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Nephrology Dialysis Transplantation

Case Report

Novel mutation in the *AVPR2* gene in a Danish male with nephrogenic diabetes insipidus caused by ER retention and subsequent lysosomal degradation of the mutant receptor

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Abstract

Mutations in the arginine vasopressin receptor 2 (*AVPR2*) gene can cause X-linked nephrogenic diabetes insipidus (NDI) characterized by the production of large amounts of urine and an inability to concentrate urine in response to the antidiuretic hormone vasopressin. We have identified a novel mutation in the *AVPR2* gene (L170P) located in the fourth transmembrane domain in a Danish NDI male. Analysis of the mutant receptor in Madin-Darby Canine Kidney cell culture revealed that AVPR2-L170P was retained in the endoplasmic reticulum, and the expression was dramatically downregulated compared to wild-type AVPR2. Inhibition of the lysosome resulted in increased intracellular accumulation of AVPR2-L170P, indicating that AVPR2-L170P is downregulated via the lysosome. Inhibition of the proteasome resulted in plasma membrane localization of AVPR2-L170P, although the overall levels of AVPR2-L170P were unchanged.

Keywords: AVPR2; ER retention; nephrogenic diabetes insipidus

Introduction

Fine-tuning of urine concentration occurs in the renal collecting duct by the actions of the antidiuretic hormone arginine vasopressin (AVP). AVP binds to arginine vasopressin receptor 2 (AVPR2) on the basolateral plasma membrane of renal collecting duct principal cells, thereby inducing apical plasma membrane insertion of aquaporin-2 leading to increased water uptake and urine concentration [1, 2]. Nephrogenic diabetes insipidus (NDI), which can be either inherited or acquired, is characterized by the excretion of large volumes of dilute urine and resistance to the action of vasopressin. Almost 90% of congenital NDI is inherited in an X-linked recessive manner due to mutations in the *AVPR2* gene residing in the Xq28 region, while 10% is autosomal NDI caused by mutations in the aquaporin 2 gene (*AQP2*) [1]. More than 200 putative disease-causing mutations within the *AVPR2* gene have been reported [3] and functional characterization of some of these mutant receptors have revealed receptor malfunction at different levels such as receptors with drastically reduced binding affinity for vasopressin (type 1), defective intracellular trafficking (type 2) or reduced receptor transcription (type 3) [1].

In the present report, we describe a novel mutation in the *AVPR2* gene in a Danish male with NDI. Cell-based experiments demonstrate that this novel mutant is likely misfolded. We propose that, in NDI, the accumulation of the misfolded protein is non-toxic since other functions of the principal cell of the collecting duct are intact.

Case history

A 30-year-old Caucasian male patient had a history of polyuria and polydipsia from infancy. During early childhood, he was followed by a paediatric clinic. Later in life, however, he only had sporadic contact with hospitals. The patient is normally developed without cognitive deficits and is gainfully employed.

After referral, further investigations revealed a daily urine output of typically 13 L with low osmolality, 75 mOsm/kg, despite high plasma AVP concentration. A water deprivation test showed an inability to produce concentrated urine before and after administration of exogenous AVP. Subsequent treatment with hydrochlorothiazide lowered the patient's daily urinary output by ~3 L [4]. Due to high mictional volumes suggesting distended urinary bladder, he was instructed to void at regular intervals,

irrespective of whether urine had accumulated or not. None of the patient's relatives (Figure 1) had a similar history of polyuria and polydipsia. The patient's mother and sister agreed to be genetically tested for the mutation. The father is adopted and healthy. The mother's sister and her three sons are clinically healthy and have therefore not been genetically tested. Blood samples were taken from the patient, his sister and mother. The mother was diagnosed as a heterozygous carrier of the mutation, while the sister was not. The mother did not wish to participate in further investigations and therefore we were not able to conduct urine sampling in order to test for possible skewed X-chromosome inactivation [5]. However, it was reported that she as a child had extraordinary thirst and suffered from enuresis nocturna until the age of 10 (Tomas M. Christensen, personal communication). The daughter of the patient is an obligate carrier of the mutation but due to her young age (1 year), genetic testing and urine analysis has not been performed.

