Avian renal proximal tubule epithelium urate secretion is mediated by Mrp4

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Birds are uricotelic and, like humans, maintain high plasma urate concentrations (~300 μM). The majority of their urate waste, as in humans, is eliminated by renal proximal tubular secretion; however, the mechanism of urate transport across the brush-border membrane of the intact proximal tubule epithelium during secretion is uncertain. The dominance of secretory urate transport in the bird provides a convenient model for examining this process. The present study shows that short hairpin RNA interference (shRNAi) effectively knocked down gene expression of multidrug resistance protein 4 (Mrp4; 25% of control) in primary monolayer cultures of isolated chicken proximal tubule epithelial cells (cPTCs). Control and Mrp4-shRNAi-treated cPTCs were mounted in Ussing chambers and unidirectional transepithelial fluxes of urate were measured. To detect nonspecific effects, transepithelial electrical resistance (TER) and sodium-dependent glucose transport (Iglu) were monitored throughout experiments. Knocking down Mrp4 expression resulted in a reduction of transepithelial urate secretion to 35% of control with no effects on TER or Iglu. Although electrical gradient-driven urate transport in isolated brush-border membrane vesicles was confirmed, potassium-induced depolarization of the plasma membrane in intact cPTCs failed to inhibit active transepithelial urate secretion. However, electrical gradient-dependent vesicular urate transport was inhibited by the MRP4 inhibitor MK-571 also known to inhibit active transepithelial urate transport by cPTCs. Based on these data, direct measure of active transepithelial urate secretion in functional avian proximal tubule epithelium indicates that Mrp4 is the dominant apical membrane exit pathway from cell to lumen.

urate; ABCC4; renal tubular secretion; organic anion transport

URATE IS A NORMAL OXIDATION product of purine metabolism (21) and serves as a potent plasma antioxidant at normal concentrations in birds as well as humans. However, hyperuricemia is associated with gout, kidney stones, insulin resistance, and cardiovascular disease. Even hypernormal plasma urate has been suggested as a contributing factor to the development of the metabolic syndrome (10, 40) characterized by cardiovascular disease, truncal obesity, glucose intolerance, hyperinsulinemia, and hypertension, most of which have been associated with high dietary fructose but not always through hyperuricemia (50). In normal rats, hyperuricemia has been shown to induce systemic hypertension, renal vasoconstriction, glomerular hypertension, and glomerular hypertrophy (38, 41, 48). In the human kidney, urate is freely filtered and then largely (>90%) reabsorbed in the proximal tubules by processes including the organic anion transporters URAT1 (urate anion exchanger1, SLC22A12) (15), organic anion transporter 4 (OAT4, SLC22A9) (39), GLUT9 [SLC2A9, a basolateral membrane (BLM) voltage-dependent urate transporter] (4, 30, 58), and OAT10, (SLC22A13) (7). In addition, urate is secreted by the proximal tubule epithelium, and in both birds and great apes ~70% of renal urate excretion is due to tubular secretion (21). Thus, although this secretory process is a major contributor to urate homeostasis, the dominance of reabsorption in human tubules (filtered: 9,000 mg/24 h; excreted: 690 mg/24 h) has made it difficult to identify processes subserving only secretion.

Urate is the major nitrogenous waste in birds, and, unlike in great apes (21), there is no significant facilitated reabsorption of urate detected in the perfused chicken renal proximal tubule (9) or in primary monolayer cultures of chicken renal proximal tubule epithelial cells (cPTCs) (13), only active secretion. URAT1 is believed by many to be the main contributor to urate reabsorption in humans; however, no similar sequence is present in the chicken genome, consistent with the lack of reabsorption in the avian system. It is known that birds begin renal urate secretion at embryonic day 5 and continue to do so in greater amounts after hatching. Urate secretion has been directly measured in the perfused renal proximal tubule of adult chicken (9). Our previous characterization study of renal proximal tubule monolayer cultures from the neonate chicken indicated equivalent transport and inhibitor effects as seen in the adult perfused tubule (13).

Proposed secretion models suggest that urate is transported across the BLM by organic anion transporters 1 and 3 (Oat1 and Oat3; both expressed in chicken kidney) (13). Urate exchange for intracellular α-ketoglutarate (α-KG) by these organic anion transporters has been implicated as the mechanism for BLM urate transport (43) and has been noted in avian-perfused tubules (9) as well as in isolated BLM vesicles from pig (59) and turkey kidney (18). This widely accepted tertiary active transport mechanism relies on the generation of a sodium gradient by Na+K+ATPase and recycling of intracellular α-KG by the Na+/dicarboxylate cotransporter. α-KG leaves the cell down its electrochemical gradient, driving urate into the proximal tubule cell via Oat1 and -3 against urate’s electrochemical gradient (11). The exit step in urate secretion, cell to lumen across the brush-border (apical) membrane (BBM) of the renal proximal tubule is uncertain. Numerous studies with isolated BBM vesicles (BBMV) from diverse species (17, 24, 27, 28, 35, 45) have demonstrated the presence of a voltage gradient-driven urate transport mechanism. However, there seems to be no direct evidence for apical membrane voltage-dependent urate secretion as a step in transepithelial secretion in any intact, homologous, functional epithelium.

