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TRANSPORT PHYSIOLOGY

Fluvastatin modulates renal water reabsorption in vivo through increased AQP2 availability at the apical plasma membrane of collecting duct cells

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Abstract X-linked nephrogenic diabetes insipidus (XNDI), a severe pathological condition characterized by greatly impaired urine-concentrating ability of the kidney, is caused by inactivating mutations in the V2 vasopressin receptor (*V2R*) gene. The lack of functional V2Rs prevents vasopressin-induced shuttling of aquaporin-2 (AQP2) water channels to the apical plasma membrane of kidney collecting duct principal cells, thus promoting water reabsorption from urine to the interstitium. At present, no specific pharmacological therapy exists for the treatment of XNDI. We have previously reported that the cholesterol-lowering drug lovastatin increases AQP2 membrane expression in renal cells in vitro. Here we report the novel finding that fluvastatin, another member of the statins family, greatly increases kidney water reabsorption in vivo in mice in a vasopressin-independent fashion. Consistent with this observation, fluvastatin is able to increase AQP2 membrane expression in the collecting duct of treated mice. Additional in vivo and in vitro experiments indicate that these effects of fluvastatin are most likely caused by fluvastatin-dependent changes in the prenylation status of key proteins regulating AQP2 trafficking in collecting duct cells. We identified members of the Rho and Rab families of proteins as possible candidates whose reduced prenylation might result in the accumulation of AQP2 at the plasma membrane. In conclusion, these results strongly suggest that fluvastatin, or other drugs of the statin family, may prove useful in the therapy of XNDI.

Keywords Nephrogenic diabetes insipidus · Aquaporins · Vasopressin · Statins · Kidney · Diuresis · Epithelial transport · Diabetes insipidus

Abbreviations

XNDI	X-linked nephrogenic diabetes insipidus
V2R	V2 vasopressin receptor
AQP2	Aquaporin-2
AVP	Arginine vasopressin
TAL	Thick ascending limb of the loop of Henle
GGPP	Geranylgeranyl pyrophosphate

Introduction

Nephrogenic diabetes insipidus (NDI) is a genetic disease characterized by the inability of kidney to concentrate urine despite normal circulating levels of the antidiuretic hormone arginine vasopressin (AVP). In the majority of

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patients (>90%) the genetic defect is an inactivating mutation in the V2 vasopressin receptor (*V2R*) gene, located on the X chromosome [5, 6, 25, 32, 42, 44]. For this reason, the disease is inherited as an X-linked trait and is referred to as X-linked NDI (XNDI).

During the past two decades, more than 200 different disease-causing *V2R* mutations have been identified [57]. Under physiological conditions, AVP binding to the V2R triggers the activation of the stimulatory G protein Gs, resulting in increased intracellular cAMP levels which, through activation of the cAMP-dependent protein kinase (PKA), eventually promote the insertion of aquaporin-2 (AQP2) water channels into the apical membrane of the collecting duct principal cells [5, 6, 25, 41, 42, 44, 64]. This mechanism allows for the passive movement of water from the tubule lumen into the kidney interstitium and eventually into the bloodstream. In XNDI patients, this water reabsorption process is impaired due to loss of V2R function, resulting in the production of large volumes (>30 ml/kg/day) of dilute urine (<250 mOsmol/kg) and excessive thirst.

The main symptoms of XNDI, including polyuria, polydipsia, dehydration, and hypernatremia, usually appear soon after birth [5, 6, 25, 41, 42, 44]. Two of the most severe long-term complications associated with XNDI are chronic renal insufficiency and mental retardation, which occur in a subgroup of XNDI patients [7, 32].

In order to reduce urine output, XNDI patients are currently treated with thiazide diuretics (e.g., hydrochlorothiazide) along with a reduction in salt intake, frequently in combination with indomethacin, a prostaglandin synthesis inhibitor, or potassium-sparing diuretics such as amiloride [6, 7, 32, 44]. However, these drugs have only limited efficacy in the treatment of XNDI and often cause severe side effects including disturbances in electrolyte balance as well as renal and gastrointestinal complications [7, 32]. Thus, there is a clear need for the development of new classes of drugs that are highly effective in the treatment of XNDI and are well tolerated.

The activation of V2R-independent intracellular pathways that can rescue AQP2 plasma membrane expression may lead to new approaches towards the effective therapy of NDI [9, 10, 39]. We have recently reported that lovastatin, a cholesterol-lowering drug, can promote the accumulation of AQP2 at the apical plasma membrane of renal cells in vitro by inhibiting AQP2 constitutive endocytosis [49]. AQP2 endocytosis could be affected either by impairing the function of regulatory protein or by perturbing the depolymerization of the actin cytoskeleton that is, in turn, regulated by the small GTPase RhoA [9, 31, 43, 61, 64]. It has been shown that statins, through inhibition of Rho proteins, can affect the polymerization state of the actin cytoskeleton [14, 17, 29, 50]

We here report that fluvastatin, another member of the statin family, exerts a similar effect on AQP2 trafficking in mouse collecting ducts in vivo, leading to increased water reabsorption and reduced urine volume. In addition, we present data indicating that fluvastatin inhibits the prenylation of Rho and Rab proteins, key regulators of AQP2 trafficking, providing a potential molecular mechanism for the ability of fluvastatin (statins) to enhance AQP2 membrane accumulation. Taken together, these findings support the novel concept that statins may become clinically useful in the treatment of XNDI.

Results

Fluvastatin treatment increases AQP2 abundance at the apical membrane of kidney collecting ducts in vivo

Several different statins were first tested on mice for their effect on AQP2 trafficking. Male wild-type (wt) C57BL/6 mice (8–10 weeks old) received a single intraperitoneal (i.p.) injection of lovastatin (20 mg/kg), cerivastatin (20 mg/kg), rosuvastatin (50 mg/kg), or fluvastatin (50 mg/kg) or saline (negative controls) and sacrificed 6 h later. Doses were chosen based on previous studies on rodents [15, 18, 58]. In parallel, another group of mice (positive controls) received two subsequent injections of 1-deamino-8-D-arginine-vasopressin, desmopressin (DDAVP, 1 μg/kg i.p.) 1 h and 30 min before being sacrificed. AQP2 expression and localization in renal collecting ducts were studied by confocal microscopy. Figure 1 shows that the four statins used differed in their efficacy to promote AQP2 accumulation at the apical plasma membrane. Whereas lovastatin and rosuvastatin had little or no effect on AQP2 localization, both cerivastatin and fluvastatin, similarly to DDAVP, greatly promoted AQP2 accumulation at the plasma membrane of collecting duct cells (see white arrows). These experiments were repeated three times with comparable results. Since fluvastatin has been approved for use in humans, we selected this drug for further experiments.

