(23*S*)-1,23,25-Trihydroxycholecalciferol, an intestinal metabolite of 1,25-dihydroxycholecalciferol

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(23*S*)-23,25-Dihydroxycholecalciferol was converted into a polar metabolite in a calciferol-deficient chick kidney homogenate. The metabolite was identified as (23*S*)-1,23,25-trihydroxycholecalciferol by absorbance spectroscopy and mass spectrometry, and by formation of derivatives. (23*S*)-1,23,25-Trihydroxycholecalciferol was also observed as a 1,25-dihydroxycholecalciferol metabolite in intestinal cells isolated from 1,25-dihydroxycholecalciferol-treated rat. The trihydroxy metabolite was 50-fold less potent than 1,25-dihydroxycholecalciferol in the chick intestinal 1,25-dihydroxycholecalciferol receptor assay.

25(OH) D_3 is the major circulating metabolite of cholecalciferol (Haddad *et al.*, 1977; Horst *et al.*, 1981). During calciferol sufficiency, 25(OH) D_3 is converted into (23*S*)-23,25(OH) $_2D_3$, amongst other metabolites. (23*S*)-23,25(OH) $_2D_3$ circulates in plasma, and is formed in kidney and in extrarenal tissue (Napoli *et al.*, 1982). (23*S*)-23,25(OH) $_2D_3$ has an apparent high turnover rate, and itself is converted into several metabolites, including 23-oxo-25(OH) D_3 (Horst *et al.*, 1982, 1983), 23,25,26-(OH) $_3D_3$ (Napoli & Horst, 1982) and (23*S*,25*R*)-25(OH) D_3 -lactone (Morris *et al.*, 1981*a,b*; Ishizuka *et al.*, 1981). It therefore appears that 23-hydroxylation is an important event in vitamin D metabolism. The present paper reports that (23*S*)-23,25(OH) $_2D_3$ undergoes 1 α -hydroxylation in kidney to produce (23*S*)-1,23,25(OH) $_3D_3$ and that intestinal cells produce (23*S*)-1,23,25(OH) $_3D_3$ from 1,25(OH) $_2D_3$.

Abbreviations used: 25(OH) D_3 , 25-hydroxycholecalciferol (calcidiol); 23,25(OH) $_2D_3$, 23,25-dihydroxycholecalciferol; 1,25(OH) $_2D_3$, 1,25-dihydroxycholecalciferol (calcitriol); 1,23,25(OH) $_3D_3$, 1,23,25-trihydroxycholecalciferol; 25(OH) D_3 -lactone, 25-hydroxycholecalciferol-23,26-lactone; e.i., electron impact; p.c.i., positive-ion chemical ionization; h.p.l.c., high-performance liquid chromatography; s.i.m., selected ion monitoring; ODS, octadecylsilane; HBSS, Hank's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Materials and methods

General methods

1,25(OH) $_2D_3$, (23*S*)- and (23*R*)-23,25(OH) $_2D_3$ and 1,24,25(OH) $_3D_3$ were supplied by M. R. Uskokovic and J. J. Partridge, Hoffmann-LaRoche, Nutley, NJ, U.S.A. (Partridge *et al.*, 1976, 1982). (23*S*)-23,25(OH) $_2$ [3H] D_3 (sp. radioactivity 90 Ci/mmol) was made from 25(OH)[26,27- 3H] D_3 (0.5 mCi/15 ml of reaction mixture) with kidney homogenates prepared from 1,25(OH) $_2D_3$ -treated chicks (Horst, 1979). E.i. mass spectra were obtained at 70 eV from the solids probe of a Finnigan model 4021 GC/MS instrument. To obtain spectra, the probe was heated from ambient temperature to 320°C over a 10 min period at a source temperature of 250°C. P.c.i. mass spectra were obtained with methane as reagent gas. U.v.-absorbance spectra were taken in propan-2-ol with a Beckman model 25 spectrophotometer. H.p.l.c. was performed with Waters Associates model ALC/GPC 204 liquid-chromatographic equipment. Normal (silica gel) and reverse-phase (ODS) h.p.l.c. columns (0.46 cm $\times$ 25 cm) were purchased from Du Pont, unless otherwise noted. The chick intestinal 1,25(OH) $_2D_3$ receptor binding assay was performed as described by Horst *et al.* (1981).

