Abstract. Four membrane calcium (Ca\(^{2+}\)) channels are known to play an important role in calcium transport: transient receptor potential cation channel subfamily V members 5 and 6 (Trpv5/6), Na\(^+/Ca^{2+}\) exchanger 1 (Ncx1) and plasma membrane Ca\(^{2+}\)-transporting ATPase1 (Pmca1b). These channels have been shown to mediate the transport of Ca\(^{2+}\) between the cytosol and bloodstream in multiple tissues, including the duodenum, kidney, and uterus. Trpv5 and Trpv6 are known to selectively import Ca\(^{2+}\) into cells, while Ncx1 and Pmca1b function to extrude Ca\(^{2+}\). The present study examined and compared expression patterns of these genes in the canine duodenum, kidney and uterus. We confirmed expression of all four genes in this canine model. To collect tissues for analysis, two adult (2.5-year-old) female dogs were euthanized and tissue samples were collected from an abdominal incision. Expression of Trpv5/6, Ncx1 and Pmca1b was analyzed by reverse transcription polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemistry. Trpv5, Trpv6 and Ncx1 mRNA were highly expressed in the kidney, while Pmca1b mRNA was primarily detected in the uterus. Protein expression was verified by Western blot analysis and localized by immunohistochemistry. Protein expression showed patterns similar to those of mRNA expression. Taken together, these results indicate that transcriptional and transitional expression of Trpv5, Trpv6, Ncx1 and Pmca1b may play a critical role in calcium transport in the canine duodenum, kidney and uterus.

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Correlation of Calcium Transport to Calciumhomeostasis in Living Organisms

Calcium plays a pivotal role in the bioregulation and homeostasis of living organisms. In its ionized form, calcium (Ca\(^{2+}\)) acts as an intracellular messenger that plays a role in muscle contraction, neurotransmission, enzyme and hormone secretion, and many other biological processes, including cell cycle regulation and programmed cell death (1-3). Ca\(^{2+}\) is a key element in the mineralization phase of teeth and bones, and maintenance of blood Ca\(^{2+}\) levels in a narrow range is essential (4). Because all Ca\(^{2+}\) in the human body is derived from the diet, the absorption and regulation of Ca\(^{2+}\) is biologically important. Extracellular Ca\(^{2+}\) is absorbed by the gastrointestinal tract and then filtered and reabsorbed in kidney, and bone tissue acts as a dynamic Ca\(^{2+}\) depot (5). Overall Ca\(^{2+}\) balance is maintained by Ca\(^{2+}\) absorption in the kidney and release from bone, both of which are under the control of calcitropic hormones that are released upon demand for Ca\(^{2+}\) (6). In addition, the uterus undergoes smooth muscle contraction and relaxation mediated by Ca\(^{2+}\), and is responsible for the maintenance of fetal blood Ca\(^{2+}\) concentration during pregnancy, which is essential for fetal bone mineralization (7). Ca\(^{2+}\) absorption by these organs is mediated by paracellular as well as transcellular transport processes. The paracellular component of epithelial Ca\(^{2+}\) transport is passive, directly connecting the luminal compartment with the blood compartment, while transcellular transport is active, involving the passage across two plasma membrane barriers, regulated by the combined actions of various hormones (5). Transcellular Ca\(^{2+}\) absorption is initially tightly regulated by the influx of Ca\(^{2+}\) across the luminal membrane of an epithelial cell. Ca\(^{2+}\) then enters the cell and is sequestered by calbindin-D\(_{28k}\) or -D\(_{9k}\) to maintain low cytosolic Ca\(^{2+}\) concentrations. Subsequently, calbindin-bound Ca\(^{2+}\) diffuses to the basolateral side of the cell, where it is extruded into the bloodstream via Na\(^+/Ca^{2+}\) exchanger 1 (NCX1) and plasma membrane Ca\(^{2+}\)-ATPase (PMCA1b). The influx of Ca\(^{2+}\) across the luminal membrane is mediated by specialized epithelial Ca\(^{2+}\) channels, the transient receptor potential cation channel subfamily V members 5 (TRPV5) and 6 (TRPV6) (8). Thus, our genes of...
interest, TRPV5, TRPV6, NCX1 and PMCA1b, appear to play critical roles in the major steps of calcium transport (9).