Fluvastatin induces water retention in mouse by increasing plasma membrane expression of AQP2 and sodium transporters in the kidney

Male C57BL/6 mice (8 weeks old) were placed into metabolic cages, and 24-h urine volume and osmolality, along with water intake and body weight, were measured for a 10-day period. Starting on the second day of observation, one group of mice received single daily i.p. injection of fluvastatin (50 mg/kg in PBS) for the next 7 days. Control littermates received a daily injection of saline during the same period. Prior to fluvastatin administration, diuresis, urine osmolality, water intake, and body weight were not statistically different between the two

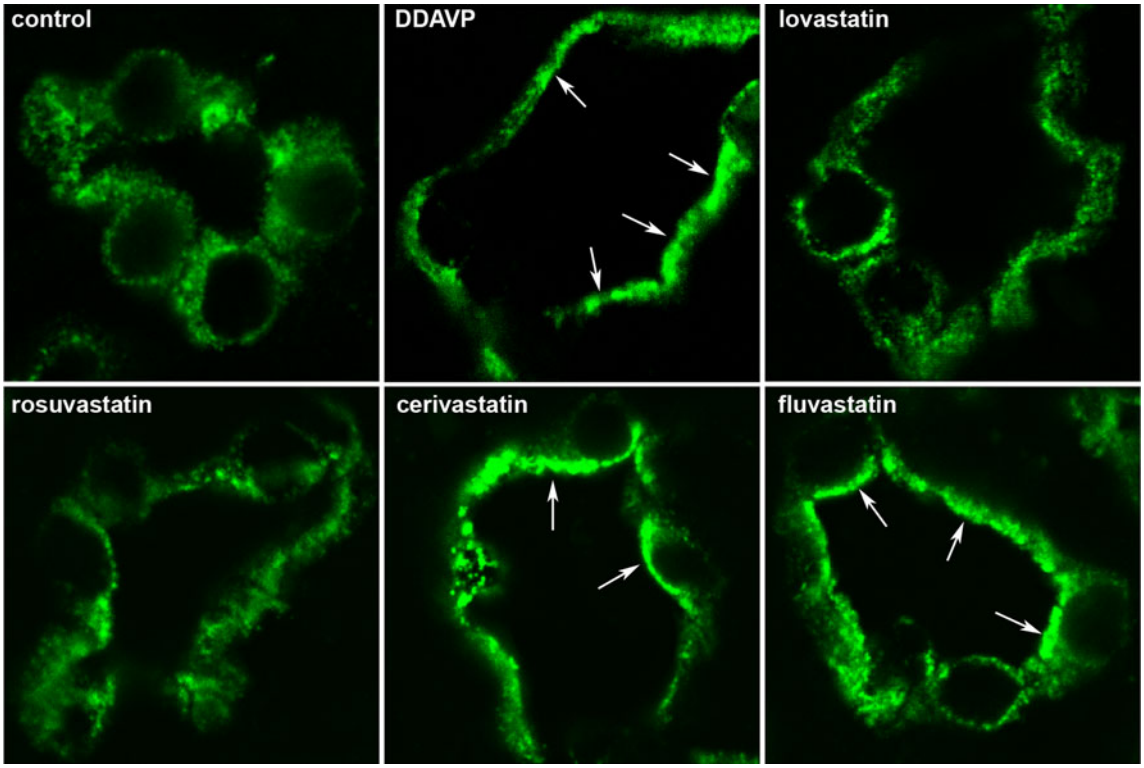

Fig. 1 Effect of acute treatment with different statins on apical AQP2 expression in collecting ducts of C57BL/6 mice. Wt C57BL/6 mice (males, 8 weeks old) were injected intraperitoneally (i.p.) with a single dose of lovastatin (20 mg/kg), cerivastatin (20 mg/kg), rosuvastatin (50 mg/kg), or fluvastatin (50 mg/kg) and sacrificed 6 h later. Control mice received the same volume of saline. Another set of mice was injected twice with DDAVP (1 μg/kg i.p.) and sacrificed 30 min after the second injection. AQP2 is visualized by immunofluorescence and confocal scanning. White arrows indicate AQP2 accumulation at the apical plasma membrane

groups (Fig. 2a). Fluvastatin injection led to a dramatic time-dependent decrease of both urine production and water intake, accompanied by a striking increase in urine osmolality (Fig. 2a). Fluvastatin-treated mice also showed a significant increase in body weight (Fig. 2a). At the end of the experiment, mice were sacrificed and blood was taken for osmolality, cholesterol, copeptin, electrolytes, and creatinine measurements.

In a parallel experiment, a group of mice (N=4) was taken under fluvastatin treatment for 7 days then kept under observation for an additional week. Urinary parameters returned to values comparable to controls in 3–4 days after the treatment suspension (data not shown).

Table 1 reports the effect of 7 days of fluvastatin treatment on the main plasma and urinary analytes measured in control and treated mice.

Fluvastatin treatment significantly reduced blood cholesterol in treated animals. Plasma electrolytes as well as copeptin levels, used as marker of vasopressin levels [37, 38], were unaffected by statin treatment. The analysis of urinary parameters indicated that fluvastatin increased creatinine and K concentrations in the urine. Surprisingly though, Na concentration in the urine was significantly reduced. Glomerular filtration rate (GFR) was not affected by the fluvastatin treatment.

Kidneys were subjected to protein analysis by both immunofluorescence and Western blotting. In particular, AQP2 accumulation at the apical plasma membrane of collecting ducts principal cells was evaluated by confocal scanning microscopy (Fig. 2b). Consistent with the changes in urinary parameters, AQP2 apical staining was more abundant in both cortical and medullary collecting ducts of fluvastatin-treated mice, as compared to control mice (Fig. 2b).

An increase in water permeability in renal collecting ducts requires not only the presence of AQP2 at the apical membrane but also depends on the existence of a hypertonic medullary interstitium generated mainly by the reabsorption of NaCl against its electrochemical gradient in the thick ascending limb (TAL) of the loop of Henle. The major electrolyte transporters involved in the process are NKCC2 and Na/K-ATPase [13, 20]. Western blotting studies showed that the abundance of AQP2, NKCC2, and Na/K-ATPase in the total renal membrane fraction was dramatically increased in fluvastatin-treated mice, as compared to control mice (Fig. 3).

