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1,25-Dihydroxyvitamin D_3-induced intestinal calcium transport is impaired in β-globin knockout thalassemic mice

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Besides being a common haematological disorder caused by a reduction in β-globin production, β-thalassemia has been reported to impair body calcium homeostasis, leading to massive bone loss and increased fracture risk. Here, we demonstrated that heterozygous β-globin knockout thalassemic mice had a lower rate of duodenal calcium absorption compared with the wild-type littermates, whereas the epithelial electrical parameters, including transepithelial resistance, were not affected, suggesting no change in the epithelial integrity and permeability. Daily subcutaneous injection of 1 $\mu g\,kg^{-1}$ 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] for 3 days enhanced the duodenal calcium absorption in wild-type, but not in thalassemic mice. Although β-thalassemia increased the mRNA level of divalent metal transporter-1, an iron transporter in the duodenum, it had no effect on the transcripts of ferroportin-1 or the principal calcium transporters. In conclusion, β-thalassemia impaired the 1,25$(OH)_2D_3$-dependent intestinal calcium absorption at the post-transcriptional level, which, in turn, contributed to the dysregulation of body calcium metabolism and β-thalassemia-induced osteopenia. Copyright © 2013 John Wiley & Sons, Ltd.

KEY WORDS—divalent metal transporter (DMT)-1; duodenum; thalassemia; Ussing chamber; vitamin D

INTRODUCTION

The globin is a polypeptide component of haemoglobin, which is important for the transport of oxygen in the blood. A decrease in or an absence of β-globin production in erythrocytes is observed in an autosomal recessive anaemic disorder known as β-thalassemia.[1] β-thalassemic patients manifest a number of serious complications, such as the iron overload-induced damage of heart and liver, increased risk of infection, enlarged spleen (splenomegaly), cardiac arrhythmia, growth retardation, osteoporosis and fracture.[1] We recently demonstrated in β-thalassemic mice carrying a heterozygous C→T mutation at nucleotide 654 of the intron 2 ($\beta^{+/IVSII\text{-}654}$ knockin) that β-thalassemia resulted in a decreased matrix mineralization and an enhanced osteoclast-mediated bone resorption, thereby leading to widespread osteopenia especially at the trabecular sites, such as tibial metaphysis and lumbar vertebrae.[2] Although the enhanced bone resorption in thalassemic mice could be explained by the thalassemia-induced increases in the circulating osteoclastogenic cytokines, such as interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α,[3–5] the underlying mechanism responsible for the decreases in bone matrix mineralization and mineral apposition rate has not been elucidated. Because the only source of calcium for bone mineralization is dietary calcium that is absorbed by the small intestine,[6,7] we hypothesized that the β-thalassemic mice might have a lower rate of intestinal calcium absorption as compared with the wild-type littermates.

In humans and rodents, the duodenum—the proximal part of the small intestine—is an important site of calcium and iron absorption.[7,8] The transcellular calcium transport in the duodenum is a three-step 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]-dependent process consisting of (i) apical calcium entry via the transient receptor potential vanilloid Ca^{2+} channels (TRPV)-5 and (TRPV)-6, (ii) facilitated cytoplasmic diffusion in a calbindin-D_{9k}-bound form, and (iii) basolateral extrusion by the plasma membrane Ca^{2+}-ATPase (PMCA)-1b.[7,9] In wild-type mice, administration of calciotropic hormone 1,25$(OH)_2D_3$ markedly stimulates the duodenal calcium transport, in part, by increasing the expression and activity of TRPV5, TRPV6, calbindin-D_{9k} and $PMCA_{1b}$.[6,10] In addition, the duodenal epithelial cells also express the apical and basolateral iron transporters, namely divalent metal transporter (DMT)-1 and ferroportin-1, respectively, both of which are responsible for the enhanced iron absorption in β-thalassemia.[11–13]

Therefore, the main objectives of the present study were to investigate whether the duodenal calcium transport rate in thalassemic mice was lower than that of the wild-type

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littermates, and whether there was an impairment of the 1,25 $(OH)_2D_3$-induced duodenal calcium transport in thalassemic mice. Because the adaptive changes in the intestinal calcium absorption, such as after 1,25$(OH)_2D_3$ administration, are known to be sex-dependent,[14] the present calcium transport study was performed in both male and female mice.