TRPV5 and TRPV6 are TRP-related, six-transmembrane spanning channels and belong to a gene subfamily with six members (10). Both genes contain 15 exons, encoding proteins of approximately 730 amino acids, and share 75% homology (9). Both function as facilitative transporters, mediating the cellular uptake of calcium down its electrochemical gradient with saturation kinetics but no obvious gating mechanism, such as ligand or voltage gating. These channels can be blocked by trivalent and divalent cations such as La3+, Gd3+, Pb2+, Cd2+ and Cu2+ (11). Both channels display strong inward rectification and are characterized by marked Ca2+ selectivity of more than 100-fold over univalent cations (12). Thus, they are considered to be the apical gatekeepers in transcellular Ca2+ transport (8). TRPV5 has been identified as the gene responsible for Ca2+ influx in epithelial cells of the renal distal convoluted tubule and is highly expressed in kidney. TRPV6 mediates Ca2+ transfer across the apical membrane in the intestine, pancreas and placenta, contributes to bone homeostasis, and tends to be highly expressed in the proximal intestine, placenta and exocrine tissues regulated by 1,25-dihydroxyvitamin D (11, 13).

NCX1 is an antipporter protein comprised of 9 transmembrane segments with a long internal loop between the 5th and 6th transmembrane segments. Of the three NCX isoforms (NCX1, NCX2 and NCX3), only NCX1 is expressed ubiquitously. PMCA1b is one of four isoforms (PMCA1-4), which are further divided into several subtypes by alternative gene splicing. Located in cellular membranes, PMCA1b acts as a Ca2+ eliminator using an ATP-dependent pump (14). NCX1 and PMCA1b collectively constitute the Ca2+ extrusion system, acting in opposition with transporter proteins mediating Ca2+ entry. In general, the Ca2+ concentration outside the cell is approximately 1,000 times higher than that inside the cell. Therefore, it is not possible for Ca2+ to freely diffuse out of cells, and a driving force is essential for Ca2+ extrusion. NCX1 is a membrane transporter that couples the export of one Ca2+ ion to the import of 3 Na+ ions. NCX1 can easily reverse the direction of this transport and bring Ca2+ into cells if the Na+ concentration gradient decreases or the membrane potential becomes less negative (15). PMCA1b is a more ubiquitous transmembrane pump that uses one molecule of ATP to transport one molecule of Ca2+ from the cytosol to the external environment. It has a much higher affinity for Ca2+ but a much lower capacity than NCX1 (16). Since PMCA1b is capable of effectively binding to Ca2+ even at low concentrations, it is better suited to the task of maintaining the very low concentrations of Ca2+ normally found within a cell. In sum, the activities of NCX1 and PMCA1b complement one another.

As mentioned above, we recognized the significance of the cooperative action among these four genes in in vivo Ca2+ transport and sought to further characterize this interaction. The intestine, kidney and uterus are three of the major organs involved in Ca2+ transport. Expression and immunolocalization of Ca2+ transport proteins in the duodenum, kidney and pancreas of the dogs has previously been reported (17). However, since a previous report showed discrepancies in the expression patterns of Ca2+ transport genes in results derived from (RT-PCR), Western blot and (IHC) analysis, we wanted to confirm these earlier results and further investigate the expression patterns of these genes. To this end, we analyzed expression of Trpv5, Trpv6, Ncx1 and Pmca1b in the canine duodenum, kidney and uterus using RT-PCR, Western blot analysis and IHC.

Materials and Methods

Experimental animals. Two 2.5-year-old female beagle dogs were sacrificed for our experiments. Both dogs were fed a commercial diet (Proplan; Nestle Purina Petcare, Korea) with free access to tap water and were kept separately in stainless steel cages in a controlled environment maintained on a 12-hour light/dark cycle (temperature 23±2°C, relative humidity 50±10%, ventilation 17±1 times/min). The dogs were euthanized and a midline incision was made to collect the three organs (the proximal duodenum, the kidney cortex, and uterine horn). All isolated organs were dissected and washed in cold sterile saline (0.9% NaCl). All tissue collection procedures were approved by the Ethics Committee of Chungbuk National University (CBNU).

