Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 174-180

A RAPID AND SENSITIVE IN VITRO ASSAY OF 25-HYDROXYVITAMIN D_3-1α-HYDROXYLASE AND 24-HYDROXYLASE USING RAT KIDNEY HOMOGENATES

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Received April 13, 1984

SUMMARY: A sensitive and rapid in vitro assay of 25-hydroxyvitamin D_3 [25-(OH)D_3]-1α- and 24-hydroxylase activities was developed using rat kidney homogenates. A potent inhibitor of the enzymes in rat plasma was removed by thoroughly perfusing rats with saline. Kidney homogenates prepared from vitamin D-deficient rats preferentially produced tritiated 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$$D_3$] from 25(OH)[^{3}H]$D_3$. Addition of 10 μl or more of rat plasma to 3 ml of 10% kidney homogenates suppressed 1α-hydroxylase activity dose-dependently. Thyroparathyroidectomy (TPTX) of vitamin D-deficient rats greatly abolished 1α-hydroxylase activity. Administration of parathyroid hormone to the TPTX rats increased 1α-hydroxylase activity and that of 1α,25(OH)$_2$$D_3$ enhanced 24-hydroxylase markedly. Since this assay is technically simple, rapid and sensitive, it will be useful in studying the regulatory mechanism in the renal metabolism of 25(OH)D_3 in mammals.

It is well known that vitamin D_3, before it exerts its physiological action in bone and intestine, is converted first in the liver to 25-hydroxyvitamin D_3 [25(OH)D_3] and then in the kidney mainly to 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$$D_3$], the active form of the vitamin, and to 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2$$D_3$] (1). Production of these two dihydroxy metabolites in the kidney is rigidly regulated by parathyroid hormone (PTH) (2, 11), 1α,25(OH)$_2$$D_3$ (3, 4), and other hormones and ions (4).

During the last decade, in vitro assays of 25(OH)D_3-1α- and 24-hydroxylase activities have been performed extensively in avian kidneys, because in rat kidney homogenates the existence of some inhibitor(s) interfered with the 1α- and 24-hydroxylation reactions of 25(OH)D_3. The inhibitor is thought to be a vitamin D binding protein in rat plasma (5). However, it has long been

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0006-291X/84 $1.50
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hoped that a sensitive in vitro assay of the 1α- and 24-hydroxylase activities could be developed for rats, the most commonly used mammalian experimental animal in research on vitamin D metabolism.

Recently, it has been possible to measure the enzyme activities in vitro in rats, using kidney mitochondria (6), isolated renal tubules (7) and kidney slices (8), which were extensively washed with buffer to eliminate the inhibitor. However, no one had succeeded in the in vitro assay of 1α- and 24-hydroxylase activities using rat kidney homogenates before the report of Tanaka and DeLuca (9). They demonstrated the method of assaying the enzyme activities by using an extremely high concentration (1×10^{-4} M) of 25(OH)D_3, with which the inhibitor was supersaturated. They determined the enzyme activities by measuring the peak height on a high pressure liquid chromatograph (HPLC) or by a competitive protein binding assay using the intestinal cytosol receptor for 1α,25(OH)$_2D_3$. We have developed a rapid and sensitive assay of the 1α- and 24-hydroxylase activities in homogenates of rat kidney extensively perfused with buffer.

MATERIALS AND METHODS

Animals: Weanling male rats (Sprague-Dawley strain) were maintained on a vitamin D-deficient diet containing 0.47% Ca and 0.30% P (10) for 6 weeks. Some vitamin D-deficient rats were either thyroparathyroidectomized (TPTX) or sham-operated surgically. After the surgery, rats were continuously infused with a nutrient solution containing 5 mM Ca for 30 h as reported previously (11). The infusion enabled vitamin D-deficient TPTX rats to survive for 2 - 3 days. Some of the vitamin D-deficient rats were given either bovine PTH (TCA powder, 181 U/mg, Inolex, Ill) at a rate of 6 U/h for 18 h during the last part of infusion, or 1.3 nmol of 1α,25(OH)$_2D_3$ intravenously once immediately after the surgery. All TPTX rats were killed 30 h after the surgery.

