Impaired body calcium metabolism with low bone density and compensatory colonic calcium absorption in cecectomized rats

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Jongwattanapisan P, Suntornsaraton P, Wongdee K, Dorkkam N, Krishnamra N, Charoenphandhu N. Impaired body calcium metabolism with low bone density and compensatory colonic calcium absorption in cecectomized rats. Am J Physiol Endocrinol Metab 302: E852–E863, 2012. First published January 24, 2012; doi:10.1152/ajpendo.00503.2011.—An earlier study reported that colonic calcium absorption contributes less than 10% of total calcium absorbed by the intestine, although the cecum has the highest calcium transport rate compared with other intestinal segments. Thus, the physiological significance of the cecum pertaining to body calcium metabolism remains elusive. Herein, a 4-wk calcium balance study in cecectomized rats revealed an increase in fecal calcium loss with marked decreases in fractional calcium absorption and urinary calcium excretion only in the early days post-operation, suggesting the presence of a compensatory mechanism to minimize intestinal calcium wasting. Further investigation in cecectomized rats showed that active calcium transport was enhanced in the proximal colon but not in the small intestine, whereas passive calcium transport along the whole intestine was unaltered. Since apical exposure to calcium-sensing receptor (CaSR) agonists similarly increased proximal colonic calcium transport, activation of apical CaSR in colonic epithelial cells could have been involved in this hyperabsorption. Calcium transporter genes, i.e., TRPV6 and calbindin-D9k, were also upregulated in proximal colonic epithelial cells. Surprisingly, elevated serum parathyroid hormone levels and hyperphosphatemia were evident in cecectomized rats despite normal plasma calcium levels, suggesting that colonic compensation alone might be insufficient to maintain normocalcemia. Thus, massive bone loss occurred in both cortical and trabecular sites, including lumbar vertebrae, femora, and tibiae. The presence of compensatory colonic calcium hyperabsorption with permissive osteopenia in cecectomized rats therefore corroborates that the cecum is extremely crucial for body calcium homeostasis.

calcium balance study; calcium-sensing receptor; colon; osteopenia; parathyroid hormone

BECAUSE THE CEcum has the highest rate of calcium transport (26, 39), it was surprising that rats subjected to massive large bowel resection were normocalcemic (41), and cecal calcium absorption was reported to contribute only ~9% of total calcium absorbed from the whole intestine (3, 4). To resolve this discrepancy and to determine the physiological significance of the cecum in body calcium homeostasis, calcium input and output were examined after surgical removal of the cecum (cecectomy) for any sign of negative calcium balance or compensatory mechanism that might have occurred in bone and other parts of the intestine.

Under normal conditions, the cecum absorbs free-ionized calcium released from insoluble complexes of calcium after exposure to small acidic molecules such as acetic, propionic, butyric, succinic, and other short-chain fatty acids, all of which are produced during fermentation of dietary fibers and prebiotics by luminal microflora (36, 37, 48). Once released, calcium traverses the cecal epithelium by both transcellular and paracellular pathways (31). We recently showed that the paracellular pathway became predominant, with the cecal paracellular calcium flux being ~10 times greater than the transcellular flux when mucosal calcium concentrations exceeded 5 mmol/l (7). Nevertheless, uphill transcellular calcium transport was still of considerable importance in view of free calcium concentrations being somewhat variable, depending on calcium intake and the presence of calcium-trapping molecules, e.g., phytate, oxalate, phosphate, and carbonate (4, 22). Since fractional calcium absorption is relatively low (~10–20% of calcium intake), in part due to the formation of insoluble complexes, the intestine uses several mechanisms to ensure adequate calcium supply to the body (9). Transcellular calcium transport in both small and large intestine is a three-step process consisting of 1) apical calcium uptake via transient receptor potential cation channel, subfamily V, member 6 (TRPV6) and voltage-dependent L-type calcium channel 1.3 (Cav1.3); 2) cytoplasmic translocation by various calcium-binding proteins, such as calbindin-D9k and parvalbumin; and 3) basolateral extrusion by plasma membrane Ca2+/ATPase-1b (PMCA) and Na+/Ca2+ exchanger-1 (NCX) (21, 29). On the other hand, paracellular intestinal calcium transport requires several tight junction proteins in the claudin family, such as claudin-2, -12, and -15, which form charge-selective tight junction pores for paracellular cation movement (1). A number of intestinal calcium transporters are directly regulated by hormones, for instance 1,25-dihydroxycholecalciferol [1,25-(OH)2D3], estrogen, and prolactin (9, 30). Certain luminal factors, e.g., glucose and fatty acids, have also been shown to increase calcium transporter expression and/or calcium absorption (5, 16, 36). Whether luminal calcium itself regulates the rate of intestinal calcium absorption is not known, but the presence of calcium-sensing receptors (CaSR) in the apical membrane of enterocytes suggests possible involvement (11, 13). Since the maintenance of normal plasma calcium is of utmost importance, negative calcium balance due to a variety of causes, e.g., low dietary calcium intake, leads to the upregulation of calcium transporters, thereby enhancing intestinal calcium absorption (4, 21). In the face of calcium wasting or inadequate intake, normocalcemia may also be maintained by an increase in osteoclast-mediated bone resorption (30), leading to osteopenia with low bone mineral content (BMC) and
density (BMD) or even overt osteoporosis. Since cecectomy should be associated with fecal calcium loss, both intestinal and skeletal compensations are expected to help restrict intestinal calcium loss and maintain normocalcemia.

