ABSTRACT Recent advances in bone and calcium (Ca) metabolism have relied upon genetically modified mice. However, although human studies have identified gender as an important modulator of Ca metabolism, its effect on Ca metabolism has not been examined in mice. Here we examined basal and vitamin D–regulated Ca absorption (in situ ligated loops) and mRNA levels for the apical membrane calcium channel, TRPV6, and the calcium binding protein, calbindin D_{9k} (CaBP) mRNA levels (real-time PCR) in duodenum of female and male mice. At 2 mo of age, females fed a 5 g Ca/kg diet had higher Ca absorption (62.3 ± 4.8 vs. 47 ± 3.6%) and TRPV6 mRNA levels than males even though plasma 1,25 dihydroxyvitamin D [1,25(OH)_{2} D] was not different. In mice fed high (20 g/kg), normal (5 g/kg), or low (0.2 g/kg) Ca diets for 7 d to alter plasma 1,25(OH)_{2} D (91 ± 12, 322 ± 25, and 587 ± 43 pmol/L, respectively), the relation between Ca absorption (slope = 0.116 vs. 0.084, P = 0.021) or duodenum TRPV6 mRNA (slope = 0.042 vs. 0.025, P = 0.034) and circulating 1,25(OH)_{2} D was steeper in females. After a single 1,25(OH)_{2} D injection (200 ng/100 g body weight), peak induction of TRPV6 mRNA was 2-fold greater (at 6 h) and CaBP mRNA was 20% higher in females (at 16 h). Duodenal vitamin D receptor mRNA levels did not differ between genders. Our data indicate that female mice are more sensitive to changes in serum 1,25(OH)_{2} D levels than males and that this must be considered when using mice to study calcium and bone biology. J. Nutr. 134: 1857–1861, 2004.

KEY WORDS: • calcium absorption • calbindin D_{9k} • TRPV6 • gender • vitamin D

In the last decade, scientists have come to rely upon the use of transgenic and knockout mice to understand the role of various proteins in physiology. In the area of vitamin D biology alone, knockout mice were developed for the vitamin D receptor (VDR) (1,2), the 1α hydroxylase (3), the vitamin D–24 hydroxylase (4), and megalin (5). Although we recognize that gender is an important determinant of osteoporotic risk (6), and although we have come to rely more extensively on mouse models for the study of calcium homeostasis, there is little information available on the influence of gender on calcium homeostasis and the response to regulators of calcium homeostasis in mice.

Intestinal calcium absorption is regulated primarily by 1,25 dihydroxyvitamin D [1,25(OH)_{2} D], presumably by vitamin D–mediated transcriptional activation through the classical VDR (7,8). Transcellular calcium absorption is a 3-step process including entry of calcium into the enterocytes through the calcium channel TRPV6 (9), translocation of calcium from the brush border membrane to the basolateral membrane by the calcium binding protein calbindin D_{9k} (10), and extrusion of calcium through the basolateral calcium ATPase PMCA1b (11). We showed previously that TRPV6 and calbindin D_{9k} mRNA levels are regulated by 1,25(OH)_{2} D in mouse duodenum and that this coincides with the induction of duodenal calcium absorption (9). In the current study, we examined the effect of gender on basal and vitamin D–regulated calcium absorption and gene expression in mice.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical unless otherwise specified below.

Animals. Normal healthy mice were obtained from our breeding colony of VDR heterozygous mice and selected on the basis of genotype as previously described (7). The mice were originally obtained from Dr. Shigeaki Kato [University of Tokyo, Japan (2)] and are from a CD-1 X C57 BL/6 background. Pups were weaned at 21 d of age. Throughout the experiments, mice were housed individually and exposed to a 12-h light:dark cycle. Food and water were consumed ad libitum. All of the animal experiments were approved by the Purdue Animal Care and Use Committee.

Experimental design

Examination of gender differences in calcium metabolism in mice. At weaning, WT and VDR null mice were weaned and
randomly assigned to an AIN-76A diet containing 5 g calcium (Ca), 4 g phosphorus (P), and 1000 IU (25 μg) cholecalciferol/kg diet (12) or an AIN76A diet modified to contain 20 g calcium, 12.5 g phosphorus, and 200 g lactose/kg diet. At 60 d of age, the mice were anesthetized and the capacity of the duodenum to absorb Ca from a test dose (2 mmol/L Ca) was determined using in situ ligated loops as previously described (7) (n = 5–6 per gender and genotype). At the end of the absorption period, blood was drawn into heparinized tubes and plasma was prepared for later analysis of 1,25(OH)2 D levels by using a 125I-1,25(OH)2 D RIA kit according to the manufacturer’s directions (IDS Diagnostics). The femur was also removed and examined for calcium content as previously described (7). Three additional anesthetized mice of each gender and genotype were killed by exsanguination and duodenal scrapings were collected for later analysis of TRPV6, calbindin Dα-k, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels by real-time PCR (RT-PCR) as previously described (9).