Generation of 1,23,25(OH) $_3D_3$ from 23,25(OH) $_2D_3$

White Leghorn cockerels (1 day old; Hy-line Indian River Co., Dallas Center, IA, U.S.A.) were fed calciferol-deficient diets (1% calcium) for

14 days, and switched to a calciferol-deficient calcium-free diet for 7 days. On day 21, a kidney homogenate was prepared. Unlabelled (23*S*)-23,25-(OH)₂D₃ (0.12 μmol/flask; five flasks), (23*S*)-23,25-(OH)₂[³H]D₃ (2 μCi in one flask) and (23*R*)-23,25-(OH)₂D₃ (0.12 μmol/flask; five flasks) were incubated with the kidney homogenate for 1.5 h at 37°C as described by Napoli & Horst (1982). The reactions were quenched with 3.75 vol. of methanol/dichloromethane (2:1, v/v). The lipids were extracted as reported by Horst *et al.* (1981). The extracts from the five flasks incubated with unlabelled (23*S*)-23,25-(OH)₂D₃ were combined with the extract from the flask in which (23*S*)-23,25-(OH)₂[³H]D₃ had been incubated. The five extracts from the incubations of unlabelled (23*R*)-23,25-(OH)₂D₃ were also combined.

(23*S*)- and (23*R*)-1,23,25-(OH)₃D₃ were individually isolated from the appropriate incubations by identical procedures. Details will be given for (23*S*)-1,23,25-(OH)₃D₃. The sample was eluted through a column (0.6 cm × 10 cm) of silicic acid and Celite (1:1, w/w) with ethyl acetate (12 ml). The recovered material was eluted from a normal-phase h.p.l.c. column with propan-2-ol/hexane (7:43, v/v). Two radiolabelled peaks were observed. One (71% of the radioactivity) migrated with authentic (23*S*)-23,25-(OH)₂D₃ (7 ml). The second (29% of the radioactivity) eluted at 22 ml and was also associated with a u.v.-absorbing peak. Authentic 1,25-(OH)₂D₃, 1,24,25-(OH)₃D₃ and 1,25,26-(OH)₃D₃ were eluted at 17, 28 and 43 ml respectively. The peak at 22 ml was rechromatographed on the same column with propan-2-ol/dichloromethane (7:93, v/v). A single radioactive peak migrating with a u.v.-absorbing peak was observed at 28 ml. Authentic, 1,25-(OH)₂D₃, 1,24,25-(OH)₃D₃ and 1,25,26-(OH)₃D₃ were eluted at 15, 33 and 58 ml respectively. The peak was re-applied to the column, and eluted with propan-2-ol/hexane (11:84, v/v) in the recycle mode for a total of three passes. Approx. 500 ng (on the basis of u.v.-absorbance measurement) of (23*S*)-1,23,25-(OH)₃D₃, and about 200 ng (on the basis of h.p.l.c. peak height) of (23*R*)-1,23,25-(OH)₃D₃ were obtained.

Generation of 1,23,25-(OH)₃D₃ from 1,25-(OH)₂D₃

Male Sprague-Dawley rats (300 g; Harlan, Indianapolis, IA, U.S.A.), fed a stock diet, were injected intraperitoneally with 1,25-(OH)₂D₃ (500 ng in 0.1 ml of propylene glycol) at 24, 12 and 6 h before decapitation. The entire small intestine was removed. The lumen was rinsed with a solution of 0.9% NaCl and 25 mM-dithiothreitol (70 ml). The intestine was inverted and the ends were tied. The intestinal sac was incubated with 20 mg of Protease