MATERIALS AND METHODS

Animals

Heterozygous $\beta^{IVSII-654}$ knockin ($\beta^{+/IVSII-654}$) and β-globin knockout ($\beta^{+/th3}$; BKO) thalassemic mice[15,16] and their age-matched wild-type littermates (*Mus musculus* C57BL/6 strain; 7-week-old, weighing 20–30 g) were obtained from the National Laboratory Animal Centre, Thailand. The genotype of each mouse was verified from the tail DNA. Because the homozygous genotype was lethal, heterozygous mice were used in the present study. Both $\beta^{+/IVSII-654}$ knockin and BKO mice had a phenotype of β-thalassemia intermedia, which was consistent with the previous report.[2,15,16] The animals were placed in polystylene shoebox cages, and acclimatized in the vivarium with room temperature of 22–25 °C and humidity of ~55% for 7 days under 12/12 h dark/light cycle. They were fed regular chow containing 1.0% calcium and 0.9% phosphorus, and reverse osmosis (RO) water *ad libitum*. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University.

Experimental design

Body weights were recorded before sample collections. In the first series of experiments, total RNA samples were collected from the duodenal epithelial cells of $\beta^{+/IVSII-654}$ knockin and wild-type mice to quantify the mRNA levels of important genes related to intestinal iron transport (i.e. DMT1 and ferroportin-1), calcium transport (i.e. TRPV5, TRPV6, calbindin-D_{9k} and $PMCA_{1b}$) and nuclear vitamin D receptor (VDR). To determine whether 1,25$(OH)_2D_3$ altered the expression of calcium transporter genes, male and female $\beta^{+/IVSII-654}$ knockin thalassemic mice and the wild-type littermates were subjected to a single-dose subcutaneous (s.c.) injection of 2 μg kg^{-1} 1,25$(OH)_2D_3$ (catalogue no. 71820; Cayman Chemical, Ann Arbor, MI, USA), and the total RNA specimens were collected from the duodenal epithelial cells at 6 h post-injection for quantitative real-time polymerase chain reaction (qRT-PCR) study.[10] This high-dose 1,25$(OH)_2D_3$ injection regimen has been reported to upregulate duodenal calcium transporter gene expression in wild-type mice within 6 h post-injection, and the 1,25$(OH)_2D_3$-enhanced calcium transport could be observed within 72 h post-injection.[10] To investigate the effect of 1,25$(OH)_2D_3$ on the duodenal calcium transport (Figure 1), BKO thalassemic mice and the wild-type littermates were injected with 1 μg kg^{-1} 1,25$(OH)_2D_3$ s.c., or 3 ml kg^{-1} 9:1 propylene glycol–ethanol s.c. (vehicle) at 72, 48 and 24 h prior to determination of duodenal calcium flux and the epithelial electrical properties by Ussing chamber technique.[10]

