Vitamin D Deficiency, Hypocalcemia, and Increased Skeletal Muscle Degradation in Rats

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ABSTRACT The myopathy associated with vitamin D deficiency was examined in vitamin D-deficient and vitamin D-supplemented rats. When compared with either vitamin D-supplemented ad lib. or pair-fed rats, weight gain and muscle mass were decreased in vitamin D-deficient hypocalcemic animals. With the exception of a modest decrease in muscle creatine phosphate levels, muscle composition was unchanged by vitamin D deficiency. Muscle protein turnover rates were determined in both in vivo and in vitro studies and demonstrated that myofibrillar protein degradation was increased in vitamin D deficiency. Normal growth rates could be maintained be feeding the rats vitamin D-deficient diets containing 1.6% calcium, which maintained plasma calcium within the normal range. In addition to its role in maintaining plasma calcium, vitamin D-supplemented rats had significantly higher levels of the anabolic hormone insulin. Vitamin D supplementation may affect muscle protein turnover by preventing hypocalcemia, as well as directly stimulating insulin secretion, rather than by a direct effect within skeletal muscle.

INTRODUCTION

The association between vitamin D deficiency states and muscle abnormalities has been noted frequently (1–4). Vitamin D deficiency is associated with a proximal myopathy and histologic evidence of muscle fiber atrophy, which rapidly improve after the administration of vitamin D_3 or its metabolites (5–8). The mechanism through which vitamin D metabolites acts is still uncertain. Within any tissue, protein turnover is determined by both rates of protein synthesis and degradation so that alterations in either or both processes could be responsible for the effects of vitamin D within muscle. Among the factors known to regulate muscle protein turnover are food intake (9), various hormones (10), and muscle work (11). Recently, in in vitro studies, calcium has also been shown to have a direct effect on muscle protein turnover rates (12, 13). Vitamin D administration increases plasma calcium levels, improves force generation by muscle, affects the secretion of other hormones, and is associated with increased food intake (5, 14–16). In addition, a direct anabolic effect within muscle has been suggested for one vitamin D metabolite. Two reports, both utilizing isolated muscle preparations, have appeared suggesting that 25-hydroxycholecalciferol [25(OH)D_3][1] performs a specific function within skeletal muscle to improve net muscle protein anabolism. In the first study, incorporation of [^{14}C]leucine was increased in epitrochlearis muscle preparations obtained from vitamin D-deficient rats acutely treated with either cholecalciferol or 25(OH)D_3 (7). In a second, preliminary study, epitrochlearis muscle preparations taken from uremic rats were incubated for 1 h with or without 25(OH)D_3 added to the media. Addition of 25(OH)D_3 led to decreased rates of alanine and glutamine release suggesting that 25(OH)D_3 decreased protein degradation in vitro (8). Unfortunately, the methods utilized in both studies are not entirely satisfactory for the assessment of protein turnover within muscle. Quantitation of protein turnover requires a marker amino acid, which is neither metabolized nor synthesized within that tissue (10). Leucine, alanine, and glutamine do not meet these requirements, since muscle is a major site of leucine oxidation (17); alanine and glu-

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[1] *Abbreviations used in this paper:* MH, N^{τ}-methylhistidine; 25(OH)D_3, 25-hydroxycholecalciferol.

tamine are formed within muscle and their release from muscle does not accurately reflect protein degradation (18).

This paper reports our attempts to delineate the effects of vitamin D deficiency on skeletal muscle protein turnover. We measured growth and muscle composition in young, vitamin D-deficient rats before and after supplementation with cholecalciferol. Independent rates of muscle protein synthesis and degradation were assessed during in vitro perfusion studies in rats using a well-characterized, physiologic muscle perfusion system. Phenylalanine, an amino acid that is not metabolized or synthesized in muscle, was used as the marker amino acid. Myofibrillar degradation rates were also assessed both in vitro and in vivo by measuring the rate of release of the amino acid N^{τ}-methylhistidine (MH). MH is formed by the posttranslational methylation of specific histidyl residues within actin and myosin (19). During myofibrillar degradation the MH released is not reutilized for synthesis but is rapidly and quantitatively excreted in urine (20). Several studies and critical reviews have demonstrated that MH release accurately reflects muscle degradation in the rat (21–23).

Finally, the importance of calcium as a moderator of muscle protein turnover was assessed through the use of diets designed to maintain normocalcemia in the face of vitamin D deficiency.

METHODS

Male Sprague-Dawley rats were obtained either at weaning (21 d old) or at 7 d of age in groups of 10 rats along with a lactating dam (Charles River Laboratories, Wilmington, MA). Immediately on arrival, all rats were housed in an area shielded from ultraviolet light, including fluorescent lighting. Deionized water was provided ad lib. and the rats were fed specially formulated synthetic vitamin D-deficient diets. Diets were identical except for their calcium and phosphate contents: 0.8% Ca/0.3% P, 0.8% Ca/0.45% P, 1.6% Ca/1.1% P (Teklad diets No. 78021, 80404, 81133, respectively). Phosphate content was altered to prevent hypophosphatemia. Rat pups were weaned at 21 d of age. Twice weekly the rats were weighed to the nearest 0.5 g. When supplementation studies were begun, male rats were first transferred to separate cages and then injected intraperitoneally with 0.1 ml of propylene glycol containing either 400 IU of vitamin D_3 (acute studies) or 75–80 IU of D_3 twice weekly (chronic studies). Ergocalciferol (D_2), Drisdol, was obtained from Winthrop Laboratories (New York); cholecalciferol was obtained from Sigma Chemical Co. (St. Louis, MO). Legends indicate which vitamin D was used. In each case the vitamin D-deficient rats received propylene glycol only. Identical stimulation of weight gain and restoration of serum calcium were achieved whether a single dose of vitamin D_3 (400 IU) or chronic therapy with vitamin D_3 was used.

