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*Am J Physiol Renal Physiol* 292:351-360, 2007. First published Jul 11, 2006;  
doi:10.1152/ajprenal.00049.2006

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## Axial heterogeneity of vasopressin-receptor subtypes along the human and mouse collecting duct

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Submitted 13 February 2006; accepted in final form 19 June 2006

**Carmosino M, Brooks HL, Cai Q, Davis LS, Opalenik S, Hao C, Breyer MD.** Axial heterogeneity of vasopressin-receptor subtypes along the human and mouse collecting duct. *Am J Physiol Renal Physiol* 292: F351–F360, 2007. First published July 11, 2006; doi:10.1152/ajprenal.00049.2006.—Vasopressin and vasopressin antagonists are finding expanded use in mouse models of disease and in clinical medicine. To provide further insight into the physiological role of V1a and V2 vasopressin receptors in the human and mouse kidney, intrarenal localization of the receptors mRNA was determined by *in situ* hybridization. V2-receptor mRNA was predominantly expressed in the medulla, whereas mRNA for V1a receptors predominated in the cortex. The segmental localization of vasopressin-receptor mRNAs was determined using simultaneous *in situ* hybridization and immunohistochemistry for segment-specific markers, including aquaporin-2, *Dolichos biflorus* agglutinin, epithelial Na channels, Tamm Horsfall glycoprotein, and thiazide-sensitive Na<sup>+</sup>-Cl<sup>-</sup> cotransporter. Notably, V1a receptor expression was exclusively expressed in V-ATPase/anion exchanger-1-labeled alpha-intercalated cells of the medullary collecting duct in both mouse and human kidney. In cortical collecting ducts, V1a mRNA was more widespread and detected in both principal and intercalated cells. V2-receptor mRNA is diffusely expressed along the collecting ducts in both mouse and human kidney, with higher expression levels in the medulla. These results demonstrate heterogeneous axial expression of both V1a and V2 vasopressin receptors along the human and mouse collecting duct. The restricted expression of V1a-receptor mRNA in intercalated cells suggests a role for this receptor in acid-base balance. These findings further suggest distinct regulation of renal transport function by AVP through V1a and V2 receptors in the cortex vs. the medulla.

*in situ* hybridization; vasopressin receptors; intercalated cell

ARGININE VASOPRESSIN (AVP) is the key hormone responsible for producing a concentrated urine and is secreted in high concentrations during antidiuresis. At least two different vasopressin receptors are expressed in the kidney: V1a and V2 receptors (37). Both V1a and V2 receptors are seven transmembrane-spanning G protein receptors. Vasopressin activates G<sub>s</sub> through the V2 receptors, stimulating adenylate cyclase and cAMP generation. In contrast, the V1a receptor is coupled to G<sub>q/11</sub> and stimulates phospholipase C, inducing PKC activation and calcium release from intracellular stores (8). The antidiuretic action of AVP is mediated mainly through adenylate cyclase-coupled V2 receptors that induce the translocation of the aquaporin-2 (AQP2) water channel to the apical membrane of collecting duct principal cells (38). Clinical utility of V2

antagonists as aquaretics in water-retaining diseases (55) supports a critical role of V2 receptors in the regulation of body fluid volume.

The distribution of V1a and V2 receptors in the kidney has been well studied in rat and rabbit by radioligand binding studies (18, 20, 48, 57) and several physiological functional assays (17, 27, 29, 31, 44). Intrarenal localization of V1a remains less well defined. It has been suggested that V1a receptors participate in the vasopressor effect of AVP and are localized in renal vascular system and glomeruli (22) and glomerular mesangial cells (32). Other reports suggest that V1a is expressed in the cortical collecting duct (CCD) (3, 16), outer (OMCD) (3) and inner medullary collecting duct (IMCD) (3), and interstitial cells (62). In contrast, Ostrowski et al. (47) showed by *in situ* hybridization that V1a is abundantly expressed in vasa recta, but that it was absent in glomeruli and IMCD. V2-receptor expression in the collecting duct is well established (3, 38, 48), whereas only small amounts of V2 receptors were found in the medullary thick ascending limbs (23, 45).

AVP may also regulate urinary acidification. Functional evidence for V1a receptors in principal cells of rabbit CCDs has also been provided (16). Luminal AVP action in rabbit CCD may be mediated via apical V1a receptors (6, 61). This apical action of vasopressin supports a physiological role for the high concentration of AVP in urine, especially during antidiuresis. V1a receptor has also been functionally linked to acid-base balance, since basolateral acetazolamide, a potent inhibitor of carbonic anhydrase, suppresses the luminal AVP-induced hyperpolarization (6). Moreover, chronic metabolic acidosis increased V1a-receptor mRNA expression in rat collecting ducts (53). Taken together, these findings suggested that V1a receptor may be involved in sodium, water, and acid-base balance in rat or rabbit kidney.

In contrast to these data in rat and rabbit, there is surprisingly little information concerning the localization of vasopressin receptors in the human or mouse kidney. To our knowledge, there are no studies mapping the V2 receptors in human kidney. Histoautoradiography, using radiolabeled agonists, suggests a predominant cortical distribution pattern of V1a in the cortex of mouse and human kidney; however, the identity of the nephron segments in which it is expressed was not determined (7). V1a-mediated Ca<sup>2+</sup> increase has been reported in mouse CCD (41), whereas immunolocalization suggests

