K-Cl cotransporter expression in the human kidney

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Liapis, Helen, M. Nag, and Deepak M. Kaji. K-Cl cotransporter expression in the human kidney. Am. J. Physiol. 275 (Cell Physiol. 44): C1432–C1437, 1998.—The K-Cl cotransporter protein KCC1 is a membrane transport protein that mediates the coupled, electroneutral transport of K and Cl across plasma membranes. The precise cell type(s) in the kidney that express the K-Cl cotransporter have remained unknown. The aim of the present investigation was to define the distribution of KCC1 mRNA in the human kidney. We used in situ hybridization with a nonradioactive digoxigenin-labeled riboprobe. We identified abundant KCC1 mRNA expression in the epithelial cells throughout the distal and proximal renal tubular epithelium. The transporter was also expressed in glomerular mesangial cells and endothelial cells of the renal vessels. These findings suggest that the K-Cl cotransporter may have an important role in transepithelial K and Cl reabsorption.

THE K-CL COTRANSPORTER is a membrane protein that mediates a secondary active, coupled transport of K and Cl across plasma membranes (4, 7, 9, 10, 22, 30–32). Like the well-known Na-K-2Cl cotransport (20), K-Cl cotransport is electroneutral (23). However, in contrast to Na-K-2Cl cotransport, it is poorly inhibited by low (micromolar) concentrations of bumetanide (22) and is independent of extracellular or intracellular Na (22). Although the Na-K-2Cl cotransporter is activated by phosphorylation, the K-Cl cotransporter is activated by dephosphorylation (21, 25).

Two isoforms of the K-Cl cotransporter protein, encoded by separate genes, have been identified so far. The KCC2 isoform is found only in the brain (38, 39), whereas the KCC1 isoform is distributed in many tissues (16, 27). The protein is a member of the cation-CI cotransporter (CCC) family. There is a low level of homology (~25% identity) between the K-Cl cotransporter protein and two other members of the CCC family: the bumetanide-sensitive Na-K-2Cl cotransporter and the thiazide-sensitive Na-K cotransporter (16).

The presence and/or role of the K-Cl cotransporter in mediating K transport in renal tubular epithelial cells have been controversial. Although some investigators have provided evidence for a K-Cl cotransporter (12, 13, 18, 19, 41–43), others have looked for and failed to find the K-Cl cotransporter in membrane preparations from renal tubular epithelial cells. Chen and Verkman (5) reported that rates of CI transport across basolateral membrane vesicles from rabbit renal cortex failed to increase with the imposition of a K gradient, both in the presence or absence of valinomycin, suggesting the absence of K-Cl cotransport. Grassl et al. (17) also measured anion transport in basolateral membrane vesicles from the renal cortex and failed to find evidence for K-Cl cotransport. In the present study, we used in situ hybridization to localize the expression of KCC1 mRNA within specific cells in the human kidney. Portions of this work have been presented and published in abstract form recently (33).

MATERIALS AND METHODS

Preparation of riboprobe. Full-length rabbit KCC1 cDNA, a gift of Gillen et al. (16) was subcloned into the EcoR I site of pcDNA3.1 (Invitrogen, Carlsbad, CA). The plasmid pcDNA3.1 containing the 5.4-kb rabbit KCC1 cDNA was linearized with BstX I. A restriction map of rabbit KCC1 cDNA revealed three BstX I restriction sites: at nt 547, 1369, and 2433. The restriction fragments generated by BstX I digestion were separated by electrophoresis with 1% agarose gel. The 822-bp KCC1 fragment (547–1369) was excised and ligated into pBluescript KS(+) vector. At the nucleotide level, this fragment is 90% identical to human KCC1 within the same region, as determined by a BLAST search (blast.ncbi.nlm.nih.gov). The excised 822-bp fragment was selected for ligation into pBluescript KS(+) vector. After amplification and purification, the size was again verified by electrophoresis with 1% agarose gel. Single-stranded RNA probes (riboprobes) were synthesized by in vitro transcription using digoxigenin (DIG)-11-dUTP (Boehringer Mannheim, Indianapolis, IN). To obtain the antisense probe, the plasmid was linearized with Sac I and transcribed using T7 polymerase. To generate the sense probe, plasmid was linearized with Pst I and transcribed using T3 polymerase. The specificity of the antisense riboprobe was confirmed by DNA dot-blot hybridization with KCC1 cDNA (see results and Fig. 1) and by Northern blot analysis of poly(A)+ mRNA from multiple human tissues (Fig. 2). In contrast, no signal was appreciated when the sense riboprobe was used for Northern blot analysis (not shown), suggesting the absence of nonspecific binding.