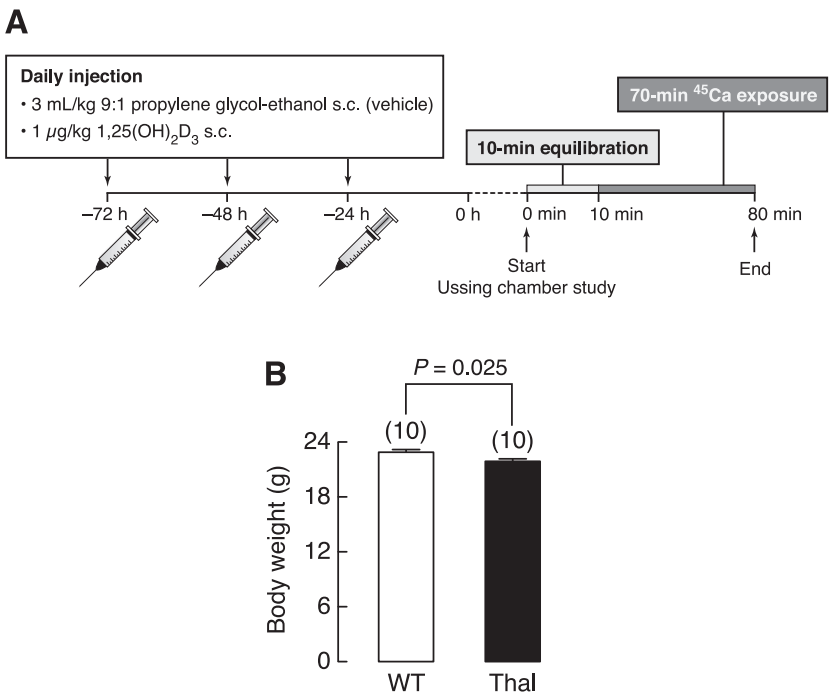

Figure 1. (A) A diagram showing the experimental design. For more details, please see Materials and Methods. (B) Body weights of 8-week-old male BKO thalassemic (Thal) and wild-type (WT) mice. Numbers in parentheses represent the numbers of animals

Sample collections

Mice were anesthetized by intraperitoneal injection of 70 mg kg^{-1} sodium pentobarbitone (Abbott Laboratory, North Chicago, IL, USA). Thereafter, 1.5-cm median laparotomy was performed, and the duodenal segment (0–5 cm from the pylorus) was removed and cut longitudinally to expose the mucosal surface. The duodenal tissue was finally mounted between the two halves of Ussing chamber. In the qRT-PCR study, duodenal mucosa was scraped with a cold glass slide to harvest the epithelial cells.[17] Peripheral blood smear and spleens were also examined for the presence of hypochromic microcytic anaemia and splenomegaly, respectively.

Measurement of epithelial electrical properties

The epithelial electrical parameters were transepithelial potential difference (PD; the apical side was negative), short circuit current (Isc), and transepithelial resistance (TER). PD and Isc indicated the electrogenic ion transport across the epithelium, whereas TER roughly represented the epithelial integrity and permeability. As described previously,[18] a duodenal tissue was mounted in Ussing chamber filled on both sides with solution containing (in mmol l^{-1}) 118 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, 2.5 L-glutamine and 2 mannitol (all purchased from Sigma) at 37 °C. The solution was continuously aerated with humidified 5% CO_2 in 95% O_2 to maintain a constant partial pressure of CO_2 and pH 7.4. Osmolality was ~291 mmol kg^{-1} H_2O as measured by an osmometer (model 3320; Advanced Instruments, Norwood, MA, USA). RO water used in this experiment had electrical resistance higher than 18.3 MΩ cm and free-ionized calcium less than 2.5 nmol l^{-1}. In Ussing chamber setup, two pairs of salt bridges made of 2 mol l^{-1} KCl and 2% wt/vol agar connected Ag/AgCl half-cells (World Precision Instrument, Sarasota, FL, USA) with the Ussing chamber and served as electrodes for measuring PD or passing electrical current. Each Ag/AgCl half-cell was connected to a preamplifier or direct current-generating unit (model ECV-4000; World Precision Instrument) and, finally, to PowerLab/4SP (operated with Chart version 5.4.1; ADInstruments, Colorado Springs, CO, USA) for digital recording. TER was calculated from PD and Isc by Ohm's equation.

Measurement of transepithelial calcium flux

As described previously,[10] the tissue was first equilibrated in the chamber with physiological bathing solution for 10 min. Thereafter, the apical chamber was filled with bathing solution containing radioactive ^{45}Ca (0.45 μCi ml^{-1}; final specific activity of 360 mCi mol^{-1}; catalogue no. NEZ013; PerkinElmer, Boston, MA, USA). Unidirectional calcium flux ($J_{H\rightarrow C}$; nmol h^{-1} cm^{-2}) from the hot side (H; apical side) to the cold side (C; basolateral side) was calculated by Equations (1) and (2).

$$J_{H\rightarrow C} = R_{H\rightarrow C}/(S_H \times A) \quad (1)$$

$$S_H = C_H/C_{To} \quad (2)$$

where $R_{H\rightarrow C}$ is the rate of tracer appearance in the cold side (cpm h^{-1}), S_H is the specific activity in the hot side (cpm $nmol^{-1}$), A is the surface area (cm^2), C_H is the mean radioactivity in the hot side (cpm) and C_{To} is the total calcium content in the hot side (nmol).