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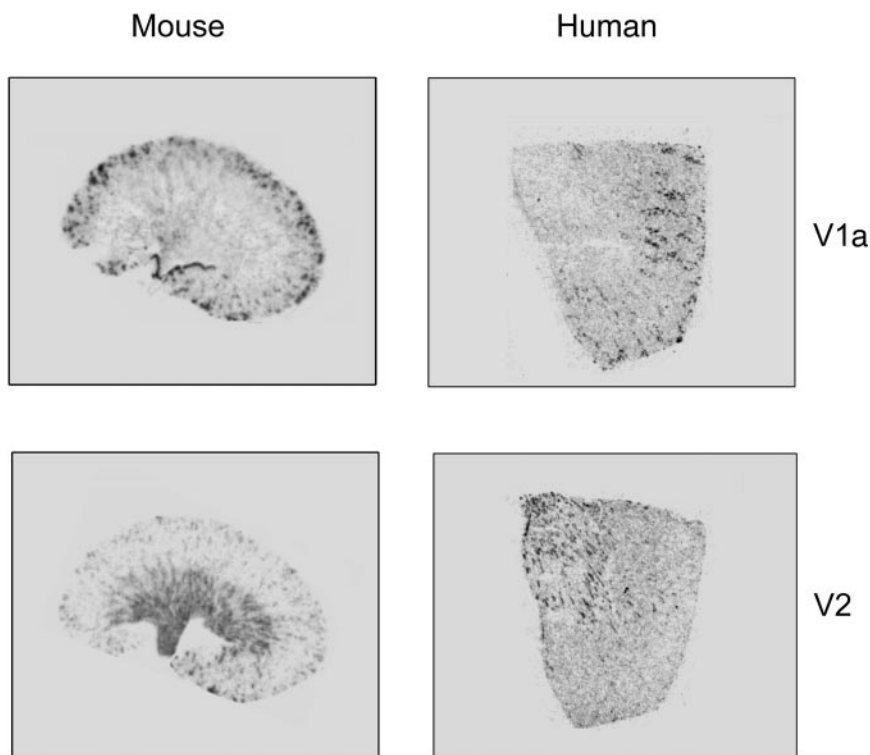


Fig. 1. Autoradiograms of in situ hybridization for V1a- and V2-receptor mRNA distribution in mouse and human kidney sections. Note the axial distribution of V1a and V2 receptors with V1a predominantly in the cortex and V2 in the medulla.

V1a may be expressed in connecting tubules (CNTs) or CCD staining in human kidney (25). The purpose of this study was to determine the localization of V1a and V2 vasopressin-receptor mRNAs in mouse and human kidney using in situ hybridization.

#### METHODS

**In situ hybridization.** Riboprobes for in situ hybridization were synthesized by in vitro transcription. A 1,257-bp *EcoRI/XhoI* fragment and 1,116-bp *EcoRI/XhoI* fragment encoding, respectively, the full length of human V1a- and V2-receptor cDNAs, were cloned into pCRII vector. The plasmid was linearized, and sense and antisense RNA was transcribed from the flanking SP6 and T7 promoters in the presence of [ $\alpha$ - $^{35}$ S]UTP, essentially as previously described (14).

A 750-bp *SallI/NotI* fragment of the 3' end of mouse V1a receptor was cloned in pSportI vector, and T7 and SP6 were used to obtain the sense and antisense RNA probes, respectively. In contrast, a 1,200-bp fragment encoding the full length of mouse V2 receptors was cloned into pBS vector, and the T7 and T3 promoter was used to transcribe the antisense probe.

Before hybridization, mouse and human kidney sections were deparaffinized, refixed in paraformaldehyde, treated with proteinase K (20  $\mu$ g/ml), washed with PBS, refixed in 4% paraformaldehyde, and treated with triethanolamine plus acetic anhydride (0.25% vol/vol). Finally, sections were dehydrated with 100% ethanol.  $^{35}$ S-labeled antisense and sense riboprobes from mouse and human V1a and V2 vasopressin receptors ( $5 \times 10^5$  cpm/ $\mu$ l) were hybridized to the section at 55°C for 18 h. After hybridization, the sections were washed at 65°C once in  $5 \times$  saline sodium citrate (SSC) plus 10 mM  $\beta$ -mercaptoethanol, once in 50% formamide,  $2 \times$  SSC, and 100 mM  $\beta$ -mercaptoethanol for 30 min. After two additional washes in 10 mM Tris, 5 mM EDTA, 500 mM sodium chloride at 37°C, sections were treated with RNase A (10  $\mu$ g/ml) at 37°C for 30 min, followed by another wash in 500 mM sodium chloride at 37°C. Sections were then washed twice in  $2 \times$  SSC and twice in  $0.1 \times$  SSC at 65°C. Slides were

dehydrated with graded ethanol containing 300 mM ammonium acetate. Slides were then dipped in emulsion (Ilford K5; Knutsford, Cheshire, UK), diluted 1:1 with 2% glycerol, and exposed for 6–7 days at 4°C. After developing in Kodak D-19, slides were counterstained with hematoxylin. Photomicrographs were taken using a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) and either dark-field (Micro Video Instruments, Avon, MA) or bright-field optics.

To define the nephron segments that labeled for vasopressin receptor, additional staining was performed using a polyclonal AQP2 antibody (Alpha Diagnostic International), a polyclonal thiazide-sensitive  $\text{Na}^+$ - $\text{Cl}^-$  cotransporter (TSC) antibody (a kind gift from Dr. Steve Hebert, Yale School of Medicine, New Haven, CT), an anti-Tamm-Horsfall (TH) protein polyclonal antibody (Cappel Immunoreagents), polyclonal  $\beta$ -epithelial Na channel (ENaC) antibody (kind gift from Mark Knepper, National Institutes of Health, Bethesda, Maryland 20892, USA), a polyclonal anion exchanger (AE)-1/2 antibody (52) (kindly provided by Seth Alper, Israel Deaconess Medical Center, Harvard University, Boston, MA), and polyclonal  $\text{H}^+$ -ATPase (Santa Cruz Biotechnology). First, the slides were briefly incubated with 3%  $\text{H}_2\text{O}_2$  to eliminate endogenous peroxidase activity and, thereafter, with the primary antibody for 60 min. The sections were rinsed with Tris-buffered saline containing 0.1% Tween 20 and incubated with a biotinylated secondary antibody against rabbit immunoglobulin for 30 min. Then sections were incubated with horseradish peroxidase-conjugated streptavidin for 20 min. Horseradish peroxidase labeling was detected by peroxidase substrate solution and counterstained with hematoxylin before being examined by light microscopy.

