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Expression of Trans- and Paracellular Calcium and Magnesium Transport Proteins in Renal and Intestinal Epithelia During Lactation

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Abstract

Significant alterations in maternal calcium (Ca\textsuperscript{2+}) and magnesium (Mg\textsuperscript{2+}) balance occur during lactation. Ca\textsuperscript{2+} is the primary divalent cation mobilized into breast milk by demineralization of the skeleton and alterations in intestinal and renal Ca\textsuperscript{2+} transport. Mg\textsuperscript{2+} is also concentrated in breast milk, but the underlying mechanisms are not well understood. To determine the molecular alterations in Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport in the intestine and kidney during lactation, 3 groups of female mice consisting of either non-pregnant controls, lactating mice, or mice undergoing involution were examined. The fractional excretion of Ca\textsuperscript{2+}, but not Mg\textsuperscript{2+}, rose significantly during lactation. Renal 1-alpha hydroxylase and 24-OHase mRNA levels increased markedly as did plasma 1,25 dihydroxyvitamin D levels. This was accompanied by significant increases in intestinal expression of Trpv6 and S100g in lactating mice. However, no alterations in the expression of cation permeable claudins (-2, -12 or -15) were found in the intestine. In kidney, increased expression of Trpv5 and Calb1 was observed during lactation, while no changes in claudins involved in Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport (-2, -14, -16 or -19) were found. Consistent with the mRNA expression, both Calbindin-D\textsubscript{28K} and TRPV5 protein expression increased. Colonic Trpm6 expression increased during lactation, while renal Trpm6 remained unaltered. In conclusion, proteins involved in transcellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport pathways increase during lactation, while expression of paracellular transport proteins remained unchanged. Increased fractional Ca\textsuperscript{2+} excretion can be explained by vitamin D-dependent intestinal hyperabsorption and bone demineralization, despite enhanced transcellular Ca\textsuperscript{2+} uptake by the kidney.
Introduction

In lactating women, an estimated 200 mg of calcium (Ca$^{2+}$) and 25 mg of magnesium (Mg$^{2+}$) are mobilized into breast milk each day (36, 51). While serum concentrations of these minerals remain unchanged from pre-pregnancy values during lactation (36), Ca$^{2+}$ and Mg$^{2+}$ concentrations in human milk are 3-fold and 2-fold greater than the serum concentrations of these electrolytes, respectively (17, 36). Stable isotope studies reveal a net negative Ca$^{2+}$ balance of 104 mg per day in lactating women with low calcium intake two months post-partum (48). Much of the Ca$^{2+}$ mobilized into breast milk derives from demineralization of bone. This occurs via increased bone resorption and results in decreased bone mineral density. Women lose 2-9% of their trabecular bone mass as well as a smaller fraction of cortical bone, during lactation (29, 43, 54). Lactation in mice results in an even greater loss of bone. Mice lose about 20% of spinal bone mineral content by the 3rd week of lactation (31, 61). In fact, the concentration of Ca$^{2+}$ in breast milk in mice may reach as high as 95 mM, a concentration 10-fold higher than that found in lactating women (52). These species differences are likely due to the larger number of offspring mice produce. Consistent with this, breastfeeding mothers of twins secrete nearly double the amount of Ca$^{2+}$ into breast milk, compared to mothers with a singleton pregnancy (35).

In addition to altered bone remodeling, changes in intestinal and renal Ca$^{2+}$ transport may occur to supply sufficient Ca$^{2+}$ to the neonate (32, 44). In women, no alteration in intestinal Ca$^{2+}$ absorption during lactation has been observed when compared to non-lactating or non-pregnant women (25, 30, 54). In contrast, fractional intestinal Ca$^{2+}$ absorption in rodents may be double that of non-lactating control animals (6, 7, 21). Increased renal reabsorption of minerals may also occur in women whereby urinary Ca$^{2+}$ is significantly decreased during lactation (29, 36, 54).
While one study suggests similar alterations in lactating female rats, these findings have not been confirmed in mice (7). The discrepancies observed between rodents and women are likely explained again by their larger number of offspring and hence an increased demand for Ca\textsuperscript{2+} in breast milk.

Although less well recognized, Mg\textsuperscript{2+} is also mobilized into human breast milk during lactation, reaching concentrations of 1.4 mM (17). The concentration of Mg\textsuperscript{2+} in rodent breast milk is 6.8 mM, 5 times that observed in milk of lactating women (17, 28). Importantly, 60-65% of total body Mg\textsuperscript{2+} is found in bone and may be crucial to achieve optimal bone mineral density of the lumbar spine (17, 47). Hence, it may be important to deliver sufficient Mg\textsuperscript{2+} to the rapidly growing skeleton of the neonate. Mg\textsuperscript{2+} might be mobilized via similar processes to Ca\textsuperscript{2+}, i.e. by bone demineralization during lactation. However, whether altered transport of Mg\textsuperscript{2+} occurs in intestinal and renal epithelia during lactation has not been examined. Studies of lactating women report conflicting results. Either decreased urinary Mg\textsuperscript{2+} excretion or no change was reported. These discrepancies may be due to differences in dietary Mg\textsuperscript{2+} intake (13, 32, 37). Conversely, balance studies in the lactating rat have suggested that urinary Mg\textsuperscript{2+} excretion remains unaltered during lactation (8).