Dietary manipulation of serum 1,25(OH)2 D. Mice were fed a commercial diet (8664, Harlan Teklad) until 90 d of age; they were then randomly assigned to 1 of 3 AIN-76A–based diets containing a normal (5 g Ca, 4 g P/kg diet), low (0.2 g Ca, 3.5 g P/kg diet), or high calcium content (20 g Ca, 200 g lactose, 12.5 g P/kg diet), (Research Diets) (n = 10 per gender and diet). After 7 d of consuming the experimental diets, anesthetized mice were killed by exsanguination and a duodenal scraping was harvested for analysis of calbindin Dα-k, TRPV6, and GAPDH mRNA levels by exsitu ligated loops. A second set of mice (n = 10 per gender and diet) was raised under the same protocol and the efficiency of intestinal calcium absorption was examined using in situ ligated loops. For both sets of mice, at killing, blood was collected into heparinized tubes by cardiac puncture and plasma was analyzed for 1,25(OH)2 D levels.

Time course and dose response to 1,25(OH)2 D injection. Mice were fed a standard diet until 90 d of age and then fed a diet containing 0.2 g Ca/kg and 8 g strontium/kg diet (Research Diet, D10373A) for 7 d to inhibit endogenous synthesis of 1,25(OH)2 D (13,14). On d 8 of strontium feeding, a time course study and a dose response to 1,25(OH)2 D study were conducted. In the time course study, anesthetized mice were injected with a single dose of 1,25(OH)2 D (Biomol) (i.p. 200 ng/100 g body weight in 0.1 mL vehicle, a 1:9 mix of ethanol and propylene glycol), and killed by exsanguination at 1, 3, 6, and 16 h after the injection (n = 6 per gender at each time point). Control mice received an i.p. injection of 0.1 mL of vehicle and were killed by exsanguination under anesthesia 16 h after the injection. In the dose response study, mice were injected with either 0.1 mL of vehicle or 1 dose of 1,25(OH)2 D (i.p. 25, 50, 100 ng/100 g body weight in vehicle, n = 5 of each gender per dose). Anesthetized mice were killed by exsanguination 6 h after the injection [a time determined to be optimal for TRPV6 mRNA induction in other experiments (9)]. In both studies a duodenal scraping was harvested, total RNA was isolated, and TRPV6, calbindin Dα-k, and GAPDH mRNA were analyzed via RT-PCR.

RNA isolation and PCR analysis. RNA was isolated from duodenal scrapings using TriReagent according to the manufacturer’s directions (Molecular Research Center). The isolated RNA was reverse transcribed into cDNA as previously described (15). RT-PCR was conducted on samples using the BioRad My iQ RT-PCR system. Parameters for TRPV6, calbindin Dα-k, and GAPDH were previously reported by our group (9), whereas the VDR primers were those previously reported by Healey et al. (16). Message levels were normalized to the expression of GAPDH within the sample and are expressed as arbitrary units or relative to a specific group mean.

Statistical analysis. Data from Expt. 1 and the time course and dose response studies were analyzed by two-way ANOVA (i.e., treatment × gender or genotype × gender) using the SAS statistical program (version 8.0, SAS Institute). When the plots of predicted values vs. residuals demonstrated that the data were not normally distributed, log transformation was conducted before statistical analysis. Comparisons of multiple group means were done using Fisher’s protected LSD. Regression analysis on the data from the dietary manipulation study was conducted using the general linear models and contrast procedures. Differences between group means or regression parameters (intercept, slope, line) were considered significant at the P < 0.05 level. Data are expressed as means ± SEM.