**Real-time PCR.** RNA was extracted from medullary and cortical regions dissected from mouse and human kidneys using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Purified total RNA was subjected to DNase I digestion (Promega). DNA-free RNA was quantified by spectrophotometry, and 10  $\mu$ g were added to a reverse transcriptase reaction using High Capacity cDNA Archive kit (Applied Biosystems), according to the manufacturer's protocol. Quantitative real-time (RT)-PCR analysis was carried out

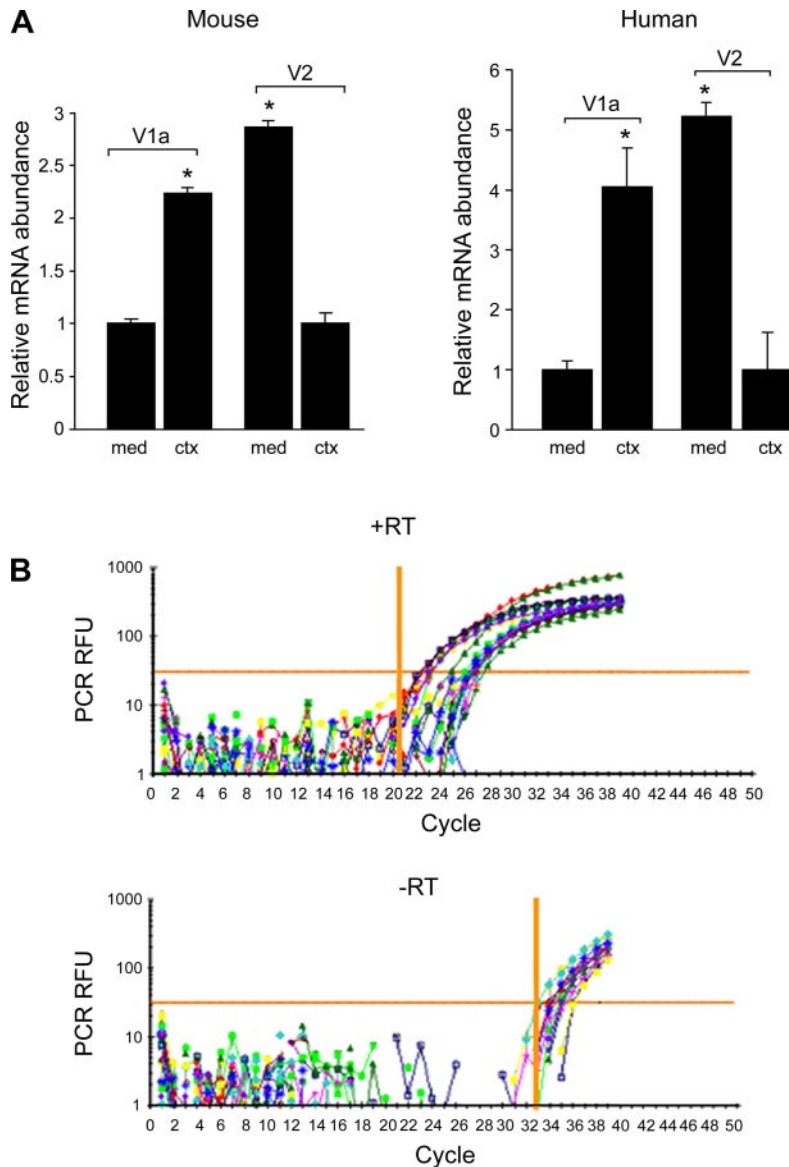


Fig. 2. A: expression of V2 and V1a receptors in cortex (ctx) and medulla (med) of mouse and human kidney as determined by real-time (RT) PCR. Results (means  $\pm$  SE) were expressed as fold increase relative to V1a in medulla and V2 in cortex (see METHODS). \* $P < 0.01$  by unpaired Student's *t*-test. B: comparison between +RT-PCR and -RT control thresholds performed as control. RFU, relative fluorescence units.

using *TaqMan* PCR Master Mix using 0.1  $\mu$ g of RNA equivalent cDNA for each reaction. Sequence-specific V1a and V2 primers and *TaqMan* probes were designed by and purchased from Applied Biosystems. RT-PCR assays were carried out in triplicate on Biorad iCycler. Thermocycling conditions were 95°C for 10 min (initial denaturation), followed by 40 cycles at 95°C for 15 s (denaturation), and 60°C for 1 min (annealing and extension). The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted CT). Gene expression values were calculated based on the comparative CT method (separate tubes) detailed in Applied Biosystems User Bulletin number 2. For each primers/probe set, validation experiments demonstrated that the efficiencies of target and reference gene amplification were approximately equal. Target genes were normalized to the reference housekeeping gene rRNA 18S.

## RESULTS

Autoradiograms generated from radiolabeled mouse V1a and V2 antisense probes, hybridized to mouse kidney, revealed that mRNA for these vasopressin receptors exhibit distinct

patterns of intrarenal expression. In contrast to inner medullary localization of V2 mRNA, V1a mRNA is predominantly expressed in the cortex, with moderate expression in the outer medulla, and is not apparent in the inner medulla (Fig. 1). Autoradiograms of human kidney sections revealed a similar pattern of receptor mRNA distribution as observed in mouse. An axial gradient of human V1a and V2 receptors was clearly present, with V1a predominating in the cortex and V2 receptor in the medulla (Fig. 1). No localized hybridization signal was detected using sense probes (data not shown).

