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The Chemical Chaperone, PBA, Reduces ER Stress and Autophagy and Increases Collagen IV α5 Expression in Cultured Fibroblasts From Men With X-Linked Alport Syndrome and Missense Mutations

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Introduction: X-linked Alport syndrome (OMIM 301050) is caused by *COL4A5* missense variants in 40% of families. This study examined the effects of chemical chaperone treatment (sodium 4-phenylbutyrate) on fibroblast cell lines derived from men with missense mutations.

Methods: Dermal fibroblast cultures were established from 2 affected men and 3 normals. Proliferation rates were examined, the collagen IV α5 chain localized with immunostaining, and levels of the intra- and extracellular chains quantitated with an in-house enzyme-linked immunosorbent assay. *COL4A5* mRNA was measured using quantitative reverse transcriptase polymerase chain reaction. Endoplasmic reticulum (ER) size was measured on electron micrographs and after HSP47 immunostaining. Markers of ER stress (ATF6, HSPA5, DDIT3), autophagy (ATG5, BECN1, ATG7), and apoptosis (CASP3, BAD, BCL_2) were also quantitated by quantitative reverse transcriptase polymerase chain reaction. Measurements were repeated after 48 hours of incubation with 10 mM sodium 4-phenylbutyrate acid.

Results: Both *COL4A5* missense variants were associated with reduced proliferation rates on day 6 ($P = 0.01$ and $P = 0.03$), ER enlargement, and increased mRNA for ER stress and autophagy (all P values < 0.05) when compared with normal. Sodium 4-phenylbutyrate treatment increased *COL4A5* transcript levels ($P < 0.01$), and reduced ER size ($P < 0.01$ by EM and $P < 0.001$ by immunostaining), ER stress (p HSPA5 and DDIT3, all P values < 0.01) and autophagy (ATG7, $P < 0.01$). Extracellular collagen IV α5 chain was increased in the M1 line only ($P = 0.06$).

Discussion: Sodium 4-phenylbutyrate increases collagen IV α5 mRNA levels, reduces ER stress and autophagy, and possibly facilitates collagen IV α5 extracellular transport. Whether these actions delay end-stage renal failure in men with X-linked Alport syndrome and missense mutations will only be determined with clinical trials.

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KEYWORDS: autophagy; chaperones; ER stress; missense mutations; unfolded protein response; X-linked Alport syndrome

Alport syndrome is an inherited form of progressive renal failure that is accompanied by hearing loss, lenticonus, and retinopathy.[1] Most patients have X-linked disease and mutations in the *COL4A5* gene.[2,3] *COL4A5* codes for the collagen IV α5 chain which forms a heterotrimer with the α3 and α4 chains.[4] The resulting α3α4α5 network predominates in the basement membranes of the glomerulus (GBM), cochlea, lens capsule, and retina, which are all abnormal in Alport syndrome.[5–8] *COL4A5* mutations also affect the collagen IV α5 chain in the α5α5α6 network of the epidermis.[9]

More than 1200 different pathogenic variants have been described in *COL4A5* (www.LOVD.nl). Forty

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percent are missense mutations, which often replace a glycine residue in the Gly-Xaa-Yaa collagenous domain.[10] Normal collagen biosynthesis includes translational modification and trimerization to form an α3α4α5 protomer that is secreted extracellularly. Missense mutations cause endoplasmic reticulum (ER) retention of the unassembled or misfolded collagen and its subsequent degradation. This is consistent with the demonstration of the collagen IV α5 chain in male Alport podocytes but its absence from affected GBM.[11] A compensatory increase in the α1α1α2 network, which is more susceptible to proteolysis,[12] leads to GBM thinning, lamellation, and vacuolation.[13]

In other collagen diseases, including those due to mutant *COL4A1* and *COL4A2* genes, missense mutations cause protein misfolding and retention within the ER.[14,15] The accumulation of misfolded protein results in the unfolded protein response (UPR) and increased ER stress. The stress response is both protective, by enhancing the capacity for protein folding and reducing translation,[16] and damaging, by activating protein degradation via the ER-associated degradation system and/or autophagy.

Missense mutations in the collagen IV α1, α2 chain[15,17] genes, and the α3 chain genes in autosomal recessive Alport syndrome and thin basement membrane nephropathy[18] all activate the UPR and increase ER stress. Other inherited diseases affecting the kidney that are caused by abnormal protein folding include Pierson syndrome (*LAMB2*) and focal segmental glomerulosclerosis due to nephrin (*NPHS1*), podocin (*NPHS2*), *CD2AP*, and α*ACTN4* mutations.[19–24]

Chaperones are small compounds that stabilize misfolded proteins and improve the protein-folding capacity of cells, thereby increasing protein secretion and reducing ER stress. Chemical chaperones represent potential treatments for disease due to missense mutations associated with the retention of misfolded proteins. Sodium 4-phenylbutyrate (PBA) increases ER folding capacity, stabilizes the folded protein, and facilitates protein trafficking[25] as well as increasing ER-associated degradation and decreasing autophagy.[26] PBA is an orally administered ammonia scavenger already in clinical use for the treatment of urea cycle disorders.[27] PBA treatment has been demonstrated to reduce collagen IV α2 chain retention and associated ER stress in *COL4A2* missense mutant cell lines.[15]

This study examined the consequences of *COL4A5* missense mutations on protein expression, ER stress, autophagy and apoptosis, and cell proliferation in primary fibroblasts from males with X-linked Alport syndrome, and the effects of treatment with the chemical chaperone PBA.

