CONTRIBUTION OF INTESTINAL CALCIUM ABSORPTION TO 1,25-DIHYDROXYVITAMIN D₃-INDUCED CALCIUM ACTION IN THE TOTAL PARENTERAL NUTRITION RAT

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ABSTRACT — To investigate the contribution of intestinal calcium (Ca) absorption to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)-induced Ca action, we assessed parameters related to Ca metabolism after a single dosing of 1,25(OH)₂D₃ in the total parenteral nutrition (TPN) solution or 5% D-mannitol (MAN) solution treatment with rats. Animals were divided into 6 groups (vehicle, 100 µg/kg p.o. and 25 µg/kg i.v.; n=8) in Experiment 1 and 8 groups (vehicle, 1, 10 and 100 µg/kg p.o.; n=6) in Experiment 2 at TPN or MAN solution treatment. In both experiments, the parameters related to Ca metabolisms, urinary Ca and urinary deoxypyridinoline on 0-24 hr or serum Ca, osteocalcin and parathyroid hormone at 24 hr were measured after 1,25(OH)₂D₃ dosing. 1,25(OH)₂D₃-related increased urinary Ca or serum Ca were observed in both experiments. Decrease rates in change of urinary Ca in TPN solution treatment rats were 36.3% (100 µg/kg p.o.) or 47.1% (25 µg/kg i.v.) of MAN solution treatment rats in Experiment 1, and 29.0% (1 µg/kg), 56.2% (10 µg/kg) or 35.3% (100 µg/kg) of MAN solution treatment rats in Experiment 2. Decrease rates in change of serum Ca at 72 hr in TPN solution treatment rats were 57.3% (100 µg/kg p.o.) or 44.5% (25 µg/kg i.v.) of MAN solution treatment rats in Experiment 1, and were 57.0% (100 µg/kg) of MAN solution treatment rats in Experiment 2. There were no differences in the change of serum Ca in the 1,25(OH)₂D₃ 1 or 10-µg/kg group in Experiment 2. Our results suggest that differences in urinary Ca or serum Ca between MAN solution treatment rats and TPN solution treatment rats express the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action in the conditions of the study.

KEY WORDS: Rats, 1,25-dihydroxyvitamin D₃, Intestinal calcium absorption, Total parenteral nutrition, Calcium metabolism

INTRODUCTION

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active form of vitamin D, was discovered in the early 1970s (Fraser and Kodicek, 1970; Norman *et al.*, 1971; Holick *et al.*, 1971). 1,25(OH)₂D₃ affects all body functions of vitamin D, including intestinal calcium (Ca) absorption and bone metabolism (Brommage and DeLuca, 1985). 1,25(OH)₂D₃ was classified as a secosteroid hormone (Norman, 1994; Bouillon *et al.*, 1995). In general, Ca metabolism in the living body is regulated by bone resorption, renal secretion and intestinal absorption, and is the principal factor controlling intestinal Ca absorption (Bouillon *et al.*, 2003;

Bronner, 2003; Christakos *et al.*, 2003). Intestinal Ca absorption can be regulated by dietary calcium intake (Bronner and Pansu, 1999; Christakos *et al.*, 2003). An increase in Ca intake is a simple method for increasing the amount of Ca absorbed (Bronner, 1987; Hurwitz, 1996). 1,25(OH)₂D₃ directly increases intestinal Ca absorption through up-regulation of Ca transport proteins (Bronner *et al.*, 1986; Hoenderop *et al.*, 2004). The role of 1,25(OH)₂D₃ in the intestinal Ca absorption is established, and 1,25(OH)₂D₃ produces two types of effects, one dependent on gene expression and protein synthesis (genomic action), and a more rapid response not dependent on gene activation (nongenomic action) (Bronner, 2003; Christakos *et al.*, 2003; Hurwitz, 1996;

Lee *et al.*, 1990; Wasserman and Fullmer, 1995).

1,25(OH)₂D₃ is widely used to manage secondary hyperparathyroidism in uremic patients because of its efficacy in decreasing elevated parathyroid hormone (PTH) concentration (Slatopolsky *et al.*, 1984; Slatopolsky and Brown, 2002). In clinical usage, however, major side effects have been reported of a hypercalcemia by a Ca action of 1,25(OH)₂D₃ (Binderup, 1993; Hartenbower *et al.*, 1977; Mortensen *et al.*, 1996). Although analyses of intestinal Ca absorption were performed *in vivo* and *in vitro* (organ, cellular, and molecular levels) (Bouillon *et al.*, 2003; Bronner, 2003; Christakos *et al.*, 2003), the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action is not clear *in vivo*. We focused our attention on the total parenteral nutrition (TPN) solution treatment rats for elucidation of the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action. TPN is a method of maintaining required nutrition for the body through intravenous infusion (Dudrick *et al.*, 1968; Steiger *et al.*, 1972). We have recently developed a new method in which TPN is administered into the posterior vena cava via the femoral vein using the tail cuff method in rats (Asanuma *et al.*, 2006). In the TPN solution treatment rats, a diet does not pass digestive organs by administering the nutrients necessary for the living body via the vein as TPN solution. Therefore, in the TPN solution treatment rats, we assumed that the intestinal absorption of Ca in the diet did not occur. Accordingly, we consider it possible to investigate the contribution of the intestinal Ca absorption to the Ca action of 1,25(OH)₂D₃ by the study in the TPN solution treatment rats in a condition without Ca absorption from the small intestine. In this investigation, we compared the contribution of intestinal Ca absorption to hypercalcemia and Ca metabolism by single and a multiple dosing of 1,25(OH)₂D₃ in the TPN solution treatment rats.

MATERIALS AND METHODS

Animals

Forty-eight female Slc:SD rats (SPF) aged 7 weeks were obtained from SLC Japan Co., Ltd. (Shizuoka, Japan) and used in each experiment. Animals were housed one per cage in a wire netting stainless steel cage before surgery and housed individually in a polycarbonate cage after surgery. All animals were acclimatized to the facility for 7 days prior to being used in the study, and maintained under controlled conditions of 24 ± 2°C air temperature, 55 ± 10% relative

humidity, 14–16 air changes/hr ventilation cycle, and a 12 hr light: 12 hr dark cycle. They were provided free access to a pelleted rodent diet (CE-2, CLEA Japan, Inc., Shizuoka, Japan) and water via an automatic watering system in the wire netting cage or water bottle in the polycarbonate cage. All procedures associated with this study were received and approved by the Institutional Animal Care and Use Committee and were performed according to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources.

Operative procedures

At the age of 8 weeks, rats were anesthetized with ketamine hydrochloride (50 mg/kg, i.p., Sankyo Yell Yakuhin Co., Ltd., Tokyo, Japan) and xylazine hydrochloride (10 mg/kg, i.p., Bayer Co., Ltd, Tokyo, Japan). The posterior vena cava via the femoral vein was cannulated with a polyurethane catheter (0.6-mm ID × 0.9-mm OD, Access Technologies, Skokie, IL, USA) under sterile conditions according to a tail cuff method (Asanuma *et al.*, 2006; Cave *et al.*, 1995).

The catheter was tunneled subcutaneously from the incision to the tail and exited through a tail cuff (Lomir Biomedical Inc., Quebec, Canada) and a tether 45 cm long (Lomir Biomedical Inc.) was attached to a 23-gauge plastic swivel (Lomir Biomedical Inc.). Rats were then placed in polycarbonate cages individually. The swivel was connected via polyethylene PE-50 tubing (Becton Dickinson and Company, Sparks, MD, USA), with a 23-gauge needle to a 0.22 µm filter (Millipore Japan Co., Ltd., Tokyo, Japan), which was connected to a 50-ml syringe. Following surgery, the rats were given buprenorphine hydrochloride (0.01 mg/kg, s.c., b.i.d., Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for pain relief during two days. After surgery, all rats were infused with saline (Terumo Corporation, Tokyo, Japan) at 4 ml/kg/hr with a syringe pump (TE-311, Terumo Corporation) for a recovery period of 14 days.

Chemicals

The TPN solution was prepared by adding vitamins for TPN (Multamin for Injection, Sankyo Co., Ltd., Tokyo, Japan) to a standard TPN solution (PNTWIN No.3, Ajinomoto Pharma Co., Ltd, Tokyo, Japan), the composition of which is shown in Table 1. The 5% D-mannitol (MAN) solution was prepared by dissolving D-mannitol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in water for injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan).

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1,25(OH)₂D₃ was synthesized in the Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). A 1 mg/ml stock solution of 1,25(OH)₂D₃ was prepared in ethanol. This stock solution was diluted in a medium chain triglyceride (Contensio chemicals GmbH, Marl, Germany) for orally dosing or ethanol (99.5%, Junsei Chemical Co., Ltd., Tokyo, Japan) for intravenous dosing.

Experimental design

At first, we compared the effect of Ca action by a toxic dose of 1,25(OH)₂D₃ dosing in Experiment 1. Second, we compared the effect of Ca action by a multiple dose of 1,25(OH)₂D₃ in Experiment 2.