Ca\textsuperscript{2+} is absorbed from the intestines via transcellular or paracellular pathways (2, 16). Paracellular permeation is controlled by tight junction proteins along the length of the small intestine and is considered the only pathway of Ca\textsuperscript{2+} flux in the ileum and jejunum (16, 26). Ca\textsuperscript{2+} can move through paracellular pores, driven by passive diffusion down its concentration gradient, by solvent drag generated via the absorption of Na\textsuperscript{+} through the Na\textsuperscript{+}-H\textsuperscript{+} exchanger.
(NHE3) (2, 9) or a combination thereof. Claudins -2, -12 and -15 are tight junction proteins that form cation selective pores, thereby facilitating paracellular cation transport, including that of the divalent ions, Ca\(^{2+}\) and Mg\(^{2+}\) (2, 11, 18, 19). Transcellular absorption of Ca\(^{2+}\) occurs in the duodenum (50) and proximal large colon (2) as well as in the cecum of rodents (27, 53). In the current model, Ca\(^{2+}\) enters the epithelial cell via the apical Ca\(^{2+}\) channel, transient receptor potential vanilloid 6 (TRPV6) (4, 62) from where it is shuttled across the cell bound to Calbindin-D\(_{9K}\) (CaBP\(_{9K}\)) (24). Basolateral extrusion is mediated by Ca\(^{2+}\)-ATPase (PMCA1) and, to a lesser extent, the Na\(^{+}\)-Ca\(^{2+}\)exchanger (NCX1) (1, 24, 55). Similar to Ca\(^{2+}\), Mg\(^{2+}\) is absorbed in the intestine via transcellular and paracellular pathways (3). Absorption in the small intestine is thought to be predominantly via the paracellular route (3, 40). In the transcellular pathway, Mg\(^{2+}\) enters the cell via the apical channel, transient receptor potential melastatin 6 (TRPM6) which is expressed predominantly in the cecum and colon while no expression is detected in the duodenum or jejunum (20). Intracellular shuttling and basolateral extrusion mechanisms are not clearly delineated for Mg\(^{2+}\), although ancient conserved domain protein 4/cyclin M4 (CNNM4) is likely to contribute significantly to extrusion (63).

In the kidneys, once filtered, Ca\(^{2+}\) and Mg\(^{2+}\) are reabsorbed by transcellular and paracellular pathways. Renal paracellular Ca\(^{2+}\) and Mg\(^{2+}\) transport likely occurs via Claudin-2 in the proximal tubule (PT) and is dependent on Na\(^{+}\) reabsorption via the Na\(^{+}\)-H\(^{+}\) exchanger (NHE3) (45, 49). In the thick ascending limb (TAL), Claudins -16 and -19 have been implicated in forming a paracellular Ca\(^{2+}\) and Mg\(^{2+}\) shunt, which facilitates their reabsorption. This process is dependent on a lumen-positive gradient established by sodium absorption via the sodium-potassium-chloride cotransporter (NKCC2) (2, 3, 34, 56). In addition, Claudin-14 in the TAL blocks Ca\(^{2+}\)
reabsorption (14). Transcellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} reabsorption occur in the distal convolution. This structure encompasses the distal convoluted tubule (DCT segments 1 & 2, the connecting tubule (CNT) and initial cortical collecting duct (iCCD) (38). Akin to the duodenum, Ca\textsuperscript{2+} transport in the distal convolution (primarily DCT2 and CNT) involves the apical Ca\textsuperscript{2+} channel TRPV5, intracellular binding and shuttling by Calbindin-D\textsubscript{28K} (CaBP\textsubscript{28K}) and basolateral extrusion via Ca\textsuperscript{2+}-ATPases (PMCA1 and PMCA4) or NCX1 (1, 16, 23, 24). Vectorial transcellular Mg\textsuperscript{2+} transport occurs in the DCT1 and is facilitated by apical uptake of Mg\textsuperscript{2+} via TRPM6 channels (20).

While regulation of select proteins involved in active transcellular Ca\textsuperscript{2+} transport during lactation has been investigated (12, 60, 65), little is known about possible changes in proteins involved in paracellular transport of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and the transcellular pathway for Mg\textsuperscript{2+} transport. Given an increased requirement for mobilizing divalent cations into breast milk of mice, as compared to humans, the rodent lactation model provides a powerful tool to elucidate changes in transport proteins occurring during lactation, as changes are likely more pronounced in mice, than in humans. Based on this reasoning, we decided to examine the specific molecular alterations in both transcellular and paracellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport pathways across intestinal and renal epithelia during lactation. We found a marked increase in expression of *Trpv6* and *S100g*, molecules involved in the transcellular Ca\textsuperscript{2+} transport, in the duodenum, cecum, and proximal colon of lactating mice. In addition, increased mRNA and protein expression of TRPV5 and *Calb1* encoding CaBP\textsubscript{28K} was observed in the kidney of lactating mice. These changes likely resulted from dramatic increases in serum 1,25 dihydroxyvitamin D (1,25(OH)\textsubscript{2}D). *Trpm6* expression was significantly increased in the colon but not kidney during lactation. No
alterations in the expression of Cldn -2, -12, or -15 in any intestinal segment were seen.

Similarly, no changes were observed in expression of Cldn -2, -14, -16 or -19, molecules mediating paracellular cation transport in the kidney. Taken together, these results suggest that intestinal hyperabsorption of Ca$^{2+}$ and Mg$^{2+}$ via transcellular pathways occur during lactation, while only selective transcellular reabsorption of Ca$^{2+}$ is seen in the kidney. These alterations, in combination with previously reported bone demineralization, enable the concentration of Ca$^{2+}$ and Mg$^{2+}$ in breast milk securing sufficient transfer of these minerals to the neonates.
Methods

Experimental animals

FVB/N mice (obtained from Janivier labs, France) were mated at 9 weeks of age with males who were removed before parturition. Animals were maintained on a rodent diet (Altromin, Germany) containing 0.7% of Ca$^{2+}$ and 0.2% Mg$^{2+}$ as well as 600 IU Vitamin D$_3$ per Kg. After parturition, litters were maintained for 12 days in the lactating group (LAC) (N=7) or removed immediately after birth to induce involution (INV) (N=6). Virgin mice (N=6) were used as control (CON). On day 11, mice were weighed and spot urine samples collected from each mouse every 90 minutes for 9 hours. The interval of collection was chosen to allow for efficient collections of urine during the light cycle given that voiding frequency of a mouse is about 6 times per day (46). The samples collected from the same mouse were subsequently pooled and stored for analysis. On day 12, mice were anesthetized using 100 mg/kg ketamine (Ketalar) and 10 mg/kg xylazine (Rompun™), opened with a laparotomy and had blood withdrawn from the abdominal vena cava. All animal experiments were conducted in accordance with Danish Law under the animal experimental permits #2014-15-0201-00043 and 2015-15-0201-00587.