METHODS

Study Subjects

Two men with X-linked Alport syndrome due to *COL4A5* missense mutations and 3 age-matched nonhematuric normal subjects were studied. Both affected men had functioning renal transplants.

This work was approved by the Northern Health Human Research Ethics Committee according to the principles of the Declaration of Helsinki, and all participants provided signed, informed consent.

Cell Culture

Primary dermal fibroblast cultures were established from 4-mm skin punch biopsies from each subject. The tissues were teased out, incubated with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin/l and 2 mM L-glutamine (Invitrogen) in 5% CO_2, and fibroblasts grown to confluence at 37 °C.

Immunocytochemistry

Fibroblasts were cultured in chamber slides (BD Falcon Slides, BD Biosciences, Bedford, MA), fixed with ice-cold acetone, and incubated with a 1:300 rabbit polyclonal anticollagen IV α5 antibody (Covalab, Villeurbanne, France), followed by a 1:300 dilution of fluorescein isothiocyanate goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO). Slides were examined using a Zeiss microscope and photographed at ×200 magnification.

Enzyme-Linked Immunosorbent Assay

Bovine GBM was purified as described previously[28] and coated on plates at 20 μg/ml in phosphate-buffered saline, pH 7.3, overnight at 4 °C (Nunc immunoplates). Plates were then blocked with 1% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany) in phosphate-buffered saline for 2 hours at room temperature. Concentrations of GBM were determined in an inhibition assay performed as follows. Twenty-five microliters of intracellular lysate prepared from the supernates after microfugation, from 5 freeze-thaws in phosphate-buffered saline, or 25 μl of the extracellular culture supernate, all corrected for cell numbers, were preincubated with 25 μl of 1:50 dilution rabbit anticollagen IV α5 antibody in phosphate-buffered saline/TWEEN (Sigma-Aldrich) at room temperature for 30 minutes and then added to GBM-coated wells in duplicate for 1 hour at room temperature. The plates were then incubated with a 1:1000 dilution of alkaline phosphatase–conjugated goat anti-rabbit IgG (Sigma-Aldrich) for 30 minutes and p-nitrophenylphosphate (Sigma-Aldrich) for 15 minutes at

room temperature. The absorbance was measured at 405 nm (Thermo Scientific Fisher plate reader), and the concentrations were determined from a standard curve of serial dilutions of GBM with final concentrations of 0.03 to 30 μg/ml.

Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using an RNA Isolation Kit (Zymo Research, Irvine, CA). RNA integrity was assessed after agarose gel electrophoresis, and the concentrations were measured spectrophotometrically (Nanodrop Technologies, Wilmington, DE). The samples were subjected to DNase treatment using a DNA-free kit (Ambion, Inc., Austin, TX), and 1 μg from each sample reverse-transcribed using oligo dT and a SuperScript III First Strand Synthesis System Kit (Invitrogen).

Samples were then assayed for *COL4A5* transcripts, using the fluorescent intercalating agent SYBR Green 1 (Qiagen, Hilden, Germany), specific primers (Life Technologies, Mulgrave, Victoria, Australia) (Supplementary Tables S1 and S2), and the ABI 7500 real-time PCR System (Applied Biosystems, Waltham, MA). Individual reactions comprised 5 μl of 2× QuantiTect SYBR Green RT-PCR Master Mix (Qiagen), 0.7 μl each of 20 ng/μl sense and antisense primer, and 2 μl of 100 ng/μl cDNA template, in a total volume of 10 μl. The threshold cycle value was calculated at the end of each run using glyceraldehyde-3-phosphate dehydrogenase as the internal control and software provided by the manufacturer. Each sample was examined in duplicate and the assays performed in triplicate. The results were compared with expression in the 3 normal male fibroblast cell lines in different experiments.

Growth Curves

Fibroblast proliferation was examined over 6 days. Five × 10^4 cells in 2 ml of Dulbecco's modified Eagle's medium were seeded in each well of a 6-well culture plate (BD Falcon, BD Biosciences) and allowed to adhere for 24 hours. Cells were counted the next day (time 0), and then each 24 hours. Cells were counted after trypsinization using a hemocytometer and an Olympus CKX41 microscope.

Quantitation of the UPR, Cell Stress, Apoptosis, and Autophagy Pathways

Levels of mRNA corresponding to markers of the UPR (cell stress), pro- and antiapoptotic pathways, and autophagy, were quantitated using primers (Supplementary Table S2), and quantitative polymerase chain reaction, as described previously.

ER Size and Colocalization Experiments

ER size was examined in the primary dermal fibroblasts using 2 methods: electron microscopy and immunocytochemistry. For electron microscopy, fibroblast cell pellets were fixed in 2.5% chilled glutaraldehyde (Sigma-Aldrich), postfixed in 2% OsO4 (Sigma-Aldrich), and prepared routinely. The grids were examined, and images were captured with a transmission electron microscope (Phillips CM 120BioTWIN, Eindhoven, The Netherlands). ER size was measured using ImageJ software (https://imagej.nih.gov/ij/), and ER size was determined in consecutive cells that were measured and averaged.

For immunostaining, fibroblasts were grown on coverslips and processed for immunohistochemistry, but with the additional antibodies (antiHSP47, 1:400, Enzo Life Sciences, Farmingdale, NY). ER volume from 50 consecutive fields were quantified again using ImageJ software.

Treatment With the Chemical Chaperone PBA

One flask of cells was left untreated, and the others were incubated with 10 mM of PBA (Sigma-Aldrich) for 48 hours at 37 °C, and the cells were then harvested. In general, data were obtained in duplicate from 3 independent experiments for each cell line.

