Chemical Chaperones Reduce Endoplasmic Reticulum Stress and Prevent Mutant HFE Aggregate Formation*

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HFE C282Y, the mutant protein associated with hereditary hemochromatosis (HH), fails to acquire the correct conformation in the endoplasmic reticulum (ER) and is targeted for degradation. We have recently shown that an active unfolded protein response (UPR) is present in the cells of patients with HH. Now, by using HEK 293 T cells, we demonstrate that the stability of HFE C282Y is influenced by the UPR signaling pathway that promotes its degradation. Treatment of HFE C282Y-expressing cells with tauroursodeoxycholic acid (TUDCA), a bile acid derivative with chaperone properties, or with the chemical chaperone sodium 4-phenylbutyrate (4PBA) impeded the UPR activation. However, although TUDCA led to an increased stability of the mutant protein, 4PBA contributed to a more efficient disposal of HFE C282Y to the degradation route. Fluorescence microscopy and biochemical analysis of the subcellular localization of HFE revealed that a major portion of the C282Y mutant protein forms intracellular aggregates. Although neither TUDCA nor 4PBA restored the correct folding and intracellular trafficking of HFE C282Y, 4PBA prevented its aggregation. These data suggest that TUDCA hampers the UPR activation by acting directly on its signal transduction pathway, whereas 4PBA suppresses ER stress by chemically enhancing the ER capacity to cope with the expression of misfolded HFE, facilitating its degradation. Together, these data shed light on the molecular mechanisms involved in HFE C282Y-related HH and open new perspectives on the use of orally active chemical chaperones as a therapeutic approach for HH.

A large number of diseases result from protein misfolding and aggregation. Hereditary hemochromatosis (HH)³ constitutes an example of such a disease. HFE, a type I transmembrane glycoprotein homologous to major histocompatibility complex I, interacts with β₂-microglobulin, and only the HFE/β₂-microglobulin heterodimer is able to reach the cell surface through the standard secretory pathway (1). The C282Y mutation in HFE prevents the formation of an intra-molecular disul-fide bridge in the α3 domain of HFE blocking β₂-microglobulin association and the trafficking of the protein to the cell surface (2). The resulting misfolded protein is retained in the endoplasmic reticulum (ER) and subjected to accelerated proteasomal degradation (3). The observation that HFE binds to transferrin receptor I implicated this protein in the regulation of iron metabolism (4), a fact supported by the finding that carriers of the HFE C282Y mutation develop HH, an iron overload disorder (1).

It has been known for some time that some compounds collectively called chemical chaperones have the ability to stabilize proteins in their native conformation contributing in some cases to rescue of the folding defect of mutant proteins (5). One well studied example of this mechanism is the restoration of the cell surface expression and function of the mutant cystic fibrosis transmembrane conductance regulator protein by chemical chaperones (6). It is thought, however, that these compounds may be effective in a number of other protein folding defects, thus providing an interesting therapeutic approach for a large number of different human diseases (7).

We have recently reported that cells expressing HFE C282Y have an active unfolded protein response (UPR) (8). This specific ER stress response enhances the levels of molecular chaperones involved in protein folding and degradation and reduces the rate of protein synthesis (9). Upon UPR activation, activating transcription factor-6 (ATF6), an ER stress-transducing protein, is cleaved (nATF6) and relocates to the nucleus where it promotes expression of UPR-responsive genes (10, 11). Another active transcription factor that promotes transcription of UPR-responsive genes is produced by the alternative splicing of X box-binding protein-1 (sXBP1) (12).

Our goal in this study is to investigate the effect of the chemical chaperones tauroursodeoxycholic acid (TUDCA) and 4PBA, 4-phenylbutyrate; ATF6, activating transcription factor-6; HA, hemagglutinin; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; Ab, antibody; mAb, monoclonal Ab; Endo H, endoglycosidase H; HSP, heat shock protein.
sodium 4-phenylbutyrate (4PBA) in the HFE C282Y-associated UPR as well as the impact of these compounds on the intracellular trafficking and localization of the HFE mutant protein. We show that chemical enhancement of the ER folding capacity results in the prevention of the UPR activation and influences the degradation of HFE C282Y. In addition, investigation of the subcellular localization of HFE C282Y revealed that this misfolded protein forms aggregates and that 4PBA is effective in preventing their formation. These findings offer a potential new strategy for therapy designed to prevent the potential toxicity of the intracellular aggregates.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—The following Abs were used: 8C-10 (mouse anti-human HFE, a kind gift from Dr. Rachel Ehrlich, Tel Aviv University, Israel); rabbit anti-HFE cytoplasmic tail (CT) (13); mouse anti-KDEL (detects mainly BiP, an GRP94) and rabbit anti-β-actin (Abcam, Cambridge, UK); mouse anti-HA (Abcam, Cambridge, UK); donkey anti-mouse fluorescein isothiocyanate, anti-rabbit fluorescein isothiocyanate, and donkey anti-mouse-Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

