Molecular Mechanisms of Primary Hypercalciuria

KEVIN K. FRICK and DAVID A. BUSHINSKY

Department of Medicine, Nephrology Unit, University of Rochester School of Medicine and Dentistry, Rochester, New York.

Nephrolithiasis, with a lifetime incidence of up to 13% (1–9), results in significant morbidity as well as substantial economic costs, not only directly from medical treatment but also indirectly through time lost from work. Approximately 70% of kidney stones are composed of calcium, generally combined with oxalate and/or phosphate (1,7). Hypercalciuria is the most consistent metabolic abnormality found in patients with calcium nephrolithiasis (1–10). Indeed, idiopathic hypercalciuria (IH), excess calcium excretion with no identifiable metabolic cause, is found in up to 40% of stone-formers (11) but has an incidence of less than 10% in the overall population (12). The elevation in urinary calcium leads to increased supersaturation with respect to a solid phase, generally calcium oxalate or calcium phosphate, which increases the propensity to kidney stone formation (13).

Idiopathic hypercalciuria is an inherited metabolic abnormality (14–17). In pediatric patients with nephrolithiasis, 73% had a family history of kidney stones in at least one first-order or second-order relative, as opposed to a prevalence of 22% in a control population of pediatric renal and urologic patients (18). Of the patients with hypercalciuria, the prevalence of nephrolithiasis in the family history was 69% (18). Coe et al. (19) found a strong inheritance pattern in patients with nephrolithiasis that they conjectured was autosomal dominant. In support of a genetic basis for hypercalciuria, we have selectively bred a strain of rats for this disorder. After almost 60 generations of inbreeding, all of the rats are hypercalciuric: they excrete approximately 8 to 10 times as much calcium as control animals and almost uniformly form kidney stones (13,16,17,20–31) (Figure 1). The ability to select for this trait, hypercalciuria, solidifies the genetic nature of this disorder.

In a normal, nonpregnant, adult, intestinal calcium absorption is precisely balanced by urine calcium excretion so that the total amount of body calcium remains constant. Consuming a typical diet of 20 mmol of calcium per day, approximately 16 mmol are lost to fecal excretion, indicating that 4 mmol are absorbed through the intestine (Figure 2) (9,32,33). The primary reservoir of body calcium, the skeleton, contains about 20 mol of calcium, of which about 14 mmol per day are exchanged through balanced bone formation and resorption. Extracellular fluid includes another 25 mmol of calcium. The kidney filters approximately 270 mmol of calcium per day, of which all but 4 mmol are reabsorbed (3,4). It is logical to assume that idiopathic hypercalciuria is caused by dysregulation of calcium transport at sites where large fluxes of calcium must be precisely controlled: these sites are the intestine, kidney, and bone.

If one could understand the molecular mechanism(s) by which primary hypercalciuria occurs, clinical investigators could then screen families of stone formers before the onset of overt disease and possibly direct treatment at the specific underlying molecular defect(s) in calcium transport. Complicating the search for genes responsible for hypercalciuria are the following caveats: mutations in several pathways can be responsible for hypercalciuria; expression of more than one gene may need to be altered to cause a discernible phenotype; and penetrance of the mutation(s) may be incomplete. A single genetic abnormality responsible for idiopathic hypercalciuria is unlikely, as there appears to be a continuum in the rates of calcium excretion between normal and hypercalciuric humans (7,34) and control and inbred hypercalciuric rats (16,17).

This focus of this review will be the delineation of potential mechanisms responsible for idiopathic hypercalciuria by studying selected known genetic disorders resulting in excess urine calcium excretion. We will exclude hypercalciuria resulting from metabolic abnormalities for which there is no known genetic defect for calcium transport, such as primary hyperparathyroidism, malignancy with production of PTHrP, renal tubular acidosis, vitamin D toxicity, immobilization, hyperthyroidism, and Paget disease (9). We will partition our discussion into disorders affecting the intestine, the kidney, and the bone, as these sites are responsible for regulation of calcium homeostasis and molecular defects in these sites of calcium transport can contribute to hypercalciuria and subsequent stone formation. Although this is an organ-based separation, we must recognize that calcium transport pathways are often generalized and not limited to one anatomic site. In addition, as studies of the genetic hypercalciuric rats have shown (see below), hypercalciuria may involve a dysregulation of multiple calcium transport systems.

Intestinal Calcium Transport

Approximately 90% of calcium absorption occurs in the small intestine, whereas the remaining 10% occurs in the cecum and ascending colon (35). Intestinal calcium absorption
proceeds through two pathways: a nonsaturable paracellular pathway thought to predominate when the diet is replete in calcium, and a saturable vitamin D-dependent transcellular pathway, which becomes the major pathway when dietary calcium is limited (Figure 3) (36). The active, transcellular pathway is downregulated by a diet replete in calcium (37).

Luminal calcium enters the enterocyte at the microvillus border of the apical membrane via facilitated translocation through an epithelial calcium channel, either CaT1 (38) or ECaC (39); diffusion of calcium through the cell is facilitated by binding to calbindin D9k (40), and extrusion of calcium through the basolateral membrane against an electrochemical gradient is achieved by plasma membrane Ca\(^{2+}\)-ATPase (PMCA) (32,33).

