Production of C-24- and C-23-oxidized metabolites of 1,25-dihydroxycholecalciferol by cultured kidney cells (LLC PK₁) and their presence in kidney *in vivo*

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1,25-Dihydroxy[³H]cholecalciferol was converted into several more-polar metabolites by a cultured pig kidney cell line (LLC PK₁). The production of metabolites was stimulated by pretreating the cells with unlabelled 1,25-dihydroxycholecalciferol. A similar profile of metabolites was observed on high-pressure-liquid-chromatographic analysis of an extract from the kidneys of rats dosed intravenously with 1,25-dihydroxy[³H]cholecalciferol. Among the metabolites detected were 1,24,25-trihydroxycholecalciferol, 1,25-dihydroxy-24-oxocholecalciferol, 1,23,25-trihydroxy-24-oxocholecalciferol and 1,25-dihydroxycholecalciferol-26,23-lactone. The results are in accord with data reported for intestinal 1,25-dihydroxycholecalciferol metabolism [Napoli, Pramanik, Royal, Reinhardt & Horst (1983) *J. Biol. Chem.* **258**, 9100–9107]. These data indicate that C-23- and C-24-oxidation of 1,25-dihydroxycholecalciferol are phenomena common to calciferol target tissues, and that regulation of 1,25-dihydroxycholecalciferol homeostasis is dependent on the rate of its metabolism in addition to the rate of its synthesis.

1,25-(OH)₂D₃ is a hormone, produced from vitamin D₃, that regulates calcium and phosphate homeostasis (Kanis *et al.*, 1982), and induces bone-marrow cell differentiation (Abe *et al.*, 1981; Miyaura *et al.*, 1981). The regulation of 1,25-(OH)₂D₃ production in kidney, its primary site of synthesis, has been well studied (Fraser, 1980). In contrast, the metabolism of 1,25-(OH)₂D₃ has not been investigated thoroughly. Metabolic pathways are just undergoing definition; the regulation of 1,25-(OH)₂D₃ metabolism is not understood. This metabolism, in target tissues such as kidney and intestine, may be involved in activating 1,25-(OH)₂D₃, directing it to specialized functions, and/or in terminating its action. Elucidating 1,25-(OH)₂D₃ metabolism would seem prerequisite to understanding regulation of its steady-state concentration, and may provide insight into its multiple functions.

Previous investigations have shown that intestine converts 1,25-(OH)₂D₃ into 1,24,25-(OH)₃D₃ (Kumar *et al.*, 1978). 1,24,25-(OH)₃D₃ serves as precursor for intestinal synthesis of 24-oxo-1,25-(OH)₂D₃ (Napoli *et al.*, 1983) and 24-oxo-1,23,25-(OH)₃D₃ (Napoli & Horst, 1983b). Intestine also produces (23*S*)-1,23,25-(OH)₃D₃ (Napoli & Horst, 1983a), a precursor of 1,25-(OH)₂D₃-26,23-lactone (Ishizuka *et al.*, 1981; R. L. Horst, G. W. Engstrom & J. L. Napoli, unpublished work). The ultimate dispositions of 24-oxo-1,23,25-(OH)₃D₃ and 1,25-(OH)₂D₃-26,23-lactone are not known, but it is possible that these metabolites undergo side-chain cleavage to yield CO₂ (Kumar *et al.*, 1976) and a C-23-carboxylic acid (Esvelt *et al.*, 1979). The site(s) of synthesis of (25*S*)-1,25,26-(OH)₃D₃, a 1,25-(OH)₂D₃ metabolite that circulates in blood, is not known (Reinhardt *et al.*, 1981a,b; Partridge *et al.*, 1981). This metabolite, however, is not likely to be an intermediate in 1,25-(OH)₂D₃-26,23-lactone or 24-oxo-1,23,25-(OH)₃D₃ synthesis, by analogy to known pathways of 25-OH-D₃ metabolism (Napoli & Horst, 1981; Horst *et al.*, 1982b; Napoli *et al.*, 1982; Yamada *et al.*, 1983). Thus there are at least three different pathways of 1,25-dihydroxycholecalciferol metabolism known, and quite probably several more exist.