Preparation of rat kidney homogenates: Rats were anesthesized with ether and completely exsanguinated from the aorta with a syringe. The animals were then perfused with 50 ml of calcium- and magnesium-free phosphate buffered saline [PBS(-)]. The color of the kidneys changed from dark violet to pale pink. The kidneys were removed and placed in ice-cold 15 mM Tris-acetate buffer (pH 7.4) containing 0.19 M sucrose, 2 mM magnesium acetate and 25 mM sodium succinate. A 10% (w/v) homogenate was prepared in the Tris-acetate buffer.

Determination of in vitro 25(OH)D_3-1α- and 24-hydroxylase activities: Graded amounts (5 to 3750 pmol) of 25(OH)[^{3}H]D_3 (80,000 cpm) (Amersham, Bucks, U.K.) dissolved in 30 μl of ethanol were added to 3 ml of the homogenates. Oxygen gas was flushed for 1 min into each flask on ice. Then the homogenates were incubated at 37°C for 20 min. The reaction was stopped by adding 10 ml of methanol/chloroform (2/1, v/v). Extraction was performed by the method of Bligh and Dyer (12). The lipid extract was mixed with 520 pmol of authentic

1α,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$ (gifts from Dr. I. Matsunaga, Chugai Pharmaceutical Co., Tokyo, Japan) and applied to a straight phase HPLC [Waters HPLC 204 equipped with a Finepak-SIL column (4.6 mm x 25 cm, JASCO, Tokyo, Japan)]. The column was eluted at a flow rate of 1.5 ml/min using a mixed solvent of hexane/isopropanol/methanol (88/6/6, v/v). Thirty 30 sec fractions were collected and the radioactivity was counted with a liquid scintillation counter. For identification of 1α,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$, the putative 1α,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ fractions from the straight phase HPLC were separately applied to a reverse phase HPLC using a μ-Bondapak C_{18} column (4.6 mm x 25 cm, Waters). The column was eluted at a flow rate of 1.5 ml/min with a solvent of methanol/water (75/25, v/v), and fifty 30 sec fractions were collected. Radioactive peaks obtained from the reverse phase HPLC were purified again by the same straight phase HPLC described above. The resulting radioactive peaks were subjected to the periodate cleavage test of Garabedian et al. (13) and mass spectrometry (JEOL-D300). Plasma calcium and phosphorus concentrations were measured by procedures previously described (14). Statistical significance was determined by analysis of variance or the Student's t test.

RESULTS AND DISCUSSION

Figure 1A shows a straight phase HPLC profile of lipid extracts of kidney homogenates obtained from vitamin D-deficient rats incubated with 3 nmol of 25(OH)[^{3}H]D$_3$ for 20 min. The homogenates metabolized 25(OH)[^{3}H]D$_3$ to a radioactive metabolite which comigrated to the same position as authentic 1α,25-