CaT1 was isolated from a rat duodenal cDNA library using expression cloning in Xenopus oocytes and assaying \(^{45}\)Ca\(^{2+}\) uptake (38). The 727-amino acid protein encoded has sequence and structural homology to the calcium store-operated channels, which include TRP and TRP-like, important in Drosophila vision; mammalian homologs include the capsaicin receptor, VR1 (43). As expressed in oocytes, the Km for Ca\(^{2+}\) was 0.44 mM, consistent with an estimated intestinal [Ca\(^{2+}\)] of 1 to 5 mM after a calcium-containing meal (38). Rat tissue screening with Northern blots indicates that a 3.0-kb band of hybridization to CaT1 was abundant in small intestine and a 6.5-kb band was present in small amounts in thymus, brain, and adrenal gland; no CaT1 hybridization was detected in kidney.

In situ hybridization to rat intestinal segments indicated a gradient of expression with traversal of the intestine: in the small intestine, CaT1 RNA was present at highest levels in duodenum, to a lesser degree in the proximal jejunum, and absent from the ileum. In the large intestine, CaT1 RNA was abundant in the cecum while present only at low levels in the colon. In all segments, CaT1 RNA was consistently expressed at higher levels in villus tips than in crypts. barley et al. have isolated a cDNA probe, ECAC2, from human duodenum that is 90% identical to rat CaT1 and distinct from human ECAC1 (44) and which they conclude is the human homologue to CaT1 (45). In biopsy samples from 20 healthy volunteers, duodenal RNA levels for ECAC2 correlated with levels of hybridization for calbindin D\(_{9k}\) (r = 0.48; P < 0.05) and PMCA1 (r = 0.83; P < 0.001) but not with 1,25(OH)\(_2\)D\(_3\) or 25(OH)D\(_3\) levels (45), consistent with the finding that rat CaT1 RNA levels are not responsive to treatment with 1,25(OH)\(_2\)D\(_3\) for 15 h (38). However, two strains of vitamin D-receptor deficient (VDR-KO) mice both showed RNA levels of CaT1 in duodenum that were less than 10% of levels in WT controls (46).

CaT1 shares sequence homology to the epithelial calcium
transporter ECaC (39,47), which was isolated by expression cloning from a rabbit connecting tubule/cortical collecting duct cDNA library (39). ECaC RNA is present in duodenum at very high levels and is found as well in the jejunum, kidney, and placenta (39). In the rabbit duodenum, ECaC protein is present in high levels in villus tips, whereas it is virtually absent from crypts, a pattern similar to that seen for CaT1 (48). Double-immunofluorescent labeling of serial sections of duodenum indicated that ECaC, calbindin D_{9k}, and PMCA colocalized to the same cells, with ECaC found apically, PMCA basolaterally, and calbindin D_{9k} distributed throughout the cells (48). Whereas RNA abundance suggests that CaT1 is the principal epithelial calcium channel in duodenum, ECaC RNA levels are also decreased in VDR-KO mice in duodenum (46).

Human diseases in which upregulation of intestinal calcium absorption is clearly the primary lesion are rare. Hypersensitivity to 1,25(OH)_{2}D3 or its metabolites can lead to increased intestinal absorption (49). Scott et al. (50) have found linkage of IH with an apparent absorptive component to microsatellite markers near the VDR locus in a cohort of 47 French-Canadian pedigrees. Jackman et al. (51) have also found a polymorphism in the VDR gene in 19 patients with hypercalcuria and a family history of nephrolithiasis. However, other studies have found no linkage to VDR in different kindreds with hypercalcuria (52,53). Imamura et al. (54) described two unrelated children with increased intestinal absorption of calcium, each with deletion of 4q33-qter and substitution of unknown chromosomal segments. However, human ECaC1 maps to 7q31.1-q31.2 (55), and ECAC2 is nearby at 7q34-q35 (45). The gene for PCMA1 is located at 12q21–23 (56), and calbindin-D_{9k} is on the X chromosome (57). Reed et al. (58) have mapped a defect in three kindreds with increased intestinal absorption of calcium to 1q23.3-q24, and they have identified a group of base substitutions in a putative gene within that locus, four of which increased the relative risk of disease 2.2-fold to 3.5-fold (59). The sequence has pronounced homology to the rat soluble adenylyl cyclase gene and transcripts were detected, although the functionality of this gene has yet to be established (59). It may be of interest that rat CaT1 has a potential protein kinase A phosphorylation site near the amino terminus that is absent from the corresponding region of ECaC (38).

