Fluconazole Increases Osmotic Water Transport in Renal Collecting Duct through Effects on Aquaporin-2 Trafficking

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ABSTRACT

Background Arginine-vasopressin (AVP) binding to vasopressin V2 receptors promotes redistribution of the water channel aquaporin-2 (AQP2) from intracellular vesicles into the plasma membrane of renal collecting duct principal cells. This pathway fine-tunes renal water reabsorption and urinary concentration, and its perturbation is associated with diabetes insipidus. Previously, we identified the antimycotic drug fluconazole as a potential modulator of AQP2 localization.

Methods We assessed the influence of fluconazole on AQP2 localization in vitro and in vivo as well as the drug’s effects on AQP2 phosphorylation and RhoA (a small GTPase, which under resting conditions, maintains F-actin to block AQP2-bearing vesicles from reaching the plasma membrane). We also tested fluconazole’s effects on water flow across epithelia of isolated mouse collecting ducts and on urine output in mice treated with tolvaptan, a VR2 blocker that causes a nephrogenic diabetes insipidus–like excessive loss of hypotonic urine.

Results Fluconazole increased plasma membrane localization of AQP2 in principal cells independent of AVP. It also led to an increased AQP2 abundance associated with alterations in phosphorylation status and ubiquitination as well as inhibition of RhoA. In isolated mouse collecting ducts, fluconazole increased transepithelial water reabsorption. In mice, fluconazole increased collecting duct AQP2 plasma membrane localization and reduced urinary output. Fluconazole also reduced urinary output in tolvaptan-treated mice.

Conclusions Fluconazole promotes collecting duct AQP2 plasma membrane localization in the absence of AVP. Therefore, it might have utility in treating forms of diabetes insipidus (e.g., X-linked nephrogenic diabetes insipidus) in which the kidney responds inappropriately to AVP.

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stimulation shifts this equilibrium toward plasma membrane localization. This shift is associated with changes in the phosphorylation of serine 256 (S256), S264, and S269 of AQP2 as well as a decrease in the phosphorylation of S261 and a reduction in ubiquitination.

The AVP-induced redistribution of AQP2 is also associated with the inhibition of the small GTPase, RhoA. RhoA maintains F-actin as a physical barrier, preventing AQP2-bearing vesicles from reaching the plasma membrane and thus, avoiding inappropriate water reabsorption under resting conditions. AVP induces the protein kinase A (PKA)–mediated phosphorylation of RhoA at S188 and provides for its inhibition. The inhibition causes depolymerization of F-actin and the removal of the barrier, facilitating the redistribution of AQP2 to the plasma membrane.

Dysregulation of AVP-mediated water reabsorption is associated with or causes disease. When AVP levels are increased, such as in the syndrome of inappropriate antidiuretic hormone secretion, late-stage heart failure, or hepatic cirrhosis, AQP2 is predominantly located in the plasma membrane and causes excessive water retention, leading to hyponatremia. *Vice versa*, defects of the system cause diabetes insipidus (DI). Patients with DI produce large amounts of hypotonic urine and polydipsia. If the hormone is absent as a consequence of mutations in the encoding gene, the disease is classified as central DI. When the collecting duct principal cells cannot respond to the hormone due to mutations in the genes encoding the V2R or AQP2, the result is nephrogenic DI. DI can also be acquired, for example, as a consequence of lithium therapy of bipolar disorder. In about 55% of the patients treated with lithium, the AQP2 redistribution is inhibited. Thiazide diuretics and a low-salt diet are prescribed to increase solute and water reabsorption in other nephron segments. However, effective treatments are not available for all forms of DI.

In the case of deficient AVP, defects in the V2R or in the presence of lithium, AQP2, and the machinery for its transport to the plasma membrane are intact. To find potential targets and molecules that might exhibit clinical utility in the treatment of such DI forms, we screened 17,700 small molecules in a cell-based assay to identify modulators of the AQP2 localization. In that study, the widely used antimycotic drug, fluconazole, emerged as a candidate. Fluconazole is the first-in-line drug for treatment of mucosal and invasive *Candida* infections. Fluconazole is also an integrated component for treating cryptococcal infections. The drug belongs to the azole family and serves as an alternative to ketoconazole because of fewer side effects. Fluconazole inhibits 11-β-hydroxylase and 17-α-hydroxylase and has been used in treating Cushing syndrome. In that regard, fluconazole was superior to ketoconazole.

In this study, we investigated the hypothesis that fluconazole can induce trafficking of AQP2 to the plasma membrane, thereby increasing osmotic water transport in the renal collecting duct and reducing water excretion.