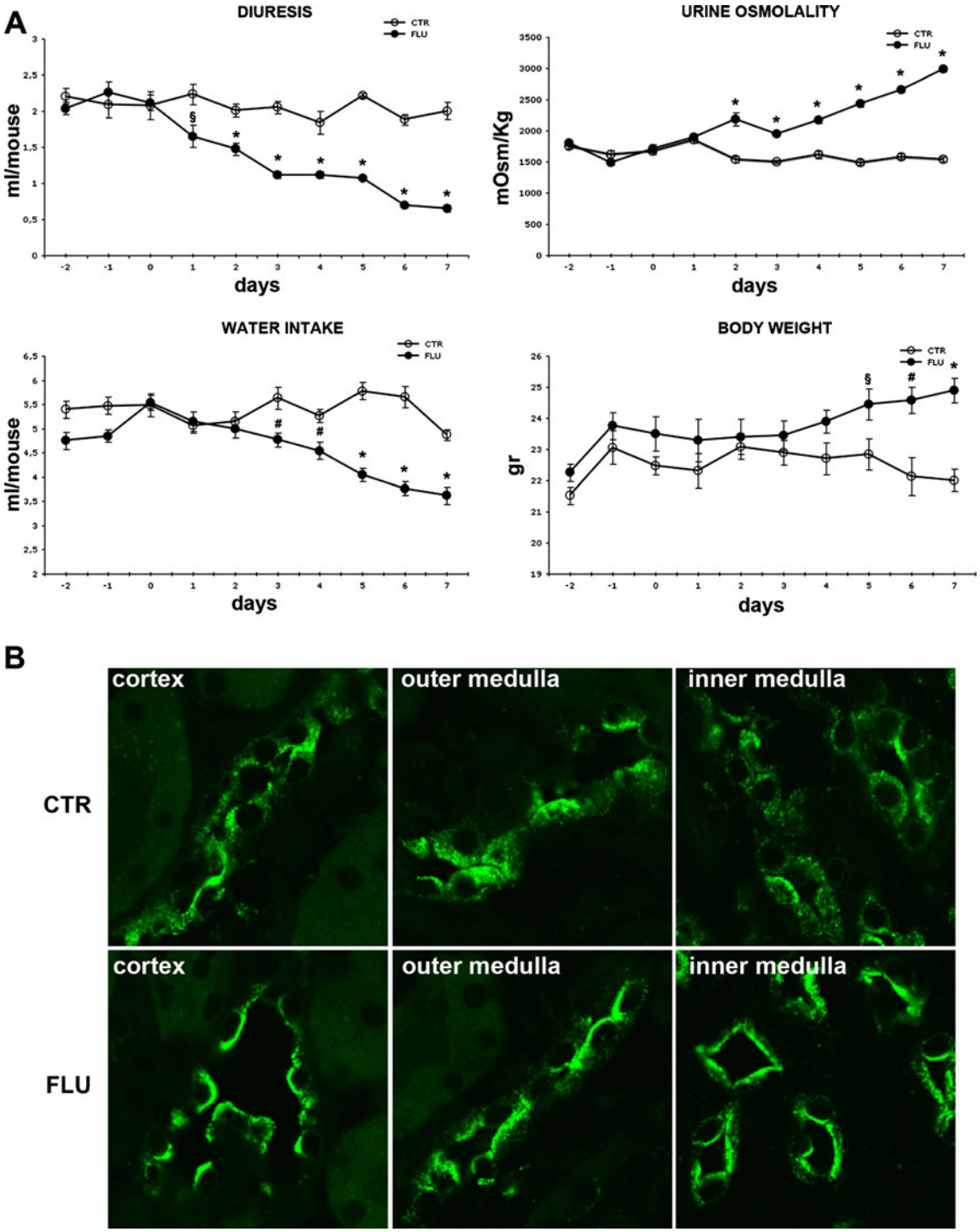

Fig. 2 Effect of prolonged treatment with fluvastatin on urinary parameters and AQP2 intracellular distribution in C57BL/6 mice. **a** C57BL/6 mice were acclimated in metabolic cages for 2 days before starting the experiment. Starting at day 0, animals were injected daily i.p. either with saline alone (CTR) or with fluvastatin (FLU, 50 mg/kg) over a period of 7 days. Diuresis and urine osmolality, along with water intake and body weight, were monitored daily. Data are shown as means±SEM (N=4 per group; *P<0.0005, #P<0.005, and §P<0.05 were the levels of significance obtained with Student's t test). Experiments were repeated three times with comparable results. **b** At the end of the experiments, mice were sacrificed and AQP2 was immunodetected in kidney sections by immunofluorescence and confocal scanning. Representative images are shown

The effect on Na transporters was also investigated in the liver that is the site of statins accumulation. As shown in supplementary Fig. 1, the abundance of Na/K-ATPase was also increased in the liver although to a less extent

Table 1 Statistical analysis of the effect of fluvastatin treatment on urinary and plasma parameters in mice

	Vehicle	Fluvastatin-treated mice	*P*
U_{Na} (mEq/L)	149.9±6.9 (*N*=19)	85±14.21 (*N*=7)	<0.0005***
U_K (mEq/L)	256.5±8.12 (*N*=19)	307.3±22.49 (*N*=7)	<0.05*
U_{Creat} (mg/dL)	37.42±1.2 (*N*=19)	121.07±6.2 (*N*=7)	<0.0001***
$P_{Total\ cholesterol}$ (mg/dL)	212.4±22.25 (*N*=4)	134±25.25 (*N*=4)	<0.05*
$P_{LDL\text{-}cholesterol}$ (mg/dL)	180.4±27.1 (*N*=4)	88.83±28.41 (*N*=4)	<0.05*
$P_{HDL\text{-}cholesterol}$ (mg/dL)	24.86±4.74 (*N*=4)	38.17±3.15 (*N*=4)	<0.05*
$P_{Triglycerides}$ (mg/dL)	35±6.67 (*N*=4)	35.17±3.82 (*N*=4)	n.s.
P_{Na} (mEq/L)	138.3±13.3 (*N*=6)	141.5±12.75 (*N*=6)	n.s.
P_K (mEq/L)	4.62±0.3 (*N*=6)	4.5±0.4 (*N*=6)	n.s.
P_{Osm} (mOsm/kg)	305.7±11.8 (*N*=6)	304.3±21.3 (*N*=6)	n.s.
P_{Creat} (mg/dL)	0.13±0.05 (*N*=6)	0.13±0.01 (*N*=6)	n.s.
$P_{Copeptin}$ (pg/ml)	1.29±0.22 (*N*=8)	0.75±0.34 (*N*=6)	n.s.
GFR (μl/min)	354.8±15.2 (*N*=6)	383.04±17.3 (*N*=6)	n.s.

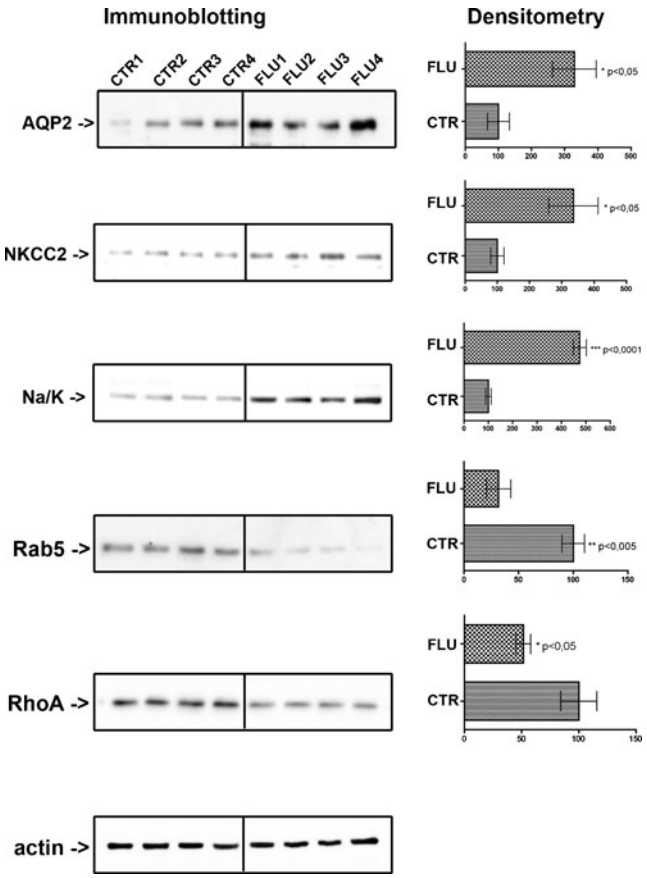

Fig. 3 Western blotting analysis of total kidney membrane fractions from C57BL/6 mice after prolonged treatment with fluvastatin. C57BL/6 mice were injected daily i.p. with either saline alone (CTR) or with fluvastatin (FLU, 50 mg/kg) over a period of 7 days. Total membrane fractions were isolated from whole kidneys and analyzed for the abundance of AQP2, NKCC2, Na/K-ATPase, Rab5, and RhoA. A summary of the Western blotting data, obtained by densitometric analysis of immunoreactive bands, is given in the *right panel*. Values represent mean band densities±SEM normalized for the actin content (*N*=4 per group)

compared to the increase observed in the kidney. The abundance of NKCC1, the NKCC isoform expressed in the liver, was not affected by fluvastatin treatment.

In the kidney, the increase in NKCC2 expression at the apical membrane of TAL of treated animals was also confirmed by immunofluorescence (see supplementary Fig. 2) using an antibody that recognizes the phosphorylated form of the protein [19]. Since phosphorylation is the main mechanism of activation of NKCC2 [19], the increased staining obtained with this antibody suggested an increased activity of NKCC2 in fluvastatin-treated mice. The abundance of aquaporin-4 at the basolateral membrane was not affected by fluvastatin treatment (supplementary Fig. 2).

