Tolvaptan inhibits ERK-dependent cell proliferation, Cl\textsuperscript{−} secretion, and in vitro cyst growth of human ADPKD cells stimulated by vasopressin

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Abstract

Purpose: ADPKD is a common inherited renal disorder characterized by the formation of renal cysts that lead to progressive decline in renal function and eventual end-stage renal disease. Tolvaptan, a nonpeptide V2 receptor antagonist, is approved for the treatment of hyponatremia in water-retaining disorders. In ADPKD cells, AVP is associated with increased cell proliferation, cAMP accumulation, and transepithelial Cl\textsuperscript{−} secretion. Here, we determined the effect of graded concentrations of tolvaptan on intracellular cAMP, ERK activity, cell proliferation, and transepithelial Cl\textsuperscript{−} secretion.

Methods: Tolvaptan, a V2 receptor antagonist, inhibits AVP-induced transepithelial Cl\textsuperscript{−} secretion by human ADPKD cystic cells. Tolvaptan inhibits ERK-dependent cell proliferation, Cl\textsuperscript{−} secretion, and in vitro cyst growth of human ADPKD cells stimulated by vasopressin.


Conclusions: Tolvaptan inhibits AVP-stimulated cell proliferation and Cl\textsuperscript{−} secretion. Tolvaptan inhibits AVP-induced ERK signaling and cell proliferation. Basolateral application of AVP to ADPKD cell monolayers grown on permeable supports caused a sustained increase in short-circuit current that was completely blocked by the Cl\textsuperscript{−} channel blocker CFTRinh-172, consistent with AVP-induced transepithelial Cl\textsuperscript{−} secretion. Tolvaptan inhibited AVP-induced Cl\textsuperscript{−} secretion and decreased in vitro cyst growth of ADPKD cells cultured within a three-dimensional collagen matrix. These data demonstrate that relatively low concentrations of tolvaptan inhibit AVP-stimulated cell proliferation and Cl\textsuperscript{−} secretory function by human ADPKD cystic cells.

Keywords: ADPKD, cystic cell, epithelial cell, MAP kinase, tolvaptan, V2 receptor

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These results provide a strong rationale for testing V2 receptor antagonists in ADPKD patients (34).

Tolvaptan, a derivative of OPC-31260, has a higher affinity for the human V2 receptor (51) and is currently being investigated for safety and efficacy in ADPKD patients (TEMPO trials). Preliminary data suggest that tolvaptan slows ADPKD cyst growth (33); however, the mechanism has not been fully elucidated. The purpose of the current study was to examine the effects of various concentrations of tolvaptan on AVP-induced proliferation, ERK-mediated cell proliferation, Cl⁻ secretion, and in vitro cyst growth of human ADPKD cyst epithelial cells. The results suggest that concentrations of tolvaptan achievable in the plasma of ADPKD patients in the TEMPO trials potently inhibit AVP-induced cell proliferation and Cl⁻-dependent fluid secretion by human ADPKD cyst epithelial cells.

METHODS

Primary cultures of ADPKD and normal human kidney cells. ADPKD kidneys were obtained from hospitals participating in the Polycystic Kidney Research Retrieval Program with the assistance of the PKD Foundation (Kansas City, MO) and from the Biospecimen Shared Resource at the Kansas University Medical Center (KUMC). ADPKD patients who donated their discarded kidneys had a median age of 53 years, ranging from 43 to 73 years (n = 13). Since the majority of ADPKD cases are caused by mutations in PKD1 and these patients have an earlier onset of end-stage renal disease compared with patients with PKD2 mutations (54 vs. 74 years) (27), it is likely that most, if not all, of the primary ADPKD cells were derived from PKD1 kidneys. Normal regions of human kidneys, confirmed by histological examination, were collected from nephrectomy specimens. Normal kidneys withheld from transplantation as a result of anomalous vasculature were also obtained from the Midwest Transplant Network (Kansas City, KS). The protocol for the use of surgically discarded kidney tissues complies with federal regulations and was approved by the Institutional Review Board at KUMC. Primary cell cultures were prepared as previously described (40, 46). Cells were seeded and grown in T75 flasks containing DMEM/F12 supplemented with 1% charcoal-stripped FBS and ITS for 24 h. The serum concentration was reduced to 0.002% FBS and ITS for 24 h and incubated in DMEM/F12 supplemented with 1% charcoal-stripped FBS, 5 ng/ml EGF, and 5 ng/ml insulin for 3 days to initiate cyst growth. After 5–7 days, the outer diameters of cross-sectional images of spherical cysts with distinct lumens were measured using a digital camera attached to an inverted microscope and analyzed with video analysis software. Surface area was calculated from the outer diameters and total surface area of the cysts was determined from the sum of individual cysts within each well. Cysts with diameters < 100 μm were excluded from measurement. These experiments were repeated in three cell preparations from different ADPKD kidneys.