^{45}Ca radioactivity was analysed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT, USA). Total calcium concentration in the bathing solution was analysed by an atomic absorption spectrophotometer (model SpectrAA-300; Varian Techtron, Springvale, Australia). In the absence of transepithelial calcium gradient (i.e. bathing solution in both hemi-chambers contained the same calcium concentration of 1.25 mmol l^{-1}), the measured transepithelial calcium flux represented the active calcium transport.[18]

Quantitative real-time PCR

The PCR technique was modified from the method of Charoenphandhu *et al.*[18] Briefly, total RNA was extracted from duodenal scrapings by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed with oligo-dT_{20} primer and iScript kit (Bio-rad, Hercules, CA, USA). Real-time PCR was performed by Bio-rad MiniOpticon PCR system with SsoFast EvaGreen Supermix (Bio-rad). Primers used in the present study are listed in Table 1.[19] Glyceraldehyde-3-phosphate dehydrogenase served as a housekeeping gene for normalization. Relative mRNA level was calculated from the threshold cycle (C_T) by $2^{-\Delta C_T}$ method. Melting curve analysis and amplicon sequencing confirmed the presence of specific PCR products. qRT-PCR experiments were performed in triplicate.

Statistical analysis

Results are expressed as means ± SE. The data were analysed by unpaired Student's *t*-test or Mann–Whitney test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The differences were considered significant when *P*-values were less than 0.05.

RESULTS

All β-thalassemic mice manifested hypochromic microcytic anaemia and splenomegaly, similar to that reported previously.[2] Body weights of BKO mice were significantly lower than those of the age-matched wild-type mice (Figure 1(B)). Although there was a twofold increase in the transcripts of DMT1—the principal iron transporter in the duodenal apical membrane[13]—in male thalassemic mice as compared with the age-matched wild-type mice (Figure 2), the expressions of ferroportin-1, calcium transporters (i.e. TRPV5, TRPV6, calbindin-D_{9k} and $PMCA_{1b}$) and VDR were not altered (Figures 2 and 3).

Table 1. *Mus musculus* primers used in the qRT-PCR experiments

Gene name (abbreviated name)	Accession no.	Primer (forward/reverse)	Product size (bp)
Iron transport			
Divalent metal transporter-1 (DMT-1)	NM_001146161.1	5′-TGTTTGATTGCATTGGGTCTG-3′ 5′-CGCTCAGCAGGACTTTCGAG-3′	73
Ferroportin-1	NM_016917.2	5′-CTACCATTAGAAGGATTGACCAGCTA-3′ 5′-ACTGGAGAACCAAATGTCATAATCTG-3′	82
Calcium transport			
Transient receptor potential vanilloid family Ca^{2+} channel-5 (TRPV5)	NM_001007572.2	5′-TGCTACTGTCCTATGATGGAGG-3′ 5′-GGAGTCAATCTCTGTGAGGTCA-3′	200
Transient receptor potential vanilloid family Ca^{2+} channel-6 (TRPV6)	NM_022413.4	5′-ACATAGCTCCTGCTCACTC-3′ 5′-TCTCCTACTTCACCTGTGG-3′	150
Calbindin-D_{9k}	AF_136283.1	5′-TGCTGTTCCTGTCTGACTCCT-3′ 5′-GGGGAACTCTGACTGAATCAG-3′	180
Plasma membrane Ca^{2+}-ATPase-1b ($PMCA_{1b}$)	NM_026482.2	5′-AATCCTGAGTGCTAATGGCG-3′ 5′-CGGTGACGACATCATTCTCA-3′	179
Vitamin D receptor			
Nuclear vitamin D receptor (VDR)	NM_009504.4	5′-CTGCTCCTTCAGGGATGGA-3′ 5′-AGCGTTGAAGTGGAAGCCC-3′	128
Housekeeping gene			
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_008084.2	5′-TTCCAGTATGACTCCACTCACG-3′ 5′-AAGACACCAGTAGACTCCACGA-3′	167