(type IX; Sigma) for 15 min at 37°C with shaking in 20 ml of buffer A [HBSS, without calcium and magnesium, containing Hepes (15 mM), glucose (5 mM), BSA (1%, w/v), penicillin-G (5 units/ml), heparin (60 USP units/ml), dithiothreitol (25 mM) and EDTA (5 mM), pH 7.4]. The mucosal cells were removed by gently pulling the intestine through the blades of a pair of scissors. The suspension was sedimented. The mucosal cells were washed with 20 ml of buffer B [buffer A with calcium (1 mM), magnesium (1.4 mM) and phenylmethanesulphonyl fluoride (1 mM)] and sedimented. Half of the cells (10⁸) from one intestine were suspended in 15 ml of buffer B. Viability was determined by Trypan Blue exclusion to be > 90%. The intestinal cell suspension (15 ml) was added to 1,25-(OH)₂[³H]D₃ (90 Ci/nmol; 2.9 nmol) in an Erlenmeyer flask (125 ml). After incubation at 37°C with gentle shaking for 30 min, the reaction was quenched with methanol/dichloromethane (2:1, v/v), and the lipids were extracted (Horst *et al.*, 1981). The residue obtained from evaporation of the organic phase was filtered through a silica gel Sep-Pak and a 0.45 μm filter with ethyl acetate. The recovered radiolabelled material was analysed with a normal-phase h.p.l.c. column eluted with propan-2-ol/hexane (1:9, v/v). In this system, 1,25-(OH)₂D₃, 1,23,25-(OH)₃D₃ and 1,24,25-(OH)₃D₃ were eluted at 38, 60 and 93 ml respectively.

Results and discussion

A kidney homogenate, prepared from calciferol-deficient chickens, converted (23*S*)-23,25-(OH)₂[³H]D₃ (2.5 nM) into a single more polar metabolite. Material for isolation was generated with separate incubations of unlabelled synthetic (23*S*)-23,25-(OH)₂D₃ and (23*R*)-23,25-(OH)₂D₃ (13 μM each). The products of each unlabelled substrate were individually isolated, but migrated identically in the h.p.l.c. systems used. Each was more polar than 1,25-(OH)₂D₃, and less polar than 1,24,25-(OH)₃D₃ and 1,25,26-(OH)₃D₃ in either hexane- or dichloromethane-based h.p.l.c. systems. The u.v.-absorbance spectrum of the product from (23*S*)-23,25-(OH)₂D₃ had a λ_{max} at 265 nm and a λ_{min} at 229 nm. This indicated the presence of a calciferol-like (5*E*)-5,7,10(19)-triene chromophore.

An e.i. mass spectrum of putative (23*S*)-1,23,25-(OH)₃D₃ (Fig. 1) had a molecular ion at *m/z* 432, consistent with the addition of a hydroxy group to (23*S*)-23,25-(OH)₂D₃. Peaks at *m/z* 414, 396 and 381 represented respective losses of water, two molecules of water and two molecules of water and a methyl group from the molecular ion. The peak at *m/z* 358 result from C-23-C-24 bond

Fig. 1. E.i. mass spectrum of (23S)-1,23,25(OH)₃D₃

cleavage, and is unique to C-23-functionalized metabolites. Loss of water from m/z 358 produced m/z 340. The side-chain fragmentation pattern was similar to that of (23S)-23,25(OH)₂D₃ (Napoli *et al.*, 1982). Placement of the additional hydroxy group in the A-ring was demonstrated by the peak at m/z 152 (A-ring plus C-6 and C-7), and the base peak at m/z 134 (m/z 152 – H₂O). These peaks, and their relative abundancies, are characteristic of 1 α -hydroxylated calciferols (Paaren *et al.*, 1977; Horst *et al.*, 1982). A p.c.i. mass spectrum, obtained in the s.i.m. mode, had peaks (with relative intensities in parentheses) at m/z 433 (5), 415 (35), 397 (22), 359 (1), 341 (35), 270 (9), 252 (15), 153 (30) and 135 (100%). These data corroborated the e.i. mass-spectral data. (P.c.i. mass spectrometry in the s.i.m. mode refers to monitoring only selected positive ions, rather than the entire spectrum.)

Putative (23S)-1,23,25(OH)₃D₃ (200 ng) was allowed to react with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (50 μ l) at 90°C for 2 h to effect persilylation. The silylated product was eluted at 15 ml with 0.1% dichloromethane in methanol from an h.p.l.c. column (Whatman ODS-3; 0.42 cm $\times$ 25 cm). An e.i. mass spectrum had a molecular ion at m/z 720 (3), indicating the presence of four hydroxy groups. Peaks at m/z 630 (10), 540 (2) and 450 (1) represented sequential losses of (CH₃)₃SiOH groups from the molecular ion. A peak at m/z 575 (1) was produced by C-23–C-24 bond cleavage. Sequential losses of (CH₃)₃SiOH from m/z 575 gave m/z 485 (1) and m/z 395 (1). The base peak at m/z 206 (100%) was the silylated counterpart of m/z 134 in the e.i. mass spectrum. A peak at m/z 131 (95) indicated C-24–C-25 bond cleavage. These data support the structural assignment as 1,23,25-(OH)₃D₃. These standards were used to determine