Statistics. Data are expressed as means ± SE. Statistical significance was determined by one-way ANOVA and Student-Newman-Keuls posttest for multiple comparisons or unpaired t-test for comparison between control and treated groups.

RESULTS

Effect of AVP on cAMP-dependent proliferation of human ADPKD cells. Previously, 100 μM AVP (∼3.7 × 10⁻⁶ M) and 100 μM desmopressin (DDAVP, 2.1 × 10⁻⁸ M), agonists that bind to V2 receptors, increased intracellular cAMP and stimulated ERK-dependent proliferation of human ADPKD cyst epithelial cells (2). Here, we tested the effect of various AVP concentrations ranging from 10⁻¹² to 10⁻⁶ M on intracellular cAMP levels in confluent ADPKD cell monolayers. Cells were incubated for 15 min with AVP and then intracellular cAMP levels were measured using a cAMP enzyme-immunoassay system, as described previously (2). Low AVP concentrations (10⁻¹² to 10⁻¹⁰ M) had no measurable effect on intracellular cAMP, whereas 10⁻⁹ to 10⁻⁶ M AVP increased intracellular cAMP levels in a concentration-dependent relationship (Fig. 1). In a composite of seven experiments, 10⁻⁹ M AVP increased cAMP levels from 0.7 ± 0.2 to 4.3 ± 0.6 pmol/monolayer (P < 0.0001), demonstrating that concentrations of AVP in the physiological range stimulate cAMP production in human ADPKD cells.

Incubation of ADPKD cells for 2 or 3 days with 10⁻¹² M AVP, a concentration 1,000-fold lower than was required to induce a measurable increase intracellular cAMP, caused a significant increase in cell proliferation (Fig. 2A). The reason for the discrepancy in the concentration relationship between the effect of AVP on intracellular cAMP and cell proliferation is unclear. It is well-documented that compartmentalization of the cAMP signal provides specificity for a cellular response to ligands binding to G protein-coupled receptors (16). It is
Fig. 1. Effect of arginine vasopressin (AVP) on intracellular cAMP levels in human autosomal dominant polycystic kidney disease (ADPKD) cyst epithelial cells. Cells cultured from a human ADPKD kidney (K223) were grown as confluent cell monolayers (n = 4 wells/condition) and treated for 15 min with AVP; concentrations ranging from 10^{-12} to 10^{-6} M. Intracellular cAMP levels were determined by an enzyme immunoassay and expressed in picromoles per cell monolayer. In a composite of 7 experiments, the addition of 10^{-9} M AVP increased intracellular cAMP from 0.7 ± 0.2 to 4.3 ± 0.6 pmol/monolayer, P < 0.0001.

possible that 10^{-12} M AVP increases cAMP in a cellular compartment in close proximity of a protein complex involved in activation of cell proliferation; however, this concentration may be insufficient to increase total cellular cAMP to a level that can be detected by standard enzyme-immunoassay methods. The difference in the incubation periods for the cAMP assays (15 min and the proliferation assays (48–72 h) may also account for the disparity. In a composite of 7 experiments, the addition of 10^{-9} M AVP increased ADPKD cell proliferation 60.6 ± 7.6% (P < 0.001) above the control-treated cells (set to 100%). The increase in cell proliferation induced by AVP was comparable to the effect of 100 μM 8-bromo-cAMP, a cell membrane-permeable form of cAMP.