Recently, multidrug resistance protein 4 (MRP4; ABCC4) has been implicated in the secretion of urate by the proximal
tubule (57). MRPs are part of an ATP-binding cassette (ABC) family of membrane export pumps that help eliminate endogenous waste products. MRP2 and MRP4 are present in the BBMs of the mammalian proximal tubule and are expressed in the chicken proximal epithelium (13). MRP2 apparently does not transport urate, but MRP4 does, when overexpressed in insect Sf9 cells or HEK-293 cells (57). However, there has been no direct evidence for Mrp4 participation in urate excretion by the kidney, which prompted the present study of Mrp4’s contribution to the secretory transport of urate by the avian proximal tubule.

Here, the voltage gradient-driven pathway for the transport of urate by avian isolated BBMV was confirmed; but, there was no effect of depolarization of the proximal tubule cells on transepithelial urate secretion. More importantly, knockdown of Mrp4 gene expression with short hairpin RNA interference (shRNAi) in the intact epithelium showed that Mrp4 was the dominant pathway for apical membrane urate secretion.

MATERIALS AND METHODS

Animals. Kidneys were isolated from 6–8 leghorn chicks (domestic Gallus gallus L., 5–7 days of age) for each cell culture preparation and 1–2 chicks for BBMV preparation. This study adheres to the newest “Guiding Principles for Research . . . ” as outlined by the American Physiological Society (2a). All investigations involving animals reported in this study were conducted in conformity with these principles, and the animal protocol was approved by the University of Connecticut IACUC (protocol no. A05-044).

Preparation of cPTCs. Chicken proximal tubule segments were isolated and dispersed as previously described by Sutterlin and Laird (53) and modified by Dudás and Renfro (14). Briefly, kidneys were removed and rinsed thoroughly in HBSS. The tissue was then teased apart to remove blood vessels, ducts, and connective tissue; minced into smaller tissue fragments; and then incubated in an enzyme solution containing collagenase A (0.13 U/ml) and dispase II (0.54 U/ml) at 37°C for 10 min. Nephron segments were further dissociated by trituration and filtration through a stainless steel sieve (380-μm) and then rinsed three times with HBSS, and resuspended in a 1:1 mixture of Percoll and 2/5 Krebs-Henseleit buffer. The mixture was centrifuged at 17,500 g and the high-density band containing small proximal tubule segments was removed, rinsed with HBSS, resuspended in culture medium with 10% serum, and plated on native rat tail collagen as previously described (12). On day 7 in culture, the collagen gels with confluent epithelial monolayers on their surfaces were released from the walls of the culture dishes. By day 14 in culture, these monolayers had contracted the floating collagen rafts from 25 mm diameter to ~12 mm, achieving their normal cuboidal-columnar morphology and reestablishing their tight junction integrity. These fully differentiated monolayers have the same glucose, sulfate, phosphate, and urate transport properties as are present in the intact tissue (9, 14, 53).

Ussing chamber studies. cPTCs were used to study transepithelial urate transport during days 15–25 of culture by mounting the collagen rafts in Ussing chambers as previously described (20). The tissues were supported in the chambers by 150-μm nylon mesh on the peritubular side, and both sides of the tissue were bathed in the physiological saline solution (in mM: 1.1 CaCl2, 4.2 KCl, 0.3 MgCl2, 0.4 MgSO4, 120 NaCl, 0.4 NaH2PO4, 0.5 Na2HPO4, 1.0 glycine, 25 NaHCO3, 330 μM uric acid, 5.5 mM glucose, pH 7.4 with 5% CO2-95% O2, 290 mosmol/kg H2O), which was kept stirring at 39°C and gassed with humidified 5% CO2-95% O2 throughout the experiment. Reference electrodes were connected to the bath solutions on both sides of the tissues by 3 M KCl-2% agar bridges. Electrode asymmetry was corrected at the beginning of each experiment. Transepithelial electrical resistance (TER) was calculated for each tissue from the change in transepithelial electrical potential caused by a 10-μA current pulse. Ag-AgCl electrodes were used to pass the current via 3 M KCl-2% agar bridges, and a pair of computer-controlled, high-impedance, automatic dual-voltage clamps (EVC400; World Precision Instruments, Sarasota, FL) were used to measure and record electrical properties. At the end of each experiment the vitality and proximal tubule-like function of the epithelium were monitored by measuring the sodium-dependent glucose current. To do this, phlorizin (1 mM), a specific inhibitor of sodium-dependent glucose transport, was added to the bath solution on the luminal side, and the change in transepithelial current recorded.