Therefore, the objectives of the present study were 1) to investigate total body calcium homeostasis in cecectomized rats by calcium balance study, 2) to demonstrate compensatory upregulation of intestinal calcium transporters and calcium absorption, and 3) to determine changes in BMD and BMC after 4 wk of cecectomy. The results indicated that the rat cecum was essential for body calcium homeostasis and should not be disregarded as a simple reservoir for indigestible dietary contents.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (8 wk old, 180–200 g) were obtained from the National Laboratory Animal Centre, Thailand. The rats were acclimatized for 14 days before the start of the experiments. They were placed in hanging stainless steel cages, fed standard chow containing 1.0% w/w calcium, 0.9% w/w phosphorus, and 4,000 IU/kg vitamin D (CP, Bangkok, Thailand) and reverse osmosis water ad libitum under a 12:12-h light-dark cycle. The room had a temperature of 20–25°C, humidity of 50–60%, and average illuminance of 150–200 lux in the daytime. Body weight and food intake were recorded daily. The study was approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Thailand. All animals were cared for in accordance with the principles and guidelines of the American Physiological Society’s “Guiding Principles in the Care and Use of Animals”.

Experimental design. Age-matched rats were randomly divided into cecectomized and sham-operated (control) groups. On day 5 after surgery, rats in both groups were first investigated for calcium intake, fecal and urinary calcium excretion, total calcium excretion, and fractional calcium absorption in the calcium balance study. At the end of the 4-wk calcium balance study (day 35), arterial blood (5 ml), L5–6 vertebrae, femora, tibiae, intestinal tissues, and colonic luminal content were collected from each rat. Intestinal absorptive cells were later harvested by mucosal scraping for total RNA purification and quantitative real-time PCR, as described previously (6). Transcellular and paracellular calcium transport in all intestinal segments was determined by Ussing chamber technique. Total calcium content in the colonic lumen was analyzed by atomic absorption spectrophotometry. Bone specimens were later analyzed for BMD and BMC by dual-energy X-ray absorptiometry (DEXA).

To investigate whether calcium-induced CaSR activation was responsible for the colonic adaptation that helped minimize fecal calcium loss after cecectomy, the proximal and distal colon were removed from normal rats and mounted in an Ussing chamber. The tissue was then exposed on the apical side to various concentrations of CaSR agonists, neomycin, and spermine (40), and the active calcium transport was measured.

Surgery and postoperative care. Under 40 mg/kg ip sodium pentobarbital (Ceva Santé Animale, Libourne, France) anesthesia, cecectomy was performed according to the modified method of Kurosawa et al. (32). The Abdominal skin (2 × 2 in.) was shaved and cleaned with povidone iodine. Thereafter, a 1.5-cm median laparotomy was performed with a pair of sterile surgical scissors. The fat pad and connective tissues were retracted to allow a clear operative field. The cecum was lifted from the abdominal cavity and kept warm and moist with sterile normal saline-soaked gauze. Blood vessels of the cecum were ligated with 5-0 polyglactin (W9501T Ethicon; Johnson & Johnson, St. Stevens-Woluwe, Belgium). The cecum was then gently ligated with 2-0 silk near the common junction of ileum, cecum, and colon (Fig. 1) before being resected from the intestinal tract. The resection wound was closed by simple continuous suturing with 5-0 polyglactin. In sham-operated groups, a small incision (5 mm) was made on the cecal sac at the area devoid of major blood vessels, and the wound was also closed by simple continuous suturing with 5-0 polyglactin. Finally, the intestine was placed back into the abdominal cavity. Peritoneum and muscular layers were closed by simple continuous suturing with sterile 2-0 catgut, and the skin was closed with 2-0 silk by simple interrupted suturing.

After the operation, all rats, which were kept warm under an overhead lamp, maintained their body temperature of 37°C and were continuously monitored for respiratory rate, color of mucous membrane, and the recovery stages of anesthesia (i.e., recovery of corneal reflex and withdrawal reflex). Each rat was also administered once daily for 3 days with analgesic drug (5 mg/kg carprofen sc; Pfizer, Scotland, UK) and antibiotic drug (10 mg/kg enrofloxacin sc; Bayer Healthcare, Leverkusen, Germany). Rats were returned to their cages when they regained consciousness without signs of bleeding or respiratory failure. The 4-wk study was begun after a 3-day recovery period.