Genetic or acquired renal phosphate wasting will lead to hypophosphatemia and an increase in 1,25(OH)_{2}D_{3}, resulting in excess intestinal calcium absorption and hypercalcuria. Hereditary hypophosphatemic rickets with hypercalcuria (HHRH) is an example of this pathophysiology (60). Patients with this disease have decreased reabsorption of phosphate, high serum levels of 1,25(OH)_{2}D_{3}, and enhanced intestinal absorption of calcium. These symptoms are similar to the phenotype of mice with constructed deletion of the gene for the kidney-specific Na-Pi cotransporter Npt2, although the bone phenotype is different; while HHRH patients display rickets and osteomalacia, the Npt2 knockout (KO) mice show a delay in bone mineralization at 21 d after birth and an increase in number of trabeculae and decrease in marrow space at 115 d (61). Npt2 KO mice display higher levels of duodenal mRNA for ECaC, CaT1, and calbindin D_{9k}, consistent with their known increase in intestinal calcium absorption (62). However, mapping of the Npt2 locus in HHRH kindreds revealed no mutations that cosegregated with the disease (63,64). To screen for mutations in Npt2, Prie et al. (65) sequenced the gene from 20 patients with urolithiasis or bone demineralization associated with idiopathic hypophosphatemia and reduced phosphate reabsorption. Two patients with Npt2 mutations were identified, one with a substitution of phenylalanine for arginine 48 (exon 3), and the second with a methionine for valine 147 substitution (exon 5); both patients were heterozygous for these mutations. Each of these mutations lies in regions conserved between human, rat, rabbit, mouse, opossum, and flounder. Sequencing of exons 3 and 5 in 120 controls excluded these mutations as common polymorphisms. Microinjection of either mutant RNA into Xenopus oocytes induced a smaller phosphate current than did injection of wild-type Npt2 RNA. Coinjection of mutant RNA plus wild-type RNA led to a smaller current than wild-type RNA alone, consistent with the dominant negative effect of Npt2 mutation inferred from the genotypes of the patients.

Another class of molecular defects resulting in hypophosphatemia includes X-linked hypophosphatemic rickets/osteomalacia (XLR) and its mouse homolog, hyp; autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR); and tumor-induced rickets/osteomalacia (TIO or OHO) (66). These hypophosphatemias are characterized by normocalcemia and normocalciuria and low to normal circulating levels of 1,25(OH)_{2}D_{3} (66). In 1995, a gene defective in XLH was discovered and named PHEx, for phosphate-regulating gene homologs to endopeptidases, on the X-chromosome (67). This gene bears the hallmarks of a membrane-bound metalloproteinase, and more than 150 PHEx mutations have been documented (68). It was hypothesized that PHEx was necessary for inactivation of an unidentified peptide, which decreased renal phosphate reabsorption, and was termed “phosphatonin”. ADHR and TIO were hypothesized to result from dysregulated expression of phosphatonin (69). The gene for ADHR was linked to chromosome 12p13 (70) and identified as a member of the fibroblast growth factor family, FGF-23 (71); FGF-23 was also identified as the substance overproduced by tumors in TIO (72). FGF-23 inhibits renal epithelial cell phosphate uptake in vitro (73). Mutations found in patients with ADHR have been shown to alter proteolytic cleavage sites of FGF-23, which do not allow it to be inactivated (72) by PHEX (73). Serum levels of FGF-23 are elevated in TIO and XLR (74). A second potential phosphatonin produced by tumors causing osteomalacia has been identified as frizzled-related protein-4 (69,75), a member of the family of Wnt receptors. The mechanism by which frizzled-related protein-4 overproduction results in hyperphosphaturia is not yet clear. Other phosphatogens are known to exist: targeted disruption of the Na\(^+/H\)^+ exchanger regulatory factor NHERF-1, a protein that binds both NHE3 and Npt2, results in hyperphosphaturia and hypercalcemia (76). Although the total amount of Npt2 was not affected in NHERF-1 null mice, immunostaining revealed that, rather than being located on the apical surface, Npt2 was internalized in vesicles.
To date, the role of the phosphatoninins, FGF-23 and frizzled-related protein-4, have not been studied in HHRH. Although HHRH is inherited as an autosomal recessive (60) the known mutations in phosphatonin pathways have X-linked or autosomal dominant inheritance (75).

**Renal Calcium Transport**

**Calcium Transport in the Proximal Tubule.** Urinary calcium excretion must equal net intestinal calcium absorption to maintain calcium homeostasis. The kidney filters approximately 270 mmol of calcium, of which more than 98% must be reabsorbed to maintain neutral calcium balance (9,32,33). Any disorder of renal calcium reabsorption leads to hypercalciuria and the potential for stone formation. Approximately 70% of reabsorption occurs in the proximal tubule, predominantly through paracellular pathways with salt and water carrying calcium from the lumen to the interstitium through the transport mechanism of solvent drag (Figure 4).

**Dent Disease (X-Linked Recessive Nephrolithiasis).** Dent and Friedman (77) reported a form of Fanconi syndrome (generalized proximal tubule transport defects) with hypercalciuria, low–molecular weight proteinuria, nephrolithiasis, and nephrocalcinosis. Positional cloning revealed that this disease, as well as X-linked recessive nephrolithiasis, X-linked hypophosphatemic rickets, and idiopathic low–molecular weight proteinuria, nephrolithiasis, and nephrocalcinosis. Positional cloning revealed that this disease, as well as X-linked recessive nephrolithiasis, X-linked hypophosphatemic rickets, and idiopathic low–molecular weight proteinuria of Japanese children, are all the results of defects in the CLC-5 chloride channel (78–81). The CLC family of chloride channels encompasses nine members, including CLC-Kb, involved in some cases of Bartter disease (see below) (82,83) and CLC-1, the muscle chloride channel, mutated in Thomsen myotonia (84). These channels are voltage-gated and outwardly rectifying, with a high selectivity for chloride. Lutckx et al. (85) have found by immunohistochemistry that CLC-5 is localized to the S3 segment of the proximal tubule and to mTAL. Subsequent subcellular fractionation studies indicate that CLC-5 is localized to endosomes (85).

