

Fluvastatin Increases AQP2 Urine Excretion in a Dyslipidemic Patient with Nephrogenic Diabetes Insipidus: An *In Vivo* and *In Vitro* Study

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Abstract

Objective: Among the pleiotropic effects of statins, we have previously reported that fluvastatin increases the amount of plasma membrane-expressed AQP2 in renal collecting duct cells both *in vitro* and *in vivo*, independently of vasopressin. This effect may be of potential clinical significance for the treatment of patients affected by nephrogenic diabetes insipidus forms caused by inactivating mutations of the vasopressin type 2 receptor. Here we report the effect of fluvastatin on AQP2 plasma membrane abundance on an adult male XNDI patient treated with statins.

Methods: An adult male NDI patient, carrying an inactivating mutation of the V2R, under conventional treatment to reduce polyuria, was also treated with fluvastatin because of high levels of blood cholesterol. AQP2 plasma membrane expression in the kidney was monitored by measuring urinary excreted AQP2 (u-AQP2), before starting fluvastatin treatment and during a three months follow-up period. The effect of fluvastatin was also tested *in vitro* in mouse kidney cortical collecting duct MCD4 cells.

Results: u-AQP2 increased in a time- and dose-dependent manner after treatment with 40 and 80 mg/day of fluvastatin for 90 days. However, at this drug dosage, increased uAQP2 was not accompanied by reduction of diuresis and increase of urine osmolality. The effect of fluvastatin on AQP2 excretion was confirmed *in vitro* in cultured renal cells.

Conclusions: We first demonstrate that the use of fluvastatin increased AQP2 plasma membrane expression in an NDI dyslipidemic patient. This observation was confirmed by *in vitro* studies using mouse cultured renal cells treated with fluvastatin. Although a clinical relevant effect of fluvastatin on total diuresis and urine osmolality was not observed at the used dosages, these results suggest further investigation on the possible role of HMG CoA reductase inhibitors to improve the efficacy of the current NDI therapy.

Introduction

The water-conserving effect of the antidiuretic hormone Arginine Vasopressin (AVP) is mediated by its interaction with the type 2 Vasopressin Receptor (V2R), a class of G protein-coupled receptors localized at the basolateral membrane of the principal cell of the kidney collecting duct. Binding of AVP to V2R triggers the cAMP signal cascade and promotes the exocytosis of storage vesicles containing the water channel aquaporin-2 (AQP2) to the apical membrane of the kidney collecting duct principal cells [1-4].

Congenital Nephrogenic Diabetes Insipidus (NDI) is a rare disease caused by mutations of either the V2R or AQP2 genes, characterized by decreased ability of the kidney to concentrate urine in response to AVP which results in severe polyuria and polydipsia [5-10]. Mutations in the V2R gene, mapping on the X-chromosome, are causative of the X-linked NDI (XNDI) accounting for 90% of all diagnosed congenital NDI cases [11,12]. Affected males are unable to concentrate urine even after administration of exogenous AVP, whereas some heterozygous females, due to skewed X-chromosome inactivation, show variable degrees of symptoms [11,13,14].

Limited therapeutic options are available to treat this condition. Since exogenous dDAVP is not effective, other than ensuring adequate water intake, urine volume may be partially decreased with the use of hydrochlorothiazide diuretics that, by increasing upstream salt and water reabsorption in the proximal tubule, reduce the urine load to the distal nephron where water reabsorption via AQP2 is impaired [10]. Combination of thiazide with the cyclooxygenase inhibitor indomethacin or with amiloride may also have an effect on reducing polyuria [6,7,10,15,16]. The above treatment may decrease urine volumes to 4-12 liters per day, which is still a large volume to void.

Many strategies have been proposed that could bypass the defective V2R signaling and restore physiological AQP2 expression and/or

trafficking [7,10,17]. These include activation of the intracellular cGMP pathway; activation of the intracellular cAMP pathway by stimulation of the Gas-coupled calcitonin or secretin receptors; activation of E-prostanoid receptors EP2 and EP4 [18-24].

Among the plethora of candidate molecules that could improve the effect of the current therapy for XNDI, we demonstrated that statins induce an AVP-independent accumulation of AQP2 at the plasma membrane of collecting duct cells *in vitro* and *in vivo* in both wild type and XNDI mice, greatly improving kidney concentrating ability [23,25-27].

