METABOLISM OF 1α-HYDROXYVITAMIN D_3 TO 1α,25-DIHYDROXYVITAMIN D_3 IN PERFUSED RAT LIVER

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Received July 16,1975

Summary: The metabolism of 1α-hydroxyvitamin D_3 (1α-OH-D_3) was studied in rat liver perfused with [^{3}H]-1α-OH-D_3. [^{3}H]-1α-OH-D_3 was converted very rapidly to a more polar metabolite, which was identified as 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] by co-chromatography with synthetic 1α,25-$(OH)_2$-D_3 as well as by gas chromatograph-mass spectrometry. [^{3}H]-1α,25-$(OH)_2$-D_3 appeared in the perfusate as early as 20 min after addition of [^{3}H]-1α-OH-D_3, and its level in the perfusate increased linearly for at least 120 min. These data strongly indicate that 1α-OH-D_3 is metabolized to 1α,25-$(OH)_2$-D_3, which exerts biological effects on bone and intestine.

INTRODUCTION

Recently, much attention has been focused on a synthetic analog of vitamin D, 1α-hydroxyvitamin D_3 (1α-OH-D_3) (1-3). This analog was found to cause as great a biologic response as the hormonal form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3], in an *in vivo* time course study, suggesting that it might act directly on bone and intestine (4). However, Brumbaugh and Haussler demonstrated clearly that 1α-OH-D_3 binds to the intestinal chromatin receptor two to three orders of magnitude less avidly than 1α,25-$(OH)_2$-D_3 (5). In addition, Zerwekh et al. isolated a fraction thought to contain 1α,25-$(OH)_2$-D_3 from intestinal chromatin of rachitic chicks after administration of 1α-OH-D_3 (6). These findings, together with the fact that dihydrotachysterol$_3$, which is similar in structure to 1α-OH-D_3, is rapidly hydroxylated at position 25 *in vivo* (7), suggest that 1α-OH-D_3 must be converted to 1α,25-$(OH)_2$-D_3 before exerting its biological effects on the target tissues. Final proof of this, however, required metabolic studies with radioactive 1α-OH-D_3.

Abbreviations used: 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1α,25-$(OH)_2$-D_3, 1α,25-dihydroxyvitamin D_3.

Very recently, tritiated 1α-OH-D_3 was successfully synthesized in our laboratory. This communication shows clearly that [^{3}H]-1α-OH-D_3 is converted to [^{3}H]-1α,25-$(OH)_2$-D_3 very rapidly in perfused rat liver.

MATERIALS AND METHODS

Weanling male rats (Sprague-Dawley) were maintained for 6-7 weeks on a vitamin D deficient diet (8). Then their livers were carefully removed by the method of Miller et al.(9) and perfused with a mixture of heparinized blood (30 ml) from vitamin D deficient rats and Krebs-Ringer bicarbonate buffer, pH 7.4 (45 ml), equilibrated with 95% O_2, 5% CO_2. The perfusion apparatus (Metaloglass, Inc., Boston) was that devised by Miller et al.(9). The bile duct was cannulated and bile flowed continuously during the experiments. When the flow of perfusate through the liver (approximately 6 g wet weight) attained 30 ml/min, 1.08 μg (411,000 dpm) of radiochemically pure [2-^{3}H]-1α-OH-D_3 (78 mCi/mmol, Tohira et al., in preparation) was added into the perfusate. Then 20, 40, 60, 90 and 120 min later, perfusion was terminated, and the perfusate and liver were each extracted by the method of Bligh and Dyer (10). The chloroform layers of the extracts were evaporated to dryness and chromatographed on a Sephadex LH-20 column (1.5 x 30 cm) using a solvent of 65% chloroform-35% hexane (11). All radioactive determinations were carried out with a Packard Tri-Carb, Model 3385 liquid scintillation spectrometer. Radioactivity in the bile was counted directly in dioxane scintillation fluid (12). The extracts of liver and perfusate, either before or after chromatography, were evaporated to dryness under a stream of air and counted in toluene scintillation fluid (11).