Isolation of intestinal segments, kidney, and mammary fat pads

Isolation of murine tissue was performed as previously described (1) and saved for biochemical and histological analysis. The first 4.6 cm of intestine distal to the pylorus was taken as duodenum. Jejunum was isolated from 10 - 14.8 cm distal to pylorus. Ileum was defined at 3.2 - 6.8 cm proximal to the ileocecal junction. The cecum was removed from the large bowel. The first 2.4 cm of large intestine from the cecocolic junction was considered proximal colon. Kidneys were excised, cut in half and saved for further analysis. One kidney half was immersion
fixed for 3 hours in 10% formaline and subsequently placed in PBS with azide at 4°C until paraffin embedding. All remaining isolated tissues were snap frozen in liquid nitrogen and stored at -80°C. Mammary fat pads were dissected out near the 4th and 5th nipple and the 1-3rd nipple and immersion fixed and processed for paraffin embedding or frozen as described above.

Serum and urine
Urine was stored at -80°C until analysis. Blood was collected in glass tubes, centrifuged at 5000 rpm for 15 min, serum was collected and stored at -80°C until analysis. Serum creatinine, Ca^{2+} and Mg^{2+} measured on an Architect c16000 Clinical Chemistry Analyzer (Abbott Laboratories, Abbott Park, Illinois, U.S.A.). Urine was acidified with 1 ul of 5M HCl per 50 µl urine. Urinary creatinine and Ca^{2+} was measured on the Architect c16000 and urinary Mg^{2+} was measured using a colorimetric endpoint method and a Roche/Hitachi Mg^{2+} kit for Modular P/D analyzers per manufacturer’s instructions (Roche Diagnostics, Switzerland). Serum 1,25 dihydroxyvitamin D was measured using a RIA kit (Immunodiagnostic Systems Ltd., Boldon, UK), as per manufacturer’s instructions (49).

Calculations of clearance and fractional excretion
Renal creatinine clearance as an estimate of glomerular filtration rate (GFR) and fractional excretion, were calculated using standard formulas as described previously (15). Briefly, creatinine clearance was calculated using the formula \( C_{\text{Crea}} = U_{\text{Crea}} \times \frac{V}{P_{\text{Crea}}} \); where \( U \) is the concentration of creatinine in urine, \( V \) is the rate of urine flow and \( P \) is the plasma concentration of creatinine. The fractional excretion was calculated by dividing the clearance of the electrolyte with the estimated GFR as follows; \( FE = \frac{C}{GFR} \).
**Determination of gene expression by quantitative PCR**

Total RNA was isolated from frozen tissues as previously described (1). Briefly, total RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, USA) and subjected to DNase treatment (Fermentas). RNA was quantified by UV spectroscopy at 260nm on a nanoPhotometer (Implen, Munich, Germany) and quality purity was assessed as the 260/280 nm ratio. 1ug of RNA was then reversed transcribed into cDNA (iScript cDNA Synthesis Kit, Bio-Rad, Copenhagen, Denmark). Quantitative PCR was performed in triplicate for each sample in TaqMan Universal Master Mix II (Applied Biosystems Inc, Foster City, CA, USA) with specific primers and probes using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA). Primer and probe sequences for Trpv5, CaBP28K, Ncx1, Pmca1, Pmca4, CaBP9K, Cldn12, Cldn15, Cldn16 and Cldn19 have been previously published (1, 49, 53). The remaining primer and probe sequences are listed in Table 1. Sequences were generated using IDT software (Integrated DNA Technologies, San Diego, CA) or Life Technology (Applied Biosystems Inc, Foster City, CA, USA) primer sets (where indicated). Quantification was performed using the relative standard curve method with a standard curve made from serial dilutions of a cDNA mixture from each tissue. Target genes expression was normalized to 18s for each sample, which had the least variability in expression between groups of three housekeeping genes examined, including GAPDH and B-Actin.

**Antibodies**

Monoclonal antibodies were raised in mice against an epitope on the human Transient Receptor Potential Vallinoid 5 Ca\(^{2+}\) channel (GLNLSEGDEGEEVYHF), encoded by the human TRPV5
gene as well as an epitope on the human Calbindin-D$_{28K}$ (KEFNKAFELYDQDGN) encoded by the human *CALB1* gene. The antibodies were generated as described in detail previously (64). In brief, NMRI mice were injected with 30 μg of peptide coupled onto diphtheria toxoid mixed with GERBU adjuvant, twice over a 14 day interval. Three days prior to fusion the mice received an intravenous booster injection with the conjugated peptide together with adrenaline. Fusion was done as described previously using the SP2 myeloma cell line as fusion partner (33). Clones were screened by ELISA and cloned by limited dilution. Positive clones were characterized further for their ability to stain mouse kidney. Two clones; clone 2 obtained from the human Calbindin-D$_{28K}$ fusion and clone 10 from the human TRPV5 was selected for the study, as they showed the expected labelling pattern in the distal convolution from mice as previously described (22, 42). Moreover, for colocalization studies rabbit polyclonal antibodies against NCX1 (Alomone Laboratories, Jerusalem, Israel) was used.

**Sectioning of tissue for microscopy.**

Tissues isolated from lactating animals and their respective control groups were immersion fixed for 3 hours in 10% formalin and stored in PBS until embedding with paraffin. Embedding was done on a Tissue-Tek Vacuum Infiltration Processor 6 (Sakura Finetek, Torrance, CA, USA). Following embedding, tissues were sectioned on a HM 355S Automatic Microtome (Thermo Scientific) microtome at 2 μm. Frozen kidney sections were cut on a cryomicrotome (CM1860 UV Cryostat, Leica).

**Immunohistochemical staining of tissue for light microscopy**
Paraffin-embedded tissue was stained as previously described with minor modifications (1). After sectioning, the tissue was rehydrated using Tissue-Clear (Tissue-Tek, Sakura Sections) and subsequently placed in a series of graded ethanol. Heat induced antigen retrieval was performed in TEG buffer (10 mM Tris, 0.5 mM EGTA, pH = 9.0). Endogenous peroxidase enzymes and free aldehyde groups were blocked using 0.6% H$_2$O$_2$ and 50 mM NH$_4$Cl in PBS respectively. Sections were incubated overnight at 4°C with primary antibody in 0.1% Triton X-100 in PBS, washed and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP, DakoCytomation, Denmark). HRP activity was visualized with the DAB+ Substrate Chromogen System (K3467, DakoCytomation) and sections were counterstained with hematoxylin. Finally, the sections mounted using Aquamount. Light microscopy was carried out using an Olympus BX51 microscope. For histological evaluation of mammary fat pads, sections were rehydrated to 70% ethanol and stained with Mayers Hematoxylin before being dehydrated and mounted.

