Effects of 1,25-dihydroxycholecalciferol administration on the rat renal vitamin K-dependent carboxylating system

Peter I. Karl, David L. Carnes* and Paul A. Friedman°

Department of Pharmacology, Harvard Medical School, Charles A. Dana Research Institute, Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine, Beth Israel Hospital, Harvard Medical School and **The Children's Hospital Medical Center, Department of Medicine, Endocrine Division, Boston, MA 02215, USA*

Received 19 July 1985

We have shown previously that the in vitro activity of the renal vitamin K-dependent γ-glutamyl carboxylase toward synthetic oligopeptide substrates is stimulated by administration of either parathyroid hormone (PTH) or 1,25-dihydroxycholecalciferol [1,25$(OH)_2D_3$] to rats [(1983) J. Biol. Chem. 258, 12783–12786]. Here we report that administration of 1,25$(OH)_2D_3$ to rats increases their levels of endogenous carboxylase substrate as well. Rats fed a vitamin D-deficient diet had highly elevated serum PTH levels while vitamin D-replete animals had undetectable levels. Furthermore, since PTH increases 1,25$(OH)_2D_3$ levels by stimulating renal 25-hydroxyvitamin D-1α-hydroxylase, it is very likely that the stimulatory effects of PTH on the renal vitamin K-dependent carboxylating system are mediated by 1,25$(OH)_2D_3$.

γ-Glutamyl carboxylase *Substrate* *Vitamin K* *Kidney* *1,25-Dihydroxycholecalciferol*

1. INTRODUCTION

Vitamin K participates in the posttranslational γ-glutamyl carboxylation of proteins other than vitamin K-dependent coagulation factors [1–3]. Prominent among these is the bone γ-carboxyglutamic acid containing protein known as bone Gla protein or osteocalcin [4,5]; this protein is synthesized and secreted from bone cells following carboxylation [6,7]. While several laboratories have carried out extensive studies on BGP, no definite physiologic role has been assigned to the protein. However, it has been demonstrated in rat osteosarcoma cell culture [8] that BGP synthesis and secretion are stimulated by 1,25$(OH)_2D_3$. A less well-characterized vitamin K-dependent protein is that found in kidney [9]. The renal vitamin K-dependent carboxylating system, which utilizes this protein as a substrate and has characteristics similar to the liver carboxylating system, has been localized to renal tubules and not to glomeruli [10], with the majority of enzyme found in the proximal tubule.

Although the physiological significance of the renal carboxylating system is unknown, we have shown that the in vitro activity of the renal vitamin K-dependent γ-glutamyl carboxylase toward synthetic oligopeptide substrates is stimulated by the prior administration of either of the calcemic hormones, PTH or 1,25$(OH)_2D_3$, to rats [11]. Here we report that administration of 1,25$(OH)_2D_3$ to rats increases their levels of endogenous carboxylase substrates as well.

° To whom correspondence should be addressed at: Clinical Pharmacology/Toxicology Unit, 330 Brookline Avenue, Boston, MA 02115, USA

Abbreviations: Gla, γ-carboxyglutamic acid; BGP, bone Gla protein; 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; PTH, parathyroid hormone; FLEEL, the pentapeptide phenylalanyl-leucyl-glutamyl-glutamyl-leucine

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. MATERIALS AND METHODS

$1,25(OH)_2D_3$ was a generous gift from Hoffman LaRoche. Triton X-100 and vitamin K were purchased from Sigma; $NaH^{14}CO_3$ (50 mCi/mmol) from ICN Pharmaceuticals; the pentapeptide FLEEL from Vega Biochemicals; Calcium Rapid Stat Diagnostic Kit from Lancer; Dowex AG 1-X8 (−400 mesh) from Bio-Rad; and Liquiscint from National Diagnostics.

In experiments designed to determine the dose-response relationship between endogenous substrate levels and $1,25(OH)_2D_3$, normal male Sprague-Dawley rats (Charles River Breeding Laboratories) were given daily injections of $1,25(OH)_2D_3$ (0.5, 1.0 or 1.5 ng/g body wt) for 7 days. The time course of the response was determined with daily injections of $1,25(OH)_2D_3$ (1 ng/g body wt) for up to 7 days. In each experiment rats were killed 1 day after the last injection, and blood samples and kidneys were obtained.

