxylase is known to require protein synthesis [2, 3] and it has been suggested [4] that both changes, i.e. repression of the 1α-hydroxylase and induction of the 24*R*-hydroxylase, are steroid receptor mediated events involving alterations in gene expression.

Potential inhibitors of the renal 1α-hydroxylase are of interest for a number of reasons. A comparison of the ability of a compound to inhibit the 1α-hydroxylase by direct interaction with the enzyme in isolated mitochondria with the ability of the same compound to repress 1α-hydroxylation and induce 24*R*-hydroxylase activity in intact cells could provide insight into the mechanisms of these processes. Similarly, comparisons of the activity of a compound in the modulation of 25-OH-D_3 metabolism with its calcitropic activity in bone or intestine may help elucidate the mechanisms of action of 1,25$(OH)_2D_3$ in these tissues. Such comparisons could also reveal compounds which could be given systemically to inhibit the formation of 1,25$(OH)_2D_3$ without causing hypercalcemia. One clinical setting in which this would be useful is hypervitaminosis D.

The current paper focuses on three A-ring analogs, whose structures are shown in Fig. 1, as such experimental probes. The synthesis of these compounds has already been reported [5, 6].

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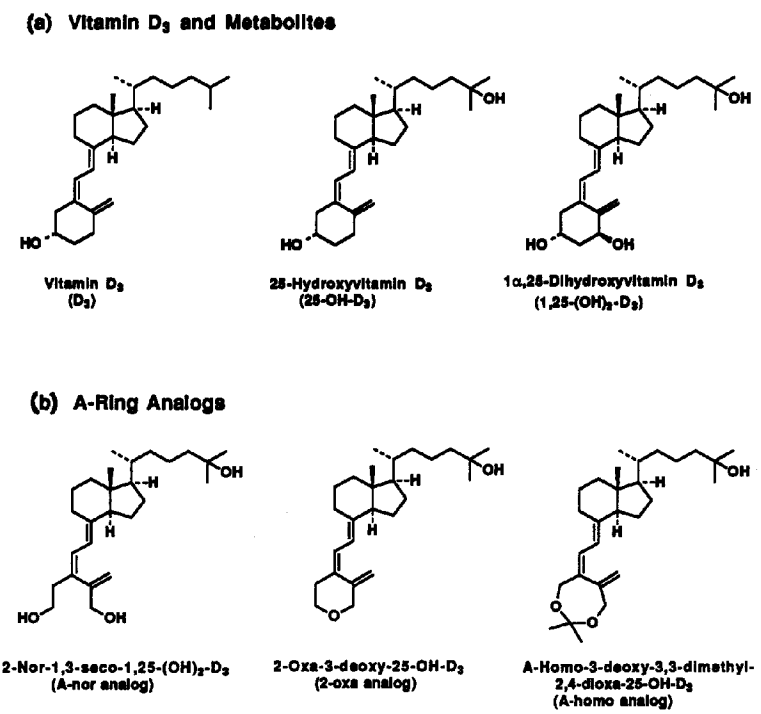

Fig. 1. The structures of vitamin D_3, its metabolites and three A-ring analogs of $1,25(OH)_2D_3$ used in these studies.

EXPERIMENTAL

Mitochondria were isolated from a 10% (in 0.25 M sucrose) homogenate of kidney tissue from 3- to 4-week-old vitamin D deficient chicks by standard differential centrifugation, as described previously [7]. Assays of 1α-hydroxylase activity were carried out in a final volume of 2.0 ml containing 25 mM Tris–Cl, 75 mM sucrose, 10 mM malate, 2 mM $MgCl_2$, 5×10^{-8} M 25-OH-[26,27-^{3}H]vitamin D_3 (40 mCi/mmol) and 2.5–3.0 mg/ml mitochondrial protein. In some experiments, the substrate concentration was varied as indicated in the figure legend. The A-ring analog of interest was added in ethanol just prior to the initiation of the reaction with substrate. Incubations were at 37°C for 10 min and were terminated by the addition of $CHCl_3$ methanol (1:2, v/v).

Kidney cells were isolated from 2- to 3-week-old vitamin D deficient chicks and cultured, as described previously [2]; 20 h prior to assay, cultures were changed to serum-free medium with 5 μg/ml insulin $1,25(OH)_2D_3$ or the A-ring analog of interest was added at this time. Incubations with [^{3}H]25-OH-D_3 were for 30 min and were terminated by scraping the cells into $CHCl_3$ methanol (1:2, v/v).

For both mitochondrial and cell culture assays, lipids were extracted by a modification of the method of Bligh and Dyer [8, 9] and samples were prepared for chromatography on a 10 μm silica radial compression cartridge. Radioactivity was quantitated either by collection of individual fractions and subsequent liquid scintillation counting or by flow-through detection of radioactivity with a Beckman Model 171 flow-through detector. The percent of total radioactivity converted to [^{3}H]$1,25(OH)_2D_3$ was determined and converted to pmol/10 min based on the known specific activity of the starting substrate.