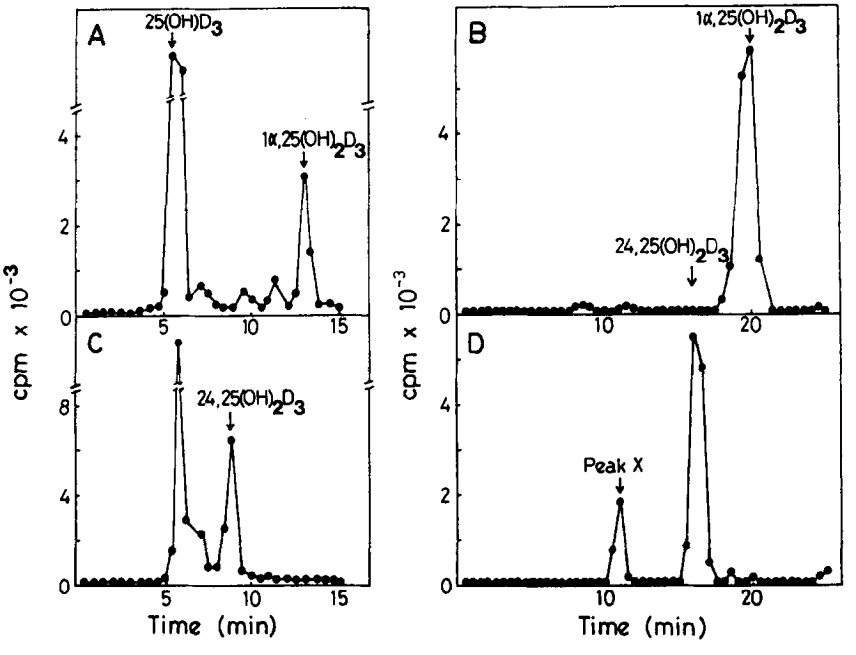

Fig. 1 HPLC profiles of lipid extracts of kidney homogenates from vitamin D-deficient rats (A, B) and 1α,25(OH)$_2$D$_3$-supplemented TPTX rats (C, D). In panel C, vitamin D-deficient rats were given 1.3 nmol of 1α,25-(OH)$_2$D$_3$ intravenously immediately after the surgery and killed 30 h later. The putative 1α,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ fractions on the straight phase HPLC (A, C) were respectively applied to reverse phase HPLC (B, D).

$(OH)_2D_3$ on HPLC. The putative $1\alpha,25(OH)_2D_3$ fraction on the straight phase HPLC column was applied to a reverse phase HPLC column. The radioactive peak which coincided with authentic $1\alpha,25(OH)_2D_3$ was the only one detected and more than 95% of the radioactivity was recovered in that fraction (Fig. 1B). Final identification of the putative $1\alpha,25(OH)_2D_3$ on the reverse phase HPLC was confirmed by mass spectrometry [m/e 416 (M^+), 398, 380, 287, 152 and 134].

Conversion of $25(OH)[^3H]D_3$ to $1\alpha,25(OH)_2[^3H]D_3$ by the kidney homogenates was linear for up to 30 min. Kidney homogenates preheated at 100°C for 5 min did not produce any radioactive metabolites. When kidney homogenates from vitamin D-deficient rats were incubated for 20 min with graded concentrations of $25(OH)[^3H]D_3$, production of $1\alpha,25(OH)_2[^3H]D_3$ was saturated at a concentration of 0.8 - 0.9 μM. The Lineweaver-Burk plot analysis indicated that the apparent Km of the 1α-hydroxylase was 2×10^{-6} M and the Vmax was 170 pmol/300 mg tissue/20 min, which were comparable with those of the chick kidney 1α-hydroxylase reported by Gray et al. (15). The inhibitory effect of rat plasma on the renal 1α-hydroxylase activity is shown in Fig. 2. Addition of 10 μl

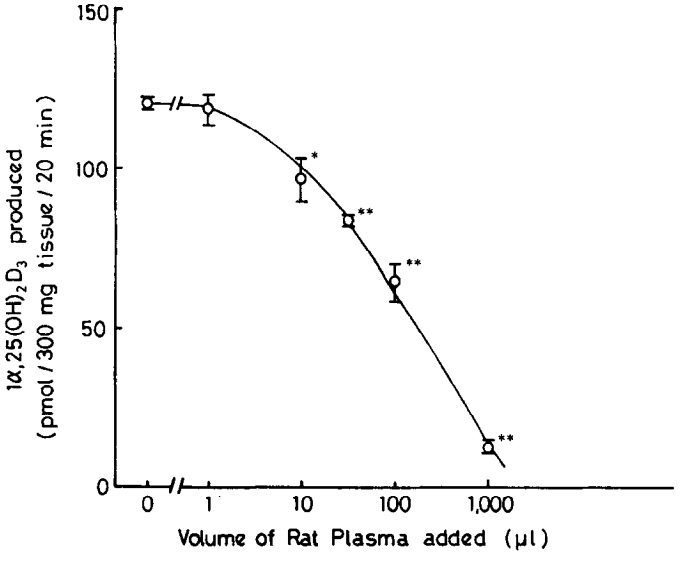

Fig. 2 Inhibitory effect on the renal 1α-hydroxylase activity of vitamin D-deficient rat plasma added to kidney homogenates. A graded volume of rat plasma was added to 3 ml of 10% kidney homogenates. Each kidney homogenate was incubated with 1.25 nmol (80,000 cpm) of $25(OH)[^3H]D_3$. Each point is the mean ± S.E.M. of three rats. Significantly different from the 1α-hydroxylase activity without rat plasma (*, $p < 0.02$; **, $p < 0.001$).

or more of plasma from vitamin D-deficient rats to 3 ml of 10% kidney homogenate suppressed 1α-hydroxylase activity dose-dependently (Fig. 2).