This effect on AQP2 trafficking has not been observed in humans and, in particular, on NDI patients. Here we report, for the first time, the effect of fluvastatin on AQP2 plasma membrane abundance on an adult male XNDI patient treated with statins because of high levels of blood cholesterol. AQP2 membrane expression in the kidney was monitored by measuring urinary excretion of AQP2 (u-AQP2), before starting fluvastatin treatment and for three months during treatment. u-AQP2 increased in a time- and dose-dependent manner after treatment, suggesting that the possible use of statins to improve

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the efficacy of the current NDI therapy is worth further investigation. Using an *in vitro* model of mouse cultured renal cells; we confirmed that the increased plasma membrane expression of AQP2 induced by fluvastatin is paralleled by a concomitant increase of AQP2 excretion in the culture medium.

Materials and Methods

Study protocol

A 48 year old man with mild intellectual disability was admitted to the hospital for evaluation of polydipsia and polyuria, obesity and diabetes mellitus. His weight was 100.5 kg, height 160 cm, BMI 38.5 kg/m²; his blood pressure was 140/85 mmHg. He drank approximately 5 liters of water per day; urine volume was approximately 5 liters. His general clinical condition was good.

At the age of 5 he was diagnosed with non-obstructive hydronephrosis and secondary bilateral mega ureter. Water restriction test and dDAVP test revealed the presence of AVP-resistant NDI. Genetic screening revealed an AVP receptor 2 (V2R) missense mutations at nucleotide c.926T>C responsible for an amino acid substitution Leu309Pro already described and responsible for XNDI [28].

He was treated with hydrochlorothiazide and amiloride with significant diuresis contraction. The current pharmacological treatment was hydrochlorothiazide 50 mg/die and amiloride 5 mg/die. He was previously diagnosed with diabetes mellitus and treated with metformin 1000 mg twice a day. Exenatide 10 µg twice a day s.c. was recently added. Metabolic control was good, no chronic diabetes complications were found. HBA1c was 5.8%, with no glycosuria.

Other laboratory investigations revealed: sodium 142 mmol/L, chloride 101 mmol/L, urine osmolality 187 mosm/kg, urine S.G. 1004. Thyroid function was normal. Total cholesterol was 262 mg/dl (normal range 110-200 mg/dl), HDL cholesterol was 33 mg/dl (35-110), triglycerides were 202 mg/dl (50-150).

Cardiovascular risk factors were estimated and the patient was considered eligible for statin therapy. Fluvastatin was chosen because of our previous report showing an effect of this drug on AQP2 plasma membrane expression in the hope that this statin could also ameliorate NDI symptoms [23,27]. Informed consent was obtained prior to entering the subject into this study. The local Ethics Committee approved this observational clinical study.

Fluvastatin dosing and urine sampling

The patient was treated with fluvastatin 40 mg/die for 30 days and then the dose was increased to 80 mg/die for the next two months. Urine samples, (24 h collection) were collected during three nonconsecutive days before initiation of the fluvastatin treatment (T0), 15 and 30 days after administration of 40 mg/die fluvastatin (T1 and T2) and 30 and 60 days after administration of fluvastatin 80 mg/die (T3 and T4). Diuresis, electrolytes, creatinine and osmolality were measured. Urine osmolality were measured using a vapor pressure osmometer (Vapor 5520; www.wescor.com). Aliquots of each urine sample were kept at -80°C until used for determination of AQP2 excretion. u-AQP2 was analyzed at each time point and values obtained at T0 considered as the baseline.

Purification of urine exosomes

A urine volume corresponding to 1 mg of urine creatinine equivalents was mixed with protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 µM leupeptin, 1 µg/ml pepstatin) and centrifuged at

3,000xg at 4°C to remove urine sediment including whole cells, large membrane fragments, and other debris. Nanomembrane concentrators Vivaspin 2, 100 kDa Molecular Weight Cut-Off (MWCO) (<http://www.generon.co.uk>) were used. Urine was added to the nanomembrane concentrator and centrifuged at 5,000xg at 4°C until the final volume obtained was approximately 20 µl. The retentate was solubilized in 1 volume of pre-heated (95°C) Laemmli's buffer with 400 mM DTT and proteins separated by SDS-PAGE.

Cell culture and drug treatments

Mouse cortical collecting duct MCD4 cells, stably expressing human AQP2, were generated as described elsewhere and maintained in DMEM/F12 1:1 supplemented with 5% cholesterol-depleted Fetal Bovine Serum (FBS) [29]. Cholesterol depletion was obtained by centrifuging the FBS at 300,000xg for 24 hours at 4°C in a Beckman-Coulter ultracentrifuge (70Ti rotor).