Statistical Analyses

Data are provided as mean ± SD, and statistical analyses were performed using an unpaired *t* test. Differential mRNA expression was analyzed by analysis of variance. Statistical analyses were performed using Graph Pad Prism, version 5. A P value <0.05 was considered significant.

RESULTS

Fibroblast cell lines were established from the skin biopsy specimens from 2 men with X-linked Alport syndrome due to Gly substitutions (Table 1). Male 1 (G908R) had early-onset renal failure together with lenticonus and central retinopathy, and in Male 2 (G624D), renal failure had developed at middle age with hearing loss but no ocular features.

Collagen IV α5 Chain Expression in Affected Fibroblasts

The collagen IV α5 chain was demonstrated predominantly in a perinuclear location, corresponding to the ER, in the normal and affected male fibroblasts (Figure 1a, left and right, respectively). There was less intracellular α5 chain in the cell line from Male 1 by immunocytochemistry (the cell line from Male 2 was not examined) and less than normal in both cell

Table 1. Clinical features in males with X-linked Alport syndrome and missense mutations

Male	Sex, age (yr)	Exon	Mutation	Renal features	Age at ESRF (yr)	Hearing loss	Lenticonus	Central retinopathy	Peripheral retinopathy
1	M, 48	32	p.G908R	RF	14	Yes	Yes	Yes	Yes
2	M, 54	25	p.G624D	RF	46	Yes	Yes	No	Yes

ESRF, end-stage renal failure; H, hematuria; N/A, applicable; P, proteinuria; RF, renal failure.

lines by inhibition enzyme-linked immunosorbent assay ($P < 0.0001$ and $P = 0.003$, respectively) (Figure 1b).

There was no difference in the levels of collagen IV α5 mRNA in the affected Male 1 and Male 2 cell lines compared with normal by quantitative polymerase chain reaction (both P values were nonsignificant) (Figure 1c).

One explanation for the reduced levels of intracellular collagen IV α5 chain but normal mRNA levels in the affected male cell lines is that the collagen IV α5 chain was degraded, at least in part, intracellularly.

Cell Proliferation

Both affected male fibroblast cell lines grew more slowly than the mean of 3 normal cell lines measured at day 6 ($P = 0.03$, $P = 0.01$, Figure 2a). The cell line from Male 1 grew more slowly than that from Male 2. This cell line corresponded to the mutation associated with earlier onset renal failure.

ER Stress, Autophagy, and Apoptosis

Affected fibroblasts in both cell lines demonstrated increased levels of mRNA coding for the UPR/ER stress (ATF6, HSPA5, and DDIT3) compared with the 3 normals by quantitative polymerase chain reaction

