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Evidence that Proteasome Inhibitors and Chemical Chaperones Can Rescue the Activity of Retinol Dehydrogenase 12 mutant T49M

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Abstract

Retinol dehydrogenase 12 (RDH12) is a microsomal enzyme that catalyzes the reduction of all-*trans*-retinaldehyde to all-*trans*-retinol when expressed in cells. Mutations in RDH12 cause severe retinal degeneration; however, some of the disease-associated RDH12 mutants retain significant catalytic activity. Our previous study (Lee et al., *FEBS Lett.* 2010 584:507–10) demonstrated that the catalytically active T49M and I51N variants of RDH12 undergo accelerated degradation through the ubiquitin-proteasome system, which results in reduced levels of these proteins in the cells. Here, we investigated whether stabilization of T49M or I51N RDH12 protein levels through inhibition of proteasome activity or improved folding could rescue their retinaldehyde reductase activity. For the T49M variant, inhibition of proteasome activity resulted in an increased level of T49M protein in the microsomal fraction. The higher level of the T49M variant in microsomes correlated with the higher microsomal retinaldehyde reductase activity. T49M-expressing living cells treated with inhibitors of proteasome activity or with dimethyl sulfoxide exhibited an increase in the conversion of retinaldehyde to retinol, consistent with recovery of functional RDH12 protein. On the other hand, accumulation of the I51N variant in the microsomes did not result in higher retinaldehyde reductase activity of the microsomes or cells. These results provide a proof of concept that, at least in the case of the T49M variant, prevention of accelerated degradation could lead to restoration of its function in the cells. This finding justifies further search for more efficient and clinically relevant compounds for stabilizing the T49M variant activity.

Keywords

retinol; dehydrogenase; photoreceptors; mutant; human

1. Introduction

Retinol dehydrogenase 12 (RDH12) is an NADP⁺-dependent short-chain dehydrogenase/reductase that is associated with the membranes of endoplasmic reticulum and functions as a retinaldehyde reductase in the cells. Mutations in RDH12 have been implicated in severe

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early-onset retinal dystrophy (arRD) [1,2], Leber congenital amaurosis (LCA) [3,4], and autosomal dominant retinitis pigmentosa [5]. The exact mechanism leading to photoreceptor cell death in patients carrying mutations in RDH12 remains unknown, but it is generally believed that accumulation of retinaldehyde in photoreceptors lacking sufficient RDH12 activity may be the major factor contributing to cytotoxicity [6,7]. As an integral membrane protein, RDH12 joins the list of many other membrane proteins that were linked to inherited human diseases [8]. Disease-causing mutations in these proteins are typically distributed throughout the whole sequence and usually result in inefficient trafficking and low cellular yields [8]. Mutations in RDH12 follow the same pattern and result in low steady-state levels of the mutant proteins in cells. Recently, we have demonstrated that RDH12 mutant variants T49M and I51N are targeted for accelerated degradation by the ubiquitin-proteasome system [9], suggesting that these proteins are recognized as misfolded by the cytosolic chaperones and are degraded through the 26S proteasome in the cytoplasm in a process known as ERAD (ER-associated degradation) [reviewed in refs. 10–12]. However, the folding defects in T49M and I51N variants appear to be rather subtle since both proteins retain significant catalytic activity [7]. Unfortunately, the degradative system is limited in its ability to discriminate between serious alterations in protein conformation and those which could have negligible effects within the cell [13]. A number of studies suggested that mutant proteins with subtle misfolding defects can be rescued from ERAD through the use of proteosomal inhibitors or small molecules called chemical chaperones. For example, missense mutants of transmembrane protein E-cadherin were shown to be rescued from ERAD by treatment of cells with MG132 or 2% dimethyl sulfoxide (DMSO) [14].

MG132 (Z-Leu-Leu-Leucinal-) is a peptide aldehyde that can readily enter cells and selectively inhibit the proteasome [reviewed in refs. 15 and 16]. Peptide aldehydes are substrate analogues and potent transition-state inhibitors primarily of the chymotrypsin-like activity of the proteasome. These inhibitors block proteolytic activity of the 26S proteasome without influencing its ATPase or isopeptidase activities and have several features that are particularly advantageous for cell biology. They are reversible and highly potent (for MG132, nanomolar K_i for the chymotryptic activity of pure 20S proteasomes, and micromolar IC_{50} for the inhibition of proteolysis in cultured cells). Importantly, cell viability and growth are not generally affected by these agents for at least 10–20 hours after exposure [15–17].