For peptide preabsorption, the peptide was incubated with the corresponding primary antibody in 0.1% Triton X-100 in PBS for 30 minutes at room temperature. Sections were incubated overnight with the preabsorbed antibody/peptide solution at 4°C. Incubation with secondary antibodies and staining were conducted as described above.

**Immunohistochemical staining of tissue for fluorescent microscopy**

Staining was done essentially as above, however, after incubation with anti-TRPV5 mouse monoclonal antibodies and secondary anti-mouse-HRP, a Cy3-coupled TSA substrate (TSA Cyanine 3, Perkin Elmer) was added instead of DAB solution. After washing, sections were boiled in TEG to remove bound primary and secondary antibodies. Thereafter another round of
immunolabeling was performed using primary mouse monoclonal antibodies recognizing Calbindin-D_{28K} followed by Alexa-labeled 488 secondary antibodies. Colocalization of NCX1 and TRPV5 was performed on kidney cryosections. Sections were immersed in pre-boiled TEG buffer and incubated for 20 minutes at room temperature, washed and incubated overnight at 4°C with mouse anti-TRPV5 and rabbit anti-NCX1 (Alomone laboratories) primary antibodies. Sections were washed and incubated for 60 minutes at room temperature with Alexa-labeled 488 secondary antibodies recognizing NCX1 rabbit polyclonal antibodies and Alexa-labeled 568 secondary antibodies recognizing TRPV5 mouse monoclonal antibodies. Sections were mounted using Fluorescent Mounting Medium (DakoCytomation).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, California, USA). Data is expressed as mean ± SEM. Group means were compared using one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test. P < 0.05 was considered significant.
Results

Confirmation of induction of lactogenesis

Female mice were maintained with their litters for 12 days after parturition (LAC) or had their pups removed immediately after birth to induce involution (INV) to serve as a control for alterations in Ca\(^{2+}\) balance occurring during pregnancy. Furthermore, virgin female mice were used as regular controls (CON). Histology of the mammary fat pads confirmed induction of lactogenesis in the lactation group with development of secretory alveoli lined with cuboidal epithelial cells (Figure 2A). Following involution, this epithelium regressed markedly. Lactating mice had significantly greater body weight (35.3 ± 0.7g), than mice in the control group (22.4 ± 0.7g) and mice undergoing involution (26.2 ± 0.6g) (Figure 2B). Mice undergoing involution had a significantly higher bodyweight than virgin controls.

Increased serum 1,25(OH)\(_2\)D and urinary excretion of Ca\(^{2+}\) in lactating mice

Results of serum and urine variables are shown in Table 2. The total 9-hour urine volume increased 2.9 times in lactating mice compared to control mice. No difference was observed in urine volume between mice in the control group and involution group. When corrected for the increased bodyweight, total urine volume remained significantly higher in the lactation group compared to the involution group, but not the control group. Estimated glomerular filtration rate (GFR) determined by creatinine clearance was not different between groups. However, when normalized to bodyweight, GFR was significantly decreased in lactating mice compared to control. While serum Ca\(^{2+}\) was similar between all groups, lactating mice had higher total urine Ca\(^{2+}\) excretion compared to mice in the involution group, but not in the control group. When
normalized to bodyweight, urinary Ca\(^{2+}\) was not different between groups. However, fractional excretion of Ca\(^{2+}\) was 2.5 times higher in lactating mice compared to control mice. No differences were observed for serum Mg\(^{2+}\), total urine Mg\(^{2+}\) excretion or fractional excretion of Mg\(^{2+}\). However, when corrected to bodyweight, mice in the control group had significantly higher urinary Mg\(^{2+}\) excretion compared to mice in the involution group, but not the lactation group. Serum 1,25(OH)\(_2\)D is known to transcriptionally regulate expression of intestinal and renal Ca\(^{2+}\) transport proteins. Here, we observed a 9-fold increase in serum 1,25(OH)\(_2\)D during lactation, while no difference was observed between control and involution mice.

**Duodenal expression of transcellular Ca\(^{2+}\) transporters increase during lactation**

Expression of genes coding for transporters mediating trans- and paracellular Ca\(^{2+}\) transport across intestinal segments was determined by quantitative real-time PCR. Expression of the apical Ca\(^{2+}\) channel *Trpv6* and *S100g* encoding the intracellular binding protein Calbindin-D\(_{9k}\), that are responsible for the inward uptake and buffering of Ca\(^{2+}\) in the duodenal enterocyte respectively, more than doubled during lactation (Figure 3A-B). The expression of *Trpv6* and *S100g* reduced towards control levels following involution, suggesting that increased Ca\(^{2+}\) transport protein expression is a direct result of lactation. The expression of *S100g* in the involution group was not statistically different from both the control and lactation groups, which is suggestive of a transition in the expression profile from that of the pre-lactation state to a control state. Expression of *Atp2b1*, encoding PMCA1, and *Slc8a1*, encoding NCX1, that facilitate transport of Ca\(^{2+}\) across the basolateral membrane of the enterocyte, were not statistically different between all groups in this intestinal segment (Figure 3C-D).
No apparent changes in mRNA expression of small bowel paracellular transporters

In an effort to investigate whether paracellular transport is subjected to regulation in intestinal epithelium during lactation, we examined changes in expression of the sodium-hydrogen exchanger 3, encoded by *Slc9a3* gene, which is known to drive paracellular Ca\(^{2+}\) absorption and likely also Mg\(^{2+}\) absorption in the small intestine (49). We also examined expression of Claudins (Cldn-2, -12, -15) that in the intestine, have been shown to form cation permeable paracellular pores. In the duodenum, there were no differences in expression of *Slc9a3* (Figure 3E) nor Cldn2, Cldn12, and Cldn 15 (Figure 3 F-H). As paracellular absorption appears to be the only pathway for Ca\(^{2+}\) and Mg\(^{2+}\) transport in the distal small bowel, we also assessed expression of these Claudins in the jejunum and ileum of lactating mice. Similar to results from the duodenum, no differences were observed in abundance of *Slc9a3*, Cldn2, Cldn12, and Cldn15 mRNA in jejunum (Figure 4A-D), and ileum (Figure 4E-H). These results suggest that intestinal paracellular absorption is not regulated at the transcriptional level during lactation.