Cells were grown to confluence in 150 cm² cell culture Petri dishes or on glass coverslips and treated overnight (16 h) with 50 µM indomethacin (www.sigmaaldrich.com) to reduce basal cAMP levels, alone (Ctr) or in combination with either 100 µM forskolin (www.sigmaaldrich.com) to stimulate cAMP production (FK), or with 5 µM fluvastatin Na (<http://www.seqchem.com>) to inhibit the mevalonate pathway (Flu).

Purification of exosomes from cell culture medium

Twenty-five ml of cell culture medium from each experimental condition of MCD4 cells were harvested and centrifuged at 17,000xg for 30 min at 4°C to eliminate dead cells and cellular debris. The supernatants were then ultracentrifuged at 200,000xg for 1 h at 4°C.

The pellets were washed in PBS to eliminate contaminants and resuspended in RIPA buffer. Protein concentration was determined and 5 µg of total proteins were solubilized in Laemmli's buffer with 100 mM DTT, denatured at 95°C and separated by standard SDS-PAGE for Western blotting analysis.

Immunofluorescence

MCD4 cells, cultured on glass coverslips, were fixed with 4% Paraformaldehyde (PFA) for 15 min and then permeabilized with 0.1% Nonidet P-40 (www.sigmaaldrich.com) 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) for 1 h and with AlexaFluor™ 488-conjugated donkey anti-rabbit (Invitrogen, 1:1,000) for 1 h. Monolayers were washed, inverted on a drop of mounting medium and analysis was performed under a confocal laser-scanning fluorescence microscope (Leica TSC-SP2; www.leica.com).

Immunoblotting

Protein samples from urine and cell culture medium, solubilized in Laemmli's buffer, were resolved on 12% SDS polyacrylamide gels and electrophoretically transferred to PVDF membranes (<http://www.millipore.com>) for Western blotting analysis.

After blocking with 3% Bovine Serum Albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), membranes were incubated overnight at 4°C with primary antibodies anti-AQP2 1:1000, and anti-Flotillin-2 1:500 (<http://www.scbt.com>) [29,30]. Membranes were washed in TBS-T and incubated with HRP-conjugated secondary antibodies (<http://www.scbt.com>) for 1 hour at room temperature. Reactive proteins were revealed with an enhanced chemiluminescent

detection system (SuperSignal West Femto Chemiluminescent Substrate, <http://www.piercenet.com>) and visualized on a ChemiDoc XRS system (<http://www.bio-rad.com>). The quantification of protein bands was performed by determination of the relative optical density using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For exosomes isolated from cell culture media, the relative band density of AQP2 was normalized with that of Flotilin-2 band.

Statistics

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

Results

In Vivo Study-Fluvastatin effect on u-AQP2 excretion

As shown by the Western blotting analysis reported in Figure 1A, and the relative densitometry analysis in Figure 1B, u-AQP2 levels were comparable in the three urine samples obtained before starting fluvastatin treatment (T0). After 15 days of fluvastatin treatment at 40 mg/die (T1), u-AQP2 excretion was still comparable to the baseline (T0). Interestingly, after 30 days of fluvastatin 40 mg/die treatment (T2) u-AQP2 increased two folds compared to the control baseline. Fluvastatin treatment at 80 mg/die for the next 30 days (T3) led to a further dramatic dose-dependent increase of u-AQP2 excretion. At this time point AQP2 levels were nearly fourfold increase compared to baseline and remained constantly high after the last 30 days of therapy

(T4). However, during the entire duration of the study the increase of AQP2 excretion was not accompanied by a parallel reduction of diuresis and/or increase in urine osmolality (Figure 1C and D, respectively).

with fluvastatin 80 mg/die (T4). However, during the entire duration of the study the increase of AQP2 excretion was not accompanied by a parallel reduction of diuresis and/or increase in urine osmolality (Figure 1C and D, respectively).

In Vitro Study-Fluvastatin Effect on AQP2 Release in the Culture Medium

In order to correlate the increased uAQP2 excretion with the increased apical membrane expression of AQP2 induced by fluvastatin, we performed *in vitro* studies in cultured renal cells. A mouse collecting duct cell line stably transfected with human AQP2, MDC4 cells, was used to further investigate the effect of fluvastatin on AQP2 exosome excretion. We previously showed that in this cell line incubation with fluvastatin nicely accumulates AQP2 at the apical plasma membrane [27,31]. Cells were either left untreated (Ctr) or treated with forskolin 100 μ M (FK) or fluvastatin 5 μ M (Flu) for 16 hours in the culture medium, then subjected to immunofluorescence protocol to visualize AQP2 cellular distribution. In the same experimental conditions, exosomes excreted in the culture medium were isolated by ultracentrifugation as described in Material and methods.