Several days before the collection of 24-h urine samples, the rats were weighed and placed in individual metabolic cages. This was done because we have consistently noted higher rates of urinary MH excretion in rats during the first 24 h after being transferred to individual cages (24). After the rats had acclimated, urine was collected (HCl preservative) for 24 h before vitamin D_3 supplementation and for 24-h periods up to 5 d thereafter. At the end of each day's urine collection, the rats were weighed and the cage bottoms washed with distilled water to insure complete urine collection. Urine volume was measured and aliquots of urine were frozen at −20°C for later determination of MH and creatinine levels.

When pair-feeding and food intake studies were conducted, rats were placed in cages with fine mesh bottoms. Each day the quantity of food remaining the previous day removed and carefully weighed and a known quantity of food was placed in the cages. The amount of food consumed by each vitamin D-deficient rat was then given to its pair-fed, vitamin D-supplemented mate. There was no attempt to correct for small amounts of food dropped through the bottom of the cage but visual inspection suggested that food wastage was no different between different groups of rats.

At various times after the administration of vitamin D_3 or vehicle, the rats were anesthetized with pentobarbital and either utilized for hemicorpus perfusion studies or killed to determine carcass weights, muscle composition, plasma MH, calcium, phosphorus, insulin, and creatinine levels. Carcass, visceral, and gastrointestinal weights were determined by weighing. Tissue muscle from gastrocnemius was frozen in situ using a Wollenberger clamp, cooled in liquid nitrogen and stored at −80°C. Gastrocnemius muscle was pulverized using a mortar and pestle cooled in liquid nitrogen. Samples of muscle powder were used for determination of RNA-phosphorus, DNA-phosphorus, protein, ATP, and creatine phosphate.

Hemicorpus perfusion was performed according to the method of Jefferson et al. (25). The rats were heparinized and anesthetized with sodium pentobarbital. Through an abdominal incision, the major arteries, veins, and viscera were ligated. The chest was opened, the aorta cannulated, the perfusion begun, and the vena cava severed. The body was transected at the level of the diaphragm and the viscera and testes removed. The perfusate consisted of Krebs-Henseleit bicarbonate buffer containing washed bovine erythrocytes (hematocrit 25%), bovine serum albumin 3% (fraction V, Miles Laboratories, Inc., Elkhart, IN), 15 mM glucose, and normal plasma levels of amino acids. For measurements of protein synthesis and degradation, 0.4 mM [^{14}C]-phenylalanine, with a specific activity of 125 μCi/mmol, was used. The perfusate was gassed with a mixture of oxygen/carbon dioxide (95:5%) and maintained at 37°C. The first 50 ml of perfusate was discarded and 100 ml of perfusate then recirculated through the preparation at a rate of 14 ml/min. At 1 and 3 h of perfusion, perfusate samples and samples of gastrocnemius muscle were removed for analyses of phenylalanine and MH concentrations in whole perfusate and muscle extract. Protein synthesis was calculated by the method of Jefferson et al. (25) as the increase in the disintegrations per minute [^{14}C]phenylalanine in gastrocnemius muscle protein between 1 and 3 h of perfusion divided by the mean intracellular specific activity of phenylalanine determined at the same two time points. Protein degradation was calculated from the change in perfusate phenylalanine specific activity between 1 and 3 h. Degradation was assessed without the use of protein synthesis inhibitors, and both synthesis and degradation rates were measured in the same preparation (25). Synthesis data are reported as nanomoles phenylalanine incorporated per hour per gram of gastrocnemius muscle. Degradation data are given as nanomoles per hour per gram hemicorpus for both phenylalanine and MH release.

Chemical analyses. Urinary creatinine was measured with a Beckman Creatinine 2 Analyzer (Beckman Instru-

ments, Inc., Fullerton, CA). Calcium was determined using a Corning calcium analyzer (Corning Medical and Scientific, Corning Glass Works, Medfield, MA) and phosphorus with a commercial kit (No. 198 Hycel, Inc., Houston, TX). Plasma and whole perfusate were precipitated with equal volumes of 10% trichloroacetic acid (TCA) and aliquots of the supernatant taken for determination of MH and phenylalanine concentrations. TCA extracts of gastrocnemius muscle and whole perfusate were analyzed for phenylalanine by a fluorometric method (26), as previously described (25). To analyze rat urine for MH, 19 vol of rat urine were precipitated with 1 vol of 70% perchloric acid. Aliquots of the supernatant mixed with equal volumes of 12 N HCl, sealed and heated at 130°C for 2 h (24). The mixture was then neutralized with 4 vol of 1.5 N NaOH and aliquots taken for analysis. Muscle protein hydrolysates were prepared and analyzed for MH as noted previously (24). The preparation and high pressure liquid chromatographic analysis of fluorescamine derivatives of MH was performed as previously reported by a separation utilizing a μBondapak C-18 reverse-phase column (Waters Associates, Milford, MA). The solvent utilized was 23–27% acetonitrile in water. Detection was by a Farrand A4 fluorometer (Farrand Optical Co., Inc., Valhala, NY) and the MH peak as quantitated by the method of external standardization (27). Protein concentration was determined by the biuret method (28) using crystalline bovine albumin as standard. RNA-phosphorus and DNA-phosphorus were determined by the method of Manchester and Harris (29). ATP and creatine phosphate levels were determined enzymatically (30, 31). Serum 25(OH)D_3 levels were determined by high-pressure liquid chromatography (32). Plasma insulin levels were measured with a radioimmunoassay kit (Amersham Corp., Arlington Heights, IL).