At the end of the experiments, rats were subjected to laparotomy under general anesthesia. The internal organs were explored prior to intestinal tissue and blood collection. None of the operated rats showed abdominal fluid, leakage of intestinal contents, interorgan adhesion, or signs of inflammation. Neither intestinal obstruction nor distension was observed.

Calcium balance study. The calcium balance study was for determining in vivo intestinal calcium absorption and body calcium reten-
tion. After recovery from the operation, ceccotomized and sham-operated rats were individually housed in metal cages (1 rat/cage, model 3701M081; Techniplast, Venice, Italy) for 4 wk. Cecotomized rats were fed the same amount of food as sham-operated rats (pair feeding) during recovery and experimental periods to ensure equal calcium intake. Volume of urine, water intake, body weight, and weight of feces were recorded. Fecal pellets were later dried in an oven at 80°C for 48 h to obtain dry weight. Dry fecal pellets were finely ashed at 800°C for 24 h in a muffle furnace (model 48000; Thermolyne, Dubuque, IA) to remove organic materials. Fecal ash (1 mg) was dissolved in 2 ml of 3 N HCl and then diluted twofold. Thereafter, fecal and urine specimens (100 µl) were diluted with a solution containing 0.38% wt/vol SrCl2 and 0.9% vol/vol HClO4 and analyzed for calcium content with an atomic absorption spectrophotometer (model SpectraAA-300; Varian Techtron, Springvale, Australia). Fecal and urinary calcium excretions were calculated from total ash weight and total urine volume, respectively. Total calcium excretion (mmol·kg⁻¹·day⁻¹) and fractional calcium absorption (%) were calculated from Eqs. 1 and 2 (45).

\[
\text{total calcium excretion} = \frac{F_{Ca} + U_{Ca}}{BW} \\
\text{fractional calcium absorption} = \frac{C_{Ca} - F_{Ca}}{C_{Ca} \times 100}
\]

where \( F_{Ca} \) is fecal calcium excretion (mmol), \( U_{Ca} \) is urinary calcium excretion (mmol), \( C_{Ca} \) is calcium intake (mmol), and \( BW \) is body weight (kg).

**Ussing chamber and calcium flux measurement.** After a median laparotomy was performed, duodenum (10 cm), jejunum (10 cm), ileum (8 cm), and proximal and distal colon (8 cm each) were removed, stripped of the muscle layer, and cut longitudinally to expose the mucosa. The tissue was then mounted in an Ussing chamber with physiological bathing solution for 20 min. Thereafter, the chamber was aerated with 5% CO₂ in 95% O₂ at 37°C and had an osmolality ranging from 300–320 mosmol/kg H₂O.

Transepithelial potential difference (PD) and short-circuit current (Isc) were determined by Ag/AgCl half-cell connected to each hemichamber through a salt bridge (4 M KCl in 4 g wt/vol agar). Two PD-sensing Ag/AgCl electrodes were placed near the mounted tissue and connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL) and finally to a PowerLab/4SP (AD Instruments, Colorado Springs, CO). The Isc-passing Ag/AgCl electrodes were placed at the distant end of each hemichamber to supply Isc, which was also measured by the EVC-4000/PowerLab system. Transepithelial resistance (TER) was calculated from PD and Isc by Ohm’s equation.

Transepithelial calcium flux was determined by the modified method of Charoenphandhu et al. (6). The tissue was first incubated in the chamber with physiological bathing solution for 20 min. Thereafter, the mucosal chamber was filled with bathing solution containing \(^{45}\)Ca (initial amount of 5 mCi/ml, final specific activity of ~450–500 mCi/mmol; Amersham). Bidirectional calcium flux \((J_{H\rightarrow C\rightarrow} \text{mmol} \cdot \text{m}^{-2} \cdot \text{cm}^{-1} \cdot \text{s}^{-1})\) from the hot side (H; mucosal side) to the cold side (C; serosal side) was calculated by Eqs. 3 and 4.

\[
J_{H\rightarrow C\rightarrow} = \frac{R_{H\rightarrow C\rightarrow} \cdot (S_H \times A)}{S_H} \\
S_H = C_H / C_{To}
\]

where \( R_{H\rightarrow C\rightarrow} \) is the rate of tracer appearance in the cold side (cpm/h); \( S_H \) is the specific activity in the hot side (cpm/mmol); \( A \) is the surface area of the tissue (cm²); \( C_H \) is the mean radioactivity in the hot side (cpm); and \( C_{To} \) is the total calcium content in the hot side (mmol). \(^{45}\)Ca was analyzed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT). Total calcium concentration in bathing solution was analyzed with an atomic absorption spectrometer (model SpectraAA-300, Varian Techtron).

In the absence of transepithelial calcium gradient (i.e., bathing solution in both hemichambers contained the same calcium concentration of 1.25 mmol/l), the measured calcium flux represented the active calcium transport (7). The calcium gradient-dependent paracellular passive fluxes were measured by determining the transepithelial calcium fluxes in the presence of various mucosal calcium concentrations (7), i.e., 1.25, 2.5, 5, 10, and 20 mmol/l (\( n = 5 \) per each calcium concentration). In the CaSR agonist experiments, each CaSR agonist (i.e., 50–800 µmol/l neomycin trisulfate or spermine Sigma) was dissolved in bathing solution, and the mounted proximal or distal colonic segments from normal rats were exposed on the mucosal side to CaSR agonist.