As shown in Figure 2A, in resting cells, AQP2 was localized in scattered intracellular storage vesicles (Figure 1A, Ctr). FK stimulation redistributed AQP2 staining toward the apical plasma membrane (Figure 2A, FK) and fluvastatin treatment promoted a comparable effect (Figure 2A, Flu).

Figure 2B and the relative densitometric analysis (Figure 2C) show the effect of forskolin and fluvastatin on AQP2 excretion in cell culture medium. Five μ g of exosome-associated proteins from each experimental condition were separated by standard SDS-PAGE and

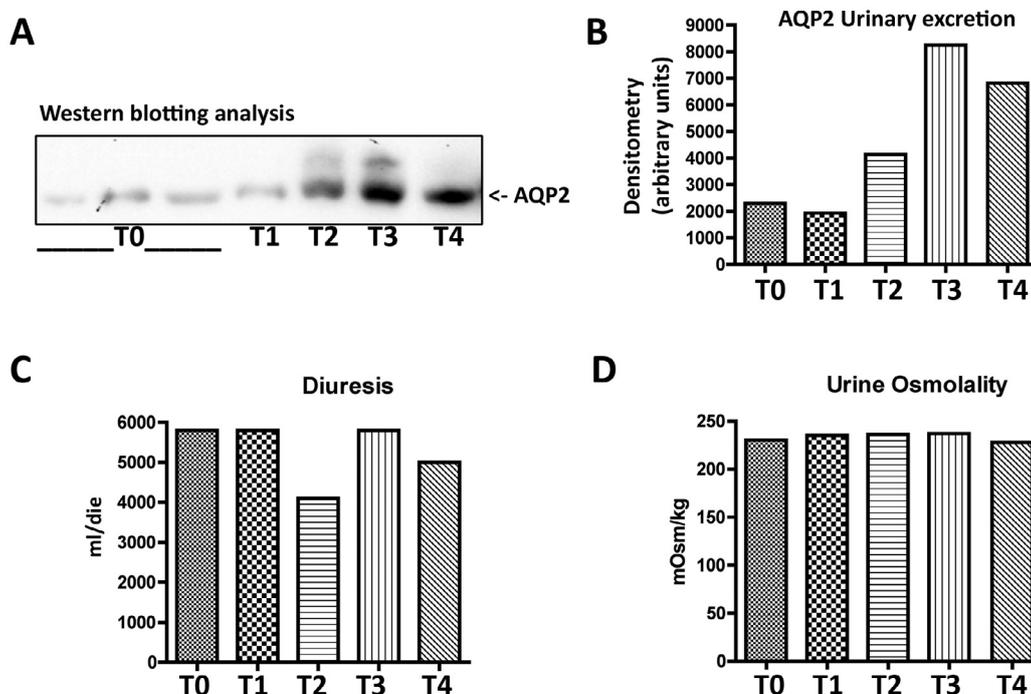
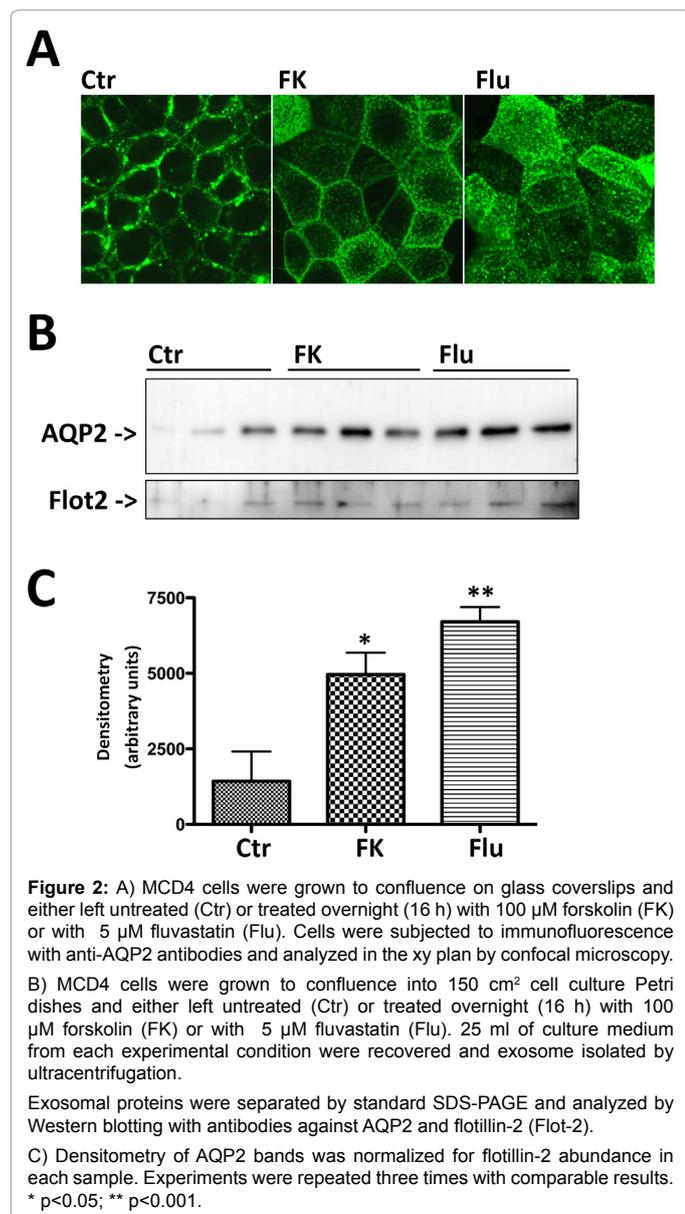