Total RNA extraction and RT-PCR. Total RNA was extracted from the duodenum, kidney and uterus using TRIzol reagent (Invitrogen ltd., Carlsbad, CA, USA) according to the manufacturer’s protocol, and the concentration of the resulting total RNA was measured by spectrophotometry at a wavelength of 260 nm. Total RNA was denatured by heating at 85°C for 10 min. Ten micrograms of total RNA from each sample was electrophoresed on a 1% formaldehyde denaturing agarose gel for 1 h at 100 V, and 18S rRNA was used as an indicator of the quantity of total RNA. For RT-PCR, total RNA (1 μg) was reverse transcribed to create first-strand complementary DNA (cDNA) using mMLV reverse transcriptase (Invitrogen ltd.) and random primers (9-mer, Takara Bio Inc., Otsu, Shiga, Japan). Trpv5, Trpv6, Ncx1, Pmca1b and β-actin (Actb), a housekeeping control gene, were amplified in a 20 μl PCR reaction containing 1U i-StarTaq™ DNA polymerase (iNtRON Bio Inc., Sungnam, Kyungki-Do, Korea), 1.5 mM MgCl2, 2 mM dNTP, and 20 pmol of primers for Trpv5, Trpv6, Ncx1, Pmca1b, or Actb. RT-PCR primers for Trpv5, Trpv6, Ncx1 and Pmca1b were dog-specific. The primer sequences used for Trpv5 were 5'-TGATGGGTGACACACACTGG-3' (sense) and 5'-GAAGCACA CTGCGAAAGGATT-3' (antisense); for Trpv6, 5'-GTGATGTCGGACACACTGGT-3' (sense) and 5'-TTCTGAGGCTTTGTCCAGGT-3' (antisense); for Ncx1, 5'-CATC TTCTGAGGCTTTGTCCAGGT-3' (sense) and 5'-GAAGCACA CTGCGAAAGGATT-3' (antisense); and for Pmca1b, 5'-GCACGAAATG ATGCTGCAGCAATG-3' (sense) and 5'-TTATCCACACAGCATCTG CAC-3' (antisense). PCR reaction parameters were 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Expression levels of the four genes of interest and Actb were then quantified and compared by electrophoresis. Cycling kinetics were performed using 26, 28 and
30 cycles to ensure linearity of PCR product detection; the final PCR conditions were 30 cycles for Trpv5, Trpv6, Ncx1 and Pmca1b, or 28 cycles for Actb. PCR products (10 μl) were separated on a 2.3% agarose gel and stained with ethidium bromide. Gel photographs taken under UV illumination were scanned using a Gel Doc EQ system (Bio-Rad Laboratories, Inc., CA, USA).

**Western blot analysis.** To obtain protein samples from the duodenum, kidney and uterus of the two female dogs, PRO-PREP™ (iNtRON Bio Inc.) was used according to the manufacturer’s instructions. As positive controls for each gene, mouse uterus (for Trpv5, Trpv6), kidney (for Ncx1) and heart (for Pmca1b) protein samples were also isolated (18, 19). Samples containing 30 μg of cytosolic protein were separated by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride membrane (PerkinElmer Co., Wellesley, MA, USA) using a TransBlot Cell (TE-22, Hoefer Co., CA, USA). The resulting blot was blocked in Tris-buffered saline-Tween 20 (TBS-T) containing 5% skim milk for 1 h and then incubated in a primary antibody of rabbit anti-Trpv5 (diluted 1:500, H-99, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-TRPV6 (diluted 1:150, C-16, Santa Cruz Biotechnology), goat anti-Ncx1 (diluted 1:500, P-13, Santa Cruz Biotechnology), rabbit anti-PMCA1b (diluted 1:500, Swant, Bellinzona, Switzerland), or mouse anti-rabbit β-actin (diluted 1:1000, Santa Cruz Biotechnology) at room temperature for 2 h and then at 4°C overnight. After washing 4 times for 1 h with TBS-T, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody against rabbit (diluted 1:5000, Santa Cruz Biotechnology) for Trpv5 and Pmca1b and against goat (diluted 1:5000, Santa Cruz Biotechnology), rabbit anti-PMCA1b (diluted 1:500, Swant, Bellinzona, Switzerland), or mouse anti-rabbit β-actin (diluted 1:1000, Santa Cruz Biotechnology) at room temperature for 2 h and then at 4°C overnight. After washing 4 times for 1 h with TBS-T, each blot was developed by incubation in ECL chemiluminescence reagent (Santa Cruz Biotechnology) and exposed to BioMax™ Light film (Kodak, Rochester, NY, USA) for 30 s (for β-actin) or 2 min (for Trpv5, Trpv6, Ncx1 and Pmca1b). To ensure the specificity of each blot, each band was stripped and re-stained without primary antibody as a negative control. Finally, to compare expression among samples, band density measurement was performed using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). The resulting data were analyzed by one-way ANOVA (Prism 4 Graph Pad; Graph Pad Software Inc., San Diego, CA, USA).