Table 2 shows the experimental design. Following the 14-day recovery period after surgery, animals were divided into 6 groups of 8 rats in Experiment 1, and were divided into 8 groups of 6 rats in Experiment 2 by body weights. MAN treatment rats were given free access to a pelleted rodent diet and deionized water. TPN treatment rats received a standard TPN

solution during 8 days. Moreover, TPN treatment rats were fasted and given free access to deionized water during 8 days.

The TPN solution was infused at 12.55 ml/kg/hr (301.2 ml/kg/day), yielding 250 kcal/kg/day as non-protein calories (Miki *et al.*, 2003; Tao *et al.*, 1979; Yeh *et al.*, 2005). The infusion speed of the TPN solution 24 hr after the start of TPN was set to half of the usual infusion speed for acclimatization to TPN, since complications are reported in early stages of the start of TPN (Silvis and Paragas, 1971). The infusion speed of the MAN treatment was set to a similar infusion speed as in the TPN treatment. The TPN solution was administered with an infusion pump (TE-171, Terumo Corporation). The MAN solution was administered with the syringe pump (TE-311, Terumo Corporation). The MAN solution served as an isotonic solution of a comparable control (Evans and Kerry, 2000). For consistency, the first day of 1,25(OH)₂D₃ dosing is referred to as study Day 0 in all sections.

Table 1. Composition of the TPN solution (PNTWIN No.3 with Multamin for Injection).

Volume (ml)	1200		
Nitrogen (g)	6.08		
Non-protein calories (kcal)	1000		
Total calories (kcal)	1160		
Non-protein calories/nitrogen ratio	164		
Glucose (g)	250.4		
Total amino acid (g)	41.44		
Amino acid (g)		Electrolytes	
L-Isoleucine	2.24	Na ⁺ (mEq)	51
L-Leucine	5.00	K ⁺ (mEq)	30
L-Lysine acetate	4.96	Mg ²⁺ (mEq)	6
L-Methionine	1.40	Ca ²⁺ (mEq)	8
L-Phenylalanine	3.74	Cl ⁻ (mEq)	50
L-Threonine	2.60	SO ₄ ²⁻	6
L-Tryptophan	0.52	Acetate (mEq)	46
L-Valine	1.80	Phosphate (mmol)	8
L-Alanine	2.48	Gluconate (mEq)	8
L-Arginine	3.16	Zn ²⁺ (μmol)	20
L-Aspartic acid	1.52		
L-Cysteine	0.40		
L-Glutamic acid	2.60	Vitamins	
L-Histidine	2.40	A, B ₁ , B ₂ , B ₆ , B ₁₂ , C, D ₃ , E, K ₂ ,	
L-Proline	1.32	Pantothenic acid, Nicotinic acid,	
L-Serine	0.88	Biotin, Folic acid	
L-Tyrosine	0.14		
Glycine	4.28		

Clinical signs and body weights

Observation of clinical signs was performed once daily for the experimental period. Body weights were recorded on Day -6, -1 and 3.

Urinalysis

Urinalysis was carried out from Day 0 to Day 1. Urine was collected individually in a polycarbonate cage with wire netting bed during approximately 24 hr. Urine volume (ml/24 hr) was measured visually with a disposable syringe. Urine specimens were stored at temperature -80°C and used for the following analyses and methods. Urinary concentrations of Ca (OCPC method), inorganic phosphorus (IP) (enzyme method), and creatinine (CRE) (enzyme method) were measured with a Hitachi 7170 auto-analyzer (Hitachi Ltd., Tokyo, Japan). The 24-hr urinary Ca and IP excretion (equivalent to mL of daily urine volume) were calculated in mg/24 hr for each rat from the urine volume and urinary Ca and IP concentrations. Urinary deoxypyridinoline (Dpd) concentration, which was used as a measure of bone resorption markers, was measured with OSTEOLINKS-DPD EIA Kit (Sumitomo Seiyaku Biomedical Co., Ltd., Osaka, Japan) in urine specimens during 0 to 24 hr after the $1,25(\text{OH})_2\text{D}_3$ administration. Values of urinary Dpd concentration were corrected for urinary CRE concentrations.

Blood biochemistry

Blood was collected from the jugular vein under conscious condition. The intention was to take samples from all animals at Day -1 (24 hr before $1,25(\text{OH})_2\text{D}_3$ dosing), Day 1 (24 hr after $1,25(\text{OH})_2\text{D}_3$ dosing), Day 2 (48 hr after $1,25(\text{OH})_2\text{D}_3$ dosing) and Day 3 (72 hr after $1,25(\text{OH})_2\text{D}_3$ dosing). Sample volume was 0.7 ml of each point by using 1.0-ml syringes fitted with 25-gauge $\times$ 25 mm needles. Blood was allowed to stand for 30 min at room temperature, centrifuged ($1870 \times g$, 4°C , 10 min), and the separated serum stored at a temperature of -80°C . Separated samples were used for the following analyses and methods. Aspartate aminotransferase (AST) (JSCC method), alanine aminotransferase (ALT) (JSCC method), alkaline phosphatase (Al-P) (JSCC method), glucose (Glucose) (hexokinase method), total cholesterol (T-Cho) (enzyme method), triglyceride (TG) (enzyme method), blood urea nitrogen (BUN) (urease-GLDH method), CRE (enzyme method), IP (enzyme method), Ca (OCPC method), total protein (TP) (Biuret's method), and albumin (ALB) (BCG method) were determined with a Hitachi 7170 auto-analyzer (Hitachi Ltd., Tokyo, Japan). Serum osteocalcin (BGP) concentration, which was used as a measure of bone formation markers, was measured with Osteocalcin Rat ELISA System (GE Healthcare Bio-Sciences Corp., Piscata-

Table 2. Experimental design.

Treatment	Group	Dosing method	Dosing concentration ($\mu\text{g/ml}$)	Dosing volume (ml/kg)	Number of animals
Experiment 1					
MAN solution	Vehicle	Single, p.o.	0	2	8
MAN solution	$1,25(\text{OH})_2\text{D}_3$ 100 $\mu\text{g/kg}$	Single, p.o.	50	2	8
MAN solution	$1,25(\text{OH})_2\text{D}_3$ 25 $\mu\text{g/kg}$	Single, i.v.	50	0.5	8
TPN solution	Vehicle	Single, p.o.	0	2	8
TPN solution	$1,25(\text{OH})_2\text{D}_3$ 100 $\mu\text{g/kg}$	Single, p.o.	50	2	8
TPN solution	$1,25(\text{OH})_2\text{D}_3$ 25 $\mu\text{g/kg}$	Single, i.v.	50	0.5	8
Experiment 2					
MAN solution	Vehicle	Single, p.o.	0	2	6
MAN solution	$1,25(\text{OH})_2\text{D}_3$ 1 $\mu\text{g/kg}$	Single, p.o.	0.5	2	6
MAN solution	$1,25(\text{OH})_2\text{D}_3$ 10 $\mu\text{g/kg}$	Single, p.o.	5	2	6
MAN solution	$1,25(\text{OH})_2\text{D}_3$ 100 $\mu\text{g/kg}$	Single, p.o.	50	2	6
TPN solution	Vehicle	Single, p.o.	0	2	6
TPN solution	$1,25(\text{OH})_2\text{D}_3$ 1 $\mu\text{g/kg}$	Single, p.o.	0.5	2	6
TPN solution	$1,25(\text{OH})_2\text{D}_3$ 10 $\mu\text{g/kg}$	Single, p.o.	5	2	6
TPN solution	$1,25(\text{OH})_2\text{D}_3$ 100 $\mu\text{g/kg}$	Single, p.o.	50	2	6

MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition solution (fasted); Vehicle, medium chain triglyceride.

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away, NJ, USA) in the jugular vein blood at Day -1 and Day 1 after the 1,25(OH)₂D₃ dosing. Serum PTH concentration, which was used as an indicator of calcium metabolism, was measured with PTH Rat ELISA System (GE Healthcare Bio-Sciences Corp.) in jugular vein blood at Day -1 and Day 1 after the 1,25(OH)₂D₃ dosing in Experiment 2.

In order to monitor the renal function, CRE clearance (Ccr) was determined by urine volume, serum and urinary creatinine concentration, and expressed in ml/min.

Necropsy

At the end of the infusion period on Day 3 (72 hr after 1,25(OH)₂D₃ dosing), all animals were anesthetized deeply with pentobarbital sodium (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), exsanguinated and subjected to necropsy.

Statistical analysis

Statistical analysis was performed by SAS pre-clinical package software (SAS version 8.2, SAS Institute Japan Inc., Tokyo, Japan). Homogeneity of variance was first assessed by Bartlett's test (Bartlett, 1937) and then significance by Dunnett's test (Dunnett, 1964) when multi-groups (vehicle group vs. 1,25(OH)₂D₃ groups) were compared. Significant difference was first assessed by F test (Snedecor and Cochran, 1967) and then by Student's *t* test (Steel and Torrie, 1980) or Aspin-Welch's *t* test (Snedecor and Cochran, 1967) when two treatments (MAN solution vs. TPN solution) were compared. *p* < 0.05 was regarded as significant.