DNA dot-blot hybridization. Plasmid pcDNA 3.1 containing full-length KCC1 cDNA was linearized with EcoR I. The full length 3.8-kb KCC1 cDNA was purified, digested with fish sperm DNA (to block nonspecific binding) in Tris-EDTA (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), denatured, spotted on a positively charged nylon membrane (maximum strength Nytran Plus; Schleicher & Schuell), and exposed to ultraviolet light for 5 min. Prehybridization was carried out for 2 h at 42°C in a prehybridization buffer (6× standard sodium citrate (SSC), 50% formamide, pH 7.4, 10× Denhardt’s reagent, 1% SDS, 10% dextran sulfate 500, 20 mM sodium maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim). Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim. Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim. Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim. Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim. Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim. Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim. Membranes were hybridized for 18 h at 43°C with the antisense riboprobe (10 ng/ml) and washed two times at room temperature with buffer containing 2× SSC, 0.1% SDS and two times at 68°C in buffer containing 0.1% maleate, pH 7.5, and 2% blocking buffer from Boehringer Mannheim.
SDS, 0.1× SSC. Blots were blocked for 3 h in 3% casein in Tris-buffered saline before adding the antibody. Membranes were incubated first in 1% BSA at room temperature for 10 min and then with anti-DIG antibody, which was conjugated to alkaline phosphatase, for 3 h, and then they were washed in buffer (0.1 M Tris, 0.15 M NaCl, pH 7.5) for 0.5 h at room temperature. After two 5-min washes with 2× SSC, blots were then dehydrated in graded alcohols and air dried at 55°C for 1 h. Two microliters of DIG-labeled probe were added to 100 µl of freshly prepared hybridization solution containing 1× SSC, 12.5× Denhardt’s solution, 50% formamide, 0.5% SDS, 0.25 mg/ml of salmon sperm single-stranded DNA, 0.5% sodium pyrophosphate and 0.01 M Tris, pH 7.4. Denhardt’s solution and salmon sperm DNA block nonspecific binding. The probe was heated at 68°C for 15 min. Hybridization solution (35 µl) was applied per slide, and sections were covered by parafilm. Slides were incubated at 53°C overnight. The next day, the slides were soaked in 5× SSC at 50°C in a water bath for 15 min, followed by a 30-min wash in 2× SSC with 50% formamide at 50°C. After rinsing in RNase buffer at 37°C for 0.5 h, slides were digested in RNase A (20 µl/ml) at 37°C for 0.5 h, followed by two 15-min washes in 0.1× SSC at 50°C. RNase A removes unhybridized probe and excess cellular RNA and improves the signal-to-noise ratio. For detection, slides were incubated in 1% BSA at room temperature for 10 min, and then with anti-DIG antibody linked to alkaline phosphatase for 3 h in a humidified chamber. Slides were washed in buffer (0.1 M Tris, 0.15 M NaCl), pH 7.5, for 0.5 h at room temperature, rinsed in the same buffer, and then incubated with alkaline phosphatase substrate (nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl-phosphate) in the dark overnight in a humidified chamber. The X-ray film was applied and developed for 15 min. 

Northern blot. The KCC1 message was detected on a human multiple-tissue Northern blot (Clontech), with the use of the protocol and the buffers described in the Clontech user manual. Prehybridization was performed at 42°C for 0.5 h with prewarmed ExpressHyb solution (Clontech). The probe was added to fresh ExpressHyb solution and mixed thoroughly. Hybridization with riboprobe-containing ExpressHyb solution was carried out at 42°C for 1 h. Detection with anti-DIG antibody and alkaline phosphatase substrate (CDP-Star) was performed as with the DNA dot-blot assay.