The HFE WT-pcDNA3 construct was a kind gift from Dr. Luisa Salter-Cid. HFE C282Y-pcDNA3 was described previously (8). β2-Microglobulin-pcDNA3.1 constructs were a kind gift from Dr. Hal Drakesmith (University of Oxford, Oxford, UK). The pEP7-nATF6-FL vector expressing a nuclear targeted and transcriptionally active fragment of ATF6 (amino acids 1–373) was described previously (8, 14). A plasmid encoding the spliced form of XBP-1 was the kind gift of Dr. K. Mori (Kyoto University, Japan) and acted as template for a 

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Transfections—293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen) in 60-mm plates according to the manufacturer’s protocol. At the time of transfection, cells were 90–95% confluent. Opti-MEM was used to dilute both DNA and Lipofectamine at a final DNA/Lipofectamine ratio of 1:2.5. After transfection cells were incubated for 48 h in DMEM with GlutaMAX medium (Invitrogen) containing 1% penicillin/streptomycin/amphotericin solution (Sigma) and 10% heat-inactivated fetal bovine serum. In these studies a plasmid encoding β2-microglobulin was co-transfected with HFE expression vectors to ensure that sufficient quantity of this protein was available for correct assembly of the HFE.

Pulse-Chase—48 h after transfection, 1 × 105 293T cells were starved for 1 h in cysteine/methionine-free DMEM (Invitrogen) supplemented with 1% l-glutamine and pulsed for 20 min with 140 μCi/ml Pro-Mix L-[35S]cysteine/methionine (Amersham Biosciences). The culture medium was then supplemented with cold cysteine and methionine and chased for the indicated times. At each time point 1 aliquot was taken, washed, and lysed in ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X, Complete EDTA-free protease inhibitor mixture (Roche Diagnostics), 10 mM iodoacetamide). Cell debris was removed by centrifugation, and the lysates were pre-cleared with 100 μl of protein A-Sepharose bead slurry (50%) for 1 h at 4°C. The lysates were incubated overnight at 4°C with anti-HA. Immunocomplexes were pulled down with protein A-Sepharose beads and washed three times in ice-cold lysis buffer. After addition of gel loading buffer solution (16) with 10% β-mercaptoethanol and boiling for 5 min, samples were loaded on 10% SDS-PAGE.

SDS-PAGE and Quantitation—10% SDS-PAGE was performed using a Bio-Rad Mini Protein II kit. Gels were fixed in 10% acetic acid, 40% methanol, incubated for 30 min with Amplify solution (Amersham Biosciences), dried, and exposed to a radioactivity storage screen. Quantitation was performed using a Typhoon PhosphorImager (GE Healthcare) with ImageQuant version 5.1 software.

Western Blot, Endo H Digestion, and Ultracentrifugation—Protein concentration in whole cell lysates was determined with RC/DC protein assay (Bio-Rad), and 30 μg were separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose Hybond-C membrane (Amersham Biosciences). After blocking at 4°C with 5% dry milk, 0.05% Tween 20 in TBS (TBS-T), the membrane was incubated with anti-HA or anti-KDEL, washed three times with TBS-T, and detected with the respective horseradish peroxidase-conjugated secondary antibody (Molecular Probes, Eugene, OR) and an enhanced chemiluminescence substrate (Pierce). To control loadings, the membrane was stripped using Restore WB Stripping Buffer (Pierce) and incubated with anti-β-actin. For the Endo H assay, whole cell lysates were digested for 4 h at 37°C with Endo H (Roche Diagnostics) or glycosidase F (Roche Diagnostics) as described by the manufacturer. The reaction was stopped by addition of gel loading buffer solution (GBL) (16) with 10% β-mercaptoethanol and boiling for 5 min.