Figure 1: A) Urine samples, (24 h collection) were collected during three nonconsecutive days before initiation of the fluvastatin treatment (T0). Urine samples were also collected at days 15 (T1) and 30 (T2) after administration of 40 mg/die fluvastatin and at days 30 (T3) and 60 (T4) after administration of fluvastatin 80 mg/die. For each time point, a urine volume corresponding to 1 mg of urine creatinine equivalents was processed as described in the methods section to isolate the exosome-enriched fraction. Total exosome proteins were resolved on 12% SDS-PAGE and analyzed by Western blotting for AQP2 abundance. B) Densitometry analysis of the bands. C, D) at each time point urine output (diuresis) and urine osmolality were measured.



analyzed by Western blotting with antibodies anti AQP2 and anti flotillin-2, an exosome marker [32,33].

As expected, FK stimulation, a classical maneuver that increases intracellular cAMP levels and promotes AQP2 exocytosis to the apical plasma membrane in renal cells, also increased AQP2 release in the culture medium *via* exosomes. Interestingly, fluvastatin treatment induced a dramatic increase of excreted AQP2 in the culture medium compared with unstimulated cells. This effect was even higher than that induced by forskolin. The density of each AQP2 band was normalized to that of the corresponding flotillin-2 band.

These results strongly suggest that fluvastatin increases AQP2 membrane expression in renal cells and does not interfere with AQP2 excretion outside the cell.

Discussion

We previously demonstrated that statins, in particular fluvastatin,

are able to promote vasopressin-independent AQP2 plasma membrane accumulation both *in vitro* in renal cell culture and *in vivo* in wt and XNDI mice [23,26,27].

In this study we provide the first experimental evidence that fluvastatin treatment is also effective in increasing AQP2 plasma membrane expression in the collecting duct renal cells in a patient affected by XNDI. This novel and interesting pleiotropic effect of statins on AQP2 trafficking may be of relevance for improving the current therapy of XNDI patients carrying inactivating mutations of the V2R receptor. We had the unique chance to observe the effect of fluvastatin treatment on AQP2 trafficking, in a hypercholesterolemic male XNDI patient subjected to fluvastatin treatment to reduce his cardiovascular risk. u-AQP2 is a well-established non-invasive urinary biomarker of collecting duct responsiveness to AVP and it is critical for the diagnosis of several disorders of renal water balance. AQP2 is excreted into the urinary space through the exosome pathway that is active at the apical membrane of most epithelial cells including the kidney tubule cells [34].

The presence of u-AQP2 reflects the abundance of AQP2 at the apical membrane of renal cells in a number of physio-pathological states [35-38]. Accordingly, u-AQP2 increases during thirsting and after dDAVP administration, and decreases after a water load [39]. To assess whether fluvastatin was able to increase AQP2 plasma membrane expression in this patient, in which AVP signaling is abolished due to an inactivating missense mutation of the V2R, we measured u-AQP2 before and after initiation of fluvastatin treatment [28].