In situ hybridization. Nonradioactive in situ hybridization was carried out as described previously (34, 35), with minor modifications. Tissues obtained from six surgically removed kidneys and two urinary bladders were cut to obtain 4-µm sections. Hematoxylin- and eosin-stained sections were examined to ensure that the selected cases had no major histopathological abnormalities. Tissues were fixed in 10% buffered formaldehyde overnight and were embedded in paraffin. Hybridization was performed as follows. Slides were deparaffinized for 30 min, then rehydrated through graded alcohols, and washed in PBS for 5 min. To increase the penetration of the riboprobe, the slides were then incubated with Proteinase K (2.5 µg/ml) in prewarmed PBS at 37°C for 0.5 h. The slides were then immersed in 0.1 M triethanolamine for 5 min at room temperature and acetylated with fresh triethanolamine containing 0.25% acetic anhydride for 10 min at room temperature. After two 5-min washes with 2× SSC (SSC: 150 mM NaCl, 15 mM sodium citrate), slides were dehydrated in graded alcohols and air dried at 55°C for 1 h. Two microliters of DIG-labeled probe were added to 100 µl of freshly prepared hybridization solution containing 1× SSC, 12.5× Denhardt’s solution, 50% formamide, 0.5% SDS, 0.25 mg/ml of salmon sperm single-stranded DNA, 0.5% sodium pyrophosphate and 0.01 M Tris, pH 7.4. Denhardt’s solution and salmon sperm DNA block nonspecific binding. The probe was heated at 68°C for 15 min. Hybridization solution (35 µl) was applied per slide, and sections were covered by parafilm. Slides were incubated at 53°C overnight. The next day, the slides were soaked in 5× SSC at 50°C in a water bath for 15 min, followed by a 30-min wash in 2× SSC with 50% formamide at 50°C. After rinsing in RNase buffer at 37°C for 0.5 h, slides were digested in RNase A (20 µl/ml) at 37°C for 0.5 h, followed by two 15-min washes in 0.1× SSC at 50°C. RNase A removes unhybridized probe and excess cellular RNA and improves the signal-to-noise ratio. For detection, slides were incubated in 1% BSA at room temperature for 10 min, and then with anti-DIG antibody linked to alkaline phosphatase for 3 h in a humidified chamber. Slides were washed in buffer (0.1 M Tris, 0.15 M NaCl), pH 7.5, for 0.5 h at room temperature, rinsed in the same buffer, and then incubated with alkaline phosphatase substrate (nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl-phosphate) in the dark overnight in a humidified chamber. The X-ray film was applied and developed for 15 min.

Fig. 1. DNA dot-blot hybridization showing the sensitivity of the K-Cl cotransporter protein (KCC1) cDNA antisense ribonucleotide probe for KCC1 cDNA.

Fig. 2. Northern blot analysis of KCC1 expression using the Clontech human multiple-tissue Northern blot. This blot has 2 µg of poly(A)+ mRNA per lane from heart, brain, placenta, lung, liver, skeletal muscle (Sk. muscle), kidney, and pancreas.
fied chamber for color development. The reaction was stopped with Tris-EDTA buffer. Slides were rinsed with Milli-Q water. Coverslips were mounted over aqueous mounting medium. The experiments were repeated four times.

Tissue sections for in situ hybridization are subjected to harsh treatment with detergents and enzymes to facilitate the penetration of riboprobe across the plasma membrane. This causes morphological distortions and retraction artifacts and makes sharp distinction between the various nephron segments difficult. Therefore, parallel (adjacent) sections stained with hematoxylin and eosin and epithelial membrane antigen (6) were obtained (not shown). The epithelial membrane antigen stains distal convoluted and collecting tubules, but not proximal tubules (6). On hematoxylin and eosin sections, proximal tubules, the most abundant type in the cortex, were identified by abundant, deeply staining eosinophilic cytoplasm and recognizable brush border; distal convoluted tubules were distinguished by the lack of brush border and less abundant, pale cytoplasm; and collecting ducts were distinguished by sharply defined intercellular margins on hematoxylin and eosin sections, unlike proximal or distal convoluted tubules.

**RESULTS**

The sensitivity and the specificity of the antisense riboprobe were assessed by DNA dot-blot hybridization. Figure 1 shows that the antisense riboprobe detected down to 2.5 pg KCC1 cDNA. In contrast, no hybridization of control plasmid DNA pBR328 (Boehringer Mannheim) was observed <2,500 pg plasmid DNA (not shown).