To examine the biologic effects of decreased CLC-5, Luyckx et al. (86) designed a hammerhead ribozyme specific for CLC-5 and produced transgenic knockdown mice (RZ). By Western blotting, kidney membranes from RZ mice had an 80% reduction in levels of CLC-5. There was no difference in serum chemistries between RZ and controls. Urine calcium/creatinine ratios were significantly higher in male RZ mice as compared with gender-matched controls, whereas calcium excretion in female RZ mice was not significantly different from female controls. Both groups of female mice had greater calcium excretion than the males. When fed a low-calcium diet, calcium excretion in the male RZ mice was not different from control male mice. As the mice aged, urine calcium excretion fell and normalized by 18 wk, hampering efforts to determine whether the hypercalciuria in the RZ mice caused nephrolithiasis or osteopenia.

To further examine the phenotype resulting from CLC-5 deficiency, two other groups made total knockouts of CLC-5 in mice, producing animals with somewhat divergent phenotypes. Piwon et al. (87) disrupted exon 5 and eliminated exon 6 of CLC-5 and substantiated that CLC-5 protein was undetectable by Western blots in kidney, liver, intestine, or testis. Both CLC-5 +/y (male knockout) and −/− (female knockout) animals were normocalcemic, normocalciuric, and hyperphosphaturic. Serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ levels were reduced in both −/− and −/−/− animals, whereas PTH levels were not significantly increased. However, urine from −/− and −/−/− animals contained higher levels of PTH relative to creatinine (PTH/Cr) and 25(OH)D$_3$/Cr, suggesting urinary loss of calcium-mobilizing proteins. SDS-PAGE analysis of the urine from both the male and female knockouts demonstrated increased levels of many urinary proteins, including vitamin D–binding protein (DBP), albumin, and retinol-binding protein, suggesting that CLC-5 is needed for endocytic retention of small proteins, an established property of proximal tubule. Gunther et al. (88) localized CLC-5 in the proximal tubule by immunocytochemistry; although diffuse CLC-5 staining can be seen throughout the cell, higher concentrations are seen in vesicles adjacent to the brush border membrane, a region known to be important for endocytic activity.

**Proximal Tubule**

<table>
<thead>
<tr>
<th>Lumen</th>
<th>Cell</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Drag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration difference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potential difference</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.** Reabsorption of calcium in the proximal tubule. Approximately 70% of filtered calcium is reabsorbed in the proximal tubule through a paracellular pathway predominantly by solvent drag (32,33).
imal tubule of \(-/y\) animals, consistent with the observed hyperphosphaturia (87). However, a low-Pi diet increased the expression of Npt2. When PTH was administered to Pi-depleted animals, Npt2 was internalized within 15 min in WT animals, but internalization took 1 h in \(-/y\) animals. In the proximal tubule, PTH binds to megalin and is endocytosed for degradation; in \(-/y\) proximal tubule, the delayed degradation causes a luminal gradient of PTH to form, such that S3 segments are exposed to higher PTH levels and show higher Npt2 internalization than do S1 segments (87).

Wang et al. (92) also inactivated CLC-5 by insertion of a neomycin resistance cassette between exon 5 and exon 6; \(-/y\) mice failed to produce CLC-5 RNA or protein. All \(-/y\) and \(-/+/y\) animals were normocalcemic, hypercalciuric, proteinuric, and \(-/y\) (males) were hyperphosphaturic. These animals had an elevation in both urinary amino acids and low–molecular weight proteins, including DBP and Clara cell protein, due to impaired endocytosis. About 7% of \(-/y\) mice had spinal deformities and backward growth of teeth consistent with abnormal calcium metabolism and skeletal growth. Thus these animals closely mimicked the clinical presentation of patients with Dent disease.

Silva et al. (83) have examined the effects of vitamin D deficiency and thyroparathyroidectomy (TPTX) in rats on CLC-5 RNA and protein levels. The combination of vitamin D deficiency and TPTX caused a fourfold to fivefold increase in urinary Ca/Cr, which was normalized by restoration of PTH. Surprisingly, serum Ca was not affected by vitamin D deficiency or TPTX. CLC-5 RNA levels from the renal cortex were decreased by about 45% in the vitamin D–deficient TPTX rats, and CLC-5 protein was not detected; PTH repletion restored the levels of CLC-5 RNA and protein. No changes in CLC-5 RNA or protein were seen in renal medulla. These studies suggest that PTH regulates CLC-5.