The three analogs (Fig. 1) described in this study were purified by HPLC and then checked for purity by ^{3}H- and ^{13}C-NMR and u.v. spectroscopic analysis and by analytical HPLC. Freshly purified samples were routinely stored under argon or nitrogen at −70°C.

RESULTS AND DISCUSSION

As shown in Fig. 2, both 2-nor-1,3-seco-$1,25(OH)_2D_3$ (2-nor analog) and 2-oxa-3-deoxy-25-OH-D_3 (2-oxa analog) are inhibitors of the 1α-hydroxylase and their relative potencies cannot be distinguished from one another from these data. At the lower concentrations, both are substantially better inhibitors than the product of the reaction itself, $1,25(OH)_2D_3$. The significant inhibitory activity of the 2-oxa analog is rather startling because, whereas the A-ring of the A-nor analog and that of the hormone $1,25(OH)_2D_3$ resemble one another (they possess A-ring hydroxyls which can assume virtually identical topographies), the A-ring of the 2-oxa analog is essentially non-polar. Moreover, it has already been shown [5, 6] that the 2-oxa analog exists in equilibrium with nearly equimolar amounts of a previtamin compound (structure not shown). These novel structural characteristics of the 2-oxa analog are thus difficult to assess in terms of its function in relation to $1,25(OH)_2D_3$ and the A-nor analog, but the data suggest that the active site of the 1α-hydroxylase does not require an intact 6-carbon A-ring. It also suggests that the side chain of the substrate, 25-OH-D_3, may be more important in binding to the active site of cytochrome *P*4501α than the A-ring.

However, the A-ring cannot be completely unimportant, since the third analog, A-homo-3-deoxy-3,3-dimethyl-2,4-deoxy-25-OH-D_3 (A-homo analog) failed to inhibit the 1α-hydroxylase at any concentration up to 1 μM. This fact reveals that the A-ring structure does have some influence on inhibitory activity. It should be noted that the A-homo analog is the acetone derived cyclic ketal of an A-dinor analog and that its lack of inhibitory activity

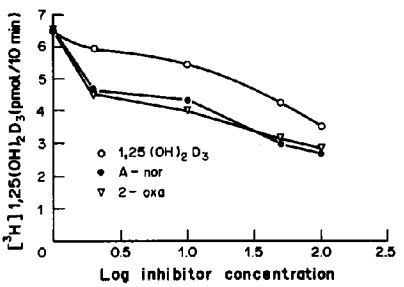

Fig. 2. Effect of the 2-nor and 2-oxa analogs, compared with that of $1,25(OH)_2D_3$, on 1α-hydroxylase activity in isolated chick kidney mitochondria. Inhibitor concentrations ranged from 0.05 to 1.0 μM. The values shown are the average of 3 determinations; SEM < 5% of the mean.

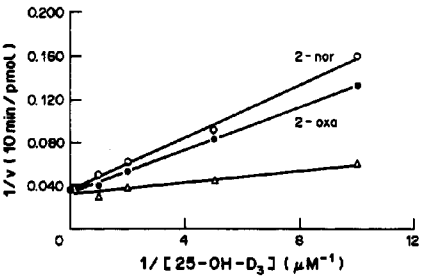

Fig. 3. Double-reciprocal plot of substrate concentration vs rate determinations in the absence of inhibitor (△) and in the presence of the 2-oxa and 2-nor A-ring analogs.

may very well be due to the presence of the bulky gem-dimethyl groups present at carbon-3 in the A-ring. Unfortunately, the analogs of the A-homo compound lacking the gem-dimethyl groups could not be synthetically accessed.

To determine the mechanism of 1α-hydroxylase inhibition by the 2-nor analog and 2-oxa analog the substrate concentration in the reaction mixture was varied and the results are shown in Fig. 3. Both compounds act as competitive inhibitors, and have K_is of 0.6 and 0.3×10^{-7} M. A similar analysis for $1,25(OH)_2D_3$ revealed a K_i of 1.7×10^{-7} M, confirming that, as seen in Fig. 2, the natural product of the 1α-hydroxylase is a less potent inhibitor of the enzyme than either of these two A-ring analogs.

We considered the possibility that the 2-oxa compound could act as a suicide inhibitor, i.e. that if it were to undergo hydroxylation at carbon-1, the result would be a hemiacetal which could then fragment into the α, β unsaturated aldehyde potentially able to covalently bind to the enzyme and inhibit it irreversibly [5, 6]. If this were the case, then there should be a time-dependent increase in the degree of inhibition of the enzyme [8] by the compound. As can be seen in Table 1, this is not the case, indicating that simple competitive product inhibition is probably at work here.