Experimental results

Sequence analysis of the *AVPR2* gene was performed as previously described [6] and revealed a novel missense mutation (L170P) in the fourth transmembrane domain of AVPR2.

Constructs

The L170P mutation was introduced into the pEGFP-V2R construct using (forward) CCTTCTCGCTCCTTCCCA-GCCTGCCCCAGC and (reverse) GCTGGGGCAGGCTG-GGAAGGAGCGAGAAGG primers and the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The mutation was confirmed by sequencing.

Immunostaining, imaging and western blotting

For transient transfections, Madin-Darby Canine Kidney (MDCK) GII cells were transfected, stained 24-h post-transfection, imaged and analysed as previously described [6].

To generate a cell line stably expressing AVPR2-L170P-GFP, a tetracycline/doxycyclin-inducible MDCK cell line was generated using the Flp-In T-REx core kit (Invitrogen) according to the manufacturer's protocol. MDCK type II cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% fetal calf serum (PAA Laboratories), ciproxin, L-glutamin and 1% non-essential amino acids. All transfection procedures were performed using calcium phosphate. Briefly, to stably introduce an FLP recognition target (FRT) recombination site, pFRT-LacZeo (Invitrogen) was linearized with XmnI and transfected into the MDCK cells. Stable transfectants were selected using 0.5 mg/mL zeocin, and insertion into the FRT site was confirmed by blue staining using the β-Gal Staining Kit (Invitrogen). To stably introduce a Tet-repressor, cells were transfected with FspI-linearized pcDNA6-TR (Invitrogen) and cells were selected using 5 µg/mL blasticidin. To identify a single clone with minimal leakiness and to identify the optimal time of induction, cell lines stably expressing green fluorescent protein (GFP) were generated by co-transfection of pcDNA5-FRT-TO-GFP plasmid with pOG44 (encoding the flp recombinase; Invitrogen) in a ratio of 1:9. Transfectants were isolated using selection with 0.1 mg/mL hygromycin and analysed via western blotting and fluorescence microscopy to determine induction. A suitable clone was chosen for subsequent experiments as described below.

AVPR2-L170P-GFP was subcloned into the pcDNA5/FRT/TO (Invitrogen) and cells were co-transfected with pcDNA5-FRT-TO-AVPR2-L170P-GFP plasmid and pOG44 using Lipofectamine 2000. Transfectants were isolated using selection with 0.1 mg/mL hygromycin and
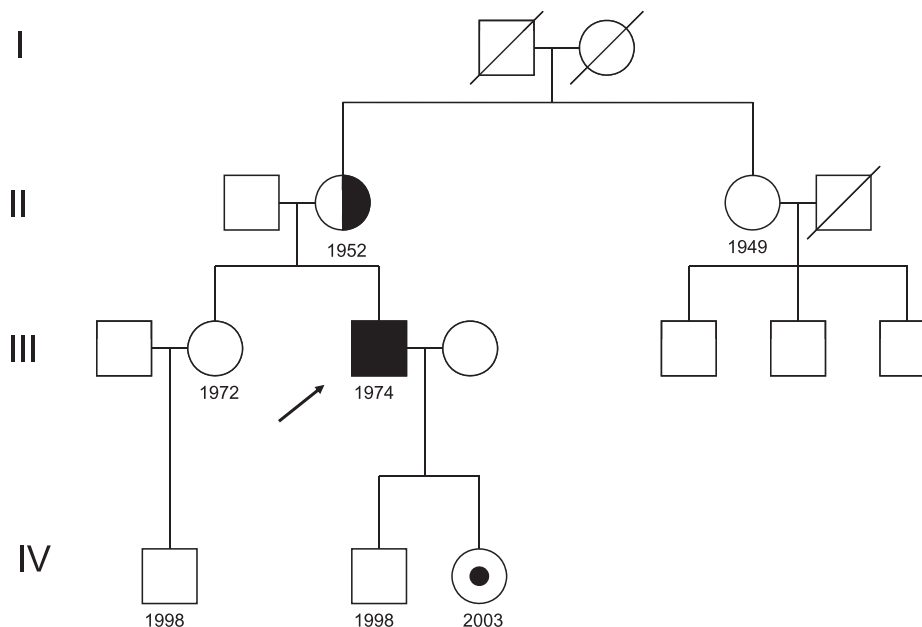
Fig. 1. Pedigree of NDI relatives. Open circles, women; open squares, men. Roman numerals, the generation; circles with a central dot, obligatory carriers; closed squares, affected subjects. A slash (/) through a symbol indicates that the subject is deceased. The proband is shown by an arrow. The year of birth is beneath the subject.

