Small interfering RNA-mediated functional silencing of vasopressin V2 receptors in the mouse kidney

Ali Hassan,1,2 Ying Tian,3 Wei Zheng,1,2 Hong Ji,1,2 Kathryn Sandberg,1,2 and Joseph G. Verbalis1,3
1Center for the Study of Sex Differences in Health, Aging and Disease; 2Department of Medicine, Division of Nephrology and Hypertension; and 3Department of Medicine, Division of Endocrinology and Metabolism, Georgetown University Medical Center, Washington, District of Columbia

Submitted 30 June 2004; accepted in final form 10 March 2005

Hassan, Ali, Ying Tian, Wei Zheng, Hong Ji, Kathryn Sandberg, and Joseph G. Verbalis. Small interfering RNA-mediated functional silencing of vasopressin V2 receptors in the mouse kidney. Physiol Genomics 21: 382–388, 2005. First published March 22, 2005; doi:10.1152/physiolgenomics.00147.2004.—The antidiuretic effects of arginine vasopressin (AVP) on the kidney are mediated by V2 subtype AVP receptors (V2R). To investigate the role of regulation of V2R in water and sodium homeostasis, we have developed a method for small interfering RNA (siRNA)-mediated inhibition of V2R expression in vivo. Three 21-nt siRNA sequences were chosen that specifically targeted the mouse V2R but shared no appreciable sequence homology to any other known mouse genes, including the vasopressin V1a, and V1b receptors. Additionally, an siRNA sequence that shared no significant matches to any known mammalian gene sequences was chosen for use as a control. Chemically synthesized siRNA was complexed with the liposomal transfection reagent DOTAP. Each mouse (male C57BL/6) received 3.6 nmol (~50 μg) of either the control (nonsilencing) or one of the V2R-targeting siRNAs via intravenous injection. Forty-eight hours after injection membranes were prepared from the inner medulla of the kidneys, and V2R expression was measured by a radioligand binding assay and Western immunoblotting. Treatment with one of the V2R-targeting siRNAs (R2) caused a 39.7 ± 8.7% reduction in V2R-specific binding compared with the control (n = 11, P < 0.05) and a 37.0 ± 23% reduction in V2R protein expression as measured by Western immunoblotting (n = 4, P < 0.001). Additionally, real-time PCR revealed that R2 siRNA treatment induced a 68.8 ± 2.2% reduction in V2R mRNA. However, this siRNA treatment did not alter the animals’ basal urine concentrating capacity under unstimulated conditions. In subsequent experiments, treatment with R2 siRNA was found to significantly attenuate the antidiuretic effects the V2R-specific AVP agonist 1-desamino-[8-D-arginine]vasopressin (dDAVP). Mice were infused with dDAVP (0.25 ng/h) for 3 days to produce maximal antidiuresis and then were injected with either the R2 siRNA or the nonsilencing control. On day 2 after treatment, urine osmolality was significantly decreased from 3,455 ± 72 in control animals (n = 12) to 3,155 ± 129 mosmol/kgH2O in R2 siRNA-treated animals (n = 12) (P < 0.05); similarly, on day 2 24-h urine volume was significantly increased from 0.86 ± 0.07 ml/day to 1.11 ± 0.06 ml/day in R2 siRNA-treated animals (P < 0.05). In summary we have demonstrated that RNA interference methodology can be used successfully in vivo to significantly reduce functional expression of the V2R in the mouse kidney.

inner medulla; in vivo gene silencing; G protein-coupled receptor

ARGININE VASOPRESSIN (AVP) is the major physiological regulator of renal water excretion. AVP acts in the kidney to increase the water permeability of the renal distal tubules and collecting ducts, thereby accelerating water reabsorption (40). The anti-diuretic effects of vasopressin are mediated by vasopressin V2 receptors (V2Rs), adenylyl cyclase-coupled members of the G protein-coupled receptor family that are present in the renal distal tubules and collecting ducts (3, 44). Vasopressin regulates the water permeability of renal collecting tubule in two ways. Short-term regulation is achieved by shuttling of aquaporin-2 (AQP2) water channels from intracellular vesicles into the apical plasma membrane (24). Long-term regulation occurs through increasing the abundance of AQP2 protein (19). The vasopressin V2R is structurally, genetically and pharmacologically distinct from the two other vasopressin receptors, the V1a receptor, expressed predominantly in the liver and vasculature (22), and the V1b receptor, expressed almost exclusively in the pituitary (5, 17, 37).