It is not clear how a loss of CLC-5 function leads to hypercalciuria; however, abnormal regulation of PTH and 1,25(OH)\(_2\)D\(_3\) synthesis appear important. The diminished recycling of luminal PTH receptors leads to increased local concentrations of PTH, as shown by the higher levels of Npt2 internalization in S3 segment as compared with S1 segment of the proximal tubule (92). As has been observed, the decreased numbers of cell surface phosphate transporters should inhibit reabsorption of phosphate and lead to hyperphosphaturia. However, as PTH induces calcium reabsorption, higher local PTH concentrations alone would not explain the observed hypercalciuria. With loss of functional CLC-5, the decrease of small protein reuptake profoundly affects vitamin D metabolism. The majority of circulating 1,25(OH)\(_2\)D\(_3\) is protein-bound and lost when the vitamin D–binding protein (DBP) is not conserved as has previously been shown in the megalin knockout mice (89,91). In addition, the proximal tubule is the site of conversion of 25(OH)D\(_3\) to 1,25(OH)\(_2\)D\(_3\) (84,93). The latter would increase intestinal absorption of calcium, resulting in hypercalciuria. CLC-5 has also been detected in mouse intestine (93) and here could play a role in calcium absorption by controlling endocytosis of epithelial calcium channels. Furthermore, it is not clear if all cellular CLC-5 is associated with endosomes or whether it may also play a role in other chloride circuits, analogous to CLC-Kb in Bartter type 3 (see below).

**Calcium Transport in the Thick Ascending Limb of Henle’s Loop.** Twenty percent of filtered calcium is reabsorbed in the thick ascending limb of the loop of Henle (TAL), via both paracellular and transcellular processes (Figure 5). In this segment, a lumen-positive voltage generated by the Na/K/2Cl transporter (NKCC2/BSC-1) provides the driving force for paracellular transport of calcium. Inhibition of NKCC2 by loop diuretics (bumetanide, furosemide) (94,95) causes a decrease in the lumen-positive voltage resulting in decreased paracellular calcium reabsorption leading to hypercalciuria. The study of inherited tubulopathies, particularly Bartter syndrome (96–98), has proven valuable for understanding the mechanisms of ion transport in the kidney.

**Figure 5.** Reabsorption of calcium in the thick ascending limb of the loop of Henle. Approximately 20% of filtered calcium is reabsorbed in the thick ascending limb of the loop of Henle. Paracellular reabsorption is driven by a lumen-positive voltage created through sodium/potassium/2 chloride reabsorption. Sodium, potassium, and chloride enter the cell through the bumetanide-sensitive transporter NKCC2, driven by low intracellular concentrations of Na\(^+\) and Cl\(^-\) maintained by the activity of Na\(^+\)/K\(^+\)-ATPase and the chloride channel CLC-Kb. Potassium reenters the tubular lumen through the ROMK channel, creating the lumen-positive voltage that drives calcium through the tight junctions, composed largely of paracellin-1 (PCLN-1), into the blood. The calcium-sensing receptor (CaSR) regulates ROMK and thus calcium reabsorption (32,33).
Barter Syndrome. Barter syndrome was first described as hypokalemic, hypochloremic metabolic alkalosis (96). The primary mechanism for this disorder is a failure to adequately reabsorb sodium in the thick ascending limb of Henle’s loop. The syndrome varies widely in the degree of severity; the most severely affected individuals present before birth with polyhydramnios as a consequence of fetal polyuria and are often delivered prematurely (99). Severe hypercalciuria often results in rapid progression of nephrocalcinosis. Other patients with Barter syndrome may be asymptomatic into adulthood. Although the condition usually appears sporadically, it can also be inherited as an autosomal recessive trait (100). The loop diuretics mimic the urinary effects of Barter syndrome (sodium wasting, kaliuresis, hypercalciuria) by inhibiting NKCC2 (94), and some Barter syndrome patients have an impaired response to furosemide (101); it was therefore a logical extension to look for NKCC2 defects in Barter syndrome patients. Simon et al. (102) demonstrated that nine children with Barter syndrome from four families had mutations in NKCC2, many of which would introduce premature truncation of the protein.

The driving force for sodium and chloride uptake in the thick ascending limb is derived from the low intracellular concentrations of these ions maintained by basolateral Na/K-ATPase and chloride channels (CLC-Kb), and the activity of NKCC2 is dependent on the presence of luminal potassium (Figure 5). Recycling of potassium ions by the ATP-regulated potassium channel ROMK provides the requisite K+ and generates the lumen-positive potential that drives paracellular calcium transport (98). In type 1 Barter syndrome, there is a mutation of NKCC2; in type 2 Barter syndrome, there is a mutation in the potassium channel ROMK (103,104); in type 3, there is a mutation in the chloride channel CLC-Kb (105,106). Each of these mutations, in NKCC2, ROMK, or CLC-Kb, leads to a decrease in the lumen-positive voltage and a reduction in calcium reabsorption; however, while all of these patients are hypercalciuric, the type 3 patients rarely present with nephrocalcinosis (105,106).

The calcium-sensing receptor (CaSR), present in TAL as well as the proximal tubule, DCT, and CCD (107,108), monitors serum calcium levels and regulates renal tubular calcium reabsorption. Inactivating mutations of the calcium-sensing receptor cause familial hypocalciuric hypercalcemia (FHH) (109). Hebert et al. (110) offer a useful model on the regulation of renal calcium reabsorption by the CaSR. In the presence of elevated basolateral levels of calcium, where reabsorption should be minimized, G_i coupled to the CaSR induces a reduction in intracellular cAMP levels, which in turn limit the activation of NKCC2. The calcium-sensing receptor can also activate phospholipase A2 to produce arachidonic acid, the metabolites of which, including 20-HETE, inhibit NKCC2 and ROMK. In support of this mechanism, Pearce et al. have found six kindreds with an autosomal dominant inheritance of hypocalciemia and hypercalciuria who proved to have activating mutations in the calcium-sensing receptor (111,112). Vezzoli et al. (113) examined four single-nucleotide polymorphisms (SNP) in exon 7 of the calcium-sensing receptor and found one SNP associated with a 13-fold higher risk of hypercalciuria.