Increased mRNA abundance of Ca\(^{2+}\) and Mg\(^{2+}\) transporters in large bowel of lactating mice

In the large bowel, transport of Ca\(^{2+}\) and Mg\(^{2+}\) appear to occur exclusively via the transcellular pathway. Expression of proteins implicated in transcellular Ca\(^{2+}\) absorption was therefore investigated in the cecum and colon of lactating mice. In the cecum, mRNA levels of *Trpv6* were 20-fold higher in lactating mice compared to mice in the control group (Figure 5A). Expression of *S100g* during lactation increased 6-fold in the cecum (Figure 5B). In the involution group, both *Trpv6* and *S100g* decreased to levels not statistically different from the control group, suggesting that the increase was a direct result of lactation (Figure 5A-B). Interestingly, mRNA expression of *Atp2b1* was 2-fold higher in the cecum of the control group compared to both the
lactation and involution groups (Figure 5C) and a similar trend was observed in expression of Slc8a1 in the cecum (Figure 5D). This suggests that this trend may result from alterations during pregnancy. In the colon, mRNA expression of Trpv6 and S100g was increased 20-fold in the lactating mice, while no difference was observed in the involution group compared to mice in the control group (Figure 5E-F). These results suggest that the increase in Trpv6 and S100g is a consequence of lactation. In contrast to results in the cecum, a 5-fold increase in Atp2b1 mRNA during lactation (Figure 5G) and a 4-fold increase in Slc8a1 mRNA during involution (Figure 5H) were observed in the colon. Expression of both of these genes was not different between lactation and involution groups, suggesting that the increases observed are a result of pregnancy, not lactation.

Transcellular Mg\(^{2+}\) absorption occurs exclusively in the colon and the inward uptake is facilitated by apical transient receptor potential melastatin 6 (TRPM6) channels. We therefore examined the expression of this channel during lactation. mRNA expression of Trpm6 was 9-fold higher in the colon of lactating mice relative to the control and involution groups (Figure 5I). There was no difference between the control and involution group suggesting the increased expression of Trpm6 was a result of lactation.

**Marked elevation in vitamin D hydroxylating enzymes during lactation**

Given the large changes in expression of transcellular mediators in the intestine, we investigated whether these changes may be due to alterations in vitamin D metabolism. We thus determined the renal expression of the 1-alpha-hydroxylase (Cyp27b1), which converts 25-hydroxy vitamin D to its active form 1,25-dihydroxyvitamin D (1,25(OH)\(_2\)D) and the 24-hydroxylase (Cyp24a1)
enzyme that inactivates vitamin D derivatives by hydroxylation at the 24 position. The expression of Cyp27b1 and Cpy24a1 mRNA increased 9-fold in lactating mice compared to control mice, while expression of these genes did not differ from controls in the involution group, suggesting that expression of these genes is increased during lactation and not pregnancy (Figure 6A-B). These results are consistent with increased serum active 1,25-dihydroxyvitamin D levels in the current study and previously observed in lactating rodents (41). No difference was observed in expression of vitamin D receptor, Vdr between groups (data not shown).

Expressions of genes involved in paracellular reabsorption of Ca\(^{2+}\) and Mg\(^{2+}\) in kidney are not altered during lactation.

Paracellular Ca\(^{2+}\) reabsorption in the proximal tubule (PT) occurs via the cation selective pore forming CLDN2 and is influenced by sodium reabsorption through NHE3, encoded by Slc9a3. Mg\(^{2+}\) permeation in this segment is likely occurring via a similar pathway. We therefore investigated the alterations of these mediators in lactating mice. No difference was observed in renal mRNA expression of Slc9a3 or Cldn2 between mice in the lactation, involution, and control groups (Figure 6C-D). The thick ascending limb (TAL) is a significant site of renal paracellular divalent cation reabsorption, driven by a large lumen positive voltage gradient, which is established by the actions of NKCC2, encoded by Slc12a1. We therefore investigated whether altered expression is observed during lactation. The mRNA abundance of Slc12a1 was not different between control mice and those in the lactation or involution groups (Figure 6E). CLDN-16 and -19 form pores selective for divalent cations. No differences were observed between groups in expression of Cldn16 or Cldn19 mRNA (Figure 6F-G). Similarly, no changes
were noted in expression of *Cldn14* mRNA whose product blocks reabsorption of Ca\(^{2+}\) (14) (Figure 6H).

*Increased expression of transcellular Ca\(^{2+}\) transport proteins in the kidney during lactation.*

Renal transcellular Ca\(^{2+}\) and Mg\(^{2+}\) reabsorption occurs in the distal convolution, encompassing the DCT, the CNT, and initial CCD segments of the nephron and collecting system (38, 42). We therefore determined the expression of genes implicated in these pathways. In lactating animals, mRNA abundance of *Trpv5* was 1.6-fold greater than that observed in control mice, while abundance of *Calb1* was 2.6-fold greater (Figure 6I-J). For both genes, the involution group was not different from the control group suggesting that the increase was during lactation only. Interestingly, mRNA expression of *Atp2b1* was 1.4-fold greater in the involution group compared to the control group (Figure 6K). No difference between groups was observed in expression of *Atp2b4, Slc8a1*, or *Slc12a3* in this segment (Figure 6L-N). Expression of *Klotho* was increased 1.5-fold in the lactation group compared to the control group (Figure 6O). Unlike results in the colon, mRNA expression of *Trpm6* in the DCT was not significantly different during lactation. However, it was higher during involution compared to control mice (Figure 6P).

To evaluate alterations in the expression of TRPV5 and CaBP\(_{28K}\) at the protein level in lactating mice, mouse monoclonal antibodies were raised against epitopes specific for these proteins. Both antibodies showed staining specific to the distal convolution, which was completely absent when the antibodies were incubated with the immunizing peptide (Figure 7A-B). Immunofluorescent staining demonstrated complete colocalization of TRPV5 and the Na\(^{+}/Ca^{2+}\) exchanger, NCX1 (Figure 7C) as well as TRPV5 and CaBP\(_{28K}\) (Figure 7D). Immunohistochemical staining of
kidney sections with antibodies specifically recognizing TRPV5 shows increased staining intensity in lactating mice compared to mice in the control and involution groups (Figure 7E).