To determine whether AVP stimulates the proliferation of noncystic tubule cells, epithelial cells were cultured from normal-appearing tissue of two early-stage ADPKD kidneys and compared with cells derived from adjacent cortical cysts (46). These ADPKD patients had relatively normal renal function; however, one kidney was removed from each patient for the treatment of severe pain. EGF stimulated the proliferation of both cystic and noncystic cells (Fig. 2B); by contrast, AVP stimulated the proliferation of only the cystic cells. Treatment with AVP had no effect on noncystic cells from kidney #182 and caused a small decrease in proliferation of noncystic cells from kidney #171. These results are consistent with previous studies showing that cAMP stimulates proliferation of cyst epithelial cells but not noncystic cells from early-stage human ADPKD kidneys (46).

Effect of tolvapatan on AVP-dependent cAMP production and ERK-dependent proliferation of human ADPKD and normal human kidney cells. ADPKD cells were incubated with tolvaptan for 30 min before the addition of 10^{-9} M AVP for an additional 15 min. Lowest concentrations of tolvaptan (10^{-12} and 10^{-11} M) caused a slight increase in intracellular cAMP above the effect of AVP alone (set to 100%); however, these increases were not significant. By contrast, higher concentrations of tolvaptan inhibited the effect of AVP with an apparent IC_{50} of ~2 × 10^{-10} M (Fig. 3) and the maximal inhibitory effect was achieved with a concentration as low as 10^{-9} M tolvaptan.

In cell proliferation assays, AVP increased ADPKD cell proliferation 52% above control (Fig. 4A) and the addition of tolvaptan inhibited AVP-induced cell proliferation with a similar dose relationship as its effect on intracellular cAMP. Stimulation of cell proliferation by AVP was decreased 30% (P < 0.05) and 43% (P < 0.001) by addition of 10^{-10} and 10^{-9} M tolvaptan, respectively, and was completely blocked by 10^{-8} M tolvaptan. In the absence of AVP, tolvaptan had a biphasic effect on cell proliferation (Fig. 4B). The lowest concentrations of tolvaptan caused a small, but significant, increase in ADPKD cell

![Graph showing effect of AVP on intracellular cAMP levels](http://ajprenal.physiology.org/)
proliferation, suggesting that the compound may be a partial agonist. By contrast, the highest concentration of tolvaptan (10^{-7} M) reduced proliferation below baseline. In NHK cells, AVP inhibited cell proliferation by 25% (Fig. 4C), consistent with the effect of cAMP (49), and the addition of tolvaptan blocked the AVP effect with cell proliferation returning to baseline with the highest tolvaptan concentration.

To determine whether the effect of tolvaptan to inhibit proliferation was associated with changes in ERK activation, we measured phosphorylated ERK (P-ERK) and total ERK in ADPKD cells incubated with 10^{-9} M AVP in the absence or presence of tolvaptan. Treatment with AVP for 15 min increased P-ERK to the same level as 10^{-4} M 8-Br-cAMP (Fig. 5A). Incubation with tolvaptan for 30 min before the addition of AVP inhibited the effect of AVP on ERK phosphorylation, whereas there was no consistent effect on total ERK levels. In a composite of three experiments, 10^{-9}M tolvaptan caused a significant decrease in P-ERK/ERK and 10^{-8} M tolvaptan completely blocked AVP-induced ERK activation (Fig. 5B).