**Immunohistochemistry.** To localize expression of the four genes, IHC was performed for Trpv5, Trpv6, Ncx1 and Pmca1b protein. To prepare samples, tissues from duodenum, kidney, and uterus were sliced by a rotary microtome and embedded in paraffin. Sections (5 μm) were deparaffinized with xylene and hydrated by successive incubation in 100%, 95%, 90%, 80% and 70% ethanol, and then in tap water. Three percent hydrogen peroxide in TBS-T was applied for 30 min to block endogenous peroxidase activity after sections were incubated in citrate buffer (pH 6.0) heated in a microwave for 10 min, and allowed to cool to room temperature. Nonspecific reactions were blocked by incubating sections in 10% normal goat serum for 2 h at room temperature. Sections were incubated with the same Trpv5, Trpv6, Ncx1 and Pmca1b primary antibodies used for Western blotting (diluted 1:200, Santa Cruz) at room temperature for 2 h and at 4°C overnight. After washing with TBS-T, sections were incubated with biotinylated secondary antibodies, rabbit IgG for Trpv5 and Pmca1b, and goat IgG for Trpv6 and Ncx1 (Santa Cruz Biotechnology; and Vector Laboratories, Burlingame, CA, USA) at 37°C for 30 min. Diaminobenzidine (DAB, Sigma-Aldrich corp., Sigma-Aldrich Corp. St. Louis, MO, USA), a chromogen, was applied to sections until they turned brown, and then hematoxylin was used to counterstain sections followed by mounting in Canada balsam.

**Results**

**Expression of Trpv5, Trpv6, Ncx1 and Pmca1b in canine duodenum, kidney and uterus.** Tissue-specific expression of TRPV5, TRPV6, NCX1 and PMCA1B in canine duodenum, kidney and uterus was analyzed by RT-PCR and Western blot analysis. Transcriptional and translational expression of TRPV5 was primarily detected in the kidney, with low levels of expression detected in the duodenum and uterus by RT-PCR and Western blot analysis (Figure 1 and 2). Ncx1 mRNA and protein appeared to be highly expressed in the kidney and expressed at lower levels in the duodenum and uterus as seen in Figures 1 and 2. Pmca1b mRNA and protein were strongly expressed in both the uterus and kidney. We found that Trpv5 and Trpv6 mRNA were more highly expressed in the kidney and duodenum than in the uterus as shown in Figure 1. In contrast, Ncx1 and Pmca1b mRNA showed much greater expression in the kidney and uterus than in the duodenum. Our Western blot analysis confirmed that the kidney was the organ that expressed all four genes most prominently (Figure 2). However, Trpv5, Trpv6, Ncx1 and Pmca1b proteins were also all detected in the duodenum and uterus as demonstrated in Figure 2. Although there is a discrepancy in the expression patterns detected using RT-PCR and Western blot analysis, we confirmed the expression of all four calcium transport genes in the canine duodenum, kidney and uterus.