Similarly, immunohistochemical staining with antibodies specific to CaBP<sub>28K</sub> revealed markedly increased expression of CaBP<sub>28K</sub> in kidneys of lactating mice compared to control and involution mice (Figure 7F).
Discussion

The present study delineates, in detail, alterations in expression of Ca\(^{2+}\) and Mg\(^{2+}\) transport pathways in the intestine and kidney of lactating mice. Our results suggest, that increased Ca\(^{2+}\) and Mg\(^{2+}\) mobilization into breast milk is mediated by a selective increase in intestinal and renal transcellular transport pathways for these minerals, in addition to bone demineralization. These alterations are driven, at least in part, by an increased production of active vitamin D in kidney. This is supported by our findings of i) increased expression of \(\text{Trpv6}\) encoding the apical Ca\(^{2+}\) channel and \(\text{CaBP9K}\) encoding the intracellular Ca\(^{2+}\) binding protein in the duodenum, cecum, and colon of lactating mice; ii) an increase in the apical Mg\(^{2+}\) channel \(\text{Trpm6}\) in the colon of lactating mice; iii) increased gene and protein expression of apical Ca\(^{2+}\) TRPV5 and intracellular binding protein \(\text{CaBP28K}\) in the kidneys of lactating mice; iv) increased renal expression of vitamin D metabolizing enzymes \(\text{Cyp27b1}\) and \(\text{Cyp24a1}\) in kidney v) increased serum 1,25-dihydroxyvitamin D and vi) no changes were observed in the paracellular transport pathways of intestinal and renal epithelia. Thus, the increased fractional excretion of Ca\(^{2+}\) observed in lactating mice is likely secondary to intestinal hyperabsorption and bone demineralization, as seen in other models of hypervitaminosis D (57). Furthermore, the increase in renal Ca\(^{2+}\) selective transport proteins clearly suggest an additional contribution of the kidney with respect to retaining enough Ca\(^{2+}\) to permit significant concentration in breast milk.

In the current study, we found that total urinary Ca\(^{2+}\) excretion was higher in lactating mice relative to mice in the involution group. When adjusting for the increased body weight in the lactating group, we found no difference between groups in total urinary Ca\(^{2+}\) excretion. Moreover, fractional excretion of Ca\(^{2+}\) was higher in lactating mice than control mice. Unlike our
findings, previous reports suggest that total urinary Ca\(^{2+}\) excretion decreases during lactation in women (29, 54). In a balance study using rats, Brommage et al. found that lactating rats had significantly less total daily urinary Ca\(^{2+}\) excretion compared to post-partum, non-lactating rats (7), however, the fractional excretion of Ca\(^{2+}\) was not determined. Moreover, these rats showed an elevated intestinal Ca\(^{2+}\) absorption as well as increased food intake. If such changes also exist in the mouse it may explain the increased fractional excretion of Ca\(^{2+}\) we observed. These differences between studies may relate to the rate of intestinal absorption between species or the relative amount of Ca\(^{2+}\) concentrated in breast milk during lactation. Ca\(^{2+}\) in breast milk of rats may indeed be less than that in mice (28, 52). In fact, studies of lactating mice have previously suggested that mice markedly increase their feeding rate and intestinal volume during lactation (the small intestinal weight almost doubles, contributing significantly to bodyweight gain) (10). This would explain the higher urinary Ca\(^{2+}\) excretion observed per mouse as well as the higher fractional excretion of Ca\(^{2+}\) recorded.

The results of the current study suggest intestinal hyperabsorption of Ca\(^{2+}\) and Mg\(^{2+}\) contribute to meeting increased requirements of these minerals during lactation. In fact, transcellular Ca\(^{2+}\) absorption is increased in the duodenum and proximal large bowel of lactating mice. We observed a greater than 2-fold increase in mRNA expression of \(Trpv6\) and \(S100g\) in duodenum during lactation, while Van Cromphaut observed a 12-fold increase in \(Trpv6\) and 3-fold increase in \(CaBP_{9K}\) at day 19 of lactation in wildtype mice (12). However, we did not observe a similar 2-fold increase in \(Pmca1b\) mRNA during lactation (12). Similarly, in rat models, increases in \(Trpv6, CaBP_{9K},\) and \(Pmca\) have been observed after 7 days of lactation (60, 65). The observed differences may be due to altered expression during the course of lactation as changes in
absorption have been observed in rats over time (5) as well as differences between rats and mice.

There is a paucity of data investigating alterations in intestinal paracellular Ca\(^{2+}\) and Mg\(^{2+}\) transport during lactation. In the duodenum of lactating rats, a 2-fold increase in Cldn12 mRNA and an increase in CLDN15 as evaluated by immunohistochemical analysis has been observed (59, 60). In our current study, we found no difference in expression of Cldn-2, -12, or -15 in any segment of the small intestine. These differences may illustrate species differences or may be due to altered paracellular absorption patterns over the course of lactation. Still, we cannot exclude small changes in mRNA due to small sample size of the current study. It is also important to note that while we did not observe differences in expression of implicated claudins, intestinal hypertrophy during lactation would increase overall intestinal paracellular absorption (10, 59). Taken together, the results from rodents suggest that the intestine compensates during lactation to increase Ca\(^{2+}\) absorption, by increasing intestinal volume and furthermore to selectively increase the transcellular pathway. Studies from breastfeeding women, however, have revealed conflicting results. In a longitudinal study of well nourished women with adequate Ca\(^{2+}\) intake, absorption of Ca\(^{2+}\) was not different from pre-pregnancy values after 2 months of lactation (54). Several others also found no difference in factional Ca\(^{2+}\) absorption between lactating and nulliparous women, although lactating women had higher mean Ca\(^{2+}\) intake prior to the study (30, 44). The discrepancies observed between rodents and women may be due to the increased demand for Ca\(^{2+}\) in breast milk in rodents because of the larger number of offspring. Consistent with this, breastfeeding mothers of twins excrete double the amount of Ca\(^{2+}\) daily in breast milk, an estimated 1000 mg (35).
Similar to expression patterns in the intestine, we observed increased gene and protein expression of renal transepithelial $\text{Ca}^{2+}$ reabsorption proteins. However, no differences were noted in genes involved in paracellular reabsorption in lactating mice. We cannot exclude that some genes that are not regulated at the RNA level, might be altered by posttranscriptional alterations in protein abundance. We observed a 1.6-fold increase in $\text{Trpv5}$ and 2.6-fold increase in $\text{Cabp1}$ mRNA expression during lactation. Van Cromphaut et al. noted similar increases in the expression of these genes in wildtype mice whereas $\text{Trpv5}$ expression was not altered and $\text{Cabp1}$ expression decreased during lactation in $\text{Vdr}$ KO mice (12). Despite the recruitment of reabsorption pathways, lactating mice had higher fractional excretion of urinary $\text{Ca}^{2+}$. This is likely secondary to hyperabsorption from the intestines and increased demineralization of trabecular bone (31, 61). In contrast to these changes observed in mice, lactating women have decreased urinary $\text{Ca}^{2+}$ excretion compared to nulliparous women (29, 32, 44, 54), however, the molecular details mediating this renal conservation are unknown.