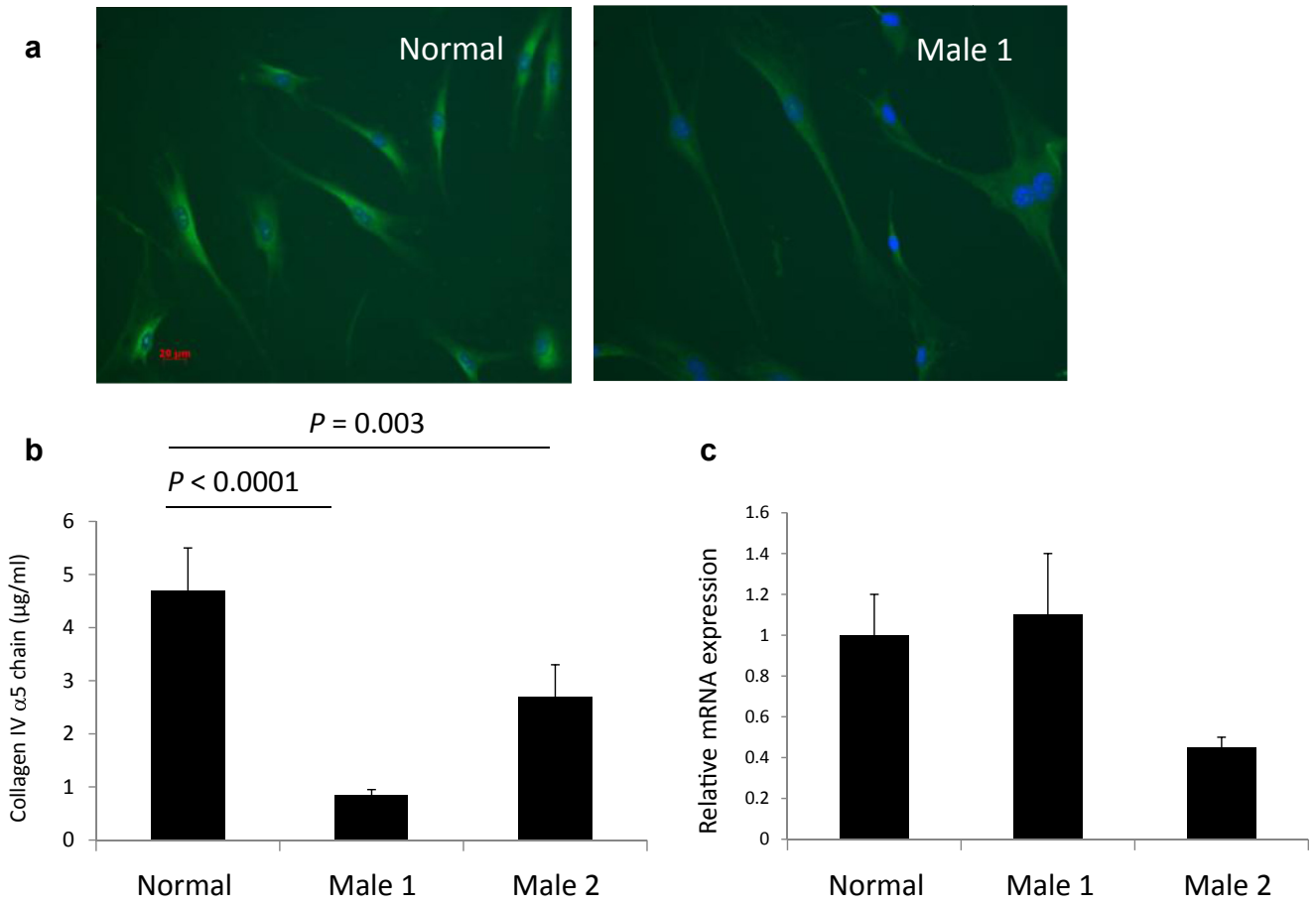

Figure 1. Effect of *COL4A5* missense mutations on collagen IV α5 chain expression in affected male fibroblasts. (a) Collagen IV α5 chain in the endoplasmic reticulum in a predominantly perinuclear distribution in normal fibroblasts (left) and less staining in fibroblasts from affected Male 1 (right). (b) Intracellular collagen IV α5 chain demonstrating reduced levels in affected Male cell lines 1 and 2 compared with normals in an inhibition enzyme-linked immunosorbent assay ($P < 0.0001$ and $P = 0.003$, respectively). (c) Collagen IV α5 mRNA levels in affected fibroblast cell lines demonstrating normal levels in cell lines from Male 1 and Male 2 (both P values not significant). The normal collagen IV α5 chain levels were derived from 3 normals. Otherwise, these results represent the mean ± SD of 3 experiments performed in duplicate. The results for mRNA were compared with a normal male standardized to 1.00.

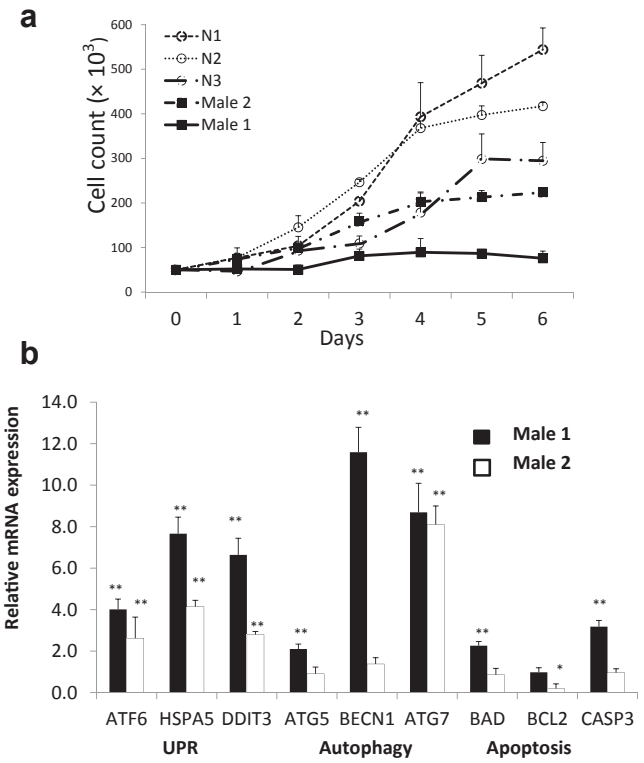

Figure 2. Effect of *COL4A5* missense mutations on cell proliferation, endoplasmic reticulum (ER) stress, autophagy, and apoptosis. (a) Affected fibroblast cell lines from Male 1 and Male 2 grew more slowly than 3 normal cell lines over 6 days ($P = 0.01$ and $P = 0.03$ compared with the mean normal value at day 6, respectively). Cell numbers were measured in duplicate. (b) Both affected cell lines had increased levels of mRNA coding for markers of the unfolded protein response (UPR)/ER stress (ATF6, HSPA5, and DDIT3), and autophagy (ATG7). The cell line from Male 1 also had increased ATG5 and BECN1 (autophagy) as well as BAD and CASP3 (proapoptosis) mRNA. These results represent the mean ± SD of 3 experiments performed in duplicate and compared with the results in a normal male standardized to 1.00.

(Male 1, $P < 0.05$ or $P < 0.01$; Male 2, all P values < 0.01) (Figure 2b).

ER stress results in activation of the protein degradation pathways such as autophagy in an attempt to restore ER and cell homeostasis. Both cell lines had increased ATG7 mRNA levels ($P < 0.05$ and $P < 0.01$) and BECN1 mRNA ($P < 0.01$ and $P < 0.05$), respectively, compared with normal.

Apoptotic marker mRNA (CASP3 and BAD) were also increased in the cell line from Male 1 ($P < 0.05$). There was no increase in the proapoptotic marker mRNA, and there was a reduction in the antiapoptotic marker BCL2 mRNA in the cell line from Male 2 ($P < 0.01$).

Both missense mutations affected autophagy markers more than markers of apoptosis.

PBA Treatment and Collagen IV α5 mRNA Level

PBA treatment increased the level of collagen IV α5 mRNA in cell lines from both Male 1 ($P < 0.001$) and Male 2 ($P < 0.001$) (Figure 3) and in a normal cell line (data not shown).