blasticidin and analysed via fluorescence microscopy to determine induction. Following 16 h of 10 µg/µL tetracycline, lysomal degradation was inhibited by the lysosomal protease inhibitor chloroquine (100 µM) for 4 h, or the proteasome was inhibited by a 4-h incubation with the proteasome inhibitor MG-132 (10 µM; Sigma), control cells were incubated with dimethyl sulfoxide (DMSO). The experiment was performed three times.

LysoTracker Red (Molecular Probes) was added 30 min before fixation. Primary antibodies were polyclonal GFP (A11122; Molecular Probes), polyclonal Calreticulin (Affinity BioReagent), monoclonal Cathepsin D (TriChem ApS) and monoclonal Clone 58K-9 (Sigma–Aldrich).

For western blotting, cells were lysed in sodium dodecyl sulfate sample buffer and western blotting was perfomed via a standard protocol. Antibody was GFP polyclonal antibody from ABCAM. Equal amounts of proteins were loaded as confirmed by Coomassie staining.

Imaging was performed on a Zeiss 200M microscope using the MetaMorph software and analysis was performed using ImageJ [7].

Localization of AVPR2-L170P-GFP in cell culture

In MDCK cells transiently transfected with AVPR2-L170P-GFP or wild-type AVPR2-GFP, wild-type AVPR2-GFP was localized to the plasma membrane, with some AVPR2-GFP localized to the Golgi as visualized by labelling with the Golgi marker Golgi 58K Protein (Figure 2A).

AVPR2-L170P-GFP could not be detected following transient transfections by normal epi-fluorescence microscopy, suggesting that the AVPR2-L170P-GFP was dramatically downregulated compared to the wild type. Increasing the signal of the mutant AVPR2-L170P-GFP with a GFP antibody revealed that the mutant was localized intracellularly but did not colocalize with the Golgi marker (Figure 2B). Co-staining with calreticulin, a marker for the endoplasmic reticulum (ER), revealed a large degree of colocalization between AVPR2-L170P-GFP and the ER (Figure 2C). Moreover, cells transfected with AVPR2-L170P-GFP had greatly increased lysosomes as detected by Lysotracker Red (Figure 2D); however, there was no colocalization between AVPR2-L170P-GFP and lysosomes. Normally, wild-type AVPR2-GFP is localized to the plasma membrane, from which it is internalized and degraded in the lysosome [8, 9]. The lack of colocalization between AVPR2-L170P-GFP and lysosomes could be due to degradation of the GFP tag. The increase in lysosomal size could be due to degradation of AVPR2-L170P-GFP or could be a secondary effect of AVPR2-L170P-GFP accumulation in the ER, which could disturb trafficking of other proteins.

Effect of lysosome and proteasome inhibition on the expression and localization of AVRR2-L170P-GFP in cell culture

To determine the cellular fate of the unstable AVPR2-L170P, inhibition of lysosomal degradation was investigated in the cell line stably expressing AVPR2-L170P-GFP under a tetracycline-inducible promoter. Some AVPR2-L170P-GFP expression was observed even without tetracycline (Figure 3B), indicating a leakiness of the promoter. AVPR2-L170P-GFP expression was induced by 16-h incubation with 10 µg/mL tetracycline, followed by a 4-h incubation with either DMSO or the lysosome inhibitor chloroquine (100 µM).