In some pathophysiological circumstances, the renal ability to concentrate urine in response to AVP is decreased. Often, this decrease in renal concentrating ability is associated with a concomitant reduction in vasopressin V2R expression. For example, the effects of 1-desamino-[8-D-arginine]vasopressin (dDAVP)-induced antidiuresis are mitigated by the “renal escape” phenomenon (7, 41). In the rat, the increased urine volume and decreased urine osmolality that indicate the beginning of the initiation of the escape process coincide with downregulation of kidney vasopressin V2R binding (39). Similarly, rats with chronic renal failure exhibit a marked decrease in vasopressin V2R density and the virtual absence of V2R mRNA without changes in other G protein-coupled receptors, suggesting that vasopressin resistance during chronic renal failure is also due, at least in part, to vasopressin V2R down-regulation (38). Furthermore, lipopolysaccharide-induced endotoxemia results in decreases in both V2R mRNA and density in the kidney inner medulla, and this downregulation is also associated with decreased renal capacity to concentrate urine (11). Since renal AQP2 expression and distribution are mainly regulated by vasopressin V2R-mediated increases in cAMP, this temporal association between decreases in urine concentrating capacity and kidney vasopressin V2R expression suggests that alterations in V2R levels play an important role in regulating renal function; however, correlative studies cannot prove a causal relationship by virtue of this temporal association. Indeed, in some instances V2R expression is reduced without any apparent defect in urine-concentrating capacity. For example, dDAVP-treated rats are able to maintain maximal AQP2 expression and urine osmolalities without manifesting escape even though vasopressin V2R binding sites are significantly reduced (7, 39). Similarly, following 72 h water deprivation, the vasopressin V2R density (Bmax) in rat renal tubular epithelial basolateral cells is reduced by 38% without affecting

382 1094-8341/05 $8.00 Copyright © 2005 the American Physiological Society
the affinity \(K_d\) of the receptor (36). This is paradoxical, since \(V_2R\) expression is reduced in a circumstance in which increased water reabsorption would be clearly advantageous. One potential explanation for these phenomena is that there is a relatively large population of “spare” vasopressin \(V_2Rs\) (i.e., a receptor reserve) in the kidney and, as a result, \(V_2R\) expression must be reduced below a certain threshold before there is any reduction in second messenger generation and thus urine concentrating capacity (36, 39). To further investigate the degree to which changes in vasopressin \(V_2R\) expression contribute to alterations in renal function, we developed a method for reducing \(V_2R\) expression in vivo.

RNA interference (RNAi) is a mechanism whereby small double-stranded RNA molecules interact with mRNA containing homologous sequences in a highly sequence-specific manner. This process is directed by endogenously expressed protein components known as an RNA-induced silencing complex (RISC), the actions of which ultimately result in the degradation of the mRNA (9). Because interaction of the siRNA with its target is highly sequence specific, typically only the target mRNA is affected and off-target effects are minimal (10). Although RNAi has been observed in a wide range of eukaryotes (31, 43), silencing of mammalian genes has only recently been demonstrated using this technique (4, 8). In mammalian cells, gene silencing can be induced by transfection of cells with 21-nt RNA duplexes or short interfering RNA (siRNA). Recently, a small number of studies have demonstrated that RNAi techniques can also be used to knock down \(V_2R\) expression in mammalian cells, gene silencing can be induced by transfection of cells with 21-nt RNA duplexes or short interfering RNA (siRNA). Several studies have demonstrated in vivo gene silencing of transgenically expressed reporter genes [e.g., green fluorescent protein (GFP) or luciferase] (15, 16, 20), but to date no study has shown that siRNA silencing of \(G\) protein-coupled receptor expression in a mammalian system has functionally significant physiological effects.

In vivo silencing of the vasopressin \(V_2R\) has posed a substantial challenge. First, the vasopressin \(V_2R\) is expressed predominantly in the inner medulla of the kidney (44). Thus it was unclear whether siRNA could be delivered to this tissue with sufficiently high efficiency to achieve a significant reduction in \(V_2R\) expression. Second, even if it were possible to reduce vasopressin \(V_2R\) expression in vivo, it was unclear whether this reduction in expression would be of sufficient magnitude to result in a physiological effect. Therefore, the aim of this study was to determine whether siRNA-mediated RNA interference could be employed successfully in vivo to reduce vasopressin \(V_2R\) expression in the mouse kidney, and if so, to determine whether this methodology could be used to investigate the role of \(V_2R\) downregulation in regulating renal function.