RESULTS

Clinical signs and body weights

There were no clinical signs considered to be related to treatment with MAN solution, TPN solution or 1,25(OH)₂D₃ dosing in Experiments 1 and 2. A decrease in body weight on Day 3 was recorded for the 1,25(OH)₂D₃ 25-μg/kg group in MAN solution treatment rats of Experiment 1 (Table 3). There were no changes in body weight considered to be related to treatment with MAN solution, TPN solution or 1,25(OH)₂D₃ dosing in Experiment 2 (Table 4).

Urinalysis

Tables 5 and 6 show the urinalysis in Experiments 1 and 2, respectively. Treatment with 1,25(OH)₂D₃ was associated with changes in several urinalysis parameters.

In Experiment 1, an increase in urine volume was observed in the 1,25(OH)₂D₃ 100-μg/kg p.o. group with MAN solution treatment rats and 25-μg/kg i.v. group with TPN solution treatment rats. An increase in urinary Ca excretion was found in the 1,25(OH)₂D₃ 100-μg/kg p.o. group and 25-μg/kg i.v. group in both treatment rats. An increase in urinary IP excretion was found in the 1,25(OH)₂D₃ 100-μg/kg p.o. group and 25-μg/kg i.v. group in MAN solution treatment rats. When compared to MAN solution treatment rats, TPN solution treatment rats showed a decrease in urinary Ca and IP excretion in all animals of the 1,25(OH)₂D₃ dosing group. TPN solution treatment rats showed an increase in Ccr in the 1,25(OH)₂D₃ 100-μg/kg p.o. group compared to the vehicle group and 1,25(OH)₂D₃

Table 3. Body weights in Experiment 1.

Treatment Time	Body weight (g)		
	Vehicle p.o.	1,25(OH) ₂ D ₃	
		100 μg/kg p.o.	25 μg/kg i.v.
MAN solution			
Day -6	220.2 ± 9.0	221.1 ± 11.8	221.2 ± 11.4
Day -1	228.2 ± 11.0	230.9 ± 12.8	229.6 ± 10.8
Day 3	227.5 ± 12.3	225.9 ± 13.4	209.7 ± 9.5*
TPN solution			
Day -6	219.6 ± 9.9	221.1 ± 11.0	220.9 ± 11.0
Day -1	222.4 ± 9.0	217.7 ± 13.3	220.9 ± 9.2
Day 3	221.1 ± 8.6	219.4 ± 13.3	219.9 ± 12.4

Data are expressed as mean ± S.D. (n = 8). MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition (fasted); Vehicle, medium chain triglyceride; Day -6, before start of TPN solution treatment or MAN solution treatment; Day -1, 24 hr before dosing; Day 3, 72 hr after dosing. There was no significant difference between the MAN solution treatment and TPN solution treatment (*p* > 0.05, Student's or Aspin-Welch's *t*-test). **p* < 0.05, Significant difference from the vehicle group (Dunnett's test).

25- $\mu\text{g/kg}$ i.v. group compared to MAN solution treatment rats.

In Experiment 2, an increase in urinary Ca excretion was found in the $1,25(\text{OH})_2\text{D}_3$ 1- $\mu\text{g/kg}$ group in MAN solution treatment rats or 10- and 100- $\mu\text{g/kg}$ $1,25(\text{OH})_2\text{D}_3$ groups in both treatment rats. An increase in urinary IP excretion was found in the 10- and 100- $\mu\text{g/kg}$ $1,25(\text{OH})_2\text{D}_3$ groups in MAN solution treatment rats. When compared to MAN solution treatment rats, TPN solution treatment rats showed a decrease in urinary Ca and IP excretion in all animals of the $1,25(\text{OH})_2\text{D}_3$ dosing group. TPN solution treat-

ment rats showed an increase in Ccr in the vehicle group and $1,25(\text{OH})_2\text{D}_3$ 25- $\mu\text{g/kg}$ i.v. group compared to MAN solution treatment rats in Experiment 2.

In Experiment 1, $1,25(\text{OH})_2\text{D}_3$ -related increased urinary Ca excretions were observed, and decrease rates in change of urinary Ca excretion in TPN solution treatment rats were 36.3% (100 $\mu\text{g/kg}$ p.o.) or 47.1% (25 $\mu\text{g/kg}$ i.v.) of MAN solution treatment rats (Table 7). In Experiment 2, $1,25(\text{OH})_2\text{D}_3$ -related increased urinary Ca excretion were observed, and decrease rates in change of urinary Ca excretion in TPN solution treatment rats were 29.0% (1 $\mu\text{g/kg}$), 56.2% (10 $\mu\text{g/kg}$)

Table 4. Body weights in Experiment 2.

Treatment Time	Body weight (g)			
	Vehicle p.o.	$1,25(\text{OH})_2\text{D}_3$ p.o.		
		1 $\mu\text{g/kg}$	10 $\mu\text{g/kg}$	100 $\mu\text{g/kg}$
MAN solution				
Day -6	213.3 $\pm$ 10.5	213.5 $\pm$ 10.8	213.6 $\pm$ 9.6	214.6 $\pm$ 11.0
Day -1	222.1 $\pm$ 36.4	222.7 $\pm$ 29.0	221.4 $\pm$ 33.2	224.1 $\pm$ 22.9
Day 3	229.1 $\pm$ 35.7	227.1 $\pm$ 28.6	227.5 $\pm$ 34.1	225.7 $\pm$ 22.7
TPN solution				
Day -6	217.1 $\pm$ 11.6	214.0 $\pm$ 8.3	214.0 $\pm$ 8.4	214.2 $\pm$ 9.3
Day -1	218.8 $\pm$ 10.9	212.7 $\pm$ 9.9	217.3 $\pm$ 32.8	215.9 $\pm$ 12.9
Day 3	230.2 $\pm$ 11.1	225.4 $\pm$ 10.7	230.7 $\pm$ 37.5	228.6 $\pm$ 13.5

Data are expressed as mean $\pm$ S.D. (n = 6). MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition (fasted); Vehicle, medium chain triglyceride Day -6, before start of TPN solution treatment or MAN solution treatment; Day -1, 24 hr before dosing; Day 3, 72 hr after dosing. There was no significant difference among the vehicle group and $1,25(\text{OH})_2\text{D}_3$ groups ($p > 0.05$, Dunnett's test). There was no significant difference between the MAN solution treatment and TPN solution treatment ($p > 0.05$, Student's or Aspin-Welch's *t*-test).

Table 5. Urinalysis on 0-24 hr after dosing in Experiment 1.

Item	Treatment	Vehicle p.o.	$1,25(\text{OH})_2\text{D}_3$	
			100 $\mu\text{g/kg}$ p.o.	25 $\mu\text{g/kg}$ i.v.
Urine volume (ml/24 hr)	MAN solution	33.8 $\pm$ 6.2	42.9 $\pm$ 9.8*	42.2 $\pm$ 5.3
	TPN solution	32.8 $\pm$ 4.8	36.2 $\pm$ 5.1	38.5 $\pm$ 2.6*
Urinary CRE (mg/dl)	MAN solution	18.18 $\pm$ 3.04	14.92 $\pm$ 3.15*	15.62 $\pm$ 1.38
	TPN solution	17.64 $\pm$ 2.30	17.54 $\pm$ 3.21	16.15 $\pm$ 1.70
Urinary Ca excretion (mg/24 hr)	MAN solution	2.77 $\pm$ 1.08	12.57 $\pm$ 2.81**	11.35 $\pm$ 1.86**
	TPN solution	3.42 $\pm$ 0.75	6.98 $\pm$ 2.06** ##	7.47 $\pm$ 2.05** ##
Urinary IP excretion (mg/24 hr)	MAN solution	22.35 $\pm$ 5.50	34.34 $\pm$ 8.14**	35.17 $\pm$ 4.65**
	TPN solution	10.60 $\pm$ 2.89##	11.30 $\pm$ 4.09##	13.12 $\pm$ 3.85##
Ccr (ml/min)	MAN solution	1.85 $\pm$ 0.30	1.69 $\pm$ 0.29	1.61 $\pm$ 0.15
	TPN solution	1.70 $\pm$ 0.15	1.89 $\pm$ 0.15*	1.75 $\pm$ 0.11#

Data are expressed as mean $\pm$ S.D. (n = 8). CRE, Creatinine; Ca, Calcium; IP, Inorganic phosphorus; MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition (fasted). Ccr (CRE clearance) was calculated by the following formula: Ccr (ml/min) = urinary CRE (mg/dl) $\times$ urine volume (ml/24 hr) / serum CRE (mg/dl)/1440 (min). * $p < 0.05$, ** $p < 0.01$, Significant difference from the vehicle group (Dunnett's test). # $p < 0.05$, ## $p < 0.01$, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

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or 35.3% (100 µg/kg) of MAN solution treatment rats (Table 8).