### Significance Statement

Dysregulation of vasopressin-induced water reabsorption in the renal collecting duct leads to diabetes insipidus, a congenital or acquired syndrome. Some forms of diabetes insipidus lack effective treatments to prevent the excessive loss of hypotonic urine that characterizes the condition. The authors previously identified the antimycotic drug fluconazole as a potential therapy, acting to modulate the effects of a water channel protein aquaporin-2 (AQP2). In this study, they show in vitro and in vivo that fluconazole induces a vasopressin-independent insertion of AQP2 into the plasma membrane of collecting duct principal cells, thereby lowering urinary output. Hence, fluconazole might have clinical utility in treating certain forms of diabetes insipidus—such as hereditary X-linked nephrogenic diabetes insipidus—in which the kidney responds inappropriately to vasopressin.

### METHODS

#### Fluorescence Microscopic Detection of AQP2 F-Aktin in Primary Inner Medullary Collecting Duct Cells and Mouse Kidneys

Primary rat inner medullary collecting duct (IMCD) cells were obtained and cultured, and AQP2 and F-actin were visualized by laser scanning microscopy (LSM780; Zeiss) as previously described. AQP2 was detected with antibody H27 (1:600) and Cy3-coupled anti-rabbit IgG (#211–165–109, 1:300; Jackson ImmunoResearch Laboratories). F-actin was visualized using Alexa Fluor 647-Phalloidin (#A22287, 1:30; Invitrogen). Nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (#10236276001, 1:100; Roche Diagnostics GmbH).

Mouse kidneys were fixed in PBS containing 4% paraformaldehyde (1 hour at 4°C), dehydrated, and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek) for cryosectioning. Immunofluorescence microscopy was carried out as described. In brief, unspecific binding was inhibited by blocking with fish skin gelatin (0.27%) for 60 minutes at 37°C. The sections were incubated with anti-AQP2 antibody H27 (1:600 dilution) overnight at 4°C, washed three times with PBS, and incubated with Cy3-coupled secondary anti-rabbit antibody (1:300; see above). Nuclei were detected with 4',6-diamidine-2'-phenylindole dihydrochloride and F-actin through incubation with Alexa Fluor 647-Phalloidin for 45 minutes at 37°C. Finally, the sections were washed with PBS three times, and signals were visualized using the LSM780.

#### Animal Experiments

Wild-type BALB/C6 mice were housed in the animal facility of the Max Delbrück Center for Molecular Medicine according to the recommendations of the Federation of European Laboratory Animal Science Associations in a specific pathogen-free environment. All procedures were carried out in accordance with ethical guidelines and permission of the local authority (Landesamt für Gesundheit und Soziales Berlin and...
Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein, Kiel).

Seven-week-old C57bl/6N male mice were water deprived for 24 hours, and they were treated with equal volumes of NaCl (0.9% saline; control), fluconazole (dissolved in 0.9% saline; Braun Melsungen), tolvaptan (Samsca), or combinations as indicated in Results and the figures. The animals were treated with intraperitoneal fluconazole injections (80 mg/kg) to achieve a concentration of 15.4 mg/L in the blood or with saline every 24 hours over a period of 96 hours. Tolvaptan was administered for the final 24 hours by intraperitoneal injection. Plasma levels of fluconazole were determined by mass spectrometry by a commercial vendor (Labor 28). Urine was collected in metabolic cages, and osmolality measured using a cryo-osmometer. At the end of the experiments, the mice were euthanized by cervical dislocation, and kidneys were harvested.

Specifically, we conducted three different experiments. The timelines are indicated in the figures and a scheme representing the regimes in Supplemental Figure 1: (1) 4 days fluconazole or saline with 24 hours of water restriction in metabolic cages, (2) 4 days of fluconazole or saline treatment without metabolic cages and water restriction, and (3) 4 days of fluconazole or saline treatment with a single dose of tolvaptan or saline.

**Western Blotting, Immunoprecipitation, and Determination of RhoA Activity**

Western blotting for the detection of AQP2, the phosphorylated forms of AQP2, RhoA, and RhoA phosphorylated at S188 and Hsp90 and Rhotekin pull downs for the detection of active GTP-bound RhoA were carried out as previously described.\(^8,11\)

For determination of RhoA activity, IMCD cells were treated with fluconazole and/or forskolin, subsequently incubated with ice-cold Rhotekin buffer (50 mM Tris, pH 7.2, 1% [wt/vol] Triton X-100, 0.5% sodium deoxycholate, 500 mM NaCl, 10 mM MgCl\(_2\), PhosSTOP EASY [Roche Diagnostics], and Complete mini EDTA-free [Roche Diagnostics]) for 10 minutes, and lysed. Lysates (300–400 μg protein) were incubated with 300 μl of Rhotekin beads. Proteins were eluted from beads with Laemmli lysis buffer and analyzed by Western blotting. Active RhoA was related to RhoA in the input fraction and the ratio to the loading control, Hsp90.