Statistical analysis was performed using, where appropriate, analysis of variance, the Newman-Keuls test modified for groups of unequal sizes, and Student's t test (33). A P value of <0.05 was considered statistically significant. All data are presented as means±SE.

RESULTS

With time, rats receiving only vitamin D-deficient diets gained less weight than treated controls. When rats were started on vitamin D-deficient diet at weaning their weights became significantly less than vitamin D-supplemented controls after ~3 wk of diet feeding, at 43 d of life (Fig. 1). Thereafter the rate

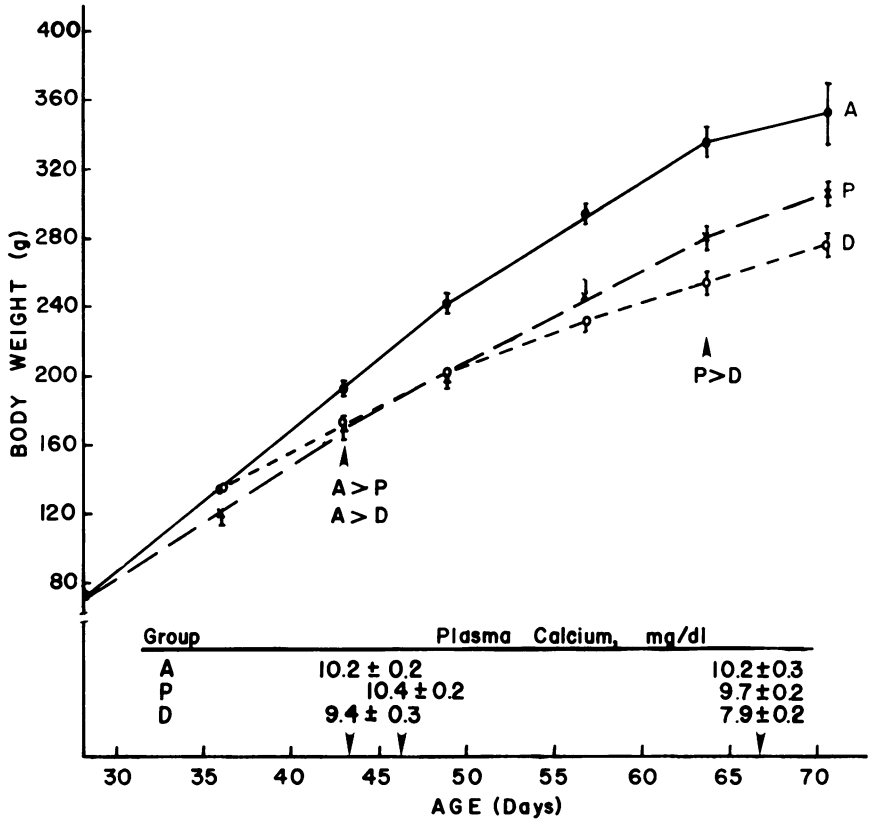

FIGURE 1 Effect of food intake and ergocalciferol on growth in rats fed a vitamin D-deficient diet. Rats were placed on a vitamin D-deficient diet containing 0.8% Ca and 0.3% P. The plasma calcium concentrations are given on the figure at the ages indicated by the downward pointing arrows. Not all points have been plotted for the body weights. Vertical lines represent the ±1 SEM of 6–12 rats. A, ad lib-fed rats treated with 75 IU ergocalciferol twice weekly; P, rats treated with ergocalciferol that were pair-fed to rats not receiving ergocalciferol; D, rats on vitamin D-deficient diet, fed ad lib.

of weight gain per day was significantly less than in vitamin D-supplemented rats. To be certain that this decreased weight gain was not solely due to decreased food intake on the 0.8% calcium diet, a second group of vitamin D-supplemented animals was pair-fed to the vitamin D-deficient animals. Weight gains in all three groups were significantly different with weight gain highest in the ad lib.-fed vitamin D-supplemented rats, intermediate in the vitamin D-supplemented pair-fed rats, and lowest in the vitamin D-deficient group, 5.3 vs. 4.5 vs. 3.4 g/d (n = 14 in each group, $P < 0.03$ by Anova) (Fig. 1). Vitamin D-deficient rats gained less weight per gram intake than either pair-fed or ad lib-fed vitamin D-supplemented rats (0.19 vs. 0.26 or 0.23 gram weight gain per gram food intake, $P < 0.01$ by Anova). Efficiency of weight gain was not significantly different between vitamin D-supplemented pair-fed or ad lib.-fed animals. Plasma calcium levels in the vitamin D-deficient group were significantly lower than those in the supplemented animals when growth began to decrease at 43 d and had declined to frankly hypocalcemic levels by the time the pair-fed rats were significantly heavier than the vitamin D-deficient animals (Table 1). Table I presents both the day when hypocalcemia was first noted and the plasma calcium levels at killing.

To produce a vitamin D-deficient state earlier in life when growth rates are normally more rapid, dams with 7-d-old rat pups were fed vitamin D-deficient diets. These vitamin D-deficient animals showed diminished weight gain (Fig. 2) that appeared to correlate with the development of hypocalcemia at ~30 d of age (Table I). After the development of hypocalcemia, it was always possible to stimulate weight gain by a single injection of cholecalciferol (Fig. 2). This was associated with a significant increase in the efficiency of weight gain, which increased from 0.34 to 0.53 gram weight gain per gram food intake ($P < 0.001$). Because these rats were studied at a younger age than rats begun on vitamin D-deficient diets at weaning, weight gain per gram food intake was higher in both vitamin D-deficient and vitamin D-supplemented groups. Within each experiment, efficiency of weight gain always improved after cholecalciferol therapy. Rats raised on 1.6% calcium diets maintained normal growth rates as long as they remained normocalcemic (Fig. 3). After the development of hypocalcemia at 61 d of life, growth rates declined (data not presented).