Blood biochemistry

Fig. 1 and 2 show the serum Ca concentration in Experiments 1 and 2, respectively. The serum Ca con-

Table 6. Urinalysis on 0-24 hr after dosing in Experiment 2.

Item	Treatment	Vehicle p.o.	1,25(OH) ₂ D ₃ p.o.		
			1 µg/kg	10 µg/kg	100 µg/kg
Urine volume (ml/24 hr)	MAN solution	35.7 ± 6.0	42.1 ± 7.6	39.2 ± 5.3	45.4 ± 9.3
	TPN solution	37.2 ± 3.1	35.6 ± 4.2	38.5 ± 6.3	41.3 ± 3.9
Urinary CRE (mg/dl)	MAN solution	17.89 ± 1.32	15.24 ± 1.64*	16.10 ± 1.31	14.27 ± 2.20**
	TPN solution	20.52 ± 1.07 ^{##}	21.38 ± 2.49 ^{##}	19.81 ± 3.67	18.29 ± 1.25 ^{##}
Urinary Ca excretion (mg/24 hr)	MAN solution	2.16 ± 1.45	6.76 ± 2.31**	11.21 ± 1.89**	15.18 ± 2.22**
	TPN solution	1.43 ± 0.64	2.76 ± 1.14 ^{##}	6.53 ± 1.64** ^{##}	6.04 ± 2.73** ^{##}
Urinary IP excretion (mg/24 hr)	MAN solution	16.48 ± 3.10	22.77 ± 5.75	27.61 ± 1.46**	32.20 ± 7.14**
	TPN solution	5.78 ± 2.05 ^{##}	3.67 ± 2.11 ^{##}	7.00 ± 3.01 ^{##}	7.37 ± 3.89 ^{##}
Ccr (ml/min)	MAN solution	1.90 ± 0.29	2.12 ± 0.58	2.02 ± 0.25	2.33 ± 0.92
	TPN solution	3.70 ± 0.94 ^{##}	3.25 ± 0.97 [#]	3.81 ± 1.20 [#]	3.11 ± 0.36

Data are expressed as mean ± S.D. (n = 6). CRE, Creatinine; Ca, Calcium; IP, Inorganic phosphorus; MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition (fasted). Ccr (CRE clearance) was calculated by the following formula: Ccr (ml/min) = urinary CRE (mg/dl) × urine volume (ml/24 hr) /serum CRE (mg/dl)/1440 (min). *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test). [#]p<0.05, ^{##}p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

Table 7. Change of urinary Ca excretion and serum Ca concentration in Experiment 1.

Item	Treatment Time	1,25(OH) ₂ D ₃	
		100 µg/kg p.o.	25 µg/kg i.v.
Change of urinary Ca excretion (mg/24 hr)			
	MAN solution		
	0–24 hr	9.80 ± 3.05	8.58 ± 2.62
	TPN solution		
	0–24 hr	3.56 ± 2.22 ^{##}	4.04 ± 2.43 ^{##}
Decrease rate (%)		36.3	47.1
Change of serum Ca concentration (mg/dl)			
	MAN solution		
	24 hr	2.31 ± 0.68	2.20 ± 0.37
	48 hr	2.29 ± 0.63	3.85 ± 0.28
	72 hr	0.96 ± 0.59	3.46 ± 0.53
	TPN solution		
	24 hr	1.92 ± 0.65	2.08 ± 0.73
	48 hr	1.18 ± 0.36 ^{##}	2.04 ± 1.06 ^{##}
	72 hr	0.55 ± 0.25	1.54 ± 1.04 ^{##}
Decrease rate (%)	24 hr	83.1	94.5
	48 hr	51.5	53.0
	72 hr	57.3	44.5

Data are expressed as mean ± S.D. (n = 8). MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition (fasted); 24 hr, 24 hr after dosing; 48 hr, 48 hr after dosing; 72 hr, 72 hr after dosing. Decrease rates were calculated by the following formula: decrease rate (%) = (Mean value in the TPN solution treatment/Mean value in the MAN solution treatment) × 100. ^{##}p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

centrations (mean $\pm$ S.D.) in Experiment 1 in the 1,25(OH)₂D₃ 100- μ g/kg p.o. group were 11.51 ± 0.24 (–24 hr), 13.83 ± 0.63 (24 hr), 13.80 ± 0.52 (48 hr) and 12.47 ± 0.53 (72 hr) mg/dl in MAN solution treatment rats and 11.04 ± 0.31 (–24 hr), 12.96 ± 0.44 (24 hr), 12.22 ± 0.45 (48 hr) and 11.59 ± 0.35 (72 hr) mg/dl in TPN solution treatment rats. In the 1,25(OH)₂D₃ 25- μ g/kg i.v. group, serum Ca concentrations were 11.69 ± 0.13 (–24 hr), 13.88 ± 0.36 (24 hr), 15.53 ± 0.29 (48 hr) and 15.14 ± 0.50 (72 hr) mg/dl in MAN solution treatment rats, and 11.23 ± 0.41 (–24 hr), 13.31 ± 0.45 (24 hr), 13.26 ± 0.77 (48 hr) and 12.77 ± 0.75 (72 hr) mg/dl in TPN solution treatment rats.

The serum Ca concentrations (mean $\pm$ S.D.) in Experiment 2, in the 1,25(OH)₂D₃ 1- μ g/kg group were 11.21 ± 0.21 (–24 hr), 11.66 ± 0.34 (24 hr), 11.67 ± 0.27 (48 hr) and 11.25 ± 0.20 (72 hr) mg/dl in MAN solution treatment rats, and 11.10 ± 0.39 (–24 hr), 11.51 ± 0.38 (24 hr), 11.60 ± 0.33 (48 hr) and 11.57 ± 0.26 (72 hr) mg/dl in TPN solution treatment rats. In the 1,25(OH)₂D₃ 10- μ g/kg group, serum Ca concentrations were 11.26 ± 0.16 (–24 hr), 12.89 ± 0.54 (24 hr), 12.42 ± 0.32 (48 hr), 11.82 ± 0.34 (72 hr) mg/dl in

MAN solution treatment rats, and 11.10 ± 0.16 (–24 hr), 12.89 ± 0.32 (24 hr), 12.32 ± 0.31 (48 hr), 11.65 ± 0.16 (72 hr) mg/dl in TPN solution treatment. In the 1,25(OH)₂D₃ 100- μ g/kg group, serum Ca concentrations were 11.45 ± 0.23 (–24 hr), 14.58 ± 0.42 (24 hr), 13.67 ± 0.37 (48 hr), 13.02 ± 0.60 (72 hr) mg/dl in MAN solution treatment rats, and 11.17 ± 0.30 (–24 hr), 13.59 ± 0.49 (24 hr), 13.39 ± 0.71 (48 hr), 12.07 ± 0.44 (72 hr) mg/dl in TPN solution treatment rats. In the 1,25(OH)₂D₃ 100- μ g/kg group, TPN solution treatment rats decreased to 13.59 ± 0.49 mg/dl at 24 hr and 12.07 ± 0.44 mg/dl at 72 hr compared to 14.58 ± 0.42 mg/dl at 24 hr and 13.02 ± 0.60 mg/dl at 72 hr in MAN solution treatment rats.

In Experiment 1, decrease rates in change of serum Ca concentration following 1,25(OH)₂D₃ dosing in TPN solution treatment rats were 83.1% (100 μ g/kg p.o.) or 94.5% (25 μ g/kg i.v.) of MAN solution treatment rats at 24 hr, and were 51.5% (100 μ g/kg p.o.) or 53.0% (25 μ g/kg i.v.) of MAN solution treatment rats at 48 hr, and were 57.3% (100 μ g/kg p.o.) or 44.5% (25 μ g/kg i.v.) of MAN solution treatment rats at 72 hr (Table 7). In Experiment 2, decrease rates in

Table 8. Change of urinary Ca excretion and serum Ca concentration in Experiment 2.