Northern blot analysis with the DIG-labeled probe detected a prominent 3.8-kb message in all tissues tested, as expected for KCC1 mRNA (Fig. 2). No signal was detected with the sense probe (not shown). The variability in the intensity of bands in various tissues does not necessarily represent variations in KCC1 mRNA abundance among the tissues and may be due to unequal loading, or varying degrees of degradation, of poly(A)⁺ mRNA from different tissues. In addition to the 3.8-kb message, a less prominent 5.6-kb message was also observed in almost all tissues. The larger 5.6-kb transcript has been reported previously in several cell lines by Larsen et al. (28) for “unknown protein X,” which was later identified as KCC1 (16). The unknown protein X is the same as KCC1 because 1) the location of coding region for protein X is identical to that of KCC1, 199 bp upstream of the lecithin cholesterol acyltransferase (LCAT) gene on chromosome 16q22.1; 2) both have 3.8-kb transcripts; and 3) both protein X and KCC1 are transcribed in the same direction as the LCAT gene (16, 28). The 5.6-kb transcript may represent another as yet unidentified isoform of the K-Cl cotransporter or a KCC1 transcript with additional sequence from the 5’ or 3’-untranslated region(s) of KCC1. The results of Northern blot analysis suggest that the antisense riboprobe was highly specific for KCC1 mRNA.

In situ hybridization revealed distinct differences in the expression of the K-Cl cotransporter message within the various cell types in the kidney. Figure 3 shows a section of the renal cortex hybridized with the antisense probe. The proximal tubules (thin arrow), distal convoluted tubules (arrowheads), and the cortical collecting ducts (thick arrow) all showed strong expression of KCC1 mRNA. The tubules from different nephron segments were identified on adjacent hematoxylin and eosin and epithelial membrane antigen-stained sections by morphological criteria described in MATERIALS AND METHODS.

Figure 4 shows a section of the renal medulla. The smaller medullary collecting ducts (thin arrows) and the epithelial cells of the larger papillary ducts of Bellini (thick solid arrows) expressed KCC1 mRNA strongly (Fig. 4). The appearance of both the smaller medullary ducts and larger ducts of Bellini in the same section is attributed to the fact that this section was cut at an angle, and therefore the depth of the section...
increased from one end of the section to the other. The epithelial cells from the papillary ducts of Bellini, with the surrounding plasma membranes, were clearly identified in the consecutive hematoxylin and eosin sections, but their morphology is not distinctive here because of retraction artifacts in the sections subjected to in situ hybridization. Staining was also observed in endothelial cells of the vasa recta (open arrows).

The specificity of the riboprobe was confirmed by the finding that kidney sections probed with the sense probe failed to hybridize (Fig. 5).

In contrast to the tubular epithelial cells, the only focal low-abundance signal for KCC1 mRNA was observed within the glomeruli (Fig. 6, also see Fig. 3). Definite identification of the cells showing the KCC1 signal in glomeruli is not possible, but the location of the hybridization signal in glomeruli (in cells located in the glomerular mesangium and outside the capillary lumen; Fig. 6, asterisks) was consistent with mesangial cell cytoplasm (arrows, Fig. 6). The KCC1 signal is also observed in distal convoluted tubules (arrowheads).

Smooth muscle cells in the interlobular arteries and arterioles (arrowheads, Fig. 7) did not express KCC1, in contrast to the tubular epithelium (Fig. 7). However, endothelial cells in medium-sized artery (open arrow, Fig. 7) stained positive with KCC1 antisense probe. The positive staining in endothelial cells is also observed in vasa recta (open arrows).

In the urinary bladder, the cells of the transitional epithelium lining the bladder lumen showed strong expression of KCC1 mRNA, whereas the cells in the submucosal and muscularis layers did not hybridize with the riboprobe (Fig. 8).

DISCUSSION

The salient feature of the present study was the abundant expression of the K-Cl cotransporter in tubular epithelial cells throughout the length of the nephron. The K-Cl cotransporter was presumably distributed throughout the nephron, since no region of the tubule was found to be devoid of KCC1 mRNA. Several investigators have suggested that a constitutively ac-

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Fig. 5. Renal cortex treated with the KCC1 sense probe as a control. Label is absent from the renal tubules, in contrast to the section treated with the antisense probe (see Fig. 3). Magnification, ×200.