**Total RNA preparation, quantitative real-time PCR, and sequencing.** Total RNA was prepared from mucosal brushings by using TRIzol reagent (Invitrogen, Carlsbad, CA), as described previously (6,10). Purity of the total RNA was evaluated by a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) reading at 260 and 280 nm, the ratio of which was in the range of 1.8–2.0. One microgram of total RNA was reverse-transcribed to cDNA with oligo(dT25) primer and iScript kit (Bio-Rad, Hercules, CA). β-Actin, a housekeeping gene, served as a control gene to check the consistency of the reverse transcription (% coefficient of variation of β-actin expression <5%; n = 10).

Primer sequences specific for the studied transcripts, i.e., TRPV5, TRPV6, Ca.1,3, calbindin-D9k, PMCA, N CX, claudin-2, claudin-15, CaSR, 25-hydroxyvitamin D3 1α-hydroxylase (1α-OHase), and Na⁺-dependent phosphate transporter (NaPi)II b, are presented in Table 1 (7,31). qRT-PCR and melting curve analysis were performed by MiniOpticon real-time PCR system (Bio-Rad) with iQ SYBR Green SuperMix (Bio-Rad). Relative expression of the studied genes over β-actin was calculated from the threshold cycle \((C_T)\) by using the 2⁻^ΔΔC_T method (35). The PCR products were also visualized on a 1.5% agarose gel stained with 1.0 µg/ml ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA). After electrophoresis, the PCR products were purified from a gel by the HiYield Gel/PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan) and sequenced to confirm the correct amplicons by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**DEXA.** Femora, tibiae, and LS-6 vertebrae were removed from cecotomized and sham-operated rats and cleaned of adhering connective tissues. As described previously by Charoenphandhu et al. (8) and Suntornsraroot et al. (42), BMD and BMC were determined in the ex vivo bone specimens by DEXA (model Lunar PIXImus2; GE Medical Systems, Madison, WI), operated with software version 2.10. The dual-energy supply was 80/35 kVp at 500 µA. The DEXA system was calibrated daily by a standard material with known BMD and BMC of 0.0690 g/cm² and 0.697 g, respectively. As for the femora, the densitometric analysis was performed on the whole bone, metaphysis (proximal 1/3 and distal 1/3 of the femur), and diaphysis (middle 1/3 of the femur).

**Blood chemistry and hormone assay.** After a median thoracotomy, cardiac puncture was performed for arterial blood collection (5 ml). Blood for plasma collection was drawn with a commercial sterile heparinized syringe (model REF364314; BD Diagnostics, Plymouth, UK) with no exposure to air. Plasma total calcium (ionized and complexed forms) was determined by modified o-cresolphthalein complexoxem complex method, while plasma inorganic phosphate was analyzed by a phosphomolybdate-based kit using a Dimension RXL analyzer (Dade Behring, Marburg, Germany). Free-ionized calcium and magnesium concentrations were measured by ion-selective electrodes (model Stat Profile CCX; Nova Biomedical, Waltham, MA) under anaerobic conditions. Plasma albumin and serum total vitamin D, i.e., cholecalciferol (vitamin D3) plus 25-hydroxycholecalciferol [25-(OH)₂D₃], were analyzed by bromocresol purple method and chemiluminescent immunoassay (catalog no. 310600; DiaSorin Liaison, Stillwater, MN), respectively. Rat total intact PTH was measured by two-site enzyme-linked immunosorbent assay kit (catalog no. 3KG024; AJP-Endocrinol Metab • doi:10.1152/ajpendo.00503.2011 • www.ajpendo.org)
Scantibodies Laboratory, Santee, CA). Each sample was analyzed in quadruplicate.

Statistical analysis. Results are expressed as means ± SE. Two sets of data were compared by unpaired Student’s t-test. One-way analysis of variance with Dunnett’s posttest was used for multiple sets of data. The level of significance for all statistical tests was P < 0.05. Differential gene expression in the qRT-PCR study was considered significant when there was a twofold or greater difference in expression between the age-matched control and cecectomized groups (10). Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

Negative calcium balance was observed only in the early days postcecectomy. On day 4 post-operation, cecectomized and sham-operated rats were placed individually in metabolic cages and pair-fed to ensure equal daily calcium intake throughout the 35-day experimental period (Fig. 2A). Data collection began on day 5 post-operation. Ccecetomized rats showed a decrease in fractional calcium absorption and urinary calcium excretion only on day 5 and days 5–9 after surgery, respectively, compared with the sham-operated rats (Fig. 2, B and C). Consistent with decreased fractional calcium absorption, fecal calcium excretion was increased (Fig. 2D), thereby leading to a significant increase in total calcium excretion (combined urinary and fecal calcium excretion; Fig. 2E). However, in the rest of the experimental period, there was no difference between sham-operated and cecectomized groups in all parameters. These results thus indicated that negative calcium balance was evident only at the very early period after cecectomy, after which compensatory mechanism(s) may have successfully restored calcium balance.