Chemical or pharmaceutical chaperones, such as 4-phenyl butyric acid (PBA), trimethylamine *N*-oxide dihydrate (TMAO), and DMSO, are a group of low molecular weight compounds known to stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins [18]. A number of these compounds have been used successfully to promote the folding of several mutant proteins associated with diseases. The goal of the present study was to determine whether treatments of cells with chemical chaperones or proteosomal inhibitors could rescue the catalytically active T49M and I51N variants of RDH12 and restore the function of RDH12 in the cells.

2. Experimental procedures

2.1. Materials

Lactacystin and calpastatin were obtained from Calbiochem (EMD Chemicals, Inc. Gibbstown, NJ). *N*-carbobenzoxyl-L-leuciny-L-leuciny-L-leucinal (MG132), pepstatin A, leupeptin, chloroquine, and NH_4Cl were all from Sigma Aldrich Co. (St. Louis, MO).

2.2 Vectors

Expression vectors for RDH12 variants were prepared in pIRES-hrGFP-2 α plasmid (Stratagene, La Jolla, CA) in frame with the C-terminal HA tag as described previously [9].

2.3 Treatment of Cells with Inhibitors of Proteases

HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C with 5 % CO₂. Cells were plated in 35-mm dishes and transiently transfected with expression constructs for wild-type or mutant RDH12 in pIRESneo vector [7] using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were incubated with protease inhibitors for 20 h.

For activity assays, the cells were incubated with 5 μ M all-*trans*-retinaldehyde. Media and cells were collected separately under reduced light. Retinoids were extracted from cells and media and separated by normal phase high performance liquid chromatography (HPLC) on a Spherisorb S3W column (4.6 mm $\times$ 100 mm) (Waters Corp., Milford, MA) in hexane/ethyl acetate/acetic acid (95:4.75:0.25, v/v/v) mobile phase at a flow rate of 0.7 ml/min using Waters Alliance 2695 HPLC system equipped with a Waters 2996 photodiode array detector. Peaks were identified by comparison to retention times of retinoid standards and evaluation of wavelength maxima and quantified as described previously [19].

2.4. Western Blot Analysis

Cell lysates were centrifuged (16,000 $\times$ g, 15 min, 4 °C) and supernatants were analyzed by Western blotting. Protein concentrations were determined using Bio-Rad *Dc* Protein Assay (Hercules, CA). Protein samples were subjected to 12 % SDS-PAGE and transferred to Hybond-P-membrane (Amersham Biosciences, Piscataway, NJ). The membrane was incubated with rabbit polyclonal RDH12 antiserum [7] at a 1:10,000 dilution. β -Actin monoclonal antibodies (Sigma-Aldrich) were used for control of protein loading at a 1:5,000 dilution. The protein bands were visualized using chemiluminescent substrate (Pierce, Rockford, IL) after incubation with goat anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (at a 1:10,000 dilution).

2.5. Isolation of Microsomes and Analysis of Retinaldehyde Reductase Activity

HEK293 cells transfected with wild-type or mutant RDH12 were incubated with or without 20 μ M MG132 for 20 h. Cells were harvested in 50 mM Tris-HCl (pH 7.4), containing inhibitors of proteases and homogenized by Teflon pestle. Microsomes were isolated through sequential centrifugations at 3,000 $\times$ g for 10 min, 10,000 $\times$ g for 35 min, and 105,000 $\times$ g for 1.5 h at 4 °C. Microsomes were resuspended in 90 mM potassium phosphate buffer (pH 7.4), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 20% glycerol. Protein concentration was determined using Bio-Rad *Dc* Protein Assay. Retinaldehyde reductase activity of microsomes was determined using 5 μ M all-*trans*-retinaldehyde as a substrate and 1 mM NADPH as a cofactor. The reactions were carried out in 90 mM potassium phosphate buffer (pH 7.4) and 40 mM KCl at 37 °C for 15 min and stopped by the addition of methanol. Retinoids were extracted twice with 2 ml of hexane and analyzed by normal phase HPLC as described above. The reaction rates were determined based on the percent substrate conversion [8].

2.6. Treatment of Cells with Chemical Chaperones

Twenty-four hours after transfection with RDH12 expression constructs, the cells were treated with 5% glycerol, 0.2% DMSO, or 2% DMSO for 24 h, and then incubated with 5

μM all-*trans*-retinaldehyde for 1 h. Retinoids were extracted from media and cells under reduced light and analyzed by HPLC as described above.