Item	Treatment Time	1,25(OH) ₂ D ₃ p.o.		
		1 μg/kg	10 μg/kg	100 μg/kg
Change of urinary Ca excretion (mg/24 hr)				
	MAN solution			
	0–24 hr	4.59 ± 1.97	9.05 ± 2.37	13.02 ± 2.14
	TPN solution			
	0–24 hr	1.33 ± 1.31 ^{##}	5.09 ± 1.63 ^{##}	4.60 ± 2.36 ^{##}
Decrease rate (%)		29.0	56.2	35.3
Change of serum Ca concentration (mg/dl)				
	MAN solution			
	24 hr	0.46 ± 0.39	1.63 ± 0.46	3.14 ± 0.50
	48 hr	0.47 ± 0.25	1.17 ± 0.27	2.22 ± 0.58
	72 hr	0.05 ± 0.27	0.57 ± 0.37	1.58 ± 0.76
	TPN solution			
	24 hr	0.42 ± 0.48	1.79 ± 0.41	2.43 ± 0.56 [#]
	48 hr	0.50 ± 0.13	1.21 ± 0.42	2.22 ± 0.51
	72 hr	0.47 ± 0.38 [#]	0.55 ± 0.23	0.90 ± 0.31
Decrease rate (%)	24 hr	91.3	109.8	77.4
	48 hr	106.4	103.4	100.0
	72 hr	940.0	96.5	57.0

Data are expressed as mean $\pm$ S.D. (n = 6). MAN, 5% D-mannitol (orally fed); TPN, Total parenteral nutrition (fasted); 24 hr, 24 hr after dosing; 48 hr, 48 hr after dosing; 72 hr, 72 hr after dosing. Decrease rates were calculated by the following formula: decrease rate (%) = (Mean value in the TPN solution treatment/Mean value in the MAN solution treatment) $\times$ 100. $^{\#}$ p<0.05, $^{##}$ p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

1,25(OH)₂D₃-induced Ca action in the TPN rat.

change of serum Ca concentration at TPN solution treatment rats in the 1,25(OH)₂D₃ 100-μg/kg group were 77.4% (24 hr) or 57.0% (72 hr) of MAN solution treatment rats (Table 8). There were no differences between TPN solution treatment rats and MAN solution treatment rats in serum Ca concentration except the above changes in Experiment 2.

Tables 9 and 10 summarize the blood biochemistry data in Experiment 1 and 2, respectively. The blood biochemistry results did not vary with MAN and TPN solution treatment rats, which is similar to our background data in orally fed rats and TPN solution treatment rats (Asanuma *et al.*, 2006; Mochida *et al.*, 1991;

Yamauchi and Kokuba, 1999).

Urinary Dpd, serum BGP and PTH concentrations

In Experiment 1, and Experiment 2 except in the 1,25(OH)₂D₃ 10-μg/kg group, no treatment related abnormalities were observed in the urinary Dpd concentration during 0-24 hr of both treatments (Fig. 3 and 4). In Experiment 2, urinary Dpd concentration during 0-24 hr was decreased in the 1,25(OH)₂D₃ 10-μg/kg group of MAN solution treatment rats compared to the vehicle group (Fig. 4). In Experiment 1 and 2, serum BGP concentrations at 24 hr were increased in a dose-related fashion in the 1,25(OH)₂D₃ dosing group in

Fig. 1. Serum calcium (Ca) concentration after a single dosing of 1,25-dihydroxyvitamin D₃ (100 μg/kg p.o. or 25 μg/kg i.v.) or vehicle (medium chain triglyceride) for at MAN (orally fed) or TPN (fasted) solution treatment to rats. Data are expressed as mean ± S.D. (n = 8). #p<0.05, ##p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

both treatments compared with the vehicle group (Fig. 3 and 4).

In Experiment 2, the serum PTH concentrations at 24 hr were decreased in a dose-related fashion at 1,25(OH)₂D₃ dosing group in both treatments compared with the vehicle groups (Fig. 4).

Necropsy

No treatment-related abnormalities were observed at necropsy in Experiments 1 and 2.

DISCUSSION

The high dose of 1,25(OH)₂D₃ increased the

intestinal Ca absorption of dietary Ca and caused chronic hypercalcemia. 1,25(OH)₂D₃-associated side effects are hypercalciuria, hypercalcemia and induction of bone resorption. The chronic hypercalcemia leads to calcification in many organs or tissues, including kidney, heart, lung, stomach and blood vessels (Binderup, 1993; Mortensen *et al.*, 1996). Secondary side effects of hypercalcemia caused severe conditions in clinical signs, decreases in food consumption and increases in water intake or body weight loss.

This study clearly demonstrates that TPN solution treatment rats following 1,25(OH)₂D₃ dosing display decreases *in vivo* Ca action compared to MAN solution treatment rats. The differences in urinary Ca

Fig. 2. Serum calcium (Ca) concentration after a single dosing of 1,25-dihydroxyvitamin D₃ (1, 10 or 100 µg/kg p.o.) or vehicle (medium chain trygriceride) for MAN (orally fed) or TPN (fasted) solution treatment to rats. Data are expressed as mean ± S.D. (n = 6). #p < 0.05, ##p < 0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

1,25(OH)₂D₃-induced Ca action in the TPN rat.**Table 9.** Blood biochemistry in Experiment 1.

Items	Time	MAN solution treatment					
		Vehicle p.o.		100 µg/kg p.o.	25 µg/kg i.v.		
AST (IU/l)	Before	104	± 19	115	± 25	90	± 18
	24 hr	102	± 15	112	± 19	110	± 19
	48 hr	96	± 22	104	± 15	95	± 22
	72 hr	98	± 15	107	± 28	105	± 20
ALT (IU/l)	Before	44	± 6	49	± 17	43	± 7
	24 hr	42	± 4	48	± 13	47	± 8
	48 hr	41	± 5	44	± 12	42	± 7
	72 hr	44	± 5	49	± 14	45	± 5
Al-P (IU/l)	Before	938	± 271	1033	± 399	936	± 166
	24 hr	833	± 226	907	± 563	749	± 153
	48 hr	884	± 178	788	± 268	708	± 125
	72 hr	891	± 193	906	± 279	802	± 116
Glucose (mg/dl)	Before	136	± 15	133	± 20	128	± 4
	24 hr	132	± 7	132	± 8	135	± 7
	48 hr	125	± 6	128	± 8	132	± 8
	72 hr	125	± 9	126	± 4	126	± 10
T-Cho (mg/dl)	Before	81	± 12	71	± 10	76	± 9
	24 hr	90	± 12	82	± 11	91	± 11
	48 hr	97	± 11	85	± 10	94	± 12
	72 hr	100	± 10	87	± 10	98	± 12
TG (mg/dl)	Before	52	± 15	51	± 13	43	± 13
	24 hr	47	± 14	43	± 19	52	± 29
	48 hr	64	± 22	58	± 25	47	± 15
	72 hr	69	± 24	67	± 30	51	± 21
BUN (mg/dl)	Before	17.2	± 2.2	17.2	± 2.4	15.3	± 1.6
	24 hr	16.7	± 1.8	19.0	± 3.7	19.4	± 2.2
	48 hr	17.4	± 1.3	20.2	± 3.3*	18.8	± 2.0
	72 hr	17.7	± 2.1	19.1	± 2.3	20.1	± 2.6
CRE (mg/dl)	Before	0.21	± 0.04	0.22	± 0.03	0.21	± 0.03
	24 hr	0.23	± 0.04	0.26	± 0.03	0.28	± 0.03**
	48 hr	0.23	± 0.04	0.26	± 0.03	0.28	± 0.01**
	72 hr	0.23	± 0.02	0.26	± 0.02*	0.27	± 0.03**
IP (mg/dl)	Before	8.16	± 1.02	8.89	± 0.54	8.38	± 0.31
	24 hr	7.48	± 0.58	8.44	± 0.56**	8.73	± 0.62**
	48 hr	7.92	± 0.49	8.02	± 0.34	8.22	± 0.20
	72 hr	7.68	± 0.47	7.65	± 0.45	8.03	± 1.23
TP (g/dl)	Before	7.32	± 0.14	7.19	± 0.21	7.05	± 0.24*
	24 hr	7.01	± 0.35	7.07	± 0.20	7.16	± 0.23
	48 hr	7.03	± 0.22	7.07	± 0.32	7.06	± 0.19
	72 hr	6.94	± 0.22	6.85	± 0.34	6.91	± 0.19
ALB (g/dl)	Before	4.15	± 0.29	4.01	± 0.25	4.06	± 0.14
	24 hr	4.05	± 0.41	4.09	± 0.37	4.20	± 0.18
	48 hr	4.00	± 0.29	3.97	± 0.33	4.06	± 0.19
	72 hr	3.99	± 0.29	3.84	± 0.35	3.96	± 0.21

Data are expressed as mean ± S.D. (n = 8). Before, 24 hr before dosing; 24 hr, 24 hr after dosing; 48 hr, 48 hr after dosing; 72 hr, 72 hr after dosing; MAN, 5% D-mannitol (orally fed); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase, Al-P, Alkaline phosphatase; T-Cho, Total cholesterol; TG, Triglyceride; BUN, Blood urea nitrogen; CRE, Creatinine; IP, Inorganic phosphorus; TP, Total protein; ALB, albumin. *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test).

Table 9. (continued). Blood biochemistry in Experiment 1.