Determination of transepithelial Urate fluxes. During flux measurements, the tissues were continuously short circuited unless otherwise noted. In these low-resistance tissues, typical short-circuit current values were 3–5 μA/cm2. The addition of 0.01 mCi [3H]urate (Moravek Biochemicals and Radiochemicals, Brea, CA) to one side of each tissue at time 0 marked the beginning of the unidirectional fluxes. The flux of radiolabeled urate was measured by taking 50-μl samples in duplicate from the bath of the nonlabeled side of the tissue every 30 min for a period of 1.5 h (unless otherwise noted). After each sample was taken, its volume was replaced by 50 μl of unlabeled saline solution. At the beginning and end of each experiment, 10-μl samples were taken from the bath of the labeled side of each tissue to determine the specific activity of the labeled solution.

Net transepithelial flux was determined by the difference between the unidirectional secretory and reabsorptive fluxes (peritubular side to lumen side and lumen side to peritubular side, respectively). Although chicks do not seem to have any means for active reabsorption of urate, there is urate flux in the reabsorptive direction due to passage through the paracellular pathway (9), which must be considered for calculation of the net transepithelial flux.

Microelectrode determinations of plasma membrane potential. Apical membrane electrical potentials of cultured epithelial cells from cPTCs were determined as described previously (32). Briefly, glass microelectrodes with tip resistances of 30–50 MΩ were inserted into cells, and recordings were obtained by an Axoclamp 2B amplifier. Several initial recordings were taken while the cPTC was in normal physiological saline solution. This solution was then replaced with a solution in which 100 mM NaCl were replaced with 100 mM KCl. After the solution change, additional recordings were taken from several cells to obtain an average apical membrane potential in that state.

Isolation of BBMV and uptake assays. The Mg2+ precipitation method used for the isolation of chick kidney BBM is a modification of the previously described Ca2+ precipitation method (28). Briefly, the kidneys were Polytron homogenized in a solution containing 50
mM mannitol and 1 mM Tris base and brought to pH 7.4 with HEPES. To isolate the BBM fragments, 30 mM MgCl2 was added to the mixture and stirred for 20 min on ice. Differential centrifugation in vesicle buffer (usually 200 mM mannitol, 20 mM Tris-HEPES, pH 7.4) was then used to remove the BLM fragments and other kidney fractions, resulting in a highly enriched BBMV suspension. The enrichment of BBMs was confirmed by marker enzyme assays for alkaline phosphatase and Na+-K+-ATPase (18.15 ± 0.58, and 1.12 ± 0.65, enrichment compared with homogenate, respectively).

The method used for measuring ion transport by the BBMV is a modification of a previously described method (25). Briefly, 10-μl samples of BBMV, containing 5 μM valinomycin, were mixed with 100 μl aliquots of incubation buffer (100 mM potassium gluconate, 20 mM Tris base, pH 7.4 with HEPES) containing 35 μM [3H]urate and vortexed to begin transport. Transport was stopped by the addition of an ice-cold solution (100 mM KCl, 5 mM proceneid, 20 mM Tris-HEPES, pH 7.6). BBMV were then collected on 0.45-μm membrane filters (12-mm diameter; Millipore) and washed with 3 ml of stop solution to eliminate any excess incubation buffer. The filters were placed in a 24-well plate with 400 μl Ultima Gold Scintillation Cocktail (Perkin Elmer) per well, and the amount of radioisotope on each filter was measured in a Hidex Chameleon Plate Reader (BioScan, Washington, DC). Filter blanks were done for each incubation buffer, and all samples were run in triplicate.

Isolated BLM preparation. The method used to isolate the chick BBM is a modification of the previously described method (47). Briefly, 8–10 g of fresh or frozen kidneys were minced with scissors in homogenization medium (250 mM sucrose, 10 mM Tris-HEPES, pH 7.4) and subjected to 20 strokes with a glass Teflon homogenizer and then Polytetrafluoroethylene homogenized at speed 8 for 3–5 s periods separated by two 1-min rest periods. After centrifugation for 20 min at 20,500 g, the crude membrane extract (the top layer) was removed and resuspended in fresh homogenization medium. These samples were then glass Teflon homogenized for 18 strokes at 10,000 rpm, combined with 9% Percoll, and homogenized for another two strokes. Centrifugation at 39,500 g was then used to separate the BLM fractions from other fractions based on density. The top six milliliters were removed and discarded; the next seven milliliters were removed, mixed with additional homogenization medium, and spun at 100,000 g to remove the Percoll. The final pellet was resuspended in an appropriate buffer for Western blot analysis. The enrichment of the BLM was confirmed by marker enzyme assays for Na+-K+-ATPase and alkaline phosphatase (38.55 ± 7.06 and 3.02 ± 0.77, enrichment compared with homogenate, respectively).