Colonic active calcium absorption was enhanced in cecectomized rats. Since fecal calcium loss was observed in cecectomized rats only in the first week post-operation, it was possible that the colon might augment its calcium transport activity in response to delivery of an excess calcium load from the small intestine. In an attempt to identify the site of a possible compensatory mechanism, we measured active calcium transport in various intestinal segments of cecectomized rats. It was found that the proximal colon (P = 0.039; cecectomized vs. sham-operated rats), but not duodenum (P = 0.081), jejunum (P = 0.083), or ileum (P = 0.243), exhibited a significant increase in active calcium transport (Fig. 3). On the other hand, paracellular calcium transport, which usually becomes predominant over transcellular transport when the mucosal calcium concentration is higher than 5 mmol/l (7), was not changed in any of the intestinal segments (Fig. 4). However, it was only at low mucosal calcium concentrations that the proximal colon showed an increase in transepithelial calcium transport (Fig. 4D). Because the mucosal calcium concentrations were 1.25 and 2.5 mmol/l, the enhanced transepithelial calcium transport must be accounted for by an increase in the transcellular active calcium transport.

Analysis of colonic calcium content by atomic absorption spectrophotometry revealed that the luminal calcium amount...
was higher in cecrectomized rats (day 35 post-surgery) compared with sham-operated rats (4.32 ± 0.08 vs. 3.77 ± 0.16 mol/g fecal ash weight; n = 8 per group; P = 0.004). This finding confirmed that the efficiency of colonic calcium absorption must be enhanced after cecectomy, since cecrectomized rats showed unaltered fecal calcium excretion despite an increased calcium load.

At the molecular level, qRT-PCR revealed approximately eight- and threefold upregulations of salient transcellular calcium transporter genes, namely TRPV6 and calbindin-D9k, respectively, in the proximal colonic epithelial cells of cecrectomized rats (Fig. 5). In contrast, the colonic expressions of other transcellular calcium transporter genes, i.e., TRPV5, Ca1.3, PMCA, and NCX, were not changed after cecectomy. The transcripts related to the paracellular calcium transport, such as claudin-2, -12, and -15 (6, 15), were not altered, consistent with no change in the colonic paracellular calcium transport. The colonic CaSR mRNA expression was also unchanged in cecrectomized rats (Fig. 5). In the small intestine, besides Ca1.3 expression, which was downregulated in the jejunum and ileum, the expression of other calcium transport-related genes remained unaltered after cecectomy (Fig. 5, A–C). In addition, there was also no change in the mRNA expression of 1α-OHase and NaPi-IIb in all studied intestinal segments of cecrectomized rats (n = 8–11; data not shown).

Hyperparathyroidism and hyperphosphatemia were evident in cecrectomized rats. Blood chemistry analysis further showed that cecrectomized rats had normal plasma pH (Fig. 6A) and albumin levels (data not shown), both of which could indirectly affect free-ionized calcium concentration (47). Although total plasma calcium, plasma ionized calcium, and magnesium concentrations in cecrectomized rats were comparable to those of sham-operated rats (Figs. 6, B–D), the plasma inorganic phosphate was significantly elevated in cecrectomized rats (Fig. 6E). Interestingly, the serum PTH level was increased about two-fold in cecrectomized rats, whereas total vitamin D levels remained unchanged (Fig. 6, F and G).

Osteopenia was observed throughout the body on day 35 post-cecectomy. Since cecrectomized rats manifested hyperparathyroidism and hyperphosphatemia, we further postulated that the compensatory stimulation of colonic calcium absorption might be insufficient to maintain the normocalcemic state in cecrectomized rats. In other words, PTH was required to induce calcium release from bone, thereby leading to massive
bone loss. As shown in Fig. 7, despite having similar tibial and femoral lengths, cecectomized rats had lower BMD and BMC in whole tibiae and whole femora as well as in L5–6 vertebrae compared with sham-operated rats. In the primarily cortical bone such as femur, which is one of the strongest weight-bearing bones in the body, cecectomy-induced decreases in BMD and BMC were observed in both cortical part (diaphysis) and trabecular parts (proximal and distal metaphyses), as depicted in Fig. 8.

Colonic CaSR activation may contribute to compensatory colonic calcium absorption. To find out whether the extra calcium load to the colon of cecectomized rats could trigger the colonic compensatory mechanism via CaSR, known to be abundantly expressed on the apical membrane of colonic epithelial cells (Ref. 13, and Charoenphandhu and Suntornsaratoon, unpublished observation), the colonic tissue was exposed on the mucosal side to CaSR agonists neomycin and spermine. As shown in Fig. 9, mucosal exposure to 200 μmol/l neomycin or 800 μmol/l spermine significantly stimulated active calcium transport in the proximal colon but not in the distal colon. Indeed, the distal colon may not be an efficient site for calcium absorption, as normal active calcium flux in this segment was relatively low compared with that in the proximal colon (14.51 ± 1.39 vs. 26.58 ± 1.89 mmol·h^{-1}·cm^{-2}; P < 0.001, n = 8 per group). CaSR agonists did not affect colonic electrical parameters, namely PD, Isc, and TER (Table 2).