For detection of protein aggregates, cells were solubilized in 1% Triton X lysis buffer for 1 h on ice. After a centrifugation step at 300 × g for 5 min to clear cell debris, whole cell lysates
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FIGURE 1. UPR activation promotes HFE C282Y degradation. A, HFE WT-HA and HFE C282Y-HA transfected cells were 35S-labeled and chased for the indicated times. HFE was recovered by immunoprecipitation (IP) with anti-HA mAb. The graph represents the percentage of remaining protein at each time point (solid line, HFE WT; dashed line, HFE C282Y). Data are from three independent experiments, (*, p < 0.05; **, p < 0.01). B, 293T cells were transfected to express either HFE WT-HA or HFE C282Y-HA plus the plasmids indicated. The left- and right-hand side blots are derived from the same experiment (representative of three independent experiments). The blots were stripped and incubated with an antibody against β-actin, and the intensity of the HFE C282Y bands was normalized to β-actin levels and plotted on a bar graph as average ± 1 S.D. of three independent experiments. The asterisks represent statistically significant differences (**, p < 0.01) compared with HFE C282Y transfected cells.

were split into 2 equal portions. One represented the total protein content, and the other was ultracentrifuged at 100,000 × g for 1 h at 4 °C in a Sorvall Ultra Pro80 centrifuge. The pellet fraction and the protein content of the supernatant fraction (obtained by a 10% trichloroacetic acid precipitation) were solubilized with GLB, 10% β-mercaptoethanol, and boiling for 5 min. Equivalent gel loading was confirmed by staining the nitrocellulose membrane with 0.1% Ponceau S.

Treatment with Chemical Chaperones—18 h post-transfection, 293T cells were washed and incubated for 30 h in DMEM with GlutaMAX medium supplemented with 1% penicillin/streptomycin/amphotericin solution and 10% fetal bovine serum in the presence of 1 mM TUDCA (Calbiochem) or 5 mM 4PBA (Sigma). The percentage of transfected cells was not affected by the treatment with the chemical chaperones (data not shown).

Real Time PCR—Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Following treatment with 2 units/sample of RQ1 DNase, in the presence of 50 units/sample of RNase inhibitor (Invitrogen), for 30 min at 37 °C, 1 μg of RNA was reverse-transcribed, using Superscript reverse transcriptase (Invitrogen), following the manufacturer’s instructions. Expression levels were evaluated by quantitative real time PCR with the ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA) using 1× SYBR Green PCR Master Mix (Applied Biosystems). Quantification of β-actin gene expression was performed as a control. Relative expression levels were calculated as 2ΔΔCt (ΔCt human β-actin − ΔCt HLA-A × 10,000) (for details see ABI PRISM 7700, User Bulletin 2). The oligonucleotides used were 5'-CTCCCTTTGGTAAGGTGACCATC-3' and 5'-ATCACAATGAGGGGCCTGTACCC-3' for HFE and 5'-CCTGGGTTGGCGGAACCCTTGATGTG-3' and 5'-CTGGACGGCCTTTCATAGTACGGC-3' for BiP.

Fluorescence Microscopy—HFE WT-GFP or HFE C282Y-GFP transiently transfected 293T cells were grown on coverslips and either left untreated or treated with 1 mM TUDCA or 5 mM 4PBA for 30 h. Cells were rinsed three times in PBS, fixed in 3.5% paraformaldehyde, and simultaneously blocked and permeabilized with 5% bovine serum albumin, 0.1% NaN3, followed by incubation at 4 °C with a saturating amount of primary Ab for 30 min in 96-well plates. After three washes cells were incubated with Cy3-conjugated secondary Ab for 30 min on ice without permeabilization. Cells were washed, and flow cytometry analysis was performed in a FACS-Calibur (BD Biosciences). The Cy3 fluorescence, representing cell surface expression of HFE, was measured in GFP-positive cells. For each sample a minimum of 15,000 events were acquired. To define the background staining, irrelevant mAbs of the same isotype were used.

RESULTS

Modulation of the UPR Interferes with HFE C282Y Stability—Previous studies have shown that HFE C282Y fails to travel beyond the ER and is subjected to accelerated proteasomal degradation in COS7 cells (3). Pulse-chase analysis performed in
293T cells confirmed these observations specifically in our transfected cell model system (Fig. 1A). In 293T cells overexpressing HFE WT or C282Y, only the WT protein can reach the cell surface as observed by flow cytometry (supplemental Fig. 1A) and by the HFE WT resistance to Endo H digestion (supplemental Fig. 1B). In contrast, no Endo H-resistant protein is observed in HFE C282Y-transfected cells, confirming that the mutant protein is unable to leave the ER through the standard secretory pathway (supplemental Fig. 1B).