In the present study, we found substantial increases in renal $\text{Cyp27b1}$ and $\text{Cyp24a1}$ expression and serum 1,25(OH)$_2$D in lactating mice. In a study of rats, Zhu et al. observed increased plasma 1,25(OH)$_2$D after 21 days of gestation that remained elevated through lactation (65). The alterations in intestinal and renal $\text{Ca}^{2+}$ channels and calbindins observed in the current study may be, at least in part, secondary to increased vitamin D. Indeed, a 4-fold increase in serum 1,25(OH)$_2$D has been observed in lactating rats (41). Furthermore, vitamin D receptor knockout mice do not display similar marked increases in transcription of $\text{Ca}^{2+}$ transporter genes $\text{S100g}$ and $\text{Atp2b1}$ in the duodenum and $\text{Trpv5}$ and $\text{Cabp1}$ in the kidneys during lactation (12). It is of note, however, that while overall $\text{Trpv6}$ mRNA abundance was markedly diminished, an increase
in *Trpv6* expression was observed during lactation in *VDR* KO mice in that study, suggesting multiple effectors may be involved in regulating *Trpv6* to meet the increased Ca\(^{2+}\) requirements during lactation (12). Conflicting results have been reported in women. Kumar *et al* noted an almost doubling of plasma 1,25(OH)\(_2\)D in pregnant and lactating women relative to age-matched controls (39). A longitudinal study of women found serum 25(OH)D and 1,25(OH)\(_2\)D were not increased in lactating women (54, 58). However, it is noteworthy that serum 1,25(OH)\(_2\)D was highest in women with the lowest Ca\(^{2+}\) intake (58) and was positively associated with fractional Ca\(^{2+}\) absorption (48). Taken together, these studies illustrate differences between rodents and humans regarding vitamin D mediated increase of intestinal and renal Ca\(^{2+}\) transport proteins that have yet to be fully delineated. The mechanism driving increased active vitamin D metabolism in rodents during lactation is unclear. It may be that transient decreases in serum Ca\(^{2+}\) while pups are feeding leads to increased parathyroid hormone (PTH) that, in turn, increases renal vitamin D activation. However, as reviewed in detail by (36), systemic concentrations of PTH have generally been reported to be in the low-normal range in women and mice during lactation.

In addition to increased expression of intestinal transcellular Ca\(^{2+}\) absorption proteins, we also observed increased expression of the Mg\(^{2+}\) channel *Trpm6* in the colon of lactating mice relative to both control and involution groups. This increase in intestinal Mg\(^{2+}\) absorption likely occurs to meet increased demands. Urinary and serum analysis revealed no compensation in fractional excretion of Mg\(^{2+}\) during lactation. Indeed, renal expression of *Trpm6* was not higher in lactating mice than control. Similarly, lactating women display urinary Mg\(^{2+}\) excretion similar to nulliparous women (32). Interestingly, we did observe a significantly greater abundance of renal *Trpm6* in mice in the involution group relative to the control group. Klein *et al.* did not observe a
significant decrease in urinary Mg$^{2+}$ excretion in post partum non-lactating women compared to lactating and nulliparous women, although post-partum non-lactating women had significantly lower Mg$^{2+}$ intake (32). It may be that, under conditions of poor intake, increased renal conservation is required. Overall, our results suggest that the colon plays a major role in meeting increased Mg$^{2+}$ requirements during lactation. In contrast to its effect on intestinal Ca$^{2+}$ absorption, active vitamin D is not likely to mediate the increased Mg$^{2+}$ absorption in the colon. Previous investigations have shown that 1,25(OH)$_2$D does not increase Trpm6 expression in the colon or Mg$^{2+}$ absorption in mice (40). Similarly, treatment with 1,25(OH)$_2$D$_3$ did not alter expression of Trpm6 in the kidney of mice (20, 40).

In conclusion, this study examined alterations in expression of known intestinal and renal Ca$^{2+}$ and Mg$^{2+}$ (re-) absorption proteins during lactation. We found selective increases in the transcellular absorption pathways of both divalent cations in the intestine during lactation, without concomitant changes in known constituents of paracellular absorption. Similarly, proteins of renal transcellular Ca$^{2+}$ reabsorption were increased, while proteins of the paracellular reabsorption pathway were not changed. These transcriptional changes were likely secondary to increased vitamin D metabolism. The interplay of hormonal influence on divalent cation balance in lactating mice has yet to be fully delineated. The results of this study will aid in understanding compensatory changes to Ca$^{2+}$ and Mg$^{2+}$ handling during lactation and contribute to further research in this area.
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Author Contributions
MRB, RTA and HD conception and design of research; MRB, IA, PS, KS, RTA and HD performed experiments; MRB, IA, RTA and HD analyzed data; MRB, IA, PS, KS, RTA and HD interpreted results of experiments; MRB and HD prepared figures; MRB, IA, KS, PS, RTA and HD edited and revised manuscript; MRB, IA, KS, PS, RTA and HD approved final version of manuscript; MRB, RTA and HD drafted the manuscript.