Inhibition of lysosomal degradation by chloroquine resulted in increased intracellular accumulation of AVPR2-L170P-GFP (Figure 3A). Moreover, cells treated with chloroquine had larger lysosomes (Figure 3, stained with Cathepsin D antibodies) and inhibition of lysosomal degradation resulted in colocalization of AVPR2-L170P-GFP with lysosomes (Figure 3, overlays). Western blot analysis of wild-type AVPR2-GFP showed bands at ~68 kDa and a broad 57-kDa band corresponding to the glycosylated forms of the protein and a 46-kDa band corresponding to the lysosomal degradation product [8]. Interestingly, western blot analysis of AVPR-L170P-GFP (Figure 3B) revealed a 68-kDa band corresponding to the full-length glycosylated protein and a 46-kDa band corresponding to the degradation product, but no bands around 57-kDa were observed, indicating that there might be a deficiency in glycosylation of AVPR2-L170P-GFP. The lack of AVPR2-L170P-GFP glycosylation could be the primary cause for ER accumulation, or more likely, it could be a secondary effect due to receptor misfolding, resulting in ER retention and subsequent degradation in the lysosome. Compared to wild-type AVPR2-GFP, the intensity of the 46-kDa degradation product was larger than the 68-kDa band, indicating rapid degradation of AVPR2-L170P-GFP. This was reversed by treatment with lysosomal inhibitor chloroquine, which resulted in increased intensity of the 68-kDa band compared to the 46-kDa band (Figure 3B)

Since ER-retained V2R mutants are often degraded in the proteasome [10], we also tested if AVPR2-L170P-GFP could be degraded in this organelle. Following incubation with an inhibitor of proteasomal degradation, MG-132, there was a significant change in the localization of AVPR2-L170P-GFP, which relocated from the ER to the plasma membrane (Figure 4A); however, there was no change in the expression levels of AVPR2-L170P-GFP following MG-132 treatment (Figure 4B) and unlike lysosomal inhibition, we did not see a change in the ratio between the 68-kDa full-length protein and the 46-kDa degradation product following proteasome inhibition. This indicates that AVPR2-L170P-GFP is not degraded via the proteasome, but inhibition of proteasome degradation could lead to a general release of proteins from the ER.

Discussion

Congenital X-linked NDI is a rare disease affecting 8.8 of 1 000 000 male live births [11]. We have identified a Danish male patient with congenital X-linked NDI due to a missense mutation in the fourth transmembrane domain of the AVPR2 (L170P).