The increased abundance of AQP2, on the apical membrane, likely results from the modulation of regulatory proteins known to regulate AQP2 trafficking. To address this point we also studied the expression levels of Rab5 and RhoA, two small GTP-binding proteins predicted to be involved in the regulation of AQP2 trafficking to and from the plasma membrane [11, 21, 61, 62]. Interestingly, the amount of Rab5 and RhoA associated with total renal membranes was significantly lower in fluvastatin-injected animals, as compared to the vehicle-injected ones (Fig. 3).

Reduced protein prenylation may explain the effect of fluvastatin on AQP2 trafficking in vivo and in vitro

By inhibiting the activity of HMG-CoA reductase, statins deplete cells of mevalonate. Enzymatic conversion of mevalonate yields the isoprenoid farnesyl pyrophosphate (FPP) that can be converted either to squalene and eventually to cholesterol, or to the isoprenoid geranylgeranyl pyrophosphate (GGPP). FPP and GGPP serve to tether important signaling proteins (e.g., Ras, Rho, Rac, and Rab)

to cell membranes via prenylation, thereby regulating their activity [33, 55]. To determine whether reduced prenylation was responsible for the observed reduction in RhoA and Rab5 abundance in kidney membranes prepared from fluvastatin-treated mice, we examined whether fluvastatin affected prenylation of members of the Rho family and Rab5 in cultured renal collecting duct cells (MCD4 cells; [48]). We treated MCD4 cells with fluvastatin (100 μM) for 12 h in culture medium in the absence or presence of either mevalonate (250 μM) or GGPP (3 μM). Simultaneous provision of mevalonate was expected to prevent any fluvastatin-dependent effect whether related to cholesterol depletion or to inhibition of protein isoprenylation. Instead, GGPP was used to specifically prevent fluvastatin effects due to inhibition of protein isoprenylation. In a different set of experiments, cells were also incubated for 12 h with perillyl alcohol (PA, 1 mM) or GGT1-298 (25 μM) in the culture medium. Untreated MCD4 cells served as control. At the end of the 12 h incubation period, cells were homogenized and total membranes were separated from the cytosol fractions by ultracentrifugation. Western blots were conducted on each fraction using antibodies against members of the Rho family of proteins (RhoA, RhoB, and CDC42) and Rab5 (Fig. 4a). Fractions were also probed with antibodies against Na/K-ATPase and GAPDH, markers of the membrane and cytosol compartments, respectively, in order to exclude cross-contamination between the two preparations.

As shown in Fig. 4a, fluvastatin treatment was associated with a significant reduction in the membrane expression of RhoA, RhoB, CDC42, and Rab5. This effect could be prevented by co-incubation of cells with mevalonate or GGPP. On the other hand, increased amounts of the four proteins could be detected in the cytosolic fraction of fluvastatin-treated cells (Fig. 4). Again, this effect was not observed when cells were co-incubated with fluvastatin and mevalonate or GGPP (Fig. 4).

In addition to decreased association to the membrane compartment, fluvastatin also perturbed the electrophoretic mobility of Rab5. As reported in Fig. 4, we observed that, after exposure of MCD4 cells to fluvastatin, a substantial amount of Rab5 was converted into the lower mobility, non-prenylated form that was only present in the cytosol. Consistent with previous reports showing that electrophoretic shift is a result of loss of protein isoprenylation [45], we observed that changes in electrophoretic protein mobility caused by statin treatment were reversed upon provision of exogenous mevalonate and GGPP demonstrating that these effects are dependent upon protein isoprenylation.

Therefore, we further assessed whether the geranylgeranyl transferase inhibitors PA and GGTI-289 would reproduce the effect obtained by fluvastatin on membrane-to-cytosol shift of RhoA and Rab5.

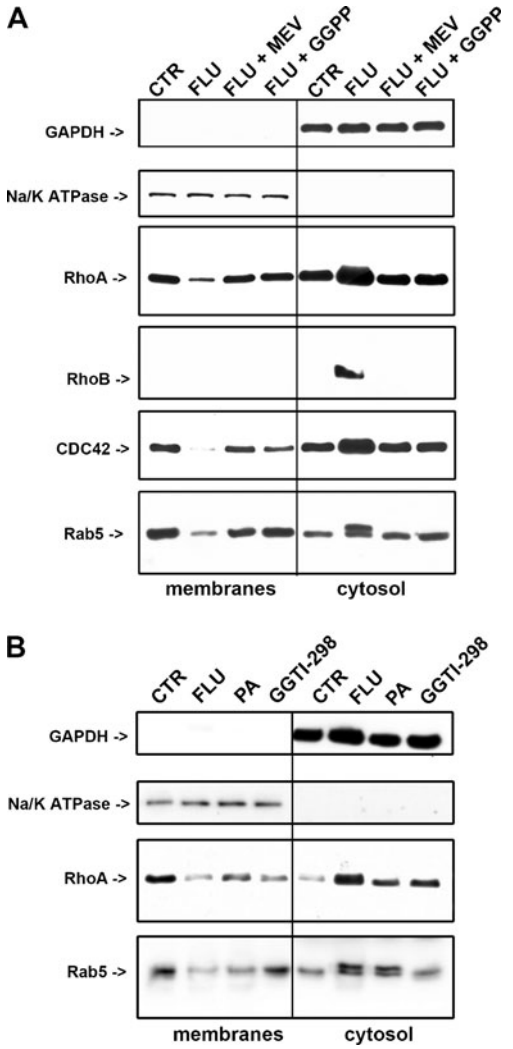

Fig. 4 Effect of treatment with fluvastatin or isoprenylation inhibitor on the membrane tethering of Rho and Rab proteins in renal MCD4 cells. **a** MCD4 cells were grown to confluence and treated overnight (16 h) with 100 μM fluvastatin, either alone (FLU) or in combination with either 250 μM mevalonate (MEV) or 3 μM geranylgeranyl pyrophosphate ammonium salt (GGPP). Untreated cells were used as control. Cells were homogenized and the post-nuclear fraction centrifuged at 110,000×*g* for 1 h to obtain the cytosol (supernatant) and membrane (pellet) fractions. Cytosolic or membrane proteins were separated by standard SDS-PAGE and analyzed by Western blotting with antibodies against GADPH, Na/K-ATPase, RhoA, RhoB, CDC42, and Rab5. Same results were obtained in three independent experiments. **b** MCD4 cells were treated with fluvastatin as described above or incubated for 12 h with either perillyl alcohol (1 mM) or GGTI-298 (25 μM). Membranes and cytosol were separated as described above; proteins were separated by standard SDS-PAGE and analyzed by Western blotting with antibodies against GADPH, Na/K-ATPase, RhoA, and Rab5. The same results were obtained in three independent experiments

In particular, PA was expected to inhibit the prenylation of both Rab and Rho [23], while GGTI-298 can only interfere with Rho prenylation [65].

Figure 4b shows that the amount of membrane-bound RhoA was reduced in the presence of both prenylation

inhibitors that also induced a parallel increase of soluble RhoA in the cytosol. As expected the amount of membrane-bound Rab5 was only affected by PA but not by GGTO-298.

Taken together, these findings strongly support the hypothesis that reduced membrane association of Rho proteins and Rab5 in fluvastatin-treated cells is a consequence of reduced prenylation of these proteins.

RhoA has been implicated in regulating AQP2 trafficking in renal cells by modulating the polymerization state of the cortical actin cytoskeleton [31, 62]. In particular, it has been shown that RhoA inhibition induces partial actin depolymerization, thus facilitating AQP2 accumulation at the apical plasma membrane of renal cells in vitro [31, 62]. On the basis of these findings, we used MCD4 cells to investigate the effect of fluvastatin on both AQP2 localization and the polymerization status of the actin cytoskeleton. Cells were either stimulated with forskolin (20 μM) for 30 min to achieve maximal AQP2 membrane expression, or treated with fluvastatin (100 μM) for 12 h in the presence or absence of mevalonate (250 μM) or GGPP (3 μM). In a similar fashion, MCD4 cells were also treated with perillyl alcohol, a nonselective inhibitor of geranylgeranyl transferases type I and II [23].