Mutations in the paracellular channel that resulted in reduced calcium reabsorption would also be expected to lead to hypercalciuria. Paracellin-1 (PCLN-1) was identified by Simon et al. (114) as the principal protein in the tight junctions of the TAL and is a member of the claudin family of tight junction proteins (claudin-16) (115). Mutations in PCLN-1 were found in cases of familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC, also known as hypomagnesemia hypercalciuria syndrome, HHS) (116). Two unrelated patients with HHS and missense mutations in PCLN-1 were confirmed to have defective reabsorption of magnesium and calcium without excessive loss of sodium (117). Furosemide infusion increased sodium excretion sixfold both in controls and in the 2 HHS patients; however, Mg²⁺ and Ca²⁺ excretion increased twofold and ninefold, respectively, in controls but did not change in patients with HHS. MgCl₂ infusion elicited a sixfold increase in fractional excretion of calcium in the controls but did not induce hypercalciuria in the patients with HHS (117).

These data support the hypothesis that primary sequence alterations in PCLN-1 affect calcium and magnesium reabsorption.

Screening of 25 European families with FHHNC, including 33 affected individuals and their nonaffected relatives, revealed that 94% of the affected patients had mutations in PCLN-1, with 48% of those being a missense Leu151Phe mutation (116). The common genetic abnormality and the finding that many of these patients lived in a single geographic region (Germany or Eastern Europe) suggested that the mutation occurred in a common ancestor many generations ago (founder effect) (116). An alternative, but less likely explanation is that DNA sequences around Leu151 are unstable. In 13 of 23 families, there was an increased incidence of hypercalciuria and nephrolithiasis in heterozygotic individuals not affected by FHHNC, suggesting that heterozygotes have a partial defect in paracellular calcium transport (gene dosage effect) (116).

Calcium Transport in the Distal Tubule. The remaining 8% of filtered calcium is reabsorbed in the distal convoluted tubule and connecting tubule; reabsorption in these segments is predominantly active, transcellular transport under hormonal regulation (Figure 6) (9,32,33,47,98,118). Similar to transport in the intestine, in the distal convoluted and connecting tubule, calcium enters the cell at the apical surface through a calcium channel and binds to the calcium binding protein calbindin, which serves as a shuttle to transport calcium across the cell. At the basolateral surface, calcium is extruded against an electrochemical gradient. However, the molecular species of the proteins involved in renal calcium transport differs from those in the intestine.

The identity of the apical calcium channel in distal tubule remains somewhat controversial; Hoenderop et al. (119) did not detect CaT1 RNA in human kidney RNA using RT-PCR but did find abundant ECaC RNA, while Peng et al. (120,121) found evidence of CaT1 RNA in human kidney RNA as well as ECaC, localized to distal nephron. Abundance studies using real-time PCR suggest that CaT1 RNA is more abundant than ECaC RNA in kidney (122). Fractionation studies with isolated rat tubules indicate that CaT1 RNA is found primarily in
Although apical calcium entry is generally considered to be the rate-limiting step in reabsorption into the blood occurs through the Na\(^+/Ca\(^{2+}\) exchanger (NCX) and plasma membrane Ca\(^{2+}\)-ATPase (PMCA) (32,33).

In the distal tubule, calbindin D\(_{28k}\) acts as the principal calcium shuttle (47,118). Calbindin D\(_{9k}\) and parvalbumin, another calcium-binding protein, are found only at the basolateral membrane and may play a role in the extrusion of calcium from the basolateral surface of the cell (125). Calbindin D\(_{28k}\) knockout mice (126) exhibit excessive hypercalciuria when fed a high-calcium diet (127). Basolateral calcium transport occurs via both the plasma membrane Na\(^+/Ca\(^{2+}\) exchanger (NCX) and Ca\(^{2+}\)-ATPase (PMCA), which have been estimated to transport 70% and 30% of calcium respectively (128,129).

Calcium transport in the distal tubule is of particular interest because it is regulated by parathyroid hormone (PTH) and 1,25(OH)\(_2\)D\(_3\). Although apical calcium entry is generally considered to be the rate-limiting step in reabsorption, these calcitropic hormones may affect one or several components of the pathway.

Hoenderop et al. (130) have examined the effects of 1,25(OH)\(_2\)D\(_3\) depletion and repletion on ECaC expression. Rats were made vitamin D-deficient and hypocalcemic by feeding a nonrachitogenic D-deficient diet, followed by a vitamin D-deficient, Ca-free diet for 2 wk. This regimen led to drop in plasma Ca\(^{2+}\) and a decline in 1,25(OH)\(_2\)D\(_3\); injection of 1,25(OH)\(_2\)D\(_3\) led to rapid repletion. Vitamin D deficiency caused a decline in mRNA for ECaC and reduced levels of immunofluorescent labeling; restoration of vitamin D levels led to upregulation of ECaC RNA and protein. Vitamin D repletion also resulted in upregulation of RNA and protein for calbindin-D\(_{28k}\). ECaC and calbindin-D\(_{28k}\) colocalize to cells in the DCT and CNT.