**MATERIALS AND METHODS**

Small interfering RNA (siRNA) constructs. Three different siRNA duplexes (R1, R2, and R3) targeting the mouse vasopressin \(V_2R\) were selected using standard siRNA design rules (9). BLAST searching showed that these siRNAs constructs did not match the \(V_{1a}\) or \(V_{1b}\) vasopressin receptor subtypes, nor did they have any significant homology to any other known mouse gene. Additionally, an siRNA construct that matched no known mammalian genes was selected for use as a nonsilencing control. The sequences of these four siRNA constructs are shown in Fig. 1. All siRNA duplexes were chemically synthesized by Qiagen (Carlsbad, CA; HPP grade).

**siRNA transfections.** Mice (adult male C57BL/6, 17–24 g; 5–9 wk; Taconic, Germantown, NY) were transfected with siRNA using a procedure similar to that described by Sorensen et al. (35). Mice were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg, Nembutal; Fisher Scientific, Pittsburgh, PA). Once anesthetized, the animals were volume loaded by intraperitoneal injection of 1.5 ml of isotonic saline to ensure adequate renal perfusion 30 min prior to siRNA infusion. No behavioral changes were associated with this intraperitoneal saline injection. Then, 3.6 nmol (equivalent to \(\sim 50 \mu\text{g}\) of siRNA) was complexed with the liposomal transfection reagent \(N\{[2,3-dioleoyloxy]propyl\}-\text{N,N,N-trimethylammonium methylsulfate} (DOTAP; Roche Diagnostics, Indianapolis, IN) in HEPES-buffered saline (pH 7.4). The final volume of the transfection mixture was 198 \(\mu\text{l}\), and the siRNA/DOTAP charge ratio was 1:1. The siRNA-DOTAP solution was injected through a catheter (PE-10 gauge; Becton-Dickinson, Franklin Lakes, NJ) inserted into the jugular vein. Following infusion of the siRNA mixture over a period of \(\sim 1\) min, the jugular was sutured and the animals were allowed to recover. Animals were monitored continuously post injection. All animals were awake and walking 45 min after siRNA injection. No behavioral changes were apparent. Mortality was low (94% survival). All experiments were carried out under the supervision of and in accordance with the regulations of the Georgetown University Animal Care and Use Committee.

**Osmotic minipump infusion of dDAVP.** Under light methoxyflurane anesthesia, mice were subcutaneously implanted with osmotic minipumps (model 1002; Alzet, Palo Alto, CA), which delivered 0.25 ng \(\text{dDAVP} (\text{Ferring Pharmaceuticals, Suffern, NY})\) per hour. Pumps were implanted at midday 72 h prior to siRNA injection.

**Preparation of kidney inner medullary tissue.** Forty-eight hours after siRNA infusion animals were killed by decapitation, the kidneys were quickly removed and rinsed in ice-cold PBS and were then sliced along the corticomedullary axis to separate the medulla from the cortex. The inner medullary region of the kidneys was dissected. The

Control (i.e., non-silencing)

5’-UCUCGCAACAGGUUCACGUDdT-3’
3’-dTdTAAAGGCGUCGACAGUCA-5’

R1 (V2 receptor-targeting)

5’-CUUGCCUCUGACUCAAdTdT-3’
3’-dTGTACCCCGGAAGAUAGUU-5’

R2 (V2 receptor-targeting)

5’-CGUUAGUGGGAAGGCAAdTdT-3’
3’-dTGTACCUACACCCUUUACG-5’

R3 (V2 receptor-targeting)

5’-GAUGAGUCCUUGGCCACAGdTdT-3’
3’-dTTCUCUCAGGAGCUGGUGC-5’