Abbreviations used: 1,25-(OH)₂D₃, 1,25-dihydroxycholecalciferol; 1,23,25-(OH)₃D₃, 1,23,25-trihydroxycholecalciferol; 1,24,25-(OH)₃D₃, 1,24,25-trihydroxycholecalciferol; 1,25-(OH)₂D₃-26,23-lactone, 1,25-dihydroxycholecalciferol-26,23-lactone; h.p.l.c., high-pressure liquid chromatography.

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The pig kidney cell line, LLC PK₁, converts 25-OH-D₃ into more-polar metabolite(s), and is an attractive model system for the study of vitamin D metabolism (Colston & Feldman, 1982). The present paper shows that LLC PK₁ cells convert 1,25-(OH)₂D₃ into polar metabolites similar to those previously identified as C-24- and C-23-oxidized intestinal products, that the metabolism is dependent on prior exposure to 1,25-dihydroxycholecalciferol, and that the metabolites also occur in rat kidney *in vivo*.

Experimental

General

1,25-(OH)₂D₃, 1,23,25-(OH)₃D₃, 1,24,25-(OH)₃D₃ and 1,25-(OH)₂D₃-lactone were gifts from Dr. M. R. Uskokovic and Dr. J. J. Partridge, Hoffmann-La Roche, Nutley, NJ, U.S.A. (Partridge *et al.*, 1976, 1982). 24-Oxo-1,25-(OH)₂D₃ and 24-oxo-1,23,25-(OH)₃D₃ were isolated from the incubation of 1,25-(OH)₂D₃ with rat small-intestinal mucosa as described previously (Napoli *et al.*, 1983; Napoli & Horst, 1983a). 1,25-(OH)₂-[26,27-³H]D₃ (90 Ci/mmol) was chemically synthesized as detailed previously (Napoli *et al.*, 1983).

H.p.l.c. was performed with Waters Associates model LC 204 equipment. Analytical normal-phase h.p.l.c. columns (0.46 cm × 25 cm) were purchased from Dupont.

Cell culture

Cultured pig kidney cells (LLC PK₁) were obtained from the American Tissue Culture Collection at passage 189. The cells were grown to confluence in Dulbecco's modified Eagle's medium (Gibco) containing pyruvate (1.25 mM), NaHCO₃ (44 mM), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (10 mM), foetal-calf serum (5%, v/v) and antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml; Fungizone, 0.25 µg/ml), pH 7.25, in 100 mm polystyrene tissue-culture dishes (Corning). Cells were incubated at 37°C in an atmosphere of air/CO₂ (19:1). Some of the dishes of cells were treated with unlabelled 1,25-(OH)₂D₃ (15 nM) in ethanol (5 µl) for 16 h before confluence. At confluence all of the dishes of cells were washed with Dulbecco's phosphate-buffered saline (Gibco) without Ca²⁺ and Mg²⁺, and were replenished with Dulbecco's modified Eagle's medium as described above, except serum was omitted. 1,25-(OH)₂[³H]D₃ (2.8 nM) was added in ethanol (5 µl). After 2 h of incubation the medium and the cells of each dish were separated, unlabelled 1,25-(OH)₂D₃ (2 µg) was added to both the medium and the cell sample, and each was extracted with methanol/dichloromethane by the

procedure of Horst *et al.* (1981). The residues obtained on evaporation of the organic phase were analysed by h.p.l.c. Extraction efficiency was determined from a standard curve relating h.p.l.c. peak height to µg of unlabelled 1,25-(OH)₂D₃.

1,25-(OH)₂D₃ metabolism *in vivo*

Male Sprague-Dawley rats (body wt. 150–200 g), fed on a stock diet, were dosed intraperitoneally with 1,25-(OH)₂D₃ (250 ng in 0.1 ml of propylene glycol) 36, 24 and 12 h before being killed, or received vehicle alone. At 6 h before being killed the rats (three per group) were anaesthetized with diethyl ether and each was given 1,25-(OH)₂[³H]D₃ (25 µCi) intrajugularly in ethanol (0.1 ml). The rats were killed by cardiac exsanguination under ether anaesthesia. The kidneys were rinsed with cold 0.9% NaCl, decapsulated and homogenized in water (10 ml). The homogenate was extracted with methanol/dichloromethane by the procedure of Horst *et al.* (1981). The organic phase was evaporated under diminished pressure. The residue was dissolved in ethyl acetate (8 ml) and filtered through a silica gel Sep-Pak (Waters Associates). The residue obtained on evaporation of the ethyl acetate was analysed by h.p.l.c.