**DISCUSSION**

In hindgut fermenters such as humans and rodents, the cecum harbors microbiota containing thousands of microfloral...
strains that not only are important for host immunocompetency and provision of essential nutrients (e.g., thiamine, riboflavin, and vitamin K) but are also capable of fermenting indigestible dietary components and prebiotics (33, 37). This fermenting process produces acidic molecules such as lactic, acetic, and succinic acids, which in turn dissolve calcium from its insoluble complexes, thus providing free-ionized calcium for absorption (14, 34, 37, 48). Some products of fermentation, e.g., short-chain fatty acids, could directly stimulate calcium transport in the cecum and colon (36). Although cecal calcium absorption was believed to contribute less than 10% of total calcium absorbed by the intestine (4), the cecum was found to be the intestinal segment with the highest rate of calcium transport, being greater than that in the duodenum, proximal colon, and distal colon by ~9, ~4, and ~20 times, respectively (24–28, 39). Consistent with its highest active calcium transport rate, the transcripts of TRPV5, TRPV6, calbindin-D9k and PMCA were most abundant in the cecum compared with other intestinal segments (43). One may, therefore, postulate that the cecum could have a significant role in the regulation of calcium homeostasis by being the final site for retrieving calcium from the intestinal contents.

In the present 4-wk calcium balance study of cecectomized rats, a significant increase in fecal calcium loss, with a marked decrease in fractional calcium absorption, was observed only in the early days post-operation before returning to normal condition, suggesting the presence of compensatory mechanisms to minimize intestinal calcium wasting. Pair-feeding for equal daily calcium intake throughout the 35-day period excluded adaptation due to a variation in dietary calcium intake. A decrease in urinary calcium excretion possibly to conserve calcium during the first week post-operation further suggested that compensation for negative calcium balance occurred at the systemic level and was likely to involve PTH. The compensatory mechanisms from the second week onward appeared to be adequate for maintaining normal calcium balance, as both sham and cecectomized rats had comparable total calcium excretion.

To find out how the intestine, in response to cecectomy, restricted fecal calcium loss, we determined the transepithelial calcium fluxes in all intestinal segments. It was found that the active calcium transport of cecectomized rats was enhanced in the proximal colon but not in the duodenum, jejunum, or ileum. Although paracellular passive calcium transport is generally predominant in most intestinal segments, including cecum (26, 31), the calcium gradient-dependent paracellular calcium transport at mucosal calcium >5 mmol/l was unaltered in all studied segments. An increase in transepithelial calcium transport at

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**Fig. 5.** Expression of CaSR and genes related to transcellular (TRPV5/6, Ca1.3, calbindin-D9k, PMCA, and NCX) and paracellular calcium transport (Cldn2, Cldn12, and Cldn15) in duodenum (A), jejunum (B), ileum (C), and proximal colon (D) of Cecec rats relative to that of Sham rats (data of Sham were normalized to 1). TRPV5/6, transient receptor potential vanilloid family Ca2+ channels 5/6; Ca1.3, voltage-dependent L-type Ca2+ channel 1.3; PMCA, plasma membrane Ca2+-ATPase isoform-1β; NCX, Na+/Ca2+ exchanger 1; Cldn, claudin; CaSR, calcium-sensing receptor. Levels of mRNA expression were determined by qRT-PCR and expressed as log2 means ± SE. Data were plotted along with corresponding fold change values. Two dashed lines indicate 2-fold upregulation or downregulation of gene expression in Cecec rats. Nos. of animals in each group are in parentheses.
mucosal calcium of 2.5 mmol/l in the proximal colon of cecectomized rats was likely to result from the markedly enhanced active calcium transport, which was normally overshadowed by bulk paracellular calcium fluxes at higher mucosal calcium concentrations. Indeed, an absence of change in the paracellular passive calcium transport was not unexpected, since without cecal fermentation free-ionized calcium in the large intestine should be too low to induce paracellular passive calcium transport.