Quantitative RT-PCR was performed on dissected mouse cortex and medulla to confirm the axial expression of vasopressin receptors. Expression of mouse V1a mRNA in the cortex was  $2.23 \pm 0.05$ -fold higher than in the medulla, while mouse medullary V2 mRNA levels were  $2.86 \pm 0.1$ -fold higher than in the cortex (Fig. 2A). RT-PCR for human samples also confirmed the axial distribution observed by in situ hybridization with human V1a mRNA  $4.02 \pm 0.65$ -fold more expressed in cortex than in medulla and human V2 mRNA

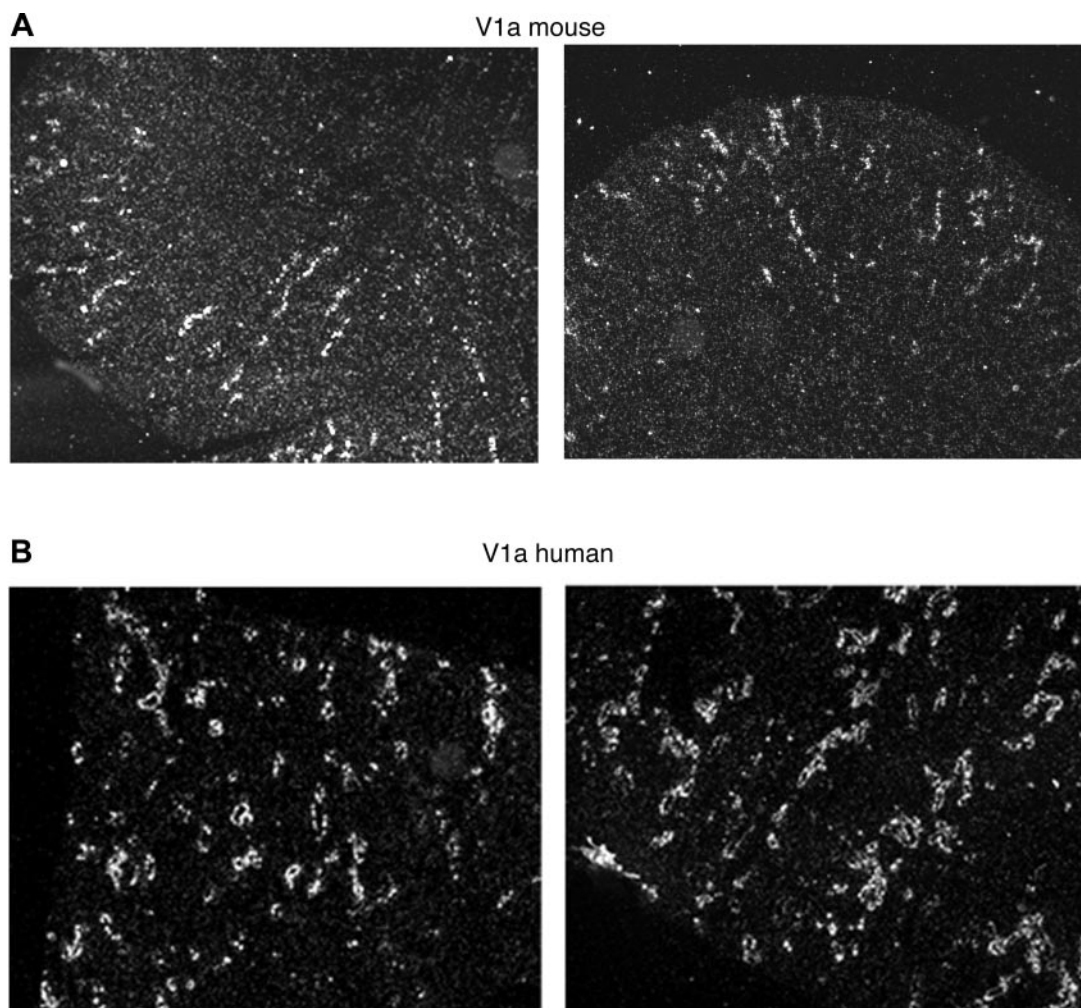


Fig. 3. Dark-field photomicrographs showing the V1a riboprobe hybridization in mouse (A) and human (B) kidney cortex. Magnification  $\times 20$ .

$5.233 \pm 0.62$ -fold more expressed in medulla than in cortex (Fig. 2A). As control, we performed no-amplification controls ( $-RT$ ) to test the contamination of RNA by genomic DNA. As shown in Fig. 2B, the threshold cycle for the vasopressin-receptor signals was typically between 18 and 24 cycles. In contrast, the threshold cycle for  $-RT$  control is 38 cycles, consistent with a negligible amount of genomic DNA in the samples (Fig. 2B).

Dark-field microscopic examination of photoemulsion-coated sections confirmed greater grain density of V1a-receptor mRNA in tubular structures of the cortex vs. medulla in both human and mouse kidney (Fig. 3).

To identify nephron segments where V1a is expressed, sections were first stained with nephron-specific immunohistochemical markers and then hybridized with V1a-receptor mRNA. Antibody against the TSC was used to identify distal convoluted tubules (DCTs). Antibody to AQP2, lectin *Dolichos biflorus* agglutinin (DBA), or ENaC was used to identify collecting duct, and TH glycoproteins were used as marker of thick ascending limb. As shown in bright-field images, in the cortical region, mouse V1a-receptor signal was restricted to AQP2-positive and TH protein and TSC-negative tubules, suggesting that V1a in mouse kidney was expressed predom-

inantly in CCD (Fig. 4A). Similar results were visible on dark-field images (*insets*). As in mouse, human V1a mRNA was detected in ENaC-positive and TH protein-negative tubules, indicating that V1a-receptor mRNA is also primarily expressed in CCDs of human kidney (Fig. 4B).

Interestingly, the V1a signal was homogeneously distributed along the CCD, in both species, suggesting that V1a receptors are present in both principal and intercalated cells (Fig. 4). In contrast, in the medulla of mouse kidney, V1a mRNA was confined to individual cells in OMCD, where the overall V1a mRNA signal is low, suggesting V1a receptors may be restricted to intercalated cells of the medullary collecting duct (Fig. 5A). To further define the intercalated cell subtype expressing V1a receptors in mouse kidney, V1a receptor in situ hybridization was performed on sections immunostained with an antibody against AE-1 (52). Coexpression of V1a-receptor mRNA and AE-1 immunoreactivity supports selective expression of V1a receptor in alpha-intercalated cells of mouse renal medulla (Fig. 5, *inset*). Similar results were obtained in human samples, where the signal for V1a mRNA was restricted to certain cells in collecting ducts of the medulla, consistent with intercalated cell labeling (Fig. 5B). In human kidney, colocalization experiment using an antibody against  $H^+$ -ATPase con-