Items	Time	TPN solution treatment		
		Vehicle p.o.	100 µg/kg	25 µg/kg
AST (IU/l)	Before	109 ± 24	106 ± 22	97 ± 21
	24 hr	108 ± 10	100 ± 21	89 ± 17 [#]
	48 hr	80 ± 12	89 ± 14	84 ± 11
	72 hr	86 ± 7	95 ± 13	91 ± 14
ALT (IU/l)	Before	41 ± 5	37 ± 8	38 ± 5
	24 hr	50 ± 10	39 ± 8*	38 ± 3* [#]
	48 hr	34 ± 6 [#]	30 ± 5 [#]	31 ± 4 ^{##}
	72 hr	37 ± 5 [#]	37 ± 9	33 ± 4 ^{##}
Al-P (IU/l)	Before	459 ± 74 ^{##}	425 ± 61 ^{##}	383 ± 69 ^{##}
	24 hr	379 ± 43 ^{##}	318 ± 33* [#]	323 ± 62 ^{##}
	48 hr	333 ± 46 ^{##}	264 ± 37* ^{##}	261 ± 65* ^{##}
	72 hr	340 ± 50 ^{##}	288 ± 44 ^{##}	258 ± 50* ^{##}
Glucose (mg/dl)	Before	182 ± 24 ^{##}	164 ± 16 ^{##}	172 ± 24 ^{##}
	24 hr	138 ± 15	149 ± 10 ^{##}	148 ± 19
	48 hr	134 ± 10 [#]	126 ± 7	140 ± 24
	72 hr	144 ± 47	144 ± 26	138 ± 11 [#]
T-Cho (mg/dl)	Before	51 ± 10 ^{##}	49 ± 5 ^{##}	51 ± 6 ^{##}
	24 hr	96 ± 16	86 ± 10	91 ± 7
	48 hr	112 ± 20	91 ± 8*	101 ± 12
	72 hr	113 ± 21	94 ± 12*	99 ± 13
TG (mg/dl)	Before	43 ± 7	46 ± 15	53 ± 10
	24 hr	30 ± 6 ^{##}	38 ± 7*	33 ± 6
	48 hr	29 ± 7 ^{##}	32 ± 5 [#]	30 ± 8 [#]
	72 hr	28 ± 8 ^{##}	38 ± 9* [#]	30 ± 5 [#]
BUN (mg/dl)	Before	14.1 ± 1.6 ^{##}	13.1 ± 2.3 ^{##}	13.7 ± 1.4
	24 hr	14.6 ± 1.1 [#]	13.9 ± 0.7 ^{##}	15.0 ± 1.2 ^{##}
	48 hr	13.8 ± 1.5 ^{##}	13.5 ± 0.9 ^{##}	14.6 ± 1.5 ^{##}
	72 hr	14.0 ± 2.0 ^{##}	12.9 ± 1.4 ^{##}	15.2 ± 2.1 ^{##}
CRE (mg/dl)	Before	0.23 ± 0.04	0.20 ± 0.03	0.22 ± 0.03
	24 hr	0.23 ± 0.01	0.23 ± 0.02 [#]	0.25 ± 0.01 ^{##}
	48 hr	0.21 ± 0.02	0.22 ± 0.02 ^{##}	0.24 ± 0.02 ^{##}
	72 hr	0.21 ± 0.04	0.21 ± 0.03 ^{##}	0.21 ± 0.02 ^{##}
IP (mg/dl)	Before	5.75 ± 0.69 ^{##}	7.57 ± 0.97* ^{##}	7.22 ± 0.94* ^{##}
	24 hr	7.00 ± 0.49	7.90 ± 0.66* ^{##}	7.53 ± 0.50 ^{##}
	48 hr	7.09 ± 0.34 ^{##}	7.75 ± 0.39* ^{##}	7.34 ± 0.35 ^{##}
	72 hr	7.27 ± 0.60	7.52 ± 0.52	7.57 ± 0.65
TP (g/dl)	Before	6.86 ± 0.33 ^{##}	6.78 ± 0.46 [#]	6.83 ± 0.32
	24 hr	6.90 ± 0.20	6.86 ± 0.47	6.90 ± 0.42
	48 hr	6.79 ± 0.20 [#]	6.59 ± 0.24 ^{##}	6.85 ± 0.20
	72 hr	6.93 ± 0.37	6.91 ± 0.40	7.01 ± 0.29
ALB (g/dl)	Before	3.86 ± 0.23 [#]	3.78 ± 0.28	3.86 ± 0.31
	24 hr	4.09 ± 0.21	3.97 ± 0.36	4.01 ± 0.23
	48 hr	4.07 ± 0.22	3.91 ± 0.33	4.02 ± 0.30
	72 hr	4.23 ± 0.31	4.13 ± 0.38	4.18 ± 0.31

Data are expressed as mean ± S.D. (n = 8). Before, 24 hr before dosing; 24 hr, 24 hr after dosing; 48 hr, 48 hr after dosing; 72 hr, 72 hr after dosing; TPN, Total parenteral nutrition (fasted); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; Al-P, Alkaline phosphatase; T-Cho, Total cholesterol; TG, Triglyceride; BUN, Blood urea nitrogen; CRE, Creatinine; IP, Inorganic phosphorus; TP, Total protein; ALB, albumin. *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test). [#]p<0.05, ^{##}p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

1,25(OH)₂D₃-induced Ca action in the TPN rat.**Table 10.** Blood biochemistry in Experiment 2.

Items	Time	MAN solution treatment			
		Vehicle p.o.	1 µg/kg	10 µg/kg	100 µg/kg
AST (IU/l)	Before	108 ± 37	90 ± 10	93 ± 9	105 ± 17
	24 hr	92 ± 14	101 ± 20	112 ± 19	119 ± 22
	48 hr	95 ± 20	91 ± 11	103 ± 35	111 ± 22
	72 hr	103 ± 18	84 ± 8	91 ± 11	104 ± 20
ALT (IU/l)	Before	43 ± 4	39 ± 6	38 ± 3	45 ± 9
	24 hr	38 ± 4	36 ± 3	35 ± 2	46 ± 6*
	48 hr	41 ± 7	36 ± 3	35 ± 3	43 ± 13
	72 hr	42 ± 6	36 ± 3	36 ± 4	44 ± 9
Al-P (IU/l)	Before	931 ± 234	1014 ± 190	1025 ± 163	1067 ± 159
	24 hr	922 ± 219	840 ± 165	805 ± 172	973 ± 247
	48 hr	944 ± 241	909 ± 184	817 ± 138	901 ± 128
	72 hr	922 ± 224	890 ± 148	900 ± 176	1012 ± 128
Glucose (mg/dl)	Before	120 ± 14	132 ± 8	122 ± 11	127 ± 9
	24 hr	116 ± 7	123 ± 6	120 ± 5	121 ± 5
	48 hr	121 ± 5	129 ± 9	125 ± 10	124 ± 7
	72 hr	121 ± 11	129 ± 6	123 ± 8	124 ± 8
T-Chol (mg/dl)	Before	95 ± 10	90 ± 11	86 ± 8	97 ± 11
	24 hr	94 ± 11	87 ± 12	84 ± 6	97 ± 9
	48 hr	99 ± 12	89 ± 9	83 ± 8*	92 ± 7
	72 hr	97 ± 14	86 ± 6	81 ± 7*	94 ± 6
TG (mg/dl)	Before	44 ± 15	33 ± 9	47 ± 9	37 ± 17
	24 hr	66 ± 21	75 ± 36	83 ± 42	75 ± 14
	48 hr	82 ± 37	62 ± 25	82 ± 20	71 ± 27
	72 hr	75 ± 26	54 ± 12	102 ± 31	84 ± 20
BUN (mg/dl)	Before	15.0 ± 1.4	15.7 ± 1.3	14.5 ± 1.2	16.4 ± 2.5
	24 hr	17.6 ± 1.7	17.8 ± 2.4	18.6 ± 2.8	20.2 ± 2.3
	48 hr	17.8 ± 2.4	16.5 ± 1.7	17.7 ± 0.6	19.6 ± 1.9
	72 hr	17.0 ± 0.9	17.4 ± 2.4	17.6 ± 1.0	19.6 ± 2.8
CRE (mg/dl)	Before	0.22 ± 0.06	0.25 ± 0.03	0.23 ± 0.03	0.21 ± 0.03
	24 hr	0.25 ± 0.03	0.22 ± 0.04	0.22 ± 0.02	0.21 ± 0.06
	48 hr	0.21 ± 0.06	0.21 ± 0.02	0.24 ± 0.02	0.22 ± 0.02
	72 hr	0.20 ± 0.03	0.24 ± 0.03	0.24 ± 0.03*	0.24 ± 0.02
IP (mg/dl)	Before	7.89 ± 0.39	7.33 ± 0.40*	7.64 ± 0.32	8.17 ± 0.41
	24 hr	7.53 ± 0.30	8.46 ± 0.52**	8.98 ± 0.36**	8.72 ± 0.40**
	48 hr	7.60 ± 0.46	7.57 ± 0.32	8.07 ± 0.67	8.05 ± 0.45
	72 hr	7.78 ± 0.49	7.15 ± 0.39*	7.79 ± 0.36	7.90 ± 0.31
TP (g/dl)	Before	7.02 ± 0.51	6.99 ± 0.25	7.00 ± 0.38	7.39 ± 0.23
	24 hr	6.79 ± 0.39	7.03 ± 0.29	7.17 ± 0.25	7.71 ± 0.46**
	48 hr	7.07 ± 0.36	7.18 ± 0.30	6.92 ± 0.26	7.50 ± 0.41
	72 hr	7.13 ± 0.49	6.89 ± 0.39	6.79 ± 0.34	7.28 ± 0.12
ALB (g/dl)	Before	3.78 ± 0.25	3.83 ± 0.13	3.81 ± 0.25	3.84 ± 0.21
	24 hr	3.73 ± 0.28	3.80 ± 0.17	3.86 ± 0.31	3.84 ± 0.28
	48 hr	3.82 ± 0.31	3.89 ± 0.16	3.78 ± 0.16	3.77 ± 0.27
	72 hr	3.80 ± 0.40	3.78 ± 0.14	3.75 ± 0.17	3.73 ± 0.26

Data are expressed as mean ± S.D. (n = 6). Before, 24 hr before dosing; 24 hr, 24 hr after dosing; 48 hr, 48 hr after dosing; 72 hr, 72 hr after dosing; MAN, 5% D-mannitol (orally fed); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase, Al-P, Alkaline phosphatase; T-Chol, Total cholesterol; TG, Triglyceride; BUN, Blood urea nitrogen; CRE, Creatinine; IP, Inorganic phosphorus; TP, Total protein; ALB, albumin. *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test).