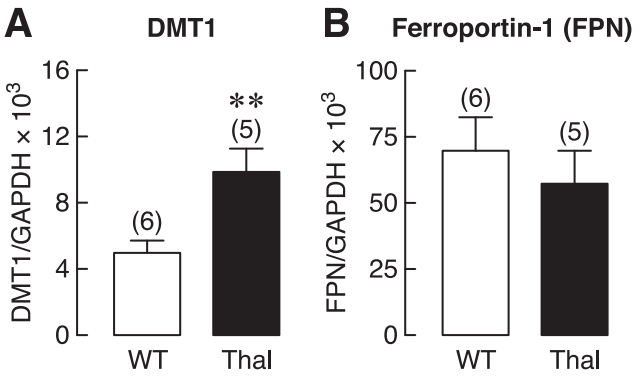

Figure 2. Expression of iron transporter genes (A) DMT1 and (B) ferroportin-1 (FPN) in the duodenal epithelial cells of 8-week-old male $\beta^{+/IVSII-654}$ knockin thalassemic (Thal) and wild-type (WT) mice, as determined by qRT-PCR. The mRNA level was normalized by GAPDH transcript. Numbers in parentheses represent the numbers of animals. $^{**}P < 0.01$ compared with WT group (Mann–Whitney test)

Interestingly, as shown in Figures 4 and 5, the transepithelial calcium transport in male and female vehicle-treated thalassemic mice was lower than that in their wild-type littermates by ~40%, indicating that β-thalassemia impaired duodenal calcium absorption despite no change in calcium transporter gene expression. β-Thalassemia had no effect on the duodenal electrical parameters, i.e., PD, Isc and TER, in both male and female mice (Table 2), suggesting no change in the epithelial integrity and permeability. After 3 days of daily subcutaneous injection of $1\,\mu g\,kg^{-1}$ $1,25(OH)_2D_3$, the transepithelial calcium transport in male and female wild-type mice was significantly increased by ~60% (Figures 4 and 5). However, $1,25(OH)_2D_3$ administration could not enhance the duodenal calcium transport in male and female thalassemic mice (Figures 4 and 5), whereas the mRNA levels of TRPV5, TRPV6, calbindin-D_{9k} and $PMCA_{1b}$ in $1,25(OH)_2D_3$-treated thalassemic mice were comparable with those of $1,25(OH)_2D_3$-treated wild-type mice (data not shown).

DISCUSSION

How β-thalassemia induces bone loss, osteoporosis and fracture is not well understood. Our recent investigation in $\beta^{+/IVSII-654}$ knockin thalassemic mice showed that β-thalassemia enhanced the osteoclast activity and bone resorption,[20] presumably by inducing bone marrow expansion and excessive production of osteoclastogenic cytokines, such as receptor activator of nuclear factor-κB ligand (RANKL), IL-1, IL-6 and TNF-α.[3–5,21] Nevertheless, the osteoclast-mediated bone resorption could not fully explain the β-thalassemia-induced osteoporosis because thalassemic mice also exhibited decreases in mineral apposition rate, mineralizing surface and mineralized volume in tibial metaphysis.[2] It was thus possible that β-thalassemia might also reduce the intestinal calcium supply for bone formation.

The proximal small intestine is the important site of $1,25(OH)_2D_3$-dependent calcium absorption.[7] In the present study, we demonstrated experimentally that β-thalassemia markedly suppressed the duodenal calcium transport in both male and female mice. A previous investigation in homozygous β-thalassemic patients subjected to oral administration of radioactive ^{47}Ca also showed that serum concentration of ^{47}Ca was lower, whereas fecal ^{47}Ca excretion was higher than those in the control group.[22] Although intestinal calcium transport was not directly determined in this ^{47}Ca study, the results suggested that homozygous β-thalassemic patients had calcium malabsorption, similar to that observed in mice with β-thalassemia intermedia.

Interestingly, despite having a decrease in duodenal calcium transport, the mRNA levels of VDR and major calcium transporters, namely TRPV5, TRPV6, calbindin-D_{9k}