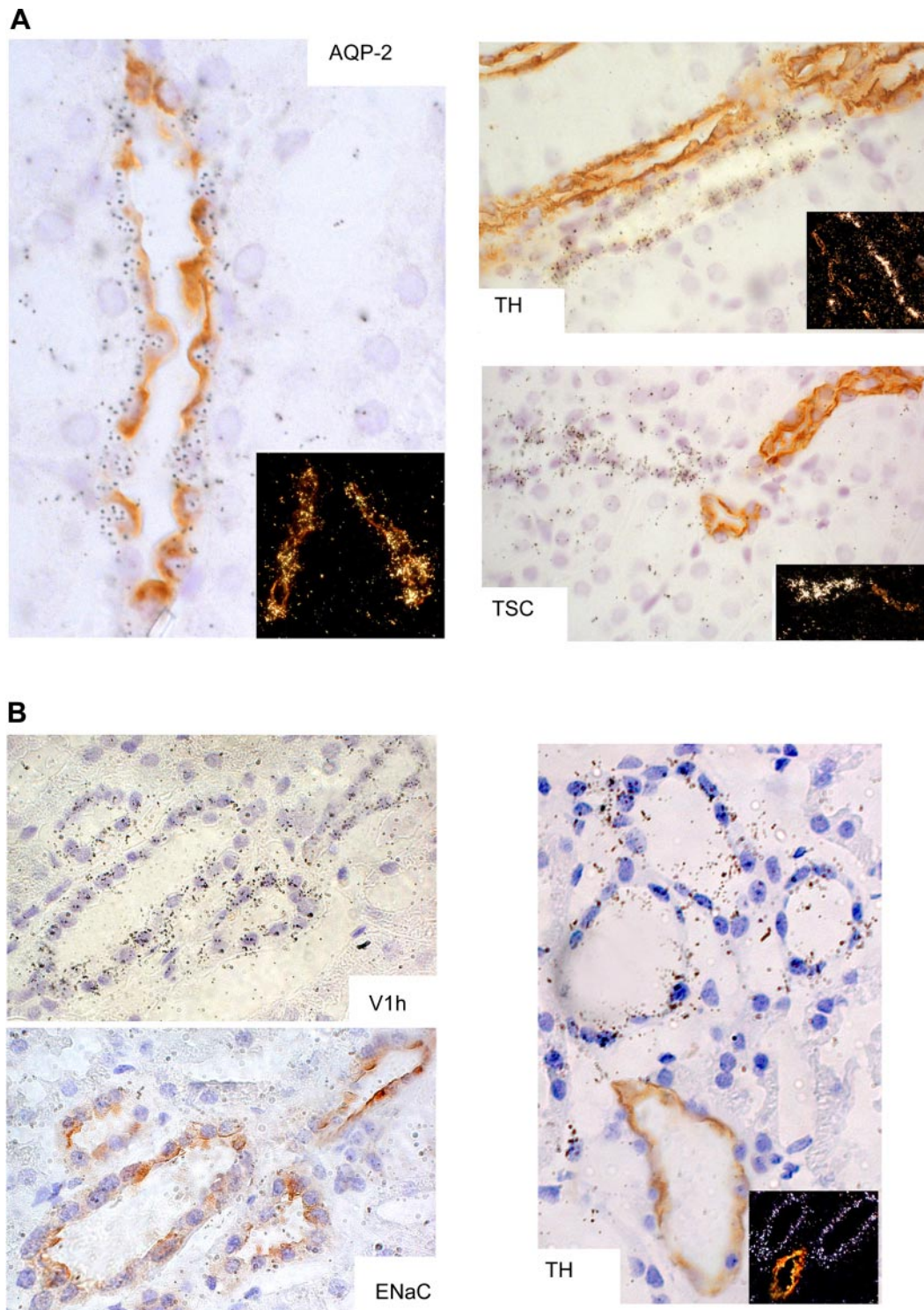


Fig. 4. Photomicrographs of V1a mRNA expression in the renal cortex. *A*: mouse kidney, coimmunostaining with nephron selective markers: aquaporin-2 (AQP2) for collecting duct, Tamm-Horsfall (TH) protein for thick ascending limb, and thiazide-sensitive cotransporter (TSC) for distal convoluted tubule (bright-field illumination). *Inset*: dark-field illumination. Magnification  $\times 60$ . *B*: human kidney, coimmunostaining in serial sections using epithelial Na channel (ENaC) as marker of collecting ducts (V1a, ENaC), and TH protein for the thick ascending limb (bright-field illumination). *Inset*: dark-field illumination. Magnification  $\times 60$ .

firmed that V1a receptors were expressed in intercalated cells also in human renal medulla (*inset*).

In contrast to the cortical predominance of V1a mRNA, V2 mRNA was most highly expressed in the medullary region in both species, manifesting as dense circular clusters of label that

occasionally extended into the cortex (Fig. 6). This pattern of labeling is consistent with V2R expression in the medullary and CCDs. This conclusion was corroborated by immunohistochemical colabeling of V2R mRNA positive tubules with collecting duct markers. AQP2 immunoreactivity and mouse