The effect of each treatment on AQP2 subcellular localization was visualized by confocal immunofluorescence (Fig. 5a, green staining). The actin cytoskeleton was visualized by incubation with phalloidin-conjugated Alexafluor™-555 (Fig. 5a, red color, inset). In parallel, changes in cell water permeability were measured with the Flexa station (Fig. 5b).

As expected, forskolin stimulation, a classical maneuver that increases intracellular cAMP levels and promotes AQP2 exocytosis, increased AQP2 abundance at the apical plasma membrane (Fig. 5a) and led to a ~2.5-fold stimulation of water permeability (Fig. 5b). Under these experimental conditions, RhoA is partially inhibited through P*K*A phosphorylation, associated with a partial depolymerization of the actin cytoskeleton [31, 62]. Similarly, fluvastatin promoted AQP2 accumulation at the apical membrane and increased water permeability in MCD4 cells (Fig. 5a, b). Interestingly, fluvastatin treatment also resulted in a partial depolymerization of the actin cytoskeleton (Fig. 5a, inset). These fluvastatin-induced changes were not observed when MCD4 cells were co-incubated with fluvastatin and either mevalonate or GGPP (Fig. 5).

In agreement with these findings, perillyl alcohol, a nonselective inhibitor of protein prenylation [23], mimicked the effects of fluvastatin in MCD4 cells (Fig. 5).

Our findings support the concept that reduced prenylation of RhoA leads to partial actin depolymerization, thus facilitating AQP2 accumulation at the apical plasma membrane. To further test this concept, we measured the specific activity of RhoA in fluvastatin-treated MCD4 cells via fluorescence resonance energy transfer (FRET) using a probe consisting of a Rho-binding domain (RBD) of Rhotekin sandwiched by YFP and CFP [66]. In this system, the binding of endogenous GTP-RhoA to RBD in the probe is expected to displace YFP and CFP, thereby decreasing FRET efficiency. The results of the FRET experiments are summarized in Fig. 5c. Similarly to forskolin, incubation with fluvastatin significantly increases NFRET signal, consistent with a decrease in the activity of RhoA (Fig.5c). In line, perillyl alcohol also reduced Rho activity, thus reproducing the effect of fluvastatin (Fig.5c). Moreover, compared with control condition, NFRET values did not change when cells were treated with fluvastatin in the presence of either mevalonate or GGPP (Fig.5c)

As described above, fluvastatin treatment led to a reduced membrane accumulation of Rab5 in vivo (Fig. 3) and in vitro (Fig. 4). To examine whether this effect contributed to enhance AQP2 expression at the apical plasma membrane in renal cells, we greatly reduced Rab5a expression in MCD4 cells by treating cells with Rab5 siRNA (Fig. 6a).

We then examined the effect of Rab5a knockdown on the intracellular localization of AQP2 by immunolocalization followed by confocal analysis in the *xy* and *xz* planes. In control cells transfected with scrambled siRNA, AQP2 staining was localized to small vesicles scattered in the perinuclear and subapical cytoplasm (Fig. 6b). On the other hand, in cells transfected with Rab5 siRNA, AQP2 staining was observed exclusively at the apical plasma membrane (Fig. 6b).

Discussion

We have previously reported that the cholesterol-lowering drug lovastatin promoted the accumulation of the water channel AQP2 at the apical plasma of renal MCD4 cells in vitro [49]. In this study, we provide the novel evidence in vivo that fluvastatin, another drug of the statin family, elicits the same effect in mouse kidney collecting ducts in a vasopressin-independent fashion resulting in water retention. Currently, six different statins (simvastatin, pravastatin, lovastatin, fluvastatin, atorvastatin, and rosuvastatin) are approved for the treatment of hypercholesterolemia in humans. Several large trials demonstrated that statins are not only safe and well tolerated but also significantly decrease cardiovascular morbidity and mortality in hypercholesterolemic patients [1, 2, 52, 56]. All statins block the conversion of HMG-CoA to mevalonic acid with consecutive attenuation of the biosynthesis of cholesterol [22].

However, additional data indicate that statins exert additional effects that are independent of their plasma