When expressed in cell cultures, the present AVPR2-L170P mutant was shown to be trapped intracellularly and colocalized with ER markers suggesting that AVPR2-L170P mutant is misfolded and degraded. The intracellular retention has been characterized as a type 2 mutation and functional studies conducted to date have shown that the majority of mutant AVPR2 receptors are of this type [1].
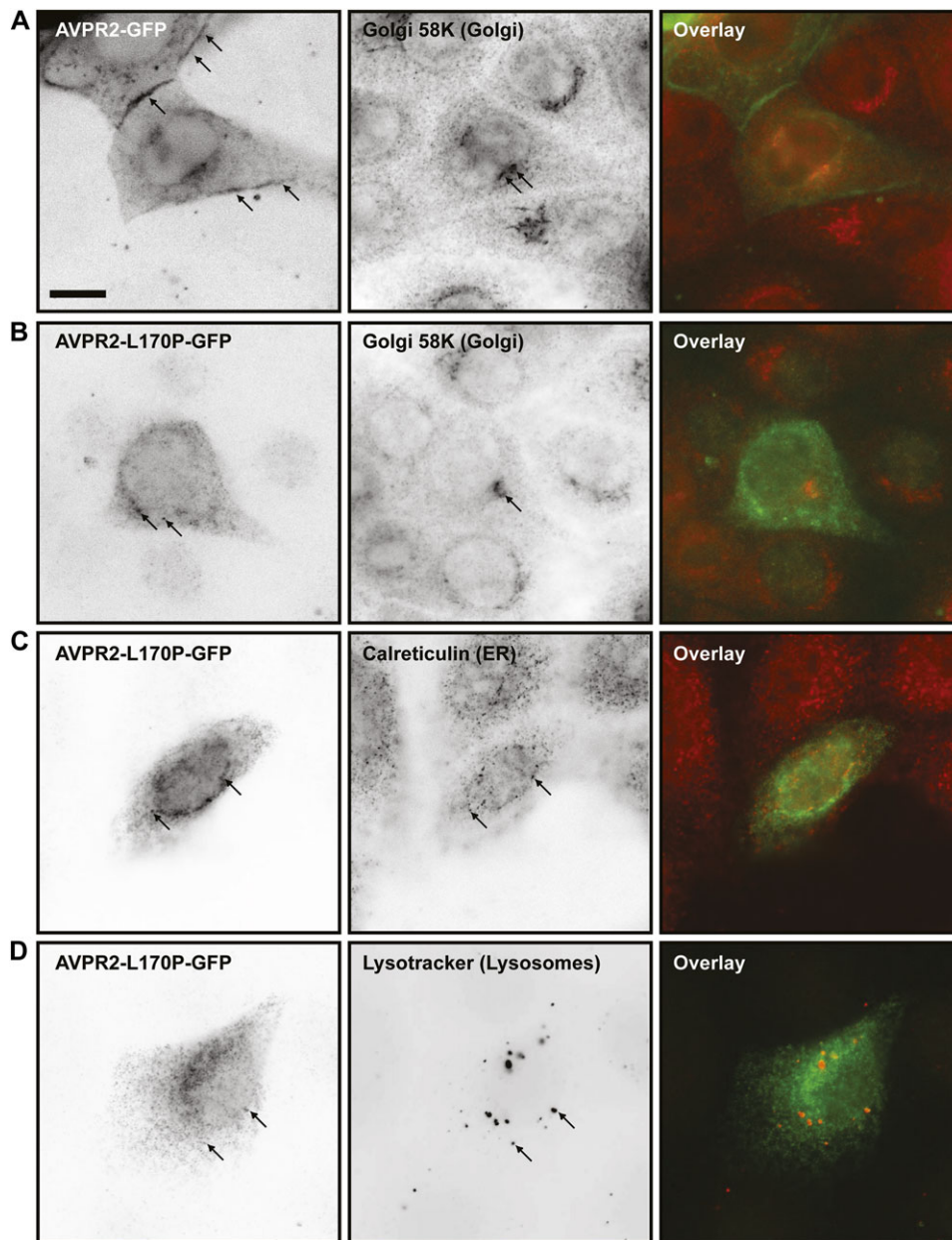
Fig. 2. (**A**) Wild-type AVPR2-GFP transiently expressed in MDCK cells and stained with the Golgi marker G58K. Wild-type AVPR2-GFP localizes to cell–cell contacts of MDCK cells and to the Golgi region. (**B**). AVPR2-L170P-GFP transiently expressed in MDCK cells and stained with the Golgi marker G58K. AVPR2-L170P-GFP does not seem to localize to the Golgi compartment. (**C**) AVPR2-L170P-GFP transiently expressed in MDCK cells and stained with the ER marker calreticulin. AVPR2-L170P-GFP localizes to intracellular structures with some overlap with the ER marker calreticulin. (**D**) AVPR2-L170P-GFP transiently expressed in MDCK cells. Cells were loaded with Lysotracker Red before fixation, which shows that cells expressing AVPR2-L170P-GFP had larger lysosomes than surrounding cells, although AVPR2-L170P-GFP did not colocalize, which could be due to degradation of the GFP-tag in lysosomes. Please note that samples B, C and D have been stained with antibody against the GFP tag since they were otherwise not bright enough for imaging. Scale bar is 10 μm.

We are proposing to classify the inherited diseases characterized by the accumulation of misfolded proteins into two groups: in the first group, there is toxic accumulation of the misfolded protein and a gain-of-toxic function mechanism now extensively explored in alpha-1-antitrypsin deficiency and in familial neurohypophyseal diabetes insipidus, both dominant disorders [12, 13].

In the second group, the function of the cell where this accumulation takes place remains intact; this is the case with AVPR2 mutations where the other important function of the principal cell of the collecting tubule, namely the reabsorption of Na^+ through the epithelial sodium channel (ENaC) channel, remains intact since there is no evidence of loss of function of the ENaC channel in male patients bearing AVPR2 mutations [14].