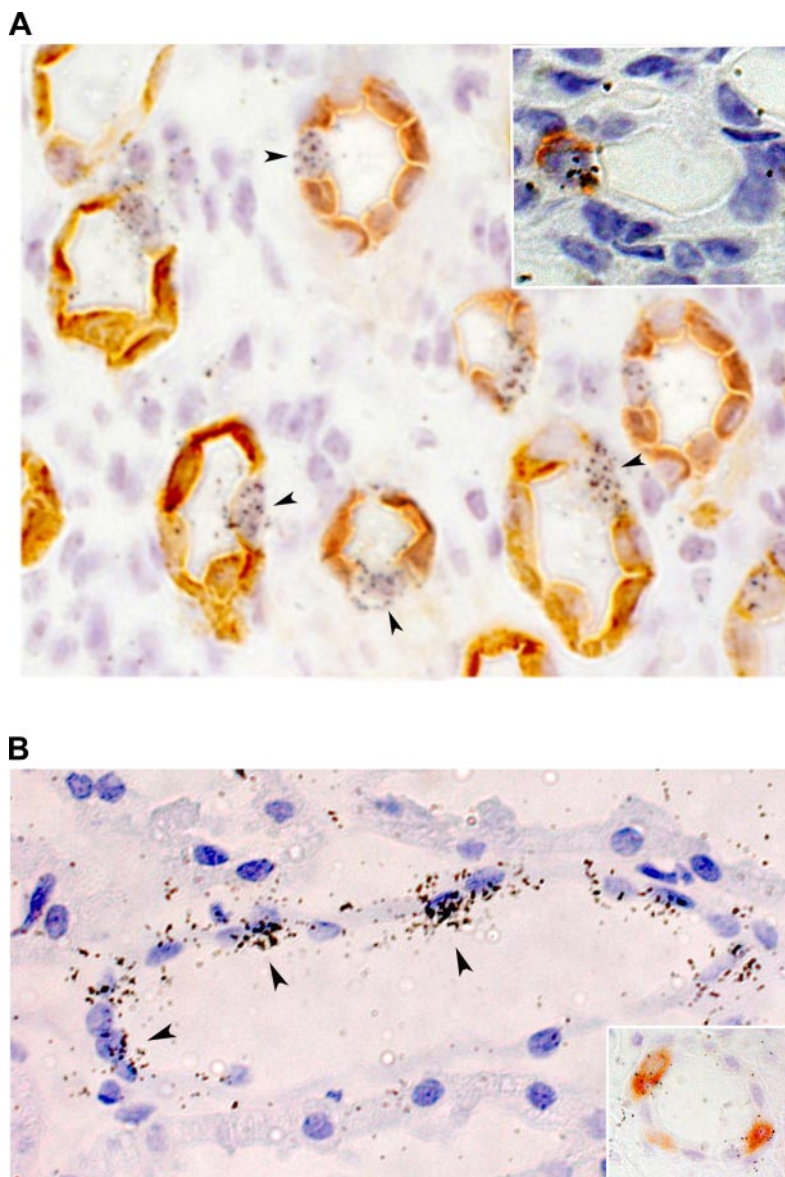


Fig. 5. Photomicrographs showing the V1a localization in the renal medulla (arrowheads). *A*: mouse kidney, coimmunostaining with AQP2 and anion exchanger-1 (AE-1) antibody (*inset*) (bright-field illumination). Magnification  $\times 60$ . *B*: human kidney, coimmunostaining with  $H^+$ -ATPase (*inset*) (bright-field illumination). Magnification  $\times 60$ .

V2-receptor mRNA completely overlap (Fig. 7A, AQP2). Moreover, silver grains were absent in TSC and TH protein-positive tubules (Fig. 7A, TH and TSC), and similar results were evident in dark-field images (*inset*). Colocalization studies in human samples also showed that V2-receptor mRNA is expressed in DBA-positive and TH protein-negative tubules, demonstrating that V2 receptors are also expressed in human collecting ducts (Fig. 7B, DBA and TH). Together, these data suggest that both V1a and V2 receptors are expressed in collecting ducts in mouse and human kidney, with V1a predominating in the cortical segment.

#### DISCUSSION

Collecting ducts are the major target for the physiological action of vasopressin in the kidney (34). At physiological concentrations of  $10^{-11}$  M, the antidiuretic action of vasopressin is mediated via V2 receptors coupled to adenylate cyclase, inducing PKA-mediated AQP2 phosphorylation and its translocation to the apical membrane of principal collecting duct

cells (43). AVP not only induces water reabsorption in collecting ducts, but also modulates  $Na^+$  reabsorption (10, 21, 42) and  $K^+$  secretion (4, 40).

Interestingly, AVP was reported to increase  $Ca^{2+}$  in CCD (5, 12, 16), IMCD (51), and cultured IMCD cells (30), although whether these effects are mediated via basolateral V1a or saturated V2 receptors remains controversial. Ando et al. (6) reported that luminal AVP hyperpolarized the transepithelial voltage in microperfused rabbit CCDs and suggested that this was mediated by V1a receptors. Ikeda et al. (28) observed that luminal AVP increased intracellular calcium in microperfused rabbit CCDs, also consistent with luminal V1a in the collecting duct.

Remarkably, molecular localization of V1a and V2 receptor in human and mouse kidney remains poorly defined. Arpin-Bott et al. (7) suggested that cortical V1a labeling is restricted to the juxtaglomerular apparatus of adult mouse and human as well as in unidentified tubular structures, possibly CCD, using binding of a radiolabeled V1a agonist. Moreover, in the outer