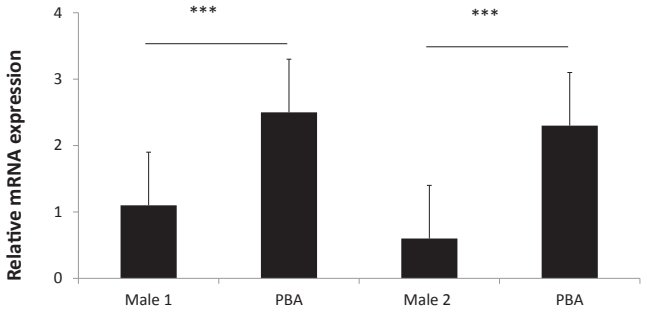

Figure 3. Effect of sodium 4-phenylbutyrate (PBA) on collagen IV α5 mRNA expression. PBA incubation increased the expression of collagen IV α5 mRNA in fibroblast cell lines from both Male 1 and Male 2 cell lines compared with normal ($P < 0.001$ in both cases). These results represent the mean ± SD of 3 experiments performed in duplicate. ***$P < 0.001$.

PBA Treatment and Collagen IV α5 Chain Location

PBA treatment produced fewer consistent effects on collagen IV α5 transport. In the cell line from Male 1, intracellular collagen IV α5 levels were reduced ($P = 0.002$), and there was a trend toward higher extracellular collagen IV α5 levels ($P = 0.06$), but this effect was not seen in the cell line from Male 2 (Figure 4).

PBA Treatment and ER Size

ER on electron micrographs were larger in affected fibroblasts from Male 1 than normal cells ($P < 0.01$) and became smaller after PBA treatment ($P < 0.01$) (Figure 5a and b). The reduction in ER size after PBA treatment was confirmed independently with HSP47 colocalization by cytochemistry (Figure 5c) in cell lines from both Male 1 and Male 2 ($P < 0.001$ and $P < 0.001$, respectively) compared with a normal cell line (Figure 5d).

PBA Treatment and ER Stress, Autophagy and Apoptosis

Levels of mRNA coding for the UPR (ATF6, HSPA5, and DDIT3) were all less in the cell lines from Male 1 after PBA treatment ($P < 0.01$) (Figure 6a and b). Levels of autophagy mRNA (ATG5 ($P < 0.05$), BECN1 ($P < 0.010$) and ATG7 (all P values < 0.01), and for the proapoptosis markers (CASP3 and BAD) were also less (all P values < 0.01).

Levels of mRNA coding for the UPR (HSPA5 and DDIT3), for autophagy (ATG5) and the proapoptosis marker (CASP3) in the cell line from Male 2 were all lower after PBA treatment (all P values < 0.01) (Figure 6a and b).

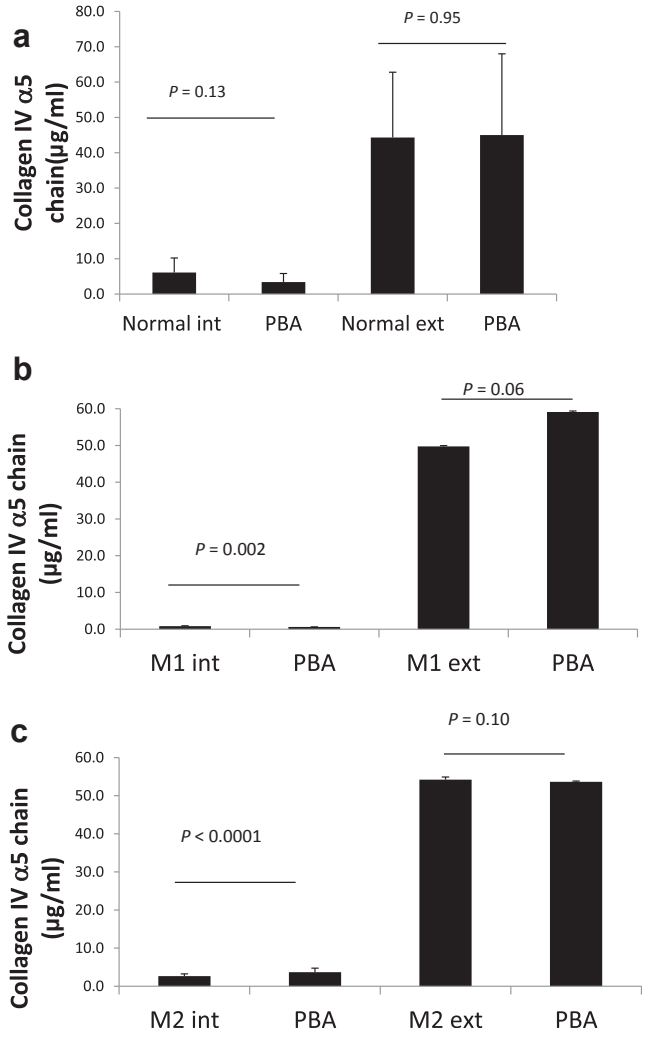

Figure 4. Effect of sodium 4-phenylbutyrate (PBA) incubation on collagen IV α5 chain levels. (a) The normal cell line demonstrated no difference in intra- or extracellular collagen IV α5 levels after PBA treatment ($P = 0.13$ and $P = 0.95$, respectively). (b) PBA treatment reduced intracellular collagen IV α5 levels ($P = 0.002$) and demonstrated an increased trend in extracellular collagen IV α5 ($P = 0.06$) in the M1 cell line. (c) PBA treatment increased intracellular collagen IV α5 levels ($P < 0.0001$), but extracellular levels were not different ($P = 0.10$) in the M2 cell line. These results represent the mean ± SD of 3 experiments performed in duplicate. ext, extracellular; int, intracellular.