Fig. 2. H.p.l.c. analysis of radioactivity migrating with 1,23,25(OH)₃D₃ produced by intestinal cells *in vitro* from 1,25(OH)₂D₃

Material obtained from an initial h.p.l.c. column was analysed on a second h.p.l.c. column. (a; 2 ml/fraction), eluted with a propan-2-ol/dichloromethane (7:93, v/v). The 1,23,25(OH)₃D₃ peak recovered from (a) was analysed on a third h.p.l.c. column (b; 1 ml/fraction), where the amino-modified normal phase was eluted with propan-2-ol/hexane (12:43, v/v). The elution positions of authentic standards are indicated: 1, 1,25(OH)₂D₃; 2, 1,23,25(OH)₃D₃; 3, 1,24,25(OH)₃D₃.

whether intestine converted 1,25(OH)₂D₃ into 1,23,25(OH)₃D₃.

Intestinal cells isolated from 1,25(OH)₂D₃-treated rat converted 1,25(OH)₂[³H]D₃ (0.2 μ M) into several tritiated peaks eluting in h.p.l.c. between 47 and 51 ml (peak A), 54 and 63 ml (peak B) and 83 and 90 ml (peak C). Peak B, the peak migrating in the area of 1,23,25(OH)₃D₃, contained 52% of the product radioactivity. Peak B was re-analysed on the same column developed with propan-2-ol/dichloromethane (7:93, v/v). A multi-component mixture was observed (Fig. 2a), containing peak B1 (46%), peak B2 (37%), peak B3 (3.5%) and peak B4 (14%). Peak B3 migrated with the 1,23,25(OH)₃D₃ standard. Peak B3 was applied to an amino-modified normal-phase h.p.l.c. column and was eluted with propan-2-ol/hexane (7:43, v/v). This column distinguished (23S)- from (23R)-1,23,25(OH)₃D₃. Most of the radioactivity in peak B3 (51%) migrated with (23S)-1,23,25(OH)₃D₃; no radiolabelled peak migrated with (23R)-1,23,25(OH)₃D₃. Contamination of B3 by the other B peaks, particularly B2, probably contributed to the remaining peaks in Fig. 2(b). Peak B3 from the amino-modified h.p.l.c. column was applied to a reverse-phase h.p.l.c. column developed with water/methanol (11:39,

v/v). The radioactivity co-eluted at 13 ml with standard 1,23,25(OH)₃D₃ in this fourth h.p.l.c. system. No other peaks were present.

(23*S*)-1,23,25(OH)₃D₃ was compared with 1,25(OH)₂D₃ in affinity for the chick intestinal cytosolic 3.7*S* 1,25(OH)₂D₃-specific receptor; 50% displacement of 1,25(OH)₂[³H]D₃ was caused by either 5.3 pmol of (23*S*)-1,23,25(OH)₃D₃ or 0.1 pmol of 1,25(OH)₂D₃.

The present paper shows that kidney is capable of 1α-hydroxylating (23*S*)-23,25(OH)₂D₃, a circulating vitamin D₃ metabolite (Napoli *et al.*, 1982) and that intestinal cells *in vitro* produce (23*S*)-1,23,25(OH)₃D₃ from 1,25(OH)₂D₃, during conditions of vitamin D₃ excess. (23*S*)-1,23,25(OH)₃D₃ has much lower affinity for the intestinal cytosolic 3.7*S* 1,25(OH)₂D₃ receptor than does the hormone 1,25(OH)₂D₃. These data suggest that induction of an intestinal 1,25(OH)₂D₃-23*S*-hydroxylase may be a mechanism of 1,25(OH)₂D₃ degradation. The low concentration of (23*S*)-1,23,25(OH)₃D₃ observed, relative to other 1,25(OH)₂D₃ metabolites, may reflect a low rate of synthesis, or a high turnover rate. These questions require further investigation.

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