The aforementioned colonic calcium hyperabsorption might have resulted from extra calcium load to the proximal colon, which in turn activated the apical CaSR in the colonic epithelial cells, especially in the proximal colon. Thus, the proximal colon, but not the distal colon, directly exposed on the apical side to CaSR agonists neomycin and spermine, showed a similar increase in calcium transport to that in the cecectomized rats (Fig. 9). Neither of the CaSR agonists affected the colonic electrical properties, including transepithelial resistance, indicating no change in paracellular permeability to ions (19). Also, a calcium load to the proximal colon did not lead to any significant change in colonic CaSR expression (Fig. 5). In fact, the function of apical CaSR is well established as a modulator of fluid and electrolyte transport in the intestine (13, 18). Under normal conditions, besides calcium with sensitivity as low as 3–10 mmol/l (17), a number of luminal nutrients such as phenylalanine and polyamines are capable of stimulating CaSR (12, 20), but it is not known whether these nutrients can modulate colonic calcium absorption after cecectomy (when the colon was flooded with luminal contents containing these nutrients). Further investigations are required to definitively test a possible role of CaSR in this colonic response and to unravel CaSR signal transduction in colonocytes of cecectomized rats.

At the molecular level, two salient transcellular calcium transporter genes encoding apical calcium channel TRPV6 and cytoplasmic calcium-binding protein calbindin-D9k were also upregulated in the colon but not in the small intestine of cecectomized rats. Expression of other calcium transporters, including PMCA, was not increased, although PMCA activity might have been enhanced later by calbindin-D9k overexpression (46). Nevertheless, it was previously elaborated in a TRPV6/calbindin-D9k double-knockout model that both proteins were not essential for vitamin D-dependent active calcium transport in the mouse duodenum (2). Perhaps in the duodenum of TRPV6/calbindin-D9k double-knockout mice certain nutrients, such as glucose and amino acids, increase sodium uptake and thus depolarize the epithelial cells, thereby enhancing calcium absorption through an alternative voltage-dependent Ca_{v}1.3 calcium channel (29). On the other hand, in the colon, which had low luminal concentrations of such depo-
larizing nutrients compared with the proximal small intestine, TRPV6 and calbindin-D9k were still required for active calcium absorption. Therefore, this upregulation of TRPV6 and calbindin-D9k mRNA expression in colonic epithelial cells was consistent with the enhanced colonic calcium transport in cecectomized rats. Furthermore, as colonic paracellular passive calcium absorption was not affected by cecectomy, there was no significant change in the mRNA expression of claudin-2, -12, and -15, all of which encode tight junction proteins capable of forming calcium-permeable pores for passive calcium transport (1, 6, 15).

What does substantial colonic compensation in cecectomized rats imply? In our opinion, it implies an important role of cecal calcium absorption in body calcium homeostasis as evidenced by body investment in energy-consuming active calcium transport in the distal segment to retrieve the otherwise lost calcium. However, although cecectomized rats appeared normocalcemic at week 4 post-operation, hyperphosphatemia and elevated PTH levels were still evident, suggesting that the normocalcemic state was indeed maintained, in part, by calcium release from bone. In other words, the colonic compensation was insufficient to normalize calcium imbalance after cecectomy. The elevated serum levels of intact PTH, a potent stimulator of osteoclast-mediated bone resorption (44), thus confirmed that the negative calcium balance had not been

Fig. 7. A and B: tibial and femoral bone lengths. Bone mineral density (BMD) and bone mineral content (BMC) of L5–6 vertebrae (C and D), whole tibiae (E and F) and whole femora (G and H) in Cecec and age-matched Sham rats. Bone specimens were collected on day 35 post-operation. Bone lengths were measured with a vernier caliper; BMD and BMC were determined by DEXA. Nos. in parentheses represent nos. of animals. ***P < 0.001 vs. Sham group.

Fig. 8. BMD and BMC in the (A and B) femoral diaphysis, (C and D) proximal (Prox.) femoral metaphysis, and (E and F) distal (Dis.) femoral metaphysis of Cecec and age-matched Sham rats. Bone specimens were collected on day 35 post-operation and later analyzed by DEXA. Nos. in parentheses represent nos. of animals. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Sham group.
normalized solely by the colonic calcium hyperabsorption. Hence, even with this colonic compensation, cecectomized rats manifested massive bone loss with decreases in BMD and BMC of various cortical and trabecular sites, including lumbar vertebrae, tibiae, femoral diaphyses, and femoral metaphyses. Our results were consistent with a previous report of decreased femoral bone strength in ceccoclonecctomized rats on day 20 post-operation (41). However, further histomorphometric analyses are required to elucidate the cecectomy-induced bone loss at the cellular level.

Generally, the trabecula-rich areas, such as vertebrae and metaphyses of long bone, have larger calcium exchange surfaces than the compact (cortical) area in diaphyses (38). Trabecular osteopenia is thus evident in conditions with mild-to-moderate negative calcium balance, e.g., during lactation (9). On the other hand, a combined cortical and trabecular osteopenia in cecectomized suggested that negative calcium balance was relatively severe, and thus it required a huge amount of released calcium from bone to help maintain plasma calcium concentration. Prolonged excessive bone resorption could eventually elevate plasma phosphate levels (23), consistent with that observed in cecectomized rats. Bone loss in the femoral diaphysis may also profoundly affect bone geometry and strength, leading to an increase in fracture risk and disability. However, during the early days post-operation, an increase in renal calcium reabsorption might partially contribute to plasma calcium correction, but it was rather inadequate, because our calcium balance study showed that the cecectomy-induced fecal calcium loss was much greater than the enhanced renal calcium reabsorption (~2.5 vs. ~0.05 mmol·kg body wt⁻¹·day⁻¹ on day 5 after cecectomy).