We have recently shown that the ER-retained HFE C282Y mutant protein leads to the activation of the UPR (8). To investigate the involvement of the ER stress pathway on the protein stability of HFE, 293T cells were co-transfected with plasmids encoding HFE WT or C282Y, and the UPR was genetically modulated by the co-expression of transcriptionally active isoforms of either ATF6 (nATF6) or XBP1 (sXBP1). Induction of the UPR was confirmed by analysis of GRP94 and BiP levels by Western blotting (supplemental Fig. 1C). Stimulation of the UPR by either sXBP1 or nATF6 resulted in highly significant decreases in steady state expression of the HFE C282Y protein compared with HFE WT (Fig. 1B).

Chemical Chaperones Block the UPR Activation and Alter the HFE C282Y Stability—To evaluate the effect of chemical chaperones on the ER stress response, HFE WT- or C282Y-expressing cells were cultured in the presence of TUDCA and 4PBA. Using BiP and GRP94 levels as markers for the UPR activation, we observed that HFE C282Y-transfected cells treated with chemical chaperones have lower levels of both proteins indicating that these compounds were effective in inhibiting the activation of the UPR in HFE C282Y-expressing cells (Fig. 2A). As an independent measure of UPR activation, real time PCR was performed to quantify BiP expression at the transcriptional level. Although no significant effect was observed in HFE WT-transfected cells, analysis of the data showed a statistically significant decrease in BiP mRNA in HFE C282Y-transfected cells cultured in the presence of TUDCA or 4PBA when compared with untreated cells (Fig. 2B). The efficiency of chemical chaperones was further confirmed by the capacity of TUDCA to alleviate ER stress in cells co-expressing HFE C282Y plus nATF6 or sXBP1 (Fig. 2C).

FIGURE 2. Chemical chaperones protect from UPR activation and modulate HFE C282Y protein levels. A, BiP and GRP94 from whole cell lysates of HFE WT-HA- or C282Y-HA-transfected cells either untreated or treated with 1 mM TUDCA or 5 mM 4PBA were detected by Western blot with anti-KDEL. The intensity of the bands was quantified, normalized to β-actin, and plotted as average ± 1 S.D. Data are from three independent experiments. The asterisks represent statistically significant differences (*, p < 0.05; **, p < 0.01) between the cells treated with the chemical chaperones and the untreated cells. B, BiP mRNA levels of 293T cells transfected with empty vector (Mock), HFE WT-HA, or HFE C282Y-HA either left untreated or treated with the chemical chaperones TUDCA and 4PBA were assessed by quantitative real time PCR. The graph represents the average ± 1 S.D. of three independent experiments. β-Actin was used as an endogenous control gene. **, p < 0.01 relative to HFE C282Y-HA untreated cells. C, cells transfected to express the indicated proteins were either treated with 1 mM TUDCA or left untreated and BiP and GRP94 levels detected by Western blot. The intensity of the bands corresponding to BiP and GRP94 was quantified, normalized to β-actin, and plotted on a bar graph as average ± 1 S.D. of three independent experiments. *, p < 0.05; **, p < 0.01 relative to untreated cells expressing the same proteins. D, HFE protein levels of HA-tagged HFE WT or C282Y transfected cells either untreated or treated with TUDCA or 4PBA were detected by Western blot with anti-HA mAb. The asterisk indicates the position of a nonspecific band. The intensity of the HFE band was normalized to β-actin and plotted on a bar graph as average ± 1 S.D. of three independent experiments. *, p < 0.05 compared with HFE C282Y-HA transfected cells.
By having shown in Fig. 1 that stimulation of the UPR lowers the levels of HFE C282Y, treatment with chemical chaperones should rescue the mutant protein from degradation. Indeed, we observed increased levels of this protein in HFE C282Y-transfected cells when TUDCA was present in the culture medium (Fig. 2D, right-hand side blot, 2nd versus 3rd lane). However, treatment with 4PBA did not result in the stabilization of the HFE C282Y protein. In the presence of this chemical chaperone, the amount of protein detected was smaller than that observed in untreated cells (Fig. 2D, right-hand side blot, 2nd versus 4th lane). HFE WT transfection did not result in the UPR activation (Fig. 2A). Neither TUDCA nor 4PBA had a significant effect on steady state expression of BiP, GRP94 (Fig. 2A), and HFE WT (Fig. 2D, left-hand side blot).

To exclude a possible effect of the chemical chaperones on the transcription efficiency of HFE, mRNA from TUDCA or 4PBA-treated and untreated cells was quantified by real time PCR. Fig. 3 shows that no significant differences were observed in the HFE mRNA levels, corroborating the hypothesis that 4PBA facilitates HFE C282Y degradation.