Results and discussion

Cultured pig kidney cells not previously exposed to 1,25-(OH)₂D₃ excreted a negligible amount of 1,25-(OH)₂[³H]D₃ metabolites into their medium (Fig. 1). Analysis of the cell extract provided a similar chromatogram. The total recovery of tritium from the cells and medium was 102%; 93% of the original tritiated substrate was present as 1,25-(OH)₂[³H]D₃. In contrast, several quantitatively significant polar metabolites of 1,25-(OH)₂[³H]D₃ were extracted from the medium of cells that had been pretreated with unlabelled 1,25-(OH)₂D₃ (Fig. 1). Analysis of the cell extract showed a qualitatively similar profile of polar 1,25-(OH)₂[³H]D₃ metabolites. Recovery of tritium from the cells and medium was 77% after correction for extraction efficiency. About 43% of the original tritiated substrate was present as 1,25-(OH)₂[³H]D₃. Unlabelled 1,25-(OH)₂D₃ had been introduced into the cells and the media of both groups at the end of the incubation with 1,25-(OH)₂[³H]D₃ to aid in determining the efficiency of extraction and h.p.l.c. analysis. Therefore recovery of only 77% of the tritium from the 1,25-(OH)₂D₃-treated cells and their medium, as opposed to 102% from the non-treated plates, was not caused by handling losses. The difference reflects the amount of radiolabel lost as a result of 1,25-(OH)₂[26,27-³H]D₃ metabolism. This could be the

Fig. 1. H.p.l.c. analysis of 1,25-(OH)₂[³H]D₃ metabolism by LLC PK₁ cells

Media from cells incubated with 2.7 nM-1,25-(OH)₂[³H]D₃ for 2 h was analysed with an analytical normal-phase column eluted with propan-2-ol/hexane (1:9, v/v). The cells received no pretreatment (-----) or were incubated with unlabelled 15 nM-1,25-(OH)₂D₃ for 16 h before addition of the radio-label (—). The elution positions of 1,25-(OH)₂D₃ metabolite standards are indicated: 1, 1,25-(OH)₂D₃; 2, 24-oxo-1,25-(OH)₂D₃; 3, 23-oxo-1,25-(OH)₂D₃; 4, 24-oxo-1,23,25-(OH)₃D₃; 5, 1,23,25-(OH)₃D₃; 6, 1,25-(OH)₂D₃-26,23-lactone; 7, 1,24,25-(OH)₃D₃. Note the change in scale between fractions 37 and 38. Fractions were 1 ml each.

Fig. 2. Re-analysis by h.p.l.c. of the peak B region shown in Fig. 1

Peak B (fractions 50–55) from the medium of LLC PK₁ cells pretreated with unlabelled 1,25-(OH)₂D₃ and then incubated with 1,25-(OH)₂[³H]D₃ was re-analysed with an analytical normal-phase h.p.l.c. column eluted with propan-2-ol/dichloromethane (3:47, v/v). The elution positions of 1,25-(OH)₂D₃ metabolite standards are numbered as in the legend to Fig. 1. Note that this h.p.l.c. system clearly distinguishes among the three peaks that migrated closely in the first system: 3, 23-oxo-1,25-(OH)₂D₃; 4, 24-oxo-1,23,25-(OH)₃D₃; 5, 1,23,25-(OH)₃D₃. Fractions were 2 ml each.

oxo-1,25-(OH)₂D₃, peak B migrated with 24-oxo-1,23,25-(OH)₃D₃, and was distinct from 1,23,25-(OH)₃D₃ (Napoli & Horst, 1983a), and 23-oxo-1,25-(OH)₂D₃ (Horst *et al.*, 1982a), as had been previously observed with the intestinal peak (Fig. 2). Peak C, recovered from the cells, migrated with 1,24,25-(OH)₃D₃ in the second analysis; peak C recovered from the medium migrated with 1,24,25-(OH)₃D₃ (42%) and 1,25-(OH)₂D₃-26,23-lactone (58%). The elution positions of 1,25-(OH)₂D₃ and its metabolites in these two h.p.l.c. systems were established with compounds that had had their structures verified by mass-spectrometric analyses. Moreover, these two h.p.l.c. systems, when used sequentially as was done here, are capable of resolving 1,25-(OH)₂D₃ and all of its known metabolites (Napoli *et al.*, 1983; Napoli & Horst, 1983b). Thus these h.p.l.c. analyses provide reliable evidence for the structural assignments proposed.

