DIRECT INVOLVEMENT OF VITAMIN D IN THE REGULATION OF 25-HYDROXYCHOLECALCIFEROL METABOLISM

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Received 22 March 1974

1. Introduction

Much evidence indicates that vitamin D_3 should be converted first in the liver to 25-hydroxycholecalciferol [25-OH-D_3] and then in the kidney to 1,25-dihydroxycholecalciferol [1,25-$(OH)_2$-D_3], before it exerts physiological actions in bone and intestine [1,2]. This active metabolite of vitamin D_3 induces a remarkable change in the levels of plasma calcium and phosphate [1]. Therefore, to maintain rigid homeostasis of these ions, an efficient feed-back control system is expected to be operating in the process of 1,25-$(OH)_2$-D_3 formation. A major site under such control appears to be the hydroxylation reaction of 25-OH-D_3 to 1,25-$(OH)_2$-D_3 [3–8]. Several modes of the control have been advocated in regards to this mitochondrial reaction in the kidney. The most conspicuous one is related to whether 25-OH-D_3 is metabolized to an active form [1,25-$(OH)_2$-D_3] or to a nearly inert metabolite [24,25-$(OH)_2$-D_3]. This type of regulation was shown to function in vivo when the animals had been placed for some time under the stress of calcium and phosphate metabolism. More 1,25-$(OH)_2$-D_3 was produced when the animals were in need of calcium, and more 24,25-$(OH)_2$-D_3 was produced in the opposite situation [3]. Several concepts have been proposed for the mechanism of this particular type of regulation, and the plasma level of calcium [3], phosphate [4], or parathyroid hormone [5–7] has been suggested as a direct factor that leads to this kind of metabolic regulation.

In the present communication, we intend to report evidence that the regulation can proceed in quite an efficient way without changes in the plasma levels of calcium, phosphate and possibly parathyroid hormone. It led us to a suggestion that the level or the action of vitamin D or its metabolite(s) in the animal is the major factor that exerts this regulation.

2. Materials and methods

2.1. *Animals and preparation of kidney mitochondria*

Twenty-eight white Leghorn cockerel chicks of one day old were maintained for 2 weeks on a vitamin D-deficient diet [9] containing 1.2% calcium and 0.45% phosphorus. Then, the chicks were divided into 4 groups; the first and the second were fed the same diet of 3.0% calcium, and the third and the fourth the same diet of 1.2% calcium, for another 2 weeks. In

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addition, the second, the third and the fourth group of the chicks were administered orally 1, 10 and 100 IU of vitamin D_3 every day for the last 2 weeks, respectively. After the feeding for 4 weeks in total, the chicks were killed by decapitation. Kidney mitochondria free from heavy contamination of calcium were prepared as described earlier [8]. Plasma calcium and phosphate concentrations were determined by means of a Perkin–Elmer Model 403 atomic absorption spectrometer and with the method of Fiske–Subbarow [10], and mitochondrial protein was assayed by the method of Gornall et al. [11].

2.2. *Incubation and extraction of samples*

In a 25 ml Erlenmeyer flask, mitochondria (approximately 6.0 mg of protein) were preincubated in air for 5 min at 30°C in 1 ml of a solution of Gray et al [8,12], to which graded concentrations of calcium or EGTA were added as indicated in the text. The reaction was started with the addition of 790 ng (0.18 μCi) of [26,27-^{3}H]-25-OH-D_3 (Amersham, Bucks, England), and stopped 20 min thereafter by the addition of 10 ml of a mixture of methanol–chloroform (2:1 v/v). Extraction was performed as reported by Gray et al. [12].

2.3. *Measurements of in vitro production of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 from 25-OH-D_3*

Chromatography of the extracts was carried out on a 1 × 30 cm column of Sephadex LH 20 using a solvent of 65% chloroform–35% hexane according to the method of Holick and DeLuca [13]. Amounts of each vitamin D_3 metabolite produced in vitro were calculated from the radioactivities of the respective metabolites and the specific activity of the isotope, as described earlier [8].

3. Results

After having tried various concentrations in the content of calcium, phosphorus and vitamin D in the diet, we arrived at a program as described under Materials and methods that allowed us to produce the chicks constantly that were rationed with graded doses of vitamin D, but had similar extent of hypercalcemia and hypophosphatemia (table 1). As a result of hypercalcemia, the animals are also expected to be identical in the state of secondary hypoparathyroidism. This enabled us to test the influence of dietary vitamin D without interference from such variables as plasma levels of calcium, phosphate and parathyroid hormone. In every case, the in vitro activity of kidney mitochondria to produce either 1,25-$(OH)_2$-D_3 (the activity of 1-hydroxylase) or 24,25-$(OH)_2$-D_3 (the activity of 24-hydroxylase) was strikingly dependent on the concentration of calcium in the reaction system. (Representative data are shown in fig. 1. See also ref. [8].) In our experimental condition, the maximal activity was obtained with 0.2 mM $CaCl_2$ for 1-hydroxylase and with 0.4 mM $CaCl_2$ for 24-hydroxylase. In fig. 2 are summarized the data on the maximal activity of the hydroxylases as a function of the dose of dietary vitamin D. Even in the absence