3. Results and Discussion

The T49M and I51N variants of RDH12 exhibit catalytic efficiency that is equal to (I51N) or greater (T49M) than that of wild-type RDH12 (wtRDH12) [7]. However, both of these proteins are expressed at markedly lower steady-state levels in the cells [2,7]. Our previous study demonstrated that inhibition of proteasome activity stabilizes the cellular levels of T49M and I51N RDH12 proteins [9]. This finding implied that it may be possible to rescue the catalytically active variants of RDH12 from degradation and to increase the retinaldehyde reductase activity of the cells. To determine whether stabilization of protein levels increases the cellular retinaldehyde reductase activity, we first examined the microsomal content of the mutant proteins after treatment with inhibitors of proteasome, since the catalytically active RDH12 is normally associated with the microsomal membranes. Western blot analysis showed that treatment with MG132 (Fig. 1A) or lactacystin (data not shown) increased the microsomal levels of all three proteins – wtRDH12, T49M, and I51N. This observation indicated that T49M and I51N mutations did not appear to affect the efficiency of RDH12 integration into the microsomal membranes. However, mutations at these positions significantly raise the apparent K_m values of RDH12 for nucleotide cofactors [7], consistent with the close proximity of the mutations to the cofactor binding site (Fig. 2).

Importantly, treatment with MG132 increased the retinaldehyde reductase activity of the microsomes: from 3 to 4 $\text{nmol min}^{-1}\text{mg}^{-1}$ for T49M microsomes; and from 1.5 to 2.2 $\text{nmol min}^{-1}\text{mg}^{-1}$ for wtRDH12 microsomes. The ~2-fold higher activity of T49M microsomes was in agreement with our previous finding that T49M variant is ~2-fold more active than wtRDH12 [7]. At the same time, no increase in activity was observed for I51N microsomes despite the overall increase in I51N protein. Thus, inhibition of the proteasome appeared to have a protein-specific effect and resulted in greater levels of functional T49M and wtRDH12 but not I51N variant in the microsomal membranes.

To determine whether the rescued RDH12 protein contributed to the overall retinaldehyde reductase activity of whole cells, we measured the formation of retinol from retinaldehyde in intact cells expressing T49M, I51N, or wtRDH12. A statistically significant improvement in the conversion of retinaldehyde to retinol after treatment with MG132 was observed in the cells containing T49M but not I51N variant of RDH12 (Fig. 1B), as would be expected based on microsomal activity assays. These results suggested that T49M variant of RDH12 is a potential candidate for therapeutic interventions targeting misfolded proteins.

Proteasomal inhibitors have shown promise as therapeutic drugs for the treatment of inflammation and various human cancers [20]. Inhibition of proteasome activity with MG132 was found to induce the expression of heat shock proteins and, in this manner, protect against cellular stress caused by misfolded proteins [21,22]. However, proteasomal inhibitors may not be appropriate for the long-term treatments of genetic diseases. A more feasible approach appears to be the use of chemical chaperones. Chemical chaperones are small molecules that have been described to bind nascent proteins, promoting the folding of mutant proteins to a folded state resembling the wild-type protein, thus allowing them to escape ERAD. In addition, chemical chaperones are thought to reduce aggregation, prevent nonproductive interactions with other resident proteins and alter the activity of endogenous chaperones [23].

To explore the possibility that chemical chaperones could improve RDH12 folding, we treated the cells expressing RDH12 variants with glycerol or DMSO (0.2% and 2%). Treatment with 5% glycerol turned out to be toxic for the cells and reduced the levels of the T49M RDH12 protein (Fig. 3A). DMSO at 0.2% had no effect on protein levels (Fig. 3A), but treatment with 2% DMSO resulted in an increase in the steady-state levels of T49M, I51N, and wild-type RDH12 (Fig. 3A). The 2% concentration of DMSO did not appear to be toxic for the cells during the 25-h incubation period.

To determine the effect of 2% DMSO on cellular RDH12 activity, we incubated intact cells with all-*trans*-retinaldehyde and examined the products of retinaldehyde metabolism. The cells expressing T49M variant exhibited a statistically greater rate of retinaldehyde conversion to retinol (Fig. 3B) and a corresponding increase in retinyl esters (data not shown) after treatment with DMSO, while the cells expressing I51N were unaffected. These results suggested that 2% DMSO improved the folding of T49M variant, stabilizing it against degradation, and increasing the levels of functional RDH12. The finding that the stabilizing effect of chemical chaperones varies for individual proteins and can also be mutation-specific is consistent with previous reports [23,24]. For example, E-cadherin mutant R749W completely recovered its plasma membrane expression and the adhesion and invasion suppressor functions after incubation with 2% DMSO (the most efficient chemical chaperone in that study), while mutant E757K was still partially retained in the perinuclear region and not fully restored at the functional level [14].