Effect of tolvaptan on AVP-induced Cl\(^{-}\) secretion by human ADPKD cell monolayers. Accumulation of fluid within the cysts of ADPKD kidneys is driven by cAMP-dependent transepithelial Cl\(^{-}\) secretion mediated by apical CFTR Cl\(^{-}\) channels (38, 40). To determine the effects of AVP and tolvaptan on anion secretion, ADPKD cell monolayers were grown on permeable supports, which were mounted in modified Ussing chambers for measurement of short-circuit current (I\(_{SC}\)), as described previously (19). Forskolin, a cAMP agonist, stimulated I\(_{SC}\) by ADPKD cells and the subsequent apical addition of CFTRinh-172, an inhibitor of the CFTR Cl\(^{-}\) channel, blocked forskolin-stimulated I\(_{SC}\) with an apparent IC\(_{50}\) of 5 \(\mu\)M (Fig. 6A). V2R is predominantly expressed on the basolateral surface of collecting duct cells. Recently, V2R was also shown to localize to the primary cilium on the apical aspect of collecting duct cells stimulated with AVP. ADPKD cells were incubated for 30 min in the absence or presence of various concentrations of tolvaptan ranging from 10^{-12} to 10^{-7} M. AVP (10^{-9} M) was added to the bathing solution for an additional 15 min, and then cAMP was extracted from the cells and measured using a cAMP enzyme immunosassay (2). The apparent IC\(_{50}\) for tolvaptan to inhibit the effect of AVP on intracellular cAMP in human ADPKD cyst epithelial cells appears to be ~0.2 nM. Data are means ± SE (n = 3 ADPKD cell preparations). **P < 0.001 compared with AVP alone (set to 100%).

**Fig. 3. Effect of tolvaptan on intracellular cAMP levels in ADPKD cells stimulated with AVP. ADPKD cells were incubated for 30 min in the absence or presence of various concentrations of tolvaptan ranging from 10^{-12} to 10^{-7} M. AVP (10^{-9} M) was added to the bathing solution for an additional 15 min, and then cAMP was extracted from the cells and measured using a cAMP enzyme immunoassay (2). The apparent IC\(_{50}\) for tolvaptan to inhibit the effect of AVP on intracellular cAMP in human ADPKD cyst epithelial cells appears to be ~0.2 nM. Data are means ± SE (n = 3 ADPKD cell preparations). **P < 0.001 compared with AVP alone (set to 100%).

**Fig. 4. Effect of tolvaptan on AVP-induced proliferation of human ADPKD and normal human kidney (NHK) cells. A: ADPKD cells (n = 11 kidney preparations) were incubated for 72 h with control media or 10^{-9} M AVP in the absence or presence of various concentrations of tolvaptan. **P < 0.001 compared with control-treated cells. †P < 0.05 and ‡P < 0.001 compared with cells treated with AVP alone. B: in the absence of AVP, low concentrations of tolvaptan caused a small increase in ADPKD cell proliferation, whereas higher concentrations inhibited proliferation below the basal rate. *P < 0.01 and **P < 0.001 compared with untreated cells. C: AVP decreased the proliferation rate of epithelial cells cultured from NHK (K208 and K253). Tolvaptan blocked the effect of AVP on NHK cell proliferation, consistent with inhibition of cAMP production induced by V2 receptor.
To investigate the effect of tolvaptan on AVP-dependent cyst formation of ADPKD cells in vitro. To investigate the effect of tolvaptan on AVP-dependent cell proliferation and fluid secretion under conditions that more closely resemble cyst growth in situ, ADPKD cells were cultured within a polymerized collagen gel and stimulated to form cysts. Cysts developed within a collagen matrix from clonal growth of individual cells treated with EGF and forskolin. After cyst formation was initiated, EGF and forskolin were removed, and AVP alone or in combination with tolvaptan were added to the bathing media. AVP increased the total surface area (SA) of the ADPKD cysts (diameter ≥100 μm) per well (Fig. 7). Addition of 10⁻⁸ M tolvaptan significantly reduced total SA, demonstrating that V₂ receptor antagonism inhibits both cell proliferation and Cl⁻-dependent fluid secretion by ADPKD cysts stimulated with AVP.

**DISCUSSION**

AVP is secreted by the posterior pituitary gland in response to changes in extracellular fluid osmolality. Binding of AVP to V₂ receptors on the basolateral membrane of principal cells of the collecting duct increases intracellular cAMP, causing insertion and activation of AQP-2 water channels into the apical membrane. Enhanced water permeability of the collecting duct allows water in the glomerular filtrate to be reabsorbed and returned to the circulation. Maintenance of water excretion by the kidneys depends on an appropriate plasma AVP level to correctly regulate osmotic water reabsorption.