In experiments designed to study the effect of vitamin D deficiency and $1,25(OH)_2D_3$ treatment on the carboxylating system, weanling male Holtzman rats (Holtzman, Madison, WI) were maintained on vitamin D-deficient diet (Bioserve) for 8 weeks. A group of these rats was supplemented with 100 IU vitamin D_3 orally 3 times per week. A second group received intraperitoneal injections of $1,25(OH)_2D_3$ (1 ng/g body wt) daily during the last 7 days of the experimental period. The last injection was given 2 h before killing and blood samples were obtained from the abdominal aorta of each rat under ether anesthesia for determinations of serum calcium, vitamin D metabolites and PTH. Vitamin D metabolites were assayed according to Shepard et al. [12] and PTH was assayed by a nonequilibrium radioimmunoassay using chicken 12 antibovine PTH antiserum which crossreacts with rat PTH [13]. The rats were then exsanguinated before the kidneys were removed for carboxylase assays.

Preparation of renal microsomes and determination of carboxylase activity with FLEEL were as described [10,11]. Protein carboxylation in intact resuspended microsomes in a final reaction volume of 500 l was also carried out as described [11]. Following a 30 min incubation at 37°C, the reactions were stopped with the addition of 25 μl of 10% Triton X-100 and 28 μl of 4.4 N acetic acid. Incubation tubes were then placed on ice for 10 min. Protein was precipitated with 10% trichloroacetic acid and pelleted by centrifugation. The pellet was washed with H_2O and then hydrolyzed in 400 μl of 2 M KOH at 110°C for 16 h in a sealed glass tube. Following hydrolysis samples were neutralized with $HClO_4$ and $KClO_4$ was removed by centrifugation. The supernatant (400 μl) was applied to a Dowex 1-X8 column (0.6× 4 cm), which was equilibrated with 0.75 M ammonium acetate, pH 4.9. Fractions (1 ml) were collected and counted for radioactivity fixed in Gla as described in [11].

Protein was determined by a modified method of Lowry et al. [14] using bovine serum albumin as

Table 1

Renal carboxylase endogenous substrate and serum calcium levels after $1,25(OH)_2D_3$ treatment

A. Time-dependent responses to $1,25(OH)_2D_3$ treatment (1 ng/g body wt)

Treatment	Day	Endogenous carboxylation[a] (cpm/mg protein ± SD)	Serum calcium (mg/100 ml ± SD)
Vehicle	0	13.5 ± 3.0	9.9 ± 0.3
	7	11.2 ± 0.7	9.8 ± 0.8
$1,25(OH)_2D_3$	1	13.9 ± 1.0	11.2 ± 1.1
	2	27.5 ± 2.8[b]	12.1 ± 1.1
	4	72.9 ± 7.2[b]	14.2 ± 0.8
	7	56.9 ± 6.8[b]	14.1 ± 1.1

B. Dose-dependent responses to $1,25(OH)_2D_3$ treatment for 7 days

$1,25(OH)_2D_3$ (ng/g)	Endogenous carboxylation (cpm/mg protein ± SD)	Serum calcium (mg/100 ml ± SD)
0.0	11.5 ± 0.5	9.4 ± 0.4
0.5	31.8 ± 2.5[b]	13.3 ± 0.7
1.0	53.6 ± 2.3[b]	14.3 ± 0.1
1.5	31.8 ± 1.4[b]	14.3 ± 0.4

[a] Carboxylation reactions were carried out as described in section 2 except that renal microsomes were pooled from 3 rats for each treatment

[b] Significantly different from control (vehicle only) as determined by the Student's t-test ($P \leq 0.001$)

Table 2
Serum PTH, vitamin D metabolites and calcium levels

Treatment	PTH (pg/ml)[b]	25(OH)D (ng/ml)	$1,25(OH)_2D$ (pg/ml)	Serum calcium[a] (mg%)
−D	3582 ± 562	Undetectable	78.6 ± 12.5	7.91 ± 1.4
+D[c]	Undetectable	12.3 ± 3.3	150.5 ± 18.6	10.10 ± 0.4
$-D + 1,25(OH)_2D_3$[d]	Undetectable	Undetectable	>500	13.50 ± 2.1