Short hairpin RNA interference. Four 66-nt DNA template sequences were designed against the chicken Mrp4 sequence (Invitrogen), inserted into the mU6pro vector using the protocol described previously (61), and cloned in DH5α competent bacterial cells (Invitrogen) according to the manufacturer’s instructions. The plasmid DNA was extracted from the bacterial cells using the Plasmid Maxi Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions, and its concentration was measured by UV spectrophotometry at 260 and 280 nm to ensure that the same amounts of total RNA from the control, control-shRNAi, and Mrp4-shRNAi-treated tissues were added to the reaction. Semiquantitative RT-PCR was performed using the Qiagen OneStep RT-PCR Kit, and the primers generated against the known sequence for Mrp4 (GenBank accession no. XM_416986) in the chicken (G. gallus) (forward primer 5'-CGAGGCACTGGTGACGTAGC3', reverse primer 5'-CGCACTCAGGTATGCTCAA3') (Invitrogen). The reverse transcription reaction was performed at 50°C for 30 min followed by 95°C incubation for 15 min to denature the RT. This was immediately followed by the PCR, which had a denaturing temperature of 94°C, an annealing temperature of 56°C, and an extending temperature of 72°C for 30 cycles (semiquantitative). Equal amounts of the PCR products were separated on a 1% agarose gel and stained with Gel-Star (Lonna Rockland, Rockland, ME). Differences between samples were determined by pixel density analysis.

Real-time and RT-PCR. RNA samples were isolated and quantified as described above, cDNA was created from the RNA samples using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantification and purity of cDNA was analyzed by UV spectrophotometry at wavelengths 260 and 280 nm. cDNA (20 ng) was generated from control and control-shRNAi and Mrp4-shRNAi-treated tissues were combined with the Fast SYBR Green Master Mix (Applied Biosystems) and primers (200 nM) designed against chicken Mrp4 for use with real-time PCR (forward primer 5'-CAAGTGGTGTTTTGTCGTGTTG-3', reverse primer 5'-TCTCGCCAGGGAGAACA-3') (Integrated DNA Technologies). In addition, control, control-shRNAi, and Mrp4-shRNAi-treated samples were combined with the master mix and primers against β-actin (200 nM) (forward primer 5'-ATGGATGGAAATCTGCTGAC-3', reverse primer 5'-CTCC-TGTGTTGTTGTTG-3'). The reaction used an initial enzyme activation step at 95°C for 20 s followed by 40 cycles of degradation at 95°C for 3 s and annealing/extension at 60°C for 30 s. Fluorescence was quantified during the annealing/extension step, and product formation was confirmed by melting curve analysis (55–95°C). Data were analyzed during the linear stage of amplification. All samples were run in triplicate. No-amplification controls (where no enzyme was added) and no-template controls (where no cDNA samples were added) were run to eliminate the possibility of false amplification or amplification of a contaminant. Relative mRNA amounts of target gene chicken Mrp4 were calculated after normalization to an endogenous reference gene, β-actin.

Transfection of cPTCs. The Magnetofection technique was used to transfet the cPTCs with the plasmid DNA. Magnetofection uses a solution of magnetic nanoparticles called the Polymag solution (Boca Scientific, Boca Raton, FL). The magnetic nanoparticles are cationic and designed to bind to DNA. Plasmid DNA was mixed with the Polymag solution and chick culture medium according to the manufacturer’s instructions and allowed to bind at room temperature for 30 min. The solution was then added to the lumen side surface of cPTC confluent monolayers on unreleased collagen, and a strong magnet (Boca Scientific) was placed under the culture dish for 20 min. The magnetic plate drew the magnetic particles, with the DNA bound to them, onto the apical cellular surfaces. By concentrating the DNA on the cells and allowing the cells to take up the DNA by endocytosis, high-efficiency transfection with no apparent toxic effects was achieved. Some control cPTCs (Polymag controls) were treated with only the magnetic nanoparticles (no-plasmid DNA) and subjected to the same magnetic treatment as the control-shRNAi and Mrp4-shRNAi (knockdown) cPTCs.

RNA isolation, RT-PCR, and gel electrophoresis. RNA was isolated from cPTCs using the Qiagen RNeasy Kit according to the manufacturer’s instructions. RNA concentration and purity were measured by UV spectrophotometry at 260 and 280 nm to ensure that the same amount of total RNA from the control, control-shRNAi, and Mrp4-shRNAi-treated tissues were added to the reaction. Semiquantitative RT-PCR was performed using the Qiagen OneStep RT-PCR Kit, and the primers generated against the known sequence for Mrp4 (GenBank accession no. XM_416986) in the chicken (G. gallus) (forward primer 5'-CGAGGCACTGGTGACGTAGC3', reverse primer 5'-CGCACTCAGGTATGCTCAA3') (Invitrogen). The reverse transcription reaction was performed at 50°C for 30 min followed by 95°C incubation for 15 min to denature the RT. This was immediately followed by the PCR, which had a denaturing temperature of 94°C, an annealing temperature of 56°C, and an extending temperature of 72°C for 30 cycles (semiquantitative). Equal amounts of the PCR products were separated on a 1% agarose gel and stained with Gel-Star (Lonna Rockland, Rockland, ME). Differences between samples were determined by pixel density analysis.