In ADPKD and ARPKD, renal cysts originate from collecting ducts, where V₂ receptors are predominantly expressed (2, 32, 37). AVP stimulates cAMP production in human ADPKD and ARPKD cells through G protein-coupled receptor activation of adenylyl cyclases. Several signaling pathways have been implicated in PKD pathogenesis; however, cAMP has been shown to play a central role in cyst growth by stimulating both cell proliferation and transepithelial fluid secretion. In PCK rats, a model orthologous to human ARPKD, suppression of plasma AVP by simply increasing water intake reduced renal cAMP and decreased the level of ERK activation, cell proliferation, and disease progression (23). OPC-31260, which antagonizes V₂ receptors, also reduced renal cAMP and dramatically halted disease progression in four different genetic models of PKD (12, 35, 42). Wang et al. (43) confirmed that the effect of the V₂ receptor antagonist was due to inhibition of the renal effects of AVP by eliminating vasopressin expression in the PCK rat. PCK rats were crossed with Brattleboro rats essentially free of cysts. Administration of DDAVP by osmotic minipump restored cystic disease in the AVP-deficient PCK rats, providing unequivocal evidence for the roles of AVP and cAMP on PKD progression.

Tolvaptan is an orally administered, potent, and highly selective V₂ receptor antagonist that increases free water clearance and has been shown to correct hyponatremia (serum sodium levels below 135 mmol/l) associated with heart failure, cirrhosis, or syndrome of inappropriate antidiuretic hormone.
secretion (4, 6, 29, 51). The affinity of tolvaptan for V2 receptors is 1.8-fold greater than AVP ($K_i$ 0.43 vs. 0.78 nM) and 29-fold greater than $V_{1A}$ receptor ($K_i$ 12.3 nM); and tolvaptan does not appear to bind to $V_{1B}$ receptors (51). In patients with heart failure, daily delivery of 15–60 mg of tolvaptan (titrated according to response) for up to 30 days improved serum sodium levels. The drug was well-tolerated with only a few adverse events such as thirst and dry mouth.

Fig. 6. Effect of tolvaptan on AVP-induced Cl$^-$ secretion by human ADPKD cell monolayers. Short-circuit current ($I_{SC}$) was measured across ADPKD cell monolayers mounted in Ussing chambers using a dual voltage-clamp device as described previously (39). A: 10 µM forskolin added to the basolateral fluid maximally stimulated $I_{SC}$, set to 100%. Addition of apical CFTR$^-$ channel blocker CFTRinh-172 inhibited cAMP-dependent anion secretion with an apparent $IC_{50}$ of 5 µM. In a composite of 3 monolayers, 5 µM CFTRinh-172 inhibited 100% of the AVP-induced $I_{SC}$. B: $I_{SC}$ tracings for 2 ADPKD cell monolayers treated with either apical (a) or basolateral (b) AVP. Apical 10 µM benzamil, an inhibitor of the epithelial Na$^+$ channel, was added to block Na$^+$ transport. Basolateral (b) addition of $10^{-9}$ M and $10^{-8}$ M AVP increased $I_{SC}$. By contrast, the addition of apical (a) $10^{-9}$ M AVP had no effect and $10^{-8}$ M AVP caused only a small increase in $I_{SC}$. Maximal current induced by 10 µM basolateral forskolin was similar between the 2 monolayers. C: various concentrations of AVP were added sequentially to the basolateral media after a new steady-state $I_{SC}$ was reached. D: anion secretion induced by $10^{-8}$ M AVP was completely blocked by 5 µM CFTRinh-172, confirming that AVP increases transepithelial Cl$^-$ secretion by ADPKD monolayers. E: in a summary of 10 monolayers, AVP-stimulated Cl$^-$ secretion was inhibited by increasing concentrations of tolvaptan sequentially added to the basolateral media. *$P < 0.05$ and **$P < 0.001$ compared with control. †$P < 0.01$ and ‡$P < 0.001$ compared with AVP-stimulated $I_{SC}$. 