Chemical Chaperones Do Not Restore HFE C282Y Cell Surface Expression—Several examples of mutant proteins whose folding defects are corrected by the action of chemical chaperones have already been described (5). As the C282Y mutation impairs the correct assembly of HFE molecules and concomitantly their trafficking beyond the ER toward the cell surface, we wanted to investigate if treatment with TUDCA or 4PBA promoted the stabilization of a conformation that restores HFE C282Y cell surface expression. To do that, 293T cells transiently expressing HFE WT-GFP or HFE C282Y-GFP were cultured in the presence or absence of TUDCA or 4PBA. Cell surface expression of HFE was then evaluated by flow cytometry analysis of the anti-HFE staining in GFP-positive cells (cells successfully transfected with HFE WT or HFE C282Y). The results revealed that treatment with the chemical chaperones had no effect on the correct cell surface expression of HFE WT when compared with untreated cells (Fig. 4A). Regarding the effect of TUDCA and 4PBA on HFE C282Y, this set of experiments revealed that none of the chemical chaperones used was able to restore the cell surface expression of the mutant protein (Fig. 4A). The failure of these compounds to promote the correct intracellular trafficking of HFE C282Y was further confirmed by the results obtained with Endo H. Following digestion with this glycosidase, analysis of protein extracts of cells expressing HFE C282Y and cultured in the presence or absence of TUDCA or 4PBA did not reveal any Endo H-resistant HFE protein, as observed by the conversion of all the protein to a deglycosylated (HFE-CHO) state when compared with untreated cells (Fig. 4A).
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A

![Image of protein localization](https://example.com/image.png)

**Figure 5.** 4PBA prevents the formation of HFE C282Y intracellular aggregates. A, 293T cells were transiently transfected with HFE WT-GFP or HFE C282Y-GFP and either left untreated or incubated for 30 h in the presence of 1 mM TUDCA or 5 mM 4PBA. Permeabilized cells were stained with FITC-labeled anti-KDEL Ab and analyzed by confocal microscopy. Merged images are shown in yellow. 4,6-Diamidino-2-phenylindole staining of the cell nucleus is shown in blue. B, cells bearing intracellular aggregates were counted and percentages calculated. At least 200 cells from each experimental condition were counted. Cells showing accumulation of the HFE-GFP fluorescence in individual loci were classified as having protein aggregates. Data are from three independent experiments. **, p < 0.01.

4PBA Prevents HFE C282Y Aggregation—To investigate the intracellular localization of the HFE C282Y protein, 293T cells expressing either HFE WT-GFP or HFE C282Y-GFP were stained with anti-KDEL Ab and analyzed by immunofluorescence microscopy. KDEL is an ER retention motif found in proteins such as BiP and GRP94. The amount of GFP-tagged HFE proteins that co-localize with KDEL-containing proteins represents the portion of HFE residing in the ER. As expected, HFE WT showed only mild localization in the ER, with most of the protein spread out within the cell (Fig. 5A, HFE wt). Contrary to this expression pattern, HFE C282Y was found in the ER (Fig. 5A, HFE C282Y). Interestingly, however, we observed that in ~37% of the cells (Fig. 5B), HFE C282Y was found in intracellular aggregates (Fig. 5A, HFE C282Y panels) that co-localized extensively with KDEL proteins. Ultracentrifugation analysis of detergent-treated protein extracts confirmed that the structures were the result of HFE C282Y aggregation (Fig. 6). Indeed, the majority of the mutant protein was recovered in the pellet (detergent-resistant) fraction, contrasting with the higher amount of HFE WT observed in the supernatant (Fig. 6A, 5th versus 8th lanes and 6th versus 9th lanes).

Treatment with TUDCA did not produce any considerable change in the localization pattern or in the percentage of HFE C282Y aggregates (Fig. 5A, HFE C282Y + TUDCA; Fig. 5B). However, although no effect on the intracellular location of HFE C282Y was found in cells treated with 4PBA, this chemical chaperone significantly reduced the percentage of cells with detectable protein aggregates (Fig. 5, A, HFE C282Y + 4PBA; Fig. 5B), suggesting that 4PBA may promote a more efficient disposal of HFE C282Y to the degradation route, preventing its aggregation.