Fig. 1. Sequences of short interfering RNA (siRNA) constructs used in this study. In addition to a control (i.e., nonsilencing) siRNA construct, three different siRNA duplexes were designed that targeted the coding region of the mouse \(V_2R\) vasopressin receptor (\(V_2R\) mRNA (GenBank accession no. NM_019404). These siRNA constructs targeted the following nucleotides within the vasopressin \(V_2R\) mRNA sequence: R1, 260–280; R2, 680–700; R3, 1202–1222.
siRNA-MEDIATED SILENCING OF VASOPRESSIN V2 RECEPTORS

remaining portion of the inner medulla was homogenized in ice-cold buffer A (50 mM Tris-HCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1.0 µg/ml leupeptin, 10 µg/ml aprotinin, and 0.04 U/ml antipain; pH 7.4) using a StedFast SL1200 homogenizer (Fisher Scientific) fitted with a Teflon pestle. The resulting homogenate was centrifuged at 25 g for 10 min at 4°C. The pellet was discarded, and the supernatant was recentrifuged in ice-cold buffer A at 15,000 g for 30 min at 4°C. The membrane preparations were gently vortexed and resuspended in ice-cold buffer A to a final concentration of 0.5–1.0 mg/ml. Membrane protein concentrations were determined by protein assay using BSA as the standard (Bio-Rad Laboratories, Richmond, CA).

**Vasopressin V2R radioligand binding assay**. V2R binding was determined in inner medulla membrane preparations as previously described by Tian et al. (39) using the V2R antagonist, d(CH2)3-[Ile2,Ile4,Tyr-NH29]AVP, which has a tyrosine iodination site at the carboxyl terminal distant from the ligand receptor binding site (courtesy of Dr. Maurice Manning, Medical College of Ohio, Toledo, OH). This ligand was iodinated using a chloramine T procedure (14), and the monoiiodinated V2R radioligand was purified by reverse-phase HPLC on a C18 column (Peptide Radiiodination Service, University of Mississippi).

**Vasopressin V2R immunoblot**. V2R protein expression was determined by immunoblotting and chemiluminescent protein detection as previously described (6). In brief, inner medullary membrane preparations (7 µg) were mixed with 5× Laemmli sample buffer (4 vol of sample to 1 vol of Laemmli buffer) and heated to 60°C for 15 min to solubilize proteins. The proteins were then separated by SDS-PAGE using Bio-Rad precast 12.5% gels (Bio-Rad Laboratories, Hercules, CA). Following electrophoresis, proteins were transferred to nitrocellulose membranes at 0.6 A for 60 min in transfer buffer (50 mM NaPO4, 150 mM NaCl, 0.01% Tween-20). The vasopressin V2R antibody used is a rabbit anti-vasopressin V2R affinity-purified polyclonal antibody (AVPR-V2; Chemicon International, Temecula, CA), the use of which has previously been described (25). The nitrocellulose membranes were incubated with the antibody (2.5 µg/ml) at 4°C overnight. Next, the membrane was incubated with the secondary antibody (1:10,000; Pierce Biotechnology, Rockford, IL) and chemiluminescence was carried out using LumiGlo reagents according to the manufacturer’s protocol (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

**Real-time PCR of vasopressin V2R mRNA**. Total RNA was extracted using the RNeasy-4PCR silica-fiber binding RNA extraction kit (Ambion, Austin, TX). First-strand cDNA was prepared from total RNA (500 ng) using the iScript cDNA synthesis kit (Bio-Rad) in accordance with the manufacturer’s protocol (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The PCR reaction mixture for vasopressin V2R detection consisted of RNase-free water, TaqMan Universal PCR Master Mix (Applied Biosystems), and 300 nM specific primers and 10 µM probe (forward primer (663F) 5'-AGC TCT TCA TTG CTC AAC GT-3'; reverse primer (755R) 5'-GTT CCG CTA GGT CCA CTG TAT-3'; probe (725T) 6FAM-CGA TTT GCA GAG CCA T-TAMRA), and cDNA samples. PCR reactions without reverse transcription were included to control for contamination by genomic DNA. Results obtained for vasopressin V2R mRNA levels were normalized to 18S ribosomal RNA.

**Measurement of urine volume and osmolality**. Animals were housed in metabolic cages, and their urine was collected and stored at −20°C. Animals were acclimated to the cages for 3 days prior to siRNA infusion. Evaporation was prevented by adding 0.5 ml mineral oil to the collection tubes. Urine samples were collected daily at midday. Urine osmolality was measured using a vapor-pressure osmometer (model 5520; Wescor, Logan, UT).

**Statistical analysis**. All results are expressed as means ± SE. Differences between treatment groups were analyzed statistically using Student’s t-test, or one-way ANOVA followed by Dunnett’s test when more than two groups were compared. All analyses were performed using the statistical analysis software package, Prism 4.0 (GraphPad Software, San Diego, CA).