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Tolvaptan inhibited AVP-induced cAMP production with an apparent IC₅₀ of ~0.2 nM (Fig. 3), similar to the reported Kᵣ for tolvaptan binding to V2 receptors. Tolvaptan (10⁻¹⁰ to 10⁻⁹ M) decreased P-ERK/ERK (Fig. 5) and inhibited ADPKD cell proliferation (Fig. 4A). These concentrations had no apparent effect on basal cell proliferation (Fig. 4B). Interestingly, 10⁻⁷ M tolvaptan caused a small, but significant, decrease in basal cell proliferation. The mechanism for the inhibitory effect of tolvaptan in the absence of AVP is unclear; however, it is possible that at high concentrations, the drug inhibits endogenous activity of the V2R or that the drug has additional anti-proliferative effects.

In HELE cells expressing cloned human V2 receptors, tolvaptan (10⁻¹⁰ to 10⁻⁹ M) had no effect on AVP-induced cAMP production, whereas higher concentrations strongly inhibited cAMP production (51). In the present study, we examined a broader range of tolvaptan concentrations in human ADPKD cells. Surprisingly, vanishingly low concentrations of tolvaptan (10⁻¹² to 10⁻¹¹ M) increased cAMP ~20% above the effect of AVP alone (Fig. 3) and stimulated basal cell proliferation (Fig. 4B), suggesting that tolvaptan at low concentrations could be a partial agonist for the V2 receptor. However, these effects of tolvaptan did not appear to prevent the concentration-dependent decrease in cell proliferation (Fig. 4A) and ERK activation (Fig. 5) when administered with AVP.

In recent studies of human subjects (TEMPO and the Study of Ascending Levels of Tolvaptan in Hyponatremia), tolvaptan administration over a range of doses consistently decreased urine osmolality and increased the rate of urine flow acutely and over the longer term. Moreover, there are no reports of unanticipated antidiuresis following the administration of tolvaptan. It would appear that doses of tolvaptan used in these trials are sufficient to chronically inhibit V2 receptors, reduce urine osmolality, and increase urine volume above baseline levels; the expected effects were tolvaptan to lower cAMP levels in collecting ducts.

The remarkable appearance of an end-stage ADPKD kidney is due to the accumulation of fluid within the hundreds or thousands of cysts that grossly expand the TKV. Fluid secretion by human ADPKD cells is associated with an increase in apically negative transepithelial potential difference (Vₑₑ) and positive Isc, consistent with active anion secretion (19). Apical application of diphenylamine-2-carboxylate, a Cl⁻ channel blocker, decreases Isc, depolarizes Vₑₑ, and inhibits net fluid secretion. Here, we show that basolateral, but not apical, AVP increases Isc across ADPKD cell monolayers (Fig. 6B) and that apical addition of the highly selective CFTR Cl⁻ channel blocker CFTRinh-172 blocks AVP-induced Isc (Fig. 6D), confirming that basolateral AVP induces Cl⁻ secretion by cystic cells via apical CFTR Cl⁻ channels (18, 19, 31, 40). Tolvaptan (concentrations ≥10⁻⁸ M) inhibits AVP-stimulated Cl⁻ secretion by human ADPKD cell monolayers (Fig. 6E) and decreases in vitro cyst growth of ADPKD cells cultured within a three-dimensional collagen matrix (Fig. 7). The capacity for tolvaptan to inhibit fluid secretion may be as important as its effect on cell proliferation. Inhibition of fluid secretion may limit the expansion of existing cysts and allow net fluid absorption to reduce cyst size and TKV.

In summary, concentrations of tolvaptan likely to be attained in the plasma of ADPKD patients by the dosing regimen of the TEMPO studies inhibit AVP-induced activation of the B-Raf/
MEK/ERK pathway and cell proliferation and decrease both AVP-stimulated Cl− secretion and in vitro cyst growth of ADPKD cells. These data in human cyst epithelial cells aid in understanding the cellular mechanisms underlying the action of tolvaptan and offer additional support for the use of tolvaptan in the treatment of renal cyst progression in patients with ADPKD.

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