With vitamin D-deficient diets containing 0.8% calcium, plasma calcium levels decreased over time in the vitamin D-deficient rats. The development of hypocalcemia was inversely related to the percent dietary calcium, requiring ~3 wk on 0.8% calcium diet, in rats started on diet at weaning and in rats obtained with lactating dams at 7 d of age (Table I). Phosphorus levels are also presented and are not significantly different between vitamin D-deficient and -treated groups.

When the rat dams were started on the 0.8% calcium diets, their pups were normocalcemic until 22–25 d on diet, although plasma calcium concentrations were lower than in vitamin D-supplemented rats. Between 25 and 30 d on the diet the plasma calcium levels

TABLE I

Effect of Age and Diet on Plasma Calcium and Phosphorus

Age diet started (days)	Dietary		Cholecalciferol treatment	Hypocalcemia, first detected (days of age)	Plasma concentrations*	
	Ca	P			Ca	P
	%				mg/dl	
7‡	0.8	0.45	−	30	6.5±0.2 (19)§	8.2±0.4 (9)
			+		10.9±0.2 (10)¶	8.0±0.2 (9)
21‖	0.8	0.30	−	43	8.0±0.3 (30)	7.5±0.2 (11)
			+		9.7±0.2 (29)¶	7.3±0.3 (10)
7‡	1.6	1.1	−	61	8.7±0.5 (8)	9.2±0.9 (8)
			+		10.6±0.3 (9)¶	7.7±0.6 (9)

Plasma calcium levels were determined at different ages. Hypocalcemia was determined by comparing rats on vitamin D-deficient diets with cholecalciferol-treated rats. Rats were killed 1–3 wk after hypocalcemia was first detected. Cholecalciferol was administered as 75 IU twice weekly or one dose of 400 IU.

* Measured at killing.

‡ Lactating mothers and pups on diet.

§ Mean±SE. Number of rats studied is indicated in parentheses.

‖ Weaned onto diet.

¶ $P < 0.005$ vs. −cholecalciferol.

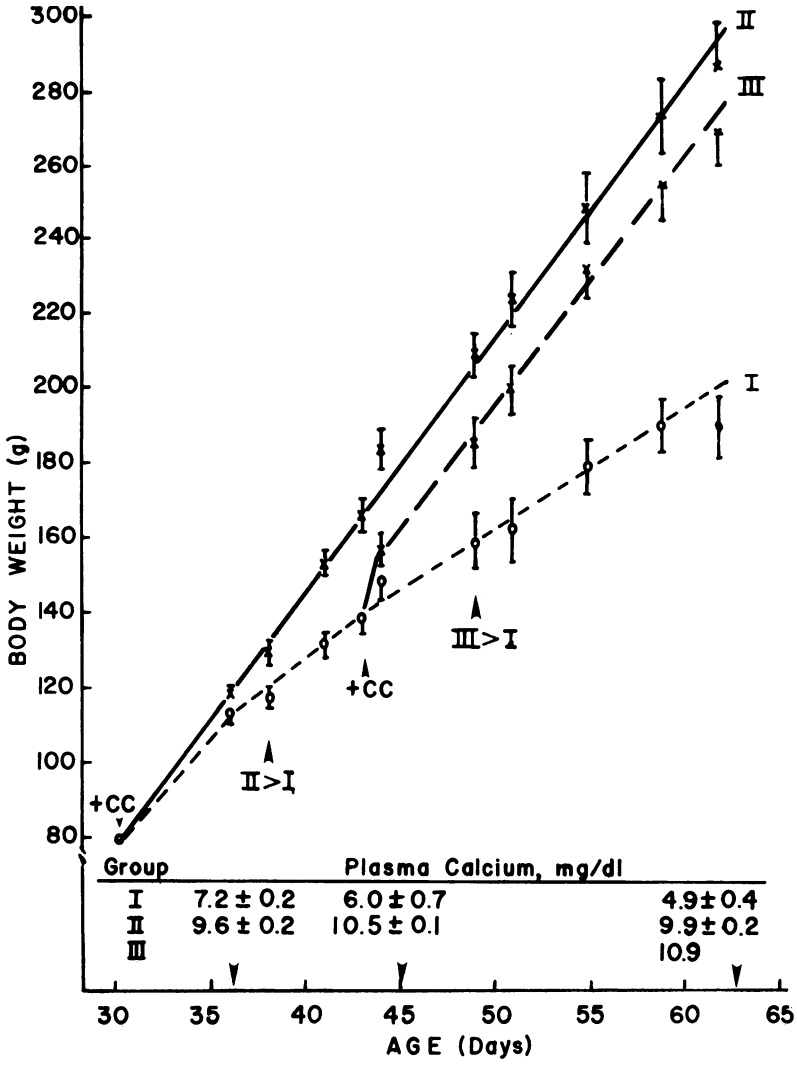

FIGURE 2 Effect of cholecalciferol administration at different ages on the growth of vitamin D-deficient rats. Dams with litters were fed 0.8% Ca and 0.45% P, vitamin D-deficient diet. Rats were divided into groups I, II, and III and were weaned onto the same diet at 24 d of age. Cholecalciferol (400 IU) was given to group II rats on day 30 and day 43; it was given to group III on day 43. Rats raised only on the vitamin D-deficient diet, group I, were hypocalcemic at 36 d of age. Points represent the means of five or more rats. The vertical bars represent ±1 SEM. +CC, point of cholecalciferol administration; II > I and III > I represent the times at which the body weights on the indicated groups were statistically different.

decreased rapidly (Table II). 25 d after starting the diet in weanling rats, plasma 25(OH)D_3 levels were undetectable in the vitamin D-deficient rats and averaged 11.7±2.4 ng/ml in rats treated with 80 IU of cholecalciferol twice weekly (Table II). For comparison, the level of 25(OH)D_3 in rats acutely treated with 400 IU of cholecalciferol 48 h previously was 16.3±3.0 ng/ml; and in chow-fed rats it was 22.8±3.2 ng/ml. At that time neither plasma calcium levels nor weight gain were significantly decreased in the vitamin D-deficient rats when compared with chronically treated controls.