Chemical chaperones like DMSO, glycerol, trimethylamine-*N*-oxide (TMAO) and 4-phenylbutyrate (4-PBA) have been shown to promote the folding of several mutant proteins associated with diseases [25]. For example, PBA increased the trafficking of the cystic fibrosis transmembrane regulator with $\Delta 508$ mutation (CFTR $\Delta 508$) and enhanced the secretion of the mutant $\alpha 1$ -ATZ protein in $\alpha 1$ -antitrypsin deficiency [26]. Oral administration of PBA to *ob/ob* mice reduced ambient blood glucose to normoglycemic levels seen in the lean wild-type controls [27]. Likewise, endogenous bile acids and derivatives such as ursodeoxycholic acid and its taurine-conjugated derivative (TUDCA) were also shown to modulate ER function [28]. PBA and TUDCA have outstanding *in vivo* safety profiles. PBA, for example, has been approved by the U.S. Food and Drug Administration for clinical use in urea-cycle disorders as an ammonia scavenger and has been in clinical trials for the treatment of other diseases such as thalassemia and cystic fibrosis [29–33]. TUDCA is a derivative of an endogenous bile acid, and it has been safely used as a hepatoprotective agent in humans with cholestatic liver diseases [32]. The ability of these chemical chaperones to alleviate ER stress, establish normoglycemia, and rescue insulin action in mice [27] demonstrates the feasibility of targeting ER function for therapeutic gain.

The goal of this pilot study was to obtain a proof of principle that the activity of certain RDH12 mutants can be improved through the use of small molecules. However, more studies are needed for screening and identification of the most efficient and clinically feasible chemical chaperones for different RDH12 variants. The therapeutic value of pharmacological chaperones in endocrine and metabolic disorders such as hyperinsulinemic hypoglycemia, hypogonadotropic hypogonadism and nephrogenic diabetes insipidus is already well established, which raises hopes that such therapy may also be successfully applied to other diseases, at least in cases where protein misfolding and ERAD degradation play a major role.

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Abbreviations

RDH12	Retinol Dehydrogenase 12
HEK293 cells	human embryonic kidney cells
DMSO	dimethyl sulfoxide
ERAD	endoplasmic reticulum associated degradation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1. Proteosomal inhibitor MG132 increases the level of functional T49M variant of RDH12
A, HEK293 cells expressing wild-type (WT), or mutant RDH12 were incubated with (+) or without (-) MG132 for 20 h and were used for isolation of microsomes. RDH12 proteins were detected in the microsomes (20 μ g) by Western blot analysis using polyclonal RDH12 antiserum. Arrowhead indicates a proteolytic fragment of T49M that accumulates in microsomes after inhibition of proteasome. **B**, Mock-transfected or RDH12-transfected cells were incubated with MG132 (dark gray) or with a vehicle (light gray) for 20 h followed by treatment with 5 μ M all-*trans*-retinaldehyde for 6 h. Retinoids were extracted from cells and media under reduced light and analyzed by normal phase HPLC as described under *Experimental Procedures*. Data are mean $\pm$ S.D., n=3. Statistical analysis was done using two-tailed unpaired Student's *t* test. *, $p < 0.05$.

Figure 2. Structural analysis of wild-type and mutant RDH12 amino acid sequence

The N-terminal transmembrane domain predicted by TMPred software (http://www.ch.embnet.org/software/TMPRED_form.html) is shown in white letters over dark gray background. α -Helices (gray cylinders) and β -strands (block arrows) are marked as predicted by NetSurfP 1.1 software package (<http://www.cbs.dtu.dk/services/NetSurfP>). Helices 2 through 5 are essential elements of the Rossmann fold structural domain. The signature motif GxxxGxxG encompasses the sites of T49M and I51N mutations (indicated by the arrow). The conserved catalytic tyrosine residue is indicated by the asterisk.

Figure 3. Chemical chaperone DMSO (2%) increases the level of functional T49M variant of RDH12

HEK293 cells expressing wild-type (WT) or mutant RDH12 were treated with the indicated chemical chaperones for 24 h. **A**, The amount of RDH12 in cell lysates (50 μ g) was evaluated by Western blot analysis using polyclonal RDH12 antiserum. The results are representative of three independent experiments. **B**, Twenty-four hours after treatment with a vehicle (light gray) or 2% DMSO (dark gray), the cells were incubated with 5 μ M all-*trans*-retinaldehyde for 1 h. Retinoids were extracted from the cells and media under reduced light and analyzed by normal phase HPLC. Data are mean $\pm$ S.D., n=3. Statistical analysis was done using two-tailed unpaired Student's *t* test. **, $p < 0.01$.