RESULTS

Intestinal calcium absorption and gene expression in WT and VDR knockout mice. When fed the 5 g Ca/kg diet from weaning, 60-d-old WT female mice had 104% higher TRPV6 mRNA and 30% greater efficiency of intestinal calcium absorption than males. Calbindin Dα-k mRNA (Fig. 1B, n = 3) and femur calcium concentration (female = 196.4 ± 2.5 vs. male = 190.4 ± 3.1 mg/g dry weight, n = 6) tended to be higher in female than in male mice (P = 0.192 and 0.148, respectively). Plasma 1,25(OH)2 D levels did not differ between male and female WT mice (362 ± 43 vs. 399 ± 55 pmol/L, n = 6). Although the efficiency of intestinal calcium absorption was significantly suppressed in VDR null mice, female mice still had significantly higher calcium absorption efficiency than male mice (Fig. 1A). TRPV6 and calbindin Dα-k mRNA levels were not higher in female VDR null mice.
(Fig. 1C). When duodenal VDR mRNA levels were measured in WT mice, male (relative value 1.0 ± 0.04) and female mice (relative value 0.93 ± 0.04) did not differ.

**Diet study.** As expected, plasma 1,25(OH)2 D levels were sensitive to dietary calcium intake (high Ca = 91 ± 12 pmol/L; normal Ca = 322 ± 25 pmol/L; low Ca = 587 ± 43 pmol/L), and the efficiency of duodenal calcium absorption reflected the plasma 1,25(OH)2 D levels (high Ca = 2.0 ± 1.9%; normal Ca = 19.8 ± 1.8%; low Ca = 57.6 ± 2.8%/10 min).

To better assess the effect of gender on duodenal responses to 1,25(OH)2 D, we took advantage of the expanded plasma 1,25(OH)2 D levels in both females and males, the expression of duodenal calcium absorption. As observed for calcium absorption, the response of TRPV6 mRNA to plasma 1,25(OH)2 D was higher in females than in males (slope  

\[ P = 0.021 \]  

and lines (  

\[ P = 0.006 \]  

of the relation differed between the genders (Fig. 2).

Duodenal TRPV6 and calbindin D9k mRNA levels were also affected by diet [TRPV6 mRNA: high Ca = 3.66 ± 0.95 arbitrary units (AU); normal Ca = 12.9 ± 1.8 AU; low Ca = 26.4 ± 2.8 AU; calbindin D9k mRNA: high Ca = 1.7 ± 0.2 AU; normal Ca = 4.7 ± 0.6 AU; low Ca = 7.7 ± 0.5 AU] and these differences were consistent with the effect of diet on the efficiency calcium absorption. As observed for calcium absorption, the response of TRPV6 mRNA to plasma 1,25(OH)2 D was higher in females than in males (slope  

\[ P = 0.034 \]  

Line  

\[ P = 0.058 \]  

Fig. 3A). Responsiveness of calbindin D9k mRNA also tended to increase although there were no differences in the slope (  

\[ P = 0.104 \]  

intercept (  

\[ P = 0.3 \]  

or line (  

\[ P = 0.194 \]  

between female and male mice (Fig. 3B).

**Time course and dose response to 1,25(OH)2 D injection.** In the time course study, plasma 1,25(OH)2 D levels were markedly increased (>5000 pmol/L) within 1 h after injection. By 16 h after injection, 1,25(OH)2 D levels fell to the basal levels (281 pmol/L). As we showed previously (9), expression of TRPV6 and calbindin D9k mRNA reflected plasma 1,25(OH)2 D levels (Fig. 4). A gender × time interaction occurred for TRPV6 mRNA (  

\[ P = 0.004 \]  

calbindin D9k mRNA (  

\[ P = 0.017 \]  

Fig. 4A and 4B). The fold inductions due to vitamin D treatment for both TRPV6 and calbindin D9k mRNA were higher in females than males (e.g., induction of TRPV6: 11-fold in females vs. 5-fold in males 3 h after 1,25(OH)2 D injection (  

\[ P = 0.027 \]  

induction of calbindin D9k: 7.2-fold in females vs. 5.3-fold in males 16 h after 1,25(OH)2 D injection,  

\[ P < 0.001 \]  

In the dose-response study, a similar analysis was performed. Six hours after a single injection with 1,25(OH)2 D, plasma levels were markedly increased at the 25 ng/100 g body weight (1094 ± 115 pmol/L), 50 ng/100 g body weight (2063 ± 305 pmol/L), and 100 ng/100 g body weight doses (3091 ± 339 pmol/L), respectively. The expression of duodenal TRPV6 increased in response to 1,25(OH)2 D injection, and this induction was influenced by gender (gender × dose interaction,  

\[ P = 0.002 \]  