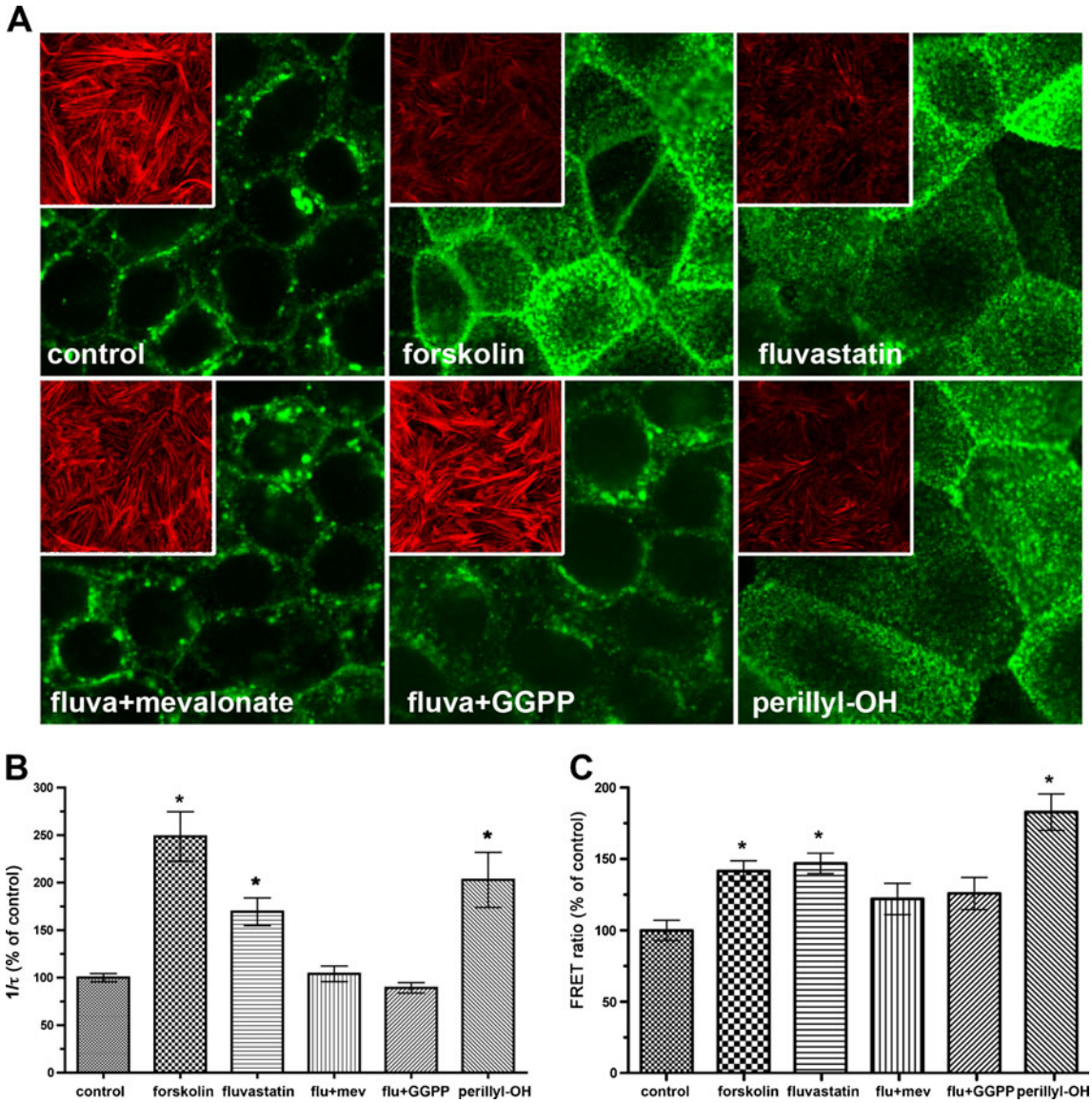

Fig. 5 Effect of fluvastatin treatment on RhoA inhibition, actin cytoskeleton remodeling, and AQP2 expression at the apical membrane in MCD4 renal cells. Cells were grown to confluence on glass coverslips and treated overnight (16 h) with 100 μM fluvastatin, either alone or in combination with 250 μM mevalonate or 3 μM geranylgeranyl pyrophosphate (GGPP), or with 1 mM perillyl alcohol. In the same experiment, cells were left untreated (control) or stimulated with 100 μM forskolin for 30 min. **a** Cells were fixed in PFA and stained with an AQP2 antibody (*green*) or phalloidin Alexafluor-555 to visualize F-actin (*red*, *inset*). Confocal pictures were taken for each experimental condition. **b** Cells were seeded in 96-well black, clear bottom microplates, treated as above and water permeability assays were carried out using a microfluorimetric assay (see the "Methods" section for details). The time constant of cell swelling induced by a hypotonic stimulus was obtained by fitting the tangent line to the first part of curve with a linear regression. The data were expressed as $1/\tau$ (s^{-1}). Values are expressed as mean±SEM; N=48. **c** Evaluation of RhoA activity by FRET analysis: Cells were treated as previously described (see above). Histogram compares changes of NFRET values between untreated cells (N=94), forskolin (N=75), fluvastatin (N=99), fluvastatin in the presence of mevalonate (N=48), fluvastatin in presence of GGPP (N=38), and perillyl alcohol (N=49). Values are expressed as mean±SEM (*P<0.05)

cholesterol-lowering properties [8]. Many of these so-called pleiotropic effects have been shown to be secondary to the inhibition of the synthesis of isoprenoid intermediates of the mevalonate pathway including FPP and GGPP [8].

Isoprenoids are important substrates for the post-translational modification of many signaling proteins including small GTP-binding proteins. These latter proteins play crucial roles in many cellular functions including cytoskeletal assembly and protein and lipid trafficking [60].

In fluvastatin-treated mice, the amount of apical AQP2 was greatly increased compared to controls. This is likely responsible for the reduced urine volume and the increased

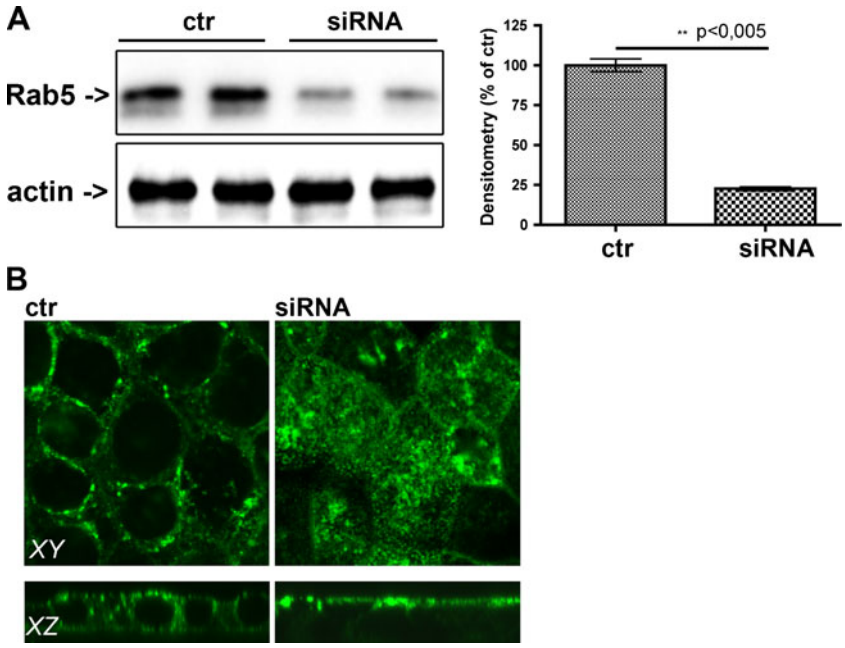

Fig. 6 Effect of Rab5 siRNA on AQP2 subcellular localization in MCD4 cells. Rab5 silencing was obtained using a custom Stealth siRNA from Invitrogen. Cells were treated with either scrambled control siRNA (ctr) or Rab5-specific siRNA (200 nM each). **a** Cells were lysed and immunoblotting was performed on total proteins using an anti-Rab5 antibody. Band intensities were normalized for actin abundance and reported as means±SEM. **b** AQP2 was visualized by immunofluorescence and confocal scanning in the XY and XZ plans

urine osmolality observed in treated mice. Reduced diuresis was not a secondary effect of reduced GFR that was unchanged in treated animals (Table 1). Moreover, measurements of plasma copeptin, a marker of endogenous vasopressin levels [37, 38], indicated that in statin-treated animals AQP2 accumulation at the apical membrane occurred through a vasopressin-independent mechanism (Table 1). Beside the accumulation of AQP2 at the apical membrane, also Na transporters (Na/K-ATPase and NKCC2) were upregulated in the kidney following fluvastatin treatment. In fluvastatin-treated animals the upregulation of Na transporters likely contributed to increase Na reabsorption that, in turn, provided the driving force for water reabsorption. Consistent with this interpretation, we observed that urinary Na concentration was significantly reduced in treated animals (Table 1). However, plasma Na concentration and osmolality were unchanged in treated mice probably as a consequence of the concomitant water retention.

The overall effect of statins on AQP2 membrane expression may be due to both increased exocytosis and decreased endocytosis of AQP2. We recently demonstrated that the constitutive endocytosis of AQP2 is attenuated in lovastatin-treated MCD4 cells [49]. However, we cannot exclude the possibility that statins also stimulate, to some extent, the constitutive exocytosis of AQP2. It is also possible that statin treatment affects the activity of other cellular proteins involved in regulating AQP2 trafficking. Clearly, these questions need to be addressed in future studies.

We also demonstrated that short-term (7 days) treatment of mice with fluvastatin enhanced the total abundance of AQP2 and the NKCC2 and Na/K-ATPase sodium transporters. NKCC2-mediated NaCl reabsorption in the TAL is crucial to generate a hypertonic medullary interstitium that provides the driving force for water reabsorption in the collecting duct. In analogy with AQP2, NKCC2 trafficking to the plasma membrane is regulated acutely by vasopressin [20] and is also subjected to a constitutive recycling mechanism [3]. Moreover, both Na/K-ATPase and NKCC2 are endocytosed at the plasma membrane through a CCV-mediated process [3, 28]. It is therefore possible that fluvastatin, by inhibiting the activity of cellular proteins that stimulate the endocytosis of Na/K-ATPase and NKCC2, promotes the plasma membrane accumulation of these two transporter proteins and, by reducing lysosomal degradation, would increase the total protein abundance. As discussed above, fluvastatin was highly effective in increasing water reabsorption in the kidney in mice. It is likely that this effect is due to the ability of fluvastatin to increase the membrane expression of both NKCC2 in the TAL and AQP2 in the collecting duct.