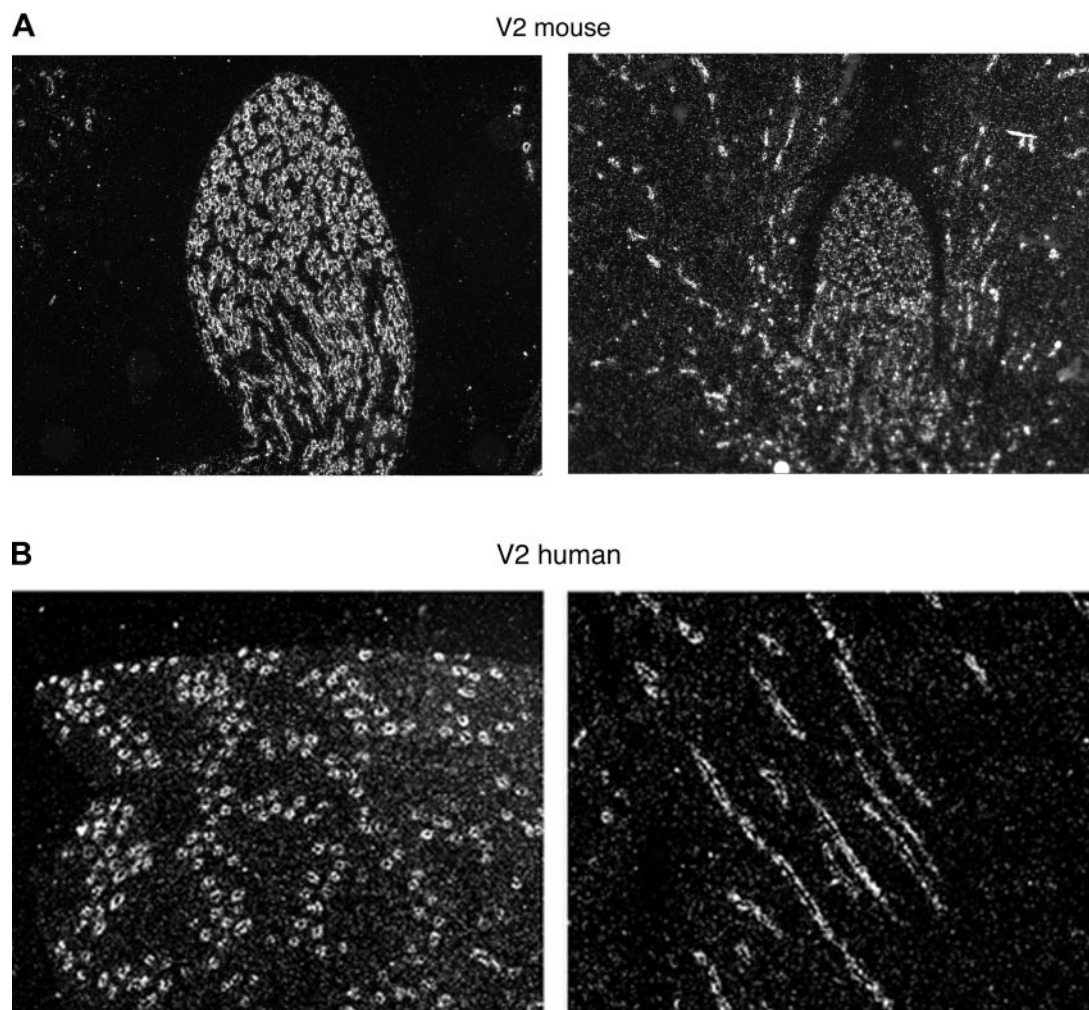


Fig. 6. Dark-field photomicrographs showing the V2 riboprobe hybridization in mouse (A) and human (B) kidney medulla. Magnification  $\times 20$ .

medulla, they found discontinuous distribution of V1a binding sites on tubular structures. The present study now clearly demonstrates that mRNA for the V1a receptors correlates with those binding studies and predominates in CCD, gradually decreasing as the collecting duct enters the medulla. Similar distribution of V1a in collecting duct has been reported in rat kidney by Ecelbarger et al. (20), who used RT-PCR on microdissected nephrons to show V1a mRNA expression in CCD and its absence in IMCD.

A novel finding of the present study is that, while V1a mRNA is diffusely expressed in the CCD, it is restricted to the intercalated cells in OMCD. This is consistent with reports using anti-V1a antibodies showing that V1a-receptor immunoreactivity is present in intercalated cells as well as in principal cells of the rat CCD (53). The present studies now confirm that a similar pattern of V1a RNA expression is also seen in human and mouse kidney. The present studies further show expression of V1a in mouse kidney colocalizes with basolateral AE-1 immunoreactivity, consistent with this cell-type being the alpha-intercalated cell (2).

Alpha-intercalated cells critically contributed to urine acidification through apically localized vacuolar H-ATPases, where the  $H^+$  produced from  $H_2O$  and  $CO_2$  via cytosolic

carbonic anhydrase II is extruded into the urine (49). The remaining intracellular  $HCO_3^-$  is released into the blood by the basolateral kidney-specific isoform of the  $Cl^-/HCO_3^-$  exchanger band 3/AE-1 (1, 15). Thus far it has not been functionally determined whether AVP can modulate these transporters through V1a receptors. Nevertheless, it has been observed that AVP interacts with luminal V1a receptors to induced transepithelial hyperpolarization via a carbonic anhydrase-dependent process, consistent with inhibition of electrogenic  $H^+$  secretion (6). Musa-Aziz et al. (39) showed that, in rat microperfused tubules, peritubular AVP stimulated  $HCO_3^-$  absorption in cortical distal tubule via V1a receptors. Moreover, chronic metabolic acidosis increased V1a-receptor mRNA expression in rat collecting ducts (53). These findings suggest a role for V1a receptors in the regulation of acid balance, consistent with the selective expression of V1a mRNA in OMCD intercalated cells seen in the present study.

At present, there are no reports describing the distribution of V2 receptors in human or mouse kidney. We found that V2 mRNA in both species was predominantly expressed in collecting duct, with greater expression in the medulla than the cortex in each species. We failed to find evidence for the expression of V2 mRNA in TH protein-positive thick limb,