After 40 d on the vitamin D-deficient diet, plasma calcium in rats receiving 0.8% calcium diet had decreased to 6.3 mg/dl (Table II). At that time, rats fed 1.6% calcium diet, without vitamin D supplementation had plasma calcium levels of 9.4 mg/dl. This latter group of rats were still growing normally in spite of undetectable levels of 25(OH)D_3.

We utilized the urinary calcium/creatinine ratio to

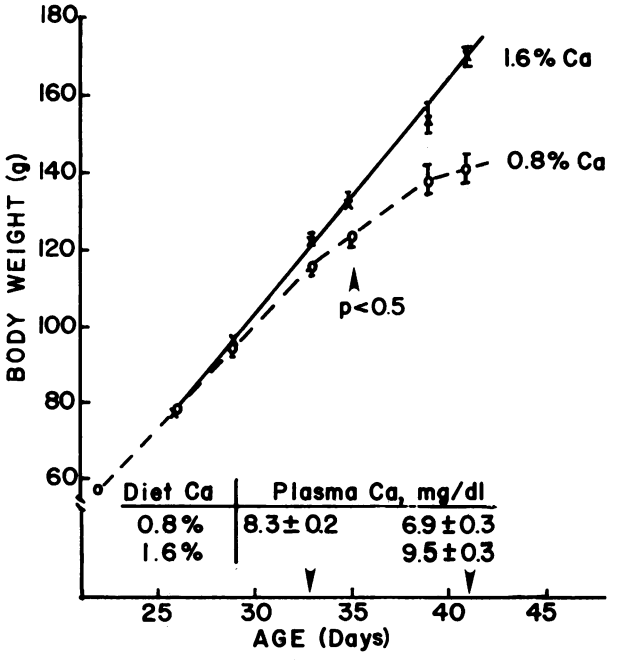

FIGURE 3 Effect of dietary calcium on the growth of vitamin D-deficient rats. Dams with litters were fed a vitamin D-deficient diet containing 0.8% Ca and 0.45% P. At 22 d of age the pups were weaned onto either that same diet or one containing 1.6% Ca and 1.1% P. Hypocalcemia was observed at 33 d of age. The body weights of the two groups were significantly different at 36 d. The points are the means of six or more rats. The vertical bars are ±1 SEM.

assess the development of vitamin D deficiency before the occurrance of hypocalcemia. The test is a convenient, noninvasive check on the development of vitamin D deficiency. Rats supplemented with vitamin D (80 IU twice weekly) had plasma calcium levels averaging 10.8 mg/dl and urinary calcium/creatinine ratios equal to 1.75±0.24 mg calcium/mg creatinine (Table II). Unsupplemented rats demonstrated reduced urinary calcium/creatinine ratios (0.27±0.019 mg calcium/mg creatinine), while still normocalcemic (9.8 mg/dl). The urinary calcium/creatinine ratio decreased further to 0.09 with the development of hypocalcemia (Table II). In rats fed a vitamin D-deficient diet containing 1.6% calcium, plasma calcium levels were normal, while the urinary calcium/creatinine ratio was as low as that seen in hypocalcemic rats (Table II). This suggested that these rats were also vitamin D deficient. To insure that our rats were indeed vitamin D deficient, all experiments on muscle composition, protein synthesis, and protein degradation were done with animals on diets for 32 d or longer.

Treatment of hypocalcemic vitamin D-deficient rats rapidly led to increased weight gain but after the initial 1–4-d period there was no further catch-up growth and weights tended to parallel those of chronically treated animals (Fig. 2). To determine whether the increase in weight also reflected increased muscle mass, the carcass weight/body weight ratios were determined in vitamin D-deficient and -treated animals, along with muscle protein, RNA, DNA, ATP, and cre-

TABLE II
Effect of Vitamin D Deficiency on Plasma Calcium, Urinary Calcium/Creatinine Ratios, and Plasma 25(OH)D_3

Days on diet	Dietary calcium	Plasma calcium	Urinary Ca/ creatinine ratios	Plasma 25(OH)D_3
	%	*mg/dl*	*mg/mg*	*ng/ml*
Vitamin D-deficient diet				
22	0.8*	9.8±0.7 (3)	0.27±0.19 (4)	
25	0.8‡	9.6±0.1 (6)		Undetectable
28–30	0.8*	7.2±0.4 (13)	0.09±0.02 (14)	
40	0.8*	6.3±0.3 (11)		Undetectable
28	1.6*	9.5±0.3 (6)	0.06±0.01 (7)	
40	1.6*	9.4±0.3 (11)		Undetectable
Vitamin D-supplemented diet				
25–30	0.8*	10.8±0.1 (14)	1.75±0.24 (6)	11.7±1.1 (5)§

Lactating dams and litters received vitamin D-deficient diet containing 0.8% Ca and 0.45% P. Pups were weaned onto vitamin D-deficient diets containing 0.8% Ca and 0.45% P or 1.6% Ca and 1.1% P. One dose of cholecalciferol (400 IU) was given 1–2 wk before urine was collected. Rats were on 1.6% Ca diet for 8–14 d before urine was collected.

* Lactating dam with 7-d-old pups placed on diet.