Immunoprecipitation of AQP2 from IMCD cells was carried out as described.\(^8\) In brief, cells were lysed with ubiquitin lysis buffer (0.15 M NaCl, 25 mM HEPES, 1% Triton X-100, 2.5 mM NEM, 0.5 mM PMSF, 1× PhosSTOP EASY, and 1× Complete mini EDTA-free); cell debris was removed by centrifugation, and the supernatants were incubated with Protein A-Sepharose, preincubated with mouse monoclonal anti-AQP2 antibody E-2 (sc-515770; Santa Cruz; 4 μl/20 mg of Sepharose beads in 1 ml of ubiquitin lysis buffer), and rotated overnight at 4°C. Beads were washed four times with ubiquitin lysis buffer. AQP2 and coprecipitated ubiquitin were detected by Western blotting (anti-AQP2 C-17 antibody; Santa Cruz) and monoclonal mouse antibody against ubiquitin (Cell Signaling).

**PKA and Para-Nitrophenylphosphate Phosphatases Activity Assays**

PKA activity was monitored using the PepTag Assay (Promega) according to the manufacturer’s instruction. For para-Nitrophenylphosphate–based evaluation, cells were lysed in lysis buffer without phosphatase inhibitors, and aliquots of 100 μg of lysates in triplicates were diluted in Colorimetric Assay Buffer (containing 10 mM para-Nitrophenylphosphate) as described.\(^28\) After 30 minutes of incubation at 30°C, the absorbance was determined at 405 nm with an xMarkMicroplate Absorbance Spectrophotometer (Bio-Rad), and phosphatase activity was calculated.

**Renal Collecting Duct Water Permeability Measurement**

Renal cortical collecting ducts (CCDs) of 12 C57bl/6J mice (male, 8–11 weeks old) were dissected and perfused for luminal fluorescence measurements.\(^27\) The CCDs were luminally perfused with solution 150 (osmolality: 150 mosm/kg; 72.5 mM NaCl, 0.2 mM KH\(_2\)PO\(_4\), 0.8 mM K\(_2\)HPO\(_4\), 0.5 mM MgCl\(_2\), 0.65 mM Ca-gluconate, and 2.5 mM glucose supplemented with 50 μM 150 kD FITC dextran) while continuously superfused (approximately 5 ml/min) with control solution (osmolality: 300 mosm/kg; 145 mM NaCl, 0.4 mM KH\(_2\)PO\(_4\), 1.6 mM K\(_2\)HPO\(_4\), 1 mM MgCl\(_2\), 1.3 mM Ca-gluconate, and 5 mM glucose, pH 7.4). After initial perfusion with fluorescent solution 150, the distal end was occluded with a holding pipette. A constant perfusion pressure at the perfusion side kept the tubule open and at constant volume. The fluorescence emission intensity from the lumen was monitored. On reaching a stable baseline of at least 90 seconds without collapse or clearly visible leakage, forskolin (30 μM), fluconazole (50 μM), or as a control, the solvent of these agents, DMSO (0.1%), was added to the bath (basolateral side of CCD; time point 0), and luminal fluorescence was monitored for another 180 seconds. A transepithelial water flow is indicated by an increment of luminal fluorescence. The increment results from water leaving the lumen along the lumen to bath osmotic gradient and an increase in luminal FITC dextran concentration caused by replenishment through the perfusion pipette. Fluorescence intensity over time was analyzed by Meta-Fluor software and normalized to the intensity at time point 5 seconds before time point 0. Relative fluorescence intensity changes as measures of water permeability were calculated after application of the indicated agents.

