

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

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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 [$1,25(\text{OH})_2\text{D}_3$] to rats [(1983) *J. Biol. Chem.* 258, 12783–12786]. Here we report that administration of $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ levels by stimulating renal 25-hydroxyvitamin D-1 α -hydroxylase, it is very likely that the stimulatory effects of PTH on the renal vitamin K-dependent carboxylating system are mediated by $1,25(\text{OH})_2\text{D}_3$.

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 $1,25(\text{OH})_2\text{D}_3$. 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 $1,25(\text{OH})_2\text{D}_3$, to rats [11]. Here we report that administration of $1,25(\text{OH})_2\text{D}_3$ to rats increases their levels of endogenous carboxylase substrates as well.

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Abbreviations: Gla, γ -carboxyglutamic acid; BGP, bone Gla protein; $1,25(\text{OH})_2\text{D}_3$, 1,25-dihydroxycholecalciferol; PTH, parathyroid hormone; FLEEL, the pentapeptide phenylalanyl-leucyl-glutamyl-glutamyl-leucine

2. MATERIALS AND METHODS

1,25(OH)₂D₃ was a generous gift from Hoffman LaRoche. Triton X-100 and vitamin K were purchased from Sigma; NaH¹⁴CO₃ (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)₂D₃, normal male Sprague-Dawley rats (Charles River Breeding Laboratories) were given daily injections of 1,25(OH)₂D₃ (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)₂D₃ (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)₂D₃ 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 D₃ orally 3 times per week. A second group received intraperitoneal injections of 1,25(OH)₂D₃ (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 μ 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 μ l of 10% Triton X-100 and 28 μ l 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 H₂O and then hydrolyzed in 400 μ l of 2 M KOH at 110°C for 16 h in a sealed glass tube. Following hydrolysis samples were neutralized with HClO₄ and KClO₄ was removed by centrifugation. The supernatant (400 μ l) 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 Glax 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)₂D₃ treatment

A. Time-dependent responses to 1,25(OH) ₂ D ₃ treatment (1 ng/g body wt)			
Treatment	Day	Endogenous carboxylation ^a (cpm/mg protein $\pm$ SD)	Serum calcium (mg/100 ml $\pm$ SD)
Vehicle	0	13.5 $\pm$ 3.0	9.9 $\pm$ 0.3
	7	11.2 $\pm$ 0.7	9.8 $\pm$ 0.8
1,25(OH) ₂ D ₃	1	13.9 $\pm$ 1.0	11.2 $\pm$ 1.1
	2	27.5 $\pm$ 2.8 ^b	12.1 $\pm$ 1.1
	4	72.9 $\pm$ 7.2 ^b	14.2 $\pm$ 0.8
	7	56.9 $\pm$ 6.8 ^b	14.1 $\pm$ 1.1
B. Dose-dependent responses to 1,25(OH) ₂ D ₃ treatment for 7 days			
1,25(OH) ₂ D ₃ (ng/g)	Endogenous carboxylation (cpm/mg protein $\pm$ SD)	Serum calcium (mg/100 ml $\pm$ SD)	
0.0	11.5 $\pm$ 0.5	9.4 $\pm$ 0.4	
0.5	31.8 $\pm$ 2.5 ^b	13.3 $\pm$ 0.7	
1.0	53.6 $\pm$ 2.3 ^b	14.3 $\pm$ 0.1	
1.5	31.8 $\pm$ 1.4 ^b	14.3 $\pm$ 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) ₂ D (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) ₂ D 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 D₃ orally 3 times/week

^d Daily intraperitoneal injections of 1,25(OH)₂D₃ (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)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ 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)₂D₃ 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) ₂ D 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 [¹⁴C]Gla are as described in section 2. Vitamin K-independent fixed ¹⁴C 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), NaH¹⁴CO₃ (2.5 µCi), and either isopropyl alcohol (5 µl) or vitamin K₁H₂ in isopropyl alcohol (5 µl). Reactions were stopped with 10% perchloric acid and soluble ¹⁴C radioactivity was assayed [11]

^c Significantly different from -D and +D as determined by the Student's *t*-test (*P* ≤ 0.01)

undetectable both in the rats fed the vitamin D-deficient diet and in those treated with $1,25(\text{OH})_2\text{D}_3$. While high levels of $1,25(\text{OH})_2\text{D}$ were found in rats treated with $1,25(\text{OH})_2\text{D}_3$ serum $1,25(\text{OH})_2\text{D}$ 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 $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ administration indicate that a maximal increase in substrate is seen at a $1,25(\text{OH})_2\text{D}_3$ 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 $25(\text{OH})\text{vitamin D-}1\alpha\text{-hydroxylase}$, a renal enzyme responsible for the conversion of $25(\text{OH})\text{D}_3$ to its active metabolite $1,25(\text{OH})_2\text{D}_3$ [16]. This study indicates that it is highly probable that the effects of PTH on the renal carboxylating system [11] are mediated via $1,25(\text{OH})_2\text{D}_3$. Since $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ treatment occurs independently of PTH.

While the rats maintained on the vitamin D-

deficient diet displayed decreased serum calcium, increased PTH and undetectable $25(\text{OH})\text{D}$, serum $1,25(\text{OH})_2\text{D}$ was still present at detectable levels. Thus, either carboxylase activity and substrate level are not altered by vitamin D deficiency or a reduction in $1,25(\text{OH})_2\text{D}$ 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.

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