Table 10. (continued). Blood biochemistry in Experiment 2.

Items	Time	TPN solution treatment			
		Vehicle p.o.	1 µg/kg	10 µg/kg	100 µg/kg
AST (IU/l)	Before	90 ± 12	131 ± 19** #	105 ± 17	130 ± 28**
	24 hr	104 ± 22	104 ± 19	117 ± 33	104 ± 23
	48 hr	87 ± 8	95 ± 22	87 ± 18	88 ± 10 [#]
	72 hr	88 ± 10	99 ± 20	86 ± 14	88 ± 6
ALT (IU/l)	Before	32 ± 4 ^{##}	37 ± 8	36 ± 10	37 ± 5
	24 hr	29 ± 6 ^{##}	30 ± 7	32 ± 8	30 ± 7 ^{##}
	48 hr	27 ± 2 ^{##}	30 ± 8	31 ± 11	26 ± 5 [#]
	72 hr	29 ± 4 ^{##}	32 ± 10	29 ± 4 ^{##}	27 ± 4 ^{##}
Al-P (IU/l)	Before	411 ± 89 ^{##}	455 ± 51 ^{##}	441 ± 58 ^{##}	493 ± 71 ^{##}
	24 hr	450 ± 60 ^{##}	434 ± 101 ^{##}	433 ± 74 ^{##}	437 ± 137 ^{##}
	48 hr	415 ± 95 ^{##}	418 ± 92 ^{##}	371 ± 65 ^{##}	383 ± 107 ^{##}
	72 hr	392 ± 79 ^{##}	440 ± 86 ^{##}	404 ± 82 ^{##}	390 ± 80 ^{##}
Glucose (mg/dl)	Before	168 ± 13 ^{##}	137 ± 44	123 ± 24	189 ± 42 [#]
	24 hr	122 ± 7	117 ± 9	117 ± 8	116 ± 5
	48 hr	136 ± 10 ^{##}	122 ± 16	130 ± 11	128 ± 7
	72 hr	140 ± 17 [#]	123 ± 21	129 ± 12	121 ± 8
T-Cho (mg/dl)	Before	81 ± 17	92 ± 16	95 ± 15	91 ± 5
	24 hr	76 ± 11 [#]	82 ± 16	88 ± 17	83 ± 10 [#]
	48 hr	83 ± 13	79 ± 13	87 ± 16	77 ± 7 ^{##}
	72 hr	78 ± 11 [#]	76 ± 12	85 ± 17	71 ± 6 ^{##}
TG (mg/dl)	Before	19 ± 6 ^{##}	22 ± 6 [#]	26 ± 11 ^{##}	20 ± 6
	24 hr	35 ± 15 [#]	29 ± 7 [#]	44 ± 15	44 ± 9 ^{##}
	48 hr	39 ± 12 [#]	35 ± 8 [#]	43 ± 13 ^{##}	42 ± 14 [#]
	72 hr	40 ± 10 [#]	37 ± 12 [#]	52 ± 25 [#]	38 ± 6 ^{##}
BUN (mg/dl)	Before	11.9 ± 1.2 ^{##}	13.9 ± 1.5 [#]	15.1 ± 1.0 ^{**}	13.6 ± 1.7 [#]
	24 hr	11.1 ± 0.8 ^{##}	11.5 ± 1.1 ^{##}	13.5 ± 2.3 ^{* #}	11.9 ± 1.3 ^{##}
	48 hr	11.4 ± 0.9 ^{##}	11.6 ± 1.4 ^{##}	12.8 ± 2.7 ^{##}	11.7 ± 1.6 ^{##}
	72 hr	11.8 ± 0.8 ^{##}	11.8 ± 1.1 ^{##}	12.6 ± 2.4 ^{##}	11.4 ± 2.2 ^{##}
CRE (mg/dl)	Before	0.21 ± 0.02	0.22 ± 0.04	0.24 ± 0.03	0.19 ± 0.03
	24 hr	0.15 ± 0.04 ^{##}	0.17 ± 0.04	0.15 ± 0.05 [#]	0.17 ± 0.02
	48 hr	0.20 ± 0.03	0.19 ± 0.02	0.20 ± 0.02 [#]	0.21 ± 0.03
	72 hr	0.19 ± 0.02	0.19 ± 0.02 [#]	0.20 ± 0.01 [#]	0.19 ± 0.01 ^{##}
IP (mg/dl)	Before	6.23 ± 0.43 ^{##}	6.49 ± 0.56 ^{* #}	5.99 ± 0.70 ^{** #}	5.72 ± 0.55 ^{** #}
	24 hr	7.23 ± 0.38	7.83 ± 0.29 [#]	8.61 ± 0.25 ^{**}	8.44 ± 0.49 ^{**}
	48 hr	7.72 ± 0.12	7.27 ± 0.23	7.96 ± 0.46	7.89 ± 0.61
	72 hr	7.54 ± 0.21	7.01 ± 0.69	7.02 ± 0.45 ^{** #}	7.23 ± 0.32 ^{** #}
TP (g/dl)	Before	6.84 ± 0.20	7.19 ± 0.33 [*]	7.04 ± 0.25	7.03 ± 0.14
	24 hr	6.84 ± 0.20	7.05 ± 0.38	7.58 ± 0.35 [#]	7.13 ± 0.27 [#]
	48 hr	7.30 ± 0.42	7.08 ± 0.26	7.10 ± 0.22	6.82 ± 0.34 ^{* #}
	72 hr	7.31 ± 0.26	7.24 ± 0.20	7.10 ± 0.39	6.68 ± 0.14 ^{** #}
ALB (g/dl)	Before	3.71 ± 0.33	3.89 ± 0.27	3.90 ± 0.40	3.82 ± 0.32
	24 hr	3.91 ± 0.33	3.90 ± 0.49	4.15 ± 0.40	4.09 ± 0.26
	48 hr	4.06 ± 0.28	3.98 ± 0.52	4.03 ± 0.40	4.05 ± 0.33
	72 hr	4.04 ± 0.30	4.07 ± 0.50	4.10 ± 0.49	4.03 ± 0.18 [#]

Data are expressed as mean ± S.D. (n = 6). Before, 24 hr before dosing; 24 hr, 24 hr after dosing; 48 hr, 48 hr after dosing; 72 hr, 72 hr after dosing; TPN, Total parenteral nutrition (fasted); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase, Al-P, Alkaline phosphatase; T-Cho, Total cholesterol; TG, Triglyceride; BUN, Blood urea nitrogen; CRE, Creatinine; IP, Inorganic phosphorus; TP, Total protein; ALB, albumin. *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test). #p<0.05, ##p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

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excretion or serum Ca concentration between MAN solution treatment rats and TPN solution treatment rats expresses the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action in the conditions of the study.

The idea of controlling the Ca by diet is not new. However, as for an approach that analyzes the contri-

bution of the intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action using the TPN solution treatment rat, our present study is the first to examine this. Similar to the present study in rats, Tsuruoka *et al.* (2000) reported the diurnal variation of the serum Ca concentration and urinary Ca excretion in rats. This report evaluated the intestinal Ca absorption *in vivo* in which

Fig. 3. Serum osteocalcin (BGP) concentration at 24 hr and urinary dioxypyridinoline (Dpd) concentration during 0-24 hr following a single dosing of 1,25-dihydroxyvitamin D₃ (100 µg/kg p.o. or 25 µg/kg i.v.) or vehicle (medium chain triglyceride) for MAN (orally fed) or TPN (fasted) solution treatment to rats. Data are expressed as mean ± S.D. (n = 8). *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test). ##p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

Fig. 4. Serum osteocalcin (BGP) concentration at 24 hr, serum parathyroid hormone (PTH) concentration at 24 hr and urinary dioxypyridinoline (Dpd) concentration during 0-24 hr following a single dosing of 1,25-dihydroxyvitamin D₃ (1, 10 or 100 µg/kg p.o.) or vehicle (medium chain triglyceride) at MAN (orally fed) or TPN (fasted) solution treatment to rats. Data are expressed as mean $\pm$ S.D. (n = 6). *p<0.05, **p<0.01, Significant difference from the vehicle group (Dunnett's test). ##p<0.01, Significant difference from the MAN solution treatment (Student's or Aspin-Welch's *t*-test).