Bone Resorption and Hypercalciuria. Many patients with hypercalciuria, especially if they are consuming a low-calcium diet, excrete more calcium than they absorb (1–10). The source of the additional urinary calcium must be the skeleton, by far the largest reservoir of calcium in the body. Several studies have confirmed that patients with nephrolithiasis generally have a reduction in the density of their skeletons compared with age-matched and gender-matched controls (131–136). Pietschmann et al. (131) examined bone density in 120 patients with nephrolithiasis and found lower spinal bone mineral density (BMD) in those who were hypercalcicuric compared with those who were normocalcicuric. Jaeger et al. (132) assayed BMD in 110 male Swiss idiopathic stone formers and compared the results with 234 controls. Stone formers were slightly shorter, had a higher BMI (body mass index), but a significantly lower BMD at the tibial diaphysis and the tibial epiphysis compared with the controls. There was no difference between normocalcicuric and hypercalcicuric stone formers at these sites. Ten of seventeen patients on a low-calcium diet for at least 1 yr had a decreased BMD and 27 stone formers reported fractures as compared with none of the controls. Giannini et al. (133) found that 49 patients with recurrent stones and IH had a lower lumbar spine Z-score than normal controls, and they also had significantly higher bone alkaline phosphatase levels and lower blood pH than controls. Misael da Silva et al. (134) examined bone formation and resorption parameters in 40 nephrolithiasis patients and classified ten as osteopenic. Forty-five percent of hypercalcicuric patients were classified as osteopenic; they had about 20% less bone mass than normocalcicuric controls. While bone volume was not different between controls and hypercalcicuric patients, the hypercalcicuric patients had increased osteoid thickness, a greater percentage of eroded surface, and increased osteoclast and osteoblast surface as a fraction of bone surface. Mineralization lag time was also greater in hypercalcicuric patients as compared with controls. Tasca et al. (135) have found a more negative Z-score in L1-L2 in hypercalcicuric patients than in controls.

In an attempt to preserve bone in patients with hypercalcicuric nephrolithiasis and osteopenia, the bisphosphonate, etidronate, was administered to seven male patients (136). Patients were also advised to eat more calcium and less animal protein and salt. At 1 yr, patients, each of whom received treatment, had a significantly higher spinal (L2-L4) T-score, although no difference was seen at 2 yr. Unfortunately, compliance was poor in the study; patients showed no net increase in calcium uptake, no decrease in protein consumption, and a significant increase in NaCl consumption. Taken as a whole, these data indicate that patients with hypercalcicuria are at risk for bone loss; as a consequence their bone density, as a proxy for calcium balance, should be monitored. A low-calcium diet is not effective in reducing the risk of stone recurrence and poses a substantial risk to maintenance of bone health (137,138).

Genetic Hypercalciuric Stone-Forming Rats

To model idiopathic hypercalciuria and spontaneous stone formation in humans, we have developed an animal model of
hypercalciuria and nephrolithiasis (13, 16, 17, 20–31). Given evidence for a genetic predisposition to hypercalciuria in both humans and rats (14, 19, 139, 140), Bushinsky and coworkers screened adult male and female Sprague-Dawley rats for hypercalciuria and used the animals with the highest urinary calcium excretion to breed the next generation, followed by subsequent selection and inbreeding of their most hypercalciuric offspring, repeating the selection for almost 60 generations (16, 20–30). By the thirtieth generation, the GHS rats (for genetic hypercalciuric stone-forming) were excreting nearly ten times as much calcium as simultaneously studied control female rats (Figure 1) (13, 16, 17, 20–31). The rats were found to have defects in calcium transport in the intestine, kidneys, and bone, similar to abnormalities found in patients with idiopathic hypercalciuria (1–10).

### Intestinal Calcium Absorption

Bushinsky and Favus (20) studied the rate of intestinal calcium transport in GHS rats. Fourth generation GHS rats not only exhibited increased urinary calcium excretion but also had significantly elevated net intestinal calcium absorption. Net duodenal calcium absorption, measured in vivo as well as in vitro, was greater in the GHS rats despite lower 1,25(OH)2D3 levels in GHS males compared with normocalciuric males, and no difference in 1,25(OH)2D3 levels among the females. When the female control and GHS rats were placed on a low-calcium diet, there was an increase in serum levels of 1,25(OH)2D3 in both groups. However, there was a greater increase in net intestinal calcium absorption in the GHS rats, even though 1,25(OH)2D3 did not increase as much as in controls. These results suggested that the GHS rats were similar to the majority of humans with idiopathic hypercalciuria; that is, there was an increase in both urine calcium excretion and intestinal calcium absorption with normal to only slightly elevated 1,25(OH)2D3 levels (141, 142).