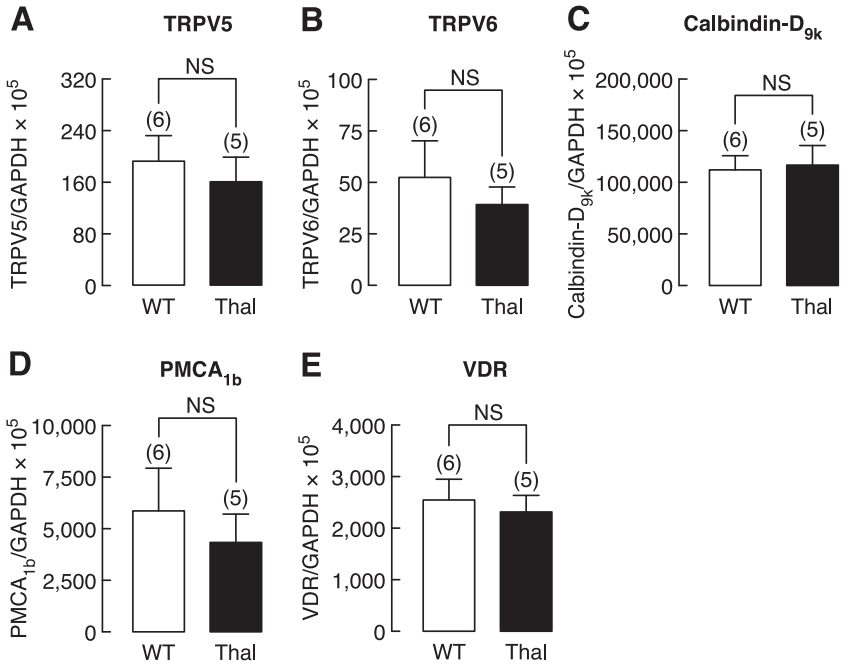

Figure 3. Expression of calcium transporter genes (A) TRPV5, (B) TRPV6, (C) calbindin-D_{9k}, (D) $PMCA_{1b}$ and (E) VDR in the duodenal epithelial cells of 8-week-old male $\beta^{+/IVSII-654}$ knockin thalassemic (Thal) and wild-type (WT) mice, as determined by qRT-PCR. The mRNA level was normalized by GAPDH transcript. Numbers in parentheses represent the numbers of animals. NS, not significant (Mann–Whitney test)

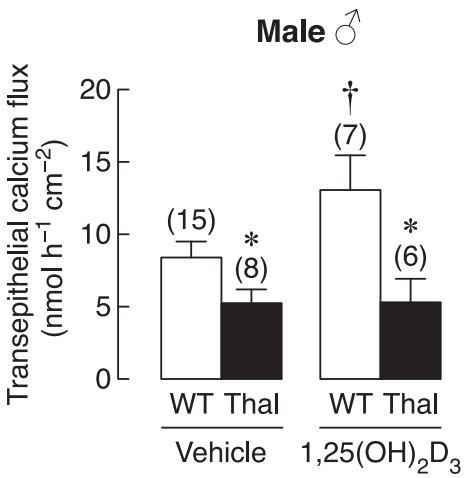

Figure 4. Transepithelial calcium transport in 8-week-old male BKO thalassemic (Thal) and wild-type (WT) mice. The vehicle-treated mice were injected with 3 ml kg^{-1} 9:1 propylene glycol–ethanol [for $1,25(OH)_2D_3$ preparation], whereas $1,25(OH)_2D_3$-treated mice were injected with 1 μg kg^{-1} day^{-1} $1,25(OH)_2D_3$ s.c. for 3 days. Thereafter, the duodenal tissue was mounted in Ussing chamber, equilibrated in physiological bathing solution for 10 min and then subjected to a 70-min calcium flux measurement. Numbers in parentheses represent the numbers of experimental animals. $^*P < 0.05$ compared with the corresponding WT group (Student's *t*-test). $^\dagger P < 0.05$ compared with the vehicle-treated WT group

and $PMCA_{1b}$, in thalassemic mice were comparable with those of wild-type littermates. Moreover, the mRNA levels of these calcium transporters were also comparable in both 1,25 $(OH)_2D_3$-treated wild-type and thalassemic mice. Therefore, the underlying mechanism by which β-thalassemia-induced calcium malabsorption should have occurred at the post-transcriptional level. Muscher *et al*. have also recently suggested a post-transcriptional regulation of TRPV6 in goats fed a reduced nitrogen and calcium diet.[23] Because there was an inverse relationship between intestinal calcium and iron absorption,[24] the enhanced intestinal iron absorption that was commonly observed in β-thalassemic mice,[12] presumably because of the thalassemia-induced upregulation of DMT1 transcription (Figure 2(A)), could partly explain the decrease in the duodenal calcium transport. The cellular mechanism of the iron hyperabsorption-induced suppression of intestinal calcium absorption remains elusive; however, the elevated intracellular iron concentration has been known to increase reactive oxygen species, which can reduce the PMCA activity.[25,26] In addition, growth retardation as indicated by relatively low body weights (Figure 1(B)) might contribute to a decrease in calcium transport in thalassemic mice since 4- and 6-week-old growth-retarded rats manifested a suppression of intestinal calcium absorption.[27]