The material in the radioactive peak was co-chromatographed with authentic synthetic 1α,25-$(OH)_2$-D_3 (Matsunaga et al., in preparation) on a Sephadex LH-20 column. The material suspected to be 1α,25-$(OH)_2$-D_3 on the Sephadex LH-20 column was further co-chromatographed with synthetic 1α,25-$(OH)_2$-D_3 on a high speed liquid chromatograph (30 cm x 6.4 mm, μ-Bondapak C_{18}, Waters, Model 440, Milford) which was developed with methanol:water (9:1, v/v). In

addition, a liver was perfused with 25 μg of [^{3}H]-1α-OH-D_3 for 120 min, and then the material suspected to be 1α,25-$(OH)_2$-D_3 on the Sephadex LH-20 column was purified on a Celite column (13). The purified metabolite was then identified with a gas chromatograph-mass spectrometer (Shimazu-LKB, Model 9000).

RESULTS

Table I shows the distribution of the total radioactivity between the perfusate, liver and bile at intervals during 120 min of perfusion. The total radioactivity in the perfusate gradually decreased, while those in the liver and bile increased during the experiment (Table I). However, the radioactivity in the bile was less than 5% of the total at each time examined, so further analyses of 1α-OH-D_3 metabolism were performed on extracts of the perfusate and liver only.

The chromatographic profiles of the extracts of perfusate and liver after

Table I. Distribution of radioactivity between the perfusate, liver and bile at intervals during 120 min of perfusion with [^{3}H]-1α-OH-D_3

Time	Perfusate	Liver	Bile	Total
min	dpm	dpm	dpm	dpm
20	294,000 (71.7 %)	114,000 (27.9 %)	1,600 (0.4 %)	409,600
40	224,000 (57.0 %)	162,000 (41.2 %)	6,900 (1.8 %)	392,900
60	218,000 (51.1 %)	198,000 (46.3 %)	11,300 (2.6 %)	427,300
90	165,000 (42.9 %)	204,000 (53.0 %)	15,600 (4.0 %)	384,600
120	151,000 (40.9 %)	199,000 (53.9 %)	19,200 (5.2 %)	369,200

1.08 μg (411,000 dpm) of [^{3}H]-1α-OH-D_3 was added to the perfusate. The amount of radioactivity recovered in the perfusate, liver and bile was always more than 90% of the isotope added. Figures in parentheses show amounts of radioactivity as percentages of the total recovered.

perfusion for 120 min are shown in Fig 1. [^{3}H]-1α-OH-D_3 was found to be converted very rapidly to a more polar metabolite. This radioactive metabolite migrated in exactly the same position as synthetic 1α,25-$(OH)_2$-D_3 on co-chromatography of the extract of perfusate with synthetic 1α,25-$(OH)_2$-D_3 on a Sephadex LH-20 column (Fig 1, bottom) as well as on high speed liquid chromatography (data are not shown). Final proof that this radioactive metabolite was 1α,25-$(OH)_2$-D_3 was obtained by gas chromatograph-mass spectrometry [m/e 416 (M^+), 398, 380, 269, 251, 134 and 59].

When perfusate containing [^{3}H]-1α-OH-D_3 was circulated in the perfusion apparatus for 120 min in the absence of liver, no radioactivity appeared in

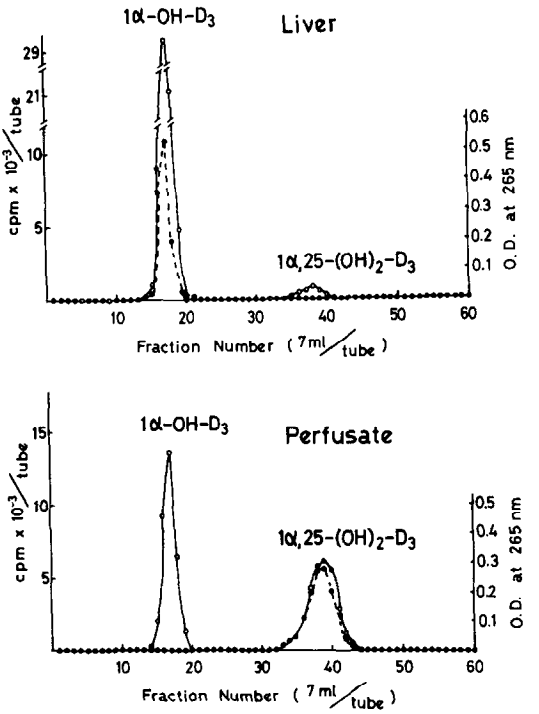

Fig 1. Chromatographic profiles on a Sephadex LH-20 column of extracts of liver (top) and perfusate (bottom) after 120 min of perfusion. Before application to the column, 200 μg of synthetic 1α-OH-D_3 were added to the liver extract (top) and 200 μg of synthetic 1α,25-$(OH)_2$-D_3 were added to the perfusate extract (bottom). The solid line shows radioactivity and the dotted line shows optical density at 265 nm.

the elution position of 1α,25-$(OH)_2$-D_3. Thus the metabolite was not produced by non-enzymatic oxidation during circulation of 1α-OH-D_3 in the perfusate equilibrated with 95% O_2, 5% CO_2.

