Selective Inhibition of the Renal Dopamine Subtype D1A Receptor Induces Antinatriuresis in Conscious Rats

Zhi-Qin Wang, Robin A. Felder, Robert M. Carey

Abstract—Both dopamine D1-like (D1A and D1B) and D2-like (D2, D3, and D4) receptor subfamilies are present in the kidney. Blockade of the intrarenal D1-like receptor family is associated with natriuresis and diuresis. Because the D1A and D1B receptor subtypes are not distinguishable by currently available dopaminergic agents, their functional role remains undefined. In the present study, the effect of selective inhibition of the renal D1A receptor with phosphorothioated antisense oligodeoxynucleotide (AS-ODN) was investigated in conscious uninephrectomized rats. After renal interstitial administration of Texas red–labeled D1A receptor AS-ODN, intense fluorescent signal was localized in the renal tubular epithelium and vasculature. In rats on normal salt intake, AS-ODN injected interstitially into the kidney reduced daily urinary sodium excretion (1.4±0.4 versus 0.8±0.2 mEq/d, n=5, P<0.05) and urine output (16.9±3.8 versus 12.5±3.6 mL/d, n=5, P<0.05). In rats on high sodium intake, continuous renal interstitial administration of D1A receptor AS-ODN transiently decreased daily urinary sodium excretion (5.4±0.5 versus 4.2±0.3 mEq/d, n=7, P<0.01) and urine output (27.6±4.5 versus 18.1±1.8 mL/d, n=7, P<0.01). Neither vehicle nor sense oligodeoxynucleotide had significant effects. Systolic blood pressure remained unchanged. The renal D1A receptor protein was significantly decreased by 35% and 46% at the end of the study in AS-ODN–treated rats on normal and high salt intake, respectively, whereas the D1B receptor and β-actin were not affected. These results provide the first direct evidence that the renal D1A receptor subtype plays an important role in the control of sodium excretion. (Hypertension. 1999;33[part II]:504-510.)

Key Words: oligonucleotides, antisense receptors, dopamine kidney sodium excretion

The renal dopaminergic system plays an important role in the regulation of blood pressure, sodium balance, and kidney function. Dopamine (DA), produced by the renal proximal tubule, acts as an intrarenal paracrine hormone mediating diuresis and natriuresis.1–3 Recent advances in molecular biology have provided the identification of at least 5 subtypes of DA receptors. These newly described DA receptors have been shown to correspond pharmacologically and biochemically to the classical D1-like (D1A and D1B in rodents, and D1 and D2 in humans) and D2-like (D2L, D2S, D3, and D4) receptor subfamilies on the basis of their ability to stimulate or inhibit adenylate cyclase, respectively.4 The expression of D1A, D1B, D2L, and D3 receptor subfamilies has been identified in the kidney.5–11 It is not clear whether these newly cloned DA receptor subtypes are functionally significant in DA-mediated renal responses. Pharmacological stimulation of D1-like receptor with D1-like receptor agonist is associated with natriuresis and diuresis by both renal hemodynamic and tubular mechanisms. Inhibition of D1-like receptor with D1-like receptor antagonists produces antinatriuresis and antidiuresis independently of hemodynamic changes.1,12 However, currently available dopaminergic agents fail to differentiate between the D1A and D1B receptor subtypes, and the role of specific D1-like receptor subtypes in D1-like receptor-mediated renal actions is yet to be determined.

Antisense oligodeoxynucleotide (AS-ODN) provides a novel strategy to inhibit the synthesis of a specific gene product.13,14 Because renal tubule epithelial cells, especially proximal tubule cells, can take up large quantities of oligodeoxynucleotide (ODN), the kidney appears to be an excellent target organ for a site-directed AS-ODN approach.15,16 In the present study, AS-ODN directed toward dopamine D1A receptor subtype mRNA was delivered directly into the renal interstitium to inhibit selectively the synthesis of the renal D1A receptor subtype, and the role of the renal D1A receptor subtype in the control of sodium excretion and blood pressure was evaluated in conscious uninephrectomized rats.

Methods

Sprague-Dawley female rats (body weight, 200 to 240 g, Harlan Sprague Dawley Inc, Madison, WI) were used. The rats were provided with free access to regular rat chow (0.28% NaCl, Bioserve) and tap water unless described otherwise. All experimental procedures on animals were approved by the University of Virginia School of Medicine Animal Care Committee.
Design and Synthesis of ODN
AS-ODN against rat D1A receptor mRNA and its control, sense oligodeoxynucleotide (S-ODN), were synthesized and purified with reverse-phase high-performance liquid chromatography (GENSET SA) as 21-mer phosphorothioate-modified ODNs (antisense: 5′-GGT AGA AGT GTT AGG AGC CAT-3′, sense: 5′-ATG GCT CCT AAC ACT TCT ACC-3′), from nucleotides 60 to 80 of the rat D1A receptor cDNA.17 The designed sequences showed no homology with other known mammalian sequences deposited in the Genbank database (GenBank accession no. M35077), as screened using the Blast program.