SDS-PAGE and immunoblotting. Samples were used with 2× Kaman buffer (2.3% SDS, 5% β-mercaptooehanol, 10% glycerol, 0.5% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8)
and used for SDS-PAGE (7% resolving gel, 4% stacking gel). The gel products were transferred to a polyvinylidene fluoride microporous membrane (Millipore). Nonspecific binding was prevented by blocking the membrane with PBS (in mM: 137 NaCl, 2.7 KCl, 4.3 Na2HPO4, 1.5 KH2PO4, pH 7.3 with HCl) containing 5% nonfat dry milk and 0.05% polyoxyethylenesorbitan monolaurate (TWEEN 20) for 2 h at room temperature. Mrp4 protein was detected by using a custom affinity-purified polyclonal antibody (antigen amino acid sequence: S'-CNGQLATDSSLDPSS-3'; mol wt 1494.55, purity 88.1%, lot no. 35838-2; Genscript, Piscataway, NJ) designed against a region near the COOH-terminal end of the chicken (G. gallus) Mrp4 sequence. The membrane was incubated in the PBS/dry milk/Tween 20 solution containing the primary antibody (1:2,000) for 1 h at room temperature. The membrane was washed 3 × 10 min in PBS/dry milk/Tween 20 solution, The polyvinylidene fluoride membrane was then incubated with the secondary antibody, goat anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich) diluted 1:2,000 in PBS/dry milk/Tween 20 solution, for 1 h at room temperature. The membrane was washed 3 × 10 min in PBS/dry milk/Tween 20 and then washed 2 times rapidly with PBS.

Detection and quantification. Signals were detected by enhanced chemiluminescence Western blotting reagents (Pierce) according to the manufacturer’s instructions and developed on medical X-ray film (Fujifilm) for analysis. Film exposure time varied depending on the strength of the signal.

Immunohistochemistry of cPTCs. Control and Mrp4-shRNAi-treated cPTCs were fixed in 2% formaldehyde in PBS at room temperature for 10 min and washed with PBS. The tissues were then permeabilized with 1% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min and washed with blocking buffer (PBS, 1% BSA). Custom polyclonal antibody for chick Mrp4 was diluted in blocking buffer 1:500, applied to the tissues and incubated at 37°C for 90 min and then washed away with blocking buffer. cPTCs were then incubated in goat anti-rabbit antibody conjugated with an Alexa Fluor 488 fluorescent label diluted 1:2,000 in blocking buffer at 37°C for 60 min and washed with blocking buffer. All washes were 4 × 5 min, then 1 × 15 min and took place at room temperature. Immunolabeled cPTCs were imaged using fluorescent microscopy (Axiovert 200 inverted microscope; Zeiss).

Statistics. Results shown are expressed as means ± SE. Experimental treatments were evaluated using one-way ANOVA followed by Tukey-Kramer comparisons. Paired comparisons of sample means were done with Student’s t-test, and significant differences were assumed if P < 0.05.

RESULTS

Knockdown of Mrp4 expression in cPTCs by Mrp4-shRNAi. Of the four potentially interfering sequences tested, only the 66-nt DNA template beginning at nucleotide 2601 (designated Mrp4-shRNAi) successfully knocked down Mrp4 mRNA levels. The representative gel in Fig. 1A shows RT-PCR products from identical quantities of control and Mrp4-shRNAi-treated cPTC total RNA. These products reveal a dense band in the control cPTCs at ~565 bp and only a faint band in Mrp4-shRNAi-treated cPTCs. The summary pixel density analysis from semiquantitative RT-PCR on 10 different paired sample sets shown in Fig. 1B indicates a 75% reduction of Mrp4 mRNA in the Mrp4-shRNAi-treated cPTCs. The samples were also run without the RT step of the reaction to confirm that the cDNA products were amplified from mRNA and not a contaminating DNA sequence present in the samples. Real-time PCR was used to further quantify the knockdown of gene expression in the Mrp4-shRNAi cPTCs compared with control and control-shRNAi-treated samples. All samples were normalized to the endogenous gene β-actin. The results of this quantitative analysis revealed a 75% reduction in chicken Mrp4 gene expression in the Mrp4-shRNAi cPTCs (Fig. 1D).

Immunoblot analysis with commercially available rat anti-human MRP4 antibody (cat. no. ALX-801-038; Axxora) and rabbit anti-human MRP4 antibody (cat. no. ALX-210-856; Axxora) did not reveal chicken Mrp4. Western blot analysis was successful with the custom polyclonal antibody raised against the chicken Mrp4 immunogen (GenScript; Fig. 2). A dense band between 140 and 155 kDa revealed the presence of Mrp4 (149.9 kDa in chicken) in the BBM (Fig. 2A). No staining was apparent with the preimmune serum (Fig. 2B) or when excess immunogen was present (Fig. 2D). Figure 2C
Fig. 2. Immunoblot analysis of isolated brush-border membrane (BBM), basolateral membrane (BLM), and acetaminophen-induced mouse liver homogenate (MLH; plus control for Mrp4) and samples. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane for blotting with antibodies. Enhanced chemiluminescence reagents were used for detection. Samples were blotted with custom polyonal rabbit anti-chicken Mrp4 antibody (A, C, and E); preimmune serum from rabbit (B); and Mrp4-immunizing antigen in addition to the rabbit anti-chicken Mrp4 antibody (D). All samples were blotted for the same amount of time, at the same temperature, and with the same goat anti-rabbit peroxidase-conjugated secondary antibody.