**Statistical Analyses**

Statistical analyses were carried out using GraphPad Prism 7 software and the Mann–Whitney U test, t test, or one-way ANOVA combined with a Bonferroni post hoc comparison test to evaluate statistical significance.
Figure 1. Fluconazole (Flu) promotes a plasma membrane localization of aquaporin-2 (AQP2), and it decreases urine output and increases urine osmolality in mice. (A) Primary inner medullary collecting duct cells were left untreated (control) or stimulated with forskolin (FSK; 30 µM, 30 minutes), arginine-vasopressin (AVP; 100 nM, 30 minutes), or Flu (50 µM, 60 minutes) alone or in combination with FSK or AVP (addition of FSK or AVP for an additional 30 minutes). AQP2 was detected by immunofluorescence microscopy using specific primary (H27) and Cy3-coupled secondary antibodies (green). Nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (blue). Shown are representative images from one of three independent experiments. (B) Intensities of intracellular and plasma membrane immunofluorescence signals arising from AQP2 were determined, related to perinuclear signal intensities, and ratios...
**Figure 1.** Continued. Ratios less than one indicate a predominant intracellular localization, and ratios greater than one indicate a predominant plasma membrane location of AQP2 (mean ± SEM; n=3 independent experiments). **P<0.001.** (C–F) Wild-type C57bl/6J male mice were treated every 24 hours over a period of 96 hours with Flu (intraperitoneal injections; 80 mg/kg body wt) or 0.9% saline (NaCl), or they were water-deprived without additional treatment. Schemes for depicting animal treatments are indicated in Supplemental Figure 1. (C) Tissue sections from kidneys were prepared, and AQP2 and nuclei were detected as indicated in A. Shown are representative images from three independent experiments each carried out with five animals per condition. The magnified views of collecting ducts are from the indicated white boxes. (D) The AQP2 localization was semiquantitatively analyzed in three animals (n=19 collecting ducts of each Flu and NaCl treated). Plotted is the AQP2 signal intensity along the indicated red line. The peak in Flu signals corresponds to the apical plasma membrane of collecting duct principal cells (mean ± SEM). ****P<0.001. (E) Baseline urine osmolality measurements with mice that had access to water ad libitum were carried out in stock cages using spot urine. (F) Water-deprived animals were kept in metabolic cages during the final 24 hours of the experiment. Urine was collected, and urine osmolality and output were monitored. n=15 animals per water-derived group and n=5 per nonwater-deprived group. Statistically significant differences are indicated (mean±SEM). ****P<0.001. (G) In vitro microperfusion of cortical collecting ducts (CCDs) isolated from wild-type C57Bl/6J mice. (Upper left panel) Combined light microscopy and fluorescence (485-nm) image of a live CCD segment in perfusion with 150 kD FITC dextran in the lumen. The CCD segments were occluded at the distal end with a holding pipette and connected on the opposite side to a perfusion pipette. (Upper center panel) The experimental setting in the control situation in the absence of treatment and on DMSO application. (Upper right panel) In the presence of FSK or Flu, water exits the lumen and is replenished via the perfusion pipette, which leads to a continuous increase of intraluminal FITC dextran. An osmotic gradient is established through the presence of 150 mosm/kg solution within the CCD lumen and 300 mosm/kg solution in the bath (basolateral). The CCDs were perfused at a constant pressure, and continuous monitoring of the fluorescence intensity from the lumen was started. On reaching a stable baseline of at least 90 seconds, FSK (30 μM), Flu (50 μM), or DMSO as a control (0.1%) was added (time point: 0 seconds). Fluorescence was measured for another 180 seconds. Results are summarized in the lower left panel. Increased water flow across the epithelia was quantitatively expressed as fluorescence increment values as a direct measure of water permeation velocity (lower right panel). Statistically significant differences are indicated (mean±SEM). For control, n=5 CCDs from five animals. For FSK, n=5 CCDs from five animals, and for Flu, n=5 CCDs from four animals. Each CCD was measured independently. **P<0.01; ***P<0.001.
RESULTS

Fluconazole Promotes the AQP2 Plasma Membrane Localization and Decreases Urine Output Independent of AVP

We tested the influence of fluconazole on the AQP2 localization in primary rat IMCD cells (Figure 1). Under resting conditions, AQP2 is located mainly in the perinuclear region of the cells. AVP or direct activation of adenylyl cyclases with forskolin leads to cAMP elevation, activation of PKA, and the redistribution of AQP2 to the plasma membrane. Fluconazole alone (50 μM) enhanced the membrane localization of AQP2, similar to forskolin and AVP, but had no additive effect with these agents (Figure 1A). Semiquantitative analyses of intracellular and plasma membrane AQP2 fluorescence signal intensities (Figure 1B) confirmed these observations. The data were surprising, because our preliminary findings had suggested an inhibitory effect of fluconazole on the forskolin-induced redistribution of AQP2.19

To test whether fluconazole promoted AQP2 redistribution and water reabsorption in vivo, we treated wild-type mice with fluconazole (Figure 1, C–F). The fluconazole dose was chosen to achieve the extracellular fluconazole concentration in patients treated for fungal infections. The patients receive 200–400 mg/d.29 We used 80 mg/kg body wt for 4 days in the mice, and the serum concentrations were 15–50 μM (data not shown). This is also similar to the concentration applied in our cell culture experiments above. Immunofluorescence microscopy (Figure 1C) and semiquantitative analysis of the AQP2 localization (Figure 1D) revealed that the renal collecting duct principal cells of fluconazole-treated mice displayed an accumulation of AQP2 at the apical plasma membrane. This situation resembled the localization of AQP2 after water deprivation, a condition associated with high plasma AVP levels. In control animals treated with equivalent volumes of 0.9% saline, AQP2 was found throughout the cytoplasm.