Uninephrectomy and Renal Interstitial Catheter Implantation
Under general anesthesia with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (4 mg/kg), the right kidney was removed. An indwelling renal interstitial catheter, constructed using an 8-mm piece of polyethylene tubing (PE-10, Clay Adams) connected by Bipax epoxy resin glue (Tra-Con) to a 4-cm PE-60 tubing, was implanted into the left kidney through a small hole made with a 26-gauge needle. The catheter tip was placed at the cortex by insertion 2 to 3 mm in depth from the outer edge of the kidney, and was anchored in place on the kidney surface with Mersilene surgical mesh (Ethicon) and a small piece of abdominal fat using Vetbond tissue adhesive (3M Animal Care Products).

Ten days after the left nephrectomy, the rats were anesthetized, and for the rat to recover from the surgery. Placement of the catheter tip was confirmed at the end of the experiment by careful visual inspection. Rats with kidney damage were excluded from the study.

Distribution of D1A Receptor AS-ODN in the Kidney and Brain
Ten days after the right nephrectomy, the rats were anesthetized, and the renal interstitial catheter was implanted as described above and filled with Ringer’s solution. Three days later, 50 µL of Ringer’s solution with or without Texas red–conjugated AS-ODN (2 µg/µL, GENSET SA) were injected during a 10-minute period into the renal interstitium through the implanted catheter. Twenty-four hours later, the kidney and brain were collected with the rat under deep anesthesia. The tissues were fixed with 2% paraformaldehyde and cryoprotected with 30% sucrose. The frozen sections were examined with a fluorescence microscope.

Effect of D1A Receptor AS-ODN on Urinary Sodium Excretion During Normal Salt Intake
Ten days after the right nephrectomy, the rats were anesthetized, and the renal interstitial catheter was implanted as described above and filled with Ringer’s solution. Three days later, 24-hour urine was collected as control. Then, 50 µL of one of the was infused into the renal interstitium during a 10-minute period (n=5/group); AS-ODN (4 µg/µL) in Ringer’s solution; S-ODN (4 µg/µL) in Ringer’s solution; or Ringer’s solution alone. Daily urine collection was continued for an additional 3 days. At the end of the study, the rat was deeply anesthetized and the kidney was quickly removed, frozen in liquid nitrogen, and stored at −70°C until protein extraction.

Effect of D1A Receptor AS-ODN on Urinary Sodium Excretion and Systolic Blood Pressure During High Salt Intake
After the uninephrectomy and renal interstitial catheter implantation, renal interstitial infusion of the Ringer’s solution was commenced through a microosmotic pump (model 2002, 0.5 µL/h, Alza) connected to the other end of the catheter. The rats were provided with regular rat chow and high salt drinking water (0.45% NaCl) ad libitum throughout the study period. Ten days later, daily urine was collected for 2 consecutive days. After systolic blood pressure was measured by noninvasive tail-cuff method (Blood Pressure Analyzer, model 179, IITC), the rats were anesthetized again as above; 50 µL of one of the following was injected into the renal interstitium during a 10-minute period (n=7/group): AS-ODN (2 µg/µL) in Ringer’s solution; S-ODN (2 µg/µL) in Ringer’s solution; or Ringer’s solution alone, followed by a constant infusion of given ODN (AS- or S-ODN, 4 µg/µL) or Ringer’s solution alone through a new microosmotic pump (model 1007D, 0.5 µL/h, Alza). One day of postsurgery recovery was allowed before daily urine collection was resumed. Blood pressure was measured again at days 2 and 5. At the end of the study, the rat was deeply anesthetized and the kidney from each animal was quickly removed, frozen in liquid nitrogen, and stored at −70°C until protein extraction.

Western Blot Analysis
The specific polyclonal rat D1A (directed against a peptide sequence 290GSEETQFEC307 on the third extracellular domain) and D1B (directed against a peptide sequence 306CRSRGAYEPDPSLR320 on the third intracellular domain) receptor subtype antibodies used in the present study have been well characterized.5,7,18–20 Western blotting was performed as previously described.18 Kidney protein samples (100 µg/rat) were electrophoretically size-separated under denaturing conditions in 7.5% SDS-polyacrylamide gels, followed by transfer of the proteins onto nitrocellulose membranes. The blots were soaked overnight at 4°C in Tris-buffered saline containing 5% nonfat dry milk and 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20). The membranes were then probed for 2 hours with either polyclonal D1A, D1B receptor antibody or monoclonal β-actin antibody (Sigma), (1:1000 dilution in Tris-buffered saline with 5% nonfat dry milk and 0.1% TWEEN 20). Blots were subsequently washed and incubated with peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibody (1:2500 dilution, Amersham). The immunoreactive protein was detected with ECL Western blotting detection kit (Amersham). Quantitative assessment of band densities was performed by scanning densitometry (ImageQuant, Molecular Dynamics). Membranes were stripped between incubations with different antibodies in a Tris-buffered solution containing 2% SDS and 100 mmol/L β-mercaptoethanol at 50°C.