In summary, PBA treatment reduced mRNA levels for the UPR (HSPA5 and DDIT3), autophagy (ATG7), and proapoptotic markers (CASP3) in both affected male cell lines (all P values < 0.01). The effect of PBA on the autophagy marker ATG7 was most pronounced and consistent in both cell lines.

DISCUSSION

This study demonstrated that missense mutations in cell lines from men with X-linked Alport syndrome are associated with increased ER size and stress, increased autophagy, and reduced cell proliferation. These effects varied with different mutations. Treatment with the chemical chaperone PBA consistently decreased ER size and stress and reduced autophagy. PBA treatment also increased collagen IV α5 transcript levels and sometimes extracellular collagen IV α5 chain levels. These results indicate novel pathogenetic mechanisms in Alport syndrome and further targets for therapy. In particular, PBA appears to have several potentially useful actions for the treatment of X-linked Alport syndrome caused by missense mutations.

As with other inherited collagen diseases, most *COL4A5* missense mutations change a Gly residue.[29] Individual mutations differ in their clinical consequences depending on their location within the gene, the substituting residue, and the effects of modifying genes.[30,31] We studied cell lines with different Gly substitutions. One was associated clinically with early-onset renal failure and extrarenal features, consistent with a severe phenotype (Male 1). In the corresponding cell line, we demonstrated increased ER stress and autophagy and less cell proliferation. These features were all more pronounced than in the cell line associated with isolated late-onset renal failure. This is consistent with missense mutations affecting the clinical phenotype through multiple mechanisms.

Disease pathogenesis in Alport syndrome has been attributed to the loss of the collagen IV α5 chain and the α3α4α5 heterotrimer and a compensatory increase in the proteolytically susceptible α1α2 network.[32] Protein leakage through the damaged GBM and reabsorption is the major initiator of tubulointerstitial damage and progressive renal failure.[32] The nearly normal collagen IV α5 mRNA levels but reduced intracellular chain in the cell line from Male 1 suggest degradation possibly by ER-associated decay or from increased autophagy. This result was unexpected because other studies of collagen missense mutations suggest intracellular collagen IV chain retention.[18] However, the collagen IV α5 reduction was confirmed independently by immunocytochemistry.

Increased ER stress has been demonstrated in other inherited collagen diseases[18,33,34] and, in particular, collagen IV diseases.[15,35] It is typically associated with autophagy more than apoptosis.[36] This was confirmed in our study.

The reduction in intracellular collagen IVα5 level but increased ER size in the cell line from Male 1 may be explained by ER size increasing even before the development of ER stress in order to counteract it.[37]

Males with *COL4A5* missense mutations not only have abnormal glomerular membranes but also abnormal membranes in the skin, aorta, Bowman's capsule, and testes. Loss of the α5α5α6 network contributes to the rare associations of aortic aneurysms and rupture in Alport syndrome.[38] Affected males with