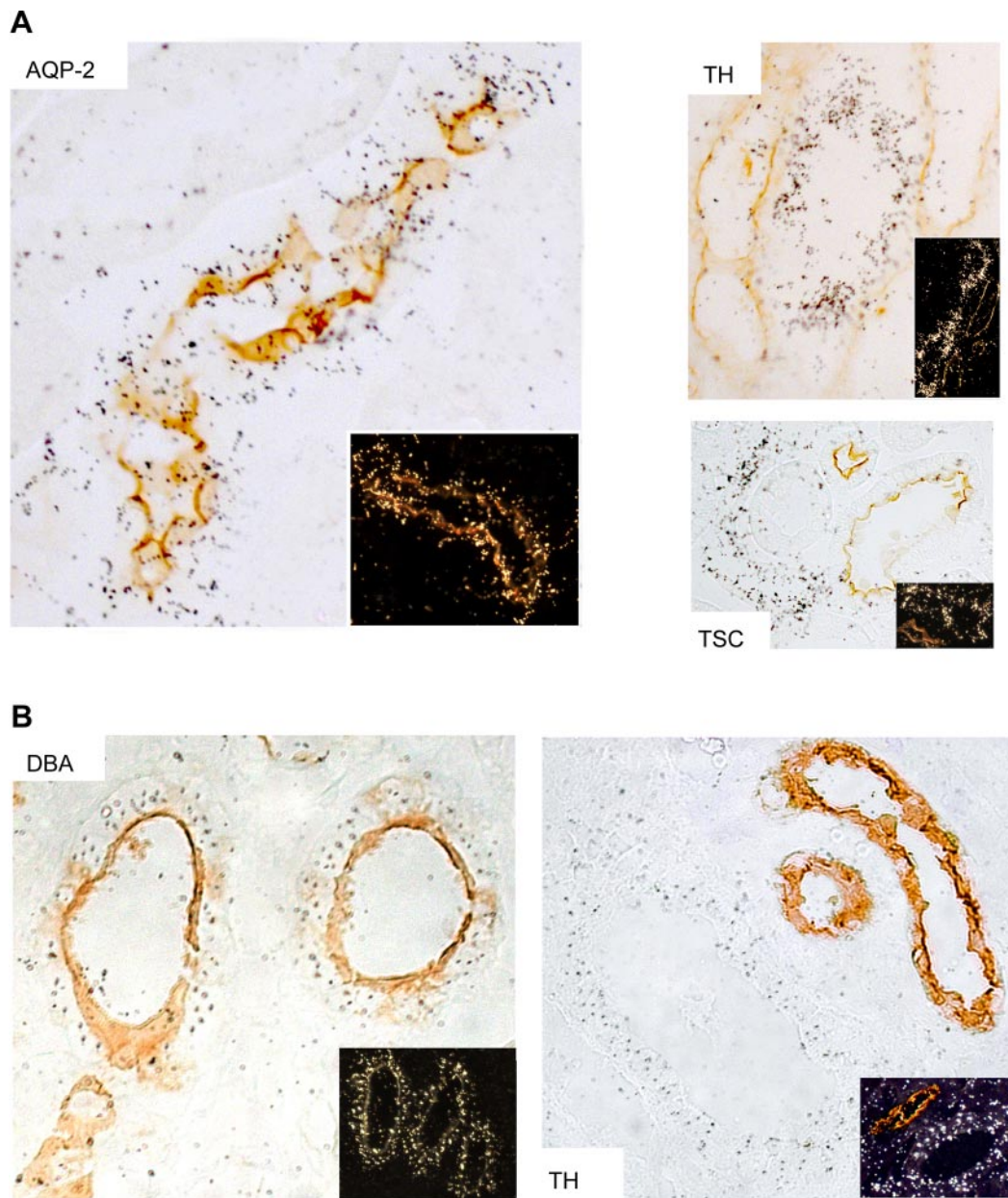


Fig. 7. Photomicrographs showing the V2 localization. *A*: mouse kidney, coimmunostaining with nephron markers: AQP2 for the collecting duct, TH protein for the thick ascending limb, and TSC for the distal convoluted tubule (bright-field illumination). *Insets*: dark-field illumination. Magnification  $\times 60$ . *B*: human kidney, coimmunostaining with *Dolichos biflorus* agglutinin (DBA) as marker of collecting ducts, and TH protein for the thick ascending limb (bright-field illumination). *Insets*: dark-field illumination. Magnification  $\times 60$ .

although we cannot exclude the possibility of mRNA expression below the detection limit of in situ hybridization in this segment. It has also been reported that V1a receptors are expressed in thick ascending limb and glomeruli in rat kidney by RT-PCR in microdissected nephron segments (54). The different results might be due to the different species analyzed or the different sensitivity between the two techniques.

Vasopressin receptors and AQP2 have also been identified in CNT in rat, consistent with emerging evidence of a role for this segment in water balance (25, 33). The present studies do not exclude the presence of vasopressin receptors in CNT in mouse and human kidney; however, given the gradual transition from DCT to CNT and from CNT to CCD in these species (11), with

overlap of  $\text{Na}^+\text{-Cl}^-$  cotransporter, ENaC, and AQP2 staining along the distal convolution, precise identification of this segment is problematic.

Functional roles for both V1a and V2 receptors in collecting duct have been well established (9, 13). Increased intracellular calcium and activation of PKC has been reported to inhibit AVP-induced water transport in rabbit CCD (5, 50). There are several potential mechanisms by which increased intracellular calcium might inhibit AVP-stimulated water flow.  $\text{Ca}^{2+}$ -inhibited adenylate cyclase isoforms are expressed in the kidney (35, 60). Alternatively,  $\text{Ca}^{2+}$  can stimulate phosphodiesterase activity in collecting ducts consistent with the presence of calcium-calmodulin-stimulated phosphodiesterase (36). A

PKC-dependent AQP2 retrieval from apical membrane of principal cells has also been observed (56). Other studies suggest that calcium/PKC-coupled signaling not only inhibits water flow but also inhibits Na<sup>+</sup> absorption and K<sup>+</sup> secretion in the collecting ducts (26, 46, 59).

In summary, the net effect of AVP on salt and water transport results from a combination of several actions of AVP on the collecting duct, involving both V2 and V1a receptors. As AVP concentration increases, a balance between the anti-diuretic effects of the hormone by V2 receptors and modulating effects by V1a receptors may occur to prevent excessive reduction in urine flow rate, thereby maintaining normal electrolyte reabsorption and secretion. Recent findings support the utility of vasopressin agonists in patients with vasodilator shock (19) and the potential use of V2 antagonists in various diseases involving altered fluid retention, such as congestive heart failure, cirrhosis, nephrotic syndrome, and syndrome of inappropriate antidiuretic hormone secretion (58). Moreover, the V2-receptor antagonist OPC31260 has been suggested to slow progression polycystic kidney disease in mouse models, suggesting clinical trials of V2-receptor antagonist in PKD (24). By defining the sites of V1a- and V2-receptor expression in mouse and human kidney, the present studies should further inform results obtained using these pharmaceuticals in mouse and human.

#### ACKNOWLEDGMENTS

We thank Reyadh Redha for expert technical support.

#### GRANTS

Support for this project was provided by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-37097 (to M. D. Breyer) and DK-071876 (to C. Hao) and the Veterans Affairs Merit Award (to M. D. Breyer).

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