The fluconazole-induced redistribution of AQP2 to the plasma membrane suggested that the drug promotes water reabsorption in collecting ducts, thereby increasing urinary osmolality. Indeed, compared with mice treated with 0.9% saline, fluconazole treatment increased urine osmolality in mice allowed free access to drinking water (Figure 1E). Under water deprivation fluconazole-treated mice displayed decreased urinary output and increased urinary osmolality compared with 0.9% saline-treated mice (Figure 1F). We calculated creatinine clearance in baseline male mice as a measure for the GFR according to Dunn et al.30 We saw no difference in creatinine clearance between fluconazole- and saline chloride–treated animals. There was also no difference in water or food intake (Supplemental Figure 2).

To test whether the fluconazole-induced redistribution of AQP2 is associated with an increase in osmotic water permeability, we measured water flow across epithelia of freshly isolated mouse CCDs. The CCDs were perfused with a hypotonic solution containing FITC dextran. When added to the bath (basolateral), fluconazole and forskolin induced a transepithelial water flow as indicated by an increment of luminal fluorescence (Figure 1G). Fluconazole and forskolin did not alter the luminal diameter, and without the lumen to bath osmotic gradient, fluconazole did not induce transepithelial water flow (Supplemental Figure 3). Thus, the fluconazole-induced redistribution of AQP2 in renal principal cells caused an increase in water reabsorption.

Fluconazole Modulates AQP2 Phosphorylation and Increases Its Abundance

To elucidate the molecular mechanisms underlying the fluconazole-induced AQP2 trafficking, we investigated signaling downstream of the V2R. As previously reported, forskolin increased the abundance of AQP2 in IMCD cells within 30 minutes (Figure 2A). Fluconazole in concentrations of 10 and 50 μM alone had a similar effect (Figure 2A). An additive effect of forskolin and fluconazole was not evident.

The increases in AQP2 abundance correlated with a decrease in the phosphorylation of S261, a region in AQP2 that is key for the regulation of AQP2 abundance in response to AVP.31 When paired with forskolin, fluconazole decreased the phosphorylation of S261 (Figure 2B). Fluconazole alone decreased the phosphorylation of S256 and S269, and these alterations were previously linked to forskolin treatment.19

In IMCD cells, we confirmed these findings in IMCD cells, MCD4 cells; MCD4 is an immortalized cell line that represents another model system for the AQP2 redistribution (data not shown). Fluconazole did not affect AQP2 mRNA levels in IMCD cells or our mice (Supplemental Figure 4). We had previously shown that a rise in cAMP and PKA activation in IMCD cells causes inhibition of p38MAPK-mediated phosphorylation of AQP2 at S261.8 This leads to a decrease in AQP2 ubiquitination and thereby, a decrease in the proteolytic degradation of AQP2.8 The result is an increase in AQP2 protein abundance.8 Fluconazole alone also mediated a decrease in the S261 phosphorylation (Figure 2B) that was associated with a decrease in the ubiquitination of AQP2, explaining the increase in AQP2 abundance (Figure 2A). Fluconazole did not enhance the forskolin effect on the S261 phosphorylation. The fluconazole-induced decrease of AQP2 ubiquitination was also observed in MCD4 cells; MCD4 is an immortalized cell line that represents another model system for the AQP2 redistribution (data not shown). Fluconazole did not affect AQP2 mRNA levels in IMCD cells or our mice (Supplemental Figure 4).