Table 1
Dietary regimen and plasma concentration of calcium and phosphate

Group	Diet			Plasma	
	Ca	P	Vitamin D_3	Ca	P
	(%)	(%)	(IU/day)	(mg %)	(mg %)
1	3.0	0.45	0	11.9 ± 0.37(7)*	5.1 ± 0.40(7)*
2	3.0	0.45	1	12.4 ± 0.36(6)	5.0 ± 0.57(6)
3	1.2	0.45	10	12.8 ± 0.30(7)	6.0 ± 0.18(7)
4	1.2	0.45	100	13.1 ± 0.50(7)	5.4 ± 0.26(7)

* Mean ± standard error (number of animals).
The concentrations of plasma calcium and phosphate of the normal chicks fed a diet containing adequate Ca (1.2%), P(0.45%) and vitamin D_3 (5 IU/day) were 10.6 ± 0.19 and 7.5 ± 0.50 mg%, respectively.

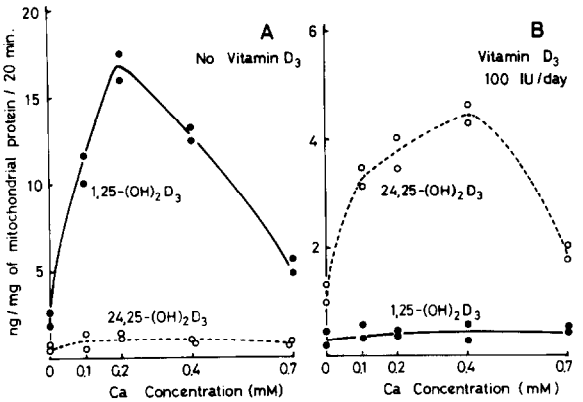

Fig. 1. Relationship between calcium concentration and the rate of in vitro synthesis of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 in kidney mitochondria taken from the chicks of the first (A^3) and the fourth group (B). Incubation was performed as described under Materials and methods in the Gray's medium of 15 mM Tris–acetate, pH 7.4, 2 mM $MgCl_2$, 5 mM succinate, 0.4 mM NADP and 0.22 M sucrose. $CaCl_2$ was added as indicated on the abscissa except that EGTA was added to 0.5 mM for 0 concentration of calcium. Each point represents one complete experiment. Note that the rate of 1,25-$(OH)_2$-D_3 production and that of 24,25-$(OH)_2$-D_3 production was maximum at the concentration of 0.2 mM and 0.4 mM calcium, respectively.

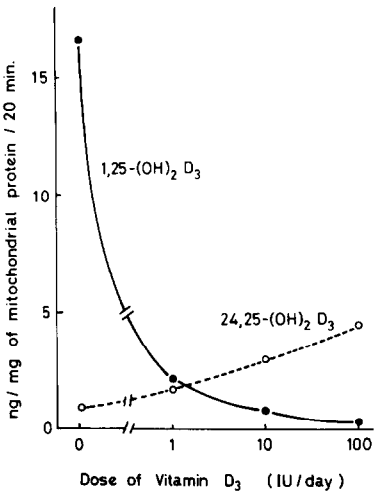

Fig. 2. Relationship between the amount of vitamin D_3 dosed to the chicks and the rate of in vitro synthesis of 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 in kidney mitochondria. Incubation was carried out as in fig. 1. The activity of 1-hydroxylase and that of 24-hydroxylase were assayed in the presence of 0.2 mM and 0.4 mM calcium, respectively. The animals allocated to the doses (0, 1, 10 and 100 IU/day) of vitamin D_3 corresponded to the animals of group 1, 2 3 and 4 in table 1. Each point represents mean of two complete experiments.

of difference in the plasma levels of calcium, phosphate and possibly of parathyroid hormone, the activity of 1 hydroxylase and that of 24 hydroxylase change inversely to each other and in relation to the dose of vitamin D in the diet. These results were highly reproducible in independent sets of replicate experiments.

4. Discussion

The present experiments employed an in vitro assay of the hydroxylases in a well-washed preparation of mitochondria so that the contamination from in vivo sources of substrates is negligible when compared with the relatively large amount of the reaction substrate added in vitro. The assay also secured the determination of the maximal activity of the respective hydroxylases. The data thus obtained clearly indicate that the regulation which directs the metabolism of 25-OH-D_3 in the kidney proceeds in the absence of a change in the plasma levels of calcium, phosphate and possibly of parathyroid hormone, and that the regulation in this case relates evidently to the dose of dietary vitamin D. This conclusion contrasts sharply to the previous concepts that the 25-OH-D_3 metabolism is directly controlled by the plasma levels of calcium [3], phosphate [4] or parthyroid hormone [5–7]. A similar conclusion has been suggested by Galante et al. [14] in a recent report, and by Tanaka and DeLuca [15] from a quite different set of in vivo experiment.

An immediate question may be how dietary vitamin D exerts this kind of regulation. Because the regulation effects inversely between the activity of 1-hydroxylase and that of 24-hydroxylase, and because these two hydroxylases may require common cofactors, it is attractive to consider that vitamin D or its metabolite(s) in the animal affects somewhat directly the amount or the activity of the enzymes.

Acknowledgements

This study was supported in part by a grant from the Ministry of Welfare of Japan and a grant from the Miura Foundation.

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