**Localization of Trpv5, Trpv6, Ncx1 and Pmca1b expression in the canine duodenum, kidney and uterus.** Tissue-specific localization of Trpv5, Trpv6, Ncx1 and Pmca1b protein was analyzed in canine duodenum, kidney and uterus using IHC. As seen in Figure 3A and B, Trpv5 and Trpv6 were detected in apical enterocytes (villous region) in the duodenum, although the expression of Trpv5 was much weaker than that of Trpv6. In contrast, both Ncx1 and Pmca1b had a tendency to be expressed together in differentiated enterocytes of the mucosa, especially in differentiated enterocytes on the lower villi of the duodenum (Figure 3C and D). In the kidney, Trpv5, Trpv6, Ncx1 and Pmca1b were all highly expressed in the distal convoluted tubules, despite regional differences between Trpv5 and Trpv6 (expressed in the apical region) and Ncx1 and Pmca1b (expressed in the basolateral region) as shown in Figure 3A-D. In the uterus, all proteins were co-expressed in the folds of the endometrial surface. However, Trpv6 was also expressed in the glandular epithelium of the uterus (Figure 3B). No positively stained cells were detected on control slides without primary antibodies against these proteins.
Discussion

TRPV5, TRPV6, NCX1 and PMCA1b are known Ca^{2+} transport genes that regulate transcellular Ca^{2+} levels in epithelial cells. Many reports have been published on Ca^{2+}-related genes in mice, rats, rabbits, humans and dogs (14, 17, 18, 20, 21). In vivo studies have demonstrated a quantitative and qualitative correlation between the expression of Ca^{2+} transport proteins and capacity for Ca^{2+} absorption (22). We selected three organs known to undergo high levels of Ca^{2+} transport, the duodenum, kidney and uterus, as our target tissues. Previous studies have investigated the expression of these four target genes in mice, rats, rabbits, sheep and humans (14, 20, 22-26). In this study, we focused on comparing mRNA and protein expression and on the immunolocalization of the four genes in the canine duodenum, kidney and uterus. Our results showed that Trpv5, Trpv6, Ncx1 and Pmca1b mRNA and protein were all detected in the canine duodenum, kidney and uterus. The four genes investigated can be divided broadly into two

Figure 1. Expression of Trpv5, Trpv6, Ncx1 and Pmca1b mRNA relative to Actb mRNA in canine duodenum, kidney and uterus as determined by RT-PCR. A: Trpv5, Trpv6, Ncx1 and Pmca1b mRNA expression was detected based on the expected sizes of the four PCR products: 207 bp (Trpv5), 189 bp (Trpv6), 201 bp (Ncx1), and 236 bp (Pmca1b). B: A graph showed the relative expressions of calcium transport genes.
groups based on the direction of Ca\(^{2+}\) transport mediated by each. The two highly homologous and selective Ca\(^{2+}\) channels, Trpv5 and Trpv6, mediate apical import of Ca\(^{2+}\) into epithelial cells of the duodenum, kidney and uterus. Ncx1 and Pmca1b mediate Ca\(^{2+}\) export across basolateral plasma membranes. Hence, we expected the expression patterns of genes in each group to show similar characteristics (27). Gene function is strongly related to the location and magnitude of expression, which will be discussed in greater detail later.