**DISCUSSION**

We have recently shown that a UPR is triggered following HFE C282Y expression (8). With this study we aimed at investigating the impact of this ER stress response, stimulated by genetic manipulation of 293T cells, on the fate of the mutant protein. In addition, the effects of two different chemical chaperones, TUDCA and 4PBA, on the HFE C282Y-associated UPR and intracellular distribution were also analyzed.

Here we describe that in HFE C282Y-expressing cells, activation of the ER stress response is associated with clearance of the mutant protein. Confirming the direct link between the UPR and the HFE C282Y degradation, we observed that this effect is amplified in the presence of nATF6, sXBP1.

Here we report that HFE C282Y forms intracellular aggregates in vitro. The co-localization pattern obtained for HFE C282Y- and KDEL-containing proteins suggests that the protein aggregates contained, besides HFE C282Y, ER resident proteins. Because we found anti-KDEL to detect BiP to a greater extent than other KDEL-containing proteins by Western blot and native immunoprecipitation experiments (8), it is reasonable to assume that BiP is one of the major components of the observed aggregates. In fact, it was previously observed that protein aggregates are enriched in molecular chaperones (17).

A common feature of almost all diseases of protein conformation is the formation of aggregates caused by destabilization of the α-helical structure with simultaneous formation of a β-sheet (18). These aggregates tend to resist degradation and accumulate in inclusion bodies, which are usually present in low copy number, most often only one per cell (19).

Chemical chaperones, such as 4PBA, are a group of compounds known to improve ER folding capacity and facilitate the trafficking of mutant proteins by stabilizing their conformation (5). 4PBA was previously shown to increase the trafficking of a mutant cystic fibrosis transmembrane regulator (CFTRΔ508) (20) and to enhance the secretion of the mutant α1-ATZ protein (21). Endogenous bile acids derivatives, such as TUDCA, can also modulate ER function protecting from UPR induction and ER stress-induced apoptosis (22, 23). In this study, we investigate whether pharmacologically active chemical chaperones alleviate the ER stress response and affect the fate of the HFE C282Y protein. We show that both TUDCA and 4PBA
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FIGURE 6. Biochemical analysis of the HFE C282Y aggregates. A, total cell lysates of HFE WT-HA- or C282Y-HA-transfected cells were solubilized with lysis buffer containing 1% Triton X and centrifuged at 100,000 × g for 1 h at 4 °C. HFE protein from total cell lysates (not centrifuged, Total), pellet fraction of the ultracentrifugation (Pellet), and the supernatant fraction (Supernatant) was detected by Western blot with anti-HA mAb. The position of the HFE band is indicated. The asterisk indicates the position of a nonspecific band. B, as a loading control, the membrane was stained with 0.1% Ponceau S before blocking. Three individual experiments were performed with similar results.

Additional text: more, as mentioned before, although the mechanism of action of chemical chaperones remains largely unknown, 4PBA has been shown to prevent aggregation of denatured α-lactalbumin and bovine serum albumin (30). Together with the results reported here, namely the decrease in the HFE C282Y protein levels and intracellular aggregates, these findings support the assumption that 4PBA protects from HFE C282Y-induced UPR by increasing the efficiency of the quality control machinery that ultimately leads the mutant protein to the degradation route thus removing the ER stress stimulus.

Previous studies have demonstrated that TUDCA has the capacity to protect from ER stress (22, 23). However, unlike 4PBA, TUDCA has not been described before to act as a chaperone that promotes the folding and trafficking of mutant proteins. Likewise, in this study we did not observe any significant effect of TUDCA on the intracellular trafficking or localization of HFE C282Y. Instead, TUDCA was found to increase the stability of the mutant protein possibly as a consequence of its influence on the UPR activation that, as shown here, enhances the clearance of the mutant HFE. Even though there was a similar outcome, the mechanism of action of TUDCA and 4PBA leading to the UPR suppression seems to diverge. TUDCA appears to prevent the UPR activation by acting directly on its signal transduction pathway. 4PBA accomplishes the same goal by modulating the ER capacity to cope with the expression of misfolded HFE, facilitating its degradation.

Despite the failure of the chemical chaperones to restore the cell surface expression of HFE C282Y, both TUDCA and 4PBA were effective in decreasing the magnitude of the HFE C282Y-associated UPR. In addition, 4PBA treatment prevented the formation of putatively toxic intracellular aggregates. However, the connection between the UPR activation and HH is recent (8). The clarification of the physiological significance and the contribution of both the UPR and the mutant HFE aggregates to the pathophysiology of HH will certainly clarify the therapeutic potential of these chemical chaperones.

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