The amount of [^{3}H]-1α-OH-D_3 in the perfusate decreased rapidly during the experiments with its rapid concomitant incorporation into the liver (Fig 2, left). The uptake of [^{3}H]-1α-OH-D_3 by the liver attained a saturated level in 90 min. The radioactivity found in the liver extract was mainly unchanged [^{3}H]-1α-OH-D_3 (Fig 1, top), and the level of [^{3}H]-1α,25-$(OH)_2$-D_3 was less than 3% of the total radioactivity recovered in the perfusate, liver and bile at each time examined (Fig 2, right). On the other hand, [^{3}H]-1α,25-$(OH)_2$-D_3 was found in the perfusate as early as 20 min after addition of [^{3}H]-1α-OH-D_3, and its level increased linearly for 120 min. At 60, 90 and 120 min after addition of [^{3}H]-1α-OH-D_3, 9, 14 and 19% of the total radioactivity was recovered as [^{3}H]-1α,25-$(OH)_2$-D_3 in extracts of perfusate, respectively (Fig 2, right). When a liver was perfused with 1.08 μg of 1α-OH-D_3 for 120 min, as much as 210 ng of 1α,25-$(OH)_2$-D_3 were recovered in extracts of the perfusate and liver. Thus the rate of 1α,25-$(OH)_2$-D_3 synthesis was approximately 17 ng/hr/g of liver.

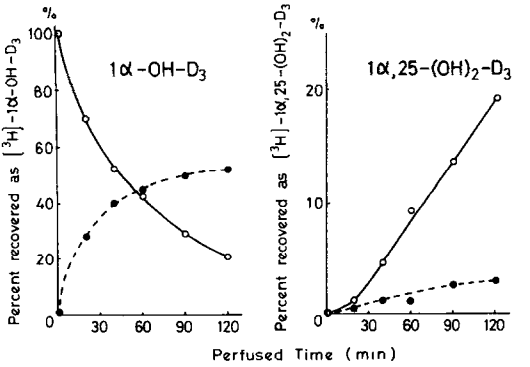

Fig 2. Time course of change in the levels of [^{3}H]-1α-OH-D_3 (left) and [^{3}H]-1α,25-$(OH)_2$-D_3 (right) in the total radioactivity recovered. The solid line shows radioactive metabolites in perfusate extracts and the dotted line shows those in liver extracts. Values are percentages of the total radioactivity recovered in the perfusate, liver and bile.

DISCUSSION

The idea that 1α-OH-D_3 must be converted to 1α,25-$(OH)_2$-D_3 before exerting its biological effects on target tissues has been suggested by several indirect lines of evidence (5-7,14). In this work we could obtain final proof of this idea using [^{3}H]-1α-OH-D_3 synthesized in this laboratory.

At least in rats, the liver is the major site of conversion of vitamin D_3 to the 25-hydroxy derivative (15,16), so we perfused the livers of vitamin D deficient rats with medium containing [^{3}H]-1α-OH-D_3. [^{3}H]-1α,25-$(OH)_2$-D_3 appeared in the perfusate as early as 20 min after addition of [^{3}H]-1α-OH-D_3 to the perfusate, and its level in the perfusate increased linearly for at least 120 min. On the other hand, [^{3}H]-1α,25-$(OH)_2$-D_3 in the extracts of liver remained within 3% of the total radioactivity recovered at each time examined, suggesting that after its production in the liver 1α,25-$(OH)_2$-D_3 is secreted into the blood very quickly. The production of 1α,25-$(OH)_2$-D_3 by the liver is rapid enough, since 1α-OH-D_3 elicits maximal effects at 6-12 hr after intravenous administration (1,3). These findings clearly show that 1α-OH-D_3 is rapidly metabolized to 1α,25-$(OH)_2$-D_3 which acts on the target tissues. The mechanism of the conversion of 1α-OH-D_3 to 1α,25-$(OH)_2$-D_3 in the liver is now under investigation.

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