‡ Weanling rats placed on diet.

§ Chronically treated with cholecalciferol.

atine phosphate levels. There were no changes in dry weight, RNA, DNA, or ATP in either acutely (1 d after therapy) or chronically (>4 d after therapy) vitamin D-supplemented rats (Table III). Creatine phosphate levels increased slightly in chronically treated rats. Since the percentage of carcass weight was unchanged after vitamin D supplementation, this suggests that muscle mass increased in parallel with weight gain after cholecalciferol therapy.

Increases in net muscle protein anabolism can be regulated through alterations in either rates of protein synthesis or protein degradation. Myofibrillar degradation rates were assessed in vivo by determining the urinary excretion of MH. Under steady-state conditions, when urinary MH excretion is divided by the simultaneously determined creatinine excretion, the MH/creatinine excretion ratio is a measure of myofibrillar protein degradation per unit muscle mass (17, 18). While MH excretion did not change significantly after cholecalciferol therapy, urinary creatinine excretion rose significantly from 19.1±0.3 to 22.1±0.4 μmol/24 h per 100 g body wt. Urinary MH/creatinine ratios were significantly decreased after cholecalciferol therapy (Table IV). This suggests that vitamin D supplementation led to either increased muscle mass, increased renal clearance of creatinine, or possibly altered rate of formation of creatinine from creatine. There were no differences in the urinary creatine/creatinine ratios, averaging 0.175±0.013, in our vitamin D-deficient or vitamin D-supplemented animals. Plasma levels of both MH and creatinine were lower after cholecalciferol administration (Table V), but the decrease was significant only for MH. The decrease in plasma MH levels suggests that the lower urinary MH/creatinine ratios seen after vitamin D supplementation truly reflects diminished myofibrillar degradation and not an increase in the body pool of free MH.

MH release from skeletal muscle protein was also

TABLE IV

Effect of Dietary Calcium and Cholecalciferol on Protein Synthesis and Degradation In Vivo and in Perfused Hemicorpus Preparations

I. MH release, in vivo and in vitro

Dietary Ca	Cholecalciferol	Urinary excretion MH/creatinine ratios	Release into perfusate
%		*mmol/mol*	*nmol MH/h/g*
A. 0.8	−	40.0±1.1 (42)	0.325±0.026 (18)
0.8	+	34.7±0.9 (42)*	0.230±0.023 (18)‡
B. 0.8	−	34.9±0.7 (9)	0.329±0.082 (5)
1.6	−	31.4±0.9 (9)‡	0.220±0.045 (5)

II. Phenylalanine incorporation and release, in vitro

Dietary Ca	Cholecalciferol	Protein synthesis	Protein degradation
%		*nmol Phe/h/g*	*nmol Phe/h/g*
0.8	−	86.0±8.6 (17)	131.1±6.5 (17)
0.8	+	96.6±8.0 (9)	122.1±9.2 (9)
1.6	−	95.8±9.6 (6)	131.6±10.2 (6)

Rats were weaned onto their respective diets. Cholecalciferol was given 1–20 d before 24-h urine collection was begun. Hemicorpus preparations of some of the same rats were perfused at the end of the 24-h urine collection period. Phe, phenylalanine.

* $P < 0.02$.

‡ $P < 0.001$.

TABLE III

Muscle Composition in Vitamin D-deficient and Cholecalciferol-treated Rats

Component	Vitamin D-deficient rats	Cholecalciferol treatment	
		Acute (1–4 d)	Chronic (>4 d)
Carcass/body weight (%)	81.1±0.6 (19)*	81.6±0.6 (11)	79.4±0.4 (8)
Dry weight (%)	24.9±0.5 (8)	24.6±0.5 (8)	24.4±0.4 (8)
Protein (*mg/g*)‡	197±1 (31)	196±6 (9)	195±5 (30)
DNA (*μg P/g*)	63.4±2.1 (23)	61.1±2.4 (7)	66.1±1.9 (23)
RNA (*μg P/g*)	154±5 (20)	170±12 (12)	160±7 (3)
ATP (*μmol/g*)	7.7±0.3 (12)	7.2±0.3 (12)	8.1±0.3 (9)
Creatinine phosphate (*μmol/g*)	18.8±0.7 (12)	17.7±0.7 (13)	22.2±0.9 (9)§

Rats were fed ad lib. the vitamin D-deficient diet containing 0.8% Ca. They were killed in the morning. The carcass weight is the weight of the body after all the internal organs have been removed; it represents mostly muscle, with skin, bone, and adipose tissue. Gastrocnemius muscles were analyzed for muscle composition. For ATP and creatine phosphate the leg muscles of anesthetized rats were frozen in situ with Wollenberger clamps cooled in liquid nitrogen.

* Mean±SE (*n*).

‡ Unit per gram wet weight gastrocnemius muscle.

§ $P < 0.01$ vs. either vitamin D-deficient or acutely treated animals.

TABLE V
Effect of Vitamin D Deficiency on Plasma MH and Creatinine

	Dietary calcium	
	0.8%	1.6%
MH (*nmol/ml*)		
− Cholecalciferol	2.48±0.14 (24)*	1.84±0.07 (14)
+ Cholecalciferol	1.93±0.11 (11)‡	1.50±0.10 (8)‡
Creatinine (*nmol/ml*)		
− Cholecalciferol	0.025±0.002 (26)	
+ Cholecalciferol	0.020±0.002 (7)	

Rats were weaned onto the indicated diets. Cholecalciferol was administered after rats became vitamin D deficient. Plasma samples were obtained at killing several days later.
* Mean±SE (*n*).
‡ $P < 0.02$ vs. −cholecalciferol.

assessed in vitro in the perfused hemicorpus. Release rates, calculated as nanomoles per hour per gram hemicorpus were determined 24 and 48 h after vitamin D administration. In rats fed 0.8% calcium diet, there was a 30% decrease in the rate of MH release from hemicorpus preparations 24 or 48 h after treatment with cholecalciferol ($P < 0.02$, Table IV). Since the muscle concentration of free MH was not significantly changed during the perfusion (3.7±0.3 nmol/g at 1 h and 3.4±0.5 nmol/g at 3 h), the MH that appeared in the perfusate must reflect ongoing actomyosin degradation during this 2-h period.