Fig. 5). Although calbindin D9k mRNA levels were 25–40% higher after 1,25(OH)2 D injection in females than in males, this difference was not significant (  

\[ P = 0.18 \]  

, data not shown).
Collectively, our data consistently demonstrated that female mice had a higher intestinal response to 1,25(OH)2 D. When fed the normal calcium AIN76A diet, the efficiency of calcium absorption from a test dose was higher in female than male mice even though plasma 1,25(OH)2 D levels were not elevated. When examining the relation between calcium absorption, duodenal TRPV6 mRNA levels, or duodenal calbindin D9k mRNA levels and plasma 1,25(OH)2 D, female mice consistently had a higher response (i.e., steeper slope). Finally, in both time- and dose-response studies, induction of TRPV6 mRNA (and to a lesser extent calbindin D9k mRNA) was greater in female than male mice.

Our findings are not consistent with data from rats. Johnson et al. (17) showed that female rats had lower mRNA and protein contents of calbindin D9k and vitamin D-24-hydroxylase than males at 2.5 mo of age, but this could be explained by lower serum 1,25(OH)2 D levels in the females. Similarly, using everted gut sacs to measure intestinal calcium absorption efficiency, Gruden et al. (18) reported that calcium transport was significantly higher in 2- to 3-mo-old male than female rats. In contrast, Dupuis et al. (19) showed that the rate of calcium absorption from in vivo ligated loops was similar during a 30-min test irrespective of gender in mature rats. In a crossover study in humans using stable isotopic tracers, Miller et al. (20) found no gender effects on calcium absorption in healthy male and female adolescents. We can conclude 2 things from our study. First, mice may not be an appropriate model for assessing human-relevant gender influences on calcium metabolism. Second, studies on calcium homeostasis (and particularly calcium absorption) using genetically modified mice should be designed to account for possible gender differences.

The mechanism of the gender effect on duodenal calcium absorption efficiency and gene expression is not clear from our studies. It was suggested that estrogen is a critical modulator of intestinal calcium absorption. Gennari et al. (21) showed that although oral calcitriol enhanced intestinal calcium absorption in premenopausal women, oophorectomy eliminated this response, and estrogen repletion returned the response to normal. Some, but not all, studies suggest that the effect of estrogen on vitamin D–mediated responses such as calcium absorption is due to effects on tissue VDR levels. For example, Chen et al. (22) found that estrogen deficiency significantly decreased total, unoccupied, and occupied VDR content in ovariectomized rats, whereas Liel et al. (23) reported that estrogen deficiency decreased VDR levels and the response of 1,25(OH)2 D target genes to 1,25(OH)2 D treatment in ovariectomized rats. In contrast, Colin et al. (24) found that ovariectomy-induced intestinal resistance to 1,25(OH)2 D action was not accompanied by reduced intestinal VDR protein levels in rats. Nevertheless, we did not find a significant difference in the level of VDR mRNA between male and female mice; thus, this simple explanation is not supported by our data.

To determine whether the effect of estrogen on intestinal calcium absorption efficiency and gene expression is mediated through the VDR, we measured calcium absorption, TRPV6 mRNA, and calbindin D9k mRNA levels in VDR KO and WT mice.
mice. WT female mice had a significantly higher calcium absorption efficiency and TRPV6 mRNA than WT male mice. A similar trend was observed in VDR KO female mice, suggesting that in addition to a gender-associated increase in the sensitivity to 1,25(OH)2 D, there is a VDR-independent effect of estrogen on intestinal calcium absorption and gene expression. This is consistent with van Cromphaut et al. (25) who showed that estrogen treatment, pregnancy, and lactation induced the expression of TRPV6 mRNA in WT and VDR KO mice, and that TRPV6 mRNA was reduced by 55% in estrogen receptor α knockout mice. These data suggest that TRPV6 gene expression is regulated by estrogen through a VDR-independent pathway. More studies are required for a better understanding of gender effects on the induction of calcium absorption and gene expression involved in this process.

In summary, we showed that gender significantly affected the induction of intestinal calcium absorption and expression of TRPV6 and calbindin D9k mRNA in response to changes in circulating 1,25(OH)2 D, i.e., female mice are more sensitive to the elevated circulating 1,25(OH)2 D than male mice. Although a definitive mechanism is not yet clear, our data suggest that it is very important to control gender in experiments involving calcium transport and intestinal gene expression in mice.

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LITERATURE CITED