Figure 1C shows a straight phase HPLC profile of lipid extracts of kidney homogenates obtained from 1α,25(OH)$_2$D$_3$-supplemented TPTX rats. The homogenates metabolized 25(OH)[^{3}H]D$_3$ to a radioactive peak which comigrated to the same position as authentic 24R,25(OH)$_2$D$_3$. The putative 24,25(OH)$_2$D$_3$ fraction on the straight phase HPLC was applied to the same reverse phase HPLC column described above. The putative 24,25(OH)$_2$D$_3$ fraction was separated into two radioactive peaks: a major peak which coincided with authentic 24,25(OH)$_2$D$_3$ and peak X (Fig. 1D). About 75% of the radioactivity was recovered in the putative 24,25(OH)$_2$D$_3$ fraction and the remaining 25% was in peak X. Almost all of the radioactivity in the putative 24,25(OH)$_2$D$_3$ fraction and in peak X was similarly transferred into the water phase after periodate cleavage. Final identification of the putative 24,25(OH)$_2$D$_3$ on the reverse phase HPLC was confirmed by mass spectrometry [m/e 416 (M^+), 398, 383, 271, 253, 136 and 118; periodate cleavage product, m/e 356 (M^+)].

Table 1 shows the effects on renal 1α- and 24-hydroxylase activities of PTH and 1α,25(OH)$_2$D$_3$ administered to vitamin D-deficient TPTX rats. Thyroparathyroidectomy of vitamin D-deficient rats markedly suppressed renal 1α-

Table 1. Effect of PTH and 1α,25(OH)$_2$D$_3$ on the renal 25(OH)D$_3$-1α- and 24-hydroxylase activities and the plasma Ca and Pi levels in vitamin D-deficient TPTX rats

Operation	Hormones administered	Number of rats	1α,25(OH)$_2$D$_3$ produced (pmol/300 mg	24,25(OH)$_2$D$_3$ produced tissue/20 min)	Plasma Ca (mg/100 ml)	Plasma Pi (mg/100 ml)
Sham	———	11	84 ± 8[b]	< 1.0	6.4 ± 0.6	8.5 ± 0.2
	———	4	8 ± 1[a]	< 1.0	4.3 ± 0.4[a]	13.4 ± 1.2[a]
TPTX	PTH	6	58 ± 5[b]	< 1.0	9.1 ± 1.1[b]	9.7 ± 0.3[a,b]
	1α,25(OH)$_2$D$_3$	5	< 1.0[a]	174 ± 8[a,b]	7.0 ± 0.7[b]	15.1 ± 2.0[a]

Each kidney homogenate was incubated with 1.25 nmol (80,000 cpm) of 25(OH)-[^{3}H]D$_3$. The amounts of 1α,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ produced were calculated from the radioactivity of the respective metabolites on the first straight phase HPLC and from the mean recoveries of the metabolites [95% as 1α,25(OH)$_2$-D$_3$ and 75% as 24,25(OH)$_2$D$_3$] on the second reverse phase HPLC.
Data are means ± S.E.M.
a. significantly different from the sham group, $p < 0.01$
b. significantly different from the TPTX control group, $p < 0.01$

hydroxylase activity, but it did not increase 24-hydroxylase activity. The surgery decreased plasma Ca and increased Pi levels significantly. When vitamin D-deficient TPTX rats were infused with bovine PTH at a rate of 6 U/h for 18 h starting 12 h after the surgery, 1α-hydroxylase activity was greatly increased. On the other hand, when vitamin D-deficient TPTX rats were given 1.3 nmol of 1α,25(OH)$_2$D$_3$ immediately after the surgery, renal 24-hydroxylase activity was strikingly stimulated 30 h later. Administration of PTH to vitamin D-deficient TPTX rats increased plasma Ca and decreased Pi levels, whereas injection of 1α,25(OH)$_2$D$_3$ significantly increased only plasma Ca levels.