Using phenylalanine as a marker amino acid, rates of synthesis and degradation of total muscle protein within the hemicorpus system were measured. After treatment with cholecalciferol, synthesis rates increased and degradation decreased. Although these changes were suggestive, they did not reach statistical significance for either process ($P = 0.14$) (Table IV). However, in determining net anabolic response to vitamin D supplementation, a small increase in synthesis (10.6 nmol phenylalamine/h per g) and a small decrease in degradation (9.1 nmol/h per g) would be additive (Table IV). There are 175 μmol of phenylalanine/g muscle protein (25), 200 mg protein/g muscle, and the proportion of muscle to body weight is the same as muscle to hemicorpus weight (34). Since the carcass/body weight ratios were the same for vitamin D-deficient and vitamin D-supplemented rats, all tissues probably grow proportionately. Therefore the +19.7 nmol/h per g net increase in protein anabolism seen after vitamin D supplementation equals an increase in growth rate of 2.0 g/d per 150 g rat, [(+19.7 nmol phenylalanine/h per g muscle) × (24 h) × (150 g rat) × (0.2 g protein/g muscle)/(175 μmol phenylalanine/g protein)]. This value is close to the increase in the weight gain observed in our vitamin D-supplemented animals (Fig. 1).

Plasma insulin levels were determined in both vitamin D-deficient and vitamin D-supplemented rats receiving 0.8 and 1.6% calcium diets. Plasma 25(OH)D_3 levels were equal in both vitamin D-supplemented groups and all vitamin D-deficient rats had undetectable 25(OH)D_3 in their plasma. Plasma insulin levels rose successively with both dietary calcium and vitamin D treatment (Table VI). When analyzed by two-way analysis of variance, only vitamin D treatment could be shown to be significantly related to the increase in plasma insulin levels.

DISCUSSION

In our studies, decreased weight gain was not directly associated with vitamin D deficiency, but rather with the development of hypocalcemia. By the time significant weight differences were apparent between vitamin D-deficient and vitamin D-supplemented pair-fed rats, our vitamin D-deficient rats were hypocalcemic. Our pair-feeding studies show that differences in weight gain between vitamin D-deficient and treated rats are in part related to decreased intake but also associated with the development of vitamin D deficiency and hypocalcemia. Prevention of hypocalcemia, either by increasing the percentage of dietary calcium as in our study or, by feeding lactose (35) to increase intestinal calcium absorption, effectively maintains growth rates, normal rates of MH release, and eliminates any effect of cholecalciferol administration on stimulation of weight gain.

Vitamin D deficiency probably developed at the same time in our animals fed 0.8%, compared to 1.6%, calcium diets for two reasons. First, urinary calcium/creatinine ratios were decreased in both groups of rats irrespective of plasma calcium, while normocalcemic animals with only modest plasma levels of 25(OH)D_3

TABLE VI
Effect of Dietary Calcium and Cholecalciferol of Plasma Insulin and Calcium

Dietary Ca	Cholecalciferol	Insulin	Calcium
%		*μU/ml*	*mg/dl*
0.8	+	65.8±11.6 (5)*	10.9±0.1 (5)
0.8	−	37.0±10.6 (5)	5.4±0.2 (5)
1.6	+	78.8±12.7 (5)	10.5±0.3 (6)
1.6	−	49.4±7.2 (5)	9.1±0.5 (5)

Lactating dams and litters were fed a vitamin D-deficient diet containing 0.8% Ca. Rats were weaned onto their respective diets at 21 d old. Cholecalciferol (400 IU) was administered at 48 d of age. Plasma samples were drawn at 60 d.
* Means±SE (*n*).

had urinary calcium/creatinine levels >10 times higher (Table II). In addition, 25(OH)D_3 levels were low in rats fed either diet for >30 d. However, plasma calcium levels were normal and growth was significantly faster in rats eating the 1.6% calcium diets.

In their classic report, Steenbock and Herting (36) noted that normocalcemic, vitamin D-deficient rats gained more weight than hypocalcemic animals, but not as much as vitamin D-supplemented controls. The calcium and phosphorus composition of the diets used by Steenbock and Herting differed from ours so that when calcium levels were maintained, all their rats were hypophosphatemic. When Steenbock and Herting (36) fed their animals vitamin D-deficient diets containing 1.7% Ca and 0.32% phosphorus, vitamin D-deficient rats gained as much weight as the controls in spite of more severe hypophosphatemia.

When vitamin D was given to hypocalcemic rats, weight gain and muscle mass rapidly increased. We tried to determine whether this was due to increased rates of protein synthesis or decreased degradation by measuring these rates in either in vivo or in vitro studies. We were able to demonstrate significant decreases in myofibrillar protein degradation rates by measuring MH release and MH/creatinine excretion ratios in vitro and in vivo, respectively. We also measured the incorporation and release of phenylalanine by the hemicorpus after both acute and chronic vitamin D administration. Our results suggest both increased phenylalanine incorporation into gastrocnemius muscle protein and decreased phenylalanine release from the hemicorpus. Our inability to demonstrate statistically significant changes in individual rates of protein synthesis or degradation in our phenylalanine studies is understandable because differences in growth rates before and after vitamin D are small when translated into percent changes in phenylalanine uptake or release for a 2-h perfusion period. Calculations based on the net increase in muscle protein anabolism can, however, account for most of the increased growth seen after vitamin D administration.