[a] All serum data are mean ± SD from 5 rats
[b] pg equivalents to bovine PTH/ml. The normal PTH level is 333 ± 38
[c] Rats on the vitamin D-deficient diet were made vitamin D replete by giving them 100 IU vitamin D_3 orally 3 times/week
[d] Daily intraperitoneal injections of $1,25(OH)_2D_3$ (1 ng/g body wt) were administered during the last 7 days on vitamin D-deficient diet

a standard [15]. Radioactivity was assayed with a Beckman LS-250 liquid scintillation system. The Student's *t*-test was used for statistical analyses.

3. RESULTS

In our earlier study dose-response and time dependency relationships for the effect of $1,25(OH)_2D_3$ on renal FLEEL carboxylase activity was reported but such relationships for the effect of the hormone on endogenous substrate levels were not [11]. Daily $1,25(OH)_2D_3$ injections (1 ng/g body wt) in vitamin D-replete rats led to an increase in serum calcium discernible after 1 day: a statistically significant increase in the carboxylase substrate level was observed after 2 days of $1,25(OH)_2D_3$ treatment and a maximal response was measured after 4 days of treatment at which time the substrate level of the treated group was 5.4-fold higher than that of the control group (table 1). Maximal elevation of serum calcium to 7 daily doses of $1,25(OH)_2D_3$ was observed at 1 ng/g body wt. Renal carboxylase substrate level was increased 2.8-fold following treatment with 0.5 ng/g body wt, while at 1 ng/g body wt the increase was 4.7-fold. At the highest dose (1.5 ng/g body wt) the increase was less than at 1 ng/g body wt, a result compatible with the occurrence of a toxic response at this level.

As shown in table 2 serum calcium was lower in rats fed vitamin D-deficient diet for 8 weeks than in vitamin D-supplemented rats; rats receiving $1,25(OH)_2D_3$ treatment during the final week of the experiment had elevated levels of serum calcium. The serum PTH level was high in rats fed the vitamin D-deficient diet but was undetectable in the other 2 groups. Serum 25(OH)D was

Table 3
Effects of vitamin D status on renal carboxylase substrate level and carboxylase activity

Treatment	Carboxylation (cpm/mg protein ± SD)	
	Endogenous[a]	FLEEL[b]
−D	10.3 ± 1.3	1023 ± 114
+D	11.7 ± 1.5	967 ± 104
$-D + 1,25(OH)_2D_3$	48.7 ± 9.8[c]	1900 ± 34[c]

[a] Rats were maintained on vitamin D-deficient diet for 8 weeks. Endogenous substrate levels were determined in triplicate in carboxylase assays using intact resuspended renal microsomes pooled from 5 rats. Each reaction mixture contained 10 mg resuspended microsomal protein. Incubations and assays for [^{14}C]Gla are as described in section 2. Vitamin K-independent fixed ^{14}C eluting at the Gla position was less than 1 cpm/mg protein
[b] Carboxylase activity was determined using FLEEL as the substrate in solubilized microsomes [17]. Incubations were in a volume of 120 μl at 17°C for 45 min. The reaction mixture contained 100 μl solubilized renal microsomes (3 mg protein), FLEEL (400 μg), $NaH^{14}CO_3$ (2.5 μCi), and either isopropyl alcohol (5 μl) or vitamin K_1H_2 in isopropyl alcohol (5 μl). Reactions were stopped with 10% perchloric acid and soluble ^{14}C radioactivity was assayed [11]
[c] Significantly different from −D and +D as determined by the Student's *t*-test ($P \leq 0.01$)

undetectable both in the rats fed the vitamin D-deficient diet and in those treated with $1,25(OH)_2D_3$. While high levels of $1,25(OH)_2D$ were found in rats treated with $1,25(OH)_2D_3$ serum $1,25(OH)_2D$ was reduced by only 50% in rats fed the vitamin D-deficient diet compared to rats fed the vitamin D-deficient diet but given oral vitamin D supplement.