**RESULTS**

**Effect of siRNA treatment on vasopressin V2R expression**. The relative efficacy of the three distinct siRNA duplexes were compared. As described above, mice were injected with 3.6 nmol (~50 µg) of each siRNA (Fig. 1), and 48 h later, V2R expression was assessed by radioligand binding assay (Fig. 2). The R2 siRNA duplex induced a marked reduction in V2R-specific binding in inner medullary membranes [39.7 ± 8.7% compared with the control siRNA, n = 11, P < 0.05 (Dunnett’s test)]. Therefore, we selected the R2 siRNA duplex for use in all further experiments. Note that R3 siRNA appeared to be of approximately equivalent potency to R2; however, due to animal variability, the R3 siRNA effect did not reach statistical significance. The effect of R1 also did not reach statistical significance and showed a smaller trend toward reduced V2R expression compared with either R2 or R3.

In addition to measuring the effect of R2 siRNA treatment on V2R binding, we also determined the effect of R2 on V2R protein expression by Western analysis. V2R protein expression was reduced by 37.0 ± 2.3% (n = 4, P < 0.001, t-test) following treatment with R2 siRNA compared with control siRNA treatment (Fig. 3, A and B).

**Effect of siRNA treatment on vasopressin V2R mRNA**. Under the same treatment conditions described above, R2 siRNA treatment induced a 68.8 ± 1.3% (n = 3, P < 0.05, t-test).

---

**Fig. 2.** Effect of V2R-targeting siRNA treatment on monoiodinated V2 radio-
ligand (125I-V2R(a)) binding in the mouse inner medulla. Binding assays were
carried out as described in MATERIALS AND METHODS. Bars are means ± SE of V2R-specific binding expressed as a percentage of the specific binding in mice treated with the control siRNA on the same day. The n values for each treatment were: control, n = 11; R1, n = 6; R2, n = 11; R3, n = 3. *P < 0.05.
reduction in V2R mRNA, as determined by quantitative real-time PCR analysis (Fig. 4).

Effect of siRNA treatment on urine volume and osmolality under unstimulated conditions. Under basal conditions we observed no significant difference in the animals’ urine-concentrating capacity following R2 siRNA treatment. Forty-eight hours after siRNA infusion both urine osmolality [control, 2,241 ± 238 mosmol/kgH2O (n = 4) vs. R2, 2,436 ± 117 mosmol/kgH2O (n = 4), not significant] and urine volume [control, 1.38 ± 0.09 ml/day (n = 4) vs. R2, 1.19 ± 0.24 ml/day (n = 4), not significant].

Effect of siRNA treatment on urine volume and osmolality during dDAVP treatment. Because we observed no alteration in urine concentrating capacity under basal conditions, we investigated whether siRNA-mediated silencing of the V2R could alter the animals’ urine-concentrating capacity under circumstances in which the V2R system was maximally stimulated. To induce maximal antidiuresis, animals were treated with the V2R-specific vasopressin agonist, dDAVP, via a continuous osmotic minipump infusion at a rate of 0.25 ng/h for the entire duration of the experiment. Three days after implantation of the dDAVP minipumps, each animal was intravenously injected with either control or R2 siRNA as described above. Twenty-four-hour urines were collected throughout the experiment.

Urine osmolality was significantly reduced following R2 siRNA treatment compared with control siRNA (Fig. 5A). Urine osmolality was significantly decreased on both day 2 [3,155 ± 129 (n = 12) vs. 3,455 ± 72 mosmol/kgH2O (n = 12), P < 0.05, t-test] and day 3 [2,861 ± 150 (n = 12) vs. 3,373 ± 128 mosmol/kgH2O (n = 12), P < 0.05, t-test] following siRNA treatment. On day 2 following infusion, urine volume was significantly increased from 0.86 ± 0.07 ml/day (n = 12) in control animals to 1.11 ± 0.06 ml/day (n = 12) in R2 siRNA-treated animals (P < 0.05, t-test) (Fig. 5B). Following this initial increase in urine volume after R2 siRNA infusion, urine volumes in the R2 siRNA-treated animals were not significantly different from the controls.