Conflict of Interest
None


56. **Simon DB, Lu Y, Choate KA, Velazquez H, Al-Sabban E, Praga M, Casari G,**


Woudenberg-Vrenken TE, Lameris AL, Weissgerber P, Olausson J, Flockerzi V, Bindels RJ, Freichel M, Hoenderop JG. Functional TRPV6 channels are crucial for transepithelial Ca2+ absorption. *Am J Physiol Gastrointest Liver Physiol* 303: G879-85,


Figure Legends

**Figure 1:** Experimental setup. FVB/N mice (n = 6-7 per group) at 9 weeks of age were mated with male mice that were removed before parturition. After pregnancy, litters were maintained for 12 days or removed immediately after birth and mice are maintained for 12 days. On day 11, spot urine was collected every 1.5 hour during a 9 hour interval.

**Figure 2:** (A) Histological evaluation of hematoxylin and eosin stained mammary fat pads from mice undergoing lactation, the control or involution groups. Note the dramatic changes in the mammary epithelium between groups. (B) Body weight on day 12 in the three groups. CON, control (N=6); LAC, lactating mice (N=7); INV, during involution (N=6). Data are presented as means ± SEM. *P <0.05.

**Figure 3:** A-D: mRNA expression of transcellular Ca\(^{2+}\) transport proteins in duodenum of virgin controls (CON, n=6), lactating mice (LAC, n=7), and mice undergoing involution (INV, n=6). Expression of duodenal *Trpv6* (A) *S100g* encoding CaBP\(_{9K}\) (B) *Atp2b1* encoding PMCA1 (C) *Slc8a1* encoding NCX1 (D) in the three groups. Note the marked increase in *Trpv6* and *S100g.*

**Figure 4:** mRNA expression profiles of genes involved in forming or driving the paracellular transport of Ca\(^{2+}\) and Mg\(^{2+}\) across the jejunum (A-D) and ileum (E-H) of virgin controls (CON,
n=6), lactating mice (LAC, n=7), and mice undergoing involution (INV, n=6). Expression in the jejunum of Slc9a3 encoding NHE3 (A) Cldn2 (B) Cldn12 (C) Cldn15 (D). Expression in the ileum of Slc9a3 encoding NHE3 (E) Cldn2 (F) Cldn12 (G) Cldn15 (H). Data are presented as means ± SEM. *P <0.05.

Figure 5: A-D: mRNA expression of transcellular Ca$^{2+}$ transport proteins in cecum and colon of virgin controls (CON, n=6), lactating mice (LAC, n=7), and mice undergoing involution (INV, n=6). Expression in cecum of Trpv6 (A) S100g encoding CaBP9k (B) Atp2b1 encoding PMCA1 (C) Slc8a1 encoding NCX (D) of the three groups. Note the increase in Trpv6 and CaBP9k. E-H: Expression in colon of Trpv6 (E) S100g (F) Atp2b1 (G) Slc8a1 (H) during lactation. I: mRNA expression of transcellular Mg$^{2+}$ transport protein Trpm6 in the colon of the three groups. Note the increase in Trpv6, S100g, and Trpm6. Data are presented as means ± SEM. *P <0.05.

Figure 6: A-B: mRNA expression of vitamin D metabolizing enzymes Cyp27b1 and Cyp24a1 during lactation in virgin controls (CON, n=6), lactating mice (LAC, n=7), and mice undergoing involution (INV, n=6). Note the 9-fold increase during lactation. C-D: mRNA expression of proteins involved in paracellular Ca$^{2+}$ reabsorption in the proximal tubule Slc9a3 encoding NHE3 (C) and Cldn2 (D). E-H: mRNA expression of proteins implicated in paracellular Ca$^{2+}$ reabsorption in the thick ascending limb Slc12a1 encoding NKCC2 (E), Cldn16 (F), Cldn19 (G), and Cldn14 (H). I-O: mRNA expression of proteins involved in Ca$^{2+}$ reabsorption in the distal convolution Trpv5 (I), Cabp1 encoding CaBP28K (J), Atp2b1 encoding PMCA1 (K), Atp2b4 encoding PMCA4 (L), Slc8a1 encoding NCX1 (M), Slc12a3 encoding
NCC (N), Klotho (O). P: mRNA expression of transcellular Mg\(^{2+}\) transport protein \textit{Trpm6}. Data are presented as means ± SEM. *P < 0.05.

**Figure 7:** Expression of proteins implicated in transcellular Ca\(^{2+}\) reabsorption in the distal convolution in virgin controls (CON, n=6), lactating mice (LAC, n=7), and mice undergoing involution (INV, n=6). Immunolabeling of kidney sections with antibody and preabsorbed antibody (+ peptide) of TRPV5 (A) and CaBP\(_{28K}\) (B). Immunofluorescent staining confirms colocalization of TRPV5 and NCX1 (C), and TRPV5 and CaBP\(_{28K}\) (D). Immunohistochemical staining of kidney sections at 200X and 400X magnification for TRPV5 (E) and CaBP\(_{28K}\) (F).
Table 1. Oligonucleotide sequences used for quantitative PCR

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Table 2. Serum and urine
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Data represented at Mean ± SEM. GFR, Glomerular filtration rate; FE Ca²⁺, fractional excretion of calcium; FE Mg²⁺, fractional excretion of magnesium; 1,25(OH)₂D, 1,25-dihydroxyvitamin D. Values with the same superscript letter are not significantly different by one-way ANOVA with Tukey’s post-test analysis.
Lactation
Parturition
Litters maintained - lactogenesis

Involution
Parturition
Litters removed at parturition

Control

Day 11
9hr urine collection

Day 12
Animals sacrificed
Figure 3
Figure 5

A

Trpv6 mRNA (fold change)

B

S100g mRNA (fold change)

C

Atp2b1 mRNA (fold change)

D

Slc8a1 mRNA (fold change)

E

Trpv6 mRNA (fold change)

F

S100g mRNA (fold change)

G

Atp2b1 mRNA (fold change)

H

Slc8a1 mRNA (fold change)

I

Tprm6 mRNA (fold change)

Legend:

CON

LAC

INV

* Indicates statistical significance.