Urinary sodium concentration was measured by flame photometry (IL943, Instrumentation Laboratory). Statistical analysis was performed with a Macintosh StatView program (Abacus Concepts). Data were expressed as mean±SEM. Comparisons were made with analysis of variance, followed by Fisher’s protected least significant difference test for multiple comparisons when appropriate. A P value <0.05 was considered statistically significant.

Results
Distribution of AS-ODN Within the Kidney and Brain
Fluorescent signal (Texas red) was detected in the cortex and medulla of the whole kidney 24 hours after renal interstitial injection. Significant uptake of the AS-ODN occurred in both tubular epithelium and intrarenal vasculature with minimal signal present in the glomerulus (Figure 1). No fluorescent signal was observed in the brain (data not shown).

Effect of AS-ODN on Urinary Sodium Excretion and Blood Pressure
In rats on normal salt intake, D1A receptor AS-ODN injected interstitially into the kidney significantly reduced 24-hour urinary sodium excretion (1.4±0.04 versus 0.8±0.2 mEq/d, n=5, P<0.05) and urine output (16.9±3.8 versus 12.5±3.6
mL/day, n=5, P<0.05) (Figure 2, left). Similarly, in rats maintained on high sodium intake, continuous renal interstitial administration of D₁A receptor AS-ODN, but not vehicle or S-ODN, decreased daily urinary sodium excretion (5.4±0.5 versus 4.2±0.3 mEq/d, n=7, P<0.01) and urine output (27.6±4.5 versus 18.1±1.8 mL/day, n=7, P<0.01) (Figure 2, right). In both cases, daily urine output and sodium excretion returned to control levels before the end of the study. Neither Ringer’s solution nor S-ODN had significant effects (Figure 2). Systolic blood pressure remained unchanged during the study (Figure 3).

Western Blot Analysis of Renal D₁A and D₁B Receptor Subtypes and β-Actin

Three days after renal interstitial injection of D₁A receptor AS-ODN to rats on normal salt intake, renal D₁A receptor subtype protein was still significantly decreased by 35% compared with rats treated with vehicle or S-ODN (Figure 4). In rats on high salt balance, 5-day continuous renal interstitial infusion of AS-ODN reduced renal D₁A receptor protein by 46% (Figure 5). However, expression of the D₁B receptor and β-actin in the kidney was not significantly altered (Figures 4 and 5).

Figure 1. Intrarenal distribution of Texas red–conjugated D₁A receptor AS-ODN 24 hours after renal interstitial injection (100 μg) in uninephrectomized rats. Conspicuous fluorescent signal in the cytoplasm was observed in the renal cortical (left) and medullary (middle) tubular epithelium (P indicates proximal tubule; D, distal tubule; and C, collecting duct) and vasculature (V), with minimal signal present in the glomerulus (G). No signal was present in the kidney injected with Ringer’s solution (right).

Figure 2. Effect of renal interstitial administration of D₁A receptor AS-ODN on daily urine output (UV) and urinary sodium excretion (UNaV) in conscious rats during normal (left) or high (right) salt intake. Left: n=5/group. *P<0.05 vs day 0; #P<0.05 vs vehicle and S-ODN. Right: n=7/group. *P<0.05, **P<0.01 vs days –1 and 0; #P<0.05, ##P<0.01 vs vehicle and S-ODN.

Figure 3. Variation of systolic blood pressure (mmHg) during the study. Left: n=5/group. *P<0.05 vs day 0; #P<0.05 vs vehicle and S-ODN. Right: n=7/group. *P<0.05, **P<0.01 vs days –1 and 0; #P<0.05, ##P<0.01 vs vehicle and S-ODN.
Figure 3. Systolic blood pressure (SBP) in conscious uninephrectomized rats on high salt intake before and during renal interstitial infusion of D1A receptor AS-ODN, S-ODN, or vehicle (n=7/group).