Under our experimental conditions, fluvastatin was the only statin that promoted a clear membrane accumulation of AQP2 in vivo. However, when administered to cultured MCD4 cells, all four statins tested induced comparable effects on protein prenylation and AQP2 membrane expression (data not shown). It is likely that differences in pharmacokinetic parameters including tissue distribution [36] are responsible for the discrepancy between the statin effects observed in vivo and in vitro.

In this study, we used fluvastatin doses that are commonly used in rat/murine studies [18, 40, 58]. However, due to the rapid upregulation of HMG-CoA

reductase during statin treatment in rodents [30], these doses are higher than those used in humans. The doses used in this work are not therefore predictive of those needed in humans to achieve the same result. A recent study examined the possible effect of atorvastatin administration on renal sodium and water handling in healthy volunteers [47]. Besides causing a modest and transitory decrease in fractional excretion of sodium, atorvastatin treatment had no significant effect on renal function. However, as already discussed, this finding does not exclude the possibility that statins endowed with different pharmacokinetic properties may affect kidney function in a fashion that could be exploited therapeutically. It is also possible that statins must be administered at higher doses to obtain the desired renal effects. The minimum effective dose of statins able of increasing AQP2 trafficking to the membrane in humans has to be determined. Since no specific pharmacological therapy exists for the treatment of XNDI at present, these observations are of potential relevant clinical interest.

In this work, we also identified intracellular pathways predicted to explain the fluvastatin-induced increases in the abundance of renal Na transporters and AQP2 at the plasma membrane in vivo.

Here we provide evidence that fluvastatin, by depleting the intracellular pool of GGPP, reduces the isoprenylation of members of the Rho and Rab families, resulting in decreased membrane tethering and reduced activity of these proteins. This effect was observed both in vivo and in vitro. Consistent with this interpretation of the data, membrane tethering was also observed upon preincubation of renal cells with specific inhibitors of geranylgeranyl transferase enzymes (GGTase). Two distinct GGTase have been identified in eukaryotic cells: protein GGTase type I (PGGT), and Rab GGTase (RGGT) [12]. Rho and Rab are prenylated by PGGT and RGGT, respectively. Specific inhibitors are available for both GGTases such as perillyl alcohol that nonselectively inhibits both classes [23] and GGTI-298 that is selective for PGGT [65].

In addition to reduced membrane tethering, we observed that both fluvastatin and perillyl alcohol affected the electrophoretic mobility of Rab5. It has been previously reported that Ras and Rab [27, 34, 45, 46], but not the Rho family proteins [16] exhibit altered electrophoretic mobility depending upon protein prenylation status. The statin-dependent change in electrophoretic mobility of these protein families likely reflects the fact that Rab family proteins have two geranylgeranyl moieties added to them, whereas Rho family proteins possess only one lipid moiety.

We observed in cultured renal cells that the fluvastatin-induced accumulation of AQP2 at the apical membrane was prevented by co-incubation with GGPP. This fluvastatin effect was mimicked by perillyl alcohol [23], a nonselective inhibitor of isoprenylation. Taken together, these findings strongly support the view that the stimulatory effect of fluvastatin on AQP2 membrane expression depends on reduced intracellular levels of GGPP, leading to reduced prenylation of important signaling molecules involved in AQP2 trafficking.

AQP2 membrane expression in renal cells is acutely regulated by AVP that promotes the short-term exocytosis of subapical storage vesicles toward the apical membrane [10, 53, 54, 64]. However, AQP2 recycles constitutively between the intracellular vesicles and the plasma membrane, even in the absence of AVP [9], suggesting that steady-state membrane accumulation of AQP2 depends on the balance between endocytosis and exocytosis processes.

In this context, RhoA and Rab5, both prenylated proteins, have been implicated in regulating AQP2 membrane expression [4, 31, 61, 62]. RhoA regulates the polymerization state of cortical actin cytoskeleton [31, 61, 62], a potential physical barrier to the spontaneous fusion of AQP2-bearing vesicles with the plasma membrane. It has been demonstrated that RhoA inhibition is part of the physiological cascade of events initiated in renal cells by vasopressin stimulation and that inhibition of RhoA promotes AQP2 accumulation at the plasma membrane in renal cells [31, 62]. The inhibition of RhoA in fluvastatin-treated cells is accompanied by actin depolymerization and AQP2 accumulation at the apical plasma membrane. We show here that the fluvastatin-dependent inhibition of RhoA is a consequence of reduced isoprenylation since it is prevented by mevalonate and GGPP and mimicked by PA. Rab5 regulates clathrin-coated vesicles (CCV)-mediated endocytosis at the plasma membrane in epithelial cells [11, 67] and may play a role in CCV-dependent AQP2 endocytosis [59]. In this study we demonstrated, for the first time, that Rab5 knockdown affects AQP2 endocytosis, thus promoting AQP2 accumulation at the plasma membrane.

In conclusion, this proof of concept study illustrates the potential of statins (fluvastatin) to modulate the expression/activity of water and solute transporters in the kidney, and their potential usefulness for the treatment of XNDI. It should be of particular interest to investigate whether fluvastatin is also effective in mice models of XNDI [35] and in patients suffering from XNDI.

Methods

Cell culture, drug treatments, and gene silencing

Mouse cortical collecting duct MCD4 cells stably expressing human AQP2 were generated as described elsewhere [26] and maintained in DMEM/F12 1:1 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin, and 5 μM dexamethasone. Cells

were grown to confluence and were treated overnight (16 h) with 1 mM perillyl alcohol (Sigma, http://www.sigmaaldrich.com) or with 100 μM fluvastatin Na (http://www.seqchem.com/), either alone or in combination with either 250 μM mevalonate (Sigma) or 3 μM GGPP ammonium salt (Sigma). GGTI-298 (Sigma) was used at the concentration of 25 μM. In all experiments, MCD4 cells were treated with 50 μM indomethacin (Sigma) overnight in the culture medium to reduce basal cAMP levels. To stimulate cAMP production, MCD4 cells were incubated with 100 μM forskolin (Sigma) for 20 min.

To reduce Rab5 expression, we used a custom Stealth siRNA from Invitrogen (www.invitrogen.com). MCD4 cells were cultured in 12-well-cell permeable inserts until 60% confluence and treated twice on two consecutive days with 200 nM stealth control (scrambled) or Rab5-specific siRNA using Lipofectamine 2000 (Invitrogen). Cells were lysed 24 h later for immunoblotting analysis or AQP2 localization assays.

Animals

All animal experiments carried out were approved by the Institutional Committee on Research Animal Care, in accordance with the Italian Institute of Health Guide for the Care and Use of Laboratory Animals.

Mice were maintained on a 12-h light/12-h dark cycle, with free access to water and food. Wt C57BL/6 mice (Harlan Laboratories, males, 8 weeks old) were injected intraperitoneally with a single dose (150 μl) of lovastatin (20 mg/kg; Sigma), cerivastatin (20 mg/kg; Sequoia Research, UK), rosuvastatin (50 mg/kg; Sequoia Research, UK), and fluvastatin (50 mg/kg; Sequoia Research, UK). Control mice received the same volume of vehicle alone (saline). The animals were euthanized by rapid cervical dislocation 6 h after statins treatment. In parallel, another group of mice was injected twice with DDAVP (1 μg/kg; Sigma) at time 0 and again at 30 min. The animals were sacrificed 30 min after the second injection.

To study the effect of short-term fluvastatin treatment, Wt C57BL/6 mice (four for each experimental group) were acclimated in rat metabolic cages adapted for mice (Techniplast) for 2 days before starting the experiment. Preweighted drinking water was provided ad libitum during the course of the studies, and urine was collected under mineral oil. Animals were injected daily i.p. with either saline or with fluvastatin (50 mg/kg) over a period of 7 days.