V2R stimulation regulates phosphorylation of AQP2 at S256 and S269, and these alterations were previously linked with its endocytosis.7,31 Fluconazole alone did not affect AQP2 S256 or S269 phosphorylation in IMCD cells (Figure 2, C and D). Consistent with our findings in IMCD cells, fluconazole treatment in mice increased the AQP2 abundance and decreased the S261 phosphorylation. The phosphorylation of AQP2 at S256 and S269 was decreased in response to fluconazole (Figure 2, E–H). V2R stimulation induces PKA activation. Therefore, we determined the effect of fluconazole on global PKA activity in IMCD cells. Although forskolin increased PKA activity compared with the basal state, fluconazole did not (Figure 3A). The decrease in the phosphorylation of S261 could be due to activation of protein phosphatases. However, fluconazole did not modulate protein phosphatase activity in IMCD cells (Figure 3B). Thus, changes of global PKA or phosphatase activity did not explain the effect of fluconazole on the phosphorylation status of AQP2.
Figure 2. Fluconazole (Flu) increases aquaporin-2 (AQP2) abundance and changes the phosphorylation status of AQP2 in primary inner medullary collecting duct (IMCD) cells and mice. (A–D) IMCD cells were left untreated or stimulated with forskolin (FSK; 30 µM, 30 minutes) or Flu alone or in combination with FSK in the indicated concentrations. If Flu and FSK were combined, FSK was added after 30 minutes of incubation with Flu for another 30 minutes. Lysates were prepared, and AQP2 was detected by Western blotting: (A) glycosylated (cg), high mannose (hm), and nonglycosylated (ng) forms of AQP2 and (B) AQP2 phosphorylated at serine 261 (pS261-AQP2), (C) S256 (pS256-AQP2), and (D) S269 (pS269-AQP2). HSP90 was detected as the loading control. Shown are representative blots from one of four independent experiments. The signals emerging from the cg, hm, and ng forms of AQP2 were semiquantitatively analyzed by densitometry, and the sums were statistically compared. Statistically significant differences are indicated (mean±SEM). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. (C, right panel) IMCD cells were left untreated or treated with FSK (30 µM, 30 minutes) or Flu (50 µM, 30 minutes). AQP2 was immunoprecipitated (IP), and AQP2 and coimmunoprecipitated ubiquitin were detected by Western blotting (polyubiquitin [pUb] and monoubiquitin [mUb]). (E–H) Wild-type C57bl/6N male mice were treated every 24 hours over a period of 96 hours with Flu (intraperitoneal injections; 80 mg/kg) or 0.9% NaCl. The renal inner medullae (n=5 per each condition) were obtained, lysates were prepared, and AQP2 was detected by Western blotting: (E) AQP2, (F) pS256-AQP2, (G) pS261-AQP2, (H) pS269-AQP2, and HSP90 as loading control. Shown are representative Western blots. (Lower panels) The signals emerging from the cg, hm, and ng forms of AQP2 were semiquantitatively analyzed by densitometry, and the sums were statistically compared. Statistically significant differences are indicated (mean±SEM). **P<0.01; ***P<0.001.
Fluconazole Decreases RhoA Activity and Depolymerizes F-Actin

We had previously shown that V2R/PKA activation in renal principal cells causes inhibition of RhoA and the depolymerization of F-actin, which could remove a physical barrier to facilitate the transport of AQP2-bearing vesicles to the plasma membrane. Similarly, inhibition of RhoA with toxins was associated with a depolymerization of F-actin and the redistribution of AQP2, indicating that RhoA is an important regulator of the localization of AQP2.11–14

In line with our previous observations,12,13 forskolin reduced RhoA activity in our primary IMCD cells (Figure 4A). Fluconazole alone had a similar effect. The two agents together did not act additively. A decrease of F-actin was evident on forskolin treatment compared with the control condition (Figure 4B). Fluconazole alone decreased F-actin, and when cells were treated with both fluconazole and forskolin, intracellular F-actin almost vanished (Figure 4B).

The effect of fluconazole was similar in mice. Fluconazole reduced RhoA activity in the renal inner medulla compared with treatment with 0.9% saline (Figure 5A). This was associated with an increase in the inactivating phosphorylation of RhoA at S188 (Figure 5B), explaining the reduction of RhoA activity. Consistently, fluconazole caused a marked reduction in F-actin (Figure 5C).

Fluconazole Ameliorates the Tolvaptan-Compromised Urinary Concentrating Ability

The V2R blocker tolvaptan causes a nephrogenic DI–like excessive loss of hypotonic urine.32,33 To assess whether fluconazole ameliorates the aquaretic effect of tolvaptan and thus, beneficially influences the DI phenotype, we treated mice with fluconazole or saline for 96 hours as indicated above and produced an aquaretic effect through single intraperitoneal injections of 20 mg/kg body wt tolvaptan. Tolvaptan was administered together with the final injection of fluconazole or saline at 72 hours. As expected, in the saline group, the urine output increased and urine osmolality decreased when tolvaptan was administered compared with controls that received only saline (Figure 6A). Compared with cotreatment with saline and tolvaptan, the cotreatment with fluconazole and tolvaptan decreased urine output while increasing urine osmolality. Tolvaptan did not affect AQP2 abundance, the phosphorylation at S256, S261, and S269 (Figure 6B); or RhoA activity as indicated by the unchanged phosphorylation at S188 (Figure 6C). However, fluconazole increased the AQP2 abundance and decreased the phosphorylation of AQP2 at S261 in the tolvaptan-treated mice (Figure 6B). No effect on the AQP2 S256 and S269 phosphorylation was observed. The increased phosphorylation of RhoA at S188 indicated RhoA inhibition (Figure 6C). Thus, fluconazole at least partially compensates for the aquaretic effect of tolvaptan by facilitating AQP2 plasma membrane localization independent of AVP-mediated V2R stimulation.