NDI is associated with a large urine production and if not recognized in early childhood, it may result in mental retardation due to dehydration. When diagnosed, urine volumes can be somewhat reduced by administering thiazide diuretics along with prostaglandin inhibitors [2, 4];
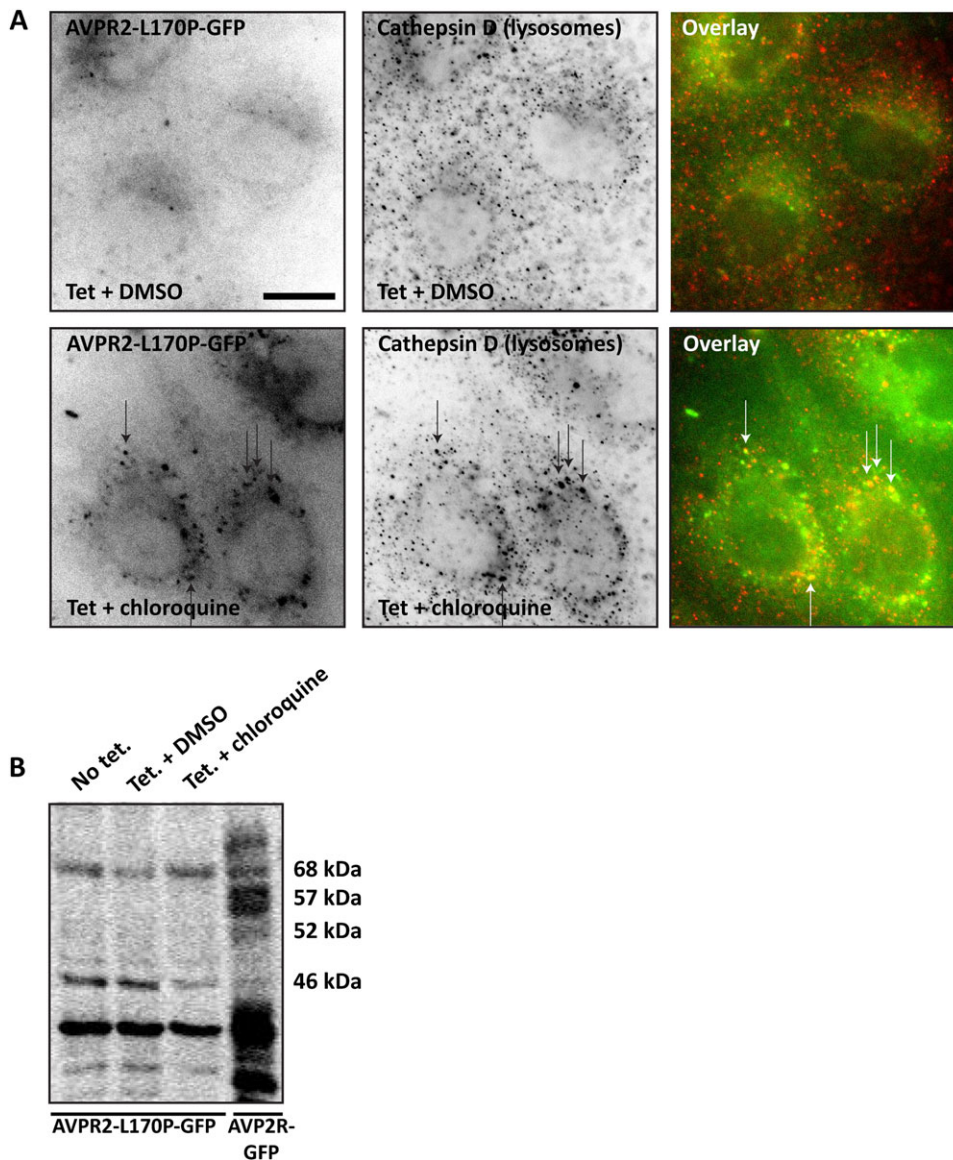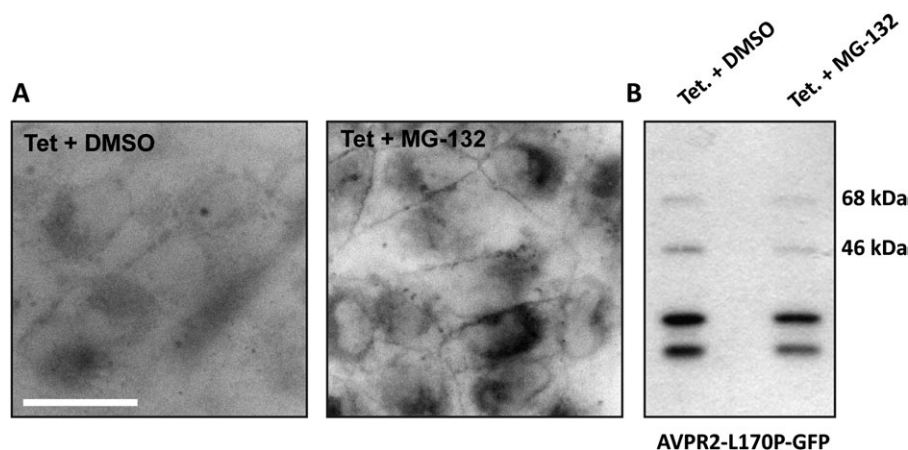
Fig. 3. (**A**) Representative images of AVPR2-L170P-GFP stably expressed in MDCK cells 16 h after induction with tetracycline followed by 4 h of incubation with DMSO or chloroquine (100 µM). There is a marked accumulation of AVPR2-L170P-GFP in cells treated with chloroquine compared to controls (DMSO). Moreover, following chloroquine treatment, AVPR2-L170P-GFP is localized in larger structures, which colocalize with the lysosomal marker Cathepsin D. (**B**) Western blot analysis of equal amount of protein from cells stably expressing AVPR2-L170P-GFP, either not induced or induced by tetracycline, and transiently expressing wild-type AVPR2-GFP probed with a GFP antibody. A 68-kDa band was observed in all samples as well as a 46-kDa band corresponding to the lysosomal degradation product. The 68-kDa band was decreased in intensity and the 46-kDa degradation product was increased in intensity in AVPR2-L170P-GFP compared to wild-type AVPR2-GFP; however, this was reversed by inhibition of lysosome degradation by chloroquine. Moreover, a broad 57-kDa band in wild-type AVPR2-GFP was completely absent from AVPR2-L170P-GFP. Cells not induced by tetracycline still show significant levels of AVPR2-L170P-GFP, indicating a leaky promoter. Scale bar is 10 µm.