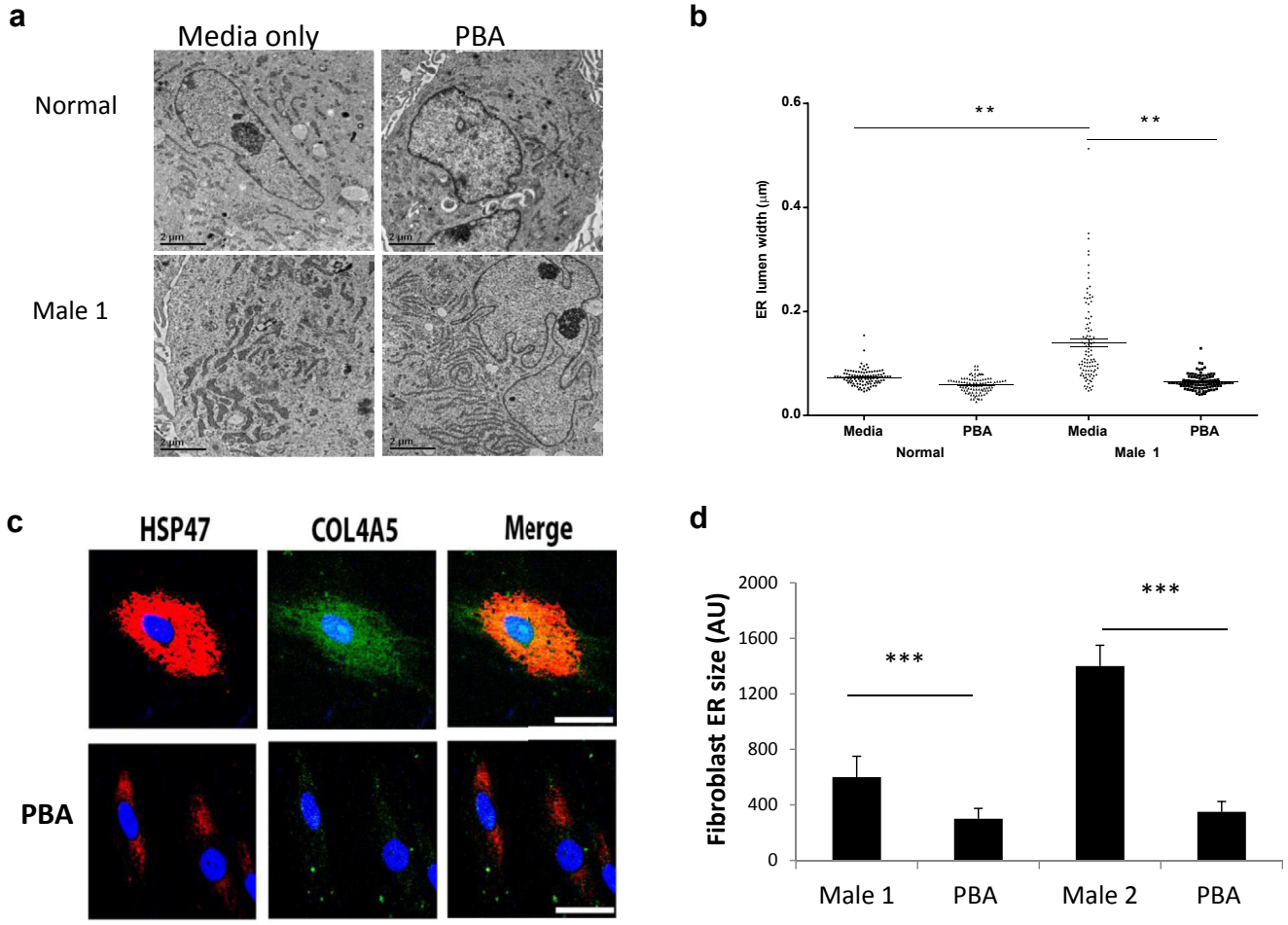

Figure 5. Effect of sodium 4-phenylbutyrate (PBA) treatment on endoplasmic reticulum (ER) size. (a,b) ERs on electron micrographs were larger in affected fibroblasts from Male 1 than normal cells ($P < 0.01$) and became smaller after PBA treatment ($P < 0.01$). (c,d) The reduction in ER size after PBA treatment was confirmed with HSP47 colocalization by cytochemistry in cell lines from both Male 1 and Male 2 ($P < 0.001$ and $P < 0.001$, respectively). These results represent the mean ± SD of 3 experiments performed in duplicate. $^{**}P < 0.01$, $^{***}P < 0.001$. AU, arbitrary units.

X-linked disease have skin abnormalities, with discontinuities in the lamina densa, epidermal thinning, duplication, and blebs.[39] This is identical to those seen with *COL4A1* and *COL4A2* mutations in which the epidermis lacks the α1α1α2 network.[15]

We studied primary human fibroblasts rather than podocytes because of their accessibility. Occasionally, fresh Alport kidneys and hence podocytes are available for study, but these are not necessarily from individuals with missense mutations nor, indeed, from individuals whose mutations are known. Males with X-linked Alport syndrome already have scarred kidneys by the time they reach renal failure in their third or fourth decade, and, later, renal transplantation means that any native podocytes in their urine are contaminated with desquamated cells from the allograft. In addition, studies using affected fibroblasts are likely to be more informative than transfected cell lines because they avoid experimentally-induced ER stress that may confuse the results. Fibroblasts also have this advantage over inducible pluripotent stem cells derived from affected skin biopsies. Although epidermal fibroblasts lack the α3α4α5 network and, thus, the effects of the *COL4A5* mutations can only be examined in the α5α5α6 heterotrimer, the mutation effects are likely to be the same in both networks.

PBA treatment had multiple different effects in the Alport cell lines. Chaperones typically facilitate the transport of the target protein to their functional sites, but collagen IV levels are also affected by both ER stress and ER-associated degradation. Collagen IV α5 mRNA expression increased in both lines, mRNA for ER stress and autophagy markers were reduced, and extracellular collagen IVα5 chain was increased in 1 cell line. The PBA-induced mRNA increase has been described previously,[40] but did not, in this study, consistently increase the extracellular collagen IV α5 levels.

Chaperone therapy represents a potential treatment for X-linked Alport syndrome caused by missense mutations through reducing ER stress. ER stress in podocytes is a major determinant of proteinuria,[41] and ER stress is pathogenetic in other diseases including