Trpv5 and Trpv6 are the most Ca\(^{2+}\)-selective channels within the TRP family, and their permeability is due to a single aspartate residue (Trpv5d542, Trpv6541) present in the pore-forming region (5). Trpv5 is known as a trafficking protein that regulates and increases renal Ca\(^{2+}\) uptake (5, 9). Trpv6 plays a role in intestinal Ca\(^{2+}\) transport (7). In addition, the maintenance of uterine Ca\(^{2+}\) balance is crucial for many physiological functions of that organ, including smooth muscle contraction and embryo implantation (18). Our studies on Trpv5 mRNA and protein expression showed that it is
Figure 3. Localization of Trpv5 (A), Trpv6 (B), Ncx1 (C) and Pmca1b (D) in canine duodenum (a-b), kidney (c-d) and uterus (e-h) by IHC. Boxes in the left hand columns are areas magnified in the middle columns. Arrows (red) indicate reactive sites. As a negative control, slides in the right hand columns (c-f, i) were stained in the absence of primary antibodies.
most highly expressed in the kidney. IHC results indicated that Trpv5 is expressed mainly in the distal convoluted and connecting tubules of the kidney (5). Trpv5 was also found to be weakly expressed in the duodenum and uterus by RT-PCR and Western blot analysis. Several previous studies have indicated that Trpv5 is only expressed in the kidney. Although our study indicated that Trpv5 is expressed in the duodenum and uterus, expression was detected at a much lower level than in the kidney (17). Another recent study did detect Trpv5 co-expressed with Trpv6 in the duodenum using RT-PCR (28). Despite the well-characterized role of Trpv6 in the intestine, we found that Trpv6 expression was higher in the kidney than in the duodenum. Through IHC analysis, we observed that Trpv6 was co-expressed with Trpv5 in apical enterocytes (in the villous region) in the duodenum, from the distal convoluted tubules to the collecting tubules of the kidney, and on the endometrial surface and glands of the uterus. These Trpv6 expression patterns in the duodenum and kidney are in agreement with another previously published report (17). In addition, Trpv5 and Trpv6 expression are regulated by the collective action of associated proteins (8). Our uterine Trpv6 expression results were in agreement with a report that Trpv6 is expressed in the apical luminal and glandular epithelium of the mouse (18). These results indicate that Trpv6 plays a substantial role in Ca2+ entry, acting cooperatively with Trpv5.

Genes mediating Ca2+ extrusion from the cell include a rather small number of transporters, including Ncx1 and Pmca1b, in contrast to the wide variety of molecules mediating Ca2+ entry, including Trpv5 and Trpv6. In general, the Ca2+ concentration outside cells is about 1,000 times higher than inside. Thus, it is not possible for Ca2+ to exit cells by diffusion, and a driving force is necessary for Ca2+ to be extruded from the cell (Ncx1 couples the export of 1 Ca2+ ion to the import of 3 Na+ ions, and Pmca1b uses one mole of ATP to power the export of each Ca2+ ion). Although Ncx1 and Pmca1b have a common functions, the relative importance of Pmca1b and Ncx1 is cell-type dependent. Ca2+ efflux in cardiac cells is mediated predominantly by Ncx1, whereas Ca2+ efflux in human erythrocytes is mediated entirely by Pmca1b, and in smooth muscle cells, both Ncx1 and Pmca1b perform this function (15). Ncx1 influences Ca2+ uptake in the duodenal mucosa and is believed to regulate Ca2+ reabsorption in the kidney (24, 25, 29). Pmca1b regulates the intracellular Ca2+ concentration by extruding Ca2+ from cells and participating in net absorptive movement (30, 31). The relatively high level of Ncx1 and Pmca1b mRNA and protein expression in the kidney, particularly along the basolateral membranes of cells in the distal convoluted tubules, these results indicate that renal Ncx1 and Pmca1 may play an important role in the active absorption of Ca2+ in the kidney (32, 33). In the uterus, Ncx1 and Pmca1b are also co-localized and expressed on the endometrial surface. Taken together these results imply that renal Ncx1 and Pmca1b may be involved in Ca2+ transport activities in these tissues, duodenum, kidney, and uterus in some species (14, 34, 35).

In this study, our results showed that the epithelial Ca2+ transport genes Trpv5, Trpv6, Ncx1 and Pmca1b are all significantly expressed at specific sites in the canine duodenum, kidney and uterus. Although one previous report indicated significant expression of these Ca2+ transport genes in the duodenum and kidney, another previous study did not detect some of our genes of interest in these tissues (17). We found that the Ca2+ influx genes Trpv5 and Trpv6 are expressed in the apical membranes of duodenal enterocytes, in renal distal convoluted tubules, and in the uterine endometrial region. The Ca2+ efflux genes Ncx1 and Pmca1b were detected in the basolateral membranes of the duodenum, as well as in the kidney and uterus. Taken together, these results imply that these calcium transport genes play a critical role in calcium transport in the canine duodenum, kidney and uterus. A further study is warranted to examine the functional role of these calcium transport genes in these tissues.

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