Figure 4. Representative immunoblots (A, B, and C, apparent molecular weight (MW) in kDa) and densitometric summary (bottom) of Western blot analysis of renal D1A (A, D1A-R) and D1B (B, D1B-R) receptor subtypes and β-actin (C) in rats on normal salt intake 3 days after renal interstitial injection of vehicle (lanes 1, 2), D1A receptor S-ODN (lanes 3, 4), or AS-ODN (lanes 5, 6) (n=5/group). **P<0.01 vs vehicle and S-ODN.
AS-ODN strategy offers the potential to suppress selectively the expression of specific genes within somatic cells in the absence of confounding systemic variables as often seen in genetically engineered animals. Once entering the cells, AS-ODNs are thought to bind to their complementary region of the target mRNA and, by a number of mechanisms, selectively inhibit gene expression. Renal epithelial cells, especially proximal tubule cells, can effectively take up phosphorothioated ODN, and the renal tubule appears to be an excellent target for gene transfer into the kidney. The utility of this strategy has been demonstrated successfully by in vivo administration of AS-ODNs against neural and inducible isoforms of nitric oxide synthase, the angiotensin subtype 1 receptor, the Na\textsuperscript{+}\textminus\textsubscript{P}\textsuperscript{i} cotransporter, intercellular adhesion molecule-1, and transforming growth factor-\(\beta\) in the kidney. In the present study, effective renal cellular uptake of AS-ODN was confirmed by distribution of Texas red–labeled ODN in renal tubular epithelium and vasculature 24 hours after renal interstitial injection.

In a recent review, Missale et al. concluded that the physiological function of the newly cloned dopamine receptor subtypes is still unknown and remains a major challenge in the coming years. Acute blockade of the intrarenal D\textsubscript{1}-like receptor family with SCH 23390 induces significant decrease of urinary flow rate, sodium excretion, and fractional sodium excretion by tubular mechanisms. Both D\textsubscript{1A} and D\textsubscript{1B} receptor subtypes are expressed in the kidney. The D\textsubscript{1A} receptor subtype has been localized in the proximal and distal tubules, collecting ducts, and intrarenal arterioles and juxtaglomerular apparatus but not glomerulus. Whole body abrogation of the D\textsubscript{1A} receptor subtype has been found to cause abnormal behavioral function with increased locomotor activity or rearing behavior and high blood pressure in D\textsubscript{1A} receptor null mice. However, the precise role of the renal D\textsubscript{1A} receptor subtype in control of sodium excretion and blood pressure has not been established.

Our research group has previously demonstrated that D\textsubscript{1A} receptor AS-ODN is able to block translation of the D\textsubscript{1A} receptor protein and the D\textsubscript{1}-like receptor agonist fenoldopam-stimulated phospholipase C expression in LTK cells stably transfected with rat D\textsubscript{1A} receptor cDNA. It also prevents fenoldopam-induced cAMP accumulation and renin secretion in cultured rat juxtaglomerular cells. In the present study, we sought to use the same AS-ODN, complementary to D\textsubscript{1A} receptor mRNA, to elucidate the possible role of renal D\textsubscript{1A} receptors in regulation of urinary sodium excretion in conscious rats either on normal salt intake or during long-term salt loading, one of the most powerful stimuli known to activate the renal DA system. The need to target the renal tubular epithelium and...
vasculature (two major sites of the D\textsubscript{1A} receptor in the kidney), together with the poor ability of phosphorothioated ODN to penetrate the blood-brain barrier, offers particular advantages for AS-ODN strategy.

In the present study, renal interstitial administration of D\textsubscript{1A} receptor AS-ODN led to a significant decrease in renal D\textsubscript{1A} receptor protein, whereas expression of the renal D\textsubscript{1B} receptor and the structural protein β-actin was not affected. Selective inhibition of the renal D\textsubscript{1A} receptor significantly reduced urinary sodium excretion in rats on either normal or high salt diet. Although the D\textsubscript{1A} receptor subtype protein in the whole kidney was significantly suppressed, the current findings do not allow us to determine whether hemodynamic or tubular mechanisms are involved or the relative importance of the tubular and vascular D\textsubscript{1A} receptor in the observed decrease in urinary sodium excretion. It is noteworthy, however, that antinatriuresis in the current study is short-lasting and is not accompanied by an increase in systolic blood pressure, despite the fact that renal D\textsubscript{1A} receptor protein was still significantly inhibited at the end of the experiment. In contrast, generalized loss (knockout) of the D\textsubscript{1A} receptor in nephrectomized rats. These results suggest that the renal D\textsubscript{1A} receptor subtype plays an important role in the short-term suppression of the renal Na\textsuperscript{+} L-transporter.

In summary, our data demonstrate that renal interstitial administration of the D\textsubscript{1A} receptor AS-ODN selectively suppressed renal D\textsubscript{1A} receptor subtype expression and produced significant antidiuresis and antinatriuresis in conscious uninephrectomized rats. These results suggest that the renal D\textsubscript{1A} receptor subtype plays an important role in the short-term control of sodium excretion.

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