This paper describes a rapid and sensitive assay of the renal 1α- and 24-hydroxylase activities using rat kidney homogenates. The method of preparation of rat kidney homogenates is essentially the same as that used for preparing avian kidney homogenates, except that the kidneys are thoroughly perfused and washed with a large volume of PBS(-). We used Sprague-Dawley strain rats in this study, whereas Holtzman strain rats have been used extensively in research on mammalian vitamin D metabolism, especially in the United States. We have also succeeded in the same in vitro assay of the enzymes in Wistar strain rats (data not shown). Thus, it is likely that the vitamin D binding protein in the plasma of Holtzman rats is a much more potent inhibitor of the 1α-hydroxylase than that in Sprague-Dawley and Wistar rats.

Using this in vitro assay system, the stimulating effect of PTH and the inhibitory effect of 1α,25(OH)$_2$D$_3$ both on the renal 1α-hydroxylase activity, and the stimulating effect of 1α,25(OH)$_2$D$_3$ on the renal 24-hydroxylase activity, all of which have already been established in avian kidney homogenates (16 - 18), were clearly confirmed in rats. Since this assay is technically simple, rapid and sensitive, it will be very useful in studying the regulatory mechanism in the renal metabolism of 25(OH)D$_3$ in mammals.

REFERENCES

1. DeLuca, H. F. (1979) J. Steroid Biochem. 11, 35-52.
2. Garabedian, M., Holick, M. F., DeLuca, H. F., and Boyle, I. T. (1972) Proc. Natl. Acad. Sci. USA 69, 1673-1676.

3. Tanaka, Y., and DeLuca, H. F. (1974) Science (Washington) 183, 1198-1200.

4. Fraser, D. R. (1980) Physiol. Rev. 60, 551-612.

5. Botham, K. M., Ghazarian, J. G., Kream, B. E., and DeLuca, H. F. (1976) Biochemistry 15, 2130-2135.

6. Vieth, R., and Fraser, D. (1979) J. Biol. Chem. 254, 12455-12460.

7. Turner, R. T., Bottemiller, B. L., Howard, G. A., and Baylink, D. J. (1980) Proc. Natl. Acad. Sci. USA 77, 1537-1540.

8. Armbrecht, H. J., Zenser, T. V., and Davis, B. B. (1981) Endocrinology 109, 218-222.

9. Tanaka, Y., and DeLuca, H. F. (1981) Proc. Natl. Acad. Sci. USA 78, 196-199.

10. Suda, T., DeLuca, H. F., and Tanaka, Y. (1970) J. Nutr. 100, 1049-1052.

11. Horiuchi, N., Suda, T., Takahashi, H., Shimazawa, E., and Ogata, E. (1977) Endocrinology 101, 969-974.

12. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917.

13. Garabedian, M., Pavlovitch, H., Fellot, C., and Balsan, S. (1974) Proc. Natl. Acad. Sci. USA 71, 554-557.

14. Horiuchi, N., Suda, T., Sasaki, S., Ezawa, I., Sano, Y., and Ogata, E. (1974) Arch. Biochem. Biophys. 171, 540-548.

15. Gray, R. W., Omdahl, J. L., Ghazarian, J. G., and DeLuca, H. F. (1972) J. Biol. Chem. 247, 7528-7532.

16. Fraser, D. R., and Kodicek, E. (1973) Nature (London) New Biol. 241, 163-166.

17. Tanaka, Y., Lorenc, R. S., and DeLuca, H. F. (1975) Arch. Biochem. Biophys. 171, 521-526.

18. Henry, H. L., Midgett, R. J., and Norman, A. W. (1974) J. Biol. Chem. 249, 7584-7592.