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diurnal conditions caused the differences in the fed condition like the freely fed or fasted. However, the change of urinary Ca excretion by 1,25(OH)₂D₃ was not significantly different between morning and evening under the freely fed condition. These findings reported that glomerular filtration rate is higher in the evening and the total filtered load of Ca is similar. Thus, there is no report which clarified the contribution of the intestinal Ca absorption to the 1,25(OH)₂D₃-induced Ca action *in vivo* in addition to the report of Tsuruoka *et al.* (2000). In order to determine the differences between MAN solution treatment and TPN solution treatment it is necessary to eliminate the factor of feeding. This can be investigated by administering the TPN solutions to the rats intravenously via continuous infusion. Therefore, we tried to evaluate the contribution to the intestinal Ca absorption following 1,25(OH)₂D₃ dosing in rats.

Under this study condition, the clear differences in the urinary Ca excretion can be attributed to the differences in treatment of MAN solution and TPN solution in Experiments 1 and 2. Alternatively, the decrease in the urinary Ca excretion at TPN solution treatment may be related to the intestinal Ca absorption-mediated 1,25(OH)₂D₃ dosing. It might be that the TPN solution treatment affects the intestinal Ca absorption by 1,25(OH)₂D₃ dosing. In Experiment 1, the differences in the serum Ca concentration can be attributed to the differences in the treatment of MAN solution and TPN solution. In Experiment 2, however, the differences in the serum Ca concentration were only observed in the 1,25(OH)₂D₃ 100-μg/kg group in the MAN solution or TPN solution treatment. These findings indicated that the dose of 1,25(OH)₂D₃ 1 and 10 μg/kg did not increase or slightly increased the serum Ca concentration in rats. In both Experiments 1 and 2, we measured the urinary IP excretion and serum IP concentration. We found that urinary IP excretion and serum IP concentration varied with MAN solution and TPN solution treatment during the experiment period.

The decrease in the serum PTH concentration in Experiment 2 is considered to be caused by the increase in serum Ca and mediated via the parathyroid gland by a negative feedback mechanism (Habener and Potts, 1990). Alternatively, the decrease in serum PTH concentration may be mediated by the 1,25(OH)₂D₃ through PTH gene transcription (Silver *et al.*, 1986; Naveh-Manly *et al.*, 1990). We found a decrease in serum PTH concentration in all the 1,25(OH)₂D₃ dosing groups for the MAN solution and TPN solution treatment, reflecting the increase in serum Ca concen-

tration of 1,25(OH)₂D₃ dosing.

We measured the bone-related parameters such as urinary Dpd and serum BGP concentrations. The urinary Dpd is one of the bone resorption markers, and serum BGP is one of the bone formation markers. The increase in serum BGP concentration in Experiments 1 and 2 is considered to be caused by the bone formation effect of 1,25(OH)₂D₃ (Nakane *et al.*, 2006). These findings in the serum BGP concentration can be attributed to the pharmacological action of 1,25(OH)₂D₃. 1,25(OH)₂D₃-induced Ca action was found in the TPN solution treatment rats, serum BGP concentrations were increased and no changes in urinary Dpd concentration were noted in the TPN solution treatment rats.

Dpd is released from bones with collagen decomposition and excreted in urine (Takahashi, 1995). Regarding the response of the urinary Dpd in rats, Dpd produced a marked continuous increase from one week to five weeks after dosing, whereas serum Ca and BGP produced a marked increase in one or 3 week (its peak) (Kirino *et al.*, 2004). These changes in Dpd reflect the high constancy of Dpd, and it was considered the marker that needs time in order to reflect change of bone resorption (Tsuruoka *et al.*, 2002). These suggest that no changes in urinary Dpd were observed since the urine sample during 0-24 hr following 1,25(OH)₂D₃ dosing were used in the present study. Furthermore, no changes in urinary Dpd could be attributed to the suggestion that the change of urinary Dpd may disappear by high values due to young rat usage in the present study. Therefore, 1,25(OH)₂D₃-induced Ca action in the TPN solution treatment rats was considered to be caused by Ca mobilization originated from bone resorption since the toxic dose of 1,25(OH)₂D₃ was administered.

In addition, the point for consideration as Ca mobilization origin in the TPN solution treatment rats is as follows. The Ca in TPN solution may be delivered into the small intestine via biliary excretion, where as the Ca could be absorbed intensively from the small intestine. Although many studies have been reported about biliary Ca, biliary Ca excretion is not fully understood. However, since bone Ca deposition rate is highest in the newborn infant (Bronner and Pansu, 1999), it is considered that most absorbed Ca shifts to the bone in the young rat. Ahrendt *et al.* (1995) suggest that hypercalcemia decreased bile flow. Therefore, since it is considered that there is little biliary Ca excretion, we hypothesize that there is little participation to Ca mobilization via biliary Ca excretion in the TPN solution treatment rats.

Since decrease rates in change of urinary Ca excretion in the TPN solution treatment rats were 36.3% (100 µg/kg p.o.) or 47.1% (25 µg/kg i.v.) in Experiment 1 and 29.0% (1 µg/kg), 56.2% (10 µg/kg) or 35.3% (100 µg/kg) in Experiment 2. From the data of urinary Ca excretion, we hypothesize that the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action may be considered to be 63.7% (100 µg/kg p.o.) or 52.9% (25 µg/kg i.v.) in Experiment 1, and 71.0% (1 µg/kg), 43.8% (10 µg/kg) or 64.7% (100 µg/kg) in Experiment 2. However, we have underestimated the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action, and we considered that urinary Ca excretion may actually be lower in the TPN solution treatment rats. Before this study, we expected that the 1,25(OH)₂D₃-induced Ca action disappeared in the TPN solution treatment rats. In fact, the condition of low Ca diet in the small intestine is a known stimulant for active intestinal Ca absorption, and this is considered to be mediated through an increase in 1,25(OH)₂D₃ production (Lee *et al.*, 1990). To monitor Ccr, it is important to assure the renal function in MAN or TPN solution treatment rats. Ccr in TPN solution treatment rats was higher than that of the MAN solution treatment rats in all groups. TPN solution treatment rats showed a decrease in urinary Ca excretion compared to MAN solution treatment rats. It was considered that TPN solution treatment rats were in a state to easily discharge Ca from the inside of the body via the kidney since the Ccr showed a high value in the TPN solution treatment rats. Therefore, we considered that results in the present study may underestimate the contribution of intestinal Ca absorption to 1,25(OH)₂D₃-induced Ca action since the 1,25(OH)₂D₃-induced Ca action was found in the TPN solution treatment rats.

Decreases in body weights of TPN solution treatment rats were recorded on Day 3 at 1,25(OH)₂D₃ 25-µg/kg i.v. group in MAN solution treatment rats of Experiment 1 (Table 3). This finding is considered to be caused by hypercalcemia of 1,25(OH)₂D₃ dosing. Since this group had serum Ca concentrations that were 15.53 ± 0.29 (48 hr) and 15.14 ± 0.50 (72 hr) mg/dl, the rats may be in a severe clinical condition. However, no abnormality in this group was observed in the clinical signs. As shown in the blood biochemistry results (Tables 9 and 10), blood biochemistry results did not vary with MAN and TPN solution treatment rats, which is similar to the our background data in orally fed rats and TPN solution treatment rats (Asanuma *et al.*, 2006; Mochida *et al.*, 1991;

Yamauchi and Kokuba, 1999).

In conclusion, we found differences of urinary Ca excretion or serum Ca concentration following 1,25(OH)₂D₃ dosing in MAN or TPN solution treatment rats. In particular, our investigations clearly demonstrate that decrease rates in change of the urinary Ca excretion at TPN solution treatment rats were 36.3% (100 µg/kg p.o.) or 47.1% (25 µg/kg i.v.) in Experiment 1, and 29.0% (1 µg/kg), 56.2% (10 µg/kg) or 35.3% (100 µg/kg) in Experiment 2. These findings may be related to differences of the intestinal Ca absorption in the conditions of the study. We hypothesize that the contribution to intestinal Ca absorption to the 1,25(OH)₂D₃-induced Ca action in rats may be considered to be 63.7% (100 µg/kg p.o.) or 52.9% (25 µg/kg i.v.) in Experiment 1 and to be 71.0% (1 µg/kg), 43.8% (10 µg/kg) or 64.7% (100 µg/kg) in Experiment 2. These observations may be helpful for better understanding of the mechanisms of 1,25(OH)₂D₃-induced Ca action in toxicological research. Further studies are required to determine this by using small bowel resection in rats, and may be useful to explain the contribution of intestinal Ca absorption to the 1,25(OH)₂D₃-induced Ca action in rats.

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