In other studies comparing phenylalanine and MH release we have been able to demonstrate larger changes in MH release in comparison to phenylalanine release in response to various stimuli such as fasting, refeeding, or addition of cycloheximide to the perfusion media (37). We hypothesized that this is due to the fact that phenylalanine is present within all body proteins while MH is restricted to actin and myosin only. Individual proteins are degraded at different rates. The release of phenylalanine and MH from protein should therefore be viewed as two measures of protein degradation, which while qualitatively similar, cannot be compared quantitatively.

We have recently demonstrated that up to 40% of a rat's daily MH production is derived from gastrointestinal tissue (34). Although the gastrointestinal tract may be the source of some of the MH produced in vivo, it cannot explain the changes in MH release seen in vitro in our eviscerated preparation. Calculating changes in growth rates based upon MH release rates determined in vitro suggest that the changes in degradation could be an important component of the improved weight gain seen after vitamin D supplementation.

In a previous report, Birge and Haddad (7) noted increased [^{14}C]leucine incorporation into muscle within 4–7 h after the administration of either cholecalciferol or 25(OH)D_3. We were unable to duplicate this increased uptake using [^{14}C]phenylalanine. There are several explanations for this discrepancy. First, we performed hemicorpus perfusion studies no earlier than 24 h after cholecalciferol supplementation so that our results are not strictly comparable. Second, protein synthesis may be stimulated for only a short time after vitamin D supplementation and may return to unstimulated levels by 1 d after therapy. We cannot rule out this possibility but suggest that this short-term stimulation could not account for the improved weight gain and increased muscle mass seen for weeks after one dose of 400 IU of vitamin D_3. The incorporation of [^{14}C]leucine (disintegrations per minute) into muscle protein was measured without correcting for intracellular specific activity. Li et al. (38) have demonstrated that the intracellular specific activity of the amino acid precursor must be taken into account when determining muscle protein synthesis rates. If protein degradation is decreased after vitamin D administration, this could lead to less dilution of the intracellular leucine-free amino acid pool, consequent higher intracellular specific activities and an apparent increase in synthesis rates.

What is responsible for decreased myofibrillar degradation rates after vitamin D supplementation? Our original hypothesis was that vitamin D had a direct anabolic effect within muscle but our ability to maintain normal weight gain in normocalcemic vitamin D-deficient rats suggests that this is not so. Our pair-feeding studies and the increased weight gain per gram of food intake rule out a primary role for diminished food intake.

Decreased intracellular ATP levels have been suggested as a possible cause of decreased protein anabolism (7). In agreement with other reports in humans (3) and chicks (14) we found normal ATP levels along with mildly decreased creatine phosphate levels in vitamin D-deficient animals. Young et al. (3) feel that the modest decreases in creatine phosphate levels seen in vitamin D deficiency could not explain the myopathy seen in this condition.

Vitamin D deficiency is associated with both hyperparathyroidism and metabolic acidosis (1, 39, 40).

The role of acidosis in the regulation of skeletal muscle protein turnover has not been studied, but Massry et al. (40) have postulated that hyperparathyroidism leads to increased protein catabolism and negative nitrogen balance. We have performed perfusion experiments using the hemicorpus system in which parathyroid hormone has been added to the perfusion media. We are unable to demonstrate any effect of highly purified parathyroid hormone on protein synthesis, degradation, or the release of MH by the hemicorpus.[2]

Calcium stimulates insulin secretion by increasing the ionized calcium concentration within the cytosol of pancreatic β-cells (41). When isolated rat pancreatic tissue is incubated or perfused with calcium-free medium, insulin release is markedly inhibited (42). This effect of hypocalcemia has been demonstrated in vivo by the finding of abnormal glucose tolerance tests in patients with hypocalcemia and hypoparathyroidism. Treatment with ergocalciferol normalized both serum calcium and the glucose tolerance tests (43). A direct effect of vitamin D on insulin secretion was noted by Norman et al. (44) who perfused isolated pancreatic tissue from vitamin D-deficient and vitamin D-treated rats. They reported a 48% decrease in insulin secretion in the vitamin D-deficient group. Clark et al. (16) treated vitamin D-deficient rats with $1,25(OH)_2D_3$ and noted significant increases in plasma insulin levels with only modest changes in plasma calcium. This same group (45) had previously reported that specific receptors exist in the pancreas for $1,25(OH)_2D_3$ and proposed that this metabolite acts directly on pancreatic β-cells to increase calcium uptake, thus stimulating insulin release in vivo. Our data also support a role for vitamin D exclusive of extracellular calcium in the control of plasma insulin levels. If this schema is correct and administration of vitamin D acts to increase free cytosolic calcium levels within the pancreas, this could explain reports in which either 1,25-dihydroxycholecalciferol, 1α-hydroxycholecalciferol, or pharmacological amounts of $25(OH)D_3$ effectively improve the myopathy of chronic renal failure, a condition in which $25(OH)D_3$ levels may be normal (46).

In summary, the administration of vitamin D to vitamin D-deficient rats leads to improved muscle protein anabolism and an increase in muscle mass and weight gain. A major effect of vitamin D administration on muscle is due to a decrease in the rate of myofibrillar protein degradation, which can be demonstrated both in vivo and in vitro. Vitamin D appears to act indirectly, either by restoring extracellular calcium levels to normal, by stimulating pancreatic secretion of insulin, or by a combination of these two factors.

[2] Wassner, S. J., and J. B. Li. Manuscript in preparation.

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