Table 2 summarizes three experiments in which the ability of the three A-ring analogs of

Table 1. Time course of inhibition of 1α-hydroxylase activity by the 2-oxa analog

Time in 2-oxa prior to assay (min)	Inhibition of 1α-hydroxylase (%)
0	56.7
5	64.5
10	62.7
30	62.0

Table 2. Effects of A-ring analogs on 25-OH-D_3 metabolism in cultured kidney cells

Steroid added	Concentration (M)	$1,25(OH)_2D_3$ (pmol/30 min/culture)	$24,25(OH)_2D_3$ (pmol/30 min/culture)
Exp. A			
None	—	10.2 + 0.3	0.5 + 0.2
$1,25(OH)_2D_3$	10^{-7}	6.2 + 0.3	4.1 + 0.3
A-homo analog	10^{-7}	7.6 + 0.2	1.3 + 0.2
A-homo analog	3×10^{-7}	4.0 + 0.1	4.0 + 0.4
Exp. B			
None	10^{-7}	25.6 + 2.2	2.1 + 0.3
$1,25(OH)_2D_3$	10^{-7}	9.2 + 0.2	15.1 + 1.1
2-nor Analog	10^{-7}	16.6 + 1.4	6.0 + 0.6
2-oxa Analog	10^{-7}	11.6 + 0.4	7.2 + 0.8
Exp. C			
None	10^{-7}	20.4 + 1.2	0.3 + 0.1
$1,25(OH)_2D_3$	10^{-7}	4.0 + 0.3	2.5 + 0.5
2-oxa Analog	10^{-7}	6.4 + 0.4	2.2 + 0.3
A-homo analog	10^{-7}	12.8 + 1.4	1.2 + 0.4

$1,25(OH)_2D_3$ to alter the pattern of hydroxylation of 25-OH-D_3 in cultured chick kidney cells. Several points are worth noting. First, all three compounds decreased 1α-hydroxylase activity in intact cells. This indicates that if all three are operating through the same mechanism, this mechanism is not direct inhibition at the active site of the 1α-hydroxylase since the A-homo analog, which was quite effective in reducing 1α-hydroxylase activity in intact kidney cells, could not under any conditions be shown to inhibit the 1α-hydroxylase when added directly to mitochondria. This interpretation is consistent with a receptor-mediated mechanism of repression of 1α-hydroxylase activity. The fact that $1,25(OH)_2D_3$ is more effective than any of the three analogs in decreasing 1α-hydroxylase activity in these cells while it is less effective in isolated mitochondria (a reproducible observation typified by the data in Fig. 2) is also consistent with a receptor mediated mechanism of steroid-induced repression of the 1α-hydroxylase in intact cells.

Neither are any of the three analogs as effective as $1,25(OH)_2D_3$ in inducing 24*R*-hydroxylase activity, when compared at equimolar concentrations. The nature of this assay makes it difficult to draw precise distinctions between the compounds, particularly when assayed in different experiments but, in general, they fell into the range of being 20–80% as effective as $1,25(OH)_2D_3$ in inducing 24*R*-hydroxylase activity, similar to the case of decreasing 1α-hydroxylase activity. This is interesting, since in the intact chick when biological activity in the intestine or brain is measured [10] these compounds are orders of magnitude less active than 1,25(OH)-D_3. This suggests either that the kidney receptor is more promiscuous than that in the intestine or bone or that there are pharmacologic interventions at work *in vivo* which prevent substantial amounts of the analogs from reaching the target tissues. For example, differences in metabolism could contribute to differences in steroid delivery to target tissues.

Figure 4 shows a typical time course of the effects of two of the A-ring analogs, along with $1,25(OH)_2D_3$, on 1α-hydroxylase activity in whole kidney cells. The similarity in the shape of the curves further suggests that $1,25(OH)_2D_3$ and the A-ring analogs tested are altering the enzyme's activity through the same mechanism. Similar time courses were also obtained for changes in 24-hydroxylase activity (data not shown).

In summary, we have tested the effects of three A-ring analogs of $1,25(OH)_2D_3$ on 1α-hydroxylase activity in isolated kidney mitochondria and on 25-OH-D_3 metabolism in intact cultured kidney cells. We conclude that two of the three, the 2-nor analog and the 2-oxa analog, act as competitive inhibitors of 1α-hydroxylase activity in isolated mitochondria. The data with intact cultured cells indicates that the three analogs act through a mechanism, which (a) is similar to that of the natural steroid hormone $1,25(OH)_2D_3$ and (b) probably is receptor mediated to alter 25-OH-D_3 metabolism in intact cells. It would be of interest to determine whether these compounds are capable of modulating circulating $1,25(OH)_2D_3$ levels *in vivo* without altering serum calcium levels in which case they could be of therapeutic interest in the management of, for example, hypervitaminosis D.

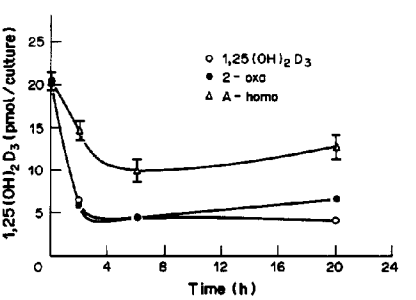

Fig. 4. Time course of the effect of $1,25(OH)_2D_3$ (○), 2-oxa (●) and A-homo (△) analogs on $1,25(OH)_2D_3$ production in cultured chick kidney cells. Values shown are the mean and error bars represent the mean of 4 determinations. Where no error bars are visible, they are smaller than the symbol.

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