The distribution of 1,25-(OH)₂[³H]D₃ and its metabolites in the cells and media of 1,25-

result of functionalization of C-26 and/or C-27, but, given the nature of the known metabolites, most probably represents side-chain cleavage of 1,25-(OH)₂D₃.

The metabolites produced by the 1,25-(OH)₂D₃-treated cultured pig kidney cells were similar to those previously generated in rat small-intestinal mucosa homogenates (Napoli *et al.*, 1983), or in cells isolated from rat small intestine as analysed by a hexane-based normal-phase h.p.l.c. system (Napoli & Horst, 1983b). To study co-specificity further, each of the three main metabolite peaks from the cells and the medium, namely A, B and C, was individually re-analysed in a second normal-phase h.p.l.c. system, based on dichloromethane, which has different chemical selectivity. In the second system, peak A again migrated with 24-

Table 1. 1,25-(OH)₂[³H]D₃ and its metabolites in LLC PK₁ cells pretreated with unlabelled 1,25-(OH)₂D₃. Cells were treated with unlabelled 15 nM-1,25-(OH)₂D₃ for 16 h, and then were incubated with 2.7 nM-1,25-(OH)₂[³H]D₃ for 2 h. For full experimental details see the text.

Metabolite	Amount of metabolite [pmol/2h per plate (% of total ³ H recovered)]		
	Cells	Media	Cells + media
1,25-(OH) ₂ D ₃ *	4.3 (60)	2.9 (50)	7.2 (55†)
1,24,25-(OH) ₃ D ₃	0.2 (2.7)	0.25 (4.2)	0.45 (3.5)
24-Oxo-1,25-(OH) ₂ D ₃	0.5 (7.0)	0.3 (5.8)	0.8 (6)
24-Oxo-1,23,25-(OH) ₃ D ₃	0.5 (7.0)	1.2 (21)	1.7 (13)
1,25-(OH) ₂ D ₃ -26,23-lactone	—	0.35 (5.8)	0.35 (2.5)

* The values for 1,25-(OH)₂D₃ represent pmol of 1,25-(OH)₂D₃ remaining. The amount of 1,25-(OH)₂D₃ added was 16.8 pmol.

† This represents 55% of the recovered radiolabelled materials and 43% of the original substrate.

Fig. 3. Metabolites of 1,25-(OH)₂D₃ occurring in rat kidney *in vivo*

Tissue was analysed 6 h after intravenous dosing of 1,25-(OH)₂[³H]D₃ to rats pretreated with unlabelled 1,25-(OH)₂D₃. H.p.l.c. conditions and standards are as described in the legend to Fig. 1. Fractions were 1 ml each. Note the change in scale between fractions 37 and 38.

(OH)₂D₃-treated kidney cells in culture is compiled in Table 1. Clearly, quantitatively significant conversion of 1,25-(OH)₂[³H]D₃ into more-polar metabolites had occurred. A small amount of 1,24,25-(OH)₃[³H]D₃ was present in the cells, whereas a slightly larger amount was present in the media. At first analysis, it may seem that a significant portion of the 1,24,25-(OH)₃D₃ synthesized was excreted from the cells. The other C-24-oxidized metabolites, however, are derived from 1,24,25-(OH)₃D₃ (Napoli *et al.*, 1983; Napoli & Horst, 1983b). Therefore most of the 1,24,25-(OH)₃D₃ produced was converted into further metabolites, and only a small portion was excreted. Since the concentration of 1,24,25-(OH)₃D₃ was

low relative to both its precursor, 1,25-(OH)₂D₃, and its metabolites, 24-oxo-1,25-(OH)₂D₃ and 24-oxo-1,23,25-(OH)₃D₃, it appears that C-24-hydroxylation is a rate-limiting step in target-tissue 1,25-(OH)₂D₃ metabolism. Likewise, more 24-oxo-1,25-(OH)₂D₃ was metabolized than was excreted. The major 1,25-(OH)₂D₃ metabolite that accumulated, especially in media, was 24-oxo-1,23,25-(OH)₃D₃, a compound that had been C-24- and C-23-oxidized. In comparison, the other C-23-oxidized metabolite detected, 1,25-(OH)₂D₃-26,23-lactone, was present at a much lower concentration. This could indicate that the C-23-hydroxylase preferentially utilizes 24-oxo-1,25-(OH)₂D₃ as substrate, rather than 1,25-(OH)₂D₃, to produce more 24-oxo-1,23,25-(OH)₃D₃ than 1,25-(OH)₂D₃-26,23-lactone. The turnover rates of the latter two metabolites, however, have not been determined. Until they are, it remains uncertain whether the rates of synthesis or the rates of degradation are the major determinants of 24-oxo-1,23,25-(OH)₃D₃ and 1,25-(OH)₂D₃-26,23-lactone concentrations.