Furthermore, intestinal malabsorption of vitamin D and systemic disturbances of calcium-regulating hormones, such as low circulating levels of parathyroid hormone, 25-hydroxyvitamin D and insulin-like growth factor-1, might partly contribute to the thalassemia-induced decrease in calcium absorption.[28–32] Although β-thalassemia did not alter the mRNA levels of VDR, low plasma levels of calciotropic hormones may decrease responsiveness of the duodenal epithelial cells of thalassemic mice to negative calcium balance, but further investigation is required to demonstrate the detailed cellular and molecular mechanisms.

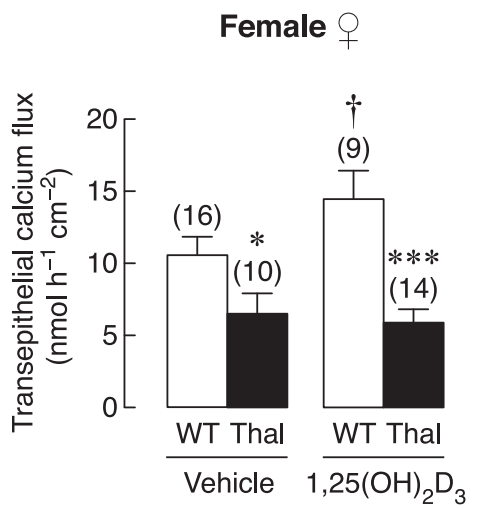

Figure 5. Transepithelial calcium transport in 8-week-old female BKO thalassemic (Thal) and wild-type (WT) mice. The vehicle-treated mice were injected with 3 ml kg^{-1} 9:1 propylene glycol–ethanol, whereas 1,25(OH)$_2$D$_3$-treated mice were injected with 1 μg kg^{-1} day^{-1} 1,25(OH)$_2$D$_3$ s.c. for 3 days. The duodenal tissue was mounted in Ussing chamber, equilibrated in bathing solution for 10 min and then subjected to a 70-min calcium flux measurement. Numbers in parentheses represent the numbers of experimental animals. *$P < 0.05$, ***$P < 0.001$ compared with the corresponding WT group (Student's *t*-test). †$P < 0.05$ compared with the vehicle-treated WT group

Table 2. Electrical parameters of the duodenal epithelia in 8-week-old BKO thalassemic and wild-type mice

Experimental groups	*n*	Electrical parameters		
		PD (mV)	Isc (μA cm^{-2})	TER (Ω cm^2)
Male				
Wild-type	16	3.36 ± 0.22	20.94 ± 2.16	179.80 ± 15.44
BKO	7	3.29 ± 0.45	21.07 ± 7.40	198.00 ± 22.51
Female				
Wild-type	17	2.69 ± 0.18	17.50 ± 1.21	162.20 ± 16.82
BKO	10	2.21 ± 0.28	16.75 ± 1.94	149.60 ± 37.28

Values are means ± SE. The apical electrical potential was negative with respect to the basolateral potential.

In conclusion, *β*-thalassemic mice exhibited lower duodenal calcium absorption as compared with the age-matched wild-type littermates, despite showing no change in the mRNA levels of calcium transporters. Moreover, exogenous 1,25(OH)$_2$D$_3$ administration enhanced duodenal calcium transport only in the wild-type mice, but not in BKO thalassemic mice. Future experiments are required to demonstrate the effects of *β*-thalassemia on the protein expression and activity of each calcium transporter. Because low intestinal calcium absorption and inadequate calcium supply deteriorated bone formation,[20,33] the present findings have corroborated that the impaired 1,25(OH)$_2$D$_3$-dependent intestinal calcium absorption did contribute to the *β*-thalassemia-induced bone loss and osteoporosis.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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