Urine output and osmolality, along with water consumption, were monitored daily. Urine osmolalities were measured using a vapor pressure osmometer (model 5520; Wescor Inc.; www.wescor.com).

Mice were anesthetized and killed by drawing blood from vena cava after fluvastatin treatment for 7 days. Plasma was separated from blood cells by centrifuging blood at 1,500×*g* for 10 min in a bench-top microcentrifuge. Sterol measurements (total-, LDL-, and HDL-cholesterol and triglycerides) were performed using an automated Beckman-Coulter chemistry analyzer AU2700®. Urinary and plasma creatinine were measured by HPLC [68]. The HPLC system consisted of a JASCO PU-2089 Plus quaternary solvent pump (JASCO Corporation, Tokyo, Japan), equipped with a Rheodyne 7525 six-port injection valve—20 l sample loop—(Rheodyne, Rohnert Park, CA, USA), a Supelcosil LC-18 (250×4.6 mm) 5 m packing column (Supelco, Bellefonte, PA, USA), and a Jasco UV-2075 Plus UV–Vis detector operating at 235 nm. A precolumn (4×2 mm; Phenomenex Inc., Torrance, CA, USA) was used to eliminate the need for sample deproteinization. All separations were performed under isocratic conditions using a mobile phase composed of 30 mM CH_3COONH_4 and methanol (94/6, *v*/*v*). Plasma and urine samples were diluted with mobile phase (1+1, and 1+99 *v*/*v*, respectively) and 20 μl directly injected.

Urinary and plasma electrolytes were measured with the ion selective electrode (ISE) method. Copeptin plasma levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kits (Uscn Life Science Inc. Wuhan, China).

Cell and tissue fractionation

MCD4 cells grown in 60-mm dishes were washed twice with ice-cold phosphate buffer saline (PBS)–calcium–magnesium (CM) and scraped into 500 μl of relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 10 mM PIPES, 1 mM PMSF, 10 μM leupeptin, 1 μg/ml pepstatin, 10 mM NaF, 1 mM sodium orthovanadate, and 15 mM tetrasodium pyrophosphate, pH 7.3). The kidneys of control and fluvastatin-treated mice were homogenized with ten strokes in a Potter-Elvehjem homogenizer at 1,250 rpm in 2 ml of the same buffer. The cells and tissue homogenates were then sonicated at 4°C using a microprobe sonicator. Unbroken cells and nuclei were pelleted by centrifugation at 500×*g* for 5 min at 4°C. The supernatants were centrifuged at 110,000×*g* for 1 h at 4°C in a Beckman-Coulter ultracentrifuge (TLA120.1 or 70Ti rotor). The high-speed supernatants represented the cytosolic fractions, while the membrane pellets were resuspended in relaxation buffer. The protein concentration of each fraction was measured using the Quant-iT™ protein assay from Invitrogen. Cytosolic or membrane proteins (20 μg) were separated by standard SDS-PAGE and analyzed by Western blotting.

Immunofluorescence

MCD4 cells cultured on permeable inserts were fixed with 4% paraformaldehyde (PFA) for 15 min and then permea-

bilized with 0.1% NP-40 in PBS for 5 min. Nonspecific binding sites were blocked with 1% BSA in PBS (saturation buffer) for 30 min at RT. Cells were then incubated with affinity-purified antibody against AQP2 (1:1,000) [63] for 1 h at 37°C in saturation buffer. After washing in PBS, cells were incubated for 1 h with AlexaFluor™ 488-conjugated donkey anti-rabbit (Invitrogen, 1:1,000) and with Phalloidin TRITC (Sigma, 1:250). Monolayers were washed, mounted in PBS/glycerol (1:1) containing 1% *n*-propylgallate, pH 8.0, and confocal images were obtained with a confocal laser-scanning fluorescence microscope (Leica TSC-SP2).

Mouse kidneys were fixed overnight with 4% PFA at 4°C, cryopreserved in 30% sucrose for 24 h, and then embedded in optimal cutting temperature (OCT) medium. Ultra-thin sections (4 μm) placed on Superfrost/Plus Microscope Slides (Thermo Scientific, Germany) were subjected to immunofluorescence analysis as described above.

Gel electrophoresis and Immunoblotting

Protein samples, diluted in Laemmli's buffer with 50 mM DTT, were heated for 10 min at 70°C, and then resolved on 12% SDS polyacrylamide gels and electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA, USA) for Western blot analysis.

After blocking with 3% BSA in TBS-T, blots were incubated overnight at 4°C with the indicated primary antibodies. Membranes were washed and incubated with HRP-conjugated secondary antibodies. Reactive proteins were revealed with an enhanced chemiluminescent detection system (SuperSignal West Pico Chemiluminescent Substrate, Pierce) and visualized on a ChemiDoc XRS system (Biorad). The quantification of protein bands was performed by determination of the relative optical density using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Antibodies and their corresponding dilutions were: mouse anti-Rab5 (1:500, Santa Cruz Biotechnology), mouse anti-RhoA (1:500, Santa Cruz Biotechnology), mouse anti-RhoB (1:500, Santa Cruz Biotechnology), rabbit anti-CDC42 (1:500, Santa Cruz Biotechnology), mouse anti-Na^+/K^+ ATPase (1:20,000, Millipore, Billerica, MA, USA), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000, Millipore, Billerica, MA, USA), goat anti-actin (1:500, Santa Cruz Biotechnology), and mouse anti-NKCC2 (1:1,000, Developmental Studies Hybridoma Bank, University of Iowa).

Fluorescence-quenching assays

MCD4 cells were seeded in 96-well black, clear bottom microplates (Corning-Costar Corp., Corning, New York) and grown to 90% confluence. Cells were treated overnight with the drugs described above. Water permeability assays were carried out using a bench-top fluorescence plate reader with integrated liquid handling (FlexStation II, Molecular Devices, MDS Analytical Technologies, USA). Cells were incubated at 37°C for 60 min with PBS containing 10 μM calcein ester (Molecular Probes, Eugene, OR) in the presence of drugs. At the end of incubation, calcein was washed out and 50 μl of isosmotic PBS-CM was added to each well. The microplate was transferred into the plate reader for fluorescence measurements. The time course of fluorescence was recorded continuously for 15 s (baseline), then for 35 s after rapid automated addition of 50 μl of distilled water (hyposmotic shock) and for 40 s after automated addition of 15 μl of 1 M mannitol to restore isosmotic conditions. Measurements were taken every 0.5 s. The fluorescence assay was carried out at 18°C. Data acquisition was performed by SoftMax Pro software, and the data were analyzed with Prism (Graphpad) software.

The time constant of cell swelling induced by the hyposmotic stimulus was obtained by fitting the tangent line to the first part of curve with a linear regression. The data were expressed as $1/\tau$ (s^{-1}). Values were expressed as % of control.

Fluorescence resonance energy transfer measurements

FRET experiments were performed as described [24, 63]. Briefly, MCD4 cells were transiently transfected with the Raichu-RBD construct previously described by Yoshizaki et al. [66] (kindly provided by Prof. Matsuda, Osaka University, Japan). ECFP and EYFP were excited at 430 or 480 nm, respectively; fluorescence emitted from ECFP and EYFP was measured at 480/30 and 545/35 nm, respectively. FRET from ECFP to EYFP was determined by excitation of ECFP and measurement of fluorescence emitted from EYFP. Corrected nFRET values were determined according to Ritter [51].

Statistics

Values are represented as average±SEM. Students' two-tailed *t* test analysis was used to evaluate differences between groups. $P<0.05$ was considered statistically significant.

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