Since intestinal phosphate transport was not measured in the present study, it remains unknown whether intestinal phosphate absorption is also enhanced in cecectomized rats, which might, in turn, contribute to hyperphosphatemia and elevated PTH levels. Nevertheless, no change in intestinal NaPi-IIb mRNA expression suggested that enhanced intestinal phosphate absorption should not be present in cecectomized rats. In conclusion, we have provided corroborative evidence to support our hypothesis that the cecum is an important site for calcium absorption and that the absence of cecal function is detrimental to the body’s calcium homeostasis, resulting in hyperparathyroidism and widespread osteopenia in as rapid as 4 wk after cecectomy. Moreover, the compensatory colonic hyperabsorption of calcium in cecectomized rats suggests that

Table 2. Electrical parameters of colonic epithelium

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>n</th>
<th>PD, mV</th>
<th>Isc, μA cm⁻²</th>
<th>TER, Ω cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>6.10 ± 0.89</td>
<td>40.30 ± 3.21</td>
<td>142.04 ± 16.94</td>
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<tr>
<td>100 μmol/L neomycin</td>
<td>5</td>
<td>3.02 ± 0.62</td>
<td>28.38 ± 8.03</td>
<td>112.52 ± 11.45</td>
</tr>
<tr>
<td>200 μmol/L neomycin</td>
<td>5</td>
<td>4.04 ± 0.63</td>
<td>29.47 ± 5.98</td>
<td>141.56 ± 19.74</td>
</tr>
<tr>
<td>200 μmol/L spermine</td>
<td>8</td>
<td>2.89 ± 1.10</td>
<td>25.46 ± 7.89</td>
<td>104.21 ± 12.82</td>
</tr>
<tr>
<td>400 μmol/L spermine</td>
<td>3</td>
<td>2.78 ± 1.04</td>
<td>16.62 ± 5.62</td>
<td>112.71 ± 5.83</td>
</tr>
<tr>
<td>800 μmol/L spermine</td>
<td>3</td>
<td>2.63 ± 0.43</td>
<td>22.80 ± 3.55</td>
<td>112.55 ± 34.10</td>
</tr>
<tr>
<td>Distal colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>8.55 ± 2.13</td>
<td>66.58 ± 15.34</td>
<td>119.42 ± 2.67</td>
</tr>
<tr>
<td>200 μmol/L neomycin</td>
<td>3</td>
<td>6.43 ± 2.39</td>
<td>55.74 ± 23.23</td>
<td>119.15 ± 28.26</td>
</tr>
<tr>
<td>800 μmol/L neomycin</td>
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<td>5.59 ± 1.74</td>
<td>54.72 ± 14.52</td>
<td>101.58 ± 17.99</td>
</tr>
<tr>
<td>200 μmol/L spermine</td>
<td>5</td>
<td>5.45 ± 1.09</td>
<td>57.43 ± 14.99</td>
<td>114.18 ± 17.98</td>
</tr>
<tr>
<td>400 μmol/L spermine</td>
<td>4</td>
<td>4.85 ± 1.64</td>
<td>51.31 ± 22.20</td>
<td>103.81 ± 12.03</td>
</tr>
</tbody>
</table>

Values are means ± SE. Epithelial electrical parameters, i.e., transepithelial potential difference (PD), short-circuit current (Isc), and transepithelial resistance (TER), of colonic tissues directly exposed on the mucosal side to calcium-sensing receptor (CaSR) agonists neomycin and spermine. All tissues were obtained from normal rats. The apical side was negative with respect to the basolateral side.

Fig. 9. Active calcium transport in the proximal colon (A) and distal colon (B) directly exposed on the mucosal side to various concentrations of CaSR agonists neomycin and spermine. Intestinal tissues were obtained from 12-wk-old normal female rats. After being mounted in Ussing chamber, the tissue was bathed on both sides with solution containing 1.25 mmol/l calcium. Nos. in parentheses represent nos. of animals. *P < 0.05, **P < 0.01 vs. control group.
the physiological significance of the large intestine with regard to calcium metabolism should be reappraised in other species, including humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.J., P.S., K.W., N.D., and N.C. performed experiments; P.J., P.S., K.W., N.D., and N.C. analyzed data; P.J., P.S., K.W., N.D., and N.C. interpreted results of experiments; P.J., K.W., and N.C. drafted manuscript; N.C. conception and design of figures; P.J., P.S., K.W., N.D., N.K., and N.C. edited and revised manuscript; and N.C. interpreted results of experiments; P.J., K.W., and N.C. prepared figurements; P.J., P.S., K.W., N.D., N.K., and N.C. approved final version of manuscript; K.W., N.K., and N.C. drafted manuscript; N.C. conception and design of research.

REFERENCES