Low-homogenate yield made Western blot analysis impractical for cPTCs. Therefore, immunohistochemistry was used to determine whether Mrp4 protein expression, like Mrp4 mRNA expression, had also been reduced in the cPTCs transfected with the Mrp4-shRNAi construct. The epithelial tissues were stained with the rabbit anti-chicken Mrp4 antibody and then stained with a green fluorescent secondary antibody for detection. cPTCs on contracted collagen gels are densely packed as shown by the phase contrast image in Fig. 3A; the scanning electron micrograph of the monolayer surface reveals the microvillar BBM surface in more detail (Fig. 3B). Figure 3C indicates that the secondary fluorescent antibody appears to stain the BBM of the control cPTCs. Figure 3E shows an Mrp4-shRNAi-transfected cPTC stained and imaged under the same conditions as the paired control. It is clear from the reduction in staining that the Mrp4-shRNAi knocked down the expression of the Mrp4 protein. Figure 3D is a control cPTC that was not stained with the Mrp4 antibody but was exposed to the fluorescent secondary antibody as in Fig. 3, C and E. No nonspecific binding of the secondary antibody to the cPTCs was apparent. An additional three identical trials revealed similar staining (data not shown).

Effect of knockdown of Mrp4 on active transepithelial urate secretion. Figure 4A is summary data of unidirectional and net active transepithelial urate fluxes in control, Polymag control, control-shRNAi, and Mrp4-shRNAi-treated cPTCs. Flux measurement was initiated at t = 0 by the addition of [14C]urate. Steady-state isotopic flux was apparent after 1 h. The unidirectional secretory flux, peritubular side to lumen side exceeded the unidirectional reabsorptive flux (also referred to as the leak flux), revealing net transport of urate in the secretory direction, a flux ratio of ~3:1 under short-circuited conditions. These results indicate that neither the magnetic nanoparticles nor shRNAi alone have an affect on active urate transport. In contrast, net urate secretion by cPTCs transfected with the Mrp4-shRNAi was less than one-third that of the paired control-shRNAi net flux (Fig. 4A). The reduction in urate secretion of ~60–70% accompanied the 75–80% reduction in Mrp4 gene expression (see Fig. 1). The electrical recordings taken at the end of each 1.5-h time course were used to evaluate the vitality of each of the cPTCs used for experimentation. The average TER of the Mrp4-shRNAi cPTCs was not significantly

Fig. 3. Fluorescence immunohistochemistry analysis of control cPTCs monolayer cultures and Mrp4 knockdown cPTCs. cPTCs were labeled with a custom polyonal rabbit anti-chicken Mrp4 primary antibody and then labeled with a goat anti-rabbit Alexa Fluor 488 conjugated secondary antibody. A: phase contrast image of proximal tubule cells grown in culture on a collagen substratum. B: scanning electron micrograph showing apical membrane microvilli of cPTCs. C: representative image of labeled control cPTCs showing the presence of Mrp4 in the apical membrane microvilli of the proximal tubule cells. D: representative image of control cPTCs stained with secondary antibody in the absence of the primary antibody. E: representative image of labeled cPTCs following knockdown of Mrp4. Labeling conditions were identical to those of C. P, paracellular space; M, microvilli. Scale bar = 5 μm.
Different from that of the controls (Fig. 4B). Glucose transport based on phloridzin-sensitive current was also not significantly different in the Mrp4-shRNAi cPTCs compared with the Polymag controls and control-shRNAi-treated cPTCs (Fig. 4C); thus, the tissues remained functionally intact following transfection.

Transepithelial urate secretion by cPTCs is not dependent on plasma membrane electrical potential. Figure 5 shows summary data comparing active transepithelial secretion of urate, TER, and sodium-dependent glucose transport (I_{glu}) of control cPTCs with that of those treated with antimycin A and those where plasma membrane potential was depolarized. Intracellular microelectrode recordings revealed that the plasma membrane electrical potential of cPTC cells in the normal physiological saline solution was −66 ± 2.2 mV (n = 9) and that the substitution of 100 mM potassium for 100 mM sodium in the bathing saline caused the cells to immediately depolarize to −16 ± 0.7 mV. Because prolonged exposure to 100 mM KCl is lethal, the flux determinations shown in Fig. 5 were limited to 30 min. Exposure of rabbit renal tubules to 10^{-5} M antimycin A has been shown to cause an 80% reduction in ATP concentration within 10 min (19). Figure 5 shows that the addition of 100 μM antimycin A completely eliminated trans-
epithelial urate secretion by control cPTCs within 30 min of exposure (Fig. 5A). Figure 5A also shows that high potassium depolarization caused no change in the transepithelial urate secretion compared with the paired control and that net transport of urate in the depolarized state was inhibited by antymycin A. None of the treatments significantly changed TER (Fig. 5B). Glucose transport was significantly decreased by the addition of antymycin A and removal of Na+ as expected (Fig. 5C).