Fig. 6. Low-abundance focal signal is evident within the mesangial space of the glomerulus outside the capillary loops (asterisks), consistent with the location of the cytoplasm of the glomerular mesangial cells (arrows). KCC1 signal is also evident in distal convoluted tubules (arrowheads). Magnification, ×400.

Fig. 7. High-power light micrograph showing KCC1 signal in the endothelial cells of medium-sized artery (arrow) but not in smooth muscle of the artery. Magnification, ×200. Arrowheads, smooth muscle cells in interlobular arteries and arterioles.

Fig. 8. Tissue section from urinary bladder treated with the antisense probe. Intense label is observed in the epithelium, whereas the label is absent from the lamina propria and the muscle layer on the top. Magnification, ×50.
K-Cl cotransporter expression in human tissues

We found prominent expression of the K-Cl cotransporter KCC1 in the medullary collecting duct cells. The renal medulla is a site where urea concentrations may reach values as high as 500–600 mM in normal human subjects (26). Urea has been shown to activate K-Cl cotransporter in human and sheep erythrocytes (8, 24). These findings raise the possibility that high urea concentrations, and in particular acute changes in urea concentrations, may modulate K-Cl cotransporter activity in the renal medulla.

A separate volume regulatory role for K-Cl cotransporter in proximal tubules was suggested by the studies of Avison et al. (3). These authors found that addition of glucose to rabbit proximal tubular suspensions led to an increase in intracellular Na content (suggesting Na-glucose cotransport). Despite the increase in intracellular Na, cell volume was maintained, because intracellular K content decreased. The net K efflux was ouabain and Ba insensitive and was inhibited by high (1 mM) but not low concentrations of furosemide or bumetanide. These findings suggest that K-Cl cotransport may play a pivotal role in maintaining cell volume during transepithelial glucose and NaCl reabsorption in proximal tubes.

Our study suggested possible localization of the KCC1 mRNA signal in glomerular mesangial cells. Mesangial cells regulate the surface area of the glomerulus. Isometric contraction of mesangial cells decreases the surface area and decreases the glomerular filtration rate (GFR). A variety of ligands, hormones, and cytokines have been shown to modulate GFR, in part by affecting mesangial cell volume (11). The possible expression of the volume-sensitive K-Cl cotransporter in the glomerular mesangial cell suggests the possibility that the transporter may play a key role in the modulation of GFR by various ligands and cytokines. However, we caution that it is not possible to be certain of the identity of the stained cells.

We also found KCC1 expression in the endothelial cells of renal arterioles. This finding extends the work of Perry and O’Neill (40) who found evidence for the K-Cl cotransporter in the endothelial cells of the aorta. It has been proposed that humoral and mechanical stimulation of endothelial cells leads to a cascade of events, which ultimately leads to modulation of smooth muscle tone and vascular permeability (40). The activation of ion transport pathways is an early participant in this cascade. Because smooth muscle tone and vascular permeability may control renal blood flow, activation of K-Cl cotransporter may be an early and key component of the pathway by which humoral stimuli regulate renal blood flow.

The epithelial cells of amphibian and mammalian urinary bladder express water channels (1, 15) and exhibit a low but measurable permeability to water (37). Localization of KCC1 in human bladder epithelial cells is described here for the first time. The K-Cl cotransporter may be important in regulatory volume decrease after cell swelling on prolonged exposure of the bladder epithelial cells to dilute urine.

In summary, this is the first report that shows strong expression of the K-Cl cotransporter KCC1 in human renal tubular epithelial cells. Taken together with earlier functional studies, it suggests that the K-Cl cotransporter plays an important role in transcellular K transport in renal tubular cells. In addition, the K-Cl cotransporter may also be involved in volume regulation, both during hypotonic challenge in the medulla and during transepithelial solute transport in the proximal tubular cells.

We thank Qinglong Hu and FuXiong Lu for technical help with the Northern blot analysis. This work was supported by a Merit Review Grant (to D. M. Kaji) from General Medical Research, Dept. of Veterans Affairs, Washington, DC.

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Received 3 April 1998; accepted in final form 31 August 1998.
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