DISCUSSION

These data demonstrate that vasopressin V2R expression in the inner medulla of the kidney can be reduced in vivo via siRNA-mediated RNA interference in the mouse. Furthermore, the reduction in V2R expression induced by this siRNA treatment was of significant magnitude to reduce the animals’ functional ability to respond to vasopressin stimulation, as evidenced by significantly reduced urine osmolality and increased urine volume during continuous dDAVP infusion. The R2 siRNA duplex potently reduced V2R-specific binding by ~40% at 48 h after transfection. Since Scatchard analysis was not performed, because of limitations in murine inner medulla membrane quantity, the reduction in specific binding could reflect a reduction in ligand affinity (Kd) or a reduction in receptor number (Bmax). (Note that to complete one radioligand saturation isotherm for Scatchard analysis, at least 3–4 mice would be required to generate sufficient tissue.) However, the reduction in specific binding most likely reflects a reduction in Bmax, since the magnitude of the reduction in binding closely correlated with the magnitude of the reduction in protein expression by Western immunoblotting. Furthermore, siRNA

![Fig. 3. Western Blot analysis of vasopressin V2R expression in the mouse inner medulla following R2 siRNA treatment. A: V2R immunoblot showing V2R immunoreactive bands following either control siRNA or R2 siRNA treatment. B: semiquantitative analysis of V2R bands. Data are means ± SE. ***P < 0.001.](http://physiolgenomics.physiology.org/)

![Fig. 4. Effect of siRNA treatment on vasopressin V2R expression in the mouse inner medulla. V2R mRNA was quantitated by real-time PCR analysis as described in MATERIALS AND METHODS. Data are means ± SE. *P < 0.05.](http://physiolgenomics.physiology.org/)
siRNA-MEDIATED SILENCING OF VASOPRESSIN V2 RECEPTORS

Fig. 5. A: R2 siRNA treatment reduces urine osmolality during dDAVP-induced antidiuresis. Animals were treated with 0.25 ng/h dDAVP throughout the experiment by osmotic minipump infusion as described in MATERIALS AND METHODS. Three days after the commencement of the dDAVP infusion, animals were injected with either control or R2 siRNA. Urine osmolality was significantly reduced on days 2 and 3 following R2 siRNA injection, compared with the control. Data are means ± SE. *P < 0.05. B: time course of changes in urine volume following R2 siRNA treatment during dDAVP-induced antidiuresis. Data are means ± SE. *P < 0.05. **P < 0.01.

silences genes by reducing mRNA expression and thus would not be expected to affect ligand affinities.

The siRNA delivery method used in this study was based on the intravenous siRNA transfection protocol previously devised by Sorensen et al. (35). Each animal was injected with 3.6 nmol of siRNA complexed with the cationic liposomal transfection reagent, DOTAP, to improve the ability of the siRNA to cross the cell membrane. This siRNA dose has previously been demonstrated to be effective in inducing siRNA-mediated RNAi of transgenically expressed luciferase (16) and GFP (35) in the mouse kidney via intravenous delivery (16, 35). One study reported siRNA knockdown of GFP expression in the kidney of transgenic GFP mice, demonstrating that the mouse kidney contains the intracellular machinery necessary for RNA interference (16). It should be noted, however, that in that study, GFP knockdown was measured in the whole kidney rather than just the inner medulla. Most V2Rs are expressed in the inner medulla, and this tissue has considerably more variable blood flow than the cortex (23). To counter this potential problem, we volume-loaded the animals with an intraperitoneal injection of isotonic saline to increase blood flow to the renal medulla. We chose to measure the effect of siRNA treatment after 48 h, since the half-life of the vasopressin receptor, similar to most G protein-coupled receptors, is relatively long; half-lives of 4–6 h have been reported for the V2R when expressed in eukaryotic transient transfection systems (18, 29).

Although evidence from in vitro and cell culture systems suggests that any mRNA can be silenced using RNA interference techniques (9), a large degree of variability in the efficacy of siRNA duplexes has been observed (27, 30). The reasons for the lack of efficacy of some siRNA sequences is only partially understood (27). Possible explanations include the sequence of the target within the mRNA (13), the secondary structure of the siRNA duplex (30), and the binding of interfering proteins to the mRNA (9). Although we were able to use established siRNA design guidelines to select sequences likely to be effective in order to maximize the probability of identifying a highly potent siRNA sequence, we designed three separate siRNA duplexes that targeted the vasopressin V2R at different positions within the coding region of the V2R mRNA sequence. We then screened these three siRNA sequences for their efficacy at reducing V2R binding in the mouse kidney inner medulla. A nonsilencing siRNA duplex was selected for use as a control since recent evidence has clearly demonstrated that mock transfection is not an appropriate control in siRNA experiments (1, 34). Although all three siRNAs showed a trend toward reducing V2R-specific binding, only the effect of R2 reached statistical significance. Although the R3 siRNA duplex appeared to be equipotent to R2, the effects of R3 did not reach statistical significance. As described above, we can only speculate as to the mechanistic reasons for the apparent differences in potency of the three siRNA sequences.