DISCUSSION

We report here that fluconazole causes the insertion of AQP2 into the plasma membrane of renal collecting duct principal
cells, and in mice, it causes a decrease in urine output and an increase of urine osmolality, indicative of enhanced water reabsorption. This is corroborated by our observation that fluconazole increases water flow across the epithelia of isolated mouse collecting ducts. Because the observed effects are independent of AVP, fluconazole could have therapeutic utility in forms of DI that are resistant to AVP, such as hereditary X-linked DI where V2R is dysfunctional.

Fluconazole decreases the phosphorylation of AQP2 at S261 and its ubiquitination. Ubiquitination is involved in the endocytic sorting of receptors and channels. AVP stimulation causes the deubiquitination of AQP2, which is associated with its plasma membrane localization in IMCD cells. In MDCK cells, ubiquitin-deficient AQP2 is predominantly located in the plasma membrane, and ubiquitination enhances its endocytosis. Thus, the fluconazole-induced reduction in ubiquitination is likely to promote the plasma membrane localization of AQP2. Inhibition of RhoA is associated with the plasma membrane localization of AQP2. Fluconazole mimics the inhibitory effect of AVP on RhoA. Therefore, with the phosphorylation of S261 and ubiquitination of AQP2 and RhoA activity, fluconazole targets critical regulatory factors controlling the AQP2 localization (Figure 7).
Fluconazole blocks fungal lanosterol 14α-demethylase (CYP51A1) and inhibits ergosterol biosynthesis, thereby causing membrane defects. In mammalian cells, CYP51A1 is involved in cholesterol synthesis. CYP51A1 is expressed in primary IMCD cells and rodent kidney (data not shown). In primary IMCD cells and the inner medullae of our mice,
**Figure 4.** Fluconazole (Flu) causes a decrease of RhoA activity and a depolymerization of F-actin in inner medullary collecting duct (IMCD) cells. (A) IMCD cells were left untreated or stimulated with forskolin (FSK), Flu alone, or Flu in combination with FSK for 1 hour in the indicated concentration. Lysates were prepared, and active RhoA was precipitated with Rhotekin beads. Precipitated active RhoA, RhoA in the lysates, and HSP90 as a loading control were detected by Western blotting using specific antibodies. The amount of active RhoA was related to normalized RhoA (RhoA in lysates to HSP90 [input]). Shown are representative blots from one of five independent experiments. Blots were semiquantitatively analyzed and statistically compared. Statistically significant differences are indicated (mean±SEM). ***P<0.001. (B) IMCD cells were left untreated or treated as indicated in A. Aquaporin-2 (AQP2) was detected by immunofluorescence microscopy using specific primary antibodies (H27) and Cy3-coupled anti-rabbit secondary antibodies (green), and F-actin was detected by using Alexa Fluor 647-Phalloidin (red). Nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (blue). The magnified views (zoom) of F-actin are from the indicated squares. Shown are representative images from one of three independent experiments.
fluconazole did not cause any obvious membrane defects. In *Candida albicans*, fluconazole caused an accumulation of mevalonate, an intermediate in cholesterol synthesis. By interfering with mevalonate conversion, fluconazole, similar to statins, most likely interferes with the formation of isoprenoids, such as farnesyl-PP, which are important for the membrane tethering and activation of small GTPases. Thus, in addition to the inhibition of RhoA, a decreased membrane association could contribute to the fluconazole-induced inhibition of RhoA that we observed in renal principal cells.

Theazole drug family to which fluconazole belongs has side effects, and our findings may explain some of these better.