Maintenance of rats on vitamin D-deficient diet for 8 weeks did not alter the carboxylase substrate level or the carboxylase activity (determined with FLEEL as the substrate) when compared to vitamin D-supplemented rats (table 3). When rats maintained on the vitamin D-deficient diet were given daily intraperitoneal injections of $1,25(OH)_2D_3$ for the last 7 days of the experiment, a nearly 5-fold increase in the substrate level and, as demonstrated in [11], a stimulation of FLEEL carboxylase activity (table 3), resulted.

4. DISCUSSION

This study extends our earlier work by demonstrating that $1,25(OH)_2D_3$ administration increases not only the renal carboxylase activity but the level of its endogenous substrate as well [11]. The dose-response and time course relationships between endogenous substrate levels and $1,25(OH)_2D_3$ administration indicate that a maximal increase in substrate is seen at a $1,25(OH)_2D_3$ dose of 1 ng/g body wt after 4 days of treatment. This differs slightly from the changes in carboxylase activity which were maximally increased at 1.5 ng/g and continued to increase up to 7 days of treatment [11].

Our previous work indicated that PTH also increases carboxylase activity [11]. PTH increases 25(OH)vitamin D-1α-hydroxylase, a renal enzyme responsible for the conversion of $25(OH)D_3$ to its active metabolite $1,25(OH)_2D_3$ [16]. This study indicates that it is highly probable that the effects of PTH on the renal carboxylating system [11] are mediated via $1,25(OH)_2D_3$. Since $1,25(OH)_2D_3$ administration results in reduction of serum PTH levels to undetectable levels, while stimulation of the carboxylating system occurs, our data also suggest that the stimulation observed with $1,25(OH)_2D_3$ treatment occurs independently of PTH.

While the rats maintained on the vitamin D-deficient diet displayed decreased serum calcium, increased PTH and undetectable 25(OH)D, serum $1,25(OH)_2D$ was still present at detectable levels. Thus, either carboxylase activity and substrate level are not altered by vitamin D deficiency or a reduction in $1,25(OH)_2D$ levels greater than the 50% reduction achieved in this study is necessary for any alterations to become manifest. Further study is needed to clarify this point.

ACKNOWLEDGEMENTS

This research was supported by grants HL25066 and HL30226 from the National Institutes of Health.

REFERENCES

[1] Stenflo, J. and Suttie, J.W. (1977) Annu. Rev. Biochem. 46, 157–172.
[2] Olson, R.E. and Suttie, J.W. (1978) Vitam. Horm. 35, 59–172.
[3] Suttie, J.W. (1980) Crit. Rev. Biochem. 8, 191–223.
[4] Hauschka, P.V., Lian, J.B. and Gallop, P.M. (1975) Proc. Natl. Acad. Sci. USA 72, 3925–3929.
[5] Price, P.A., Otsuka, A.S., Poser, J.P., Kristaponis, J. and Kaman, N. (1976) Proc. Natl. Acad. Sci. USA 73, 1447–1451.
[6] Lian, J.B. and Friedman, P.A. (1978) J. Biol. Chem. 253, 6623–6626.
[7] Price, P.A., Williamson, M.K. and Lothringer, J.W. (1981) J. Biol. Chem. 256, 12760–12766.
[8] Nishimoto, S.K. and Price, P.A. (1980) J. Biol. Chem. 255, 6579–6583.
[9] Hauschka, P.V., Friedman, P.A., Traverso, H.P. and Gallop, P.M. (1976) Biochem. Biophys. Res. Commun. 71, 1207–1213.
[10] Friedman, P.A., Mitch, W.G. and Silva, P. (1982) J. Biol. Chem. 257, 11037–11040.
[11] Karl, P.I. and Friedman, P.A. (1983) J. Biol. Chem. 258, 12783–12786.
[12] Shepart, P.M., Horst, R.L., Hamstra, A.J. and DeLuca, H.F. (1979) Biochem. J. 182, 52–69.
[13] Carnes, D.L., Anast, C.S. and Forte, L.R. (1978) Endocrinology 102, 45–51.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Markwell, M.A.K., Hans, S.M., Biebar, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[16] DeLuca, H.F. (1976) Adv. Exp. Med. Biol. 81, 195–209.
[17] Friedman, P.A. and Griep, A.E. (1980) Biochemistry 19, 3381–3386.