The level of reduction of vasopressin V2R mRNA (68%) that we observed following R2 siRNA treatment suggests that delivery of the siRNA to the mouse inner medulla was highly efficient. It is likely that delivery of the siRNA to other tissues using this technique was also efficient; however, because expression of the vasopressin V2R is confined almost exclusively to the kidney, it is difficult to assess delivery to other tissues.

Although we were able to markedly reduce expression of the vasopressin V2R protein by 40% in the inner medulla of mice using the R2 siRNA construct, it is interesting that the basal urine-concentrating capacity of the mice was not altered under these conditions: 48-h treatment with R2 siRNA did not significantly alter either urine osmolality or volume. This result was not surprising, since, as described above, evidence suggests that there is a large reserve of vasopressin V2Rs in the rodent kidney. Therefore, under these basal, unstimulated conditions, sufficient V2Rs likely remained to mediate the level of signal transduction required to maintain adequate AQP2 membrane insertion and normal urine concentration capacity.

We hypothesized that a functional effect of silencing of the vasopressin V2R would be most easily observed under conditions of maximally stimulated antidiuresis. Therefore, we treated the mice with the vasopressin V2R-specific agonist, dDAVP, and then infused either the control or the R2 siRNA. Under these conditions, we observed a transient but significant decrease in urine osmolality and increase in urine volume in R2 siRNA-treated animals. Urine osmolality was significantly reduced on days 2 and 3 and urine volume was significantly increased on day 2 following R2 siRNA infusion. The transient
naturally of these changes in urine volume and osmolality are not entirely surprising, given the nature of siRNA molecules. While the double-stranded structure of siRNA may provide some protection against degradation by RNases, siRNA molecules are rapidly degraded in plasma (2) and somewhat more slowly in cells (12). Therefore, any knockdown in receptor expression induced by siRNA-mediated RNAi is bound to be transient, with levels of the V2R mRNA returning to normal as the receptor-targeting siRNA is degraded. Subsequently, V2R protein expression would be expected to return to control levels as new receptors are translated from newly synthesized mRNA. Longer-term reductions in vasopressin V2R expression might be achieved through sequential injections of the siRNA construct or alternatively via transfection with vectors expressing the R2 sequence as part of a short-hairpin RNA (shRNA; Ref. 26). A number of shRNA vector expression systems have been demonstrated to be effective in vivo including plasmids (21), adenoviruses (42), and lentiviruses (28). These vector expression systems are particularly attractive because the shRNA duplex is continuously expressed in situ, and thus even relatively poor delivery of the vector to the target tissue could potentially result in long-term silencing.

In addition to the transient nature of the functional changes in urine osmolality and volume, the magnitude of the changes produced were not large. This might indicate that the induced decreases in V2R expression were just at the threshold for impairing V2R-mediated signal transduction, but further studies using alternative dosing and/or transfection strategies to produce larger graded knockdowns of the V2R will be required to ascertain this. Nonetheless, these studies constitute a clear “proof of concept” that siRNA methodology is capable of addressing important questions regarding the relation between G protein-coupled receptor expression and ligand-stimulated signal transduction in genetically intact in vivo systems.

In summary, the data presented demonstrate that siRNA-mediated RNA interference can be used to significantly reduce kidney vasopressin V2R expression in vivo and that this reduction in receptor expression is of sufficient magnitude to reduce the ability of the mice to sustain maximal antidiuresis during vasopressin stimulation. The data provide evidence that alterations in the level of genetically normal vasopressin V2Rs in the kidney can modulate maximal urine concentrating capacity. This represents, to our knowledge, the first demonstration of the use of siRNA silencing of a G protein-coupled receptor in a mammalian system to produce functionally significant physiological effects.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) Grant DK-38094 and a National Kidney Foundation (National Capital Area) grant to J. G. Verbalis and NIH Grant HL-57502 and a grant from the Center for Biological Modulators, Korea Research Institute of Chemical Technology (South Korea), to K. Sandberg. A. Hassan is the recipient of an American Heart Association Jocelyn Beard Moran postdoctoral fellowship.

REFERENCES