however, at present, no cure for congenital NDI is available.

In working toward a potential cure for NDI, a great advance in potentially treating NDI comes from work by Suga *et al.* [15]. They successfully used *in vivo* gene therapy to transfer *AQP2* to rat collecting ducts, which temporarily and partially rescued lithium-induced NDI. This is the first time a gene has successfully been incorporated into the renal collecting duct and this technique shows great promise for potentially treating patients with different forms of NDI caused by mutations in *AVPR2* or *AQP2*.

Recent studies in cell cultures have shown that different pharmacological chaperones are able to rescue some AVPR2 folding mutants by restoring their plasma membrane expression [16] or inducing their intracellular activation [17]. The list of specific chemical chaperones is rapidly expanding and chaperones able to rescue AVPR2 folding mutants from both the ER (type A) [18] and the ER/Golgi intermediate compartment (type B) [19] have been identified and potentially, these chaperones can be used *in vivo* to rescue patients carrying AVPR2 mutations leading to NDI [20, 21]. Since AVPR2-L170P-GFP relocalized from the ER to the plasma membrane upon treatment with the proteasome inhibitor

Fig. 4. (**A**) Representative images of AVPR2-L170P-GFP stably expressed in MDCK cells 16 h after induction with tetracycline followed by 4 h of incubation with DMSO or MG-132. There is a marked relocalization of AVPR2-L170P-GFP from the ER to the plasma membrane following inhibition of the proteasome with MG-132 compared to DMSO. (**B**) Representative western blot analysis using GFP antibodies. MDCK cells stably expressing AVPR2-L170P-GFP induced by tetracycline followed by a 4-h treatment with either DMSO or the proteasome inhibitor MG-132. A 68-kDa band corresponding to the non-glycosylated form of AVPR2-L170P-GFP was present in all lanes as well as a 46-kDa band presumed to be a degradation product. There was no significant difference in the intensity of the bands between DMSO and MG-132-treated cells. Scale bar is 10 μm.

MG-132, it is very likely that chaperones could aid in correct folding of the mutant, thereby bypassing lysosomal degradation leading to a rescue of the mutant receptor.

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