Figure 5. Fluconazole decreases RhoA activity and causes depolymerization of F-actin in renal inner medullae of mice. Wild-type C57bl/6N male mice were treated with fluconazole intraperitoneal injections (80 mg/kg) or 0.9% NaCl every 24 hours over a period of 96 hours. (A) Lysates were prepared from renal inner medullae (n=5 per each condition), and active RhoA was precipitated with Rhotekin beads. Precipitated active RhoA, RhoA, and HSP90 (loading control) in the lysates were detected using specific antibodies. Shown are representative Western blots. Blots were semiquantitatively analyzed, and signals were statistically compared. The signals arising from active RhoA were related to normalized RhoA (RhoA to HSP90 in lysates). Statistically significant differences are indicated (mean±SEM). **P<0.01. (B) Mice were treated as indicated in A, and lysates from one inner medulla from each animal were prepared (n=5 per condition). Total RhoA, inactive RhoA phosphorylated at serine 188 (S188; pS188-RhoA), and HSP90 as a loading control were detected by Western blotting. Blots were semiquantitatively analyzed and statistically compared. Statistically significant differences are indicated (mean±SEM). Shown are representative Western blot results. *P<0.05. (C) The second inner medullae of kidneys of each animal were subjected to fluorescence microscopic analysis of aquaporin-2 (AQP2), F-actin (Phalloidin), and nuclei (DAPI). Shown are representative images. Lower panels show magnified views of the areas indicated in upper panels. Scale bars, 10 μm.
Figure 6. Fluconazole (Flu) decreases urine output in tolvaptan (Tol)-treated wild-type mice. Wild-type C57bl/6N male mice were treated every 24 hours over a period of 96 hours with Flu (intraperitoneal injections; 80 mg/kg) or 0.9% NaCl. (A) Twenty-four hours before euthanasia, the mice were placed in metabolic cages, and single intraperitoneal injections (20 mg/kg) of Tol were administered to the 0.9% NaCl– and Flu-treated mice (Supplemental Figure 1); 24-hour urine osmolality and output were monitored (n=5 per each group). Statistically significant differences are indicated (mean±SEM). (B and C) Aquaporin-2 (AQP2), RhoA, and phosphorylation of AQP2 and RhoA were detected by Western blotting. The Western blots were quantified as described in Figures 2, 4, and 5. Shown are results from three mice for each condition. *P≤0.05; **P≤0.01; ***P≤0.001.
Azoles modulate actions of other drugs, because they inhibit cytochrome P450 (CYP) enzymes\(^40\) and thereby, the extent of the half-lives of drugs that are metabolized by the targeted CYPs. For example, tolvaptan is metabolized by CYP3A4, which in turn, is inhibited by ketoconazole (threefold more potently than by fluconazole). As a result, ketoconazole enhanced the aquaretic effect of tolvaptan.\(^41\) However, this enhancing effect of ketoconazole on the tolvaptan action also shows that the antidiuretic effect of fluconazole is apparently not a common property of azoles. Moreover, azoles also differ in other pharmacologic properties. Ketoconazole is extensively metabolized in the liver, increasing the risk of hepatotoxicity.\(^42\) Fluconazole is minimally metabolized in the liver, and 80% of it is excreted unmodified with urine.\(^43\) Together, the known differences between the azoles and our observations may serve as a guide to new chemical structures mimicking the antidiuretic effect of fluconazole but that have more favorable pharmacologic properties.

Earlier strategies attempted to increase AQP2 surface expression. The PDE5 inhibitor Sildenafil\(^44\) and the antidiabetic drug rosiglitazone increase urine osmolality and decrease urine output in DI animal models\(^45\) and isolated patient reports.\(^46\) Another antidiabetic drug metformin (and metformin-mediated AMP-activated protein kinase activation)\(^47\) and inhibition of the EGF receptor with the anticancer drug erlotinib\(^48\) promoted the plasma membrane localization of AQP2 and were beneficial in DI animal models. However, findings with regard to effects of AMPK activation are controversial.\(^49\) Statins have been tested in mouse models for nephrogenic DI.\(^50,51\) We had identified a small molecule, FMP-API-1, that globally activates PKA and uncouples it nonselectively from its interactions with A-kinase anchoring proteins.\(^52\) This molecule induced the plasma membrane insertion of AQP2 and was suggested as a starting point for a new treatment strategy for DI.\(^52,53\)

In conclusion, we elucidate an antidiuretic effect of the approved and widely used drug fluconazole. Fluconazole could be repurposed for treating AVP-resistant forms of DI where AQP2 and its trafficking machinery are intact. Moreover, fluconazole’s chemical structure and pharmacologic properties may serve as a guide to new antidiuretic drugs that target renal principal cells and cause fewer side effects than fluconazole.

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DISCLOSURES

None.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2018060668/-/DCSupplemental.

Supplemental Figure 1. Scheme showing animal treatment.
Supplemental Figure 2. Fluconazole does not affect (A) creatinine clearance, (B) water, or (C) food intake of mice.
Supplemental Figure 3. (A) Without transepithelial osmotic gradient, the application of 50 μM fluconazole does not induce transepithelial water flow across epithelia of isolated CCDs; (B) diameter of mouse CCDs during water flux measurements at a given osmotic gradient (300/150 mosm/kg) does not change in response to DMSO, fluconazole (50 μM), or forskolin (30 μM).
Supplemental Figure 4. Fluconazole does not alter AQP2 mRNA abundance in primary IMCD cells and mice.

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