Confirmation that urate transport by BBMV can be driven by an electrical potential. Figure 6 shows representative (inset) and summary data of [14C]urate uptake into chicken kidney BBMV in the absence and presence of a potassium-valinomycin-generated electrical driving force (vesicles electrically positive inside) confirming prior observations in another galliform (17). Summary data are 5-s uptakes expressed as a percentage of equilibrium (60 min) values. It is clear that a large electrical gradient (100 mM potassium, out > in) can drive urate uptake into the vesicles above equilibrium but that a weaker gradient (42 mM potassium, out > in) cannot (Fig. 6). Interestingly, MK-571 (40 μM), an MRP4 inhibitor, effectively inhibited electrical gradient driven urate uptake by BBMV.

**Fig. 6.** [14C]urate uptake in BBM vesicles (BBMVs) with varying initial electrical gradients. A representative plot of urate uptake by BBMV in the presence and absence of a potassium gradient, demonstrating an electrical gradient dependent “overshoot,” is shown in the inset. Bars are summary data of urate uptake expressed as a percentage of the equilibrium values for various initial potassium gradients. Uptake was stopped and measured at 5 s or 1 min and equilibrium values were measured at 60 min. The same external solution was used for the 3 different electrical gradients (100 mM potassium gluconate, 10 mM HEPES, 1 M KOH to pH 7.4). Internal solutions varied to obtain the different electrical gradients (100 mM potassium gluconate, 10 mM HEPES, 1 M KOH to pH 7.4; 58 mM potassium gluconate, 84 mM mannitol, 10 mM HEPES, 1 M KOH to pH 7.4; and 200 mM mannitol, 20 mM Tris-HEPES, pH 7.4). All vesicles contained valinomycin at 5 μM and all extravesicular solutions contained 35 μM [14C]urate. MK-571 was used at 40 μM in the external solution. *Significantly different from no-gradient control at P < 0.05, n = 3. **Significantly different from 1 min uptake by BBMV in 0 Kout vs. 100 Kout at P < 0.05, n = 3.

**DISCUSSION**

RNA interference directly decreased Mrp4 gene expression, and a newly developed polyclonal antibody confirmed the knockdown in the apical membrane of chicken renal proximal epithelium. This reduction of Mrp4 resulted in a proportional reduction of active urate secretion. Prior isolated membrane vesicle uptake studies had implicated the apical membrane electrical gradient in the cell-to-lumen exit step for urate. Since the present data supported a major role for Mrp4 in secretion, the effect of the electrochemical driving force for urate at the apical membrane was reexamined. In the functional epithelium, no effect was seen on urate secretion upon reducing the plasma membrane electrical potential more than fourfold.

The phenomenon of renal urate secretion by the avian proximal tubule was unequivocally demonstrated by micropuncture studies in starlings (29), confirming the result of overall net secretion of urate previously reported for birds (37). Further characterization of this powerful secretory mechanism was done in isolated perfused and nonperfused chicken proximal tubules. Net secretion was confirmed, and the steps in secretion at the BLM were consistent with those seen in mammals (9). In chicken proximal tubule fragments, cell-to-medium ratios for urate were 2.5 and indicated urate uptake across the BLM was against the electrochemical gradient and competitively inhibited by para-aminohippurate (PAH) (9). Dependence of urate uptake on α-KG exchange, as seen in mammals (43), is present (9); however, there may be multiple systems for urate uptake at the BLM. No facilitated reabsorption was ever observed in perfused chick proximal tubules, and the investigators concluded that leak flux is paracellular (9).

More recent transport studies with cPTCs have demonstrated active urate secretion by the cPTCs under short-circuited conditions (13) and reabsorptive flux consistent with paracellular leak. Application of PAH to the interstitial side of the cPTCs totally inhibited urate secretion (13), supporting a common entry path at the BLM. Both Oat1 and Oat3 mRNA are expressed in the chicken kidney proximal tubule epithelium (13). Peritubular addition of lithium, an inhibitor of Na+/dicarboxylate cotransport, and therefore an inhibitor of the recycling of intracellular α-KG, also inhibited urate secretion (13). Estrone sulfate, a specific substrate of Oat3 in the rabbit (51), inhibited urate and PAH secretion (13), further supporting the involvement of OATs in these BLM steps in secretion. In their recent review, Hediger et al. (23) suggested that OAT1 and OAT3 are likely to be the primary transporters responsible for urate uptake (interstitium to cell) at the BLM energized by the outwardly directed α-KG gradient generated by the Na+/dicarboxylate cotransporter and Na+-K+-ATPase. Data from knockout mice indicate that Oat1 and Oat3 are involved only in organic anion secretion and do not participate in reabsorption (16).