Quite interestingly, the amount of u-AQP2 was time- and dose-dependently increased during fluvastatin treatment. At the end of the three months observation period, u-AQP2 was 3- to 4-fold increase compared to baseline. Next, we performed *in vitro* experiments in cultured renal cells to verify the correlation between plasma membrane expression and extracellular release of AQP2 in the presence of fluvastatin as observed in our patient. The possible interaction of statin with the both exosome pathway formation and release of plasma membrane protein outside epithelial cells is poorly understood. We took advantage of the MCD4 cells that recapitulate the major features of cortical collecting duct renal cells and are useful to study the regulated trafficking of AQP2 [29].

Overnight treatment with fluvastatin accumulates AQP2 at the apical plasma membrane of MCD4 cells to an extent comparable to that elicited by treatment with the cAMP-elevating agent forskolin, as previously reported [27]. In the same experimental conditions, we compared the amount of AQP2 released in the culture medium through the conventional exosome pathway. AQP2-containing exosomes were isolated from the culture medium by ultra-speed centrifugation and checked for the presence of the exosome marker flotillin-2 [39,40]. In cultured renal cells, fluvastatin-induced AQP2 accumulation at the apical plasma membrane is accompanied by a dramatic AQP2 release in the culture medium (Figure 2). This data strongly supports the conclusion that increased uAQP2 is a consequence of increased AQP2 plasma membrane abundance in our patient.

This is the first report showing the effect of statins treatment on AQP2 trafficking in an X-linked NDI patient. In those patients, due to inactivation of the AVP signaling, AQP2 transcription levels are very low and the amount of the channel at the plasma membrane is barely detectable [41,42]. The observation that fluvastatin robustly increase plasma membrane expression of AQP2, as indirectly showed by AQP2 urinary excretion, represents the first proof-of-principle in humans that statins might deserve further investigation as potential tools to improve

the current pharmacological treatment of XNDI. In fact, none of the drug prescribed for handling XNDI, although effective in reducing the polyuria, are targeted to reestablish AQP2 plasma membrane expression.

However, in our patient, despite the encouraging results on AQP2 membrane expression, neither urine output nor urine osmolality ameliorated, even at late point during the observation period. The lack of effect of fluvastatin on XNDI clinical signs might indicate that the pharmacological doses given in this study might be insufficient to elicit the maximal effect on AQP2 trafficking. Higher statin doses might be required to achieve pleiotropic effects beyond cholesterol lowering, especially in the kidney. To this respect, it is worth mentioning that all the statins currently available on the market have been selected for their specific liver targeting, to maximize their cholesterol-lowering potential and reduce extra hepatic tissues effects. Different molecules of the same family, with a preferential kidney targeting, might prove more effective in promoting AQP2 trafficking.

All statins block the conversion of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) to mevalonic acid with consequent attenuation of the biosynthesis of both cholesterol and isoprenoid intermediates of the mevalonate pathway, including farnesyl pyrophosphate and geranylgeranyl pyrophosphate [43,44]. Isoprenoids are important substrates for the post-translational modification (prenylation) of many signaling proteins including small GTP-binding proteins.

It has been proposed that one of the pleiotropic effect of statins, responsible for AQP2 plasma membrane accumulation in renal cells, is the inhibition of prenylation, and hence membrane anchoring, of key molecular switches regulating AQP2 trafficking in renal cells. In particular, reduced prenylation of RhoA, regulating the dynamics of cortical actin cytoskeleton polymerization and AQP2 exocytosis, might explain AQP2 accumulation at the apical plasma membrane [27,45,46]. In analogy, reduced prenylation of Rab5, facilitating AQP2 endocytosis at the apical plasma membrane, might stabilize AQP2 at the plasma membrane [27,47].

In this scenario, specific inhibitors, blocking the mevalonate pathway downstream the first step inhibited by statins, might selectively inhibit the production of Isoprenoids without interfering with cholesterol biosynthesis. To this respect, we previously demonstrated that an inhibitor of the isoprenylation process, perillyl alcohol, mimics the effect of statins *in vitro* [27].

Overall, this proof-of-concept study demonstrates that fluvastatin is effective in increasing AQP2 membrane availability in the kidney of an XNDI patient. In principle, new classes of statins or higher doses of the commercially available, might achieve beneficial effects further reducing the polyuria and other clinical signs of XNDI. In conclusion, the observations described here support the hypothesis that statins or other downstream inhibitors of the mevalonate pathway might prove useful, in the future, for improving the current pharmacological treatment of XNDI.

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