### The Vitamin D Receptor

The finding of increased intestinal calcium absorption without an elevation in 1,25(OH)2D3 levels led Li et al. to hypothesize that alteration of the receptor for vitamin D might be responsible for the abnormal regulation of calcium by enterocytes (22, 143). The intensity of 1,25(OH)2D3 action correlates with receptor number and saturation (144, 145) both in rats in vivo (146–148) and in cell culture studies in vitro (149, 150). The vitamin D receptor-rich cytosolic fractions from GHS rat proximal duodenum bound more [3H]1,25(OH)2D3 than similar fractions prepared from normocalciuric controls (22). Using Scatchard analysis, we demonstrated that this increase in binding of 1,25(OH)2D3 by the vitamin D receptor was due to an increase in the number of intestinal binding sites rather than enhanced affinity of the vitamin D receptor for its ligand. Northern analysis of GHS and control rat mRNA revealed no increased expression of the vitamin D receptor gene to account for the increase in receptor number. Gene transcription of the vitamin D receptor was comparable for both groups of rats, as was synthesis of the vitamin D-dependent calcium-binding protein, calbindin D9K. Using western blot analysis, however, more calbindin D9K was detected in intestinal protein from the GHS rats than from controls. There is thus an increase in 1,25(OH)2D3 action in GHS rats despite normal serum levels of 1,25(OH)2D3.

Yao et al. (30) found that the vitamin D receptor in the GHS rats hyperresponded to minimal doses of 1,25(OH)2D3. The hyperresponsiveness occurred through an increase in vitamin D receptor stability without involving alterations in VDR gene transcription, de novo protein synthesis, or mRNA sequence. 1,25(OH)2D3 administration also led to an increase in duodenal and renal calbindin mRNA levels in GHS rats, whereas levels were either suppressed or unchanged in wild-type animals. Thus this hyperresponsiveness appears to be of functional significance in that it affects vitamin D receptor-responsive genes in 1,25(OH)2D3 target tissues.

### Response to a Low-Calcium Diet

To determine if, in addition to enhanced intestinal calcium absorption, other mechanisms were contributing to hypercalciuria in the GHS rats, nineteenth-generation GHS and normocalciuric control rats were placed on a diet nearly devoid of calcium (0.02%) and compared with similar rats eating a normal calcium (0.6%) diet (21). On the normal-calcium diet, both groups of rats were in positive calcium balance, despite marked hypercalciuria in the GHS rats. On both the normal-calcium and low-calcium diets, intestinal calcium absorption was greater in the GHS rats compared with controls. When placed on the low-calcium diet, both normal and GHS rats had a decrease in urinary calcium excretion; however, urinary calcium excretion in the GHS rats was eight times greater than that in controls. This persistent calcium excretion in the GHS rats when fed a low-calcium diet placed many of them, but not the controls, in negative calcium balance.

### Defective Renal Tubular Calcium Reabsorption

To determine if GHS rats have a defect in renal calcium reabsorption, Tsuruoka et al. (28) performed 14C-inulin clearance studies on female GHS and control rats. Some GHS and control rats were fed standard rat chow, and others were fed a similar amount of a low-calcium diet. Each rat was parathyroidectomized and infused with calcium chloride to maintain normal concentration of serum calcium. After equilibration, urine was collected for three periods with blood ultrafiltrable calcium and 14C-inulin levels obtained at the midpoint. We found that both GHS and control rats had similar GFR and the same ultrafiltrable calcium concentrations resulting in similar filtered loads of calcium (28). Despite the consistency of calcium presented to the proximal tubule, the GHS rats had approximately three times the fractional calcium and urinary calcium excretion compared with control rats. The results were similar whether the rats were fed a normal-calcium or a low-calcium diet.

### Primary Bone Resorption

The increased sensitivity to 1,25(OH)2D3 observed in enterocytes, which is presumably due to the increase in number of vitamin D receptors (22), may be important in bone, as well. To determine if GHS rat bones are more sensitive to exogenous 1,25(OH)2D3 compared with bone from control rats, we cultured calvariae from neonatal GHS and control rats with or without 1,25(OH)2D3 or PTH for 48 h (25). There was significant stimulation of calcium efflux from GHS calvariae at 1 and 10 nM 1,25(OH)2D3, while control calvariae showed no significant response to 1,25(OH)2D3 at any concentration tested. In contrast, PTH induced a similar degree of bone resorption in control and GHS
Null Mutation Terminology: Introduction of an altered transgene into the germline of mice can, by the process of homologous recombination, lead to disruption of the native gene, producing a null mutation, which is also known as a knockout animal. Animals with a null mutation for both copies of the allele (homozygotes) are designated by the genotype $\sim /\sim$. Animals with a null mutation for one copy of the allele (heterozygotes) are designated by the genotype $\sim /+$. If the disrupted gene is on the X chromosome, male knockouts have genotype $\sim /y$, while female knockouts are $\sim /$.

Paracellular absorption: transport through cell-to-cell junctions

PCLN1: paracellin-1, a major component of TAL tight junctions

PMCA: plasma membrane calcium ATPase

ROMK: outwardly rectifying membrane K⁺ channel

Transcellular absorption: transport through the cytoplasm of the cell

References


68. Fukumoto S, Yamashita T: Fibroblast growth factor-23 is the phosphaturic factor in tumor-induced osteomalacia and may be phosphatonin. *Current Opin in Nephrol Hypertens* 11: 385–389, 2002


146. Favus MJ, Mangelsdorf DJ, Tembe V, Coe BJ, Haussler MR: Evidence for in vivo upregulation of the intestinal vitamin D

Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/