At the apical membrane, the cell-to-lumen exit step in secretion of urate is less well defined. Previous work by others with renal proximal tubule BBMV from a variety of species, as well as the results presented above on the chicken kidney vesicles, indicates a voltage gradient-driven transport of urate across the apical membrane (17, 27, 35, 45). Several transporters have been implicated in this apical membrane voltage gradient-driven urate secretion (23). Uric acid transporter (UAT or galectin 9), a multimeric protein identified in the rat

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kidney cDNA library, was suggested to function as an electrogenic urate efflux transporter/channel in the apical membrane (31). NPT1, a member of the sodium-phosphate cotransporter family, has been suggested as a possible urate transporter in the renal proximal tubule. NPT1 has 60–65% amino acid sequence identity to a more recently implicated apical membrane urate transporter, Oatv1. Oatv1 was isolated from a porcine kidney cDNA library and characterized as a voltage-driven PAH transporter. It was shown to transport urate in a voltage-driven manner when overexpressed in Xenopus oocytes (24). It is notable that voltage-dependent urate transport has only been detected in isolated membrane vesicles, oocytes, and other heterologous systems and has never been demonstrated in the intact, homologous, functional proximal tubule epithelium. A BLAST search of the chicken (G. gallus) genome reveals no evidence for UAT- (galectin 9) (accession no. P97840); however, chicken proteins with 54% and 56% amino acid sequence similarity, respectively, to NPT1 (accession no. NP_005065) and Oatv1 (accession no. NP_999509) are present. Nevertheless, the present study indicated that transepithelial urate secretion by cPTCs was insensitive to plasma membrane depolarization. Such a phenomenon was noted by Ullrich and Rumrich in their study of rat renal proximal tubule, which showed no effect of cell depolarization on PAH secretion in situ, even though rat BBMV contain NPT1 and exhibit a pronounced voltage-driven PAH uptake (55).

In addition to UAT, NPT1, and Oatv1, Hediger et al. (23) reviewed evidence for another likely urate efflux transporter, Mrp4, which has been identified in the human and localized to the apical membrane of the renal proximal tubule cells (56). The protein sequence for Mrp4 has also been identified in the chicken and is 86% homologous to the human sequence (determined by BLAST query using human MRP4, accession no. NP_001098985 into the G. gallus genome). Human MRP4 has been shown to transport urate by an ATP-dependent mechanism when overexpressed in insect sf9 membrane vesicles and HEK-293 cells (57). Previous examination of secretion of urate by cPTCs showed that MRP4 substrates methotrexate (46) and MK-571, a leukotriene C4 receptor antagonist and a known substrate for MRP4 (52). The molecular basis for the electrical gradient dependence in BBMV requires further exploration; however, Mrp4 is a member of the ABC family of membrane export pumps, and at least one member, cystic fibrosis transmembrane conductance regulator, is an ion channel.

In conclusion, urate secretion by the intact avian proximal tubule epithelium is unchanged by depolarization of the cells, even though urate transport in isolated BBMV is voltage gradient driven. Furthermore, Mrp4 is required for active urate secretion.

Perspectives

Absence of the urate degrading enzyme uricase in birds and humans results in high plasma urate concentrations proposed to play a role in lengthening life span (22), free radical scavenging (3), and activation signaling for the immune system (49), as well as the adverse effects mentioned above. Renal excretion is a major factor in controlling plasma urate, accounting for about 70% of daily elimination, and tubular secretion accounts for 70–80% of renal excretion in both birds and humans (8, 60). Tubular secretion was recently confirmed in URAT1-deficient patients where urate excretion levels exceeded filtered amounts (26). Although there have been many assumptions regarding renal transport mechanisms of urate based on information obtained from isolated membrane vesicles, overexpression systems, and other heterologous models, the distinct pathway of urate secretion in the renal proximal tubule remains uncertain. There is still no evidence in vivo or in situ for apical membrane electrical gradient-driven urate secretion. Based on the data presented here, where Mrp4 expression was knocked down in a homologous system, it can be concluded that Mrp4 plays a role in apical membrane urate secretion, probably directly mediating transport. Further exploration of the role of Mrp4 in this process is essential to understanding how urate homeostasis is achieved. Already there are several known factors, such as nitric oxide and endothelin (36, 42, 54), testosterone (33), and hepatocyte nuclear factor E2-related factor 2 (Nrf2) (2, 34) that alter MRP4 levels and modulate its activities. The amount

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of urate that is eliminated by tubular secretion may be critical in the regulation of plasma urate levels and possibly in the alleviation of complications caused by hyperuricemia, specifically metabolic syndrome and cardiovascular disease.

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**GRANT**

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