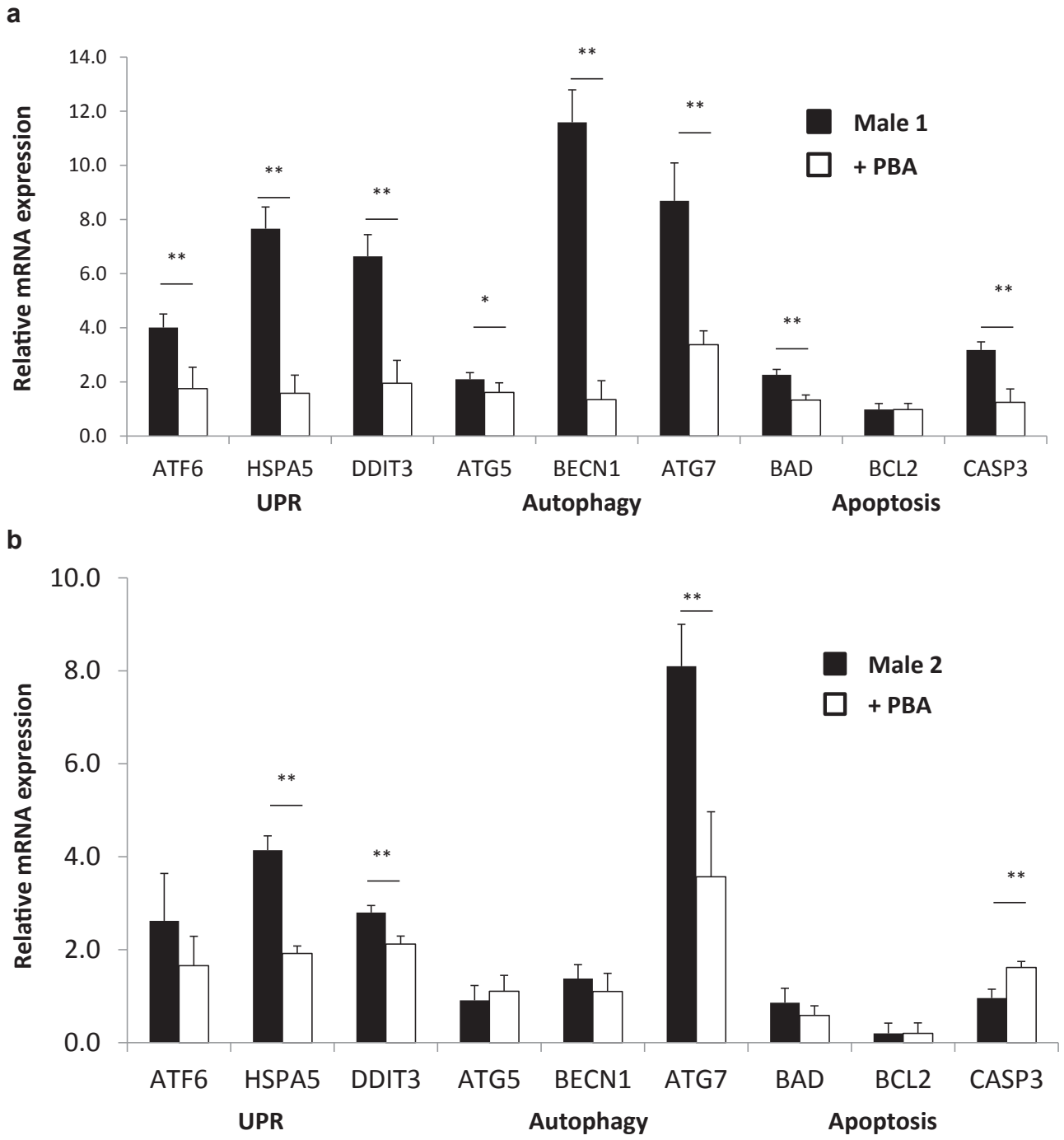

Figure 6. Effect of sodium 4-phenylbutyrate (PBA) treatment on endoplasmic reticulum (ER) stress, autophagy, and apoptosis. (a) Levels of mRNA coding for markers for the unfolded protein response (UPR)/ER stress (ATF6, HSPA5, and DDIT3) and autophagy (ATG, BECN1, and ATG7) were reduced after PBA treatment in cell lines from both Male 1 and Male 2. Levels of mRNA for CASP3 and BAD (proapoptosis markers) were also reduced after PBA treatment. (b) Levels of mRNA for the UPRs (HSPA5 and DDIT3) and autophagy (ATG7) and the proapoptosis marker (CASP3) were all reduced after PBA treatment in the cell line derived from Male 2. These were also corrected with PBA treatment. These results represent the mean ± SD of 3 experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

hereditary angiopathy with nephropathy, aneurysms, and muscle cramps syndrome and metaphyseal chondrodysplasia, which are caused by collagen IV and X mutations, respectively.[42] Treatment with PBA is likely to reduce ER stress *in vivo* in Alport syndrome and, hence, proteinuria, and further delay the onset of end-stage renal failure.

PBA is probably the most commonly used chaperone experimentally and clinically, but other chaperones may be equally or more efficacious.[22] Chaperones typically vary in their effects for different mutations, depending on the amount of protein misfolding.[20] This study suggests that even similar mutations, such as glycine substitutions in the collagenous domain, have

different effects on ER stress and different levels of responsiveness to PBA. It may be difficult to predict who will respond to chaperone treatment.

Chemical chaperone treatment may still have deleterious effects, for example, where the incorporation of an abnormal collagen IVα5 chain damages the basement membrane structure. There are no apparent consequences from coincidental effects on passenger missense mutations from experience in the treatment of the urea cycle disorders. Animal studies indicate that treatment can partially reverse the Alport glomerular membrane defect, even after damage is established.[43]

Understanding the direct clinical outcomes of manipulating ER stress signals encourages a more rational approach to the development of therapies for inherited glomerular disease. The major risk of Alport syndrome is renal failure, but this can be treated relatively safely with transplantation, and any new therapy must be evaluated against this standard. We already have safe, effective treatments in the form of angiotensin-converting enzyme inhibitors to delay end-stage renal failure.[44] However, chaperones may further extend the time to renal failure and delay the need for dialysis, which in itself is a highly valuable outcome. Chaperone treatment may also be useful as adjunctive therapy for other currently untreatable manifestations of Alport syndrome, such as the rare aortic aneurysms,[38] retinal maculopathy,[45] and rapidly progressive hearing loss in children.

DISCLOSURE

All the authors declared no competing interests.

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Part of this work was presented in poster form at the American Society of Nephrology Annual Meetings in San Diego in 2012 and in Philadelphia in 2014 and in San Diego in 2015.

SUPPLEMENTARY MATERIAL

Table S1. Primers for quantitation of *COL4A5* and *GAPDH* transcripts.

Table S2. Primers for quantitation of unfolded protein response, autophagy, and apoptosis pathways.

Supplementary material is linked to the online version of the paper at www.kireports.org.

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