To ascertain whether these kidney cells in culture are a reliable model for the disposition of 1,25-(OH)₂D₃ *in vivo*, the kidney metabolites *in vivo* of 1,25-(OH)₂[³H]D₃ were examined. The profile of 1,25-(OH)₂[³H]D₃ metabolites extracted from the kidneys of rats that had been pretreated with exogenous unlabelled 1,25-(OH)₂D₃ before being dosed with 1,25-(OH)₂[³H]D₃ was qualitatively similar to that developed from kidney cells in culture (Fig. 3). The relative amounts of the metabolites migrating with 24-oxo-1,25-(OH)₂D₃ and 24-oxo-1,23,25-(OH)₃D₃ were 5 and 10% of the amount of recovered 1,25-(OH)₂[³H]D₃ respectively. The kidneys of rats not treated with unlabelled 1,25-(OH)₂D₃ before receiving 1,25-(OH)₂[³H]D₃ contained metabolites migrating with 24-oxo-1,25-(OH)₂D₃ and 24-oxo-1,23,25-

(OH)₃D₃ at 3 and 7% respectively of the amount of recovered 1,25-(OH)₂[³H]D₃. Thus the metabolites of 1,25-(OH)₂D₃ in the LLC PK₁ kidney cell line apparently reflect those found in kidney *in vivo*.

The present findings indicate that kidney, like the small intestine, converts 1,25-(OH)₂D₃ into C-24- and C-23-oxidized products. Logically, the metabolism of 1,25-(OH)₂D₃ in kidney should occur along the same pathway as that in the small intestine. That is, 1,24,25-(OH)₃D₃ would be precursor to 24-oxo-1,25-(OH)₂D₃, which would be precursor to 24-oxo-1,23,25-(OH)₃D₃. Therefore significant pathways of kidney and intestinal 1,25-(OH)₂D₃ metabolism seem to involve initial oxidation at C-24 followed by C-23-oxidation of the C-24-oxidized products. A second pathway is initiated by direct C-23-oxidation of 1,25-(OH)₂D₃ to produce 1,23,25-(OH)₃D₃ (Napoli & Horst, 1983a). 1,23,25-(OH)₃D₃ has been converted into 23-oxo-1,25-(OH)₂D₃ with chick kidney homogenates (Horst *et al.*, 1982a), but this derivative has not been detected as a quantitatively significant metabolite of 1,25-(OH)₂D₃ *in vivo* (Napoli *et al.*, 1983; Napoli & Horst, 1983b). 1,23,25-(OH)₃D₃ is precursor to 1,25-(OH)₂D₃-26,23-lactone *in vitro* and *in vivo* (R. L. Horst, G. W. Engstrom & J. L. Napoli, unpublished work). In the LLC PK₁ cells, the amount of 1,25-(OH)₂D₃-26,23-lactone observed was quantitatively minor relative to the C-24-oxidized metabolites. It is possible, therefore, that formation of cholecalciferol-26,23-lactones depends on substrate excess, and that C-23-oxidation of C-24-oxidized metabolites represents the more physiological pathway. Alternatively, cholecalciferol-26,23-lactones may be rapidly metabolized. These questions require further study.

Apparently, regulation of 1,25-(OH)₂D₃ homeostasis by 1,25-(OH)₂D₃ itself is dependent on induction of renal 1,25-(OH)₂D₃ 24- and 23-hydroxylases in addition to feedback inhibition of the renal 25-OH-D₃ 1 α -hydroxylase. The induction of the C-23- and C-24-hydroxylases also occurs in small intestine and perhaps in other cholecalciferol target tissues. Consequently, cholecalciferol target tissues seem to exert extra-renal control over 1,25-(OH)₂D₃ concentrations through several metabolic pathways.

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